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Caracterización genética y determinación del potencial biotecnológico de las cepas del género *Arthrospira* Stizenberger (Cyanophyceae) depositadas en tres Colecciones de Cultivo de Microalgas: UTEX (USA), CCAP (UK) y CCM-UdeC (Chile)

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RESUMEN

El género *Arthrospira* destaca por su importancia biotecnológica, debido a que posee metabolitos con actividad antiinflamatoria, antitumoral y propiedades hepatoprotectoras. El género *Arthrospira* estuvo fusionado con el género *Spirulina*, razón por la cual aún se les denomina erróneamente como "*Spirulina*" a especies de *Arthrospira.* A través de análisis genotípicos se determinó que ambos géneros no están filogenéticamente relacionados. Por otra parte, muchos taxa del género *Arthrospira* muestran variabilidad morfológica y plasticidad de los caracteres taxonómicos. A través del análisis de la secuencia de la región ITS se han obtenido árboles filogenéticos que, en la mayoría de los estudios, agrupan a las cepas de *Arthrospira* en dos clados principales ("especies filogenéticas"). Hasta la fecha no se registran estudios que analicen la existencia de variabilidad intraespecífica en las especies filogenéticas de *Arthrospira*. Parámetros fisiológicos como crecimiento, producción de pigmentos y proteínas o las propiedades funcionales de estas, son atributos de interés biotecnológico, que pueden variar entre cepas, favoreciendo el mejoramiento genético mediante simple selección. Por otro lado, las Colecciones de Cultivo de Microalgas son reservorios de biodiversidad que mantienen depositadas cepas aisladas en distintos momentos y lugares geográficos, las cuales son registradas con el nombre taxonómico indicado por el depositante, sin que medie una confirmación por parte de la Colección, lo cual puede llevar a errores de denominación.

En esta tesis se analizaron 11 cepas denotadas como *Arthrospira* y *Spirulina* provenientes de tres colecciones de cultivo de microalgas: CCAP (Escocia), UTEX (USA) y CCM-UdeC (Chile), a través de la secuencia del espaciador interno transcrito ribosomal (región ITS), con el fin de verificar su clasificación taxonómica. El análisis filogenético mostró la existencia de cepas erróneamente clasificadas en sus colecciones de cultivo de origen; siete cepas se agruparon en una de las dos especies genéticas descritas previamente en *Arthrospira* (clusters I y II), mientras que las restantes cepas se agruparon dentro del género *Spirulina,* formando dos clústers no descritos previamente. Además, el análisis morfológico y fisiológico (crecimiento, producción de biomasa, pigmentos, proteínas y lípidos) reflejó la presencia de variabilidad intraespecífica dentro de las especies genéticas de *Arthrospira*. Las cepas UTEX 2342 (cluster I) y CCM-UdeC 040 (cluster II) serían las más promisorias para un escalamiento comercial, considerando principalmente sus

atributos de crecimiento, capacidad de acumular ficocianina y proteínas y propiedad antioxidante de sus extractos acuosos crudo e hidrolizado.

Esta investigación aporta valiosa información práctica sobre cepas actualmente depositadas en colecciones de cultivo de distintos países, lo cual resulta un incentivo para el desarrollo de iniciativas que fomenten el cultivo comercial de *Arthrospira*.

ABSTRACT

The genus *Arthrospira* is broadly cultivated worldwide due to its biotechnological importance, since it has metabolites with anti-inflammatory, antitumor and hepatoprotective properties. Formerly *Arthrospira* was merged with the genus *Spirulina*, reason why *Arthrospira* species are still erroneously called "Spirulina". Through genotypic analysis it was determined that both genera are not phylogenetically related. On the other hand, many taxa of the genus *Arthrospira* show morphological variability and plasticity on taxonomic characters. By analyzing the sequence of the ITS region, most studies have obtained phylogenetic trees that group *Arthrospira* strains into two main clades ("phylogenetic species"). To date there are no studies that explore the existence of intraspecific variability in the phylogenetic species of *Arthrospira*. Physiological parameters such as growth, production of pigments and proteins or their functional properties are attributes of biotechnological interest, which can vary between strains, facilitating genetic improvement through simple selection. On the other hand, microalgae culture collections are biodiversity reservoirs that maintain isolated strains deposited at different times and geographical locations, which are registered with the taxonomic name indicated by the depositor, without confirmation by the collection, which can lead to naming errors.

In this study, eleven strains designated as *Arthrospira* or *Spirulina* in three microalgae culture collections, were analyzed using their ITS sequence, to verify their taxonomic classification. Phylogenetic analysis showed that there were misclassified strains in these collections: seven strains were grouped in one of the two genetic clusters previously described for *Arthrospira*, while remaining strains were grouped within *Spirulina* genus, forming two not previously described genetic clusters. In addition, morphological and physiological (growth, production of biomass, pigments, proteins and lipids) analysis, reflected the presence of intraspecific variability between the genetic species of *Arthrospira*. Based on the information obtained, the most promising *Arthrospira* strain was selected, its cultivation was scaled and its potential biotechnological application was analyzed considering the generation and obtention of antioxidant bioactive peptides.

INTRODUCCIÓN

I.1. Aspectos generales de las cianobacterias

Las cianobacterias constituyen una división formada por organismos cuyo metabolismo está basado en la fotosíntesis oxigénica (Dick et al., 2018; Jorquera et al., 2010). Además de contribuir de manera significativa a la producción global de biomasa, desempeñan un rol importante en los ciclos biogeoquímicos del carbono, nitrógeno y oxígeno (Karl et al., 2002; Sánchez-Baracaldo et al., 2022). Las cianobacterias agrupan a un conjunto muy heterogéneo de procariontes fotosintéticos que incluyen formas unicelulares, cenobiales y filamentosas, las cuales se agrupan en cerca de 150 géneros y 2000 especies (Hamouda et al., 2017; Thajuddin & Subramanian, 2005). Son organismos ubicuos, que están ampliamente distribuidos en ambientes acuáticos y terrestres, algunos tan extremos como aguas termales, hipersalinas, desiertos y regiones polares (Stal, 2007; Whitton & Potts, 2000). Adicionalmente, son capaces de realizar la fotosíntesis a bajas irradiancias y utilizar ion bicarbonato a altos valores de pH; algunas realizan un proceso biológico único: combinan la fijación del N_2 con la fotosíntesis oxigénica (Goebel et al., 2010; Tripp et al., 2010), mientras otras pueden utilizar diversas fuentes de azufre y poseen eficientes mecanismos de adquisición de fósforo. Por estas razones, las cianobacterias son consideradas como modelos en el estudio de las respuestas fisiológicas a estrés ambiental (Chang et al., 2017; Hamouda et al., 2017; Kirsch et al., 2019; Yang et al., 2020).

La fisiología versátil y amplia plasticidad ecológica de las cianobacterias, reflejadas en habilidades que les permiten tolerar altas temperaturas, radiación UV, estrés hídrico y estrés salino; son consecuencias de modificaciones funcionales y estructurales experimentadas durante su historia evolutiva (Gröniger et al., 2000; Veaudor et al., 2020; Whitton & Potts, 2000). Parte de su éxito se debe a la producción de una amplia gama de biomoléculas que apoyan el crecimiento bajo esas condiciones (Kirsch et al., 2019; Lima et al., 2020; Srivastava et al., 2020). En este sentido son consideradas ricas fuentes de metabolitos primarios como polisacáridos, proteínas, grasas, aceites, vitaminas y pigmentos (Hamouda et al., 2017). Igualmente, sintetizan metabolitos secundarios como péptidos, alcaloides, policétidos y terpenos; los cuales pueden actuar como hormonas, agentes quelantes de hierro, compuestos antibióticos, antifúngicos, antitumorales, antiinflamatorios y por lo tanto

constituyen una potencial fuente de moléculas biotecnológicamente importantes (Gademann & Portmann, 2008; Morone et al., 2019; Paliwal et al., 2017).

Uno de los géneros que destaca por su importancia biotecnológica es el género *Arthrospira,* perteneciente al Orden Oscillatoriales. Durante siglos, se ha utilizado a *Arthrospira* como fuente de proteínas en muchos países del mundo y en la actualidad existe un importante desarrollo comercial en torno a su cultivo masivo. *Arthrospira* es considerado como "el alimento del futuro" y está certificado por la FDA (Administración de Alimentos y Medicamentos) como GRAS (Generalmente Reconocido como Seguro) (Ajayan et al., 2012). Por lo que los consumidores perciben a *Arthrospira* como una materia prima saludable, segura, nutritiva y sustentable (Lafarga et al., 2021). Paralelamente, su cultivo ofrece importantes ventajas, como su rápido crecimiento, escaso riesgo de contaminación (por la alta alcalinidad y salinidad que requiere su cultivo), posibilidad de adaptarse a crecer en agua de mar y no existen reportes de toxicidad asociada a su consumo en humanos ni animales (Cheevadhanarak et al., 2012). El mercado internacional de *Arthrospira* fue evaluado en $335.2 millones en el 2021 y se espera que alcance los $466.9 millones en 2026 (https://www.researchandmarkets.com/reports/5503433/global-spirulina-market-2021-2026-by-type).

I.2. Situación taxonómica del género *Arthrospira*

La identificación de especies de cianobacterias es una tarea compleja, debido a la falta de consenso en la definición del concepto de especie. Las cianobacterias son organismos que no cumplen muchos de los requisitos que definen a una especie biológica, según el Concepto Biológico de Especie propuesto por Mayr (1957), en gran medida, porque sólo se reproducen asexualmente (Johansen & Casamatta, 2005). Entre los conceptos clásicos de especie, uno de los más ampliamente aceptados (de acuerdo al número de artículos de taxonomía que emplean este concepto bajo el Código Botánico) es el Concepto Monofilético de Especie, el cual es derivado del Concepto Filogenético de Especie postulado por Nixon & Wheeler (1990).

El Concepto Monofilético de Especie define a las especies como: el grupo monofilético más pequeño con autopomorfia (carácter único para un taxón particular) reconocible (Johansen & Casamatta, 2005). Estos caracteres pueden ser morfológicos, bioquímicos o moleculares.

La utilización de sólo marcadores morfológicos en la identificación de especies de cianobacterias carece de resolución a nivel de especie, así como no tiene en cuenta las especies crípticas (organismos que no están estrechamente relacionados, por lo que no comparten historia evolutiva, pero que son indistinguibles morfológicamente), las cuales están presentes en la mayoría de los géneros. Además, algunos de estos caracteres morfológicos (e.g. formación de la vaina y la presencia de heterocistos), pueden perderse en los cultivos y en ambientes altamente variables. En este sentido, los autores recomiendan la utilización de marcadores moleculares para aumentar la certeza de las revisiones taxonómicas.

El gen del ARNr de 16S, es uno de los genes más utilizados en estudios de sistemática molecular procarionte, debido a que es altamente informativo, su función es universal y tiene un grado de variación adecuado a nivel jerárquico alto (Ludwig & Klenk, 2001). En la actualidad el ARNr 16S es considerado como el "Gold-standard" en la sistemática bacteriana (Dvořák et al., 2015); sin embargo, debido a su bajo grado de variabilidad, no se recomienda en estudios a nivel de especie o inferior (Nelissen et al., 1994). En ese sentido, se han utilizado otros marcadores como *rpo*, *gyr*B, *het*R, *rbc*LX y secuencias variables no codificantes, como las regiones espaciadoras internas transcritas del ADN ribosomal (ITS), específicamente entre los genes de ARNr 16S–23S o los espaciadores intergénicos del operón de la ficocianina, entre los genes de las subunidades cpcB y cpcA (PC-IGS) (Baurain et al., 2002; Manen & Falquet, 2002).

En la selección de un marcador molecular se debe tener en cuenta el fenómeno de la transferencia genética horizontal (HGT: Horizontal Gene Transfer, de sus siglas en inglés), el cual se basa en la transferencia de genes entre células divergentes. Los eventos de HGT constituyen el principal mecanismo evolutivo en los procariontes y se producen a través de tres mecanismos: transformación (captación directa de un fragmento de ADN foráneo), transducción (inserción de genes foráneos mediada por un virus) y conjugación (transferencia de material genético vía contacto directo entre dos células) (Huang & Gogarten, 2006; Yutin, 2013). La transferencia de genes está mediada por plásmidos, transposones y bacteriófagos y persisten sólo si proporcionan una ventaja adaptativa para el organismo que los adquiere (Huang & Gogarten, 2006; García-Aljaro et al., 2017, Touchon et al., 2017).

El intercambio de genes entre organismos distantemente relacionados produce

distribuciones aberrantes en el genoma, las cuales contrastan con las relaciones inferidas a partir de los árboles filogenéticos de secuencias de proteínas heredadas verticalmente (Shi & Falkowski, 2008). En el caso de las cianobacterias, se han identificado cianófagos a través de los cuales ocurre la transferencia vía transducción. Generalmente, los genes que codifican para sistemas grandes y complejos, como las proteínas fotosintéticas y ribosomales, tienen mayor número de interacciones macromoleculares y están menos sujetas a HGT; a diferencia de los genes que codifican para pequeños ensambles de pocos productos génicos, según postula la hipótesis de la complejidad (Jain et al., 1999). En este sentido, los genes o secuencias del genoma que participan en el proceso de traducción de las proteínas, constituyen marcadores moleculares potencialmente robustos, ya que dicho proceso requiere del ensamble coordinado de al menos 100 productos génicos, lo que disminuye la posibilidad de eventos de transferencia horizontal (Shi & Falkowski, 2008). Precisamente, mediante el análisis de datos obtenidos de estas secuencias, específicamente las secuencias variables no codificantes de las regiones espaciadoras internas transcritas del ADN ribosomal (ITS), entre los genes de ARNr 16S–23S y datos morfológicos y ecológicos, se ha realizado la revisión de diversos géneros de cianobacterias (Komárek, 2016), entre ellos los géneros *Arthrospira* y *Spirulina.*

En el pasado, el género *Arthrospira* estuvo fusionado taxonómicamente con el género *Spirulina,* razón por la cual hasta hoy se les denomina erróneamente como "*Spirulina"* a cepas de *Arthrospira*. Un ejemplo de ello son las especies *A. maxima* y *A. fusiformis,* las cuales se cultivan con fines comerciales bajo la denominación incorrecta de *Spirulina maxima* y *Spirulina platensis*, respectivamente (Komárek & Anagnostidis, 2005). En la revisión de *Cyanophyceae* realizada por (Geitler, 1925, 1932), se unificó dentro del género *Spirulina* Turpin 1829, a todos los organismos oscilatoriales, con tricomas helicoidalmente enrollados a lo largo de filamentos multicelulares, independiente de la visibilidad de las paredes transversales al microscopio óptico. Previamente Stizenberger (1854) y Gomont (1892–1893) habían agrupado a los organismos con filamentos regularmente enrollados y septo visible, dentro del género *Arthrospira* Stizenberger 1852, y a los organismos con septo invisible dentro del género *Spirulina*. La diferencia en la elección de los dos criterios de clasificación conduce a la fusión o no de *Arthrospira* junto al género *Spirulina* (Tomaselli, 1997). En *Spirulina*, el grado de enrollamiento de los tricomas es generalmente superior a *Arthrospira*, las paredes transversales no son visibles al microscopio óptico y las

dimensiones de las células son generalmente menores. Mientras que los miembros del género *Arthrospira* poseen paredes transversales visibles y los filamentos pueden perder su grado de enrollamiento y convertirse en rectos (Jeeji Bai & Seshadri, 1980). Las especies de *Spirulina* habitan en aguas dulces, marinas, salobres hasta aguas termales; mientras *Arthrospira* está restringida a aguas marinas, salobres y lagos salinos de regiones tropicales y semitropicales (Castenholz, 2015; Nelissen et al., 1994). Otro aspecto distintivo es la presencia de ácido γ-linolénico en *Arthrospira*, el cual está ausente en *Spirulina,* por lo que funciona como marcador químico entre ambos géneros (Romano et al., 2000).

Los primeros estudios genéticos enfocados en la distinción de los géneros *Arthrospira* y *Spirulina*, evidenciaron diferencias en el contenido de G+C (%) en el ADN (Herdman et al., 1979; Nelissen et al., 1994). Posteriormente se han realizado análisis filogenéticos a partir de la información de la secuencia del gen de ARNr de 16S, espaciador ITS (entre los genes de ARNr 16S–23S) o los espaciadores intergénicos del operón de ficocianina (entre la subunidad cpcB y cpcA: PC-IGS) (Baurain et al., 2002; Manen & Falquet, 2002). Uno de los primeros estudios que demostró la eficacia de la utilización de las secuencias ADNr 16S e ITS en la discriminación de ambos géneros fue realizado por Nelissen et al. (1994), cuyos resultados determinaron que ambos géneros no están filogenéticamente relacionados y que las secuencias ITS son menos conservadas que los genes de ARNr 16S (Ballot et al., 2004; Nelissen et al., 1994). A su vez, las secuencias de la región ITS de ambos géneros muestran diferencias estructurales: en *Arthrospira* la región ITS está interrumpida por los genes de $ARNt^{Ile}$ y $ARNt^{Ala}$, mientras que en *Spirulina* sólo está interrumpida por el gen de $ARNt^{Ile}$ (Barriga et al., 2006; Baurain et al., 2002; Nelissen et al., 1994). Las secuencias PC-IGS también han sido empleadas en el análisis genotípico de muchas cepas de *Arthrospira* y *Spirulina*, los cuales han confirmado la separación de estos géneros (Ballot et al., 2004; Manen & Falquet, 2002).

