UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE ODONTOLOGÍA

TESIS DOCTORAL

Estudio del efecto antimicrobiano de diferentes soluciones y extractos de origen natural en un modelo *in vitro* de *biofilm* subgingival multiespecies

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Honorato José Ribeiro Vidal

DIRECTORES

Mariano Sanz Alonso
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A mi familia

“We shall defend our island, whatever the cost may be,
we shall fight on the beaches,
we shall fight on the landing grounds,
we shall fight in the fields and in the streets,
we shall fight in the hills;
we shall never surrender.”

Winston Churchill

Agradecimientos

Cuando terminé la carrera y empecé a trabajar como *Médico Dentista* en Portugal, estaba muy lejos de pensar que algún día estaría escribiendo los agradecimientos de una tesis doctoral… Recién salido de la facultad solo pensaba en empezar a trabajar y en seguir el rumbo calificado como "normal" de la vida… Pero no ha sido así, y por eso escribo estas palabras…

He vivido casi 8 años en España. Si mi lado pragmático me hace pensar que fue solamente la cuarta parte de mi vida, mi lado más sentimental me hace ver que ha sido un 25% "muy grande" e, incluso, fundamental de ella.

Mi larga estancia en tierras de Cervantes me ha hecho crecer, no solo a nivel profesional e investigador, sino también como persona. Así, me gustaría empezar por agradecer a España, la nación que me acogió como si de un hijo suyo se tratara.

No obstante, aunque no podré mencionar a todas las personas que han influido en mí, como autor de esta tesis doctoral, a algunas de ella sí voy tener el placer de hacerlo:

Me gustaría empezar por mi tutor y director, el Profesor Mariano Sanz, que desde que lo conocí no ha parado de dar vueltas con mi vida. Si, a veces, disparar a una diana parecía difícil, su capacidad de distanciar más y más la diana ha influenciado toda mi forma de vivir. A pesar de haber sido capaz de incrementar siempre la dificultad de los objetivos propuestos, también ha sido siempre capaz de caminar delante de mí, guiando mis pasos a lo largo de todos estos años. Lo que para mí parecía inalcanzable, es ahora muchas veces perfectamente razonable. Jamás podré agradecerle todo lo que ha hecho por mí.

Quiero también agradecer de forma muy especial a mi director, el Profesor David Herrera. No sé si lo sabrá, pero durante mucho tiempo se me cortaba la respiración siempre que lo veía. No le ocultaré mi constante miedo de no cumplir con sus expectativas. Jamás olvidaré el momento en que no llegué a tiempo, por primera vez, a una reunión/clase: *"Puedes hacer todos los días las cosas correctamente, y eso parece que se olvida, pero haces una sola vez una cosa mal, y eso parece que nunca se olvida"*. Pero la verdad es que, por detrás de ese aspecto tan serio y exigente, hay una bella persona que siempre ha estado disponible para ayudarme y guiarme durante todo este largo camino.

A Ricardo Faria Almeida, el gran responsable de que conociera y me pudiera unir a este “camino”. Si no fuera por haberle acompañado a Madrid ese día de clases en el Máster, no me habría dado cuenta a tiempo de que ese quería que fuera mi camino. *Muito obrigado!*

A María Sánchez, ¡sin ella simplemente este trabajo no se habría podido realizar! ¡Muchas gracias por todo el trabajo, aliento y amistad a lo largo de todos estos años!

Muchas gracias también al resto del gran equipo del laboratorio: Profesora Elena Figuero, Arancha, Leire, Ana, Patricia, Oksana, Mariajo, Itziar, Estela y Marta, que me han ayudado, acompañado y aguantado tantos días a lo largo de estos años.

A la familia del Máster de Perio y, en especial a todos los Profesores. De todos he aprendido y de todos me llevo un trocito de vuelta a Portugal.

A todos los compañeros de las promociones con las que he convivido en el Máster y, en especial, a la mía, que han compartido conmigo algunas lágrimas de amargura y muchas de felicidad, ¡superando todos los obstáculos y exámenes de revisión de literatura! Myros, a ti, mi nuevo lusitano, tengo que decirte: дуже дякую!

A todos los otros amigos que hice en Madrid y, en especial, en la Facultad de Odontología de la Universidad Complutense de Madrid. Profesores, personal auxiliar en la clínica, y en toda la facultad, mantenimiento y cafetería. ¡Entre tantas otras cosas, llevaré muchas *“saudades”* y lentejas estofadas en mi corazón!

A los doctorandos Andrea, Jaime y David, cuyas contribuciones para el desarrollo en esta tesis han sido muy importantes. Andrea, entre muchísimas otras cosas, estoy seguro de que jamás te olvidarás de las noches y domingos pasados en el laboratorio.

A los diferentes grupos de investigación que han participado en los estudios de la tesis, y que ha tenido una gran relevancia en el desarrollo de esta tesis doctoral. Hay que destacar a las Doctoras María V. Moreno-Arribas y Begoña Bartolomé, del Instituto de Investigación en Ciencias de la Alimentación (CIAL) y a los Profesores María José Ciudad y Luis Collado, del Departamento de Medicina de la Facultad de Medicina de la Universidad Complutense.

A los Profesores de la carrera en la Universidade Católica Portuguesa, cuya dedicación y trabajo me han inspirado a seguir la Periodoncia y la Investigación. Professora Maria José, siempre recordaré mis primeros cultivos de *Candida albicans*.

A los amigos y profesores de la Universidade de Aveiro y, en especial, a la Profesora Hermínia Castro, el Profesor Pedro Pombo y a mi amigo Emanuel por haber sido las primeras personas en introducirme, con 11-12 años, en el mundo de la ciencia. Los siete veranos pasados en el Departamento de Física han modelado mi personalidad y mi forma de encarar la vida y la ciencia.

A todos mis grandes amigos de Viseu que compartieron conmigo tantas noches y tantos días de *lucha por la academia*. Gustavo y Miguel, habéis estado siempre cuando más os he necesitado, y no solo en los momentos felices de la vida…

A todos mis grandes amigos de infancia que, desde la más tierna juventud hasta el día de hoy, me han acompañado en los mejores y en los peores momentos… Diogo y Pedro, jamás olvidaré el incansable apoyo y ayuda que siempre me habéis dado, pero en especial durante mi difícil recuperación.

A mi amigo Orlando, que ha sido capaz de abrirme los horizontes y de enseñarme que no podemos dejar de avanzar, incluso cuando creemos que hemos ido ya muy lejos…

A la familia Magalhães Alves, ahora también mi familia, por todo su apoyo incondicional.

A mis abuelos y al resto de mi familia que, a pesar de no haber comprendido inicialmente mis ganas de aprender más y la necesidad de alejarme todos estos años para lograrlo, han estado siempre a mi lado aportándome todo el apoyo que necesité.

A mis padres, que desde niño me han educado y apoyado. Antes creía que tenía unos padres algo rígidos y seguro diferentes a los de todos mis compañeros. Pero hoy sé que, sin esas enseñanzas de respeto, trabajo, amor y dedicación, no estaría escribiendo los agradecimientos de esta tesis doctoral. Imagino que es difícil ver a un hijo crecer, muchas veces lejos y de forma diferente de lo que imaginábamos, pero me habéis apoyado en esta aventura, a pesar de los obstáculos encontrados.

A mi hermano que, a pesar de llegar tarde, llegó a este mundo para brindarme todo su apoyo. Luís, el espíritu y la fuerza con la que naciste, y que sigues demostrando, han inspirado mi vida. Espero que la educación y la vida que compartimos te sirva para lograr todo lo que desees. Muchas gracias por tus abnegadas atenciones conmigo.

A ti, Marta, ahora mi esposa…

Me has acompañado incansablemente en todos los momentos de este viaje. Imagino que para ti este parecía no tener fin… el mejor ejemplo de eso es que estoy depositando esta tesis escasos días tras nuestro matrimonio… Muchas gracias por haber aceptado y comprendido siempre mis constantes ausencias y por haber estado siempre a mi lado, cuando más te necesita. Tu apoyo ha sido esencial. Espero que ahora consiga compensar todos esos momentos en que estuve lejos.

Gracias a los demás que, a pesar de haber sido importantes para mi en esta etapa, no he podido encontrar el espacio para nombrarlos en estas breves líneas…

A família é a mais pura fonte dos factores morais da produção"

Prefacio

La presente tesis doctoral está basada en los siguientes tres estudios publicados:

Estudio #1: M. C. Sánchez, H. Ribeiro-Vidal, A. Esteban-Fernández, B. Bartolomé, E. Figuero, M. V. Moreno-Arribas, M. Sanz, D. Herrera (2019). "Antimicrobial activity of red wine and oenological extracts against periodontal pathogens in a validated oral biofilm model." BMC Complementary and Alternative Medicine **19**(1): 145. doi: 10.1186/s12906-019-2533-5

Estudio #2: M. C. Sánchez, M. C., H. Ribeiro-Vidal, B. Bartolomé, E. Figuero, M. V. Moreno-Arribas, M. Sanz, D. Herrera (2020). "New Evidences of Antibacterial Effects of Cranberry Against Periodontal Pathogens." Foods **9**(2): 246. doi: 10.3390/foods9020246.

Estudio #3: H. Ribeiro-Vidal, M. C. Sánchez, A. Alonso-Español, E. Figuero, M. J. Ciudad, L. Collado, D. Herrera, M. Sanz (2020). "Antimicrobial Activity of EPA and DHA against Oral Pathogenic Bacteria Using an In Vitro Multi-Species Subgingival Biofilm Model." Nutrients **12**(9): E2812. doi: 10.3390/nu12092812.

Índice

I
Resumen

Resumen

Antecedentes: Las periodontitis son enfermedades multifactoriales, crónicas, de origen inflamatorio, asociadas a un *biofilm* dental disbiótico, caracterizada por la destrucción progresiva de los tejidos periodontales de suporte. Los *biofilms* dentales subgingivales se caracterizan por ser comunidades bacterianas, estructuradas en matrices de polisacáridos extracelulares organizados adheridas, en la cavidad oral, a las superficies, en un medio líquido. Los dientes, al no sufrir descamación, permiten el desarrollo de *biofilms* bacterianos complejos, que pueden incluir varios centenares de especies bacterianas diferentes.

Debido a los problemas asociados al uso de antimicrobianos, incluida la creciente preocupación con las resistencias bacterianas frente a antimicrobianos, existe en la actualidad un creciente interés en el estudio de los productos de origen natural para el tratamiento y prevención de diferentes enfermedades, incluidas las enfermedades periodontales. Entre estos productos potencialmente útiles como compuestos antibacterianos alternativos, se encuentran sustancias como los polifenoles y los ácidos grasos.

Hoy en día, la evidencia científica que estudia el efecto de dichas sustancias frente a patógenos orales es escasa y la mayoría de la información disponible estudia sus efectos frente a bacterias en estado planctónico o en *biofilms* mono especie. Así, este trabajo se propone evaluar dichas sustancias en un modelo *in vitro* de *biofilm* subgingival multiespecies, previamente validado

Objetivos: Evaluar la capacidad antimicrobiana de los diferentes productos y/o extractos de origen natural analizados en cada uno de los tres estudios que forman parte del presente trabajo, en un modelo *in vitro* de *biofilm* subgingival multiespecies.

Materiales y métodos: Se ha utilizado un modelo *in vitro* de *biofilm* subgingival, estático y multiespecies, previamente validado. En este modelo están incluidas bacterias representativas de los colonizadores primarios, intermedios y secundarios (*Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* y

Aggregatibacter actinomycetemcomitans). Tras la formación de los *biofilms*, estos eran expuestos a los diferentes extractos de origen natural testados.

La evaluación de la capacidad antimicrobiana se ha llevado a cabo midiendo el impacto de los productos testados sobre el número de bacterias medidas mediante reacción en cadena de la polimerasa cuantitativa (qPCR), complementado con la evaluación morfológica de los biofilms y su vitalidad mediante microscopía láser confocal (CLSM) y microscopía electrónica de barrido (SEM).

Resultados: Estudio #1: Se ha demostrado que el uso de vino tinto y extractos derivados del vino tuvo un impacto antimicrobiano, aunque moderado, en el total de recuentos bacterianos y en los recuentos de *F. nucleatum, P. gingivalis* y *A. actinomycetemcomitans*. **Estudio #2:** Los extractos del arándano rojo tienen un efecto antibacteriano moderado frente a patógenos periodontales en *biofilms*, aunque afectó la adhesión de bacterias en las primeras 6 horas de desarrollo del *biofilm*. **Estudio #3:** Los extractos de ácidos grasos, ácido eicosapentaenoico (EPA) y ácido docosahexaenoico (DHA) mostraron efectos antimicrobianos relevantes (de 2 o más ordenes de magnitud) frente a todas las cepas bacterianas testadas durante su crecimiento en *biofilms* maduros.

Conclusiones: Los diferentes productos y/o extractos de origen natural probados en los tres estudios incluidos en este trabajo han demostrado, de diferentes formas y con diferentes magnitudes, capacidad de afectar a *biofilms* subgingivales, en el modelo *in vitro* multiespecie utilizado.

Abstract

Background: Periodontitis are chronic multifactorial diseases, of inflammatory origin, associated with a dysbiotic dental *biofilm,* characterized by the progressive destruction of the supporting periodontal tissues. *Biofilms are* characterized by being bacterial communities structured in matrices of organized extracellular polysaccharides attached, in the oral cavity, to surfaces in a liquid medium. As teeth do not suffer shedding, allow the development *of complex biofilms* that can include several hundred different bacterial species.

Due to the problems associated with the use of antimicrobials and the growing concern with antimicrobial resistances, there is currently a growing interest in the study of naturally sourced products for the treatment and prevention of different diseases, including periodontal diseases. Among these potential useful antimicrobial compounds, substances like polyphenols and fatty acids can be listed.

Today, the scientific evidence studying the effect of these substances on oral pathogens is scarce and most of it is based on studies assessing bacteria in planktonic state or in mono species biofilms. Thus, the present work aims to test these substances in a validated *in vitro* multi-species subgingival biofilm model.

Objectives: To evaluate the antimicrobial capacity of the different products and/or extracts of natural origin tested in the three studies included in the present work, in the selected *in vitro* biofilm model.

Materials and methods: The *in vitro* static biofilm model included six different bacterial species, representing primary, intermediate and secondary colonizers (*Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* y *Aggregatibacter actinomycetemcomitans*). After the formation of the biofilms, they were exposed to the different extracts, of natural origin, tested.

The assessment of antimicrobial capacity has been carried out by measuring the impact of the products tested on the number of bacteria, evaluated by means of quantitative polymerase chain reaction (qPCR),

complemented by the morphological evaluation of biofilms and their vitality through confocal laser microscopy (CLSM) and scanning electron microscopy (SEM).

Results: Study #1: The use of red wine and wine-derived extracts have been shown to provide an antimicrobial impact, even though moderate, in total bacterial counts and in counts of *F. nucleatum, P. gingivalis* y *A. actinomycetemcomitans*. **Study #2:** Cranberry extracts had a moderate antibacterial effect against periodontal pathogens in biofilms, although it affected the adhesion of bacteria in the first 6 hours of biofilm development. **Study 3:** The fatty acids extracts, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), showed relevant antimicrobial effects (2 or more orders of magnitude) against all bacterial strains during growth in mature biofilms.

Conclusions: The different products and/or extracts of natural origin tested in the three studies included in the present work have shown, in different ways and with different magnitudes, the ability to affect biofilms in the *in vitro* multispecies subgingival biofilm model used.

II
Introducción

Introducción

Periodontitis y *Biofilms* Bacterianos

Las periodontitis son enfermedades inflamatorias crónicas de causa multifactorial asociada a un *biofilm* subgingival disbiótico, caracterizadas por la destrucción progresiva de los tejidos periodontales de suporte. Estas enfermedades se definen por la pérdida de la inserción de los tejidos periodontales a la raíz dentaria y se diagnostica por la presencia de pérdida de inserción clínica y pérdida ósea radiográfica, asociado a presencia de bolsas periodontales y el sangrado gingival (1).

La periodontitis es actualmente una de las patologías más comunes que afectan al ser humano (2, 3) y contribuye significativamente a la pérdida de dientes en sujetos adultos (4-6). En 2017, Frencken y colaboradores han publicado que la periodontitis severa es la sexta condición más prevalente en todo el mundo (7).

La encuesta *National Health and Nutrition Examination Survey* (NHANES) 2009-2012 reportó una prevalencia de periodontitis del 46%, en adultos mayores de 30 años en los Estados Unidos de América, lo que corresponde a 64,7 millones de pacientes afectados por esta patología. Además, 8,9% de los casos fueron considerados como periodontitis severa. En pacientes con más de 65 años, la prevalencia se eleva al 85% (8, 9). Igualmente, en Europa se estima que alrededor de mitad de la población adulta presenta periodontitis, siendo alrededor de un 10% los afectados por las formas severas de la enfermedad (10-12).

El aumento de la esperanza de vida en los países desarrollados, por ejemplo, 83 años en España (13), junto con la disminución en el número de pacientes desdentados y de dientes perdidos (13, 14), hace indicar que la prevalencia de la periodontitis seguirá aumentando en los próximos años (15). Esta alta prevalencia de las formas severas de periodontitis se considera como un importante problema de salud pública, no sólo por sus implicaciones en la salud bucal (pérdida dental y función de masticación, entre otras) sino también por sus efectos en la vida social y la calidad de vida de quienes la padecen (1,

16, 17).

Durante el *World Workshop* de 2017, fue adoptada una nueva clasificación de las periodontitis utilizando un sistema de “estadios” y “grados”, de un modo similar a otros sistemas de clasificación utilizados en medicina (por ejemplo, en procesos oncológicos). Mientras que el “estadio” depende principalmente de la severidad de la destrucción periodontal, así como de la complejidad en su tratamiento, el “grado” proporciona información sobre su evolución y riesgo de progresión futura, así como la presencia de factores de riesgo que condicionen dicha progresión (1).

El principal factor etiológico de las periodontitis es la presencia de comunidades bacterianas disbióticas estructuradas en matrices de polisacáridos extracelulares organizados en *biofilms* (*biofilm* subgingival) adheridos a la superficie radicular expuestas al medio externo (18, 19). Las características de las superficies en las que se forman estos *biofilms* y la respuesta tisular condicionada por la respuesta inflamatoria e inmune del hospedador, influyen significativamente en el tipo de comunidades bacterianas existentes, incluyendo las proporciones de bacterias adheridas a la superficie, su fenotipo bacteriano o la expresión de factores de virulencia y/o polisacáridos extracelulares (20-22).

El *biofilm* subgingival, por lo tanto, es un *biofilm* complejo y dinámico, que puede estar compuesto por más de 800 especies bacterianas distintas, de las que alrededor de la mitad se pueden identificar con las tecnologías microbiológicas actuales (23). La interacción entre las diferentes especies y cepas bacterianas que forman estos *biofilms* no ocurre aleatoriamente, sino que se desarrolla de un modo secuencial y reproducible (24), existiendo asociaciones específicas entre las especies bacterianas que se pueden reproducir de un modo experimental (25). Partiendo de una superficie dentaria completamente limpia, en una primera fase se forma una película de origen salival sobre la superficie del esmalte que contiene receptores (mucinas, aglutininas, proteínas ricas en prolina, proteínas ricas en fosfatos y enzimas) a los que se unen los microorganismos colonizadores primarios (26). Estos son principalmente bacterias de los géneros *Actinomyces, Neisseria, Prevotella,*

Streptococcus y *Veillonela* (24). A continuación, intervienen los colonizadores secundarios, entre los que destaca *Fusobacterium nucleatum*. Esta especie bacteriana Gram-negativa se encuentra en grandes cantidades, tanto en localizaciones donde existe enfermedad como en localizaciones sanas, y es capaz de co-agregar tanto con colonizadores primarios como tardíos, sirviendo como un ''*puente*'' entre ambos grupos de bacterias. Los colonizadores tardíos tienen un alto potencial de virulencia, estando en este grupo incluidas las especies bacterianas denominadas periodonto-patógenos, como *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* y *Tannerella forsythia* (26, 27).

Es importante tener en cuenta que las relaciones entre las bacterias incluidas en el *biofilm* subgingival pueden ser simbióticas, cuando existe una relación beneficiosa entre las bacterias que la componen, y entre éstas y el huésped, o puede ser disbiótica, que implica una alteración en las proporciones relativas de las comunidades bacterianas promoviendo una respuesta inflamatoria crónica, lo que resulta en el desarrollo de una patología (18). Específicamente, la disbiosis, a nivel subgingival, pero no en otras localizaciones del cuerpo, se caracteriza por un aumento de la diversidad de microorganismos; lo que puede ser el resultado de una respuesta inmune local débil, o un aumento en la disponibilidad de nutrientes y condiciones físico-químicas del medio ambiente local favorables para el sobre crecimiento de las bacterias más patogénicas (ambiente subgingival) (28, 29). De hecho, aunque la presencia de microorganismos periodonto-patógenos puede ser compatible con estados de salud periodontal (30), los cambios medio-ambientales favorables o una débil respuesta inmune condiciona cambios en sus proporciones relativas en relación a las bacterias asociadas a salud, lo que incide con la iniciación y progresión de un proceso inflamatorio crónico (29, 31). En particular, *Porphyromonas gingivalis* ha sido identificada como un ejemplo de patógeno clave (*key-stone pathogen*), con capacidad de aumentar la virulencia de toda la comunidad microbiana a través de interacciones interbacterianas específicas (una característica del llamado "*quorum sensing*" de los *biofilms* con la expresión de ciertas moléculas, actuando como factores de virulencia, tales como enzimas proteolíticas, u otras

moléculas pro-inflamatorias), que inducirán un estado de disbiosis, promoviendo una respuesta inflamatoria crónica del hospedador. También debe tenerse en cuenta que las diferencias intrínsecas en la respuesta individual influyen en la iniciación y progresión de esta enfermedad (18, 32).

Es importante tener en cuenta que la presencia en altas proporciones de estos patógenos periodontales en pacientes con periodontitis no ocurre únicamente a nivel de la cavidad bucal, sino que tienen capacidad de invadir los tejidos periodontales y pasar al torrente sanguíneo. Este hecho (bacteremias) se asocia principalmente con intervenciones periodontales, pero también tras actividades como la masticación (33), el acto de cepillarse los dientes (34), o usar seda dental (35). Estas especies bacterianas han sido detectadas no solo en el sistema circulatorio, sino colonizando sitios distantes, y se ha asociado a un mayor riesgo de co-morbilidad con múltiples enfermedades sistémicas (diabetes, enfermedades cardiovasculares, artritis reumatoide, etc.) (36).

A pesar de la asociación directa entre la formación y maduración de *biofilms* subgingivales y la iniciación y progresión de la periodontitis, la destrucción de los tejidos periodontales que define esta enfermedad ocurre como consecuencia de la respuesta inflamatoria e inmune desencadenada en el huésped frente a la agresión bacteriana. Por lo tanto, aunque las bacterias organizadas en *biofilms* son necesarias, el daño tisular y como consecuencia, las características clínicas y radiológicas que definen esta enfermedad, son consecuencia de la desregulación de la respuesta inmunitaria del huésped (37, 38). En la mayoría de los individuos, dicha respuesta, mediada fundamentalmente por la inmunidad innata, limitará el grado de destrucción de los tejidos y permitirá un retorno a la homeostasis (39). Sin embargo, si la agresión microbiana no es controlada adecuadamente por la respuesta inflamatoria del huésped se iniciará el proceso de destrucción del soporte periodontal (40).

A esta compleja interacción entre las bacterias y la respuesta del sistema inmunitario del huésped se añade la influencia de factores de riesgo genéticos y adquiridos, como la susceptibilidad del individuo, los mecanismos de defensa del huésped o la presencia de factores de riesgo ambientales, para el comienzo

y la progresión de la enfermedad, entendiéndose la periodontitis como una enfermedad multifactorial (41-44).

En relación con el tratamiento de la periodontitis, este se basa en la eliminación del agente causal (control de la infección), eliminando el *biofilm* bacteriano supra- y sub-gingival existente. Esto se puede lograr mediante distintas intervenciones mecánicas (instrumentación subgingival), que dependiendo de la severidad de la enfermedad, pueden ser no quirúrgicas (45) o quirúrgicas (46) y podrán necesitar terapias coadyuvantes antiinflamatorias o antimicrobianas. Por lo tanto, el propósito del tratamiento periodontal es reducir las bacterias patógenas presentes en el *biofilm* subgingival, para hacerlas compatibles con un estado de salud (homeostasis con la respuesta del hospedador) (45, 47, 48). Igualmente, la prevención de la recidiva de la periodontitis tras el tratamiento dependerá del control de los *biofilms* (49). Por esta razón, la eficacia a largo plazo de la terapia periodontal convencional depende del mantenimiento periodontal regular y de una adecuada motivación del paciente para mantener un alto grado de higiene bucal (50-52).

Aunque las mencionadas intervenciones mecánicas habitualmente disminuyen la inflamación y mejoran los parámetros clínicos (45), una proporción de pacientes no responde al tratamiento, siendo susceptible a la progresión de la enfermedad y posterior pérdida dentaria (53). Esto se debe a la dificultad del control de los patógenos periodontales mediante tratamiento mecánico en pacientes susceptibles (54-59), bien por no disminuir adecuadamente su número y proporciones (60), o por su rápida recolonización después de la terapia (61), sobre todo aquellos patógenos con capacidad de invasión de otros nichos orales, como la lengua, las amígdalas o las mucosas (62-64). Por estas razones, se desarrollaron estrategias antiinflamatorias y antimicrobianas complementarias, para así complementar la eficacia de la instrumentación mecánica subgingival (65).

Las estrategias coadyuvantes antiinflamatorias, entre las que se han investigado una amplia gama de agentes moduladores de la respuesta del huésped, incluidos los inhibidores selectivos de la cyclooxygenase-2 (COX-2) (66, 67), bifosfonatos (68-70), estatinas (71), tetraciclinas (72, 73) y macrólidos

(74, 75) no han dado resultados satisfactorios, aportando un valor añadido limitado.

Sin embargo, el uso coadyuvante de antibióticos locales y/o sistémicos o antimicrobianos no específicos, ha demostrado valor añadido significativo para combatir la infección y la recolonización por bacterias periodonto patógenas, aunque la magnitud del efecto es pequeño y de poca duración (76, 77). No obstante al efecto positivo significativo de su uso coadyuvante, sobre todo con antibióticos sistémicos, su uso indiscriminado se ha asociado a efectos secundarios relevantes (78, 79), y al desarrollo de aumento en las resistencias bacterianas (80), lo que representa una amenaza para la salud pública mundial y un problema económico importante (81). Específicamente son relevantes los efectos adversos de antibióticos del punto de vista gastrointestinal, renal o hepático (82), así como la irritación de las mucosas orales, pigmentación de las superficies dentales o la formación acelerada de cálculo asociado al uso de antisépticos (83). Asimismo, el descubrimiento de que la resolución de la fase de inflamación es un proceso activo desencadenado por mediadores lipídicos endógenos, ha puesto en duda el uso de agentes que actúan a través del bloqueo no específico de las vías inmunoinflamatorias del huésped (84).

Este análisis destaca la necesidad de desarrollar nuevos enfoques y estrategias para prevenir y tratar las enfermedades periodontales de una manera más eficiente y segura (85-88) y ampliar la necesidad por la búsqueda, mejora y desarrollo de compuestos antimicrobianos naturales capaces de inhibir la proliferación de patógenos bacterianos (83, 86-88). Por otro lado, también se están buscando nuevas estrategias antibacterianas, como, por ejemplo, el control de las enfermedades infecciosas utilizando principios activos que actúen sobre mecanismos de adhesión bacteriana (89, 90). Por ello, existe en la actualidad un creciente interés en el estudio de los productos de origen natural para el tratamiento y prevención de las enfermedades periodontales.

Entre dichos productos potencialmente útiles por su posible efecto antibacteriano y antiinflamatorio, se encuentran sustancias como los polifenoles y los ácidos grasos.

Polifenoles

Los polifenoles son productos naturales con una estructura compleja que se encuentran en más de 8000 compuestos naturales, tales como en frutas, verduras, nueces, semillas, tallos, chocolates, flores y bebidas como café, té y vino tinto (81, 91). Forman parte de un amplio grupo de compuestos químicos, llamados fitoquímicos, presentes en las plantas, que les aportan sus características al nivel del color, sabor, aroma y textura. Estos compuestos se han desarrollado a lo largo de miles de años de evolución, para defender los organismos de los efectos de los radicales libres, virus, bacterias y hongos. Los polifenoles y otros componentes de origen vegetal son capaces de afectar a la microbiota humana, ya sea promoviendo el crecimiento de microorganismos beneficiosos o actuando contra patógenos (119). De hecho, estos compuestos pueden dañar la membrana bacteriana, suprimir ciertos factores de virulencia (enzimas y toxinas) e inhibir la formación de *biofilms* bacterianos (81).

Su clasificación depende principalmente de su composición química (92), pudiendo ser divididos en cinco grupos principales: taninos hidrolizables, ácidos fenólicos, amidas polifenólicas, flavonoides y otros, con sus subgrupos correspondientes (93, 94). El grupo más grande de polifenoles son los flavonoides, que se dividen en siete subgrupos. Estos comparten una estructura similar, que consiste en dos anillos aromáticos (A y B) y tres átomos de carbono que unen los anillos A y B, formando un anillo C heterociclo oxigenado (95).