En la actualidad, las especies del género *Arthrospira* Stizenberger. ex Gomont 1892, se encuentran ubicadas en la subfamilia Phormidioideae de la familia Phormidiaceae perteneciente al Orden Oscillatoriales (Komárek & Anagnostidis, 2005). Los representantes de este género son cianobacterias filamentosas, constituidas por tricomas (agrupación de células) cilíndricos multicelulares helicoidalmente enrollados y cuyas dimensiones pueden variar entre las especies desde 2,5 a 16µm, para el ancho del tricoma. Su reproducción es estrictamente asexual, donde las células cilíndricas que forman los tricomas se dividen por

fisión binaria en un solo plano (perpendicular al eje longitudinal). La elongación del tricoma se produce a través de la múltiple división celular intercalada a través del filamento entero y la formación de nuevos filamentos ocurre por fragmentación del tricoma (Torzillo & Vonshak, 2013). La situación taxonómica dentro del género *Arthrospira* es confusa y compleja, debido a la variabilidad morfológica de muchos taxa en la naturaleza y en cultivo (Anagnostidis & Komárek, 1988; Komárek & Lund, 1990). El grado de espiralización, el arreglo de los espirales, las dimensiones del tricoma y de las células, la presencia de aerotopos, así como la forma de los ápices del tricoma, se consideran los criterios taxonómicos principales para separar especies (Anagnostidis & Komárek, 1988). A partir de estos caracteres morfológicos se han identificado diez especies tipo del género *Arthrospira*. La Tabla 1 muestra información de las especies tipo del género *Arthrospira* recopilada por (Vonshak & Tomaselli, 2000).

Tabla 1. Listado de especies tipo del género *Arthrospira* Stizenberger 1852 registrado por Vonshak & Tomaselli (2000).

Especies tipo	Primera descripción	Referencias
A. fusiformis Komárek 1990	Lago Tunatan, Siberia, Rusia	Voronichin, 1934
A. gomontiana Setchell 1895	Agua estancada, Norte América	Geitler, 1932
A. indica Desikachary & Jeeji Bai 1992	Piscina natural, India	Desikachary & Jeeji Bai, 1992
A. jenneri Stizenberger 1852	Agua estancada, Europa	Gomont, 1892
A. khannae Drouet & Strickland 1942	Piscina natural, Birmania	Desikachary, 1959
A. massartii Kufferath 1914	Agua de manantial, Luxemburgo	Geitler, 1932
A. maxima Setchell & Gardner 1917	Piscina salina, California	Gardner, 1917
A. platensis Gomont 1892	Agua estancada, Uruguay	Wittrock & Nordstedt, 1844
A. spirulinoides Chose 1923	Agua estancada, Paquistán	Geitler, 1932
A. tenuis Briihl & Biswas 1922	Cuenca artificial, India	Geitler, 1932

Muchos de estos caracteres muestran significativa plasticidad, especialmente el grado de espiralización, ya que se ha detectado la aparición espontánea de tricomas rectos que previamente eran enrollados (Tomaselli et al., 1996). Basado en estos caracteres

morfológicos, el número de especies de *Arthrospira* varía según la fuente: Jeeji-Bai (1999) informó al menos 12 especies pertenecientes a *Arthrospira* (*A. funiformis, A. fusiformis, A. geitleri, A. gomontiana, A. indica, A. jenneri, A. khannae, A. massartii, A. maxima, A. miniata, A. platensis y A. tenuis)*; mientras que otros autores registran 17 especies reconocidas y más de 20 especies sin reconocer (Komárek & Anagnostidis, 2005), lo que demuestra la falta de consenso y la subjetividad de la clasificación taxonómica dentro del género *Arthrospira.*

Es importante mencionar que las especies de *Arthrospira* que se cultivan comercialmente como *Arthrospira fusiformis* y *Arthrospira maxima* fueron recientemente agrupadas dentro de un nuevo género denominado como *Limnospira* Nowicka-Krawczyk, Mühlsteinová & Hauer. El establecimiento de este nuevo género se realizó basado en un análisis filogenético del gen 16S, características ultraestructurales y ecológicas (Nowicka-Krawczyk et al., 2019). Nelissen et al. (1994) analizaron las secuencias ITS de *Arthrospira* PCC 7345 y *Arthrospira* PCC 8005 y encontraron sólo un 83,8% de similitud; por lo que se concluyó que representaban dos especies diferentes. Posteriormente, Scheldeman et al. (1999) utilizaron los mismos marcadores genéticos y analizaron las secuencias de 51 cepas de *Arthrospira* de cuatro continentes y obtuvieron un árbol filogenético en el que todas las cepas se agruparon en dos clústers genotípicos principales, denominados: I y II; a su vez el clúster I estaba subdividido en subcluster: I.A y I.B. Además, detectaron morfoespecies como *A. platensis*, *A. maxima*, *A. indica* y *A. fusiformis* en ambos clústers. Baurain et al. (2002), también confirmaron la agrupación de las cepas de *Arthrospira* en dos clústers principales, a partir de las secuencias completas de la región ITS de 21 cepas de cuatro continentes, tres muestras secas de Chad incluida un dihé (mezcla de varias cepas *Arthrospira* secada al sol) comprada hace 30 años atrás. Todas las cepas se agruparon en los dos clústers principales I y II, donde el clúster I estaba dividido en dos subclusters (I.A y I.B) al igual que el clúster II (II.A y II.B).

En un estudio realizado por Ballot et al. (2004) se aislaron cepas de *Arthrospira* en Kenia e India, correspondientes a *A. fusiformis* y *A. indica*, según la clasificación tradicional (morfológica). Sin embargo, los análisis de las secuencias de la región PC-IGS de las cepas colectadas y las depositadas en la base de datos, demostraron que todas las cepas se agrupaban en tres clústers; donde las cepas de Kenia e India mostraron un alto porcentaje de similitud y se agruparon juntas en un mismo clúster, lo cual que serían representativas

de una sola especie (Castenholz et al., 2001). La diferencia en el número de clústers o grupos que se definen a través de la secuencia ITS (dos clústers) y la secuencia de la región PC-IGS (tres clústers), pudiera estar dada por la ocurrencia de eventos de transferencia genética horizontal; puesto que se ha detectado la presencia de una distribución en mosaico de los sitios informativos de la secuencia PC-IGS. Esto ha hecho que algunos autores cuestionen el uso de este último marcador en estudios filogenéticos y taxonómicos (Choi et al., 2012; Manen & Falquet, 2002).

En la mayoría de los estudios publicados no se detectó correlación entre las morfoespecies y el origen geográfico de las mismas (Ballot et al., 2004; Baurain et al., 2002; Scheldeman et al., 1999). Sin embargo, Dadheech et al., (2010) analizaron las secuencias de la región ITS de cepas colectadas en hábitats naturales y obtuvieron que las cepas colectadas en América se agruparon en el clúster I, mientras que las cepas de África y Asia se ubicaron en el clúster II.

Basado en el Concepto Filogenético de Especie postulado por Nixon y Wheeler (1990), el cual plantea que “las especies constituyen la menor y más pequeña agregación de poblaciones (sexual) o linajes (asexual) diagnosticables por la combinación única de los estados del carácter en individuos comparables” y, teniendo en cuenta los resultados de los análisis de las secuencias ITS; varios autores hipotetizan que el género *Arthrospira* estaría formado por sólo dos especies (filogenéticas) (Scheldeman et al., 1999; Baurain et al., 2002; Dadheech et al., 2010).

La presencia de cepas de *Arthrospira* en un amplio rango de hábitats, algunos de ellos extremos (e.g. salinos, alcalinos y con elevadas temperaturas), evidencia las adaptaciones metabólicas específicas que han adquirido, las cuales son el reflejo de divergencias genotípicas (Margheri et al., 2003; Nübel et al., 2000). Existen pocos estudios relacionados con la variabilidad intraespecífica en microalgas con potencial biotecnológico y menos aún en cianobacterias. Los estudios existentes se restringen a haptófitas y diatomeas como fuentes de ácidos grasos (Talebi et al., 2013) y a especies carotenogénicas como *Dunaliella salina* y *Haematococcus pluvialis* (Gómez et al., 2016; Gómez & González, 2005). Los análisis comparativos entre cepas permiten la identificación y evaluación de la variación natural; en especies biotecnológicamente importantes, estos estudios resultan de gran utilidad para la realización de programas de mejoramiento genético, mediante simple selección (Gómez et al., 2016).

I.3. Metabolitos de interés biotecnológico en *Arthrospira*

Algunas cepas de *Arthrospira* pueden contener de 50% a 70% de su peso seco en proteínas de alta calidad, ya que contienen todos los aminoácidos esenciales; además de un alto contenido en vitaminas, minerales, así como ácidos grasos insaturados, polisacáridos y compuestos fenólicos. Muchos de estos compuestos tienen actividad antiinflamatoria, antioxidante, antiviral, antibacteriana, antifúngica, antitumoral, estimulan el sistema inmunológico y reducen la hiperlipidemia y la obesidad (Borowitzka, 2013; Chang et al., 2017; Hirahashi et al., 2002).

Las especies del género *Arthrospira* son importantes fuentes de pigmentos como C-ficocianina y aloficocianina, los cuales poseen demostrada actividad antioxidante, antinflamatoria, antitumoral y propiedades hepatoprotectoras (Nagaraj et al., 2012; Reddy et al., 2003, Qiang et al., 2021). La C-ficocianina es un pigmento proteico azul que se ha utilizado como colorante natural en alimentos y cosméticos; además posee efectos sobre la hipertensión y la diabetes (Ichimura et al., 2013; Moraes et al., 2010); mientras que la aloficocianina posee actividad antioxidante y anti-enterovirus (Eriksen, 2008; Mandal et al., 2020). Adicionalmente, ambos pigmentos se utilizan en técnicas bioquímicas como sonda fluorescente (Qiang et al., 2021; Mandal et al., 2020).

El contenido de lípidos en *Arthrospira* es de alrededor de 6–13% con respecto a su peso seco, de los cuales la mitad son ácidos grasos poliinsaturados (Cohen et al., 1993). El principal ácido graso presente es el gamma-linolénico (GLA), un ácido graso poliinsaturado omega 6 efectivo en el tratamiento de enfermedades cardíacas, Parkinson y esclerosis múltiple; además actúa como precursor de prostaglandinas (Dyerberg, 1986; Kapoor & Huang, 2006).

Dentro de las biomoléculas acumuladas por *Arthrospira*, destaca su alto contenido de proteínas, el cual es mayor que cualquier otra fuente vegetal (Becker, 2007). Recientemente ha aumentado el interés en las propiedades de los péptidos bioactivos derivados de la hidrólisis de proteínas nutricionales, debido a sus efectos terapéuticos (Daliri et al., 2018). Estos péptidos son generalmente pequeños (de 3 a 20 residuos de aminoácidos) y se encuentran inactivos dentro de la secuencia de la proteína hasta que son liberados por enzimas proteolíticas, fermentación microbiana o durante el procesamiento de los alimentos (Korhonen & Pihlanto, 2006). La hidrólisis enzimática es el proceso comúnmente empleado

en la obtención de péptidos y se realiza en condiciones controladas de pH, temperatura, concentración del sustrato y actividad enzimática. Debido a su elevada especificidad y a la ausencia de residuos de solventes orgánicos y químicos tóxicos en el resultado final, constituye el proceso más utilizado en la industria alimentaria y farmacéutica (Ulug et al., 2021).

Las proteasas utilizadas se extraen de tejidos vegetales (ficina, papaína, bromelina); animales (pepsina, quimotripsina, tripsina) y de fuentes microbianas (proteinasa K, pronasa, colagenasa, alcalasa, flavourenzima) (Jakubczyk et al., 2020; Ulug et al., 2021; Peredo-Lovillo et al., 2022). En la actualidad se han registrado 4300 péptidos bioactivos en la base de datos de péptidos bioactivos BIOPEP-UWM^{TM} (BIOPEP), los que están clasificados en base a la bioactividad que producen (antimicrobiana, antitrombótica, antihipertensiva, opioide, inmunomoduladora, antioxidante) (Minkiewicz & Darewicz, 2019; Peredo-Lovillo et al., 2022).

La mayoría de los hidrolizados proteicos y péptidos bioactivos se han obtenido a partir de leche y productos lácteos, carne, pescado, algas y cereales (Nielsen et al., 2017, Lafarga et al., 2021). Otras fuentes menos explotadas son los residuos de la industria alimentaria, insectos y hongos comestibles, así como microalgas eucariontes y cianobacterias (Peredo-Lovillo et al., 2022).

Uno de los factores que determinan la efectividad de los péptidos bioactivos en los órganos diana es su tamaño. Generalmente, los péptidos pequeños (di-tri péptidos) son absorbidos de manera más eficiente que los grandes, los que son propensos a la hidrólisis por peptidasas de los enterocitos (Bouglé & Bouhallab, 2017). Además, los péptidos pequeños son menos alergénicos que sus proteínas parentales (Wang et al., 2021). En la generación de péptidos de pequeño tamaño con actividad biológica se emplea la hidrólisis secuencial, a través de la cual se utiliza una combinación de enzimas proteolíticas (Ulug et al., 2021).

Se han obtenido péptidos con potente actividad antioxidante a partir de proteínas de microalgas como *Navicula incerta* y *Chlorella vulgaris* (Ejike et al., 2017; Elias et al., 2008; Sheih et al., 2009). También se han identificado péptidos antihipertensivos en *Chlorella* sp. y *Nannochloropsis oculata* (Ejike et al., 2017; Sheih et al., 2009; Suetsuna & Chen, 2001), los cuales actúan a través de la inhibición de la enzima ACE en el sistema renina-angiotensina-aldosterona (RAAS) (Hernández-Ledesma et al., 2011).

Debido al alto contenido de proteínas presentes en *Arthrospira,* esta constituye una

potencial y tentativa fuente de péptidos bioactivos muy poco explorada. Los péptidos derivados de *Arthrospira* tienen actividad biológica tanto *in vitro* como *in vivo*, además muchos están identificados y disponibles en la base de datos BIOPEP-UWM^{TM} (Lafarga et al., 2021). Específicamente, la hidrólisis de proteínas de *A. platensis* y *A. maxima* originó péptidos con significativa actividad antihipertensiva y antiinflamatoria (Suetsuna & Chen, 2001; Vo et al., 2013; Lafarga et al., 2021). Estas actividades están relacionadas con la prevención y tratamiento de múltiples Enfermedades no Transmisibles (ENT) como cáncer, desórdenes inmunes y enfermedades cardiovasculares (Wang et al., 2021; Peredo-Lovillo et al., 2022).

El crecimiento, producción de pigmentos y proteínas, o las propiedades funcionales de estas biomoléculas, son atributos de gran interés para las aplicaciones biotecnológicas del género *Arthrospira*; estas podrían variar significativamente entre cepas de la misma especie, favoreciendo el mejoramiento genético mediante simple selección.

I.4 Colecciones de cultivos de microalgas

Las colecciones biológicas son repositorios biológicos que mantienen organismos vivos. En estas instalaciones se preserva el material biológico con propósitos de conservación, investigación, educación y biotecnología; actuando como depósitos de un invaluable patrimonio de biodiversidad (Boundy-Mills et al., 2015; Lo Giudice & Rizzo 2020; Lourenço, 2020). Las colecciones de cultivo de microalgas son instalaciones de almacenamiento de cepas de microalgas aisladas de un lugar específico en un momento específico; son esenciales para promover el conocimiento y el uso sostenible de la biodiversidad dentro de este grupo (Day et al., 1999, Friedl & Lorenz, 2012). Sin embargo, y a pesar de los numerosos esfuerzos realizados por los depositantes, y por las propias colecciones, muchas cepas disponibles en colecciones privadas y públicas de cultivo de microalgas siguen sin identificarse correctamente (Friedl & Lorenz, 2012). Por lo tanto, una de las principales preocupaciones relacionadas con el manejo de colecciones de cultivo de microalgas en todo el mundo es la identificación correcta de las cepas.

En este estudio se determinó a qué especies filogenéticas pertenecen las cepas del género *Arthrospira/Limnospira* disponibles actualmente en dos de las principales Colecciones de Cultivo de microalgas a nivel mundial (CCAP, de Escocia y UTEX, de Estados Unidos) y en

la Colección de Cultivo de Microalgas de la Universidad de Concepción, Chile (CCM-UdeC); se evaluó además la presencia de variabilidad inter e intraespecífica, así como el potencial biotecnológico de las cepas de este género.

Hipótesis de trabajo:

Los estudios filogenéticos realizados a partir de la secuencia ITS han mostrado que es una secuencia lo suficientemente variable y robusta, que ha permitido la identificación de dos clústers principales en el género *Arthrospira*. Teniendo en cuenta el concepto filogenético de especie y considerando los clústers formados a partir de la secuencia ITS como caracteres autopomórficos; se evaluaron las siguientes hipótesis:

Hipótesis 1:

El análisis de la secuencia de la región ITS del ADNr, permitirá confirmar la clasificación taxonómica, a nivel de género, de las cepas de *Spirulina* y *Arthrospira* depositadas actualmente en las Colecciones de Cultivo de Microalgas: UTEX (USA), CCAP (Escocia) y CCM-UdeC (Chile)

Hipótesis 2:

El análisis de la secuencia de la región ITS del ADNr, permitirá clasificar a todas las cepas de *Arthrospira* depositadas en las Colecciones de Cultivo de Microalgas: UTEX (USA), CCAP (Escocia) y CCM-UdeC (Chile), en alguna de las dos especies filogenéticas descritas para el género.

La variabilidad intraespecífica en las microalgas está muy poco explorada y estudiada. Sin embargo, los escasos estudios en microalgas de interés biotecnológico han mostrado la presencia de significativa variabilidad intraespecífica. La presencia de cepas de *Arthrospira/Limnospira* en un amplio rango de hábitats, algunos de ellos extremos (e.g. salinos, alcalinos y con elevadas temperaturas), evidencia las adaptaciones metabólicas específicas que han adquirido, las cuales son el reflejo de divergencias genotípicas y por

tanto de potencial variabilidad intraespecífica.

Hipótesis 3:

Dentro de las especies filogenéticas de *Arthrospira*, existe variabilidad intraespecífica en sus atributos fisiológicos como crecimiento y composición bioquímica de su biomasa, lo cual permite la selección de cepas de interés biotecnológico.

Para abordar las hipótesis anteriormente señaladas, se plantean los siguientes objetivos:

Objetivo general:

Identificar a qué especies filogenéticas pertenecen las cepas del género *Arthrospira* depositadas en las Colecciones de Cultivo de Microalgas UTEX, CCAP y CCM-UdeC y determinar su potencial biotecnológico.

Objetivos específicos

Objetivo específico 1:

Confirmar y/o corregir la clasificación taxonómica, a nivel de género, de las cepas de *Spirulina* y *Arthrospira* depositadas actualmente en las Colecciones de Cultivo de Microalgas: UTEX (USA), CCAP (Escocia) y CCM-UdeC (Chile).

Objetivo específico 2:

Determinar si las cepas de *Arthrospira*, depositadas actualmente en las Colecciones de Cultivo de Microalgas: UTEX (USA), CCAP (Escocia) y CCM-UdeC (Chile), pertenecen a alguna de las dos especies filogenéticas descritas para el género.

Objetivo específico 3:

Determinar si existe variabilidad intraespecífica en las especies filogenéticas de *Arthrospira*,

en cuanto a sus atributos de interés biotecnológico (crecimiento y composición bioquímica de su biomasa) y evaluar la capacidad antioxidante de cepas seleccionadas.

Objetivo específico 4:

Evaluar la capacidad antioxidante de los hidrolizados proteicos obtenidos a partir de la hidrólisis enzimática del extracto acuoso de cepas seleccionadas de *Arthrospira* con proteasas comerciales.

I. CAPÍTULO 1. Comparison of two strains of the edible cyanobacteria *Arthrospira*: Biochemical characterization and antioxidant properties

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Highlights

- Two *Arthrospira* strains were identified as belonging to the same genetic species.
- Morphological, biochemical and functional attributes were different between strains.
- Protein hydrolysis increased antioxidant properties of *Arthrospira*'s extracts.
- Strain CCM-UdeC 040 had higher potential as raw material for food and nutraceuticals.
- Next step is optimizing biomass production process for strain CCM-UdeC 040.

Abstract

Arthrospira is a cyanobacteria broadly cultivated worldwide due to its high nutritional value, metabolites with therapeutic properties, non-toxic character and easy culture. The genus *Arthrospira* has been historically confused with the genus *Spirulina* (with potential toxicity) therefore, an accurate taxonomic identification of strains for food applications, is essential. In this study we compared two strains of *Arthrospira* with remarkable morphological differences, preliminary classified as *Arthrospira maxima* (CCM-UdeC 040) and *Arthrospira platensis* (CCM-UdeC 136). Despite of their morphological differences, we confirmed that both strains belong to the same genetic species, according to their ribosomal ITS (internal transcribed spacer) sequences. We also compared the biochemical composition of both strains, including analysis of antioxidant properties of their aqueous protein extracts, both crude, as well as, hydrolyzed with commercial proteases. Biotechnological potential of these strains as raw material for nutraceutical and functional food markets is discussed. Strain CCM-UdeC 040 showed the highest biotechnological potential so further studies should be conducted for optimizing its culture conditions.

Keywords: *Arthrospira* strains; ITS sequences; Biochemical composition; Protein hydrolysates; Antioxidant properties

1. Introduction

Arthrospira is an edible cyanobacteria that was recognized by ancient civilizations because of its nutritional value. It is a rich source of “single cell” proteins, fatty acids, carbohydrates, vitamins, minerals and pigments and, because it is easy to grow on large-scale, 60% of current global microalgal production account for *Arthrospira* (Furmaniak et al., 2017; Mitra & Mishra, 2019). In addition to its nutritional properties, *Arthrospira*’s biomass has health positive effects including: immunomodulation, antioxidant, anti-inflammatory, anticancer, antiviral and antibacterial activities, among others (reviewed in Sotiroudis & Sotiroudis, 2013). The pigment phycocyanin (PC) is a phycobiliprotein of intense blue color characteristic of cyanobacteria and it has been used for functional food applications because of its therapeutic attributes. PC has shown anti-inflammatory, antiplatelet, anti-cancer, nephroprotective and hepatoprotective properties that can be explained, in part, because of its antioxidant activity (Fernández-Rojas et al., 2014; Hsieh-Lo et al., 2019).

Antioxidant compounds promote health by removing reactive oxygen species (ROS) that may exert harmful metabolic effects, which are implicated in aging and in the pathogenesis of many chronic disorders, including cancer, diabetes and cardiovascular and neurodegenerative diseases (Lobo et al., 2010; Sonani et al., 2017). Synthetic and natural antioxidants are used routinely in food, feed, medical and cosmetic industries in order to protect their products against oxidation (Lobo et al., 2010). Undesired physical properties of some synthetic antioxidants (e.g. high volatility, instability), as well as probable carcinogenic effects, explains the increasing consumer’s preference for natural antioxidants (Ito et al., 1985; Papas, 1999). Many antioxidant compounds, occurring naturally in fruits and vegetables, are recommended for dietary intake in order to prevent diseases related with oxidative stress (Lobo et al.,2010). *Arthrospira* has been highlighted as a potential source of natural antioxidants with different cellular mechanisms such as activation of antioxidant enzymes, inhibition of lipid peroxidation and DNA damage and scavenger of free radicals (Rastogi & Sinha, 2009; Sonani et al., 2017; Wu et al., 2016).

Arthrospira has been used as a food source by human for centuries and it is now recognized as one of the top superfoods (Ma et al., 2019). Its market generated 346 million dollars in 2018 and is projected to reach 779 million dollars by 2026 (https://markets.businessinsider.com). Many strains currently named as “Spirulina”,

including all those grown commercially and marketed as “Spirulina”, actually belong to the genus *Arthrospira* (Belay, 2007). It is important to differentiate both genera because, true *Arthrospira*, has been recognized as a “superfood” (= “a nutrient rich food considered to be especially beneficial for health and well-being” according to the Oxford Dictionary), classified by the American Food and Drug Administration (FDA) as GRAS (Generally Recognized As Safe) in 2002 and assigned with Class A safety level as dietary supplement by the commission of United States Pharmacopeia in 2011 (Furmaniak et al., 2017; Marles et al., 2011). On the other hand, cyanotoxin type microcystin has been reported in *Spirulina* “sensu stricto” (Gantar et al., 2009), therefore, an accurate taxonomic identification of strains for food applications is essential.

Conventionally, the arrangement of spirals, as well as cells and apices sizes of trichome have been considered as main taxonomic attributes for species of *Arthrospira* classification (Komárek & Anagnostidis, 2005); however, some of those characters are highly variable and may change according to environmental conditions (Tomaselli, 1997). The sequence of the ITS (Internal Transcribed Spacer), situated between 16 S and 23 S rRNA genes, is used in molecular taxonomy of prokaryotic organisms at species level (Johansen et al., 2011). Based on ITS sequences analysis, strains of *Arthrospira* genus from four continents were divided into two main genetic variants: I y II. These results lead to the hypothesis that all morphological species of *Arthrospira* currently described, could be grouped in just two main genetic groups (Baurain et al., 2002; Scheldeman et al., 1999).

Arthrospira represents the most remarkable source of protein because its dry biomass may contain up to 70% of (good nutritional quality) protein. Other plant sources like soybean (38%), rice (~10%), pea (2.8%), or even animal sources like milk (4%) or eggs (13%), contain protein percentages significantly lower (reviewed in Sotiroudis & Sotiroudis, 2013; Torres-Tiji et al., 2020).

On the other hand, at present, there is an increasing interest in the utilization of food-derived biologically active peptides as nutraceuticals or nutritional supplements. Bioactive peptides usually contain 2–20 amino acid residues that confer biological functions, beyond their nutritional value, like antimicrobial, antioxidant, antihypertensive and immunomodulatory activities (Hou et al., 2017; Lopez-Barrios et al., 2014; Sarmadi & Ismail, 2010). These peptides are inactive within the sequence of the parent protein molecule and can be liberated by gastrointestinal digestion by proteolytic enzymes or during fermentation processes

(Korhonen & Pihlanto, 2006).

Most studies on *Arthrospira*-derived peptides focus on antihypertensive (Aiello et al., 2019; Anekthanakul et al., 2019), anti-inflammatory (Vo & Kim, 2013), antitumor (Wang & Zhang, 2016), antidiabetic (Aiello et al.,2019; Hu et al., 2019) and anti-obesity peptides (Fan et al., 2018); on the other hand, protein hydrolysates with antioxidant properties have been very little explored yet.

Research on microalgal biotechnology includes permanent search of new strains, its correct taxonomic classification and careful characterization in terms of physiological attributes, chemical composition and functional properties. In this study we compared two strains of cyanobacteria with remarkable morphological differences, preliminary classified as *Arthrospira* maxima (CCM-UdeC 040) and *Arthrospira platensis* (CCM-UdeC 136). We also compared the biochemical composition of both strains, including analysis of antioxidant properties of their aqueous protein extracts: both crude, as well as, hydrolyzed with commercial proteases. Biotechnological potential of these strains, as raw material for nutraceutical and functional food markets, is discussed.

2. Materials and methods

2.1. Strains and culture conditions

Arthrospira maxima (strain CCM-UdeC 040) and *Arthrospira platensis* (strain CCM-UdeC 136) were obtained from the Microalgal Culture Collection at the Universidad de Concepcion (CCM-UdeC), Chile. Both strains are of unknown geographical origin. The strains were maintained and cultivated in Zarrouk medium (Zarrouk, 1966) with slight modifications (B6 solution was not added). The strains were scaled to volume of 100 mL and then to 500 mL in Erlenmeyer flasks. Experimental cultures were initiated at a cell density of 300 µg chlorophyll a L^{-1} in 20 L volume and maintained at 21 ± 1°C, photon flux density of 100 µmol m^{-2} s^{-1} and 12:12 (light/night) photoperiod for 48 days. Cultures were continuously aerated with 0.22 µm pore-filtered air.

2.2. Morphological analysis

Living trichomes of both strains were examined under an Olympus CX31 microscope fitted with bright-field and phase-contrast optics. The photomicrographs were taken with an Olympus C3040 camera attached to the microscope. Morphological characterization of the strains was performed according to Komárek and Anagnostidis (2005) and trichome width and cell size were considered as main characters. Image J software was used for photomicrographs analysis.

2.3. Molecular analysis

ITS sequence of strain CCM-UdeC 040 was previously sequenced by Barriga et al. (2006) and deposited in the Genbank under the code AY724776. ITS sequence of strain CCM-UdeC 136 was amplified and sequenced as was previously described by Barriga et al. (2006).

Consensus ITS sequence was obtained from forward and reverse raw sequences data using the Assembling sequence tool of the Geneious Prime program, version 2019. Sequence was properly deposited in the Genbank. Phylogenetic analysis included 13 strains of *Arthrospira*, available in Genbank, belonging to both genetic species previously described (Baurain et al., 2002; Scheldeman et al., 1999). Besides, one strain of each *Limnothrix* and *Spirulina* genus were included as outgroups as in Barriga et al. (2006). Alignment of the ITS region (including coding ($tRNA^{Ile}$ and $tRNA^{Ala}$) and non-coding (ISRs = intergenic spacer regions) of these 16 strains, included 501 aligned base positions and was performed using the Bayesian criterion of the Geneious Prime program, version 2019 (https://www.geneious.com). jModeltest 2 (Darriba, 2012) was used to determine the best model of sequence evolution.

Bayesian phylogenetic analysis was conducted using MrBayes 3.2.7a (Ronquist & Huelsenbeck, 2003), with 30 million generations Markov Chain Monte Carlo (MCMC) sampled every 3,000 generations. Tree was visualized and edited using the Fig Tree v1.4.4 program by A. Rambaut (http://tree.bio.ed.ac.uk).

2.4. Growth estimation and biomass production in 20 L volume cultures

Growth was monitored every two days by chlorophyll *a* concentration: 10 mL of each culture was filtered using nitrocellulose filters of 0.45-µm pore size (Durapore® Membrane Filter) and frozen at −80°C for 1 h, then 1 mL of 90% acetone was added and grinded with one ceramic sphere (6.3 mm size coated with zirconium oxide) in the Fastprep-24 homogenizer for 2 intervals of 20 s and centrifuged at 13,000×g for 2 min. The supernatant was transferred to a 5 mL flask and acetone extraction was repeated five times. The volume of pigment extraction was adjusted to 5 mL with 90% acetone. Chlorophyll *a* was determined spectrophotometrically, according to Wegmann and Metzner (1971). When stationary phase was reached (day 48) the cultures were harvested by filtration with a phytoplankton net of 0.55 µm pore size. Cell pellets were frozen for 24 h at −20°C and dried by lyophilization at −50°C and 100 militorr pressure for 48 h. Dried samples were stored at −20°C until use. Lyophilized biomass was used for proteins, pigments, lipids and fatty acids analyses and for aqueous extracts preparation.

2.5. Biochemical characterization of lyophilized biomass

Protein content was determined as described by Lowry et al. (1951) after hydrolysis of 10 mg of dry biomass in 5 mL of NaOH at 100°C for 1 h. Bovine serum albumin in a range of 15–150 µg mL^{-1} was used as protein standard for the calibration curve.

For chlorophyll *a* and total carotenoids quantification: 1 mL of 90% acetone was added to 50 mg of dry biomass and grinded with one ceramic sphere (6.3 mm size coated with zirconium oxide) in the Fastprep-24 homogenizer for 2 intervals of 20 s and centrifuged at 13,000 x g for 2 min. The process was repeated five times, the pigment's extract volume was adjusted to 5mL with 90% acetone and chlorophyll *a* and total carotenoids were spectrophotometrically determined according to Wegmann and Metzner (1971) as in 2.4.

The phycobilin pigments: allophycocyanin (APC), phycocyanin (PC) and phycoerythrin (PE) were extracted from 20 mg of algal biomass, resuspended with 1 mL of buffer phosphate (100 mM) at pH 7 and grinded with one ceramic sphere (6.3 mm size coated with zirconium oxide) in the Fastprep-24 homogenizer for 2 intervals of 20 s and stored at −80°C for 10 min. Then, samples were thawed at room temperature, vortex agitated and centrifuged at 1,392×*g*

for 5 min. The supernatant was transferred to a volumetric flask and the extraction procedure was repeated until extraction solvent became colorless. Extracts' absorbances at 650, 620 and 565 nm were recorded for APC, PC and PE, respectively, and their concentrations were calculated using the equations of Bermejo et al. (2002).

Total lipids were extracted from 30 mg of algal biomass according to Bligh and Dyer (1959) and analyzed by the lipid charring technique (Marsh & Weinstein, 1966), using tripalmitin as the standard for the calibration curve.

Fatty acids preparation and analysis were performed according to Haro et al. (2017) using a Claurus 600 chromatograph (PerkinElmer) equipped with a SPB-PUFA column and FID detector. Fatty acid methyl esters (FAMEs) were identified by comparison of their retention time with those of a mix of fatty acids standards (Sigma-Aldrich).Relative quantification was performed by estimating the area of each individual peak compared to the sum of all peaks' areas. Tridecanoic acid and nonadecanoic acid (Sigma-Aldrich Co.) were added to the samples, as internal standards, for absolute quantification of fatty acids.

2.6. Aqueous extracts preparation and characterization

1.35 g of lyophilized biomass were transferred into 50 mL Falcon tubes and 45 mL of autoclaved distilled water was added. Hydrated biomass was kept at 4°C for 30 min and then vortexed and freezed at −80°C for 20 min. Then, the mixture was crushed using a homogenizer Ultra-Turrax for 15 s and centrifuged at 4,752×g for 10 min. The supernatants were separated and frozen for 24 h at − 20°C and dried by lyophilization at −50°C and 100 militorr pressure for 48 h. Dried aqueous extracts were stored at −20°C until use.