El uso de productos naturales, como los compuestos polifenólicos, para el tratamiento y la prevención de enfermedades, ha mostrado efectos positivos en los últimos años. Los estudios epidemiológicos, clínicos y experimentales en animales apoyan el papel de los polifenoles como posibles agentes en la prevención de diversas enfermedades crónicas, incluidas las enfermedades cardiovasculares, inflamatorias, metabólicas y neurodegenerativas, y ciertos tipos de cáncer (96-100). Además, estos compuestos han atraído interés debido a su uso potencial en la prevención y tratamiento de caries y enfermedades periodontales (101). De hecho, una revisión sistemática reciente reporta que los

flavonoides y, en particular, las pro-antocianidinas y flavan-3-oles, parecen ser los compuestos más prometedores en la prevención o tratamiento de enfermedades periodontales (102).

Muchos estudios han puesto de relieve las actividades inhibitorias contra una amplia gama de bacterias orales, principalmente vinculadas a caries dentales, por ciertos agentes polifenólicos derivados de arándanos, hojas de loto, algas o semillas de perilla (103-118).

Con respecto a los patógenos periodontales, dos estudios han encontrado actividad de la quecertina frente a *A. actinomycetemcomitans, P. gingivalis* y *F. nucleatum,* entre otras bacterias (103, 104). Por otro lado, algunos polifenoles de semillas de perilla y de extractos de té verde han mostrado una fuerte actividad antimicrobiana contra *P. gingivalis* y *Prevotella intermedia* (105, 106). Otra investigación ha reportado que la naringina, un componente del flavonoide comúnmente encontrado en el pomelo, tiene una actividad antimicrobiana frente a los Actinomycetales (107); la alcina, presente en el ajo, se ha mostrado también eficaz frente *A. actinomycetemcomitans, P. gingivalis* y *F. nucleatum* (108). Además, otra investigación ha estudiado el efecto inhibitorio de los polifenoles presentes en el arándano en formación de *biofilm* y su efecto sobre las proteasas de la cisteína de *P. gingivalis* (109).

Con respecto a los extractos enológicos, estudios previos describen cómo el desarrollo de varias cepas de *Streptococcus* spp. pueden ser inhibidos por el vino tinto y el extracto de uva. Este efecto también ha sido encontrado con otras bacterias relacionadas con la caries dental (110-113).

También se investigaron los efectos de los enjuagues orales con diferentes polifenoles derivados de bebidas (incluidos diferentes tipos de té y de vino) en bacterias adheridas a la cavidad oral y en la inhibición del crecimiento de patógenos orales (114). Otra investigación ha también demostrado que los extractos de pasas fueron capaces de inhibir el crecimiento de patógenos orales (115). Más recientemente, en otro estudio, también se ha observado que el vino tinto y sus componentes tienen una acción antimicrobiana contra la microbiota oral utilizando modelos de *biofilm* generados *in vitro,* incluyendo especies

bacterianas como *Actinomyces oris, F. nucleatum, Streptococcus oralis, Streptococcus mutans* y *Veillonella dispar* (116).

Sin embargo, son muy pocos los estudios que han descrito los efectos de los extractos naturales en modelos de *biofilm in vitro* multiespecie o frente a patógenos periodontales incluidos en *biofilms* orales (109, 112, 113, 116-119).

Hay evidencia de que los polifenoles del arándano (*Vaccinium macrocarpon*) inhiben selectivamente el crecimiento de patógenos intestinales, como las cepas de *Staphylococcus* spp. y *Salmonella enterica* (120); reducen la colonización de *Escherichia coli* en las vías urinarias (121, 122); disminuyen la virulencia de *Pseudomonas aeruginosa* (123, 124*);* y que tienen un potencial antioxidante (125), antiadhesión (89, 90), antimotilidad (126-129) y anti-cariogénico (130, 131).

Por estas razones, las propiedades antibacterianas y antifúngicas del arándano contra los microorganismos orales han ganado mucha atención. Varios investigadores han realizado estudios *in vitro* e *in vivo* evaluando si ciertos derivados de compuestos de arándano podrían interferir en la formación de un *biofilm* cariogénico*.* En este sentido, se está demostrando que ciertos componentes del arándano pueden restringir las caries dentales mediante la inhibición del desarrollo de ácidos orgánicos por bacterias cariogénicas; la formación de *biofilms* con *S. mutans* y *Streptococcus sobrinus;* y la adhesión y co-agregación de un número considerable de otras especies bacterianas orales del genero *Streptococcus* (132-137). Con respecto a las enfermedades periodontales, se ha encontrado que la fracción constituyente no dializable de arándano (NMD) inhibe la formación de *biofilms* por *P. gingivalis* (138) y *F. nucleatum* (109). Dicha fracción NMD también puede inhibir la adhesión *de P. gingivalis* a varias proteínas, incluyendo el colágeno tipo I (138), y reducir la co-agregación de bacterias periodonto patógenas (135).

Sin embargo, así como en los otros grupos anteriormente descriptos de polifenoles, la evidencia sobre las propiedades antibacterianas de los extractos de arándano frente a modelos de *biofilm in vitro* multiespecie es escasa (139, 140). De la misma forma, a pesar de los efectos anteriormente descriptos de

interferencia en la adhesión y co-agregación bacteriana, tampoco hay evidencia científica de dichos efectos estados en modelos de biofilm multiespecie.

Ácidos grasos

Los ácidos grasos poliinsaturados de cadena larga (PUFAs) (también conocidos como LCn3PUFAs) tienen 18-22 átomos de carbono a lo largo de la longitud de su cadena, con el primero de muchos enlaces dobles comenzando con el tercer átomo de carbono (cuando se cuenta desde el extremo metilo, es decir, el extremo de la molécula de ácidos grasos) (141). Dos de los principales miembros de este grupo son el ácido docosahexaenoico (22:6n-3, DHA) y el ácido eicosapentaenoico (20:5n-3, EPA).

Estas sustancias están presentes en altos niveles en muchos organismos marinos (147-149), siendo los pescados grasos una fuente importante de PUFAs. Por ejemplo, una ración de salmón estándar suministra 1,5 g de EPA, además de DHA, y una ración de caballa estándar suministra hasta 3 g de estos ácidos grasos (150). Son considerados componentes dietéticos esenciales, ya que su síntesis es limitada en los humanos y es necesaria una cierta proporción dietética (151-152). Esto es de especial interés, teniendo en cuenta que las dietas occidentales son altas en fuentes de n-6 PUFA linoleicos (como maíz y semillas de girasol), que se convierten en ácido araquidónico en el cuerpo (153, 154) y son relativamente bajas en PUFAs. De hecho, en una dieta occidental común, alrededor del 10-20% de los ácidos grasos es ácido araquidónico, 0.5-1% es EPA y 2-4% es DHA (155).

El efecto de la consumición de PUFAs en la dieta ha sido evaluado en una serie de enfermedades inflamatorias crónicas, como la aterosclerosis, la artritis, el asma, la psoriasis y la enfermedad inflamatoria intestinal, y se ha demostrado que la ingesta de PUFAs proporciona una variedad de beneficios para la salud, debido a sus diferentes mecanismos de acción descritos: antiarrítmicos, antitrombóticos, anti-ateroscleróticos, hipotensores o antiinflamatorios (142-145). De hecho, el estudio *Diet and Reinfarction Trial* (DART) investigó los efectos de las intervenciones dietéticas en la prevención

secundaria de infarto de miocardio (IM) y reportó una reducción del 29% en la mortalidad total en hombres que consumieron aceite de pescado dos veces por semana (un equivalente a 500-800 mg/día de PUFA) (160). Los resultados del ensayo DART fueron confirmados posteriormente por un gran estudio multicéntrico que abarcó a 11.323 pacientes con antecedentes de infarto de miocardio, en el que EPA y DHA (850-882 mg) produjeron una reducción clínica y estadísticamente significativa en los riesgos de muerte total y, específicamente, aquella asociada a la patología cardiovascular (146).

Otra parte considerable de la investigación sobre la ingesta de estos compuestos, también se ha centrado en sus efectos sobre la artritis reumatoide (AR), en la que la destrucción de los tejidos es impulsada por citoquinas proinflamatorias y eicosanoides derivados del ácido araquidónico (147). En este sentido, una reciente revisión sistemática con 23 ensayos controlados aleatorizados reportó un pequeño pero importante beneficio en los resultados para la AR, incluyendo inflamación y dolor en las articulaciones, rigidez matutina, así como una potenciación de los efectos de los antiinflamatorios no esteroideos (AINEs), tras la ingesta de suplementos de aceite de pescado (148).

Por otro lado, también se han llevado a cabo investigaciones centradas en los efectos al nivel neurológico, ya que EPA y DHA han demostrado influir positivamente en un gran número de aspectos de la función y la disfunción a este nivel, ya que pueden modular la excitabilidad neurológica a través de sus efectos en los canales de sodio y calcio (163, 164) o mediante la activación de canales TREK de potasio (165).

A pesar de estas evidencias, los mecanismos de acción para muchos de estos efectos estudiados siguen siendo desconocidos, aunque se han descrito gran número de mecanismos celulares en los que intervienen EPA y DHA.

Los PUFAs han atraído además una considerable atención como potenciales agentes antimicrobianos de amplia acción, ya que han demostrado efectos inhibitorios frente a varias bacterias Gram-positivas y Gram-negativas, sin ocasionar incremento en resistencias a antibióticos (149-155). A pesar de esto, la forma en que los PUFAs ejercen estas actividades antibacterianas sigue sin estar clara, pero el objetivo principal de su acción parece ser la membrana

plasmática y varios procesos esenciales que se producen en ella. Por ejemplo, gracias a su estructura anfíptica, algunos de estos efectos se pueden atribuir a la capacidad de los PUFAs para actuar como detergentes; esto les permite comunicarse con las membranas celulares para crear poros permanentes o transitorios de varios tamaños. Cuando se encuentran en altas concentraciones, los PUFAs dietéticos se integran en los fosfolípidos de la membrana celular, donde sirven como precursores de los mediadores de lípidos implicados en la señalización celular, la expresión genética y los procesos inflamatorios, y contribuyen a la integridad y fluidez de la membrana celular (155, 179), lo que puede alterar la producción de energía al interferir en la cadena de transporte de electrones y la descomposición de la fosforilación oxidativa (173-178). También se han descrito procesos de lisis celular, inhibición de la actividad enzimática, desajuste de ingesta de nutrientes, y la producción de peróxido tóxico y oxidación. Por otro lado, todavía no se han descubierto resistencias bacterianas a los PUFAs, y no ha sido posible inducir un fenotipo resistente en ellas (180, 183-185).

Específicamente, se ha descrito eficacia de EPA frente a dos cepas aisladas de *Staphylococcus aureus* (*methicillin-resistant Staphylococcus aureus*, MRSA) (156). En 2017, Clarke y colaboradores han observado en un modelo murrino que la inyección de ácidos grasos podría ser eficaz en el tratamiento de las infecciones sistémicas por MRSA (157). También se ha descrito recientemente que tanto EPA como DHA han mostrado actividad antibacteriana frente a varios patógenos orales, incluyendo *S. mutans*, *C. albicans* y *P. gingivalis* (a una concentración inhibitoria del 50% de 1 a 10 g ml^{-1}) (205) o frente a patógenos periodontales como *P. gingivalis*, *F. nucleatum* y *P. intermedia* (206).

Además del potencial antimicrobiano, su efecto beneficioso sobre la salud periodontal puede deberse a su actividad antiinflamatoria, ya que los PUFAs presentan un importante efecto en la producción de eicosanoides. Se ha demostrado que un aumento de la ingesta dietética de PUFAs como el EPA y el DHA da como resultado una menor proporción de ácido araquidónico (AA) y fosfolípidos celulares inflamatorios (150). Los PUFAs compiten con el AA a dos

niveles: reduciendo la proporción del sustrato disponible para los eicosanoides derivados de AA y compitiendo cómo sustrato para las vías de la cicloxigenasa (COX) y la lipoxigenasa (LOX) (158). Además, sus efectos sobre el estrés oxidativo, disminuyendo las especies reactivas de oxígeno (ROS) y antioxidantes, potencian el mencionado efecto antiinflamatorio (159). Los suplementos de aceite de pescado con niveles elevados (equivalentes a 5,4 g de EPA y 3,2 g de DHA) mostraron una disminución en la liberación y síntesis de ROS por neutrófilos humanos (160, 161). Además, también se han descrito sus efectos sobre las citoquinas proinflamatorias, inhibiendo la producción de IL-1β, TNF-α e IL-6 en una amplia variedad de células (148, 162, 163). Estos efectos antiinflamatorios gracias a la capacidad de los PUFAs para reducir los niveles de ácido araquidónico, $PGE_{2,}$ citoquinas proinflamatorias y eicosanoides (192, 193, 198) y de inhibición de los procesos de destrucción tisular, tal como los procesos de destrucción ósea, la supresión de la activación de NF-kB y secundariamente inhibir la función del osteoclasto, al reducir los niveles RANKL (200), podrían ser potencialmente relevantes en el uso terapéutica coadyuvante con PUFAs (164). Estudios experimentales en ratas infectadas con *P. gingivalis* y alimentadas con pescado o aceite de maíz, han demostrado una pérdida ósea significativamente menor al compararse con un grupo control con dieta a base de maíz (165, 166).

Sin embargo, el conocimiento sobre la actividad antimicrobiana de los PUFAs, cuando las bacterias están organizadas en *biofilms*, es muy escaso. Tampoco se conocen mecanismos celulares y moleculares subyacentes de dicha actividad (167-169). No obstante, la combinación de la actividad antiinflamatoria, antioxidante y antimicrobiana de los PUFAs (146, 187), podría tener un gran potencial como coadyuvante en la terapéutica periodontal.

III
Justificación

Justificación

Teniendo en cuenta la alta prevalencia de la periodontitis, así como sus implicaciones tanto en la salud oral como en la salud general, es de especial importancia su tratamiento y prevención. Para que esto sea posible es fundamental en control de los *biofilms* orales. Entre otras estrategias, los productos antisépticos se presentan como una posible herramienta tanto para la prevención primaria como secundaria de la periodontitis.

Debido a los efectos secundarios de los antisépticos orales más utilizados, y debido al desarrollo de mecanismos de resistencia frente a los antibióticos, hay un interés creciente por la búsqueda de nuevos agentes antimicrobianos, preferiblemente productos y/o extractos de origen natural, que carezcan de efectos secundarios. Justificamos este proyecto de tesis doctoral en base a la falta de estudios que utilicen modelos validados de *biofilm* subgingivales multiespecie *in vitro,* para evaluar la actividad microbiana de estos compuestos naturales.

IV
Hipótesis
y
Objetivos

Hipótesis y Objetivos

Hipótesis

Hipótesis general

Los diferentes productos y/o extractos de origen natural probados en los tres estudios incluidos en este trabajo podrán ser capaces de afectar la vitalidad y organización de *biofilm* subgingival multiespecie en un modelo *in vitro*, previamente validado.

Hipótesis específicas

Estudio #1:

El vino tinto alcoholizado y desalcoholizado y los extractos enológicos Provinols™ y Vitaflavan® reducen los recuentos bacterianos de *F. nucleatum*, *P. gingivalis* y *A. actinomycentemcomitans,* así como el recuento total de bacterias incluidas en un modelo validado de *biofilm* subgingival multiespecie *in vitro*.

Estudio #2:

Los extractos de arándano reducen los recuentos bacterianos de *F. nucleatum*, *P. gingivalis* y *A. actinomycentemcomitans,* así como el recuento total de bacterias incluidas en un modelo validado de *biofilm* subgingival multiespecie *in vitro,* y también reducen la incorporación de las mismas cepas bacterianas en biofilms en formación.

Estudio #3:

Los PUFAs, EPA y DHA, reducen los recuentos bacterianos de las especies bacterianas de *S. oralis*, *A. naeslundii*, *V. parvula*, *F. nucleatum*, *P. gingivalis* y *A. actinomycetemcomitans,* incluidas en un modelo validado de *biofilm* subgingival multiespecie *in vitro*.

Objetivos

Objetivo general

Evaluar la capacidad antimicrobiana de los diferentes productos y/o extractos de origen natural evaluados en los tres estudios de este proyecto, en un modelo validado de *biofilm* subgingival multiespecie *in vitro*.

Objetivos específicos

Estudio #1

Estudiar la capacidad antimicrobiana del vino tinto alcoholizado y desalcoholizado y los extractos enológicos Provinols™ y Vitaflavan® frente a *F. nucleatum, P. gingivalis* y *A. actinomycentemcomitans,* así como el recuento total de bacterias incluidas en un modelo validado de *biofilm* subgingival multiespecie *in vitro*.

Estudio #2:

Estudiar la capacidad antimicrobiana y antibiofilm de extractos de arándano rojo frente a *F. nucleatum, P. gingivalis* y *A. actinomycentemcomitans,* así como el recuento total de bacterias incluidas en un modelo validado de *biofilm* subgingival multiespecie *in vitro*.

Estudio #3:

Estudiar la capacidad antimicrobiana de EPA y DHA frente a las especies bacterianas *S. oralis, A. naeslundii, V. parvula, F. nucleatum, P. gingivalis* y *A. actinomycetemcomitans,* incluidas en un modelo validado de *biofilm* subgingival multiespecie *in vitro*.

V
Material, Métodos y Resultados

Material, Métodos y Resultados

La sección de Material y Métodos, así como los Resultados de cada uno de los tres estudios incluidos en esta tesis doctoral, han sido descritos y publicados como artículos científicos. Todos los detalles referentes a los mismos pueden ser encontrados en las siguientes referencias:

Estudio #1: M. C. Sánchez, H. Ribeiro-Vidal, A. Esteban-Fernández, B. Bartolomé, E. Figuero, M. V. Moreno-Arribas, M. Sanz, D. Herrera (2019). "Antimicrobial activity of red wine and oenological extracts against periodontal pathogens in a validated oral biofilm model." BMC Complementary and Alternative Medicine **19**(1): 145. doi: 10.1186/s12906-019-2533-5

Estudio #2: M. C. Sánchez, M. C., H. Ribeiro-Vidal, B. Bartolomé, E. Figuero, M. V. Moreno-Arribas, M. Sanz, D. Herrera (2020). "New Evidences of Antibacterial Effects of Cranberry Against Periodontal Pathogens." Foods **9**(2): 246. doi: 10.3390/foods9020246.

Estudio #3: H. Ribeiro-Vidal, M. C. Sánchez, A. Alonso-Español, E. Figuero, M. J. Ciudad, L. Collado, D. Herrera, M. Sanz (2020). "Antimicrobial Activity of EPA and DHA against Oral Pathogenic Bacteria Using an In Vitro Multi-Species Subgingival Biofilm Model." Nutrients **12**(9): E2812. doi: 10.3390/nu12092812.

Artículo #1

Artículo #1

Actividad antimicrobiana de vino tinto y extractos enológicos frente a patógenos periodontales en un modelo de *biofilm* oral validado

Antecedentes: Los hallazgos previos de la investigación apoyan un efecto antimicrobiano de los polifenoles frente a diferentes patógenos, pero no hay evidencia de este efecto contra patógenos periodontales en *biofilms* complejos. El objetivo de este estudio fue evaluar la actividad antimicrobiana del vino tinto y los extractos enológicos, ricos en polifenoles, frente a los patógenos periodontales *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* y *Fusobacterium nucleatum,* así como en el número total de bacterias incluidas en un modelo estático de *biofilm* oral *in vitro*.

Métodos: Se desarrolló un modelo de *biofilm,* previamente validado, incluyendo *Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *F. nucleatum*, *P. gingivalis* y *A. actinomycetemcomitans* en discos de hidroxiapatita estéril. Vino tinto (y vino desalcoholizado), y dos extractos ricos en polifenoles (de vino y semilla de uva) se aplicaron a *biofilms* de 72h sumergiendo los discos durante 1 y 5 min en las soluciones de vino y durante 30 s y 1 min en los extractos enológicos. Los *biofilms* resultantes fueron analizadas por microscopía láser confocal y las bacterias viables (unidades formadoras de colonias/ml) se midieron por reacción cuantitativa en cadena de la polimerasa combinada con propidio monoazida. Se construyó un modelo lineal generalizado para determinar el efecto de los productos probados en los recuentos bacterianos viables de *A. actinomycetemcomitans*, *P. gingivalis* y *F. nucleatum*, así como en el número total de bacterias viables.

Resultados: Los resultados mostraron que el vino tinto y el vino tinto desalcoholizado causaron una reducción de la viabilidad de las bacterias totales dentro del *biofilm*, con reducciones estadísticamente significativas en el número de *P. gingivalis* viables después de 1 min ($p = 0,008$) y en *A. actinomycetemcomitans* después de 5 minutos de exposición ($p = 0,011$) con vino tinto. No se observó evidencia de efecto antibacteriano relevante con los extractos enológicos, con reducciones estadísticamente significativas de *F.*

nucleatum después de 30 s de exposición a ambos extractos enológicos (p = 0,001).

Conclusiones: Aunque moderado, el impacto antimicrobiano observado en el recuento total de bacterias y recuentos de *A. actinomycetemcomitans*, *P. gingivalis* y *F. nucleatum*, fomentan la realización de nuevas investigaciones sobre el uso potencial de estos productos naturales en la prevención y tratamiento de las enfermedades periodontales.

Sánchez *et al. BMC Complementary and Alternative Medicine* (2019) 19:145
https://doi.org/10.1186/s12906-019-2533-5

BMC Complementary and Alternative Medicine

RESEARCH ARTICLE Open Access

Antimicrobial activity of red wine and oenological extracts against periodontal pathogens in a validated oral biofilm model

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Abstract

Background: Previous research findings support an antimicrobial effect of polyphenols against a variety of pathogens, but there is no evidence of this effect against periodontal pathogens in complex biofilms. The purpose of this study was to evaluate the antimicrobial activity of red wine and oenological extracts, rich in polyphenols, against the periodontal pathogens *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* and total bacteria growing in an in vitro oral biofilm static model.

Methods: A previously validated biofilm model, including *Streptococcus oralis, Actinomyces naeslundii*, *Veillonella parvula*, *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* was developed on sterile hydroxyapatite discs. Red wine (and dealcoholized wine), and two polyphenols-rich extracts (from wine and grape seeds) were applied to 72 h biofilms by dipping the discs during 1 and 5 min in the wine solutions and during 30 s and 1 min in the oenological extracts. Resulting biofilms were analyzed by confocal laser scanning microscopy and viable bacteria (colony forming units/mL) were measured by quantitative polymerase chain reaction combined with propidium monoazide. A generalized linear model was constructed to determine the effect of the tested products on the viable bacterial counts of *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*, as well on the total number of viable bacteria.

Results: The results showed that red wine and dealcoholized red wine caused reduction in viability of total bacteria within the biofilm, with statistically significant reductions in the number of viable *P. gingivalis* after 1 min ($p = 0.008$) and in *A. actinomycetemcomitans* after 5 min of exposure ($p = 0.011$) with red wine. No evidence of relevant antibacterial effect was observed with the oenological extracts, with statistically significant reductions of *F. nucleatum* after 30 s of exposure to both oenological extracts ($p = 0.001$).

Conclusions: Although moderate, the antimicrobial impact observed in the total bacterial counts and counts of *A. actinomycetemcomitans, P. gingivalis* and *F. nucleatum,* encourage further investigations on the potential use of these natural products in the prevention and treatment of periodontal diseases.

Keywords: Red wine, Oenological extracts, Polyphenols, Periodontal diseases, *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum*

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Background

Dental biofilms located at the interface between the teeth and the gingiva are mainly composed of microbial communities encompassing hundreds of different bacterial species. In gingival health, these biofilms are typically comprised of Gram-positive facultative aerobic bacteria, while in presence of gingival inflammation, such as in gingivitis and periodontitis, these biofilms increase in volume and complexity [1]. These changes result in an increment of Gram-negative bacteria and well-recognized pathogens such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* or *Treponema denticola*, as well as *Aggregatibacter actinomycetemcomitans* [2, 3]. In fact, the etiology of periodontal diseases is currently conceived as a dysbiosis between the bacteria present in dental biofilms and the host response against this bacterial challenge, which would be responsible of the clinical expression of either gingivitis or periodontitis [4].

Prevention and treatment of periodontal diseases mainly consist on strategies to eliminate or reduce these biofilms, either mechanically or chemically (antiseptic and/or systemic or locally applied antimicrobial agents) [5, 6]. However, the widespread use of antibiotics has several unwanted effects, such as the development of bacterial resistances, alterations of the gut microbiota or even direct renal and hepatic damage [7, 8]. Similarly, certain commonly used antiseptics can cause irritation of the oral mucosa, tooth staining or increased dental calculus formation [9]. All these facts indicate the need to develop novel antimicrobial strategies useful for the management of periodontal diseases.

In recent years, scientific evidence has emerged on the potential use of naturally derived phenolic compounds in the prevention/treatment of many chronic diseases, such as cardiovascular, metabolic, or neurodegenerative diseases and, to a lesser extent some cancers [10–13]. Most of these diseases have an inflammatory base and some may be triggered by bacteria. Consequently, there is potential for the use of natural polyphenols, that may exhibit both anti-bacterial and anti-inflammatory properties. It can be therefore hypothesized that phenolic compounds, such as polyphenols could be potentially effective in the prevention and treatment of oral diseases [14].

The anti-inflammatory properties of natural polyphenols have been extensively studied [10], even in relation to the periodontal diseases [14]. In regards to their possible anti-antibacterial effect, previous studies have described different ways of actions; either associated with the ability of polyphenols to generate hydroxyl radicals which would produce H_2O_2 and subsequent damage in the bacterial DNA and in its membrane integrity [15], or by altering the structure of the bacterial cell membrane leading to exit of intracellular components, or by changing the intracellular exchange of protons and potassium and phosphate ions [16–18].

Polyphenols are naturally occurring compounds largely found in fruits (i.e., grape, apple, pear or cherry), in cereals, dry fruits, chocolate, and also in beverages (i.e., wine, coffee, beer and tea) [19]. Red wine and grapes are rich sources of phenolic compounds [20]. Its antibacterial action has been evaluated with evidence of inhibitory action on the growth of different *Streptococcus* spp. strains and other bacteria associated with dental caries [21–25]. Also, the effect of polyphenol-rich foods (including several types of tea and wine), lead up as mouth rinses, has been investigated by assessing their inhibitory activity on oral pathogens and on the bacterial adherence to oral tissues [26–28]. However, there are few studies assessing the possible effect of phenolic natural extracts on multi-species biofilms, or specifically on the periodontal pathogens associated with the etiology of periodontal diseases [22, 23, 28–32].

Therefore, the present work aims to evaluate the antimicrobial potential of red wine and dealcoholized red wine, as well as of two oenological extracts (a red wine extract and a grape seed extract), on an in vitro multispecies biofilm model that emulates subgingival biofilms and includes periodontal pathogens such as *P. gingivalis, A. actinomycetemcomitans* and *F. nucleatum*.

Methods

Red wines

A young red wine was used in this investigation (var. Pinot Noir, vintage 2010), provided by Bodegas Miguel Torres S.A. (Vilafranca del Penedès, Barcelona, Spain). The phenolic content present in the wine include: total anthocyanins = 0.447 mg of malvidin-3-glucoside mL^{-1}, total catechins = 1.612 mg of (+)-catechin mL^{-1} and total polyphenols = 1.758 mg of gallic acid equiv. mL^{-1}. The principal individual phenolic compounds found in this wine were flavan-3-ols, flavonols, alcohols, anthocyanins, stilbenes and hydroxycinnamic acids, determined by Ultra-High-Performance Liquid Chromatography-ElectroSpray Ionization-tandem Mass spectrometry (UHPLC-ESI-MS/MS) for other studies [33].

A rotary evaporator was used for the preparation of dealcoholized red wine, removing the EtOH and adding distilled water to reconstitute it until the original volume.

Oenological extracts

Two commercially available oenological phenolic extracts were used: Provinols™, a red wine extract, kindly supplied by Safic-Alcan Especialidades S.A.U. (Barcelona, Spain) and a grape seed extract, Vitaflavan®, kindly provided by Piriou (Les Derives Resiniques & Terpeniques S.A., France). The total phenolic content of the extracts was 474 mg of gallic acid equiv. g^{-1} for Provinols™ and 629 mg

of gallic acid equiv. g^{-1} for Vitaflavan®. The phenolic compositions of both oenological extracts has been determined by UHPLC-ESI-MS/MS in previous studies [34, 35]. Both the wine extract and grape seed extracts were dissolved in distilled water containing 4% dimethyl sulfoxide (DMSO) (v/v), until reaching a final concentration of 20 mg mL^{-1}.

Bacterial strains and culture conditions

Six bacterial strains, including *Streptococcus oralis* CECT 907 T, *Veillonella parvula* NCTC 11810, *Actinomyces naeslundii* ATCC 19039, *F. nucleatum* DMSZ 20482, *A. actinomycetemcomitans* DSMZ 8324 and *P. gingivalis* ATCC 33277 were used. Bacteria were cultured in blood agar plates (Blood Agar Oxoid No 2; Oxoid, Basingstoke, UK), supplemented with 5% (v/v) sterile horse blood (Oxoid), 5.0 mg L^{-1} hemin (Sigma, St. Louis, MO, USA) and 1.0 mg L^{-1} menadione (Merck, Darmstadt, Germany) at 37 °C for 24–72 h in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2).