Total protein and phycocyanin content of dried extracts were determined as was described in 2.5. except for the initial extraction step. Oxygen radical absorbance capacity (ORAC) assay of aqueous extracts was performed by Functional Life SpA Company, according to Ou et al. (2013). Results were expressed as µmol Trolox Equivalents (TE)/g of aqueous extract and calculated as mean ± SD of three technical replicates of each strain. Cytotoxicity of aqueous extracts was evaluated against THP-1 human leukemia cell line at different concentrations (0.312; 0.625; 1.25; 2.5 and 5 mg mL^{-1} of microalgae extract). The inhibitory effect (IC_{50} value) of each extract was calculated considering the required concentration to inhibiting cells growth by 50% compared to untreated cells. Three technical replicates of

each strain were analyzed. Cytotoxicity assays were performed by Functional Life SpA Company.

2.7. Enzymatic hydrolysis of aqueous extracts and characterization of protein hydrolysates

A sequential hydrolysis of aqueous extracts was performed using commercial proteases. Lyophilized aqueous extracts were dissolved in distilled water (0.1 g mL^{-1}). 1 mL of this solution was diluted with sodium phosphate buffer (0.1 M, pH 7.5, with cysteine 3 mM) at a final concentration of 2 mg extract/mL and hydrolyzed with papain (E/S of 1/100) at 45°C. The hydrolysis reaction was monitored for 4 h and the hydrolysis solution was pipetted out for blocking the reaction at 30, 60, 90, 120, 240 min of incubation times. After the enzyme inactivation at 95°C for 15 min, pH was adjusted to 2 (using 2 M HCl) and then the hydrolysates were digested with pepsin (E/S of 1/100) at 37°C for 4 h. The reaction was stopped at 95°C for 15 min. For the trypsin digestion, the pH of the protein extracts was adjusted to pH 8 by adding 2 M NaOH and hydrolyzed with trypsin (E/S of 1/100) at 37°C for 4 h. The hydrolysis reaction was stopped by heating at 95°C for 15 min. After each enzymatic hydrolysis step, the solutions containing the protein hydrolysates were centrifuged at 13,000×g at 4°C for 30 min and supernatants were used for degree of hydrolysis (DH) measurements.

Degree of hydrolysis (DH) was employed as parameter for monitoring the extent and success of proteolysis (Wang & Zhang, 2016). DH was determined by OPA assay following the methodology of Church et al. (1983) and calculated according to Spellman et al. (2003). Hydrolysis controls were the aqueous extracts subjected to the same conditions (temperature, pH change, incubation time) as the hydrolyzed samples but without the addition of proteases.

Peptides profiles of protein hydrolysates were analyzed by size exclusion chromatography on FPLC system (SEC-FPLC), according to their molecular size, where high molecular weight proteins or peptides will elute with smaller elution volumes, while low molecular weight peptides will elute at larger volumes. Quantification of proteins and peptides is based on the UV absorbance due the presence of tryptophan and tyrosine measured at 280 nm and by the peptide backbone at 215 nm (Hesse & Weller, 2016). Samples were filtered through 0.20

μm.

Minisart SFCA sterile filters (Sartorius Stedim Biotech GmbG, Germany) and 2 mL were loaded onto a Sephacryl S-100 HR column 16 × 350 mm (GE Healthcare Biosciences, Sweden) connected to an AKTA Purifier FPLC system (GE Healthcare Life Science, Canada). Elution was performed with 50 mM Tris-HCl buffer (pH 8) at a flow rate of 0.8 mL min^{-1}. Peptides were detected by measuring the absorbance at 280 and 215 nm. The data processing was completed with the UnicornTM software (GE). Antioxidant property of the hydrolyzed extracts was determined by the ABTS+ radical scavenging activity according to Cotabarren et al. (2019).

2.8. Statistical analysis

Statistical data analyses were performed using R software version 3.2.3. The normality and homogeneity of variances analyses were performed using the Kolmogorov–Smirnov and Levene tests, respectively. In order to assess the statistical significance of differences of measured parameters between strains, t-Student test was conducted. Multifactor ANOVA was used to evaluate DH of aqueous extracts with proteases among different incubation times. Significant differences among means were assessed by Dunn and Tukey test. All analyses were performed with significance level of $\alpha = 0.05$ and using technical replicates.

3. Results and discussion

3.1. Morphology and molecular characterization of strains

Strains CCM-UdeC 040 and CCM-UdeC 136 displayed the typical arrangement of multicellular cylindrical trichomes with evident cross-walls, which are the main morphological features of genus *Arthrospira* (Komárek & Anagnostidis, 2005); however, both strains showed morphological differences: strain CCM-UdeC 040 (Fig. I.1 a, b and c) exhibited a trichome's arrangement as an open helix, while trichomes of strain CCM-UdeC 136 (Fig. I.1 d, e and f) were straight. In spite of these remarkable differences, trichome's arrangement is not considered a good taxonomic attribute in the genus *Arthrospira*, because both the straight and helical forms may exist even in natural monospecific populations (Tomaselli, 1997).

Cell length of CCM-UdeC 040 strain ranged between 2.24 and 4.62 µm and cell diameter between 5.19 and 7.925 µm, while for CCM-UdeC 136, cell length ranged between 3.26 and 7.87 µm and cell diameter between 8.04 and 10.70 µm. In spite of strain CCM-UdeC 136 had a higher cell size than strain CCM-UdeC 040 ($p < 0.05$), the huge variability observed within each strain makes this attribute not useful for species discrimination; in fact size cell is considered a "notoriously untrustworthy diagnostic feature" for *Arthrospira* according to Komárek and Lund (1990). On the other hand, considering the cell diameter (=trichome diameter), both strains were on the expected range for the genus *Arthrospira*, whose cell diameter ranges between 3 and 16 µm (Vonshak, 2002).

Hence morphological attributes have to be complemented with molecular data for taxonomic characterization of *Arthrospira* strains. In this study, consensus ITS sequence of strain CCM-UdeC 136 was obtained from forward and reverse raw sequences data and deposited in the Genbank under the code MN628414.

Bayesian analysis produced the topology showed in Fig. I.1 (left). Sequence alignment defined two clusters (namely cluster I and cluster II) (Fig. I.1) in agreement with Baurain et al. (2002). Analysis of consensus ITS sequence of CCM-UdeC 136 showed similarity with several *Arthrospira* strains, including strain CCM-UdeC 040 (sequenced previously by Barriga et al., 2006) and both were grouped in the same genetic cluster.

According to monophyletic species concept exposed by Johansen and Casamatta (2005): "species is the smallest monophyletic group, where monophylly is recognized by the presence of autopomorphies (i.e., unique derived characters present only in that species)" and considering consensus ITS sequence as an autopomorphy, both strains: CCM-UdeC 040 and CCM-UdeC 136, would belong to the same (genotypic) species. Additionally, our results show that strains CCM-UdeC 040 and CCM-UdeC 136 are not related to *Spirulina* "sensu stricto" genus.

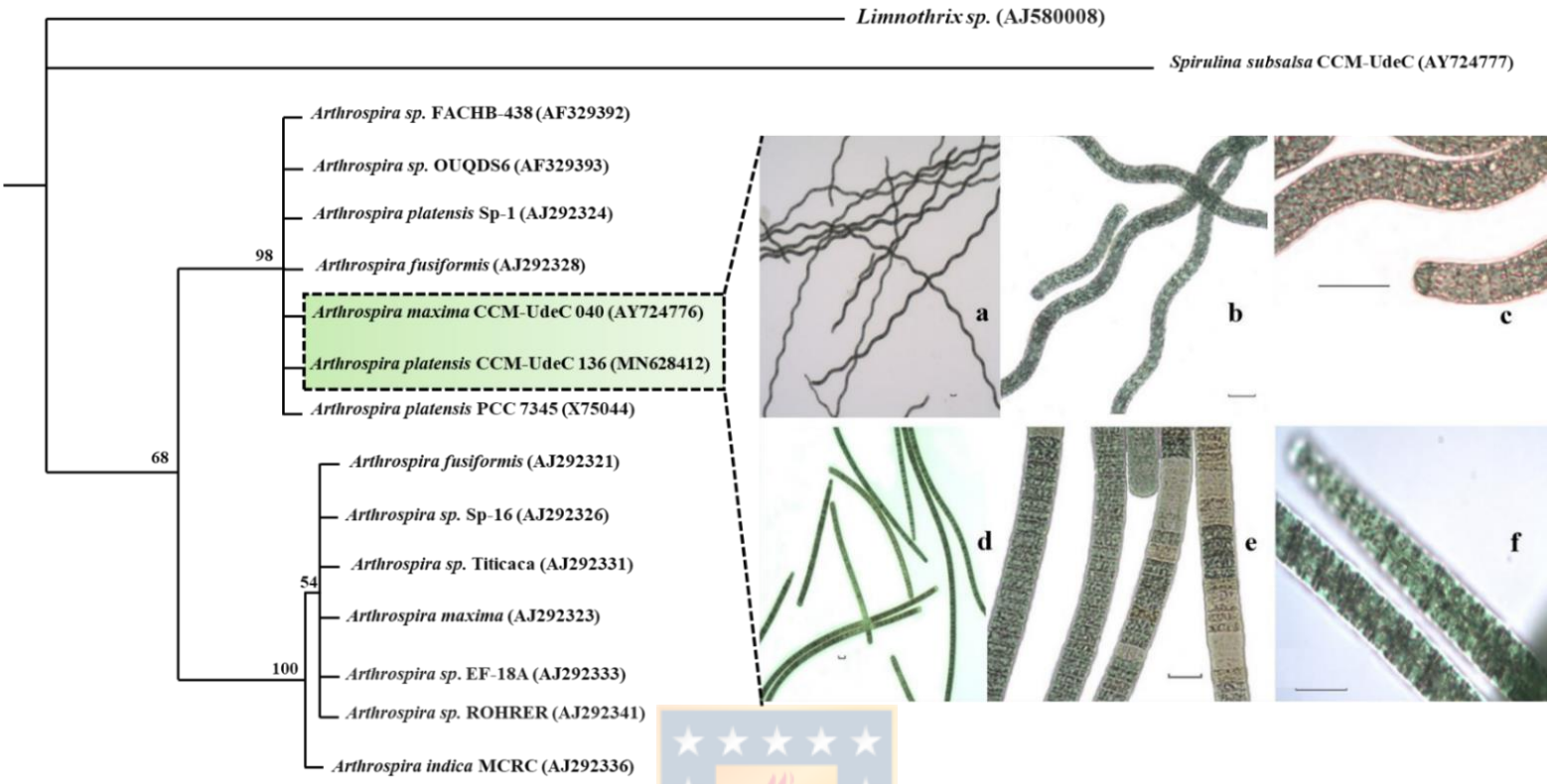

Figure I.1. Left: Rooted phylogram representation of the tree generated by the Bayesian inference method with ITS sequences of 14 taxa of *Arthrospira*, included strains CCM-UdeC 040 (AY724746) and CCM-UdeC 136 (this paper). Bootstrap values are indicated to the left of each node. Right: Photomicrographs showing the morphology of strains CCM- UdeC 040 (a, b and c) and CCM- UdeC 136 (d, e and f) under optical microscope at three magnifications. Scale bars = 10 μm.

3.2. Biochemical characterization of microalgal biomass

Considering that traditionally *Arthrospira* has been used as an edible cyanobacteria due to its high nutritional value and therapeutic properties, biochemical characterization of strains CCM-UdeC 040 and CCM-UdeC 136 is relevant for future nutraceutical and functional food applications.

Biochemical characterization of CCM-UdeC 040 and CCM-UdeC 136 biomass included analyses of pigments (chlorophyll *a*, total carotenoids, phycocyanin, allophycocyanin and phycoerythrin), total proteins, total lipids and fatty acids (Tables I.1 and I.2).

Algal cultures are influenced by various abiotic variables such as temperature, irradiance, light quality, nutrients availability, all of which play a significant role in regulating

photosynthetic activity, biomass composition and overall productivity (Delrue et al., 2017).

Table I.1. Biochemical composition of strains CCM-UdeC 040 and CCM-UdeC 136 biomass grown in 20-L batch regime.

Compounds	CCM- UdeC 040	CCM- UdeC 136
Chlorophyll *a* by dry biomass (mg g^{-1})	16,39 ± 0,73[a]	16,88 ± 0,55[a]
Carotenoid by dry biomass (mg g^{-1})	5,49 ± 0,25 [a]	6,42 ± 0,21 [b]
APC by dry biomass (mg g^{-1})	103,24 ± 5,96 [a]	85,45 ± 5,22 [b]
PC by dry biomass (mg g^{-1})	143,66 ± 4,59 [a]	111,02 ± 14,69 [b]
PE by dry biomass (mg g^{-1})	37,19 ± 2,36 [a]	26,55 ± 2,13 [b]
Total protein (% by dry weight)	43,05 ± 4,66 [a]	36,90 ± 3,15 [a]
Total lipids (% by dry weight)	3,74 ± 1,43 [a]	5,65 ± 0,27 [a]

The average values ± standard deviations of 4 technical replicates are shown. Different letters indicate significant differences (t-Student Test, $p < 0.05$).

Chlorophylls and carotenoids have colorant and therapeutic properties and they can be sold for high prices in the market (Mishra et al., 2011; Nabi et al., 2020). *Arthrospira* is considered one of the organisms with the highest chlorophyll contents in nature, ranging between 1 and 1.4% (Ajayan et al., 2012), although commercial production reports lower ranges between 0.5 and 0.9% (Ma et al., 2019). In this study high chlorophyll *a* contents were reached: 1.6% (16.39 mg g^{-1}) for CCM-UdeC 040 and 1.7% (16.88 mg g^{-1}) for CCM-UdeC 136, without significant difference among strains ($p \geq 0.05$) (Table I.1). Lima et al. (2018), using different lighting conditions for growing *Arthrospira platensis*, consisting of blue and red LEDs in different proportion, did not detect significant difference in chlorophyll *a* content, ranging between 18.85 and 25.31 mg g^{-1}, among treatments. Other experiments have evaluated the effect of organic sources of nitrogen (urea) or carbon (glycerol) on chlorophyll *a* accumulation in *A. platensis* (mixotrophic regime), obtaining no differences between nitrogen sources (Ajayan et al., 2012) but a significant decrease (47%) when glycerol is used as alternative carbon source (Markou et al., 2012). On the other hand, phosphate deprivation did not affect chlorophyll *a* content in *A. platensis* (Markou et al., 2012).

Carotenoids are a wide group of lipophilic isoprenoids synthesized by all photosynthetic organisms and also by some non-photosynthetic bacteria and fungi. They are essential

pigments in light harvesting and energy transfer during photosynthesis and in the protection of the photosynthetic apparatus against photooxidative damage (Sasso et al., 2012). Carotenoids identified in *A. platensis* include β-carotene and zeaxanthin (as the major carotenoids), and myxol 2′-methylpentoside, oscillol 2,2′-dimethylpentoside, 3′-hydroxyechinenone and β-cryptoxanthin (the minor ones) (Giorgis et al., 2017; Sugiyama et al., 2017). In this study, strain CCM-UdeC 136 accumulated more carotenoids than CCM-UdeC 040 ($p < 0.05$): 6.42 mg g^{-1} vs 5.49 mg g^{-1}, respectively (Table I.1). These values are similar to those reported for *A. platensis* using blue and red LEDs as light source, with values ranging between 4.89 mg g^{-1} and 6.38 mg g^{-1} (Lima et al., 2018) and higher to value reported by Ajayan et al. (2012) using urea as nitrogen source: 3.12 mg g^{-1}. On the other hand, carotenogenesis is enhanced by reactive oxygen species (ROS) under stress conditions, such as high light intensity, salt stress or nutrient deficiency (Reviewed in Minhas et al., 2016). However, dos Santos et al. (2019) reduced ten times the nitrate availability in an *A. platensis* culture, without a remarkable increase on its carotenoids content (increase from 2.5 to 3.6 mg g^{-1}), while Mary Leema et al., 2010 did not detect significant increase in β-carotene concentration (3.54 mg g^{-1} vs 3.76 mg g^{-1}), when used seawater, instead of freshwater, for the culture of *A. platensis*.

Cyanobacteria has high photosynthetic efficiency because they are able to absorb energy in a wide wavelength range, through special accessory pigments: phycobilinic pigments. Quality and intensity of light strongly affect the accumulation of pigments in this group, by means of a mechanism named “complementary chromatic adaptation”, that allows to regulate pigments proportion according to prevailing environmental light conditions (Bennett & Bogorad, 1973; Kehoe, 2010). In this study, the analysis of phycobilinic pigments (PC, APC and PE) showed differences between both strains being values for CCM-UdeC 040 higher than for CCM-UdeC 136 ($p < 0.05$) Table I.1.

Phycocyanin is the most important phycobilinic pigment for cyanobacteria’s market. Health beneficial properties of *Arthrospira* are mainly attributed to the phycobilinic pigment C-phycocyanin (Fernandez-Rojas et al., 2014; Liu et al., 2016). This pigment is also used as a natural blue colorant for food and cosmetics and as fluorescent marker for laboratory techniques (Fernandez-Rojas et al., 2014). The market price of phycocyanin depends on its purity ranging between US$ 500/kg and US$ 15/mg for food grade and analytical grade, respectively (reviewed in Deprá et al., 2020). Light is the most relevant parameter affecting

PC content in cyanobacteria. Low irradiance has a positive effect on PC accumulation; however it negatively affects biomass production (Chen et al., 2010; Lee et al., 2016), so, we must attempt to find a correct balance between biomass productivity and biochemical quality. It seems that blue light is the most suitable quality light for the production of PC (reviewed in Hsieh-Lo et al., 2019). Phycocyanin content of *A. platensis* grown under blue light was 2.9-fold higher than for the other quality light combinations (Lee et al., 2016). Using LED technology, mean production of phycocyanin by dry biomass of 152 mg g^{-1} (Chen et al., 2010) and 121 mg g^{-1} (Lima et al., 2018) were reported for *Arthrospira*. On the other hand, commercially produced *Arthrospira* reports PC content between 20 and 80 mg dry $weight^{-1}$ (Ma et al., 2019). In our study, using traditional fluorescent lamps, we obtained values of 143.66 mg g^{-1} and 111.02 mg g^{-1} for strains CCM-UdeC 040 and CCM-UdeC 136, respectively (Table I.1).

Because of its protein nature, phycobilinic pigments are intracellular nitrogen sources that can be used by the cyanobacteria under nitrogen starvation conditions. During nitrogen deprivation, usually a decrease in PC content is observed in *A. platensis* (Boussiba & Richmond, 1980; Deschoenmaeker et al., 2014); however, a ten times reduction in the nitrogen supply was not enough to decrease the PC accumulation in another strain classified as *A. platensis* (dos Santos et al., 2019), which could be economically convenient for large scale cultivation of *Arthrospira* for PC production.

Table I.2. Fatty acids analysis of the strains CCM-UdeC 040 and CCM-UdeC 136 biomass. Values are expressed as mg of each fatty acid by g of dry biomass (quantitative analysis) and as percentage of each fatty acid with respect to the total fatty acids detected in the respective strain (fatty acids profile).

Fatty acids	**CCM-UdeC 040**		**CCM-UdeC 136**	
	mg g^{-1}	**% of total fatty acids**	**mg g^{-1}**	**% of total fatty acids**
Lauric acid (C12:0)	0,05	0,72	0,60	3,42
Myristic acid (C14:0)	0,01	0,16	0,02	0,10
Palmitic acid (C16:0)	2,06	29,14	6,42	36,64
Palmitoleic acid (C16:1)	0,62	8,80	1,21	6,90
Estearic acid (C18:0)	0,05	0,64	0,12	0,68
Oleic acid (C18:1n9c)	0,17	2,47	0,88	5,03

Linoleic acid (C18:2n6c)	1,24	17,57	3,07	17,50
Gamma-linolenic acid (C18:3n6)	1,99	28,12	4,92	28,11
Alfa-linolenic acid (C18:3n3)	0,01	0,11	0,03	0,18
cis-11,14-eicosadienoic acid (C20:2n6)	0,04	0,52	0,11	0,61
cis-8,11,14-eicosatrienoic acid (C20:3n6)	0,06	0,78	0,13	0,71
cis-5,8,11,14,17-eicosapentaenoic acid (C20:5n3)	0,09	1,25	Nd	Nd
Nervonic acid (C24:1n9)	0,69	9,71	Nd	Nd

*nd: Not detected

Reported average protein content of *Arthrospira* is 60%, ranging between 50 and 60% (Aouir et al., 2017; Ma et al., 2019; da Rosa et al., 2015). This high value, as well as the fact of *Arthrospira*'s protein includes all the essential amino acids, makes this cyanobacteria a highly valuable nutritional source of vegetal protein (reviewed Lupatini et al.,2017). In this work, total protein contents were not different between both strains ($p \geq 0.05$) and values were lower than expected for the genus: 43.05% and 36.90% of dry weight for CCM-UdeC 040 and CCM-UdeC 136, respectively (Table I.1); nevertheless not optimization of culture conditions for protein accumulation was carried out in this study.

The protein content of *A. platensis*' biomass could achieve up 70% if grown under non-limiting nitrogen conditions (Sassano et al., 2010). Another strain, classified as *Spirulina* sp., reached 60.8% of protein when grew with CO_2, instead of sodium bicarbonate, as carbon source (da Rosa et al., 2015) and 69.78% when the culture was supplemented with 0.05 mol L^{-1} glycerol (Morais et al., 2019). Irradiance also affects protein content in *Arthrospira*, which can be explained, in part, because of the protein nature of phycobilinic pigments. Kilimtzidi et al. (2019) grew *A. platensis* at 100 µmol m^{-2} s^{-1} and 516 µmol m^{-2} s^{-1} and obtained biomass with 58.3% and 30.7% of protein, respectively.

The average lipid content of *Arthrospira* is difficult to determine due, mainly, to the differences in extraction and quantification methods used by different authors (Ciferri, 1983). Total lipid content in *Arthrospira* sp. cultivated in Zarrouk medium, at 80 µmol m^{-2} s^{-1} and 30 °C was 9.0%; however, according to salinity, phosphate and, mainly, nitrate availability, lipid

content varied from 5.45% to 15.33% of dry biomass (Chentir et al., 2018). Nitrogen's starvation, as well as salinity stress, promote lipid accumulation in *Arthrospira*, for example, *A. platensis* UTEX 2340 accumulated 15.4% lipids when grew at NaCl 0.428 M (Mata et al., 2016). In our work no significant difference ($p \geq 0.05$) was detected between both strains in total lipids content and values were in the lowest range expected: 3.74% and 5.65% of dry weight for CCM-UdeC 040 and CCM-UdeC 136, respectively.

It is worth mentioning that different values of biochemical parameters reported in literature for *Arthrospira*, compared between them and with those obtained in this study, may be derived from different cultivation conditions as well as different strain/species used. Fourteen fatty acids were identified in strain CCM-UdeC 040 and twelve in strain CCM-UdeC 136 (Table I.2). Palmitic acid was the most predominantly occurring fatty acid in both strains, followed by gamma-linolenic and linoleic acids. These three fatty acids corresponded to 74.83% and 82.25% of total fatty acids in strains CCM-UdeC 040 and CCM-UdeC 136, respectively (Table I.2). Consistent with these results, Mühling et al. (2005) reported that the sum of palmitic, gamma-linolenic and linoleic acids represented 88–92% of the total fatty acids in two strains: *A. platensis* Sp-1 (AJ292324) and *A. platensis* PCC 7345 (AJ292322), which belong to the same genetic species than CCM-UdeC 040 and CCM-UdeC 136 (Fig. I.1).

Gamma-linolenic acid (GLA) is a precursor of the nutritionally very important eicosapentaenoic and docosahexaenoic acids, two essential omega 3 fatty acids for animals. GLA has potent anti-inflammatory effects so it plays an important role in the treatment of a wide variety of pathologies such as atherosclerosis, heart diseases, Parkinson's disease, arthritis, multiple sclerosis, among others (Choopani et al., 2016; Hoorn et al., 2008). *Arthrospira* has been proposed as a potential nutritional source of this fatty acid because some strains, grown under optimized conditions, could accumulate about 1% of GLA in their dry biomass (Choopani et al., 2016). According to our results, GLA, expressed as a percentage of total fatty acids, was equal in both strains (~28%) (Table I.2). Mühling et al. (2005) reported variations in GLA between 12.9 and 29.4% of total fatty acids among 35 *Arthrospira* strains, so our results are close to the higher expected value. In spite of both strains showed similar relative content of GLA, strain CCM-UdeC 136 accumulated almost 2.5 times more GLA by dry biomass than strain CCM-UdeC 040 (Table I.2); however these values are low compared with previously reported which ranged between 10 and 15 mg g^{-1}

(Ronda & Lele, 2008).

On the other hand, even though both strains exhibited very similar fatty acids profiles, fatty acids C20:5n3 (eicosapentaenoic acid) and C24:1n9 (nervonic acid) were detected in CCM-UdeC 040 but not in CCM-UdeC 136 (Table I.2). Nervonic acid (NA), is a monounsatured omega 9 fatty acid essential for the growth and maintenance of brain physiology. Clinical studies have revealed that NA can be a marker of neurodegeneration and its intake could improve brain development (Lewkowicz et al., 2019). Because the aim of this study was the comparison of both strains rather than optimizing their growing conditions (which includes nutrients availability, light conditions, temperature, culture regime), in a second step of this research it is necessary to address this challenge in order to optimize the accumulation of metabolites of commercial interest, especially in the most promising strain.

3.3. Aqueous extract characterization

The aqueous extracts of CCM-UdeC 040 and CCM-UdeC 136 biomass showed differences on their chemical composition and antioxidant capacity (Table I.3). Total protein and PC contents were higher in CCM-UdeC 040 than in CCM-UdeC 136 ($p < 0.05$). In spite of both strains had equal protein content by dry biomass (Table I.1), probable differences on the identity (amino acids sequence) of hydrosoluble proteins of each strain would explain this result.

The antioxidant activity of aqueous extracts was measured using the Oxygen Radical Antioxidant Capacity (ORAC) assay, which can detect both hydrophilic and hydrophobic antioxidants (Schaich et al., 2015). Aqueous extract of strain CCM-UdeC 040 had a higher ORAC capacity than CCM-UdeC 136, coincident with its higher protein and PC content (Table I.3). This result was expected considering the recognized antioxidant properties of phycobilinic pigments (Pagels et al., 2019; Sonani et al., 2015); besides, fraction of aqueous extracts of *Arthrospira*, non-containing phycocyanin, also have demonstrated a remarkable antioxidant activity (Jensen et al., 2015). Despite differences between antioxidant properties of aqueous extracts of both strains (Table I.3), both were still high compared with ORAC values of fruits high in antioxidants like *Morus nigra* (black mulberry) (132.21 ± 8.88 µmol TE/g DW), *Vaccinium meridionale* (wild bilberries) (416.8 ± 53.4 µmol TE/g DW), *Aristotelia chilensis* (maqui) (285–789 µmol TE/g DW) and the Thai indigenous plant *Kadsura* spp.

(Garzón et al., 2020; Quispe-Fuentes et al., 2018; Sritalahareuthai et al., 2020; Suttisansanee et al., 2020).

Table I.3. Antioxidant capacity (ORAC test), total protein and phycocyanin content of aqueous dry extracts prepared from the biomass of strains CCM-UdeC 040 and CCM-UdeC 136.

	CCM-UdeC 040	**CCM-UdeC 136**
ORAC capacity (µmol Trolox Equivalents g dry biomass extract^{-1})	376,50 ± 20,92 [a]	263,42 ± 26,24 [b]
Total protein (% by dry aqueous extract)	47,30 ± 4,68 [a]	19,61 ± 1,56 [b]
Phycocyanin (mg dry aqueous extract g^{-1})	122,63 ± 11,82 [a]	76,18 ± 5,99 [b]

Data are the means and standard deviation of three replicate experiments. Different letters indicate statistical differences assessed by t-Student Test at $p < 0.05$.

When dealing with novel raw material for future food applications it is important to demonstrate lack of toxicity (Niccolai et al., 2017). In this study in vitro cytotoxicity of aqueous extracts of CCM-UdeC 040 and CCM-UdeC 136 was evaluated against THP-1 human leukemia cell lines and the inhibitory effect was calculated considering the concentration of extract required to inhibiting cells growth by 50% (IC_{50}). According to GRAS category of *Arthrospira* (Furmaniak et al., 2017; Marles et al., 2011), the IC_{50} values of aqueous extract were 5.0 and 4.48 mg mL^{-1} for CCM-UdeC 040 and CCM-UdeC 136, respectively; thus both extracts are classified as “potentially non-toxic” (IC_{50} > 1000 µg mL^{-1}), according to categories described by Kollar et al. (2016).

3.4. CCM-UdeC 040 and CCM-UdeC 136 protein hydrolysates

The hydrolysis of intact proteins produces bioactive peptides with potentially higher bioactivities than the parent protein itself (Korhonen, 2009). Degree of hydrolysis (DH) of aqueous extracts of strains CCM-UdeC 040 and CCM-UdeC 136, obtained from sequential hydrolysis with three proteases, and monitored for 240 min, showed notable differences between strains (Fig. I.2). This result is an evidence of differences in the identity, and therefore amino acid composition, of the hydrosoluble proteins into each extract.

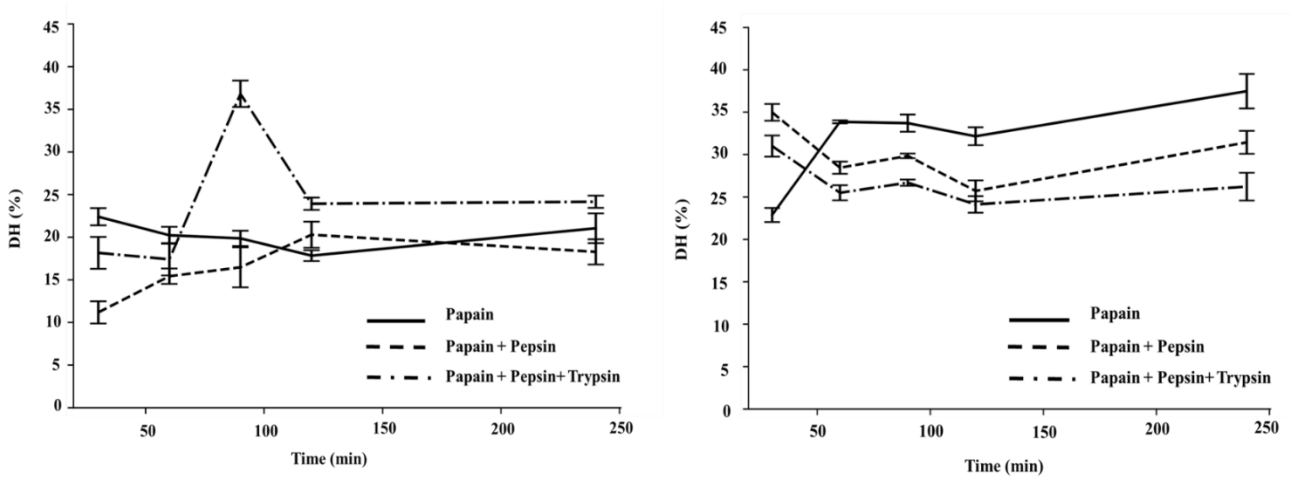

Figure I.2. Degree of hydrolysis (DH) obtained through sequential hydrolysis with papain, pepsin and trypsin of aqueous extract of CCM-UdeC 040 (A) and CCM-UdeC 136 (B) measured by OPA method.

Thirty minutes incubation of aqueous extracts of CCM-UdeC 040 and CCM-UdeC 136 with papain produced a remarkable DH value (22.40% and 22.88%, respectively) (Fig. I.2). In spite of 90 min sequential hydrolysis with papain-pepsin-trypsin of CCM-UdeC 040 extract produced the highest DH for this strain (38.54 ± 4.62%) (Fig. I.2A) and longer incubation times with papain produced higher DH for CCM-UdeC 136 extract (Fig. I.2B), we decided to continue the characterization of 30-min papain hydrolyzed extracts in order to continue comparison (more than optimization) of both strains.

Papain is a protease extracted from the tropical plant *Carica papaya* and it has GRAS status by the US federal agencies (CFR 1999, 2009). Compared with other industrial proteases, those of plant origin are much less used. The use of proteases from animal, microbial or recombinant sources is a common subject in question due to ethical discussion, religious arguments, regulatory restrictions and changes in people's eating habits (e.g., vegetarians, vegans) (Feijoo-Siota & Villa, 2011; Mazorra-Manzano et al., 2018), which provides new opportunities for the use of plant proteases in industrial processes, that include enzymatic hydrolysis of proteins. In this study, just a 30 min hydrolysis of aqueous extract of CCM-UdeC 040 and CCM-UdeC 136 with papain produced higher DH values than previously reported for aqueous extracts of Chinese *Arthrospira*'s commercial biomass for 8 and 10 h, where DH values ranged between 11.12% and 18.3%, respectively (Fan et al., 2018; Wang & Zhang, 2016).

Fast Protein Liquid Chromatography (FPLC) was used to separate hydrolysis products according to their molecular size. Differences on the elution patterns between crude (C) and hydrolysated (H) aqueous extract of strains CCM-UdeC 040 and CCM-UdeC 136 were detected (Fig. I.3). These results reinforce the differences in the proteins identity within each aqueous extracts.

For CCM-UdeC 040, fractions 1 and 3 were completely hydrolyzed by papain, while fraction 2 was significantly hydrolyzed (about three-fold of fraction 2), as was evidenced by the fall on the signal of H1 chromatograms at 215 nm, which correspond to peptide backbone absorbance. Fraction 5, corresponding to smaller components, showed a twofold higher absorbance of the hydrolysate at 215 nm and hence reflects an increase on small molecular weight peptides (Fig. I.3A).