Biofilm development

A multi-species in vitro biofilm model was developed as previously described by Sánchez and colleagues [36]. For the inoculum preparation, the microorganisms were individually cultivated in anaerobic conditions on a protein rich medium containing brain-heart infusion (BHI) (Becton, Dickinson and Company, USA) supplemented with 2.5 g L^{-1} mucin (Oxoid, Thermo Scientific, Hampshire, UK), 1.0 g L^{-1} yeast extract (Oxoid, Thermo Scientific, Hampshire, UK), 0.1 g L^{-1} cysteine (Sigma-Aldrich, Barcelona, Spain), 2.0 g L^{-1} sodium bicarbonate (Merck, NJ, USA), 5.0 mg L^{-1} hemin (Sigma-Aldrich, Barcelona, Spain), 1.0 mg L^{-1} menadione (Merck, NJ, USA) and 0.25% (v/v) glutamic acid (Sigma-Aldrich, Barcelona, Spain). The bacterial cultures were harvested at mid-exponential phase (measured by spectrophotometry), and a mixed bacteria suspension in modified BHI medium containing 10^3 colony-forming units (CFU) mL^{-1} for *S. oralis*, 10^5 CFU mL^{-1} for *V. parvula* and *A. naeslundii*, and 10^6 CFU mL^{-1} for *F. nucleatum, A. actinomycetemcomitans* and *P. gingivalis* was prepared. The biofilms were grown on sterile calcium hydroxyapatite (HA) discs of 7 mm of diameter and 1.8 mm (standard deviation, SD = 0.2) of thickness (Clarkson Chromatography Products, Williamsport, PA, USA) discs deposited in 24-wells cell culture plates (Greiner Bio-one, Frickenhausen, Germany), inoculating each well with 1.5 mL of mixed bacteria, for 72 h at 37 °C in anaerobic condition. All assays were performed independently at least three times and in triplicate ($n = 9$).

Antimicrobial activity

The antimicrobial activity of wines and oenological extracts was examined on 72 h biofilms by determining the reduction in the number of viable CFU mL^{-1} using the quantitative polymerase chain reaction (qPCR). For the oenological extracts, 30 and 60 s were selected as exposure times since they are bioactive products, commercially available, and for them, the standard exposure times established for other antimicrobial commercially available products (e.g. products with chlorhexidine), was selected [37–39]. On the other hand, in the case of wine solutions, the product was considered as a new possible bioactive agent, evaluated for the first time, therefore, not only the standard 60 s interval was selected as exposure time, but also an "extreme" exposure time of 5 min, with the aim of detecting any possible effect of red wine solutions (dealcoholized or not). Two different protocols were performed:

- For red wine (dealcoholized or not), biofilms were dipped during 1 and 5 min in the wine solutions at room temperature. Phosphate buffer saline (PBS) was used as negative control and, in order to discard a bactericidal effect of the EtOH contained in the wine, also 12% ethanol was applied.
- For the oenological extracts, biofilms were dipped during 30 s and 1 min at room temperature, due to their high phenolic content. PBS was used as negative control, and in order to discard a bactericidal effect of the DMSO used for dissolve the extracts, 4% DMSO solution was also tested.

Microbiological outcomes

After the antimicrobial treatment, biofilms were sequentially rinsed in 2 mL of sterile PBS three times (immersion time per rinse, 10 s), in order to remove possible remains of the oenological solutions or extracts and unbound bacteria. Then, biofilms were disrupted by vortex for 2 min in 1 mL of PBS. To discriminate between DNA from live and dead bacteria, propidium monoazide (PMA) (Biotium Inc., Hayword, CA, USA) was used. The use of this PMA dye combined with qPCR has shown the ability to detect the DNA from viable bacteria [40]. For this, 100 μM of PMA was added to 250 μL of disaggregated biofilm. Following an incubation period of 10 min at 4 °C in the dark, the samples were subjected to light-exposure for 30 min, using PMA-Lite LED Photolysis Device (Biotium Inc.), and then centrifuged at 12,000 rpm for 3 min prior to DNA extraction.

Bacterial DNA was isolated from all biofilms using a commercial kit ATP Genomic DNA Mini Kit® (ATP biotech. Taipei, Taiwan), following manufacturer's instructions and the hydrolysis 5'nuclease probe assay qPCR method was used for detecting and quantifying the bacterial DNA. The qPCR amplification was performed following a protocol previously optimized by our research

group, using primers and probes targeted against 16S *rRNA* gene [obtained through Life Technologies Invitrogen (Carlsbad, CA, USA)] [41].

Each DNA sample was analysed in duplicate. Quantification cycle (Cq) values, describing the PCR cycle number at which fluorescence rises above the baseline, were determined using the provided software package (LC 480 Software 1.5; Roche Diagnostic GmbH; Mannheim, Germany) . Quantification of viable cells by qPCR was based on standard curves. The correlation between Cq values and CFU mL^{-1} was automatically generated through informatics analysis (LC 480 Software 1.5; Roche).

All assays were developed with a linear quantitative detection range established by the slope range of 3.3–3.5 cycles/log decade, $r^2 > 0.998$ and an efficiency range of 1.9–2.0.

Confocal laser scanning microscopy (CLSM)

Non-invasive confocal imaging of fully hydrated biofilms was carried out using a fixed-stage Ix83 Olympus inverted microscope coupled to an Olympus FV1200 confocal system (Olympus; Shinjuku, Tokyo, Japan). LIVE/DEAD® BacLight™ Bacterial Viability Kit solution (Molecular Probes B. V., Leiden, The Netherlands) was used to stained the biofilms at room temperature. The fluorochromes were incubated (ratio 1:1) during 9 ± 1 min to obtain the optimum fluorescence signal at the corresponding wave lengths (Syto9: 515–530 nm; Propidium Iodide (PI): > 600 nm. The CLSM software was set to take a z-series of scans (xyz) of 1 μm thickness (8 bits, 1024 × 1024 pixels). Image stacks were analyzed by using the Olympus® software (Olympus). Image analysis and live/dead cell ratio (i.e. the area occupied by living cells divided by the area occupied by dead cells) was performed with Fiji software (ImageJ Version 2.0.0-rc-65 / 1.52b, Open source image processing software).

Statistical analyses

The selected outcome variables to study the antibacterial effect of wine solutions and oenological extracts were the counts of viable bacteria present on the biofilms, expressed as viable CFU mL^{-1} of *A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum* and total bacteria by qPCR, and the live/dead cell ratio of the whole biofilm by CLSM. An experiment-level analysis was performed for each parameter of the study ($n = 9$ for qPCR and $n = 3$ for CLSM results). Shapiro–Wilk goodness-of-fit tests and distribution of data were used to assess normality. Data were expressed as means ± SD.

In the case of the experiments with red wine, the effect of each solution [red wine (dealcoholized or not), PBS and 12% EtOH], the time of exposure (1 or 5 min) and their interaction with the main outcome variable (counts expressed as CFU mL^{-1} or live/dead cell ratio), was compared by means of a parametric ANOVA test for independent samples, and a general linear model was constructed for each bacterium (*A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*) and for total bacteria for qPCR results and for total bacteria for live/dead cell ratio of whole biofilm obtained by CLSM, using the method of maximum likelihood and Bonferroni corrections for multiple comparisons. A similar model was constructed in the case of the experiments with oenological extracts, in order to compare the effect of each solution (wine extract, grape seed extract, PBS and DMSO), the time of exposure (30 s or 1 min) and their interaction with the main outcome variable (CFU mL^{-1} and live/dead cell ratio of whole biofilms).

Results were considered statistically significant at $p < 0.05$. A software package (IBM SPSS Statistics 24.0; IBM Corporation, Armonk, NY, USA) was used for all data analysis.

Results

Antimicrobial effect of red wine

Table 1 depicts the effects of red wine solutions, dealcoholized or not, compared to PBS and 12% EtOH, on the counts of viable cells of *A. actinomycetemcomitans, P. gingivalis, F. nucleatum* and total bacteria.

After 1 min of exposure to red wine or dealcoholized red wine, no statistically significant effect was measured on the viable counts of *A. actinomycetemcomitans* (CFU mL^{-1}) ($p > 0.05$) when compared to control biofilms (exposed to PBS). Conversely, after 5 min a significant reduction of viable *A. actinomycetemcomitans* (CFU mL^{-1}) occurred with wine ($p = 0.053$) and dealcoholized red wine ($p = 0.011$) when compared to control biofilms. No statistically significant differences were observed between the two wine solutions at any time ($p > 0.05$). The effect of exposure time (between 1 and 5 min) was however, statistically significant for both red wine ($p = 0.030$), and dealcoholized red wine ($p = 0.006$).

After 1 min exposure to red wine solutions, there were statistically significant reductions in the viable counts of *P. gingivalis* (CFU mL^{-1}) ($p = 0.008$). Measurable reductions also occurred after 5 min of exposure with both red wine and dealcoholized red wine, although no significance differences were observed when compared to biofilms exposed to PBS ($p > 0.05$ in all cases). No statistically significant differences were observed in the effectiveness comparing the two wine solutions at applied times or when comparing exposure times ($p > 0.05$ for all cases).

For *F. nucleatum*, reductions in viable counts were not statistically significant after both 1 and 5 min of exposure (Table 1). No statistically significant differences were observed between the two wine solutions at any time ($p > 0.05$). The effect of exposure time (between 1 and 5

Table 1 Effect of red wine and dealcoholized red wine on the number of viable bacteria in the in vitro multi-species biofilm [colony forming units, CFU mL^{-1}, obtained by quantitative real-time polymerase chain reaction (qPCR)]. Data are expressed as mean ± standard deviation (SD). PBS: phosphate buffer saline, EtOH: ethanol

	Exposure time (min)	Viable CFU mL^{-1} [mean (SD)] in the biofilm			
		Treatment with PBS	Treatment with the corresponding antimicrobial agent		
			Red wine	Dealcoholized red wine	12% EtOH
A. actinomycetemcomitans	1	$1.9x10^6$ ($7.6x10^5$)	$1.8x10^6$ ($6.5x10^5$)†	$1.9x10^6$ ($1.2x10^6$)†	$8.9x10^5$ ($5.6x10^5$)
	5	$2.0x10^6$ ($2.0x10^6$)	$7.7x10^5$ ($5.1x10^5$)*†	$5.1x10^5$ ($4.5x10^5$)*†	$1.1x10^6$ ($8.6x10^5$)
P. gingivalis	1	$1.5x10^5$ ($1.3x10^5$)	$3.4x10^4$ ($3.5x10^4$)*	$5.9x10^4$ ($5.1x10^4$)	$1.2x10^5$ ($1.4x10^5$)
	5	$4.2x10^4$ ($3.3x10^4$)	$5.2x10^3$ ($3.2x10^3$)	$5.3x10^3$ ($5.6x10^3$)	$3.9x10^4$ ($1.4x10^4$)
F. nucleatum	1	$1.4x10^5$ ($7.1x10^4$)	$1.1x10^5$ ($9.1x10^4$)†	$1.2x10^5$ ($1.0x10^5$)†	$8.1x10^4$ ($8.5x10^4$)
	5	$9.2x10^4$ ($9.5x10^4$)	$3.4x10^4$ ($3.1x10^4$)†	$1.9x10^4$ ($1.6x10^4$)†	$6.5x10^4$ ($5.6x10^4$)
Total bacteria	1	$8.2x10^6$ ($4.2x10^6$)	$3.7x10^6$ ($2.7x10^6$)	$3.3x10^6$ ($2.7x10^6$)	$5.9x10^6$ ($7.9x10^6$)
	5	$7.2x10^6$ ($3.1x10^6$)	$3.9x10^6$ ($7.2x10^6$)	$4.0x10^6$ ($3.1x10^6$)	$3.0x10^6$ ($1.9x10^6$)

*$p < 0.05$, significant differences when compared viable CFU mL^{-1} to control biofilms (exposed to PBS)
†$p < 0.05$, significant differences when comparing exposure times for an antimicrobial agent

min) was however, statistically significant for both red wine ($p = 0.035$), and dealcoholized red wine ($p = 0.004$).

In regards to biofilm total bacteria, reductions in viable counts were measured (Table 1) after 1 and 5 min of exposure with both solutions, red wine (45.1 and 54.2%, respectively, of viable bacteria after the exposure when compared to control biofilms) and dealcoholized red wine (40.2 and 55.5%, respectively), but differences were not statistically significant (Table 1). No statistically significant differences were observed in the effectiveness when comparing red wine and dealcoholized red wine at 1 or 5 min or when comparing the exposure times ($p > 0.05$ for all cases).

Due to the possible antibacterial activity of EtOH present in the red wine, its effect over the three pathogens and total bacteria was evaluated. Although the treatment with 12% EtOH, emulating the alcoholic content of the wines, resulted in a decrease in total counts (Table 1), no statistically significant differences were observed when compared with PBS ($p > 0.05$ in all cases). No exposure time effect was observed for red wine or dealcoholized red wine, except for *P. gingivalis*, for which the effect of time of exposure (1 min versus 5 min) was statistically significant ($p = 0.027$).

After 72 h of incubation, CLSM observation revealed the control HA discs were covered by a mature biofilm, with multicellular aggregates well spread through the surface, showing a structural organization based bacterial communities forming microcolonies, with a live/dead cell ratio of 2.04 ± 0.43 when dipped in PBS for 1 min and 1.10 ± 0.42 for 5 min (Fig. 1 a, b). When biofilms were dipped in red wine for 1 min, a significant decrease in cell viability of the whole biofilm could be observed ($p < 0.001$; Fig. 1 e; Table 2), demonstrating a 0.74 ± 0.05 of live/dead cell ratio, which continued to decrease to 0.53 ± 0.12 after 5 min (Fig. 1 f; Table 2). A significant effect was also observed when exposed to dealcoholized red wine for 1 min (0.84 ± 0.23 of live/dead cell ratio; $p < 0.001$; Table 2) and 5 min (0.52 ± 0.03; $p > 0.05$) (Fig. 1 g, h; Table 2). Visual changes were also appreciated when applying the 12% EtOH solution for 1 and 5 min (live/dead cell ratio of 1.31 ± 0.26 and 0.93 ± 0.12, respectively; $p = 0.018$ after 1 min of exposure) (Fig. 1 c, d; Table 2). No statistically significant differences were observed when comparing red wine and dealcoholized red wine for 1 or 5 min or when comparing exposure times ($p > 0.05$ for all cases).

Antimicrobial effects of oenological extracts

Table 3 depicts the effects of the two polyphenol-rich extracts, compared to the negative control (PBS) and 4% DMSO, on the number of viable cells of *A. actinomycetemcomitans, P. gingivalis, F. nucleatum* and total bacteria.

After 30 s and 1 min exposure to the wine and grape seed extracts, there was a reduction in the viable counts of *A. actinomycetemcomitans,* although statistically significant differences were not detected (Table 3). Comparisons between both extract solutions or between the times of exposure for each extract were not statistically significant ($p > 0.05$ in all cases).

Similarly, no significant effect on viable counts of *P. gingivalis* was observed after exposure to the wine and grape seed extracts during 30 s (Table 3). The number of viable *P. gingivalis* showed reductions when biofilms were treated for 1 min with the wine extract, but not with the grape seed extract ($p > 0.05$ in both cases). No statistically significant differences were observed between the effect reached by the two oenological extracts at any time ($p > 0.05$ in both cases). The effect of time of exposure (30 s *versus* 1 min) was statistically significant for the wine extract

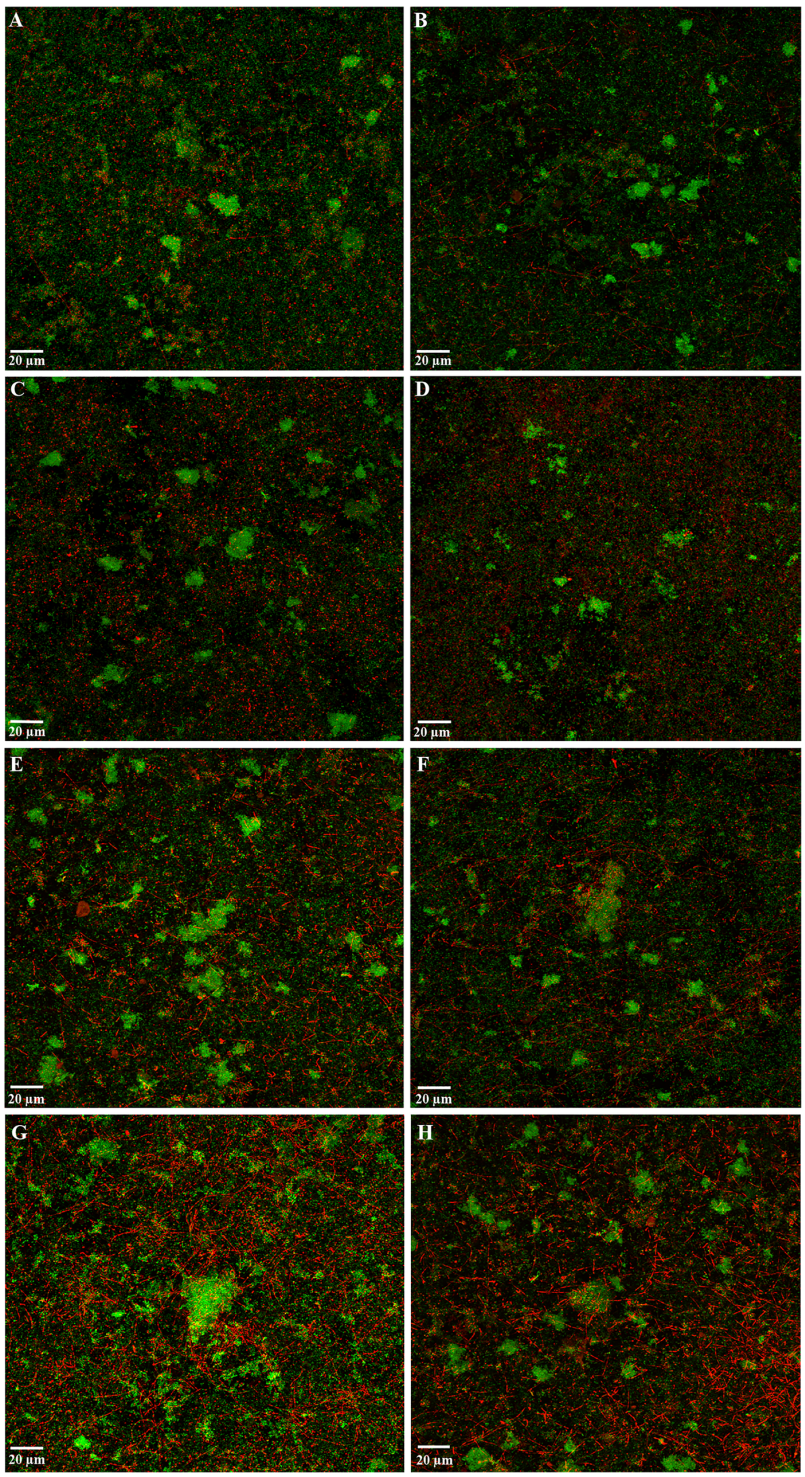

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 Maximum projection of Confocal Laser Scanning Microscopy (CLSM) images of 72 h biofilms, growth over hydroxyapatite surfaces, stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit, after exposure to: (**a, b**) negative control 1 and 5 min, respectively (phosphate buffer saline, PBS); (**c, d**) 12% ethanol solution 1 and 5 min, respectively; (**e, f**) red wine 1 and 5 min, respectively, and (**g, h**) dealcoholized red wine 1 and 5 min, respectively. Scale bar = 20 μm

($p = 0.014$), but not for the grape seed extract ($p = 0.395$).

For *F. nucleatum*, 30 s of exposure to both oenological extracts significantly reduced viable counts ($p = 0.001$, in both cases) (Table 3). However, after 1 min of exposure to both, although the reduction was maintained, no statistically differences were reached; although the oenological extract showed more effect on *F. nucleatum*. Similarly, no significant effect was observed when comparing the effect of both extracts at any time or the time of exposure for each one ($p > 0.05$ in all cases).

Regarding the total counts of bacteria included in the biofilm, 30 s and 1 min of contact with both, the wine and grape seed extracts, caused a slight reduction in the number of viable counts, but differences were not statistically significant. Similarly, no significant differences were observed between the two extracts at any time. The effect of time of exposure (30 s versus 1 min) was statistically significant for the wine extract ($p = 0.005$), but not for the grape seed extract ($p = 0.057$).

Due to the possible antibacterial activity of DMSO, its effect on the tested bacterial species and total bacteria

Table 2 Effect of red wine and dealcoholized red wine on the live/dead cell ratio (i.e. the area occupied by living cells divided by the area occupied by dead cells) of the whole biofilm obtained by Confocal Laser Scanning Microscopy (CLSM). PBS: phosphate buffer saline, EtOH: ethanol

Exposure time	Chemical treatment		Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
1 min	PBS	12% EtOH	0.727	0.208	0.018[a]	0.101	1.352
		Red wine	1.300	0.208	0.000[a]	0.674	1.926
		Dealcoholized wine	1.197	0.208	0.000[a]	0.571	1.822
	12% EtOH	PBS	-0.727	0.208	0.018[a]	-1.352	-0.101
		Red wine	0.573	0.208	0.084	-0.052	1.199
		Dealcoholized wine	0.470	0.208	0.229	-0.156	1.096
	Red wine	PBS	-1.300	0.208	0.000[a]	-1.926	-0.674
		12% EtOH	-0.573	0.208	0.084	-1.199	0.052
		Dealcoholized wine	-0.103	0.208	1.000	-0.729	0.522
	Dealcoholized wine	PBS	-1.197	0.208	0.000[a]	-1.822	-0.571
		12% EtOH	-0.470	0.208	0.229	-1.096	0.156
		Red wine	0.103	0.208	1.000	-0.522	0.729
5 min	PBS	12% EtOH	0.177	0.208	1.000	-0.449	0.802
		Red wine	0.577	0.208	0.082	-0.049	1.202
		Dealcoholized wine	0.593	0.208	0.069	-0.032	1.219
	12% EtOH	PBS	-0.177	0.208	1.000	-0.802	0.449
		Red wine	0.400	0.208	0.435	-0.226	1.026
		Dealcoholized wine	0.417	0.208	0.374	-0.209	1.042
	Red wine	PBS	-0.577	0.208	0.082	-1.202	0.049
		12% EtOH	-0.400	0.208	0.435	-1.026	0.226
		dealcoholized wine	0.017	0.208	1.000	-0.609	0.642
	Dealcoholized wine	PBS	-0.593	0.208	0.069	-1.219	0.032
		12% EtOH	-0.417	0.208	0.374	-1.042	0.209
		Red wine	-0.017	0.208	1.000	-0.642	0.609

Based on estimated marginal means
[a]The mean difference is significant at the 0.05 level
[b]Adjustment for multiple comparisons: Bonferroni

Table 3 Effect of the red wine phenolic extract (Provinols™), rich in anthocyanins, and the oenological extract from grape seeds (Vitaflavan®) on the number of viable bacteria in the *in vitro* multi-species biofilm [colony forming units, CFU mL^{-1}, obtained by quantitative real-time polymerase chain reaction (qPCR)]. Data are expressed as means ± standard deviation (SD). PBS: phosphate buffer saline; DMSO: dimethyl sulfoxide

	Exposure time	Viable CFU mL^{-1} [mean (SD)] in the biofilm			
		Treatment with PBS	Treatment with the corresponding antimicrobial agent		
			Wine extract	Grape seeds extract	4% DMSO
A. actinomycetemcomitans	30 s	$7.2x10^6$ ($6.4x10^6$)	$5.8x10^6$ ($3.8x10^6$)	$5.2x10^6$ ($5.2x10^6$)	$5.6x10^6$ ($3.0x10^6$)
	1 min	$5.2x10^6$ ($3.7x10^6$)	$5.0x10^6$ ($5.8x10^6$)	$2.4x10^6$ ($1.2x10^6$)	$5.2x10^6$ ($4.9x10^6$)
P. gingivalis	30 s	$1.7x10^6$ ($7.0x10^5$)	$1.8x10^6$ ($1.5x10^6$)†	$1.3x10^6$ ($1.5x10^6$)	$1.6x10^6$ ($1.8x10^6$)
	1 min	$8.9x10^5$ ($6.8x10^5$)	$5.0x10^5$ ($2.6x10^5$)†	$8.5x10^5$ ($4.7x10^5$)	$1.0x10^6$ ($7.0x10^5$)
F. nucleatum	30 s	$3.8x10^5$ ($3.1x10^5$)	$1.0x10^5$ ($4.6x10^4$)*	$1.1x10^5$ ($9.2x10^4$)*	$3.5x10^5$ ($1.3x10^5$)†
	1 min	$1.5x10^5$ ($1.0x10^5$)	$3.3x10^4$ ($2.7x10^4$)	$5.4x10^4$ ($4.2x10^4$)	$1.8x10^5$ ($1.5x10^5$)†
Total bacteria	30 s	$3.6x10^7$ ($2.3x10^7$)	$2.5x10^7$ ($1.5x10^7$)†	$1.9x10^7$ ($1.7x10^7$)	$1.9x10^7$ ($1.9x10^7$)
	1 min	$1.3x10^7$ ($1.1x10^7$)	$5.5x10^6$ ($4.7x10^6$)†	$5.6x10^6$ ($3.4x10^6$)	$1.1x10^7$ ($6.2x10^6$)

*$p < 0.05$, significant differences when compared viable CFU mL^{-1} to control biofilms (exposed to PBS)
†$p < 0.05$, significant differences when comparing exposure times for an antimicrobial agent

was evaluated. It was observed that the treatment with 4% DMSO (v/v), concentration used to solubilize the extracts, had no effect on the bacterial cell viability (Table 3). There were no statistically significant differences when compared with PBS ($p > 0.05$ in all cases), or between exposure time ($p > 0.05$ in all cases) except for *F. nucleatum*, for which the effect of exposure time (30 s versus 1 min) was statistically significant ($p = 0.012$).

The CLSM analysis showed that, after 72 h of incubation on HA surfaces, the biofilm covered the disc surface as multicellular aggregates, exhibited a live/dead cell ratio of 1.13 ± 0.50 when dipped for 30 s and 1.10 ± 0.16 for 1 min in PBS (Control biofilms; Fig. 2 a, b). It could be observed that after 30 s exposures to both oenological extracts, cell vitality slightly decreased in the biofilms (live/dead cell ratio of 0.77 ± 0.24 for wine extract and 1.20 ± 0.20 for the grape seed extract; $p > 0.05$ in both cases) (Fig. 2 e, g; Table 4). In the same way, after 1 min exposure to the wine extract (Fig. 2 f) and the grape seed extract (Fig. 2 h), no reduction in viability was measured by CLSM (viability ratio 1.21 ± 0.30 and 1.30 ± 0.47, respectively; $p > 0.05$; Table 4). No visual changes were observed when applying 4% DMSO solution for 30 s and 1 min (viability ratio of 0.87 ± 0.30 and 1.07 ± 0.09, respectively) (Fig. 2 c, d; Table 4). No statistically significant differences were observed when comparing wine and grape seed extracts at 30 s or 1 min or when comparing the exposure times ($p > 0.05$ for all cases).

Discussion

In the present study, the effect of red wine and oenological extracts in a validated oral biofilm model has been studied, demonstrating that wine solutions (dealcoholized or not) had a greater antimicrobial effects against *A. actinomycetemcomitans* and *P. gingivalis* when compared to the polyphenol rich oenological extracts. When comparing the oenological extracts, wine extract was more active against *P. gingivalis* and *F. nucleatum,* and the grape seed extract against *F. nucleatum.* In regards to the effects on total biofilm bacteria, wine solutions (dealcoholized or not) showed significant reductions in the live/dead cell ratios, in contrast, the oenological extracts did not evidence a relevant antibacterial effect.