On the other hand, for strain CCM-UdeC 136, fraction 1 was partially hydrolyzed, as observed when C2 and H2 chromatograms measured at 215 nm are compared; while fractions 2, 3 and 4 increased their intensities on hydrolysated sample measured at 215 nm. Fraction 4 showed a higher absorbance at 215 nm and 280 nm, which could be due to an increase on small molecular weight peptides, consistent to an effective hydrolyzation process (Fig. I.3B).

Unlike other functional properties, including antihypertensive (Aiello et al., 2019; Anekthanakul et al., 2019), anti-inflammatory (Vo & Kim, 2013), antitumor (Wang & Zhang, 2016), antidiabetic (Aiello et al., 2019; Hu et al., 2019) and anti-obesity (Fan et al., 2018), antioxidant capacity of *Arthrospira*-derived peptides is just beginning to be studied (Wang et al., 2020).

In order to evaluate if hydrolysis treatment had an effect on antioxidant capacity of aqueous extracts of the strains studied, $ABTS^{+}$ radical scavenging assay was conducted. This in vitro method is rapid, simple and it can be used over a wide range of pH values, in both aqueous and organic solvent systems and is considered a good predictor of ORAC activity (Awika et al., 2003). For both strains, ABTS scavenging activity was significantly higher for 30 min-papain hydrolyzed than crude extracts, which would demonstrate that proteolytic digestion certainly produced peptides with antioxidant properties in both strains (Fig. I.4), just like it had been previously demonstrated for the cyanobacteria *Lyngbya* and *Microcoleus* (Ghosh et al., 2016). Antioxidant capacity of CCM-UdeC 040 hydrolysate was higher than CCM-UdeC 136 (87.47% vs 75.91%, respectively).

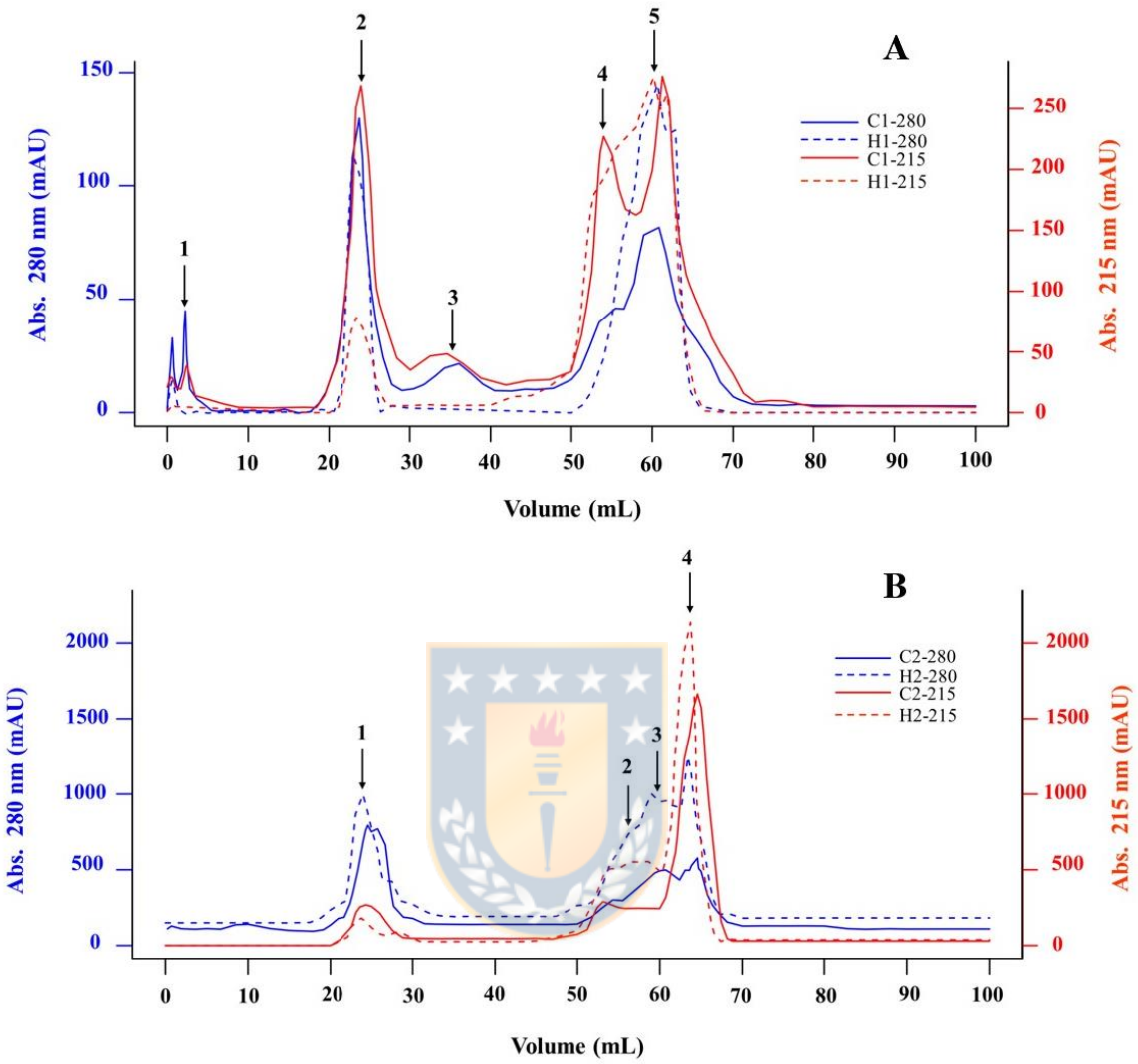

Figure I.3. Size-exclusion chromatography elution profiles of papain 30 min hydrolysates of strains CCM-UdeC 040 and CCM-UdeC 136. Arrows indicate fractions described in the text. A: C1-280, C1-215: CCM-UdeC 040 control extract measured at 280 nm and 215 nm, respectively; H1-280, H1-215: CCM-UdeC 040 hydrolysated extract measured at 280 nm and 215 nm, respectively. B: C2-280, C2-215: CCM-UdeC 136 control extract measured at 280 nm and 215 nm, respectively; H2- 280, H2-215: CCM-UdeC 136 hydrolysated extract measured at 280 nm and 215 nm, respectively.

These differences can be explained by differences in the mixtures of free amino acid and peptides resulting of proteolytic digestion of proteins of different amino acids sequence. In fact antioxidant activity of peptides has been related with the presence of specific amino acids on them acting as metal chelators or radical scavengers (Ren et al., 2008).

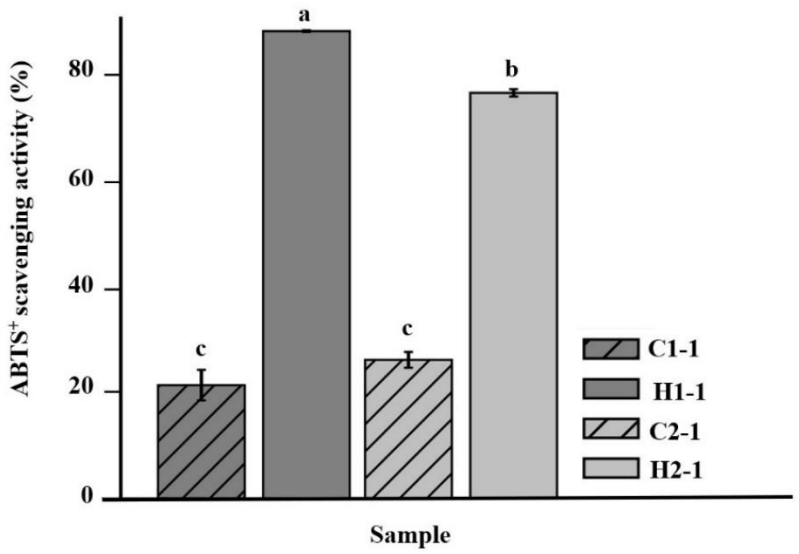

Figure I.4. ABTS assay of papain-30 min hydrolysates of strains CCM-UdeC 040 and CCM-UdeC 136. C1-1, H1-1: control and hydrolysated extracts of strain CCM-UdeC 040, respectively; C2-1, H2-1: control and hydrolysated extracts of strain CCM-UdeC 136, respectively. The average values ± standard deviations of three replicates per treatment are shown. Different letters indicate significant differences ($p < 0.05$) assessed by Dunn Test.

Phycobilinic pigments give account of an important fraction of hydrophilic proteins included in an aqueous extract of *Arthrospira*, as was also demonstrated in this study (Table I.1). Nevertheless, an important contribution of peptides derivated of hydrolysis of proteins, other than phycobiliproteins, to the antioxidant activity of an *Arthrospira* aqueous extract has been already demonstrated (Jensen et al., 2015). Future studies should include further characterization of hydrolyzed aqueous extracts obtained from strain CCM-UdeC 040, including the use of single or combined proteases treatment in order to detect new bioactive properties in this interesting strain.

For food market, genetically modified organisms (GMO) are usually questioned because there is concern about its safety, both for the environment and human health (Fayyaz et al., 2020; Hallmann, 2007). Intraspecific diversity is a widely distributed phenomenon in microalgae, including species of biotechnological value like PUFAs producers (e.g., Liang et al., 2005; López Alonso et al., 1994), carotenogenic green algae (e.g., Gómez et al., 2016; Gómez & González, 2005) and strains belonging to the same genetic species of *Arthrospira*

(this study). Intraspecific variability is a key attribute in microalgal biotechnology, because it allows simple selection of new strains with high biotechnological potential (e.g., CCM-UdeC 040), which is the simplest (and most natural) way to perform genetic improvement.

4. Conclusion

Two strains, preliminary classified as *Arthrospira maxima* (CCM-UdeC 040) and *Arthrospira platensis* (CCM-UdeC 136), were identified as belonging to the same genetic species of *Arthrospira* genus, according to their ribosomal ITS (internal transcribed spacer) sequences. Some morphological, biochemical and functional attributes showed differences between both strains, grown under the same culture conditions, which could be explained by the presence of intraspecific variability.

Our results reinforce the necessity to detailed characterization of a strain before its scaling up for commercial production, demonstrating the importance of the intrinsic attributes of each strain, beyond its taxonomic classification. Strain CCM-UdeC 040 showed the highest biotechnological potential as a source of commercially important metabolites and functional properties for food and nutraceutical markets. Considering that the culture conditions as temperature, irradiance, pH, nutrients and culture regime, certainly induce changes on the biochemical composition of *Arthrospira* biomass, it would be crucial to optimize those parameters for CCM-UdeC 040, the most promising strain in this study.

Author statement

The present article is the part of Ariadna López-Rodríguez PhD thesis work. She participated in the conceptualization, carried out the experiments and contributed to visualization and writing the original draft. Jaen Mayorga and David Flaig participated in the investigation and provided technical support. Glenda Fuentes contributed to formal analysis of DNA sequences for phylogenetic analysis. Juliana Cotabarren and Walter David Obregón made a contribution to conceptualization, investigation and resources of protein hydrolysates assays. Patricia I. Gómez participated in the conceptualization, provided lab facilities and resources and contributed to writing the original draft.

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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II. CAPÍTULO 2. Genetic characterization and assessment of the biotechnological potential of strains belonging to the genus *ArthrospiralLimnospira* (Cyanophyceae) deposited in three microalgae culture collections: UTEX (USA), CCAP (UK) and CCM-UdeC (Chile).

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Highlights

- Microalgae culture collections are invaluable biodiversity reservoirs.
- ITS sequence of 11 strains named as *Arthrospira/Limnospira* or *Spirulina* was analyzed.
- Seven strains were identified as *Arthrospira/Limnospira* and four strains as *Spirulina*.
- *Arthrospira/ Limnospira* strains showed intraspecific variability.
- Strains with desirable properties are currently available in culture collections.

Abstract

Culture collections of microalgae preserve viable material for conservation, research and biotechnology purposes; however, in the absence of routine authenticity checking, the strains deposited may become inexact references or inadequate starting material for a commercial development. The edible cyanobacteria *Arthrospira*/*Limnospira* is recognized as one of the most important industrially cultivated microalgae. *Arthrospira*/*Limnospira* strains have been frequently confused and incorrectly denoted as *Spirulina*, therefore, an accurate taxonomic identification of strains available in culture collections worldwide is imperative. In this study, eleven strains, originally designated as *Arthrospira*/*Limnospira* or *Spirulina* in three microalgae culture collections, were analyzed using their ITS sequence, to verify their taxonomic classification. Phylogenetic analysis showed that there were misclassified strains in these collections: seven strains were grouped in one of the two genetic clusters previously described for *Arthrospira*, while remaining strains were grouped within *Spirulina* genus, forming two well-supported not previously described genetic clusters. Morphological characterization of *Arthrospira* strains showed typical characters of the genus; while their physiological and biochemical characterization showed intraspecific variability in growth attributes and pigments, proteins, lipids and fatty acids contents.

Keywords: *Arthrospira* strains, ITS sequences, genetic species, intraspecific variability.

1. Introduction

Microalgae are a highly diverse group of photoautotroph unicellular organisms, comprising eukaryotic as well as prokaryotic representatives, of great environmental relevance. They contribute to approximately 50% of global photosynthetic activity, assimilating CO_2 ten to fifty times more efficiently than vascular plants [1]. Microalgae are ubiquitous having the ability to survive in different, and frequently extreme, environments, due in part to the production and accumulation of numerous secondary metabolites whose biosynthesis pathways are affected by biotic and abiotic environmental conditions [2, 3].

The biodiversity of microalgae is huge and poorly explored. Microscopic nature of these resources has provoked vagueness and underestimation of the number of species; in fact, it has been estimated that more than one million of microalgal species exist although just 166.325 species are currently included in the global on-line taxonomic database AlgaeBase [4, 5].

Culture collections are biological repositories keeping living organisms; they act as depositaries of an invaluable patrimony of biodiversity, since they preserve viable biological material for education, research, conservation and biotechnology purposes [6]. Microalgae culture collections are safe storage facilities of strains isolated from a specific place at a specific time; they are essential for promoting the knowledge and sustainable use of biodiversity within this group because they provide pure, stable, identified and certified algal resources to users [2, 7].

In spite of the numerous efforts made by the depositors and the own collections, many microalgae strains available in private and public collections are incorrectly identified and classified. In fact, it has been estimated that up to 50% of strains deposited in culture collections do not correspond to the diagnoses of the taxa to which they were originally assigned [7, 8]. Consequences of mistaken classification may include wrong reference material for users or inadequate starting material available for a commercial development; therefore, a major concern related with the handling of microalgae culture collections worldwide, is the correct strain identification.

Despite the tremendous diversity of microalgae in nature, and the enormous advantages of these resources for the production of metabolites of commercial interest, only a few genera have been exploited for biotechnological purposes [3]. The edible cyanobacteria *Arthrospira*

is one of the most important industrially cultivated microalgae [9], with an estimated global production of 15,000 t dry biomass per year [10]. The international market for *Arthrospira* was valued at $335.2 million in 2021 and is projected to reach $466.9 million by 2026 [11].

Arthrospira has gained attention due to its high nutritional value and wide pharmaceutical applications; in 2002 was recognized by the American Food and Drug Administration (FDA) as GRAS (Generally Recognized As Safe) and, in 2011, was assigned with Class A safety level as dietary supplement by the commission of United States Pharmacopeia and distinguished as one of the best “superfoods” [12, 13]. In the past, *Arthrospira* Stizenberger [14] was incorrectly unified with the genus *Spirulina* Turpin by Geitler [15]. Based on phenotypic data, as differences in helicity and trichome size, cell wall structure and pore pattern, gas vesicles, thylakoid pattern, trichome motility and fragmentation, combined to molecular information; is now accepted that *Arthrospira* and *Spirulina* are two distinct genera [16-18]. However, currently *Arthrospira* is commercialized under the name ‘Spirulina’ as a food supplement for humans and animals [13], which represents a potentially dangerous taxonomic mistake, because cyanotoxin type microcystins may appear in *Spirulina* [19], but not in *Arthrospira*; where toxicity has not been reported [12, 20-22].

Main taxonomic criteria for species delimitation within *Arthrospira* genus include: tightness and arrangement of spirals, cells and apices dimensions and trichome proportions [17]. However, some of these phenotypic characters are unstable under different environmental conditions, which would partly explain the morphological variability observed in *Arthrospira* [16, 23, 24]. The analysis of biochemical and physiological parameters has been used for the differentiation of cyanobacteria [25] and for the identification of *Arthrospira* species. It is known that *Arthrospira* strains contain high amounts of protein (ranged from 50 and 70% by dry weight), significant proportion of polyunsaturated fatty acids (PUFAs) (including γ-linolenic acid, which is absent in the genus *Spirulina*); also they are rich in vitamins and pigments such as chlorophyll *a*, phycocyanin, xanthophyll, β-carotene and zeaxanthin [26-28].

According to Nelissen and co-workers [29], sequences of the internally transcribed spacer (ITS), located between 16S and 23S ribosomal genes, exhibit sufficient variability to discriminate between *Spirulina* and *Arthrospira*. A remarkable structural feature in the ribosomal ITS spacer, that differentiates *Spirulina* from *Arthrospira*, is the presence of only one $_{t}$RNA gene ($_{t}$RNA^{Ile}) in *Spirulina* while two genes ($_{t}$RNA^{Ile} and $_{t}$RNA^{Ala}) are interrupting

the ITS spacer of *Arthrospira*, being the last one the most common configuration in cyanobacteria [30, 31].

Recently, using a polyphasic approach that included analyses of morphology, ultrastructure, ecological distribution and 16S rRNA gene sequence, the establishment of the new genus *Limnospira* Nowicka-Krawczyk, Mühlsteinová & Hauer was proposed. This genus would comprise the *Arthrospira* species of commercial interest: *L. (A.) maxima*, *L. (A.) fusiformis*, *L. (A.) indica* and, probably, *A. platensis*; being *Limnospira fusiformis* the type species [32].

Through ITS sequence analysis, *Arthrospira*/*Limnospira* strains from four continents were grouped into two main groups denoted as cluster I and cluster II [18, 31, 33]. From these studies it has been hypothesized that these clusters represent the two existing *Arthrospira* genotypes. In this sense, if the consensus ITS sequence is considered as autopomorphic character and monophyletic species concept exposed by Johansen and Casamatta [34] is applied, all strains of *Arthrospira* would belong to one of two (monophyletic) species.

In this study we acquire all the available strains, classified as *Arthrospira*/*Limnospira* or *Spirulina* and deposited in three culture collections of microalgae: the Culture Collection of Algae and Protozoa (CCAP) in United Kingdom, the Culture Collection of Algae at the University of Texas at Austin (UTEX) in USA and the Microalgal Culture Collection at the Universidad de Concepción (CCM-UdeC) in Chile, in order to verify their taxonomic classification. Additionally, for those strains confirmed as “true” *Arthrospira*/*Limnospira*, we carried out a physiological (growth) and biochemical (proteins, pigments, lipids and fatty acids contents) characterization, to assess their biotechnological potential.

2. Materials and methods

2.1. Strains and culture conditions

Eleven strains of cyanobacteria, originally designated as *Arthrospira/Limnospira* or *Spirulina*, were obtained from three microalgae culture collections in America and Europe: Culture Collection of Algae at The University of Texas, USA (UTEX) (https://utex.org); Culture Collection of Algae and Protozoa, United Kingdom (CCAP) (https://www.sams.ac.uk/facilities/ccap) and Microalgal Culture Collection at the Universidad de Concepción, Chile (CCM-UdeC) (https://www.ficolab.cl) (Table II.1).

Table II.1. Code, species name and maintaining culture medium of the strains in their original culture collections: CCAP, UTEX and CCM-UdeC. Last column indicates culture medium selected for each strain in this study.

Code of strains	Designed species name by the original culture collection	Culture medium used in the original culture collection	Culture medium used in this study
CCAP-1475-2	*Spirulina versicolor*	Artificial Seawater (Soil Extract) + BG11	Erdschreiber Medium
CCAP-1475-3	*Spirulina major*	Artificial Seawater (Soil Extract) + BG11	Erdschreiber Medium
CCAP-1475-9	*Limnospira maxima*	Artificial Seawater (Soil Extract) + BG11	Zarrouk Medium
UTEX 552	*Spirulina major*	Enriched Seawater Medium	Erdschreiber Medium
UTEX 1926	*Spirulina platensis*	Enriched Seawater Medium	Zarrouk Medium
UTEX 1928	*Spirulina platensis*	Enriched Seawater Medium	Zarrouk Medium
UTEX 1954	*Spirulina subsalsa*	Enriched Seawater Medium	Erdschreiber Medium
UTEX 2342	*Spirulina maxima*	Spirulina Medium	Zarrouk Medium
UTEX 2721	*Arthrospira fusiformis*	Spirulina Medium	Zarrouk Medium
CCM-UdeC 040	*Arthrospira maxima*	Zarrouk Medium	Zarrouk Medium
CCM-UdeC 136	*Arthrospira platensis*	Zarrouk Medium	Zarrouk Medium

In order to determine the most suitable culture medium to grow each strain, seven culture media were assayed: BG 11 medium [35], Walne medium [36], Erdschreiber medium [37], F medium [38], Bristol medium [39], Spirulina medium [40] and Zarrouk medium [41]. This first screening was performed in 10 mL volume. Strains were maintained at 20 ± 2°C, under a photon flux density of 50 µmol m^{-2} s^{-1} and 12:12 (L:D) photoperiod. Cultures appearance and cells morphology were monitored during a 30-days period. The culture medium that sustained a healthy growth of each strain was selected (Table II.1) and strains were maintained in 100 mL volume under light and temperature conditions indicated above.

2.2. Molecular analyses

ITS sequences of strains were amplified and sequenced as previously described by Barriga et al. [42]. Consensus ITS sequences were obtained from forward and reverse raw sequences data using the Assembling sequence tool of the Geneious Prime program, version 2019. ITS sequence of strains CCAP-1475-2, CCAP-1475-3, CCAP-1475-9, UTEX

552, UTEX 2721, UTEX 1954, UTEX 1928, UTEX 1926 and UTEX 2342 were properly deposited in the Genbank. Bayesian phylogenetic analysis of ITS sequences from the nine sequences generated in this study, plus 29 sequences available in the GenBank for *Arthrospira* or *Spirulina*, was conducted. *Desertifilum tharense* and *Oxynema thaianum* were included as outgroups. The phylogenetic analysis and the visualization of the resulting tree were performed on the Softwares: jModeltest 2, MrBayes 3.2.7a and FigTree v1.4.4; according to López-Rodríguez et al. [43].

2.3. Growth and biochemical analyses of *Arthrospira* strains

Strains confirmed as belonging to the genus *Arthrospira*/*Limnospira* were cultured in Zarrouk medium. Experimental cultures were initiated at a cell density of 1000 µg chlorophyll *a* L^{-1} in 800 mL volume, using 1 L Erlenmeyer flasks and maintained at 20 ± 2°C, photon flux density of 80 µmol m^{-2} s^{-1} and 12:12 (L:D) photoperiod. Cultures were continuously aerated with 0.22 µm pore-filtered air. Four replicates of each strain were cultured and grown until the stationary growth phase. The evaporated water during the culture period was replenished by the addition of sterile distilled water.

2.3.1. Confocal microscopy

Images of selected *Arthrospira* strains were obtained with a spectral confocal microscopy (LSM780 NLO, Zeiss) equipped with a Plan-Apochromat 63x/1.40 oil DIC M27 objective at the Center for Advanced Microscopy of Universidad de Concepción (CMA Bio-Bio, Concepción, Chile). Intracellular distribution of pigments was observed by scanning using 405, 488 and 633 nm argon lasers and light emissions were recorded in the blue (374-471 nm), green (490-560 nm) and red channels (629–758 nm) to monitoring fluorescence signals. For DNA visualization, the samples were stained with Hoechst 33342 (Sigma Aldrich) and observed using excitation and emission wavelengths of 405 and 374-471 nm, respectively. Images were processed using the ZEN 2011 software v8.0 (Carl Zeiss)

2.3.2. Growth

Growth of strains was monitored through chlorophyll *a* measurement, according to López-

Rodríguez et al. [43]. Every two days, 2-5 mL samples were collected from each flask for chlorophyll *a* content determination and these values were used for determining maximum specific growth rate (μ_{max}, d^{-1}), according to Vonshak [26]. The biomass of each replicate was harvested in stationary phase by centrifugation at 8000 rpm for 10 min or filtration with a phytoplankton net filter of 0.55 µm pore size. Cell pellets were frozen for 24 h at −20 ºC and dried by lyophilization at −50 ºC and 100 militorr pressure for 48 h. Dried biomass was stored at −20 ºC until analyzes (total proteins, chlorophyll *a*, carotenoids, phycobilin pigments, total lipids, fatty acids).

The biomass production of each replicate, expressed as algal dry weight, was determined at harvesting day by filtering 5 mL of culture through a pre-dried and pre-weighted 0.45 µm-pore filter and drying it at 80°C until it reached a constant weight.

2.3.3. Biochemical analysis

Total proteins, chlorophyll *a*, carotenoids, phycobilin pigments, total lipids and fatty acids contents in the lyophilized biomass of each replicate, were measured according to López-Rodríguez et al. [43].

2.4. Statistical analysis

Statistical data analyses were performed using R software version 4.0.2. The normality and homogeneity of variances analyses were performed using the Kolmogorov–Smirnov and Levene tests, respectively. In order to assess the statistical significance of differences on measured parameters between strains, one-way ANOVA was performed. Significant differences among means were assessed by Tukey test. All analyses were conducted considering a significance level of $\alpha = 0.05$ and using four biological replicates.

3. Results

The strains obtained from the culture collections UTEX, CCAP and CCM-UdeC were maintained in the most favorable culture medium for each one (Table II.1). Morphology of the strains under light microscope showed evident differences, mainly in the width and coiling degree of trichomes. Strains CCAP-1475-9, UTEX 1926, UTEX 1928, UTEX 2342, UTEX 2721, CCM-UdeC 040 and CCM-UdeC 136 had wide trichomes with a more or less

pronounced zig-zag shape, except for strain CCM-UdeC 136 that has straight trichomes. On the other hand, strains CCAP-1475-2, CCAP-1475-3, UTEX 552 and UTEX 1954 exhibited thin and tightly coiled trichomes (Figure II.1).

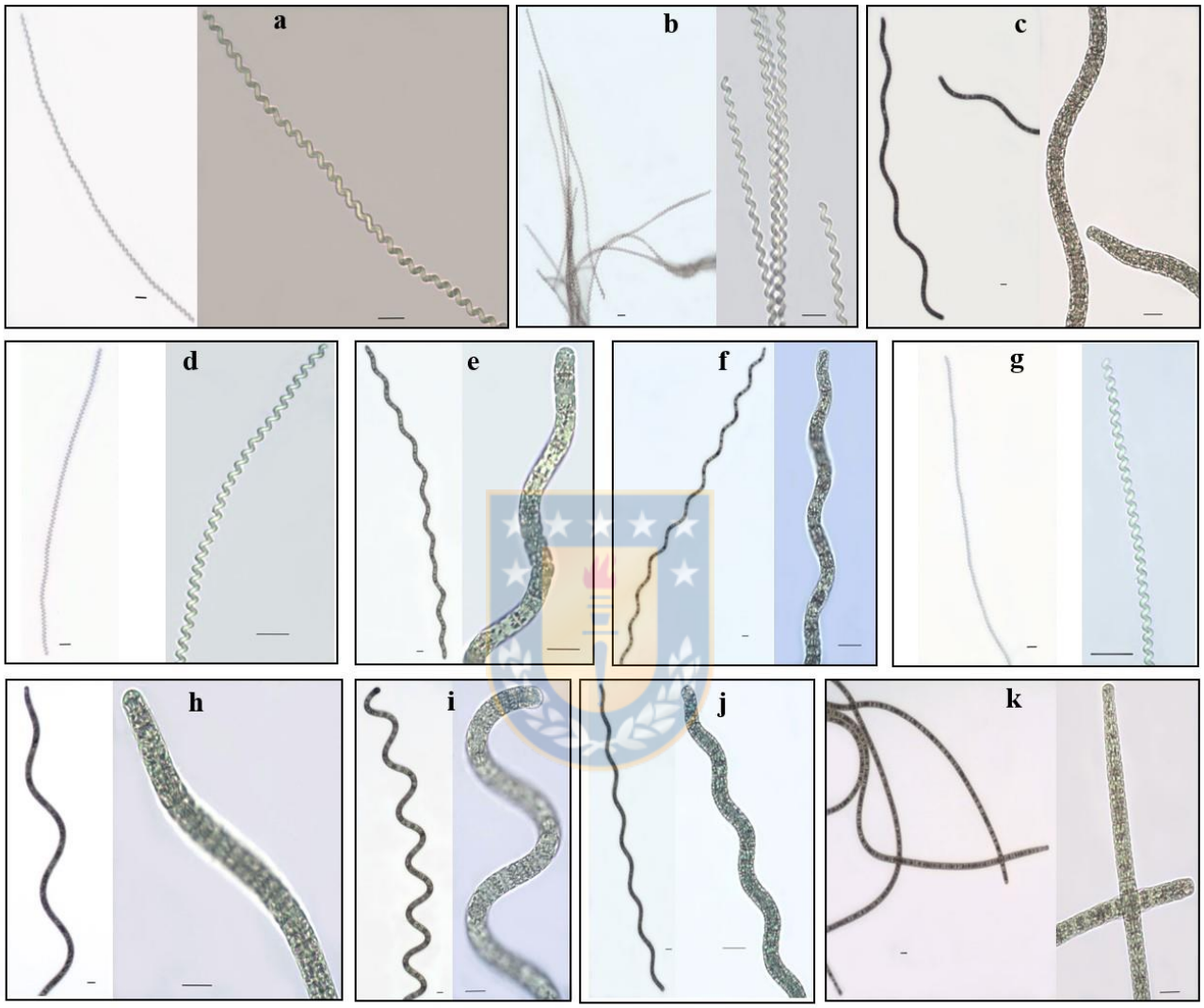

Figure II.1. Images of light microscopy of strains deposited as *Arthrospira*/*Limnospira* or *Spirulina* in the culture collections CCAP, UTEX and CCM-UdeC. (a) CCAP-1475-2, (b) CCAP-1475-3, (c) CCAP-1475-9, (d) UTEX 552, (e) UTEX 1926, (f) UTEX 1928, (g) UTEX 1954, (h) UTEX 2342, (i) UTEX 2721, (j) CCM-UdeC 040 and (k) CCM-UdeC 136. Bars are 10 μm. See Table 1 for details.

Strains were cultivated in 100 mL volume and subsequently scaled to 1 or 2 L volume, until determining true *Arthrospira* strains, through ITS sequence analysis. The biomass of all the strains was blue-green in color, except for strains UTEX 1928 and UTEX 2721 whose

biomasses were light green in color. The strains exhibited different growth behavior: strains CCAP-1475-2, CCAP-1475-3 and UTEX 1954 grew slowly, with a clumpy appearance and did not reach significant densities, while strains UTEX 552, CCAP-1475-9, UTEX 1926, UTEX 2342, CCM-UdeC 040 and CCM-UdeC 136 grew faster, in a homogeneous way and reached higher cell densities (Figure II.2).

Figure II.2. Strains growing in 100 ml (not aerated) and 1 or 2 L (aerated) volumes. (a) CCAP-1475-2, (b) CCAP-1475-3, (c) CCAP-1475-9, (d) UTEX 552, (e) UTEX 1926, (f) UTEX 1928, (g) UTEX 1954, (h) UTEX 2342, (i) UTEX 2721, (j) CCM-UdeC 040, (k) CCM-UdeC 136.

In this study, ribosomal internal transcribed spacer (ITS) of nine strains classified as *Spirulina* or *Arthrospira*/*Limnospira* in the CCAP and UTEX culture collections, were sequenced. Consensus ITS sequence of strains was obtained from forward and reverse raw sequences data. ITS sequences of CCAP-1475-2, CCAP-1475-3, CCAP-1475-9, UTEX 2721, UTEX 1954, UTEX 552, UTEX 1926, UTEX 1928 and UTEX 2342 were deposited in the Genbank (Table II.2). ITS sequence of strains CCM-UdeC 040 and CCM-UdeC 136 had been sequenced previously by us [42, 43] and corroborated as *Arthrospira*/*Limnospira.*

Table II.2. GenBank accession numbers of ITS sequences of strains investigated in this study.

Code of strains	Designed name by the original culture collection	Genbank Code
CCAP-1475-2	*Spirulina versicolor*	ON362132
CCAP-1475-3	*Spirulina major*	ON362125
CCAP-1475-9	*Limnospira maxima*	ON362133
UTEX 552	*Spirulina major*	ON362126
UTEX 1926	*Spirulina platensis*	ON362127
UTEX 1928	*Spirulina platensis*	ON362129
UTEX 1954	*Spirulina subsalsa*	ON362130
UTEX 2342	*Spirulina maxima*	ON362128
UTEX 2721	*Arthrospira fusiformis*	ON362131
CCM-UdeC 040	*Arthrospira maxima*	AY724776
CCM-UdeC 136	*Arthrospira platensis*	MN628414

Bayesian phylogenetic analysis of ITS sequences from 40 strains, aligned in 560 nucleotides long, was conducted (Figure II.3). The tree showed two main branches where most of *Spirulina* strains are separated from *Arthrospira*/*Limnospira* strains. *Spirulina* cluster is better supported than *Arthrospira*/*Limnospira* cluster (95 vs 78% bootstrap values, respectively). *Spirulina* strains were grouped in: cluster A and cluster B, both strongly supported by maximum bootstrap values. Four strains included in this study, and classified as *Spirulina* in their original culture collections (UTEX 552, UTEX 1954, CCAP-1475-2, CCAP-1475-3),

where grouped in the cluster A, along with a strain of *Spirulina* major, previously sequenced and deposited in the Genbank (X75045).