Previous in vitro studies evaluating the antimicrobial effect of phenolic compounds from wines and oenological extracts have demonstrated significant effects against selected Gram-positive and Gram-negative pathogenic bacteria [42], enteric pathogens [43], pathogenic bacteria associated with respiratory diseases [44], or gut commensal, probiotic and pathogenic bacteria [45]. In the oral cavity, Toukairin and colleagues [46] reported that polyphenols, extracted from seeds and skin of wine grapes, had antibacterial effects against certain cariogenic bacteria, mainly through inhibition of the adherence of *S. mutans* and other streptococci. Similarly, Cueva and colleagues [44] incubated planktonic pure cultures of *S. mutans* and *S sobrinus* with flavan-3-ols precursors, (+)-catechin and (−)-epicatechin (compounds present in the grape seed extract employed) and reported significant inhibition of bacterial growth. Daglia and colleagues studied the antiseptic effect of dealcoholized red wine in comparison with white wines, demonstrating a stronger action of red wines against oral streptococci, what reinforces the possible role of anthocyanins as bacteriostatic agents [25]. Recently, Esteban-Fernández and colleagues [21] showed antimicrobial activity against *P. gingivalis, F. nucleatum* and *S. mutans* growing planktonically when exposed to two wine phenolic compounds (caffeic and

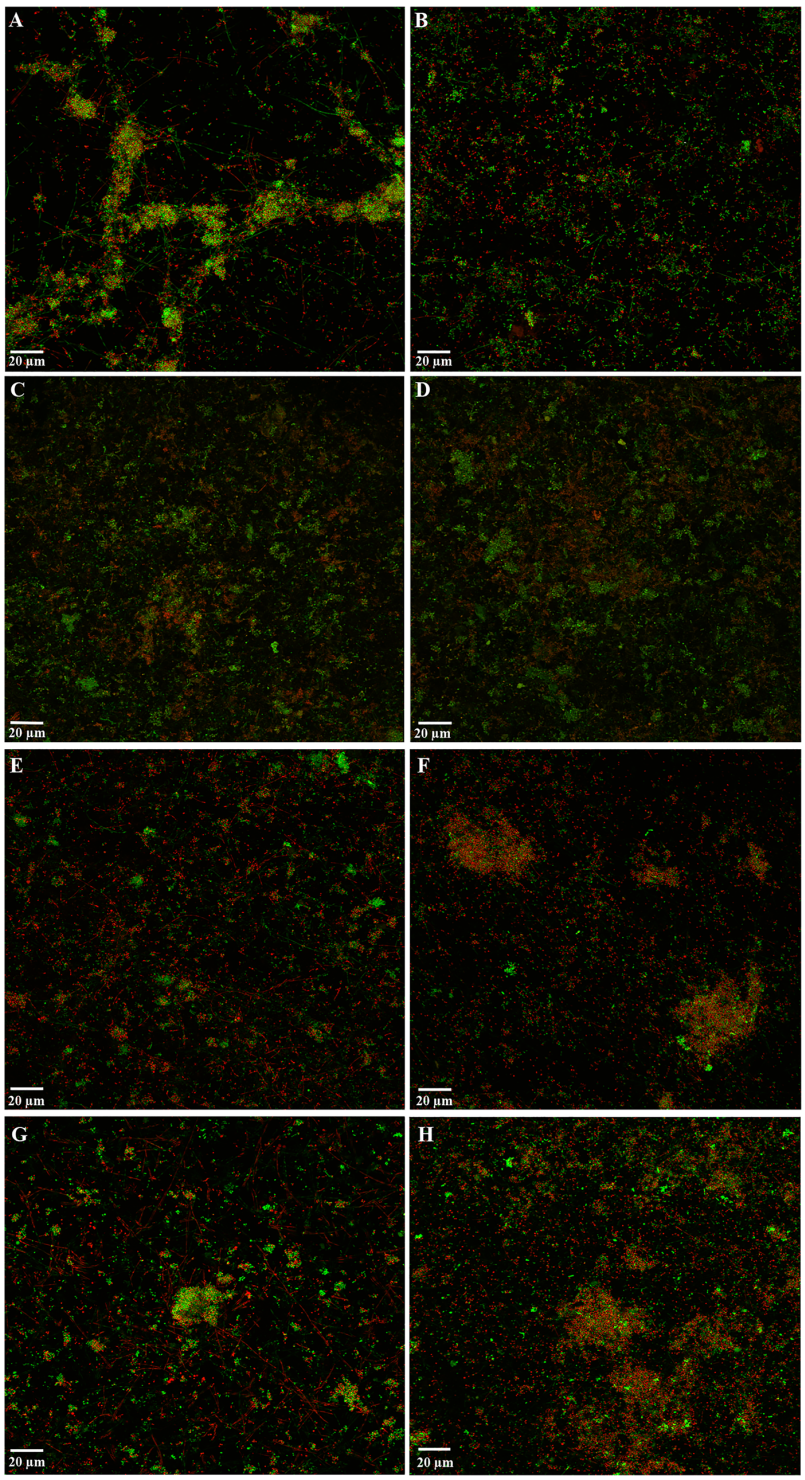

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Maximum projection of Confocal Laser Scanning Microscopy (CLSM) images of 72 h biofilms, growth over hydroxyapatite surfaces, stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit, after exposure to: (**a, b**) negative control 30 s and 1 min, respectively (phosphate buffer saline, PBS); (**c, d**) 4% dimethyl sulfoxide (DMSO) solution 30 s and 1 min, respectively; (**e, f**) wine extract 30 s and 1 min, respectively (Provinols™, 20 g L^{-1}); and (**g, h**) grape seed extract (Vitaflavan®, 20 g L^{-1}). Scale bar = 20 μm

p-coumaric acids) and the same red wine and grape seed extracts (Provinols™ and Vitaflavan®, respectively) used in the present study.

These studies, however, have focused the study of their antimicrobial effect on species commonly detected in supragingival plaque, such as *S. mutans, S. sobrinus* or *Lactobacillus* spp., but not against the periodontal pathogens usually present in the subgingival microenvironment. Furthermore, most have used planktonic pure cultures and therefore, the reported effects could not be easy to be transferred to the oral environment, where bacteria live in highly complex communities, forming biofilms [47]. As mentioned above, Esteban-Fernández and colleagues [21], established the minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations (MIC/MBC) for *P. gingivalis* to Provinols™ and Vitaflavan® of 500/≥1000 μg mL^{-1} for both extracts, and for *F. nucleatum* of 500/1000 μg mL^{-1} also for the referred extracts. However, in the present study, the observed antimicrobial activity can be considered as moderate against *P. gingivalis* and only statistically significant for *F. nucleatum* with both extracts, even at a high concentration (20,000 μg mL^{-1}). These findings reinforce the importance of using biofilms models when testing antimicrobial

Table 4 Effect of the red wine phenolic extract (Provinols™), rich in anthocyanins, and the oenological extract from grape seeds (Vitaflavan®) on the live/dead cell ratio (i.e. the area occupied by living cells divided by the area occupied by dead cells) of the whole biofilm obtained by Confocal Laser Scanning Microscopy (CLSM). PBS: phosphate buffer saline, DMSO: dimethyl sulfoxide

Exposure time	Chemical treatment		Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
						Lower Bound	Upper Bound
30 s	PBS	4% DMSO	0.250	0.256	1.000	-0.520	1.020
		Wine extract	0.353	0.256	1.000	-0.416	1.123
		Grape seeds extract	-0.130	0.256	1.000	-0.900	0.640
	4% DMSO	PBS	-0.250	0.256	1.000	-1.020	0.520
		Wine extract	0.103	0.256	1.000	-0.666	0.873
		Grape seeds extract	-0.380	0.256	0.941	-1.150	0.390
	Wine extract	PBS	-0.353	0.256	1.000	-1.123	0.416
		4% DMSO	-0.103	0.256	1.000	-0.873	0.666
		Grape seeds extract	-0.483	0.256	0.463	-1.253	0.286
	Grape seeds extract	PBS	0.130	0.256	1.000	-0.640	0.900
		4% DMSO	0.380	0.256	0.941	-0.390	1.150
		Wine extract	0.483	0.256	0.463	-0.286	1.253
1 min	PBS	4% DMSO	0.030	0.256	1.000	-0.740	0.800
		Wine extract	-0.110	0.256	1.000	-0.880	0.660
		Grape seeds extract	-0.197	0.256	1.000	-0.966	0.573
	4% DMSO	PBS	-0.030	0.256	1.000	-0.800	0.740
		Wine extract	-0.140	0.256	1.000	-0.910	0.630
		Grape seeds extract	-0.227	0.256	1.000	-0.996	0.543
	Wine extract	PBS	0.110	0.256	1.000	-0.660	0.880
		4% DMSO	0.140	0.256	1.000	-0.630	0.910
		Grape seeds extract	-0.087	0.256	1.000	-0.856	0.683
	Grape seeds extract	PBS	0.197	0.256	1.000	-0.573	0.966
		4% DMSO	0.227	0.256	1.000	-0.543	0.996
		Wine extract	0.087	0.256	1.000	-0.683	0.856

Based on estimated marginal means
[a]Adjustment for multiple comparisons: Bonferroni

activity, since bacterial cells in biofilms express different phenotypes, with greater resistance to antimicrobial agents [47–50]. Some studies have reported that the MIC of a bacteria can increase between 2 and 1000 times in a biofilm, when compared to the planktonic state [50], while other authors described 250 times greater MIC values for the same species growing in a biofilm when compared to planktonic state [51]. Sedlack and colleagues [51] described that bacterial resistance to antimicrobials appeared to be related to the maturation of the biofilms, since they demonstrated a progressive increase in resistance to the antibiotics as they matured, with a maximum resistance coinciding with the stationary phase of the growth of the biofilm. Therefore, the current work represents a further step in the study of the possible effects of polyphenols from red wine and oenological extracts in the management of periodontal diseases.

The results from the present study agree with those reported by Furiga and colleagues evaluating the activity of various extracts obtained from *Vitis vinifera* (Vitaceae) on a biofilm model composed of *S. mutans, S. sobrinus, Lactobacillus rhamnosus, P. gingivalis*, and *F. nucleatum* [22, 23]; and with those published by Muñoz-Gonzalez and colleagues [28], describing the beneficial the bactericidal activity against *A. oris, F. nucleatum*, or *S. oralis*. of red wine and dealcoholized red wine.

Conclusions

This investigation has shown that the use of red wine and wine-derived extracts had a moderate antimicrobial impact in the total bacterial counts and counts of *A. actinomycetemcomitans, P. gingivalis* and *F. nucleatum,* when tested in an in vitro multi-species biofilm model. Although the antibacterial effects of red wine and wine-derived extracts was observed, at least 2 to 3-log reduction of bacterial count would be necessary to ascertain the efficacy and/or availability of these tested agents as antibacterial agents. These results encourage further investigations on the potential use of natural agents in the prevention and treatment of periodontal diseases.

Abbreviations

BHI: Brain heart infusion culture medium; CFU: Colony-forming units; CLSM: Confocal laser scanning microscopy; DMSO: Dimethyl sulfoxide; DNA: Deoxyribonucleic acid; EtOH: Ethanol; HA: Hydroxyapatite; MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration; PBS: Phospate buffer saline; PMA: Propidium monoazide; qPCR: Quantitative polymerase chain reaction; UHPLC-ESI-MS/MS: Ultra-high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry method

Acknowledgements

The authors would like to thank A. Cortés, from the Centre of Microscopy and Cytometry, University Complutense of Madrid, for his support in the technical microscopy services. A.E-F is the recipient of fellowship from the FPI-MINECO programme.

Authors' contributions

MCS, HRV and AEF contributed to conception and design of the study with the aid of EF, BB, MVMA, MS and DH, analysis and interpretation of data and drafted the manuscript. EF performed the statistical analyses. EF, BB, MVMA, MS and DH critically revised the manuscript. All authors reviewed the original draft and read and approved the final manuscript.

Funding

This work was funded by the Spanish MINECO (AGL2015–64522-C2-R project) and Comunidad de Madrid (ALIBIRD-CM S2013/ABI-2728). None of the funders had any role in designing and/or conducting of the study; collection, management, analysis and interpretation of the data; and preparation, review or approval of the manuscript.

Availability of data and materials

The data sets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Received: 30 August 2018 Accepted: 29 May 2019
Published online: 21 June 2019

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Publisher's Note

Artículo #2

Artículo #2

Nuevas evidencias de efectos antibacterianos del arándano rojo frente a patógenos periodontales

Resumen: El preocupante aumento en las resistencias a los antibióticos pone de relieve la necesidad de buscar nuevos enfoques para tratar y prevenir las enfermedades periodontales. El propósito de este estudio fue evaluar la actividad antibacteriana y *antibiofilm* del arándano en un modelo de *biofilm in vitro* validado. Después de la caracterización química de un extracto de arándano seleccionado rico en compuestos fenólicos, sus valores para la concentración mínima inhibitoria y la concentración mínima bactericida se calcularon para las seis bacterias que forman el *biofilm* (*Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, y *Aggregatibacter actinomycemcomitans*). La actividad antibacteriana del extracto de arándano en los *biofilm* formados se evaluó midiendo la reducción de la viabilidad bacteriana, utilizando la reacción cuantitativa en cadena de la polimerasa (qPCR) combinada con *propidio monoazida* (PMA), y mediante microscopía láser confocal (CLSM); y la actividad *antibiofilm,* mediante el estudio de la inhibición de la incorporación de diferentes especies de bacterias en los *biofilm* formados en presencia del extracto de arándano, utilizando qPCR y CLSM. En el estado planctónico, la viabilidad de las bacterias se redujo significativamente ($p < 0.05$). Al crecer en *biofilm*, se observó un efecto significativo contra los colonizadores iniciales y tempranos [*S. oralis* ($p \leq 0.017$), *A. naeslundii* ($p = 0.006$) y *V. parvula* ($p = 0.010$)] después de 30 o 60 s de exposición, mientras que no se detectaron efectos significativos contra patógenos periodontales [*F. nucleatum*, *P. gingivalis* o *A. actinomycetemcomitans* ($p > 0.05$)]. Por el contrario, el arándano significativamente interfirió ($p < 0.001$ en todos los casos) con la incorporación de cinco de las seis especies de bacterias durante el desarrollo de *biofilms* de 6 h, incluido *P. gingivalis*, *A. actinomycetemcomitans* y *F. nucleatum*. Se concluyó que el arándano tiene un efecto antibacteriano moderado contra patógenos periodontales en *biofilms*, pero propiedades relevantes frente a *biofilms*, al afectar la adhesión de bacterias en las primeras 6 horas de desarrollo de *biofilms*.

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New Evidences of Antibacterial Effects of Cranberry Against Periodontal Pathogens

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Received: 20 January 2020; Accepted: 20 February 2020; Published: 24 February 2020

Abstract: The worrying rise in antibiotic resistances emphasizes the need to seek new approaches for treating and preventing periodontal diseases. The purpose of this study was to evaluate the antibacterial and anti-biofilm activity of cranberry in a validated in vitro biofilm model. After chemical characterization of a selected phenolic-rich cranberry extract, its values for minimum inhibitory concentration and minimum bactericidal concentration were calculated for the six bacteria forming the biofilm (*Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans*). Antibacterial activity of the cranberry extract in the formed biofilm was evaluated by assessing the reduction in bacteria viability, using quantitative polymerase chain reaction (qPCR) combined with propidium monoazide (PMA), and by confocal laser scanning microscopy (CLSM), and anti-biofilm activity by studying the inhibition of the incorporation of different bacteria species in biofilms formed in the presence of the cranberry extract, using qPCR and CLSM. In planktonic state, bacteria viability was significantly reduced by cranberry ($p < 0.05$). When growing in biofilms, a significant effect was observed against initial and early colonizers (*S. oralis* ($p \leq 0.017$), *A. naeslundii* ($p = 0.006$) and *V. parvula* ($p = 0.010$)) after 30 or 60 s of exposure, while no significant effects were detected against periodontal pathogens (*F. nucleatum*, *P. gingivalis* or *A. actinomycetemcomitans* ($p > 0.05$)). Conversely, cranberry significantly ($p < 0.001$ in all cases) interfered with the incorporation of five of the six bacteria species during the development of 6 h-biofilms, including *P. gingivalis*, *A. actinomycetemcomitans*, and *F. nucleatum*. It was concluded that cranberry had a moderate antibacterial effect against periodontal pathogens in biofilms, but relevant anti-biofilm properties, by affecting bacteria adhesion in the first 6 h of development of biofilms.

Keywords: polyphenols; cranberry; periodontal diseases; dental biofilm; antibacterial activity; anti-biofilm activity; *F. nucleatum*; *P. gingivalis*; *A. actinomycetemcomitans*

1. Introduction

Dental biofilm-organized periodontal pathogens (including *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*) are the primary etiological factor of periodontal diseases, which are one of the most prevalent conditions affecting human beings [1]. These conditions have not only a relevant impact in the mouth [1], but also in systemic health [2] and in quality of life indicators [3]. Due to the infectious nature of periodontal diseases, antimicrobials are widely used in their management (prevention and treatment) [4,5]. However, the worrying rise in antibiotic

resistances, including those in periodontal pathogens [6] and unwanted effects of antiseptics/antimicrobials compounds [4,5] emphasize the need to seek new approaches for treating and preventing periodontal diseases. Therefore, attention is given to the need of finding, developing, and improving antimicrobial natural compounds, capable of inhibiting the proliferation and/or adhesion of bacteria pathogens in dental/oral biofilms [5,7–10].

In previous studies, it has been shown that polyphenols, and other compounds derived from plants have an influence on human microbiota, either by promoting the growth of beneficial microorganisms or by acting against pathogens [11,12]. Cranberry (*Vaccinium macrocarpum*) compounds, including phenolic acids, proanthocyanidins (particularly, A-type proanthocyanidins), anthocyanins, organic acids, and their microbial-derived metabolites [13], selectively inhibit the growth of intestinal pathogens such as *Staphylococcus strains* and *Salmonella enterica* [14], reduce *Escherichia coli* colonization of the urinary tract [15–17], restrict the virulence of *Pseudomonas aeruginosa* [18,19], present anti-oxidant potential [20], anti-adhesion of Gram-negative and Gram-positive bacteria [21,22], and anti-motility [23,24]. Furthermore, they may be associated with relevant health benefits, including a decreased risk of cardiovascular disease-related mortality [25], prevention of type 2 diabetes mellitus [26], and potential anti-cancer properties [27,28].

The antibacterial and anti-adhesion features of cranberry against oral bacteria have drawn wide attention [22,29–31]. Several in vivo and in vitro studies have evaluated how certain cranberry derived compounds could interfere with formation of a cariogenic biofilm. In this regard, it has been demonstrated that certain components of cranberries may limit dental caries by inhibiting the production of organic acids by cariogenic bacteria, the formation of biofilms by *Streptococcus mutans* and *Streptococcus sobrinus*, and the adhesion and coaggregation of a considerable number of other oral species of *Streptococcus* [32–36]. Focusing on periodontal diseases, the non-dialyzable constituent fraction of cranberry (NDM) inhibits the formation of *P. gingivalis* [37] and *Fusobacterium nucleatum* [38] biofilms, two bacteria species associated with periodontitis. The NDM fraction may also inhibit the adhesion of *P. gingivalis* to various proteins, including type I collagen [37] and may reduce bacterial coaggregation involving periodontal pathogens [32]. However, the information on the antibacterial and anti-biofilm capacity of natural extracts from cranberry against relevant periodontal pathogens, growing in complex multi-species biofilms, is scarce.

Therefore, the aim of the present study was to evaluate the antibacterial and anti-biofilm activity of cranberry extracts in a multispecies in vitro biofilm model, including six bacteria species (*Streptococcus oralis*, *Veillonella parvula*, *Actinomyces naeslundii* and the periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans*, and *F. nucleatum*). The specific objectives were to assess (1) the antibacterial activity of a cranberry extract against bacteria species in formed biofilms, by assessing the reduction in bacteria viability, and (2) the anti-biofilm activity, by studying the inhibition of the incorporation of different bacteria species in biofilms formed in the presence of the cranberry extract.

2. Materials and Methods

2.1. Cranberry Extract

The cranberry extract used in this study was provided by Triarco Industries Inc. (Cranbury, New Jersey, USA). For determination of its total polyphenols content, the extract (0.05 g) was dissolved in 10 mL of methanol/HCl (1000:1, *v*/*v*), sonicated (120 W) for 5 min followed by an extra 15 min resting period, centrifuged, and filtered through a 0.22-μm membrane filter. For analysis of individual phenolic compounds, the extract (0.50 g) was dissolved in 10 mL of MeOH/H_2O (20:80, *v*/*v*) containing 0.2% HCl, sonicated for 10 min, centrifuged, and filtered through 0.22 μm. In both cases, sample preparations were performed in duplicate.

2.2. Analysis of Phenolic Compounds in the Cranberry Extract

Total polyphenols content was measured by the Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) and using gallic acid (25–500 mg L^{-1}) as a calibration standard. Analysis of individual phenolic compounds was carried out by UPLC-DAD-ESI-TQ MS, as previously described in

Sanchez-Patán et al. [39]. Different phenolic acids (including phenylpropionic, phenylacetic, mandelic, benzoic, and cinnamic acids), flavan-3-ols (monomers, B-type procyanidin dimers and trimers, and A-type procyanidin dimers and trimers), and anthocyanins (peonidin, cyanidin, and malvidin derivatives) were targeted [39]. Commercial standards of these phenolic acids were used to construct calibration curves for sample quantification [39].

2.3. Bacteria Strains and Culture Conditions

Reference strains of *S. oralis* CECT 907T, *V. parvula* NCTC 11810, *A. naeslundii* ATCC 19039, *F. nucleatum* DMSZ 20482, *A. actinomycetemcomitans* DSMZ 8324, and *P. gingivalis* ATCC 33277 were used. These bacteria were grown on blood agar plates (Blood Agar Oxoid No 2; Oxoid, Basingstoke, UK), supplemented with 5% (*v*/*v*) sterile horse blood (Oxoid), 5.0 mg L^{-1} hemin (Sigma, St. Louis, MO, USA) and 1.0 mg L^{-1} menadione (Merck, Darmstadt, Germany) in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37 °C for 24–72 h.

2.4. Antibacterial Assays

Figure 1 shows the experimental design followed for the study of the antibacterial effects of cranberry against planktonic bacteria and in an oral biofilm model.

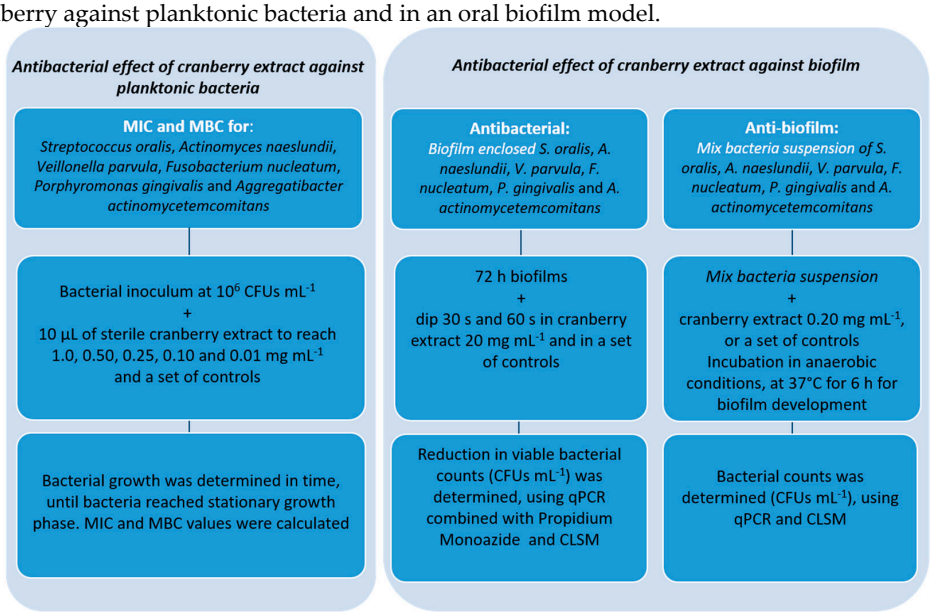

Figure 1. Scheme of the antibacterial assays carried out in this study.

2.4.1. Antibacterial Effect of Cranberry Extract Against Planktonic Bacteria

Pure cultures of the bacteria species were grown anaerobically in a protein rich medium containing brain-heart infusion (BHI) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 2.5 g L^{-1} mucin (Oxoid), 1.0 g L^{-1} yeast extract (Oxoid), 0.1 g L^{-1} cysteine (Sigma), 2.0 g L^{-1} sodium bicarbonate (Merck), 5.0 mg L^{-1} hemin (Sigma), 1.0 mg L^{-1} menadione (Merck), and 0.25% (*v*/*v*) glutamic acid (Sigma). The bacteria growth was harvested at mid-exponential phase (measured by spectrophotometry). Microtitre plate-based antibacterial assays were carried out in a 96-wells plate, adding 190 µL of each bacteria inoculum at a final concentration of 10^6 colony forming units (CFUs) mL^{-1}, and 10 µL of the sterile cranberry extract at a final concentration of 1.0, 0.50, 0.25, 0.10, and 0.01 mg mL^{-1}. Plates had a set of controls: negative control (culture media without any inoculum/cranberry extract), positive control (bacteria without any treatment) as well as blanks (cranberry extract or dimethyl sulfoxide (DMSO) dissolved in the culture media), to ensure the validity of the assay, 4% DMSO (to identify a possible bactericidal effect of DMSO, used as a

solvent for the cranberry extract), and 0.2% chlorhexidine (CHX), in order to compare with the reference of known antibacterial effect. A measurement (optical density, $O.D._{595}$) as t = 0 absorbance was taken in a microtitre plate reader (Optic Ivymen System 2100-C; I.C.T.; La Rioja, Spain). The microplates were incubated for 48 h at 37 °C under anaerobic conditions, and absorbance was measured at selected intervals (1 h during the first 12 h, and every 12 h to complete 48 h), in order to determine the bacteria growth in time, until the bacteria reached stationary growth phase. MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values were calculated and confirmed by microbial plate counting on blood agar media. Accordingly, the lowest concentration of the cranberry extract showing growth inhibition was considered as the MIC, whereas the lowest concentration of the cranberry extract that showed zero growth in blood agar plates, after spot inoculation and incubation for 72 h, was recorded as the MBC. All experiments were performed in triplicate with appropriate controls.

2.4.2. Antibacterial Effect in an Oral Biofilm Model in Vitro

In order to optimize the method for evaluating the antibacterial effect of the cranberry extract against the bacteria species growing in biofilms, a range of cranberry concentrations were initially tested (from MBCs to stock solution of cranberry extracts at 20 mg mL^{-1}). A dose of 20 mg mL^{-1} yielded the higher antibacterial effect (data not shown).

The multi-species in vitro biofilm model was developed as previously described by Sánchez et al. [40]. Briefly, pure cultures of each bacteria specie were grown anaerobically in the supplemented BHI medium. The bacteria growth was harvested at mid-exponential phase (measured by spectrophotometry), and a mixed bacteria suspension in modified BHI medium containing 10^3 CFUs mL^{-1} for *S. oralis*, 10^5 CFUs mL^{-1} for *V. parvula* and *A. naeslundii*, and 10^6 CFUs mL^{-1} for *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis* was prepared (different concentrations based on the different growth rates of each bacteria species). Sterile calcium hydroxyapatite (HA) discs of 7 mm of diameter and 1.8 mm (standard deviation, SD = 0.2) of thickness (Clarkson Chromatography Products, Williamsport, PA, USA) were coated with sterile saliva for 4 h at 37 °C in sterile plastic tubes to allow the formation of the acquired pellicle [40], and then placed in the wells of a 24-well tissue culture plate (Greiner Bio-one, Frickenhausen, Germany). Each well was inoculated with 1.5 mL mixed bacteria suspension prepared and incubated in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37 °C for 72 h.

After 72 h, biofilms were dipped during 30 s and 60 s in the cranberry solution (20 mg mL^{-1}), at room temperature. Exposure time of 30 and 60 s were selected since cranberry extracts are bioactive products, commercially available, and for them, the standard exposure times established for other antimicrobial commercially available products (e.g., chlorhexidine mouth rinses), were selected [41–43]. Phosphate buffered saline solution (PBS) was used as negative control and, in order to discard a bactericidal effect of DMSO used to dissolve the extracts, a 4% DMSO solution was also tested.

The antibacterial activity in 72 h biofilms was examined by determining the reduction in the number of viable bacteria counts (expressed as CFUs mL^{-1}), using quantitative polymerase chain reaction (qPCR) combined with Propidium Monoazide (PMA), and by Confocal Laser Scanning Microscopy (CLSM). Assays were conducted in triplicate (with trios of biofilms per replica).

2.5. Anti-Biofilm Assay

In order to optimize the method for evaluating the anti-biofilm effect of cranberry extracts against the selected bacteria species, different concentrations were tested, based on MICs of each bacteria species in planktonic state (data not shown), and it was finally concluded that a dose of 0.20 mg mL^{-1} provided the largest anti-biofilm impact, without affecting bacteria viability in planktonic state.

For the anti-biofilm assay, the mixed bacteria suspension in modified BHI medium containing 10^3 CFUs mL^{-1} for *S. oralis*, 10^5 CFUs mL^{-1} for *V. parvula* and *A. naeslundii*, and 10^6 CFUs mL^{-1} for *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis* was prepared as previously described. HA discs were coated with treated saliva for 4 h at 37 °C in sterile plastic tubes, and then placed in the wells of

a 24-well tissue culture plates. Each well was inoculated with 1.5 mL mixed bacteria suspension prepared and the cranberry extract at 0.20 mg mL^{-1}, or with PBS and DMSO in control biofilms, were added. Plates were incubated in anaerobic conditions, at 37 °C for 6 h.

The anti-biofilm activity was examined by determining bacteria counts in biofilms, as CFUs mL^{-1} by means of qPCR, and by CLSM. Assays were conducted in triplicate (with trios of biofilms per replica).

2.6. Microbiological Outcomes

After antibacterial and anti-biofilm assays, biofilms were recovered and sequentially rinsed in 2 mL of sterile PBS (immersion time per rinse, 10 s) three times, in order to remove possible remnants of the extracts and non-adherent bacteria. Then, biofilms were disrupted by vortex for 2 min in 1 mL of PBS. In the case of antibacterial activity, and to discriminate between DNA from live and dead bacteria, PMA was used (Biotium Inc., Hayword, CA, USA). The use of this PMA dye has shown the ability to distinguish between viable and irreversibly damaged cells and hence when combined with qPCR to detect the DNA from viable bacteria [44]. PMA was added to sample tubes containing 250 μL of disaggregated biofilm cells, at a final concentration of 100 μM. Following an incubation period of 10 min at 4 °C in the dark, the samples were subjected to light-exposure for 30 min, using PMA-Lite LED Photolysis Device (Biotium Inc.). After PMA photo-induced DNA cross-linking, the cells were centrifuged at 15,000 rcf for 3 min prior to DNA isolation.

Bacteria DNA was isolated from all biofilms using a commercial kit ATP Genomic DNA Mini Kit® (ATP biotech. Taipei, Taiwan), following manufacturer's instructions and the hydrolysis 5'nuclease probe assay qPCR method was used for detecting and quantifying the bacteria DNA. The qPCR amplification was performed following a protocol previously optimized by our research group, using primers and probes targeted against 16S rRNA gene (obtained through Life Technologies Invitrogen (Carlsbad, CA, USA) and Applied Biosystems (Carlsbad, CA, USA)) [44].

Each DNA sample was analyzed in duplicate. Quantification cycle (Cq) values, previously known as cycle threshold (Ct) values, describing the PCR cycle number at which fluorescence rises above the baseline, were determined using the provided software package (LC 480 Software 1.5; Roche Diagnostic GmbH; Mannheim, Germany). Quantification of viable cells by qPCR was based on standard curves. The correlation between Cq values and CFUs mL^{-1} was automatically generated through the software (LC 480 Software 1.5; Roche).

All assays were developed with a linear quantitative detection range established by the slope range of 3.3–3.7 cycles/log decade, $r^2 > 0.998$, and an efficiency range of 1.9–2.0.

2.7. Confocal Laser Scanning Microscopy (CLSM) Analyses

After antibacterial and anti-biofilm treatment referred above, and before CLSM analysis, the discs were sequentially rinsed in 2 mL of sterile PBS (immersion time per rinse, 10 sec) three times, in order to remove possible remnants of the extract and non-adherent bacteria. Non-invasive confocal imaging of fully hydrated biofilms was carried out using a fixed-stage Ix83 Olympus inverted microscope coupled to an Olympus FV1200 confocal system (Olympus; Shinjuku, Tokyo, Japan). Specimens were stained with LIVE/DEAD® BacLight™ Bacteria Viability Kit solution (Molecular Probes B. V., Leiden, The Netherlands) at room temperature. The 1:1 fluorocrome ratio with a staining time of 9 ± 1 min was used to obtain the optimum fluorescence signal at the corresponding wave lengths (Syto9: 515–530 nm; Propidium Iodide (PI): >600 nm). At least three separate and representative locations on the discs covered with biofilm were selected for these measurements (based on the presence of stacks or "towers" identified in the confocal field view). The CLSM software was set to take a z-series of scans (xyz) of 1 μm thickness (8 bits, 1024 × 1024 pixels). Image stacks were analyzed by using the Olympus® software (Olympus). Image analysis and live/dead cell ratio (i.e., the area occupied by living cells divided by the area occupied by dead cells) was performed with Fiji software (ImageJ Version 2.0.0-rc-65/1.52b, Open source image processing software).

2.8. Statistical Analyses

The selected outcome variables to study the antibacterial effect of cranberry extracts were the counts of viable bacteria present on the biofilms, expressed as viable CFUs mL^{-1} of *S. oralis*, *V. parvula*, *A. naeslundii*, *F. nucleatum*, *A. actinomycetemcomitans*, and *P. gingivalis* by qPCR, and the live/dead cell ratio of the whole biofilm by CLSM. An experiment-level analysis was performed for each parameter of the study (n = 9 for qPCR and n = 3 for CLSM results). Shapiro–Wilk goodness-of-fit tests and distribution of data were used to assess normality. The effect of each solution (cranberry extracts, PBS and 4% DMSO), the time of exposure (30 or 60 s), and their interaction with the main outcome variable (counts expressed as CFUs mL^{-1} or live/dead cell ratio), was compared by means of a parametric ANOVA test for independent samples, and a general linear model was constructed for each bacterium for qPCR results and for total bacteria for live/dead cell ratio of whole biofilm obtained by CLSM, using the method of maximum likelihood and Bonferroni corrections for multiple comparisons.

To study the anti-biofilm effect of the cranberry extract, the selected outcome variables were the counts of bacteria present on the biofilms, expressed as CFUs mL^{-1} of *S. oralis*, *V. parvula*, *A. naeslundii*, *F. nucleatum*, *A. actinomycetemcomitans*, and *P. gingivalis* by qPCR, and the live/dead cell ratio of the whole biofilm by CLSM. Shapiro–Wilk goodness-of-fit tests and distribution of data were used to assess normality. An experiment-level analysis was performed for each parameter of the study (n = 9 for qPCR and n = 3 for CLSM results). The effect of each solution (cranberry extract, PBS, and 4% DMSO) on the main outcome variables (CFUs mL^{-1} or live/dead cell ratio), was compared by means of a parametric ANOVA test for independent samples, using the method of maximum likelihood and Bonferroni corrections for multiple comparisons.

Data was expressed as means ± SD and as the mean percent inhibition that was calculated by Equation: Percent inhibition = (CFUs mL^{-1} of negative control–CFUs mL^{-1} of test / CFUs mL^{-1} of negative control) × 100.

Results were considered statistically significant at $p < 0.05$. A software package (IBM SPSS Statistics 24.0; IBM Corporation, Armonk, NY, USA) was used for all data analysis.

3. Results

3.1. Phenolic Composition of the Cranberry Extract

A phenolic characterization of the cranberry extract was initially carried out to ensure its susceptibility for this study. The content in total polyphenols content of the extract resulted in 219 mg of gallic acid equivalents g^{-1}. Concerning phenolic composition, Table 1 details the main phenolic compounds found in the extract, as determined by UPLC-DAD-ESI-TQ MS. Among phenolic acids (benzoic and cinnamic acids), the extract was especially rich in benzoic acid (8.38 mg g^{-1}), followed by others such as p-coumaric acid (0.84 mg g^{-1}) and protocatechuic acid (0.73 mg g^{-1}) in considerable less content. Concerning flavan-3-ols, main MS signals corresponded to A-type trimers (1.58 mg g^{-1}), followed by A-type (0.23 mg g^{-1}) and B-type (0.20 mg g^{-1}) dimers, monomers (0.065 mg g^{-1}), and B-type trimers (0.034 mg g^{-1}). In relation to anthocyanins, peonidin-3-arabinoside (0.32 mg g^{-1}) and cyanidin-3-arabinoside (0.15 mg g^{-1}) showed the highest content (Table 1). These compositional data were in accordance to others commercial cranberry extracts [39].

3.2. Antibacterial Assays

3.2.1. Antibacterial Effect of Cranberry Extract Against Planktonic Bacteria

MICs and MBCs values against the six bacteria species selected in planktonic state were determined for the selected cranberry extract. MICs indicated an average bacteriostatic concentration of 0.10 mg mL^{-1} against *P. gingivalis* and *F. nucleatum*, 0.25 mg mL^{-1} for *A. naeslundii* and *A. actinomycetemcomitans*, 0.50 mg mL^{-1} for *V. parvula*, and >1.00 mg mL^{-1} for *S. oralis*. MBCs tests showed similar results, with bactericidal concentrations of 0.25 mg mL^{-1} against *P. gingivalis*, 1.00 mg mL^{-1} against *F. nucleatum*, and >1.00 mg mL^{-1} for *S. oralis, A. naeslundii, V. parvula,* and *A.*

actinomycetemcomitans. According to these results, the cranberry extract exhibited antibacterial activity, displaying the largest antibacterial properties against the periodontal pathogens *P. gingivalis* and *F. nucleatum.*

Table 1. Phenolic compounds present in the cranberry extract used in this study. Data are expressed as mean and standard deviation (SD).

Compounds group	Phenolic compound	Concentration (μg g^{-1} ± SD)
Benzoic acids	Benzoic acid	8317.88 ± 222.31
	Protocatechuic acid	735.12 ± 17.76
	Vanillic acid	262.54 ± 10.16
	Gallic acid	136.16 ± 1.50
	4-Hydroxybenzoic acid	94.81 ± 2.23
	Salycilic acid	91.05 ± 2.16
	4-Hydroxymandelic acid	30.84 ± 1.14
	3-O-methylgallic acid	30.05 ± 0.64
	4-Hydroxy-3-methoxymandelic acid	14.33± 0.45
	Syringic acid	11.80 ± 1.18
	3-Hydroxybenzoic acid	11.58 ± 0.01
	3-(3,4-Dihydroxyphenyl)-propionic acid	9.61 ± 0.16
	4-Hydroxy-3-methoxyphenylacetic acid	6.55 ± 0.66
	3,4-Dihydroxy mandelic acid	3.56 ± 0.31
	4-Hydroxypheny lacetic acid	3.20 ± 0.41
	Hippuric acid	1.14 ± 0.10
	3,4-Dihydroxy phenylacetic acid	1.05 ± 0.10
	3,4,5-Trimethoxy benzoic acid	0.32 ± 0.03
Cinnamic acids	*p*-Coumaric acid	844.16 ± 15.20
	trans-Cinnamic acid	260.55 ± 0.04
	Caffeic acid	133.67 ± 2.52
	Ferulic acid	111.92 ± 4.38
	Trimethoxycinnamic acid	2.72 ± 0.27
Flavan-3-ols	∑ A-type trimers	1579.04 ± 27.31
	∑ A-type dimers	230.95 ± 18.11
	∑ B-type dimers	201.87 ± 17.21
	∑ Monomers	65.81 ± 5.20
	∑ B-type trimers	34.1 ± 0.91
Anthocyanins	Peonidin-3-arabinoside	32.73 ± 3.27
	Cyanidin-3-arabinoside	15.01 ± 0.05
	Peonidin-3-glucoside	4.84 ± 0.48
	Malvidin-3-arabinoside	1.16 ± 0.02
	Peonidin-3-galactoside	1.03 ± 0.09
	Cyanidin-3-glucoside	0.31 ± 0.02
	Cyanidin-3-galactoside	0.19 ± 0.01

3.2.2. Antibacterial Effects in an in Vitro Biofilm Model: Bacteria Counts

Table 2 depicts the effect of cranberry extracts (20 mg mL^{-1}), compared to the negative control (PBS) and 4% DMSO control solution, on the mean number of viable bacteria counts in 72 h biofilms. After an exposure of 30 or 60 s to the cranberry extract, significant reductions in viable counts in biofilms were observed for initial and early colonizers. *S. oralis* showed significant reductions after 30 ($p < 0.001$) and 60 s ($p = 0.017$) when compared to negative control (PBS), reaching in both cases a decrease of 98.9% of viable CFUs (Table 2). Significant differences ($p < 0.001$ after 30 s of exposure) were also observed when the effects of DMSO solution was compared to PBS, with percentages of decrease of 93.1% and 58.8% for 30 and 60 s exposures, respectively (Table 2). For *A. naeslundii* and *V. parvula*, a significant impact of the cranberry extract was observed after 30 s (65.7% of reduction, $p = 0.006$ and 66.7% of reduction, $p = 0.010$, respectively), but not after 60 s. No significant reductions were observed after exposure to DMSO ($p > 0.05$), after 30 or 60 s (Table 2). No statistically significant differences were observed between the cranberry extract and DMSO at any time ($p > 0.05$). The effect of exposure time (30 s versus 60 s) was not statistically significant for both solutions ($p > 0.05$ in all cases) in *S. oralis, A. naeslundii* and *V. parvula*.

For the secondary colonizer *F. nucleatum*, some effects on viable counts were observed after 30 s ($p = 0.164$) and after 60 s (decrease of 75.3%, $p = 0.448$), although not statistically significant. Additionally, no statistically significant reductions in viable counts were observed for DMSO ($p > 0.05$) (Table 2). No statistically significant differences were observed between the cranberry extract and DMSO at any time ($p > 0.05$). The effect of exposure time was however, statistically significant for the cranberry extract ($p = 0.022$) and DMSO ($p = 0.035$).

For the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis*, no significant reductions in viable counts after 30 s or 60 s of exposure to the cranberry extract ($p > 0.05$) were observed when compared to negative control: reductions of 11.5% for *A. actinomycetemcomitans* and 39.3% for *P. gingivalis* after 60 s. The same was true for DMSO ($p > 0.05$) (Table 2). No statistically significant differences were observed in the effectiveness comparing the two solutions at applied times or when comparing exposure times ($p > 0.05$ for all cases).

3.2.3. Antibacterial Effects in an in Vitro Biofilm Model: CLSM

The CLSM analysis showed that, after 72 h of incubation on HA surfaces, control biofilms covered the entire disc surface as a flat layer of cells combined with stacks of bacteria aggregations, showed a live/dead cell ratio (i.e., the area occupied by living cells divided by the area occupied by dead cells) of 1.43 (SD 0.10) and 1.25 (SD 0.15), after exposure of 30 and 60 s, respectively, to PBS (Figure 2a, b). Table 3 depicts the effects of the cranberry extract on the live/dead cell ratio of the whole biofilm obtained by CLSM. It could be observed that, after exposure of 30 s to cranberry extracts and to the 4% DMSO solution, cell vitality significantly decreased in the biofilms, showing, respectively, live/dead cell ratios of 0.67 (SD 0.07) and 0.77 (SD 0.04) for 4% DMSO ($p < 0.001$ in both cases, when compared to negative control biofilms) (Figure 2c, e). After 60 s of exposure (Figure 2f), reductions in viability were also statistically significant for cranberry extracts (live/dead cell ratio of 0.56 (SD 0.02), $p < 0.001$; Figure 2f) and for DMSO solution (live/dead cell ratio of 0.78 (SD 0.05), $p < 0.001$; Figure 2d), when compared to control biofilms (live/dead cell ratio of 1.25 (SD 0.15)). These results are consistent with those observed by means of qPCR, with significant differences in viable counts of initial and early colonizers, after exposure to cranberry extracts and DMSO solution, when compared to negative control biofilms. Statistically significant differences were observed between the cranberry extract and DMSO after 60 s of exposure ($p = 0.027$) (Table 3).

Table 2. Antibacterial effects of the cranberry extract on the mean number of viable bacteria counts in the in vitro multi-species biofilm model (in colony forming units, CFUs mL^{-1}, determined by quantitative polymerase chain reaction (qPCR)). Data are expressed as mean and standard deviation (SD). PBS: phosphate buffer saline; DMSO: 4% dimethyl sulfoxide solution.

	Exposure time (seconds)	Viable CFUs mL^{-1} [mean (SD)]			*p*-value when compared to negative control		% of reduction of viable CFUs mL^{-1} as compared with negative control	
		Negative control (PBS)	Cranberry extract	DMSO	Cranberry extract	DMSO	Cranberry extract	DMSO
S. oralis	30	$1.2 \times 10^6 \pm 1.1 \times 10^6$	$1.3 \times 10^4 \pm 1.1 \times 10^4$	$8.3 \times 10^4 \pm 1.4 \times 10^5$	0.000	0.000	98.9	93.1
	60	$6.8 \times 10^5 \pm 4.3 \times 10^5$	$7.3 \times 10^3 \pm 4.4 \times 10^3$	$2.8 \times 10^5 \pm 2.3 \times 10^5$	0.017	0.282	98.9	58.8
A. naeslundii	30	$6.7 \times 10^4 \pm 5.6 \times 10^4$	$2.3 \times 10^4 \pm 1.3 \times 10^4$	$3.4 \times 10^4 \pm 2.1 \times 10^4$	0.006	0.050	65.7	49.2
	60	$2.2 \times 10^4 \pm 1.3 \times 10^4$	$3.2 \times 10^4 \pm 2.4 \times 10^4$	$2.0 \times 10^4 \pm 1.4 \times 10^4$	1.000	1.000	45.4	9.1
V. parvula	30	$3.6 \times 10^6 \pm 2.8 \times 10^6$	$1.2 \times 10^6 \pm 1.4 \times 10^6$	$2.0 \times 10^6 \pm 2.1 \times 10^6$	0.010	0.147	66.7	44.4
	60	$1.6 \times 10^6 \pm 8.6 \times 10^5$	$4.4 \times 10^5 \pm 3.6 \times 10^5$	$1.3 \times 10^6 \pm 1.3 \times 10^6$	0.395	1.000	72.5	18.7
A. actinomycetemcomitans	30	$7.2 \times 10^6 \pm 6.4 \times 10^6$	$6.8 \times 10^6 \pm 4.7 \times 10^6$	$5.6 \times 10^6 \pm 3.0 \times 10^6$	1.000	1.000	5.6	22.2
	60	$5.2 \times 10^6 \pm 3.5 \times 10^6$	$4.6 \times 10^6 \pm 4.4 \times 10^6$	$5.2 \times 10^6 \pm 4.9 \times 10^6$	1.000	1.000	11.5	0.0
P. gingivalis	30	$1.7 \times 10^6 \pm 7.0 \times 10^5$	$1.1 \times 10^6 \pm 5.2 \times 10^5$	$1.6 \times 10^6 \pm 1.8 \times 10^6$	0.434	1.000	35.3	5.9
	60	$8.9 \times 10^5 \pm 6.8 \times 10^5$	$5.4 \times 10^5 \pm 1.8 \times 10^5$	$1.0 \times 10^6 \pm 7.0 \times 10^5$	1.000	1.000	39.3	12.3
F. nucleatum	30	$3.8 \times 10^5 \pm 3.1 \times 10^5$	$2.3 \times 10^5 \pm 1.5 \times 10^5$†	$3.5 \times 10^5 \pm 1.3 \times 10^5$	0.164	1.000	39.5	7.9
	60	$1.5 \times 10^5 \pm 1.0 \times 10^5$	$3.7 \times 10^4 \pm 3.0 \times 10^4$†	$1.8 \times 10^5 \pm 1.5 \times 10^5$	0.448	1.000	75.3	0.0

† $p < 0.05$, significant differences when comparing exposure times for an antimicrobial agent.

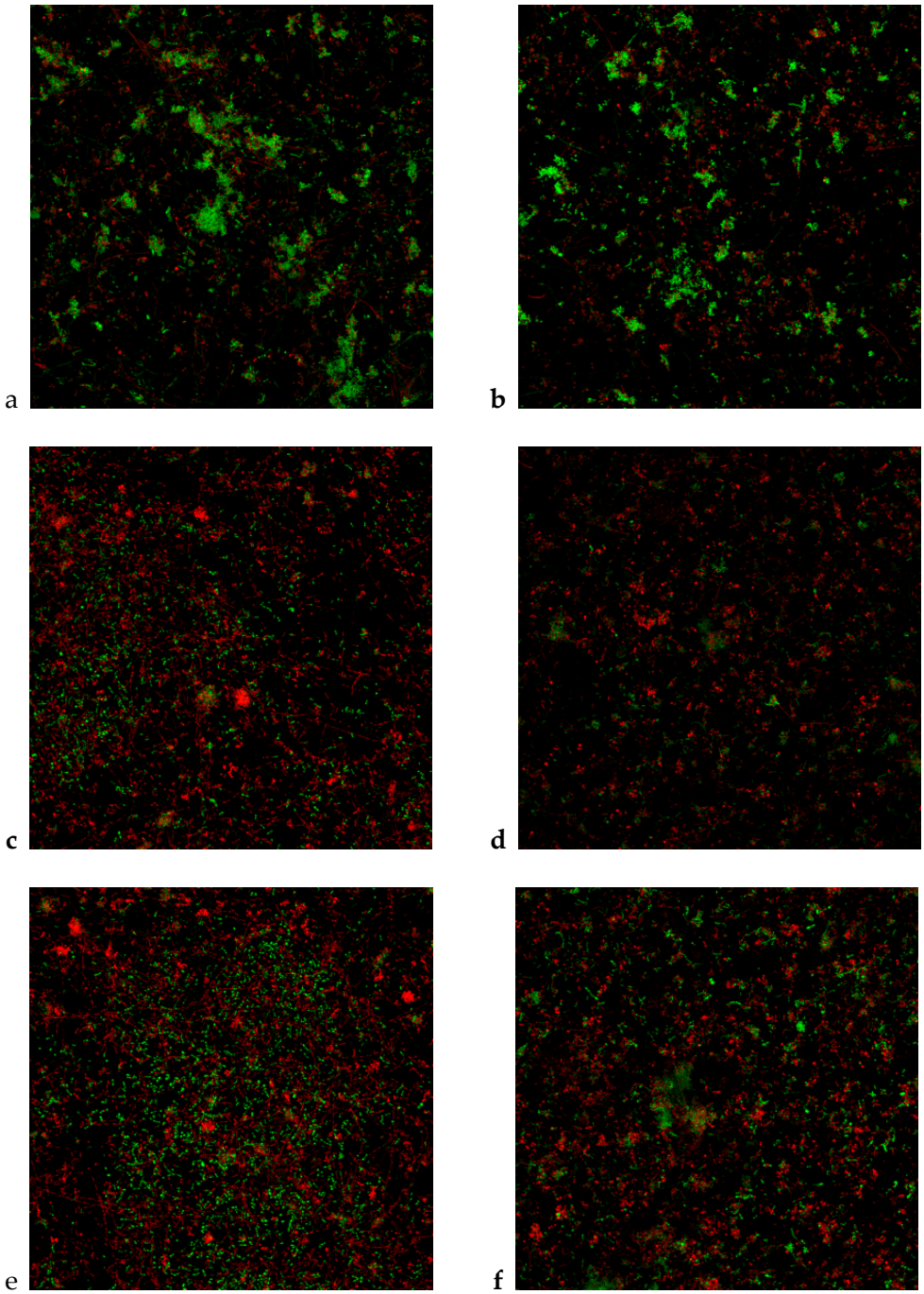

Figure 2. Maximum projection of confocal laser scanning microscopy (CLSM) images of the whole biofilm, grown 72 h over hydroxyapatite surfaces, and stained with LIVE/DEAD® BacLight™ Bacteria Viability Kit, after exposure to: (**a**,**b**) negative controls, 30 and 60 s, respectively (phosphate buffer saline, PBS); (**c**,**d**) 4% dimethyl sulfoxide (DMSO) solution, 30 and 60 s, respectively; (**e**,**f**) cranberry extracts (20 g L^{-1}), 30 and 60 s, respectively. (Scale bar = 100 μm).

Table 3. Effect of the cranberry extract on the live/dead cell ratio (i.e., the area occupied by living cells divided by the area occupied by dead cells) of the whole biofilm obtained by confocal laser scanning microscopy (CLSM). PBS: phosphate buffer saline; DMSO: 4% dimethyl sulfoxide solution.

	Treatment		Mean Difference (I–J)	Standard Error	Sig.[a]	95% Confidence Interval for Difference	
						Lower Bound	Upper Bound
Antimicrobial effect							
30 s	PBS	Cranberry	0.763	0.071	0.000	0.567	0.960
		DMSO	0.663	0.071	0.000	0.467	0.860
	Cranberry	DMSO	−0.100	0.071	0.550	−0.297	0.097
60 s	PBS	Cranberry	0.687	0.071	0.000	0.490	0.883
		DMSO	0.467	0.071	0.000	0.270	0.663
	Cranberry	DMSO	−0.220	0.071	0.027	−0.417	−0.023
Anti-biofilm effect							
6 h	PBS	Cranberry	0.35000	0.14575	0.160	−0.1292	0.8292
		DMSO	0.40000	0.14575	0.101	−0.0792	0.8792
	Cranberry	DMSO	0.05000	0.14575	1.000	−0.4292	0.5292

Based on estimated marginal means

[a] *p* value, adjustment for multiple comparisons (Bonferroni).

3.3. Anti-Biofilm Assay

3.3.1. Anti-Biofilm Assay: Bacteria Counts

The cranberry extract, at a concentration of 0.20 mg mL^{-1}, significantly inhibited the incorporation of five of the six studied bacteria species in the in vitro biofilm model (Table 4). After 6 h of contact, and compared to negative control biofilms, two of the three initial and early colonizers were significantly reduced on the HA surfaces: 98.9% for *S. oralis* ($p < 0.001$) or 90.9% for *V. parvula* ($p < 0.001$), when exposed to cranberry extracts. No significant impact was observed for *A. naeslundii*.

Periodontal pathogens showed a similar trend. *P. gingivalis* showed the largest impact of cranberry extracts: 97.2% ($p < 0.001$), with counts of 1.1×10^3 (SD 1.1×10^3) CFUs mL^{-1}, compared to 4.0×10^4 (SD 2.9×10^4) CFUs mL^{-1}, in negative control biofilms. Reductions *A. actinomycetemcomitans* (84.0%) and *F. nucleatum* (75.4%) were statistically significant ($p < 0.001$ in both cases).

For DMSO, a significant impact was observed for the three periodontal pathogens and for *S. oralis*, when compared to control biofilms ($p < 0.005$ in all cases; Table 4).

Significant differences were observed in the effectiveness comparing the cranberry extract and DMSO solution after 6 h of biofilm evolution in *V. parvula* ($p < 0.001$) and *A. actinomycetemcomitans* ($p = 0.024$).

Table 4. Anti-biofilm effects of the cranberry extract on the mean number of bacteria counts, incorporated during the 6 h of devolvement in the in vitro multi-species biofilm model (in colony forming units, CFUs mL^{-1}, determined by quantitative real-time polymerase chain reaction (qPCR)). Data are expressed as mean and standard deviation (SD). PBS: phosphate buffer saline; DMSO: 4% dimethyl sulfoxide solution.

	Viable CFUs mL^{-1} [mean (SD)]			*p*-value when compared to negative control		% of reduction of viable CFUs mL^{-1} respect to negative control	
	Negative control (PBS)	Cranberry extract	DMSO	Cranberry extract	DMSO	Cranberry extract	DMSO
S. oralis	$1.2 \times 10^5 \pm 2.5 \times 10^4$	$1.3 \times 10^3 \pm 5.3 \times 10^2$	$5.5 \times 10^2 \pm 2.6 \times 10^2$	0.000	0.000	98.9	99.5
A. naeslundii	$4.8 \times 10^4 \pm 3.1 \times 10^4$	$7.8 \times 10^4 \pm 7.6 \times 10^4$	$6.4 \times 10^4 \pm 1.9 \times 10^4$	0.608	1.000	-	-
V. parvula	$2.3 \times 10^4 \pm 1.5 \times 10^4$	$2.1 \times 10^3 \pm 2.2 \times 10^3$	$2.0 \times 10^4 \pm 7.3 \times 10^3$	0.000	1.000	90.9	13.0
A. actinomycetemcomitans	$7.5 \times 10^5 \pm 2.8 \times 10^5$	$1.2 \times 10^5 \pm 9.5 \times 10^4$	$3.8 \times 10^5 \pm 1.4 \times 10^5$	0.000	0.001	84.0	50.7
P. gingivalis	$4.0 \times 10^4 \pm 2.9 \times 10^4$	$1.1 \times 10^3 \pm 1.1 \times 10^3$	$1.0 \times 10^4 \pm 9.9 \times 10^3$	0.000	0.0047	97.2	75.0
F. nucleatum	$1.1 \times 10^5 \pm 3.8 \times 10^4$	$2.7 \times 10^4 \pm 2.0 \times 10^4$	$5.9 \times 10^4 \pm 2.0 \times 10^4$	0.000	0.005	75.4	46.4

3.3.2. Anti-Biofilm Assay: CLSM

CLSM analysis showed that, after 6 h of incubation on HA surfaces, formed biofilms showed the typical features of bacteria communities in their first steps, with a high percentage of live cells versus dead cells, that was evidenced by a live/dead cell ratio of 1.44 (SD 0.01) (Figure 3a,b). The effect of the exposure of the biofilms for 6 h to the cranberry extract, at a concentration of 0.20 mg mL^{-1}, was evident as it was not possible to observe well-structured biofilms, contrary what happened in the control samples. Although the biomass was reduced, no significant differences in bacteria vitality were observed when compared respect to controls (live/dead cell ratio of 0.99 (SD 0.01), $p = 0.160$; Table 3; Figure 3c,d), suggesting a limited antiseptic effect, and highlighting a desired effect on bacteria adhesion. Conversely, DMSO showed a similar live/dead cell ratio (1.047 (SD 0.14); Figure 3e,f), and biofilms were normally formed, with no significant differences when compared to control biofilms ($p = 0.101$; Table 3). These results are consistent with those observed by qPCR, which showed significant differences in bacteria counts of the tested bacteria species when biofilms formed in the presence of the cranberry extract where compared with control biofilms.

No statistically significant differences were observed in the effectiveness comparing the cranberry extract and DMSO at applied time ($p = 1.000$) (Table 3).

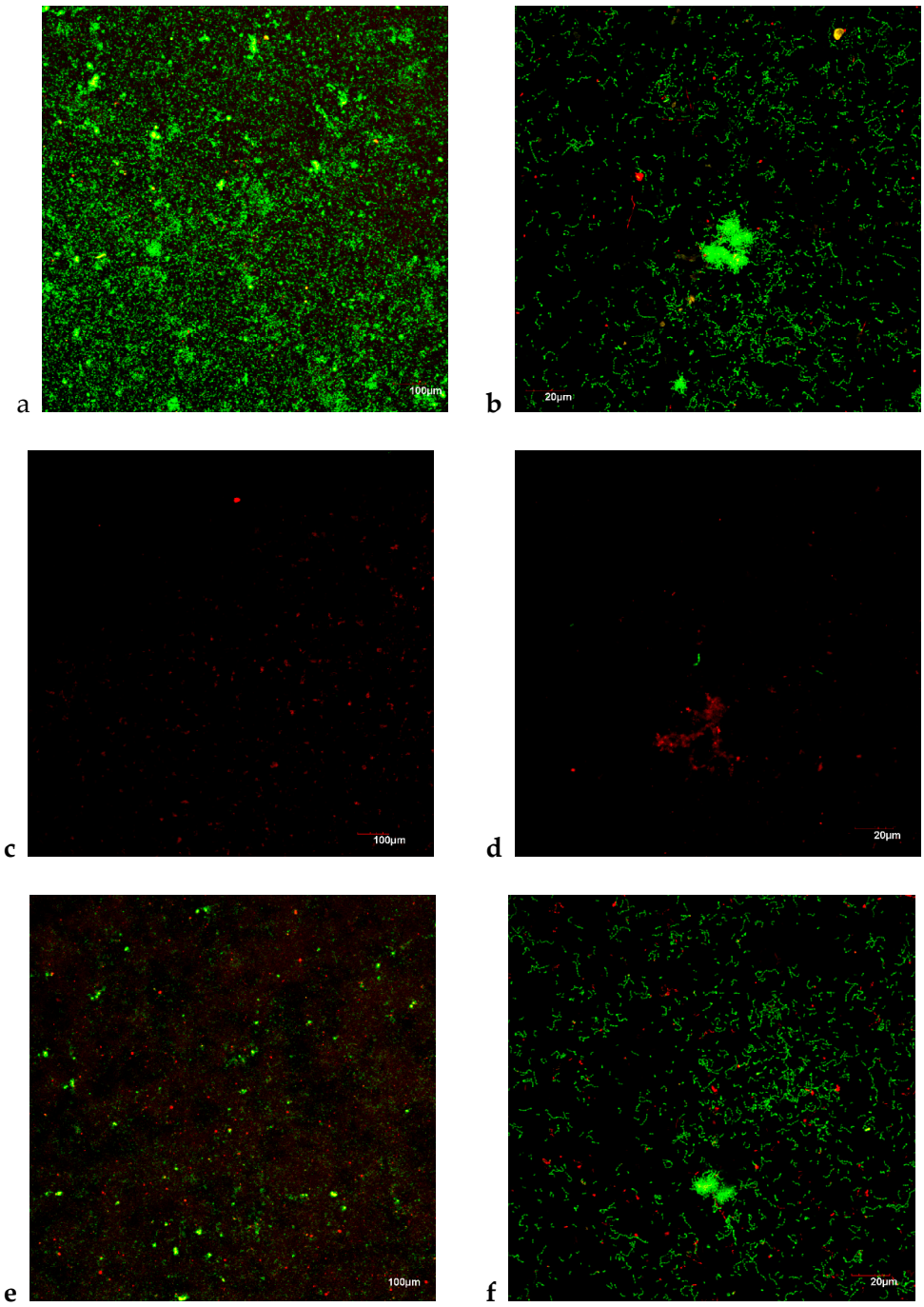

Figure 3. Maximum projection of confocal laser scanning microscopy (CLSM) images of the whole biofilm after 6 h of development, growing in the presence of 0.20 mg mL^{-1} of cranberry extract, over hydroxyapatite surfaces, and stained with LIVE/DEAD® BacLight™ Bacteria Viability Kit, after exposure to: (**a**,**b**) negative control (phosphate buffer saline, PBS); (**c**,**d**) cranberry extract; (**e**,**f**) 4% dimethyl sulfoxide (DMSO) solution.

4. Discussion

Since bacteria resistance to antibiotics is becoming an increasing health threat worldwide, alternative strategies to prevent or limit biofilm formation are a relevant goal. A growing body of evidence has demonstrated that plant extracts offer relevant antimicrobial and anti-biofilm potentials, with no significant risk of increasing antibiotic resistances. A vast number of phytochemicals have been recognized as valuable alternatives and complementary products to manage bacterial infections [45,46]. Cranberry (*Vaccinium macrocarpum*) fruits are particularly rich in biologically active phenolic compounds and organic acids [13], as it has also been confirmed from our results (Table 1). Numerous in vivo and in vitro studies have showed that different cranberry compounds/fractions/extracts possess antibacterial properties (against both Gram-positive and Gram-negative bacteria species) on various pathogenic bacteria in urinary tract infections and other diseases [33,47–49]. In this context, the present study has confirmed the antibacterial capacity of cranberry extracts against the six bacteria species (*S. oralis* CECT 907T, *V. parvula* NCTC 11810, *A. naeslundii* ATCC 19039, *F. nucleatum* DMSZ 20482, *A. actinomycetemcomitans* DSMZ 8324, and *P. gingivalis* ATCC 33277) tested in planktonic state; this findings contradict, at least partially, those of La and co-workers [50], who concluded that A-type proanthocyanidins did not present any effect on *P. gingivalis* in planktonic state. In view of our results from the UPLC-DAD-ESI-TQ MS analysis of the cranberry extract, a possible explanation for this disagreement could be that the antiseptic effect comes, not only from these A-type proanthocyanidins, but also from some of the other components of cranberry extracts, such as phenolic acids. In this context, the necessity of carrying out previous compositional characterization of cranberry extracts should be noted—as we have done in our study—in view of the diversity in this kind of products that affects their bioactivity [51].

However, bacteria are normally arrange as biofilms, predominating these sessile communities in most of the environmental, industrial, and medical habitats [52]. In fact, these highly structured bacteria communities are found in the mouth, allowing bacteria cells to withstand the natural defence mechanisms, as well as the host's immune defences or the effects of antimicrobial agents [53–55]. Therefore, the study of the antimicrobial of cranberry extracts should be performed against bacteria organized in biofilms. The results of the present study indicate that, when testing bacteria organized in biofilms, bacteria viability was affected by exposure to the cranberry extract at 20 mg mL^{-1} after 30 and 60 s of exposure. However, a significant effect was only observed for initial and early colonizers (*S. oralis, A. naeslundii*, and *V. parvula*), but, in agreement with other studies, not for periodontal pathogens (*F. nucleatum, P. gingivalis*, and *A. actinomycetemcomitans*). Philips and coworkers [56], in a recent investigation assessing the inhibitory effects of berry fruit extracts on *S. mutans* biofilms, indicated that bacteria viability was not significantly affected, as also concluded by Koo and coworkers [33]. Biofilms are an intriguing structure which demonstrate greater resistance to antimicrobial agents when compared to organisms in planktonic form [31]. A previous study using a cranberry juice concentrate formulated as a thermoreversible gel [11], showed antibacterial properties against *A. actinomycetemcomitans* and *P. gingivalis*, in contrast to the results of our study. The variability of the results may be due to the different types of samples and formulations used.

Besides the antibacterial effects, this investigation highlights new possible features regarding the anti-biofilm activity of cranberry extracts against periodontal pathogens. Bacteria adhesion to oral surfaces is the initial and crucial step in dental biofilm development and, therefore, in the pathogenesis of periodontal diseases. The cranberry extract, at a concentration of 0.20 mg mL^{-1}, inhibited the colonization of the six tested bacteria species in the in vitro biofilm model, especially for periodontal pathogens *P. gingivalis* (97.2% of reduction), *A. actinomycetemcomitans* (84%), and *F. nucleatum* (75.4%), being the impact statistically significant ($p < 0.001$ in all cases), when compared to control biofilms. Additionally, initial and early colonizers were significantly affected: *S. oralis* (98.9%, $p < 0.001$) or *V. parvula* (90.9%, $p < 0.001$). Different studies have described the role of cranberry constituents in bacteria adhesion and biofilm development: Philips and coworkers [56] indicated that cranberry extracts were the most effective extract in disrupting *S. mutans* biofilm integrity and structural architecture, without significantly affecting bacteria viability; La and co-workers [50] observed that A-type cranberry proanthocyanidins did not have any effect on *P. gingivalis*

planktonic growth, but they did inhibit biofilm formation. The anti-biofilm effect of cranberry extracts in our biofilm model was also confirmed by CLSM, with a significant disturbance on biofilm structure, a qualitative assessment that was consistent with the quantitative data provided by qPCR.

Labreque and coworkers [37] and Yamanaka and coworkers [38] observed that the non-dialyzable constituent fraction of cranberry (NDM) interfered with the colonization of *P. gingivalis* and *F. nucleatum* in the gingival crevice, reducing bacteria coaggregation in periodontal diseases [37,38,57,58]. Moreover, Polak et al. [58] found that NDM adhesion of *P. gingivalis* and *F. nucleatum* onto epithelial cells, and NDM consumption by mice attenuated the severity of experimental periodontitis, compared with a mixed infection without NDM treatment. Furthermore, NDM increased the phagocytosis of *P. gingivalis*. In addition, cranberries were described to restrain the proteolytic activity of the red complex, specifically the gingipain activity of *P. gingivalis*, trypsin-like activity of *Tannerella forsythia*, and chemotrypsin-like activity of *Treponema denticola* [59]. Cranberry extracts have also demonstrated the inhibition of the productions some cytokines: Bodet et al. [59] or Polak et al. [58] observed that NDM eliminated TNF-a expression by macrophages that were exposed to *P. gingivalis* and *F. nucleatum*, without impairing their viability.

The hydrophobic character of the cranberry extract has made the experiments difficult, requiring the use of the organic solvent DMSO in the tests, in order to overcome such complications. However, some antimicrobial activity of DMSO at the selected concentration (4%) was observed, and therefore, it may have a possible contribution in the antibacterial activity of the extract under investigation. In this way, studies have tested different concentrations, ranging up to 10% [60–63]. However, when used as a solvent, there is no established criteria as to which is the most appropriate concentration, and the interpretation of its effects on the microorganisms with which it interacts is of great importance in view of its widespread use as solvent in therapeutic and pharmacological studies [60–63]. In the present study, the 4% DMSO concentration was selected as the one that ensured complete solubilisation of the cranberry extract with minimum antimicrobial effects. However, in any case, the results obtained in the present study make evident the need to standardize an appropriate concentration of DMSO, suitable for bacterial experiments, considering that there is a discrepancy in the findings of different studies on the antimicrobial effects of different concentrations of DMSO.

5. Conclusions

This study has demonstrated that the incorporation of bacteria into the biofilm was significantly interfered, including relevant periodontal pathogens, such as *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum*. Our results support the hypothesis that cranberry components may interfere in the phase of bacteria adherence, disabling or inhibiting the adherence of periodontal pathogens and, therefore, preventing bacterial colonization. This fact could interfere with biofilm formation and possibly helping to maintain homeostasis and, thus, to prevent periodontal diseases. Anti-biofilm activity of cranberry extracts in the present study could be attributed to the presence of polyphenols, specifically phenolic acids and A-type proanthocyanidins, which are known to inactivate glucosyl-transferase and fructosyl-transferase that catalyse the formation of glucan and fructan, respectively, which play prime roles in biofilm formation and maturation [31]. It has also been reported that the polyphenols in cranberries led to desorption of biofilm by interfering with bacteria coaggregation [64]. Moreover, cranberries are supposed to reduce periodontal-related symptoms by suppressing inflammatory cascades as an immunologic response to bacteria invasion.

Despite the limitations of the study, and the great effect caused by the DMSO solvent, the research performed has identified an important anti-biofilm effect of cranberry on periodontal bacteria and serve as a support for the development of further studies, assessing the most effective vehicle and the ideal concentration to be used, without causing adverse effects on oral tissues.

Author Contributions: MCS and HRV contributed to conception and design of the study with the aid of EF, BB, MVMA, MS, and DH, analysis and interpretation of data and drafted the manuscript.

EF performed the statistical analyses. EF, BB, MVMA, MS, and DH critically revised the manuscript. All authors reviewed the original draft and read and approved the final manuscript.

Funding: This work was funded by the Spanish Ministry of Industry, Economy and Competitiveness (AGL2015–64522-C2-R project) and the Comunidad de Madrid (ALIBIRD-CM 2020 P2018/BAA-4343). It was also part of the activities of Dentaid Extraordinary Chair in Periodontal Research (*Cátedra Extraordinaria Dentaid en Investigación Periodontal*, University Complutense of Madrid, Spain).

Acknowledgments: We thank for their technical assistance to Luis M. Alonso and Alfonso Cortés, from the Centre of Microscopy and Cytometry from the Complutense University of Madrid.

Conflicts of Interest: The authors declare no conflict of interest. None of the funders had any role in designing and/or conducting of the study; collection, management, analysis and interpretation of the data; and preparation, review, or approval of the manuscript.

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Artículo #3

Artículo #3

Actividad antimicrobiana de EPA y DHA frente a bacterias patógenas orales utilizando un modelo de *biofilm* subgingival multiespecies *in vitro*

Resumen: En la búsqueda por productos naturales con propiedades antimicrobianas para su uso en la prevención y tratamiento de la periodontitis, el objetivo de esta investigación fue evaluar la actividad antimicrobiana de dos ácidos grasos omega-3, ácido docosahexaenoico (DHA) y ácido eicosapentaenoico (EPA), utilizando un modelo de *biofilm* subgingival multiespecies *in vitro* que incluye *Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* y *Aggregatiba actinomycetemcomitans*. Las actividades antimicrobianas de los extractos de EPA y DHA (100 μM), y los controles respectivos, se evaluaron en *biofilms* de 72 h por su inmersión durante 60 segundos. La actividad antimicrobiana fue evaluada por reacción cuantitativa en cadena de la polimerasa (qPCR), microscopía de láser confocal (CLSM) y microscopía electrónica de barrido (SEM). Se utilizó ANOVA con correcciones de Bonferroni para evaluar la actividad antimicrobiana de cada uno de los ácidos grasos. Tanto DHA como EPA redujeron significativamente ($p < 0.001$ en todos los casos) las cepas bacterianas utilizadas en este modelo de *biofilm*. Los resultados con CLSM fueron consistentes con los reportados con qPCR. Por SEM se evaluaron daños estructurales en algunas de las bacterias observadas. Se concluyó que, tanto DHA como EPA, tienen una actividad antimicrobiana significativa frente a las seis especies bacterianas incluidas en este modelo de *biofilm*.

nutrients

MDPI

Article

Antimicrobial Activity of EPA and DHA against Oral Pathogenic Bacteria Using an In Vitro Multi-Species Subgingival Biofilm Model

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Received: 31 July 2020; Accepted: 10 September 2020; Published: 14 September 2020

Abstract: In search for natural products with antimicrobial properties for use in the prevention and treatment of periodontitis, the purpose of this investigation was to evaluate the antimicrobial activity of two omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), using an in vitro multi-species subgingival biofilm model including *Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans*. The antimicrobial activities of EPA and DHA extracts (100 μM) and the respective controls were assessed on 72 h biofilms by their submersion onto discs for 60 s. Antimicrobial activity was evaluated by quantitative polymerase chain reaction (qPCR), confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). ANOVA with Bonferroni correction was used to evaluate the antimicrobial activity of each of the fatty acids. Both DHA and EPA significantly reduced ($p < 0.001$ in all cases) the bacterial strains used in this biofilm model. The results with CLSM were consistent with those reported with qPCR. Structural damage was evidenced by SEM in some of the observed bacteria. It was concluded that both DHA and EPA have significant antimicrobial activity against the six bacterial species included in this biofilm model.

Keywords: omega-3 fatty acids; docosahexaenoic acid; eicosapentaenoic acid; oral biofilms; confocal laser microscopy; PUFAs; prevention; periodontal diseases

1. Introduction

Periodontitis is a chronic inflammatory disease associated with dysbiotic subgingival biofilms and characterised by the progressive destruction of the tooth supporting apparatus. Its primary features include the loss of periodontal attachment to the tooth, manifested by clinical attachment loss, radiographic bone loss, and presence of periodontal pockets and gingival bleeding [1].

Although the majority of the microorganisms that colonise the oral cavity and dental surfaces are compatible with periodontal health [2], there are specific pathobionts that have shown pathogenicity by disrupting the host immune tolerance and causing a chronic unresolved inflammation in the periodontal tissues, leading to destructive changes in the connective and bone tissue metabolism [3,4]. In particular, *Porphyromonas gingivalis* has been identified as an example of keystone pathogen, with the capacity to augment the virulence of the entire microbial community through specific inter-bacterial

interactions, a characteristic feature of the "biofilm quorum sensing" [5,6], and the expression of certain molecules acting as virulence factors, like proteolytic enzymes or other pro-inflammatory molecules, that will induce a dysbiosis state by modifying the biofilm towards a pro-inflammophilic environment, thus promoting a non-resolving chronic inflammatory host response, what is characteristic of the subgingival biofilm in periodontitis. It should also be taken in consideration that differences intrinsic to the host response of each individual might influence the establishment and progression of the disease [7,8].

The current strategies to prevent and treat periodontitis are based in the reduction of the subgingival *biofilm* below a threshold compatible with homeostasis and health [9]. These strategies are based on mechanical root instrumentation, either non-surgically [10] or surgically [11], with or without adjunctive therapies, such as the use of locally and/or systemically delivered antimicrobials [12,13]. The use of adjunctive systemic antibiotics, although demonstrating a significant additional effect [13], may also cause unwanted effects [13–15], mainly associated with the development of bacterial resistances [16], which may represent a threat to global public health [17]. The use of antiseptics can also cause side effects, such as irritation of the mucous membranes, tooth staining or accelerated formation of dental calculus [18]. To overcome these limitations, the search for natural products with antimicrobial properties has been fostered and investigated [18–21].

One of these strategies has been the study of long-chain polyunsaturated fatty acids (PUFAs), fish and fish oil derivatives. PUFAs have demonstrated antimicrobial activity, with a broad spectrum inhibitory effect against various Gram-positive and Gram-negative bacteria [22–28]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have shown antibacterial activity against different oral bacterial pathogens, such as *Streptococcus mutans*, *Candida albicans*, *P. gingivalis*, *Fusobacterium nucleatum* and *Prevotella intermedia* [29]. Their mechanism of action seems to be through blocking essential bacterial processes at the level of the plasmatic membrane, such as the electron transport chain and the oxidative phosphorylation [30–35].

In addition to this antimicrobial effect, PUFAs are substrates for the cyclooxygenase and lipoxygenase pathways, actively promoting the lipoxygenase pathway, thus stimulating the synthesis of lipoxins involved in the resolution of inflammation, and blocking the cyclooxygenase pathway, thus inhibiting the secretion of prostaglandins, potent pro-inflammatory and bone resorting molecules [36]. This double potential activity (antimicrobial and anti-inflammatory) has increased attention to these natural compounds as possible adjunctive alternatives in the prevention and treatment of periodontitis. However, although there are reports on the antimicrobial activity of PUFAs using planktonic bacteria or monospecies biofilms [37], there are no reports using validated multispecies subgingival models which better resemble real conditions [38]. It was, therefore, the purpose of this investigation to to evaluate the antimicrobial activity of pure EPA and DHA against against oral pathogenic bacteria, using a validated multispecies in vitro biofilm model [38].

2. Materials and Methods

2.1. Omega 3 Fatty-Acids

The PUFAs independently evaluated in this investigation were EPA and DHA, obtained already solubilized in ethanol (Cerilliant®, Sigma-Aldrich, Barcelona, Spain).

2.2. Bacterial Strains and Culture Conditions

Reference strains of *Streptococcus oralis* CECT 907T, *Veillonella parvula* NCTC 11810, *Actinomyces naeslundii* ATCC 19039, *F. nucleatum* DMSZ 20482, *Aggregatibacter actinomycetemcomitans* DSMZ 8324, and *P. gingivalis* ATCC 33277 were used. These bacteria were grown on blood agar plates (Blood Agar Oxoid No 2; Oxoid, Basingstoke, UK), supplemented with 5% (*v*/*v*) sterile horse blood (Oxoid), 5.0 mg L^{-1} hemin (Sigma, St. Louis, MO, USA) and 1.0 mg L^{-1} menadione (Merck, Darmstadt, Germany) in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37 °C for 24–72 h.

2.3. Antibacterial Effect of EPA and DHA against Planktonic Bacteria

For determining which concentration of each of EPA and DHA was appropriate for the biofilm model assays, we undertook independent previous microtiter plate-based antibacterial assays for each of the studied fatty acids. In brief, pure cultures of the six selected bacterial strains were grown anaerobically in a protein rich medium containing brain–heart infusion (BHI) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 2.5 g L^{-1} mucin (Oxoid), 1.0 g L^{-1} yeast extract (Oxoid), 0.1 g L^{-1} cysteine (Sigma), 2.0 g L^{-1} sodium bicarbonate (Merck), 5.0 mg L^{-1} hemin (Sigma), 1.0 mg L^{-1} menadione (Merck), and 0.25% (*v*/*v*) glutamic acid (Sigma). At mid-exponential phase of bacterial growth (measured by spectrophotometry), bacteria were placed on a 96-well microtitre plates adding 200 µL of a mixture of each bacteria inoculum at a final concentration of 10^6 colony-forming units (CFUs) mL^{-1}, and EPA or DHA for a final concentration of 12.5, 25, 50, 100 and 200 µM. Plates had a set of controls: phosphate-buffered saline (PBS) was used as negative control; ethanol controls (adjusted to match the ethanol concentration present in each of the fatty acids (EtOH)); positive control (bacteria without any treatment). A measurement (optical density, O.D.595) as t = 0 absorbance was taken in a microtitre plate reader (Optic Ivymen System 2100-C; I.C.T.; La Rioja, Spain). The microplates were incubated for 48 h at 37 °C under anaerobic conditions, and absorbance was measured each 2 h, in order to determine the bacterial growth until reaching a stationary growth phase. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were calculated and confirmed by microbial plate counting on blood agar media. Accordingly, the lowest concentration of the DHA or EPA showing growth inhibition was considered as the MIC, whereas the lowest concentration of the DHA or EPA that showed zero growth in blood agar plates, after spot inoculation and incubation for 72 h, was recorded as the MBC. All experiments were performed in triplicate with appropriate controls.

2.4. Biofilm Development

A multispecies in vitro biofilm model was developed, as previously described by Sánchez and co-workers [38]. Briefly, pure cultures of each bacteria were anaerobically grown in a modified BHI liquid medium. Bacterial cultures were harvested at mid-exponential phase (measured by spectrophotometry), and a mixed bacteria suspension in modified BHI medium, containing 10^3 CFU mL^{-1} for *S. oralis*, 10^5 CFU mL^{-1} for *V. parvula* and *A. naeslundii*, and 10^6 CFU mL^{-1} for *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis*, was prepared. Sterile calcium hydroxyapatite (HA) discs, of 7 mm of diameter and 1.8 mm (standard deviation, SD = 0.2) of thickness (Clarkson Chromatography Products, Williamsport, PA, USA), were coated with treated saliva for 4 h at 37 °C in sterile plastic tubes, and then placed in the wells of a 24-well tissue culture plate (Greiner Bio-one, Frickenhausen, Germany). Each well was inoculated with 1.5 mL of mixed bacteria inoculum and incubated in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37 °C for 72 h. At 37 °C for 72 h, the timepoint in which the biofilm model reach maturity, containing all bacterial species at an optimal concentration to carry out the assay [38–40]. The plates employed for assessing the sterility of the culture medium were used as controls.

2.5. Antimicrobial Activity on Biofilms

The antimicrobial activities of EPA and DHA extracts (100 µM) were assessed independently on 72 h biofilms by their submersion onto discs for 60 s. Phosphate-buffered saline (PBS) was used as a negative control; ethanol controls (at the same concentration of the one in commercial DHA or EPA) were used to rule out the bactericidal effect of the solvent (EtOH); and 0.2% chlorhexidine (Sigma-Aldrich) was used as positive control.

All the independent sets of experiments for each of the PUFAs were repeated three times on different days using fresh bacterial cultures with trios of biofilms for each analysis.

2.6. Microbiological Outcomes: Quantitative Polymerase Chain Reaction (qPCR) Analysis

After the application of the tested products, treated biofilms were sequentially rinsed in 2 mL of sterile PBS three times (immersion time per rinse, 10 s), disrupted by vortex for 2 min in 1 mL of PBS and treated with a 100 μM concentration of propidium monoazide (PMA) (Biotium Inc., Hayword, CA, USA) to discriminate between DNA from live and dead bacteria [41]. Following an incubation period of 10 min at 4 °C in the dark, the samples were subjected to light-exposure for 30 min, using a PMA-Lite LED Photolysis Device (Biotium Inc.). After PMA photo-induced DNA cross-linking, the cells were centrifuged at 12,000 rpm for 3 min prior to DNA isolation. To avoid any influence of the experimental process on bacterial viability, the same procedure (incubation at 4 °C and exposure to light source) but without the exposure to PMA, was used as a negative control.

Bacterial DNA was isolated from all biofilms using a commercial kit ATP Genomic DNA Mini Kit® (ATP Biotech. Taipei, Taiwan), following manufacturer's instructions, and the hydrolysis 5'nuclease probe assay qPCR method was used for detecting and quantifying the bacterial DNA. The qPCR amplification was performed following a protocol previously optimized by our research group, using primers and probes targeted against 16S *rRNA* gene (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) [42]. Each DNA sample was analysed in duplicate. Quantification cycle (Cq) values, describing the PCR cycle number at which fluorescence rises above the baseline, were determined using the provided software package (LC 480 Software 1.5; Roche Diagnostic GmbH; Mannheim, Germany). Quantification of viable cells by qPCR was based on standard curves. The correlation between Cq values and CFU mL^{-1} was automatically generated by the software (LC 480 Software 1.5; Roche Diagnostic GmbH; Mannheim, Germany). All assays were run with a linear quantitative detection range established by the slope of 3.3–3.6 cycles/log decade, $r^2 > 0.997$ and an efficiency range of 1.9–2.0.

2.7. Confocal Laser Scanning Microscopy (CLSM) Analysis

The CLSM analyses were performed at the Centre for Cytometry and Fluorescence Microscopy of the Complutense University of Madrid (UCM), Spain. Hydroxyapatite containing the grown biofilms were washed three times sequentially in 2 mL sterile PBS (10 s immersion per wash) to remove any remnants of the extracts and non-binding bacteria. Three separate, representative locations were selected on the HA discs covered with fully hydrated biofilms (based on the presence of columns or towers of bacterial communities, identified in the confocal field of vision) and analysed with non-invasive confocal microscopy using an Ix83 Olympus fixed-phase microscope coupled to an Olympus FV1200 confocal system (Olympus, Shinjuku, Tokyo, Japan). The specimens were stained with the LIVE/DEAD® BacLight™ Bacterial Viability Kit solution (Molecular Probes B.V., Leiden, The Netherlands) at room temperature. Fluorochrome at a ratio of 1:1 was used with a staining time of 9 ± 1 min in order to obtain the optimal fluorescence signal at the corresponding wavelengths (Syto9: 515–530 nm, propidium iodide (PI): >600 nm). The CLSM software was programmed to perform a series z of scans (xyz) 1 μm thick (16 bits, 2048 × 2048 pixels). The images were analysed using Olympus® software (Olympus). Using the Fiji software (ImageJ Version 2.0.0-rc-65/1.52b, Open source image processing software), a live/dead analysis was performed in order to access the live/death ratio (green cells divided by the sum of green and red cells). Data were expressed as mean and SD.

2.8. Scanning Electron Microscopy (SEM) Analysis

The SEM analyses were performed at the National Centre of Electronic Microscopy (UCM, Madrid, Spain). Firstly, the samples were washed sequentially with 2 mL sterile PBS to remove non-binding bacteria on the HA disc and this process was repeated three times consecutively (10-s immersion per wash). After this, the samples were fixed with a solution of 4% paraformaldehyde (Panreac. Química, Barcelona, Spain) and 2.5% glutaraldehyde (Panreac. Química) for 4 h at 4 °C. Next, the samples were once again washed with PBS and sterile water (10 min immersion time per wash) and dehydrated

through a graduated series of ethanol solutions (30, 50, 70, 80, 90 and 100%; 10-min immersion time for each series). Then, the samples were dried by critical points, coated with gold by sputtering and analysed using electron microscopy, using a JSM 6400 electron microscope to do so (JSM 6400; JEOL, Tokyo, Japan), with a backscattered electron detector and an image resolution of 25 kV.

2.9. Statistical Analyses

The primary outcome variable was the number of viable bacteria present in the biofilm, measured by qPCR, for each tested bacterial species: *S. oralis*, *A. naeslundii*, *V. parvula*, *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*, expressed as CFU mL^{-1}. Form the mean values of each group, the percentage of reduction was calculated for DHA or EPA, EtOH and CHX when compared to the negative control (PBS) value [43]. As a secondary outcome, the live/death ratio obtained through the CLSM analysis was compared among the groups.

To determine the data distribution, box plots, asymmetry coefficients and Shapiro–Wilk tests were used. Data were expressed as means and SD. Two ANOVA tests with post-hoc Bonferroni corrections were performed in order to independently compare DHA or EPA versus PBS, EtOH and CHX. Data analysis was carried out using a dedicated computer software (IBM SPSS Statistics 24.0; IBM Corporation, Armonk, NY, USA) and the results were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Antibacterial Effect of DHA and EPA on Planktonic Bacteria

MICs and MBCs values against the six bacterial strains selected in planktonic state were determined for each of the fatty acids. In the case of DHA, the MICs were 50 µM for *S. oralis*, *A. naeslundii* and *V. parvula*; 100 µM for *F. nucleatum*; and 25 µM for *P. gingivalis* and *A. actinomycetemcomitans*. The MBCs were 100 µM for all the six bacterial species.

In the case of EPA, the MICs were 50 µM for *S. oralis* and *V. parvula,* and 100 µM for *A. naeslundii* and *F. nucleatum*, and 25 µM for *P. gingivalis* and *A. actinomycetemcomitans*. The MBCs were 100 µM for all six bacterial species. Based on these results, the 100 µM concentration was selected in the biofilm experiments with the two fatty acids.

3.2. Antibacterial Effect of the DHA Extract on Biofilm

3.2.1. Analysis by qPCR

The results of the antimicrobial effect of DHA extracts, compared with the negative control (PBS), EtOH and the positive control (CHX), are depicted in Table 1 and expressed as average counts of viable bacteria recovered from the 72 h biofilms (viable CFU mL^{-1}). Compared with the negative control (PBS), a statistically significant reduction ($p < 0.001$) in viable bacterial counts of the initial and early colonizers was observed. These reductions reached three orders of magnitude, amounting to 99.96% for *S. oralis*, 99.82% for *A. naeslundii* and 99.80% for *V. parvula* (Table 1). No statistically significant differences were found when comparing DHA with EtOH or CHX, although the magnitudes of reduction achieved by these controls were lower than with DHA (Table 2).

For the secondary coloniser *F. nucleatum*, statistically significant reductions ($p < 0.001$) were observed when comparing DHA to the negative control (99.92%). Differences were also statistically significant when comparing DHA with CHX ($p < 0.002$), with CHX showing a lesser effect compared with DHA. When comparing EtOH and CHX with the negative control, smaller differences were observed (Table 2).

For the periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans*, statistically significant reductions occurred after exposure with DHA, EtOH and CHX ($p < 0.001$, in all cases), when compared to the negative control. With DHA, these reductions were up to three orders of magnitude for

P. gingivalis (99.92%) and for *A. actinomycetemcomitans* (99.90%), while after exposure to EtOH or CHX, the reductions were of a single order of magnitude.

3.2.2. CLSM Analysis

In control biofilms, after 72 h of incubation, CLSM images showed the entire surface of the HA discs covered by bacteria, with a live/death ratio of 0.74 (SD = 0.07). These biofilms showed the morphological characteristics of multi-species bacterial communities grouped in "towers" (Figure 1A). After 60 s of exposure to the DHA extracts, the reduction in cell viability was pronounced (Figure 1C), with a live/death ratio of 0.11 (SD = 0.08). This reduction also occurred in the biofilms exposed to EtOH (Figure 1B), although it was of a smaller magnitude (0.49, SD = 0.08). Similar reductions were also observed when exposed to 0.2% CHX (Figure 1D), with a live/death ratio of 0.37 (SD = 0.07).

3.2.3. SEM Analysis

Biofilms were clearly identified by SEM on the HA discs after treatment with PBS, DHA, EtOH or CHX (Figure 2).

Table 1. Antibacterial effects of docosahexaenoic acid (DHA), as observed in the mean number of viable bacteria counts [colony forming units per mL (CFUs mL^{-1}), determined by quantitative polymerase chain reaction], evaluated in an in vitro multi-species biofilm model. Data are expressed as mean and standard deviation (SD). Differences are considered statistically significant at $p \leq 0.05$. PBS: phosphate buffer saline; DHA: 100 μM docosahexaenoic acid; EtOH: ethanol at the same concentration as the present in DHA; CHX: 0.2% chlorhexidine.

	Treatments	Mean (SD)	Global *p*	% of Reduction of Viable CFUs mL^{-1} as Compared with PBS
S. oralis	PBS	7.80×10^7 (3.46×10^7)	<0.001	
	DHA	3.49×10^4 (4.53×10^3)		99.96%
	EtOH	1.99×10^7 (1.23×10^7)		74.49%
	CHX	1.39×10^6 (3.81×10^5)		98.22%
A. naeslundii	PBS	3.22×10^6 (1.04×10^6)	<0.001	
	DHA	5.88×10^3 (1.51×10^3)		99.82%
	EtOH	1.58×10^6 (9.42×10^5)		51.04%
	CHX	3.23×10^5 (1.88×10^5)		90.07%
V. parvula	PBS	4.56×10^7 (9.86×10^6)	<0.001	
	DHA	8.83×10^4 (2.60×10^4)		99.80%
	EtOH	1.68×10^7 (5.40×10^6)		63.16%
	CHX	8.18×10^6 (9.59×10^6)		82.07%
F. nucleatum	PBS	2.24×10^6 (1.04×10^6)	<0.001	
	DHA	2.01×10^3 (8.68×10^2)		99.91%
	EtOH	7.36×10^5 (6.70×10^5)		67.15%
	CHX	1.62×10^6 (1.15×10^6)		27.68%
P. gingivalis	PBS	2.32×10^7 (8.31×10^6)	<0.001	
	DHA	1.77×10^4 (2.71×10^3)		99.92%
	EtOH	2.37×10^6 (1.20×10^6)		89.79%
	CHX	1.82×10^6 (3.13×10^6)		92.16%
A. actinomycetemcomitans	PBS	1.14×10^7 (4.84×10^6)	<0.001	
	DHA	1.12×10^4 (4.71×10^3)		99.90%
	EtOH	4.62×10^6 (2.25×10^6)		59.48%
	CHX	2.10×10^6 (5.64×10^5)		81.58%

Table 2. Comparisons between docosahexaenoic acid (DHA) and the controls used as observed in the mean number of viable bacteria counts (colony forming units per mL (CFUs mL^{-1}), determined by quantitative polymerase chain reaction) evaluated in an in vitro multi-species biofilm model. Differences are considered statistically significant at $p \leq 0.05$. PBS: phosphate buffer saline; DHA: 100 μM docosahexaenoic acid; EtOH: ethanol at the same concentration as the present in DHA; CHX: 0.2% chlorhexidine.

	Comparisons	Mean Difference	95% Confidence Interval for Difference		Post Hoc *p*
			Lower Bound	Upper Bound	
S. oralis	PBS-DHA	7.80×10^7	5.36×10^7	1.02×10^8	<0.001
	PBS-EtOH	5.81×10^7	3.37×10^7	8.25×10^7	<0.001
	PBS-CHX	7.66×10^7	5.23×10^7	1.01×10^8	<0.001
	EtOH-DHA	1.99×10^7	-4.47×10^6	4.43×10^7	0.170
	CHX-DHA	1.35×10^6	-2.30×10^7	2.57×10^7	1.000
	EtOH-CHX	1.86×10^7	-5.82×10^6	4.29×10^7	0.240
A. naeslundii	PBS-DHA	3.22×10^6	2.28×10^6	4.15×10^6	<0.001
	PBS-EtOH	1.64×10^6	7.05×10^5	2.58×10^6	<0.001
	PBS-CHX	2.90×10^6	1.96×10^6	3.84×10^6	<0.001
	EtOH-DHA	1.57×10^6	6.31×10^5	2.51×10^6	<0.001
	CHX-DHA	3.17×10^5	-6.23×10^5	1.26×10^6	1.000
	EtOH-CHX	1.25×10^6	3.14×10^5	2.19×10^6	0.004
V. parvula	PBS-DHA	4.55×10^7	3.57×10^7	5.53×10^7	<0.001
	PBS-EtOH	2.88×10^7	1.90×10^7	3.86×10^7	<0.001
	PBS-CHX	3.74×10^7	2.76×10^7	4.72×10^7	<0.001
	EtOH-DHA	1.67×10^7	6.89×10^6	2.65×10^7	<0.001
	CHX-DHA	8.09×10^6	-1.70×10^6	1.79×10^7	0.160
	EtOH-CHX	8.59×10^6	-1.21×10^6	1.84×10^7	0.115
F. nucleatum	PBS-DHA	2.24×10^6	1.12×10^6	3.36×10^6	<0.001
	PBS-EtOH	1.50×10^6	3.86×10^5	2.62×10^6	0.004
	PBS-CHX	6.15×10^5	-5.03×10^5	1.73×10^6	0.789
	EtOH-DHA	7.34×10^5	-3.84×10^5	1.85×10^6	0.445
	CHX-DHA	1.62×10^6	5.05×10^5	2.74×10^6	0.002
	EtOH-CHX	-8.89×10^5	-3.84×10^5	1.85×10^6	0.194
P. gingivalis	PBS-DHA	2.32×10^7	1.73×10^7	2.92×10^7	<0.001
	PBS-EtOH	2.09×10^7	1.49×10^7	2.68×10^7	<0.001
	PBS-CHX	2.14×10^7	1.55×10^7	2.73×10^7	<0.001
	EtOH-DHA	2.36×10^6	-3.58×10^6	8.29×10^6	1.000
	CHX-DHA	1.81×10^6	-4.13×10^6	7.74×10^6	1.000
	EtOH-CHX	5.50×10^5	-5.39×10^6	6.49×10^6	1.000
A. actinomycetemcomitans	PBS-DHA	1.14×10^7	7.81×10^6	1.49×10^7	<0.001
	PBS-EtOH	6.76×10^6	3.20×10^6	1.03×10^7	<0.001
	PBS-CHX	9.28×10^6	5.73×10^6	1.28×10^7	<0.001
	EtOH-DHA	4.61×10^6	1.06×10^6	8.17×10^6	0.006
	CHX-DHA	2.08×10^6	-1.47×10^6	5.64×10^6	0.654
	EtOH-CHX	2.53×10^6	-1.03×10^6	6.08×10^6	0.325

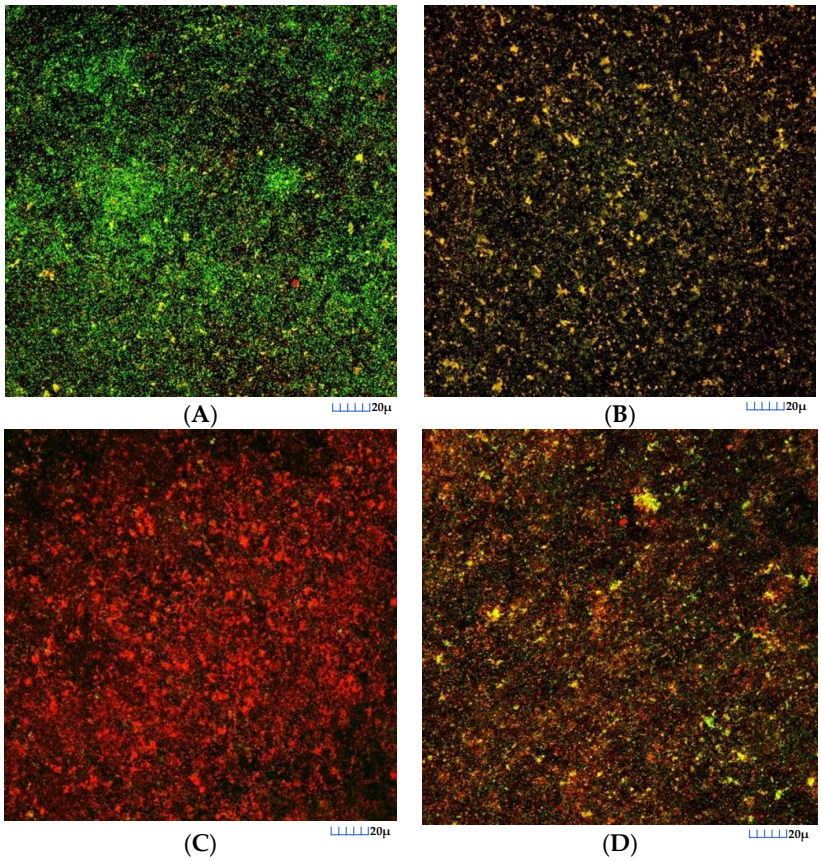

Figure 1. Maximum projection of images obtained by confocal laser scanning microscopy (CLSM) of the 72 h biofilms, where the growth of these biofilms was observed on the surfaces of the hydroxyapatite discs after 60 s of exposure: (**A**) to the negative control (phosphate buffer saline); (**B**) to the ethanol solution; (**C**) to the docosahexaenoic acid (DHA) extracts (100 µM concentration) and (**D**) to 0.2% chlorhexidine. Specimens were stained with the LIVE/DEAD® BacLightTM Bacterial Viability Kit solution, containing SYTO 9 and Propidium Iodide nucleic acid stains. Cells with a compromised membrane that are considered to be dead or dying were stain red (PI), whereas cells with an intact membrane were stain green (SYTO9).

On the HA discs treated with PBS (Figure 2A), bacterial growth on the surface of the discs was evident, with bacterial cells forming chains (characteristic of *Streptococcus* and *Aggregatibacter* species), or as multicellular aggregates, with a structural organisation based primarily on cell-to-cell co-aggregation. The fusiform bacilli, characteristic of *F. nucleatum* species, were also recognizable, forming three-dimensional structures resembling sessile communities.

The analysis of the discs treated with DHA (Figure 2B) showed marked differences compared to the control discs, such as a lower bacterial density in the surface, and a high proportion of bacteria with evident structural damage. The discs treated with EtOH (Figure 2C) or CHX (Figure 2D) also showed a reduction in bacterial density, but it was not as marked as in the discs treated with DHA (Figure 2B).

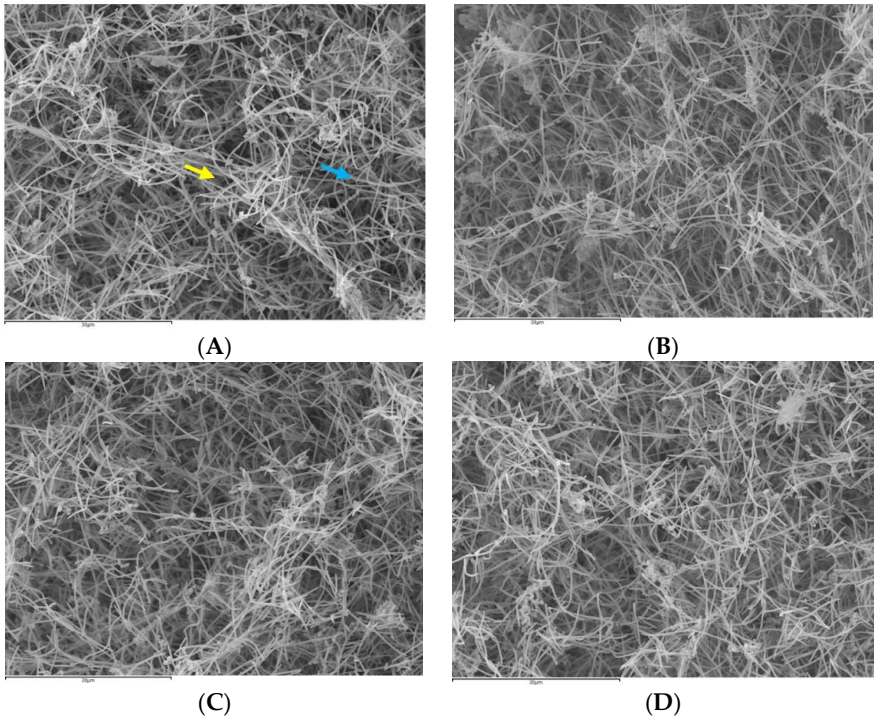

Figure 2. Scanning electron microscopy (SEM) of biofilms with an evolution of 72 h in hydroxyapatite (HA) discs treated with the negative control: phosphate buffer saline (PBS) (**A**), with docosahexaenoic acid (DHA) at 100 µM (**B**), with EtOH (**C**) or with the positive control: 0.2% chlorhexidine (CHX) (**D**). A dense bacterial population could be observed on the HA discs treated with PBS (**A**), forming discontinuous layers of bacteria bonded to the discs. Meanwhile, on the biofilms of the discs treated with DHA (**B**), a lower density of cells distributed across the surface of the disc could be seen, and some of these exhibited structural damages. Likewise, on the discs treated with EtOH (**C**) or CHX (**D**), a reduction in the bacterial density present on the surface of the disc could also be observe, although it was lower than that on the discs treated with DHA (**B**). Chains of *Aggregatibacter* and/or *Streptococcus* (blue arrow) and fusiform bacilli of the *F. nucleatum* genus (yellow arrow) could be identified. Magnification (**A**–**D**): 1500×. The samples were dried by critical points and coated with gold by sputtering.

3.3. Antibacterial Effect of the EPA Extract

3.3.1. qPCR Analysis

The antimicrobial effects of the EPA extracts, compared to the negative control (PBS), EtOH and the positive control (CHX), are depicted in Table 3, expressed as average counts of viable bacteria recovered from the 72 h biofilms.

Compared to the negative control (PBS), statistically significant reductions in viable bacteria counts of *S. oralis* were seen after the exposure to EPA ($p < 0.001$), reaching a reduction of 97.16% in CFUs mL^{-1} (Table 3). Compared to EtOH ($p = 0.144$) and CHX 0.2% ($p = 1.000$), no statistically significant differences were observed (Table 4).

For *A. naeslundii* and *V. parvula*, statistically significant reductions (98.36% and 95.43%, respectively) occurred after exposure to EPA ($p < 0.001$). With EtOH and CHX, the bacterial counts were also significantly reduced, albeit to a lesser magnitude. For *V. parvula*, statistically significant differences were also found when comparing the effect of EPA with EtOH ($p < 0.003$). For *F. nucleatum*, statistically significant reductions (98.67%) occurred with the exposure to EPA ($p < 0.001$), with a similar lesser effect when EtOH and CHX 0.2% were applied. Furthermore, the effect of EPA versus CHX was

statistically significant ($p < 0.001$). For *P. gingivalis* and *A. actinomycetemcomitans*, statistically significant reductions occurred after the exposure to EPA (97.51% and 91.36%, respectively). Similar significant reductions were observed with EtOH and CHX ($p < 0.001$), when compared with PBS. For *P. gingivalis*, statistically significant differences were found when comparing exposure to the EPA extracts versus EtOH ($p = 0.002$) and versus CHX ($p = 0.014$). For *A. actinomycetemcomitans*, no differences were found, either when comparing exposure to EPA versus EtOH ($p = 0.676$) or versus CHX ($p = 0.423$) (Table 4).

3.3.2. CLSM Analysis

In control biofilms, after 72 h of incubation, CLSM images showed that the entire surface of the HA discs was covered by live bacteria, with a live/death ratio of 0.75 (SD = 0.08). Biofilms depicted the characteristic morphology of bacterial communities grouped in multi-species "towers" (Figure 3A). In contrast, after 60 s of exposure to the EPA extracts, the reduction in cell viability was very pronounced (Figure 3C) being the live/death ratio 0.15 (SD = 0.09). This reduction also occurred in the biofilms exposed to EtOH (Figure 3B), although it was of a smaller magnitude 0.53 (SD = 0.09). Similar reductions were observed when applying 0.2% CHX (Figure 3D) where the live/death ratio 0.39 (SD = 0.06). These results were consistent with the qPCR results.

Table 3. Antibacterial effects of eicosapentaenoic acid (EPA) as observed in the mean number of viable bacteria counts (colony-forming units per mL (CFUs mL^{-1}), determined by quantitative polymerase chain reaction), evaluated in an in vitro multi-species biofilm model. Data are expressed as mean and standard deviation (SD). Differences are considered significant at $p \leq 0.05$. PBS: phosphate buffer saline; EPA: 100 µM eicosapentaenoic acid; EtOH: ethanol at the same concentration as that present in EPA; CHX: 0.2% chlorhexidine.

	Treatments	Mean (SD)	Global *p*	% of Reduction in Viable CFUs mL^{-1} as Compared with PBS
S. oralis	PBS	4.71×10^7 (1.38×10^7)	<0.001	
	EPA	1.34×10^6 (5.12×10^5)		97.16%
	EtOH	9.17×10^6 (2.46×10^6)		80.54%
	CHX	1.64×10^6 (7.58×10^5)		96.52%
A. naeslundii	PBS	3.63×10^6 (1.47×10^6)	<0.001	
	EPA	5.98×10^4 (1.82×10^4)		98.36%
	EtOH	6.08×10^5 (1.91×10^5)		83.25%
	CHX	3.66×10^5 (1.30×10^5)		89.92%
V. parvula	PBS	6.43×10^7 (1.66×10^7)	<0.001	
	EPA	2.94×10^6 (8.30×10^5)		95.43%
	EtOH	2.10×10^7 (3.14×10^6)		67.34%
	CHX	1.80×10^7 (1.08×10^7)		72.01%
F. nucleatum	PBS	2.16×10^6 (9.67×10^5)	<0.001	
	EPA	2.88×10^4 (1.45×10^4)		98.67%
	EtOH	6.66×10^5 (5.88×10^5)		69.17%
	CHX	1.24×10^6 (4.58×10^5)		42.60%
P. gingivalis	PBS	1.27×10^7 (1.60×10^6)	<0.001	
	EPA	3.16×10^5 (1.55×10^5)		97.51%
	EtOH	1.92×10^6 (5.04×10^5)		84.88%
	CHX	1.63×10^6 (2.18×10^5)		87.17%
A. actinomycetemcomitans	PBS	6.84×10^6 (3.40×10^6)	<0.001	
	EPA	5.91×10^5 (3.18×10^5)		91.36%
	EtOH	2.07×10^6 (7.91×10^5)		69.74%
	CHX	2.29×10^6 (1.62×10^6)		66.52%

Table 4. Comparisons between eicosapentaenoic acid (EPA) and the controls used as observed in the mean number of viable bacteria counts (colony-forming units per mL (CFUs mL^{-1}), determined by quantitative polymerase chain reaction), evaluated in an in vitro multi-species biofilm model. Differences were considered significant at $p \leq 0.05$. PBS: phosphate buffer saline; EPA: 100 μM eicosapentaenoic acid; EtOH: ethanol at the same concentration as the present in EPA; CHX: 0.2% chlorhexidine.

	Comparisons	Mean Difference	95% Confidence Interval for Difference		Post Hoc *p*
			Lower Bound	Upper Bound	
S. oralis	PBS-EPA	4.57×10^7	3.64×10^7	5.50×10^7	<0.001
	PBS-EtOH	3.79×10^7	2.86×10^7	4.72×10^7	<0.001
	PBS-CHX	4.54×10^7	3.61×10^7	5.47×10^7	<0.001
	EtOH-EPA	7.83×10^6	-1.47×10^6	1.71×10^7	0.144
	CHX-EPA	2.96×10^5	-9.00×10^6	9.59×10^6	1.000
	EtOH-CHX	7.53×10^6	-1.76×10^6	1.68×10^7	0.177
A. naeslundii	PBS-EPA	3.57×10^6	2.59×10^6	4.56×10^6	<0.001
	PBS-EtOH	3.02×10^6	2.04×10^6	4.01×10^6	<0.001
	PBS-CHX	3.27×10^6	2.28×10^6	4.25×10^6	<0.001
	EtOH-EPA	5.48×10^5	-4.39×10^5	1.54×10^6	0.768
	CHX-EPA	3.06×10^5	-6.81×10^5	1.29×10^6	1.000
	EtOH-CHX	2.42×10^5	-7.45×10^5	1.23×10^6	1.000
V. parvula	PBS-EPA	6.14×10^7	4.81×10^7	7.47×10^7	<0.001
	PBS-EtOH	4.33×10^7	3.00×10^7	5.66×10^7	<0.001
	PBS-CHX	4.63×10^7	3.30×10^7	5.96×10^7	<0.001
	EtOH-EPA	1.81×10^7	4.82×10^6	3.14×10^7	0.003
	CHX-EPA	1.51×10^7	1.81×10^6	2.84×10^7	0.019
	EtOH-CHX	3.00×10^6	-1.03×10^7	1.63×10^7	1.000
F. nucleatum	PBS-EPA	2.13×10^6	1.32×10^6	2.94×10^6	<0.001
	PBS-EtOH	1.50×10^6	6.88×10^5	2.31×10^6	<0.001
	PBS-CHX	9.27×10^5	1.18×10^5	1.74×10^6	0.018
	EtOH-EPA	6.37×10^5	-1.72×10^5	1.45×10^6	0.205
	CHX-EPA	1.21×10^6	3.98×10^5	2.02×10^6	0.001
	EtOH-CHX	-5.70×10^5	-1.38×10^6	2.39×10^5	0.336
P. gingivalis	PBS-EPA	1.23×10^7	1.12×10^7	1.35×10^7	<0.001
	PBS-EtOH	1.07×10^7	9.61×10^6	1.19×10^7	<0.001
	PBS-CHX	1.10×10^7	9.90×10^6	1.21×10^7	<0.001
	EtOH-EPA	1.60×10^6	4.77×10^5	2.73×10^6	0.002
	CHX-EPA	1.32×10^6	1.93×10^5	2.44×10^6	0.014
	EtOH-CHX	2.84×10^5	-8.41×10^5	1.41×10^6	1.000
A. actinomycetemcomitans	PBS-EPA	6.25×10^6	3.70×10^6	8.81×10^6	<0.001
	PBS-EtOH	4.77×10^6	2.21×10^6	7.33×10^6	<0.001
	PBS-CHX	4.55×10^6	2.00×10^6	7.11×10^6	<0.001
	EtOH-EPA	1.48×10^6	-1.07×10^6	4.04×10^6	0.676
	CHX-EPA	1.70×10^6	-8.55×10^5	4.26×10^6	0.423
	EtOH-CHX	-2.18×10^5	-2.77×10^6	2.34×10^6	1.000

3.3.3. SEM Analysis

Biofilm formation was clearly observed on the HA discs at 72 h after treatment with PBS, EPA, EtOH or CHX (Figure 4).

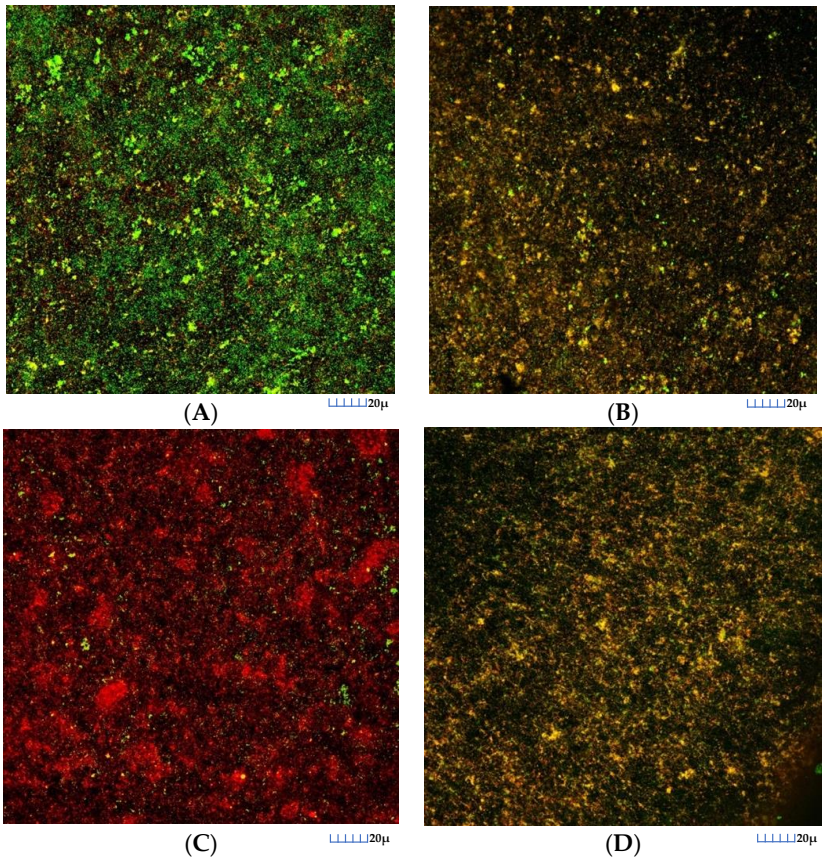

Figure 3. Maximum projection of images obtained by confocal laser scanning microscopy (CLSM) of the 72 h biofilms, where the growth of these biofilms was observed on the surfaces of the hydroxyapatite discs, stained with LIVE/DEAD® BacLightTM Bacterial Viability Kit, after 60 s of exposure: (**A**) to the negative control (phosphate buffer saline); (**B**) to the ethanol solution; (**C**) to the docosahexaenoic acid (EPA) extracts (100 µM concentration) and (**D**) to 0.2% chlorhexidine. Specimens were stained with the LIVE/DEAD® BacLightTM Bacterial Viability Kit solution, containing SYTO 9 and Propidium Iodide nucleic acid stains. Cells with a compromised membrane that are considered to be dead or dying were stained red (PI), whereas cells with an intact membrane were stained green (SYTO9).

On the HA discs treated with PBS (Figure 4A), there was evidence of bacterial growth on the surface of the discs with identification of bacterial cells forming chains (*Streptococcus* and *Aggregatibacter* species) and the characteristic fusiform bacilli (*F. nucleatum* species), forming three-dimensional sessile communities. The analysis of the discs treated with EPA (Figure 4B) showed a lower bacterial density in the surface, with the presence of bacteria with evident structural damage. The discs treated with EtOH (Figure 4C) or CHX (Figure 4D) also showed a reduction in bacterial density, but it was not as marked as in the discs treated with EPA (Figure 4B).

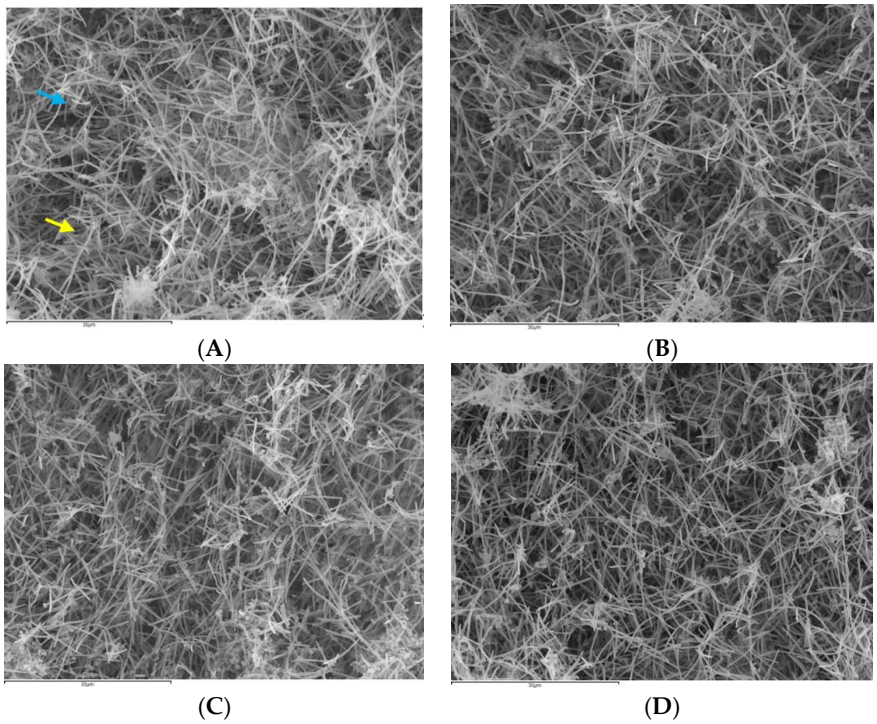

Figure 4. Scanning electron microscopy (SEM) of biofilms with an evolution of 72 h in hydroxyapatite (HA) discs treated with the negative control: phosphate buffer saline (PBS) (**A**), with docosahexaenoic acid (EPA) at 100 µM (**B**), with ethanol (**C**) or with the positive control: 0.2% chlorhexidine (CHX) (**D**). A dense bacterial population could be observed on the HA discs treated with PBS (**A**), forming discontinuous layers of bacteria bonded to the discs. Meanwhile, on the biofilms of the discs treated with EPA (**B**), a lower density of cells distributed across the surface of the disc could be seen, and some of these exhibited structural damages. Likewise, on the discs treated with EtOH (**C**) or CHX (**D**), a reduction could also be observed in the bacterial density present on the surface of the disc, although this reduction was slighter than that on the discs treated with EPA (**B**). Chains of *Aggregatibacter* and/or *Streptococcus* (blue arrow) and fusiform bacilli of the *F. nucleatum* genus (yellow arrow) could be identified. Magnification (**A**–**D**): 1500×. The samples were dried by critical points and coated with gold by sputtering.

4. Discussion

The results of the present investigation provide evidence that the application of EPA or DHA extracts, on mature biofilms grown on HA discs, significantly reduced the bacterial counts and the cell viability in the six bacterial strains used in this in vitro multispecies biofilm model (*S. oralis*, *A. naeslundii*, *V. parvula*, *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans*).

These results are concordant with previous investigations that have shown an antimicrobial antiseptic effect of PUFAs extracts [33], mainly EPA and DHA extracts found naturally in many marine organisms [44,45]. In addition, and beyond their antimicrobial activity (confirmed in the present study), it should be highlighted that PUFAs have also demonstrated anti-inflammatory [25–28,44–47] and antioxidant [48] properties.

These previous investigations reporting antibacterial effects of EPA and DHA used single-bacteria biofilm models in planktonic growth [29,49]. Sun et al., in 2016, investigated the potential effects of EPA and DHA against periodontal bacteria as mono-species biofilms in planktonic state, demonstrating significant antimicrobial activity against *P. gingivalis* and *F. nucleatum* [37]. However, it is widely known that microorganisms have different properties when growing within multispecies biofilms,

compared to their planktonic state, such as resistance to antimicrobial agents [50–55]. A number of studies indicate that the MIC of an organism may increase 2- to 1000-fold when growing within a biofilm [52], being at least 250 times greater than the MIC of the same species in a planktonic state [56]. In fact, the bacterial resistance to antimicrobials appears to be related to the maturation of the biofilm, with maximum resistance coinciding with the stationary growth phase of the biofilm [56,57]. For these reasons, an in vitro multi-species biofilm model was used in the present investigation. This model has been previously validated by Sanchez et al. and it included six species commonly present in subgingival biofilms (*S. oralis*, *A. naeslundii*, *V. parvula*, *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans*) [38]. In the present investigation, EPA and DHA were evaluated independently, compared with negative and positive controls, demonstrating that DHA extracts may have a superior effect, against these controls, although a direct comparison between the extracts was not provided. The superior effect showed by DHA may be due to its smaller three-dimensional molecular structure, which may facilitate its diffusion through the biofilm extracellular matrix.

The mechanisms that underline the observed bacterial inhibitory effect of EPA or DHA against oral pathogens are still unknown. Possibly—as described for other PUFAs—the fatty acids are incorporated into the cell plasma membrane, resulting in greater membrane fluidity and permeability, thus affecting its integrity, which leads to cell death [23,29]. This effect could be exacerbated by the presence of unsaturated double bonds that exert a direct toxic effect on the bacterial cell membrane [25]. At concentrations of 100 μM, DHA and EPA are not cytotoxic to human oral tissue cells [37], or to C2C12 myoblasts [58], but maintain their antibacterial activity. In the present investigation, we have used a concentration of 100 μM and the SEM observations have shown not only a reduction in the number of bacteria present, but also distinct morphological changes with observable structural damage. These observations coincide with previous observations, also using 100 μM of EPA and DHA [37]. In addition, similar events have been described for other bacterial species upon exposure to DHA, such as *Burkholderia cenocepacia*, [28], or *Helicobacter pylori* [25].

The quantitative analysis showed that EPA and DHA, when compared to the controls, significantly reduced the bacterial viability of the tested species ($p < 0.001$). In the case of *V. parvula*, *F. nucleatum* or *P. gingivalis*, they showed greater antimicrobial activity than CHX, an antiseptic considered as the gold standard for use in oral mouthwash formulations [18]. The effect of the tested extracts in reducing cell viability was also observed in the CLSM analysis. The discs treated with both DHA and EPA, although having a more pronounced effect with DHA, showed a low cell viability. These CLSM results were fully congruent with the qPCR results. It was noticeable the higher antimicrobial effect of DHA and EPA, when compared with CHX, has shown a marked antibacterial effect in multispecies biofilm models, both in vitro and in vivo [40,59]. One of the reported advantages of CHX molecules is their binding capability to oral tissues, which allows its slow and continuous release [60,61] for up to 12 h (substantivity) [62]. The possible substantivity and pharmacokinetics of EPA and DHA extracts are currently unknown, and therefore further investigations are required before considering these extracts as real alternatives to currently used oral antiseptics.

Although the study is the first to demonstrate the antibacterial effect of EPA or DHA extracts in a validated multi-species in vitro biofilm model [38], there are some limitations that should be highlighted. Firstly, only six bacterial species were used, in comparison with the hundreds present in naturally occurring subgingival biofilms. Furthermore, the model used represents a static biofilm model, and in its current design, the effect of the immune system cells is not involved. Although this model has a mixed population of six bacterial strains representing the initial, early and late colonizers present in subgingival biofilms, it does not have the diversity usually found in natural subgingival biofilms, as these can reach 200 species. Our model also only includes one strain of each bacterial species, which can provide different results, especially when regarding eventual evolutionary mechanisms of response to the PUFAs. In regard to the extracts analysed, their commercial formulation entails their dissolution in ethanol at different concentrations. In order to rule out the possibility that the potential antiseptic effect of these agents was due to ethanol, rather than to the extracts tested, ethanol was

included in the experiments as a control (at the same concentration found in each of the commercial products), thus demonstrating the specific antibacterial effect of the PUFA extracts. On the other hand, the findings of this research are expected to be comparable to other artificial surfaces used in dentistry, for example titanium and zirconia [40].

Besides the presented limitations of the study, this kind of models should be regarded as a primary step in the research process, identifying possible candidate molecules to be investigated in studies with a higher level of scientific evidence.

5. Conclusions

The results of the present study provide evidence that EPA or DHA extracts demonstrated a relevant antibacterial capacity against six bacterial species in a validated in vitro biofilm model. Specifically, EPA or DHA extracts (at 100 μM) resulted in statistically significant reductions in the CFU mL^{-1} of *S. oralis*, *A. naeslundii*, *V. parvula*, *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* after 60 s of exposure.

Further research is needed in order to evaluate the possible use of PUFAs in the chemical plaque control during the management of periodontitis.

Author Contributions: H.R.-V. and M.C.S. contributed to conception and design of the study with the aid of A.A.-E., E.F., M.J.C., L.C., D.H., and M.S., analysis and interpretation of data and drafted the manuscript. E.F. performed the statistical analyses. M.J.C., L.C., D.H., and M.S. critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was part of the activities of Dentaid Extraordinary Chair in Periodontal Research (Cátedra Extraordinaria Dentaid en Investigación Periodontal, University Complutense of Madrid, Spain).

Acknowledgments: We thank for their technical assistance to: Luis M. Alonso and Alfonso Cortés, from the Center for Cytometry and Fluorescence Microscopy of the Complutense University of Madrid (UCM); Ana M. Vicente Montaña from the Spanish National Centre for Electron Microscopy (ICTS).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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VI
Discusión

Discusión

Los resultados de los estudios incluidos en este trabajo proporcionan valiosos conocimientos que podrían ser utilizados en el futuro para estudiar, en estudios *in vivo,* el efecto de los productos naturales estudiados sobre las enfermedades periodontales, ya que los diferentes productos probados en este trabajo han demostrado, con mayor o menor intensidad, un efecto antimicrobiano en el modelo de *biofilm* multiespecies empleado.

En el primer estudio (Estudio #1, (170)), se evaluó la capacidad antibacteriana del vino tinto y el vino tinto desalcoholizado, así como la de los extractos enológicos ricos en antocianina Provinols™, y Vitaflavan®, un extracto de semilla de uva enológica, frente a un modelo de *biofilm* subgingival *in vitro* multiespecie.

Los resultados obtenidos en el modelo de *biofilm in vitro* utilizado han demostrado que, tanto el vino tinto alcohólico como el desalcoholizado, presenta cierta capacidad antimicrobiana frente a los patógenos periodontales *F. nucleatum, P. gingivalis* y *A. actinomycetemcomitans*. Se necesitaron 5 minutos de exposición a las soluciones de vino tinto para tener un efecto significativo en la vitalidad de las cepas bacterianas incluidas en *biofilm*, excepto con *P. gingivalis*, que presentaba una reducción significativa en el número de UFC mL^{-1} después de sólo 1 minuto de exposición. Este efecto también se reflejó en el número total de bacterias en *biofilm*. Al comparar los efectos antimicrobianos del vino tinto con alcohol y del vino tinto desalcoholizado, después de 1 y 5 minutos de exposición respectivamente, no se observaron diferencias estadísticamente significativas entre ellos.

Estas observaciones concuerdan con los hallazgos descritos por Furiga y colaboradores, que estudiaron la actividad *antibiofilm* de diversos extractos obtenidos de *Vitis vinifera* (Vitaceae) frente *biofilms* artificiales, compuestos principalmente por *S. mutans*, *S. sobrinus*, *L. rhamnosus*, *P. gingivalis* y *F. nucleatum* (112, 113). Los resultados también coinciden con los descritos por Muñoz-González y colaboradores, en 2014, quienes describieron el efecto antibacteriano beneficioso de los vinos en la cavidad oral, con un alto nivel de

actividad bactericida frente, entre otros, *A. oris*, *F. nucleatum* y *S. oralis* (116). Otro grupo de investigación ha estudiado también el efecto antiséptico del vino tinto desalcoholizado (110), observando su poderosa actividad frente a diferentes estreptococos orales, que era incluso mayor que el obtenido con los vinos blancos, que no tienen antocianinas. Este hallazgo refuerza la teoría de la actividad antimicrobiana de los componentes del vino tinto distintos del etanol, y centra la atención en el efecto potencial sobre las antocianinas (sólo presentes en la variedad de vino tinto).

Además, en este estudio (Estudio #1,(170)) también se han estudiado los efectos antibacterianos de un extracto de polifenoles del vino (Provinols™), particularmente rico en antocianinas, y otro extracto enológico obtenido a partir de semilla de uva (Vitaflavan®). Después de 30 segundos de exposición, se confirmó que la vitalidad bacteriana se vio significativamente afectada, con la excepción de *P. gingivalis*. Después de 1 minuto de tratamiento con los extractos enológicos, todas las bacterias experimentaron una reducción en la vitalidad, que disminuyó significativamente en comparación con el control de PBS. Una vez más, la excepción fue *P. gingivalis* cuando expuesta a Vitaflavan®. Los extractos polifenólicos del vino (Provinols™) mostraron un mayor efecto, pero no se encontraron diferencias estadísticamente significativas, en ninguno de los casos, en la actividad antimicrobiana de ambas soluciones, cuando se comparó después de 30 segundos y 1 minuto de exposición. Otro grupo de investigadores obtuvieron resultados similares, que demostraron las propiedades antibacterianas frente a *A. actinomycetemcomitans* (171).

Aunque no se realizó una comparación directa del vino tinto (tanto con como sin alcohol) con los extractos de polifenoles del vino, los datos de reducción de las UFC mL^{-1} sugieren que el vino tinto tiene un efecto más potente sobre el modelo de *biofilm* estudiado, lo que sugiere el posible papel clave desempeñado por las antocianinas. Estos resultados difieren, sin embargo, de los reportados en 2012 por Cueva y colaboradores, que mostraron que los extractos de semilla de uva y Vitaflavan® y su fracción oligomérica jugaron un mayor papel antimicrobiano contra varios patógenos orales que los extractos restantes estudiados (extracto de vino tinto y extracto de la semilla de la uva)

(172).

La justificación de la actividad antimicrobiana demostrada en el Estudio #1 (170) se basa en que las uvas y el vino son una fuente abundante de componentes polifenólicos, incluyendo ácidos hidroxibenzoicos e hidroxicinnámicos, alcoholes fenólicos, monómeros Flavan-3-ole, procianidinas poliméricas y oligoméricas, flavonoles, estilbenos y antocianinas (que sólo están presentes en la variedad de vino tinto) (173) con un potencial efecto antimicrobiano. Este efecto ha sido justificado por la secreción de bacteriocinas durante la fermentación alcohólica y maloláctica, dotándolas de un papel antimicrobiano (174). Se ha investigado también la actividad antibacteriana *in vitro* del vino tinto y blanco contra las Enterobacteriaceae patógenas (175). También se ha descrito la actividad antimicrobiana de diferentes vinos frente a seis cepas de *Helicobacter pylori* a partir de biopsias gástricas (176, 177). En 2005, un otro grupo de investigación llevó a cabo una investigación sobre la actividad antibacteriana contra las bacterias patógenas Gram-positivas y Gram-negativas de los vinos caseros e industriales (178). Más recientemente, se observó el efecto de los polifenoles del vino y los extractos enológicos en el crecimiento de bacterias patógenas asociadas a enfermedades respiratorias (172).

Una consideración importante en experimentos basados en polifenoles es la solubilidad de estos compuestos. Muchos polifenoles son insolubles en agua, por lo que normalmente se utilizan solventes como el dimetil sulfóxido (DMSO), metanol o etanol. Además, la composición del vino tinto incluye el etanol, normalmente entre 10-12%, lo que le confiere un papel antibacteriano en sí mismo. Al analizar los datos, el hecho de que la mayoría de los disolventes tengan una capacidad antibacteriana puede ser un problema. Los resultados obtenidos confirman que DMSO a una concentración del 4,0% (v/v), utilizada para solubilizar los extractos, tiene algún efecto sobre la vitalidad de las cepas incluidas en el estudio. Por el contrario, el tratamiento con un 12%de etanol, emulando el contenido de alcohol del vino tinto, dio lugar a una disminución considerable de la población de bacterias vitales. Estas observaciones se corresponden con las descritas por Muñoz-González y colaboradores, en 2014

(116), quienes estudiaron los efectos antimicrobianos del vino tinto con y sin alcohol, en un modelo de *biofilm* supragingival, y muestra como *F. nucleatum*, después de la exposición al 12% de etanol, experimentó una reducción significativa en su cantidad. La conclusión extraída por estos autores, como en nuestro caso, es que otros componentes del vino también deben tener esta capacidad frente a esta especie bacteriana, además de la capacidad antimicrobiana intrínseca del 12% de etanol.

Esta investigación (Estudio #1,(170)) ha demostrado que el vino tinto, con o sin alcohol, así como los extractos de origen enológico, tienen capacidad de reducir la vitalidad bacteriana en las cepas bacterianas presentes en el modelo de *biofilm* utilizado. No obstante, son necesarios más estudios para comprender mejor las vías por las cuales hay interacciones entre los diferentes polifenoles presentes en el vino y su actuación sobre los *biofilms* orales. La mejor comprensión de estas vías podrá un día permitir el desarrollo de herramientas que puedan ayudar en la prevención y tratamiento de las enfermedades periodontales.

En el segundo estudio incluido en esta tesis (Estudio #2,(179)) se analizó la capacidad antimicrobiana y anti-adhesión de extractos de arándano rojo, ricos en polifenoles utilizando un modelo *in vitro* de *biofilm* subgingival multiespecie.

El arándano rojo es un fruto que es especialmente rico en compuestos biológicamente activos. Entre ellos se incluyen ácidos fenólicos, flavonoides, antocianinas, proantocianidinas tipo A y ácidos orgánicos (180).

Diferentes estudios *in vivo* y *in vitro* han demostrado que los diferentes compuestos presentes en este fruto presentan capacidades antimicrobianas frente a diversas bacterias patogénicas, tanto Gram-positivas como Gram-negativas. Entre ellas, algunas responsables por infecciones urinarias y otros tipos de infecciones sistémicas (132, 181, 182).

Los resultados del Estudio #2 (179) indican que la vitalidad bacteriana se ve afectada por la exposición a extractos de arándanos (20.000 g mL^{-1}) durante

30 y 60 segundos, cuando las seis especies bacterianas están organizadas en *biofilms*. Sin embargo, aunque se logra un efecto sustancial contra los colonizadores primarios *S. oralis*, *A. naeslundii* y *V. parvula,* nuestros resultados, en línea con otros estudios, no demuestran ningún efecto significativo frente a *F. nucleatum*, *P. gingivalis*, o *A. actinomycetemcomitans*. Estos resultados concuerdan con el estudio de La y colaboradores, en 2010, en el que las proantocianidinas tipo A no tuvieron ningún impacto en el crecimiento de *P. gingivalis* en estado planctónico (139).

La capacidad antimicrobiana de diferentes frutos rojos frente a *biofilms* monoespecie de *S. mutans,* cepa bacteriana asociada a la caries, ha sido evaluada en diferentes estudios, pero sin encontrar efectos significativos (137, 183). Este tipo de hallazgos puede estar justificado por la presencia de *biofilms* maduros cuya biomasa dificulta la penetración de los productos testados (184). Sin embargo, la reducción de la vitalidad bacteriana observada en el presente trabajo, aunque discreta (y no estadísticamente significativa), podría respaldar observaciones *in vivo* anteriores, que mostraron que los extractos de arándano (sumo concentrado de arándano, en forma de gel termorreversible) poseen propiedades antimicrobianas contra *P. gingivalis* y *A. actinomycetemcomitans* (185). Por otro lado, las diferencias entre la investigación anteriormente citada y la nuestra (Estudio #2, referencia) pueden deberse a la distinta metodología para estudiar el efecto antibacteriano y las diferencias en las formulaciones de arándano utilizadas.

Es de destacar por otro lado, que en nuestra investigación (Estudio #2,(179)), además de la capacidad antibacteriana, se ha evaluado también la capacidad anti-adhesión. Para que se puedan formar los complejos *biofilms* asociados con la periodontitis, es esencial la adhesión de los colonizadores a las superficies dentales. En este campo, los extractos de arándano con la concentración de 200 μg mL^{-1} han sido capaces de interferir significativamente con la formación de *biofilm* y de la incorporación de las seis cepas bacterianas incluidas en el modelo utilizado. No solamente los colonizadores primarios se vieron afectados (especialmente en la evaluación de las 6 h), sino que este hallazgo ha sido especialmente notorio al evaluar la incorporación de *F.*

nucleatum, *P. gingivalis* y *A. actinomycetemcomitans*. Numerosos estudios han investigado el papel de los componentes del arándano en la adhesión bacteriana y en el desarrollo de *biofilms* (109, 134, 138, 183, 186, 187). La investigación de Philip y colaboradores, en 2019, ha concluido que el extracto de arándano es el extracto más eficaz para interrumpir la integridad y la arquitectura estructural del *biofilm* monoespecie de *S. mutans*, sin afectar significativamente la viabilidad bacteriana (183). En nuestro estudio (Estudio #2, referencia), el análisis CLSM demostró el efecto antiadhesivo del arándano en el modelo *in vitro* de *biofilm* usado. En esas observaciones se ha podido constatar alteraciones en la morfología estructural de los *biofilms* expuestos. Este hallazgo ha sido consistente con los datos cuantitativos obtenidos por qPCR. La y colaboradores, en 2010, a pesar de observar que las AC-PAC no tenía ningún efecto en el crecimiento de *P. gingivalis* en un estado planctónico, sí inhibía la formación de *biofilms* lo que coincide con nuestros resultados de adhesión bacteriana (139).

La fracción no dializable (NDM, por las siglas en inglés de *non-dialyzable material*) del arándano, ha sido descrita como capaz de interferir con la colonización de *P. gingivalis* y *F. nucleatum* en el surco gingival en dos investigaciones (109, 138). Esta misma fracción es también capaz de evitar la adhesión de *P. gingivalis* a diferentes proteínas como, por ejemplo, el colágeno tipo I y de esta forma reducir la co-agregación bacteriana (109, 138, 186, 187). En un modelo *in vivo* murino, donde los ratones consumían la fracción NDM, se ha observado también que esta inhibe la adhesión de *F. nucleatum* y *P. gingivalis* a células epiteliales (187), reduciendo la severidad de la periodontitis experimental. Bodet y colaboradores, en 2006, han descrito también el papel de esta fracción en el aumento de fagocitosis de *P. gingivalis*; en la reducción de la actividad de gingipainas producidas por *P. gingivalis*; la actividad de tripsina producida por *T. forsythia* y la actividad de la quimiotripsina de *T. denticola* (188). La actividad de la fracción NDM no se restringe al efecto en las bacterias, si no que interfiere también en mediadores producidos por el huésped, como es el caso de mediadores pro-inflamatorios producidos por macrófagos (IL-1β y TNF-α) previamente expuestos a *F. nucleatum* y *P. gingivalis* (187, 189).

Los resultados de nuestra investigación (Estudio #2,(179)), al mostrar la capacidad de los extractos de arándano rojo en alterar la formación de *biofilm,* relanzan la hipótesis de que algunos de los compuestos presentes en estos extractos podrían ayudar a evitar la formación de los *biofilms* maduros y complejos asociados con la periodontitis. Este efecto puede deberse a diferentes compuestos, como los polifenoles, y proantocianidinas, que inactivan la glucosiltransferasa y la fructosiltransferasa, enzimas que catalizan la formación de glucano y fructano, respectivamente, y que desempeñan un papel importante en la formación y maduración los *biofilms* (190). Hay también evidencia de que los polifenoles de arándano pueden inhibir la formación de *biofilms* interfiriendo con la co-agregación bacteriana (135).

En el tercer estudio (Estudio #3,(191)) incluido en esta tesis se analizó la capacidad antimicrobiana de los PUFAs: EPA y DHA. Los resultados de esta investigación dan pruebas de que la aplicación de EPA o DHA en *biofilms* maduros, crecidos en discos de HA, redujo significativamente los recuentos y la viabilidad celular de las seis cepas utilizadas en el modelo de *biofilm* (*S. oralis, A. naeslundii, V. parvula, F. nucleatum, P. gingivalis* y *A. actinomycetemcomitans*).

Estos resultados son concordantes con investigaciones anteriores que han demostrado un efecto antimicrobiano antiséptica de los extractos de PUFAs (192), principalmente extractos de EPA y DHA, encontrados naturalmente en muchos organismos marinos (193, 194). Además, y más allá de su actividad antimicrobiana (confirmada en el Estudio #3,(191)), cabe destacar que los PUFAs también han demostrado propiedades antiinflamatorias (141, 149, 150, 152, 155, 193-195) y antioxidantes (196), lo que les convierte en productos con gran potencial en su uso coadyuvante en la terapia periodontal. Aun así, poco se sabe sobre su espectro antimicrobiano de actividad o sobre sus mecanismos de acción frente a patógenos periodontales, particularmente cuando están organizados en *biofilms*. Dos estudios previos han investigado el potencial antibacteriano de EPA y DHA frente a patógenos orales, aunque evaluando dichas bacterias en crecimiento planctónico (167, 168). Sin embargo, Sun y

colaboradores, en 2016, evaluaron el efecto antibacteriano de EPA y DHA, utilizando cepas de *F. nucleatum* y *P. gingivalis* tanto en estado planctónico como en *biofilms* monoespecie, y demostrando actividad antimicrobiana en los dos tipos de crecimiento bacteriano (197).

Sin embargo, es ampliamente conocido que los microorganismos tienen diferentes propiedades cuando crecen dentro de *biofilms* multiespecies, en comparación con su estado planctónico, como la resistencia a los agentes antimicrobianos (198-203). Varios estudios indican que la concentración mínima inhibitoria (MIC, por las siglas en inglés de *Minimum Inhibitory Concentration)* de un organismo puede aumentar de 2 a 1000 veces cuando crece en *biofilm* (200), siendo al menos 250 veces mayor que el MIC de la misma especie en estado planctónico (204). De hecho, la resistencia bacteriana a los antimicrobianos parece estar relacionada con la maduración del *biofilm*, con la máxima resistencia coincidiendo con la fase de crecimiento estacionario del *biofilm* (204, 205). Por estas razones, en la presente investigación se utilizó un modelo de *biofilm* multiespecie *in vitro*.

En la presente investigación, el EPA y el DHA se evaluaron de forma independiente, en comparación con sus controles negativos y positivos. Por esa razón no es posible hacer una comparación directa entre el efecto de cada uno de ellos. No obstante, por la observación de los datos presentados, parece ser que el efecto del DHA es superior al del EPA. Este efecto superior mostrado por DHA puede deberse a su estructura molecular tridimensional más pequeña, lo que puede facilitar su difusión a través de la matriz extracelular de *biofilm*.

Todavía se desconocen los mecanismos que fundamentan el efecto inhibitorio bacteriano observado de EPA o DHA contra patógenos orales. Posiblemente (así se ha descrito con otros PUFAs) los ácidos grasos se incorporan a la membrana plasmática celular, lo que resulta en una mayor fluidez y permeabilidad de la membrana, afectando así su integridad, lo que conduce a la muerte celular (154, 168). Este efecto podría verse exacerbado por la presencia de dobles enlaces insaturados que ejercen un efecto tóxico directo sobre la membrana celular bacteriana (149). A concentraciones de 100 μM, DHA y EPA no son citotóxicos para las células de tejido oral humano (197), o frente a

mioblastos C2C12 (206), pero mantienen su actividad antibacteriana. En la presente investigación, hemos utilizado una concentración de 100 μM y las observaciones por SEM han demostrado no sólo una reducción en el número de bacterias presentes, sino también cambios morfológicos distintos con daños estructurales observables. Estas observaciones coinciden con observaciones anteriores, utilizando también 100 μM de EPA y DHA (197). Además, se han descrito eventos similares para otras especies bacterianas tras la exposición a DHA, como en *Burkholderia cenocepacia* (155), o *H. pylori* (149).

El análisis cuantitativo mostró que, tanto EPA como DHA, en comparación con sus controles, redujeron significativamente la viabilidad bacteriana de las especies utilizadas en este modelo. En el caso de *V. parvula*, *F. nucleatum* o *P. gingivalis*, los PUFAs mostraron una mayor actividad antimicrobiana que la clorhexidina, un antiséptico considerado como la referencia para su uso en formulaciones orales de enjuague bucal (83). El efecto de los extractos probados en la reducción de la viabilidad celular también se observó en el análisis CLSM. Los discos tratados con DHA y EPA, aunque tenían un efecto más pronunciado con DHA, mostraron una baja viabilidad celular. Estos resultados de CLSM fueron totalmente congruentes con los resultados de qPCR. Se notó el mayor efecto antimicrobiano de DHA y EPA, en comparación con clorhexidina, que ha demostrado un marcado efecto antibacteriano en modelos de *biofilm*, tanto *in vitro* como *in vivo* (207, 208). Una de las ventajas reportadas de la clorhexidina es su capacidad de unión a los tejidos orales, lo que permite su liberación lenta y continua (209, 210) hasta 12 h (sustantividad) (211). Actualmente, se desconoce la posible sustantividad y farmacocinética de los extractos de EPA y DHA, por lo que se requieren más investigaciones antes de considerar estos extractos como alternativas reales a los antisépticos orales utilizados actualmente.

Aunque este estudio (Estudio #3,(191)) es el primero en demostrar el efecto antibacteriano de los extractos de EPA y DHA en un modelo de *biofilm in vitro* multiespecies (212), hay algunas limitaciones que deben destacarse, no solamente para este estudio, sino también para los dos primeros trabajos presentados (Estudios #1 y #2,(170, 179)). En primer lugar, en el modelo de

biofilm utilizado sólo se utilizaron seis especies bacterianas, al revés de los cientos presentes en los *biofilms* subgingivales de origen natural. Además, el modelo utilizado representa un modelo de *biofilm* estático, y en su diseño actual, el efecto de las células del sistema inmunitario no se puede tener en cuenta. Aunque este modelo tiene una población mixta de seis cepas bacterianas que representan los colonizadores iniciales, tempranos y tardíos presentes en *biofilms* subgingivales, no tiene la diversidad que se suele encontrar en las *biofilms* subgingivales naturales, ya que pueden llegar a 200 especies diferentes. Nuestro modelo también incluye sólo una cepa de cada especie bacteriana, que puede afectar los resultados, en especial cuando se trata de mecanismos evolutivos eventuales de respuesta a los PUFAs (o a los productos probados en los Estudios #1 y #2,(170, 179)). En cuanto a los PUFAs analizados, su formulación comercial implica su disolución en etanol en diferentes concentraciones. Con el fin de descartar la posibilidad de que el efecto antiséptico potencial de estos agentes se deba al etanol, en lugar de a los extractos probados, el etanol se incluyó en los experimentos como control (en la misma concentración que se encuentra en cada uno de los productos comerciales), demostrando así el efecto antibacteriano específico de los PUFAs. Por otro lado, se espera que los resultados de esta investigación sean comparables a los obtenidos en otras superficies artificiales utilizadas en odontología, por ejemplo, titanio y zirconio (207).

No obstante las limitaciones presentadas relativas a los tres estudios (Estudios #1, #2 y #3 (170, 179, 191)), este tipo de modelos deben considerarse como un paso primario e inicial en el proceso de investigación, identificando posibles moléculas candidatas a ser investigadas en estudios con un mayor nivel de evidencia científica.

VII
Conclusiones

Conclusiones

De manera general, se puede concluir que:

Los diferentes productos y/o extractos de origen natural probados en los tres estudios incluidos en este trabajo han demostrado, de diferentes formas y con diferentes magnitudes, la capacidad de afectar a *biofilms* subgingivales multiespecie en el modelo *in vitro* utilizado.

Los resultados observados justifican la realización de nuevas investigaciones sobre el potencial uso de los diferentes productos de origen natural, en el desarrollo de nuevas formulaciones que puedan servir de herramienta de cara a la prevención y tratamiento de las enfermedades periodontales.

De manera específica, se puede concluir que:

- Se ha demostrado que el uso de vino tinto y extractos derivados del vino tuvo un impacto antimicrobiano, aunque moderado, en los recuentos bacterianos totales y en los recuentos de *F. nucleatum, P. gingivalis* y *A. actinomycetemcomitans*.

- Los extractos del arándano rojo tienen un efecto antibacteriano moderado contra patógenos periodontales en *biofilms*, pero propiedades relevantes frente a los *biofilms*, al afectar la adhesión de bacterias en las primeras 6 horas de desarrollo de *biofilms*.

- Tanto el EPA como el DHA han demostrado efectos antimicrobianos relevantes (de 2 o más ordenes de magnitud) frente a todas las cepas bacterianas organizadas en *biofilms* maduros.

VIII
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Bibliografía

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