The *Arthrospira*/*Limnospira* cluster was divided into two subclusters designated as cluster I and cluster II. Three strains classified as *Spirulina* in their original collections grouped in one of the subclusters of *Arthrospira*/*Limnospira*: strain UTEX 2342 in cluster I and strains UTEX 1926 and UTEX 1928 in cluster II. *Arthrospira* strains CCM-UdeC 040 and CCM-UdeC 136 (previously sequenced by us) grouped in cluster II, while strains CCAP-1475-9 and UTEX 2721 (this work) grouped in cluster I.

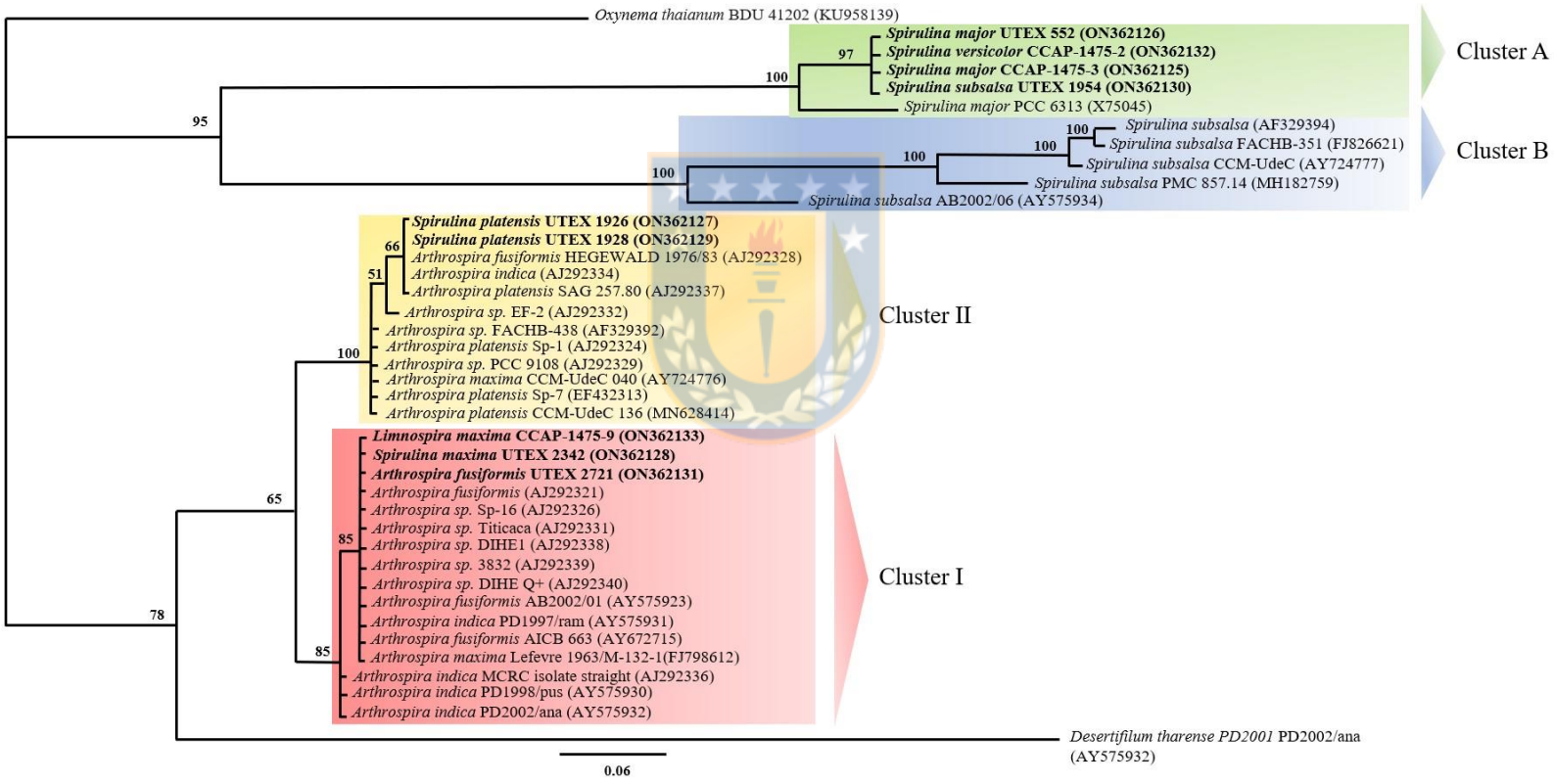

Figure II.3. Rooted phylogram representation of the tree generated by the Bayesian inference method with ITS sequences of nine strains of *Spirulina* and *Arthrospira*/*Limnospira* generated in this work (Table 1) and 29 sequences available in the GenBank. ITS sequences of *Desertifilum tharense* (AY575932) and *Oxynema thaianum* (KU958139) were included as outgroups. GenBank code of each sequence is included in brackets. Bootstrap values are indicated to the left of each node.

Considering the phylogenetic analysis results, CCM-UdeC 040, CCM-UdeC 136, UTEX 1926, UTEX 1928, UTEX 2342, UTEX 2721 and CCAP-1475-9 are true *Arthrospira*/*Limnospira* (Figure II.3); therefore, further analyzes focused on these strains. One strain of *Arthrospira*/*Limnospira*, belonging to each of the two clusters, was selected to obtain confocal images. Strain UTEX 2342 was chosen as representative of cluster I and strain UTEX 1928 as representative of cluster II (Figure II.4).

Figure II.4. Confocal fluorescence images of *Arthrospira*/*Limnospira* strains: (a) UTEX 2342, (b) UTEX 1928. From left to right: Autofluorescence of photosynthetic pigments in the red range (chlorophyll *a*, PC and APC), autofluorescence of carotenoids signal emitted in the green range (490-560 nm), Hoechst stained DNA (blue) and merged image (right). Bars correspond to 20 µm.

Both strains presented the typical arrangement of multicellular cylindrical trichomes in an open helix with evident cross walls, characteristic of *Arthrospira*; however, trichomes of strain UTEX 2342 had higher cell size than trichomes of strain UTEX 1928. Four types of fluorescence signals were detected when both strains were excited with a 405, 488 and 633 nm laser (Figure II.4, from left to right): autofluorescence signal emitted from photosynthetic pigments such as chlorophyll *a*, PC and APC, in the red range (629-758 nm); autofluorescence emitted by carotenoids pigments within the green range (490-560 nm); fluorescence signal emitted in the blue range (374-471 nm) from DNA stained with Hoechst and a mixture of the fluorescence signals. The distribution of fluorescence in the cells showed that pigments and DNA appears to be uniformly distributed, occupying a significant area of the cells; besides, the presence of gas vesicles was evident in both strains, showing up as grayish-blackish structures spread throughout the cell (Figure II.4).

3.1. Growth results

Seven strains were molecularly confirmed as belonging to the genus *Arthrospira/Limnospira*: CCM-UdeC 040, CCM-UdeC 136, UTEX 1926, UTEX 1928, UTEX 2342, UTEX 2721 and CCAP-1475-9 (Figure II.3); however, strains UTEX 1928 and UTEX 2721 were discarded for growth and biochemical analyses because they exhibited an unhealthy growth performance (monitored through cultures macroscopic appearance, cells morphology and chlorophyll *a* measurement) (Figure II.2). The growth curves of strains CCM-UdeC 040, CCM-UdeC 136, UTEX 2342, UTEX 1926 and CCAP-1475-9 are shown in Figure II.5.

Cultures were harvested at day 21 or 30 according to their growth curves. The maximum growth rate ranged between 0.08 (strain UTEX 1926) and 0.16 (strains CCM-UdeC 136 and UTEX 2342) d^{-1} (Table II.3). No significant difference in dry biomass obtained from the cultures of the *Arthrospira*/*Limnospira* strains was observed (Table II.3).

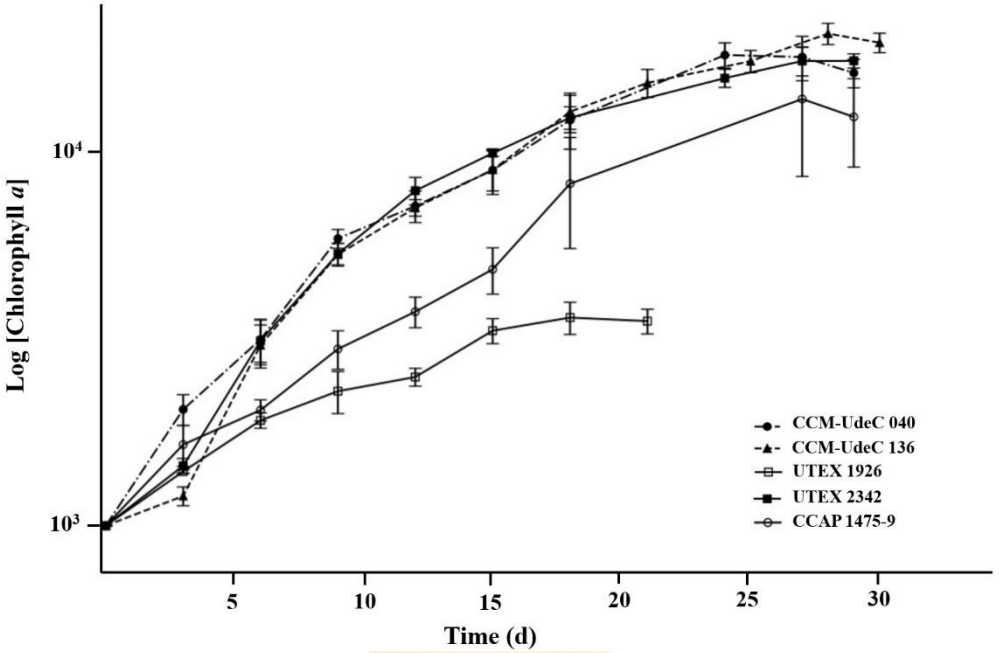

Figure II.5. Growth curves of five *Arthrospira*/*Limnospira* strains (CCM-UdeC 040, CCM-UdeC 136, CCAP-1475-9, UTEX 2342 and UTEX 1926) in Zarrouk medium. Logarithm of chlorophyll *a* concentration (µg chlorophyll a L^{-1}) is plotted as a function of time (days). Bars represent standard deviation of four biological replicates.

Table II.3. Parameters of growth (specific growth rate and biomass dry weight at harvesting day) and biochemical composition (pigments: chlorophyll *a*, total carotenoids, allophycocyanin (APC), phycocyanin (PC), phycoerythrin (PE), total protein and total lipids) of the biomass of *Arthrospira*/*Limnospira* strains. The average values ± standard deviation of four replicates per strain are shown. Different letters for the same column indicate significant differences among strains (ANOVA, Tukey test, $p < 0.05$).

Strains	Specific growth rate (μ_{max}) (d^{-1})	Biomass dry weight (g L^{-1})	Chlorophyll *a* by dry biomass (mg g^{-1})	Carotenoid by dry biomass (mg g^{-1})	APC by dry biomass (mg g^{-1})	PC by dry biomass^{-1} (mg g^{-1})	PE by dry biomass^{-1} (mg g^{-1})	Total protein (% by dry weight)	Total lipids (% by dry weight)
CCM-UdeC 040	0.119 ± 0.01^{b}	1.56 ± 0.21^{a}	14.81 ± 0.16^{b}	3.37 ± 0.02^{b}	53.98 ± 7.47^{a}	69.90 ± 9.32^{a}	17.85 ± 2.06^{ab}	55.13 ± 4.08^{ab}	3.65 ± 0.25^{b}
CCM-UdeC 136	0.158 ± 0.01^{a}	1.40 ± 0.08^{a}	16.59 ± 0.85^{a}	4.31 ± 0.31^{a}	51.78 ± 11.8^{a}	57.50 ± 13.8^{a}	14.44 ± 2.85^{b}	42.28 ± 2.19^{c}	5.00 ± 0.92^{a}
UTEX 1926	0.077± 0.01^{c}	1.31 ± 0.14^{a}	4.41 ± 0.29^{e}	1.50 ± 0.07^{d}	12.86 ± 0.76^{b}	9.48 ± 0.84^{b}	6.91 ± 0.32^{c}	17.59 ± 2.11^{d}	2.00 ± 0.22^{c}
UTEX 2342	0.161 ± 0.01^{a}	1.38 ± 0.18^{a}	13.10 ± 0.41^{c}	3.18 ± 0.13^{bc}	66.85 ± 3.47^{a}	69.15 ± 3.97^{a}	20.28 ± 1.15^{a}	62.25 ± 2.15^{a}	4.50 ± 0.01^{ab}
CCAP-1475-9	0.105 ± 0.02^{b}	1.52 ± 0.14^{a}	10.89 ± 0.49^{d}	2.82 ± 0.14^{c}	57.32 ± 1.11^{a}	62.32 ± 0.77^{a}	19.14 ± 0.37^{a}	49.13 ± 4.91^{bc}	3.96 ± 0.70^{ab}

3.2. Biochemical characterization

Biochemical characterization of the biomass of *Arthrospira*/*Limnospira* strains included pigments (chlorophyll *a*, carotenoids, phycocyanin, allophycocyanin and phycoerythrin), total protein, total lipids and fatty acids analyses (Tables II.3 and II.4).

Chlorophyll *a* content was significantly different among strains and ranged between 16.59 mg g^{-1} (CCM-UdeC 136) and 4.41 mg g^{-1} (UTEX 1926) (Table II.3). On the other hand, total carotenoid content in biomass was much lower than chlorophyll *a* content in all the strains; this parameter ranged between 4.31 mg g^{-1} and 1.50 mg g^{-1}, in strains CCM-UdeC 136 and UTEX 1926, respectively (Table II.3).

Phycocyanin (PC) and allophycocyanin (APC) contents were similar ($p \geq 0.05$) among the strains, except for strain UTEX 1926 that accumulated significantly lower amounts of all the phycobilin pigments ($p < 0.05$) (Table II.3).

The protein content by dry biomass varied significantly among the strains compared. Four of the five strains exhibited values from 42.28 % (CCM-UdeC 136) to 62.25% (UTEX 2342) in this parameter, while the fifth strain (UTEX 1926) accumulated only 17.59% of total protein by dry biomass (Table II.3).

The highest total lipid content was obtained for the biomass of strains CCM-UdeC 136 (5%), while strain UTEX 1926 accumulated the lowest lipid amount (2%) (Table II.3).

The main fatty acids detected in the *Arthrospira* strains were: palmitic acid (C16:0), gamma-linolenic acid (C18:3n6=GLA) and linoleic acid (18:2n6c). The most important polyunsaturated fatty acid in all the strains was GLA, with values ranging between 22.23 and 29.30 %, in strains CCAP-1475-9 and CCM-UdeC 040, respectively, and 2.05 and 3.97 mg GLA per g of dry biomass, in strains UTEX 1926 and CCM-UdeC 040, respectively (Table II.4).

Table II.4. Fatty acids analysis of the biomass of *Arthrospira*/*Limnospira* strains. Values are expressed as the percentage of each fatty acid with respect to the total fatty acids detected in the respective strain (fatty acids profile) and as mg of each fatty acid by g of dry biomass (quantitative analysis). The average values ± standard deviation of four replicates per strain are shown. Different letters for the same fatty acid and unit (% or mg g^{-1}) indicate significant differences among strains (ANOVA, Tukey test, $p < 0.05$).

Fatty acids	CCM-UdeC 040		CCM-UdeC 136		UTEX 2342		CCAP-1475-9		UTEX 1926	
	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}
Lauric acid (C12:0)	1.05 ± 0.09^{a}	0.14 ± 0.01^{a}	1.50 ± 0.66^{a}	0.15 ± 0.02^{a}	1.02 ± 0.01^{a}	0.13 ± 0.01^{ab}	0.86 ± 0.11^{ab}	0.11 ± 0.01^{b}	0.43 ± 0.09^{b}	0.04 ± 0.01^{c}
Myristic acid (C14:0)	0.12 ± 0.01^{a}	0.02 ± 0.01^{a}	0.10 ± 0.01^{ab}	0.01 ± 0^{b}	0.12 ± 0.01^{a}	0.02 ± 0.01^{ab}	0.08 ± 0.02^{b}	0.01 ± 0^{b}	0.09 ± 0.01^{b}	0.01 ± 0^{b}
Palmitic acid (C16:0)	37.10 ± 0.33^{a}	5.01 ± 0.51^{a}	38.37 ± 0.79^{a}	4.75 ± 0.54^{a}	37.43 ± 0.16^{a}	4.79 ± 0.17^{a}	34.69 ± 0.56^{b}	4.39 ± 0.58^{a}	38.59 ± 1.17^{a}	3.04 ± 0.22^{b}
Palmitoleic acid (C16:1)	8.59 ± 0.11^{a}	1.15 ± 0.12^{a}	8.55 ± 0.20^{a}	1.06 ± 0.13^{ab}	6.53 ± 0.09^{b}	0.84 ± 0.02^{c}	7.09 ± 0.21^{c}	0.90 ± 0.10^{bc}	6.22 ± 0.14^{c}	0.49 ± 0.03^{d}
Estearic acid (C18:0)	0.613 ± 0.05^{c}	0.08 ± 0.01^{b}	0.74 ± 0.04^{bc}	0.09 ± 0.01^{b}	0.87 ± 0.05^{b}	0.11 ± 0.01^{a}	0.74 ± 0.08^{b}	0.10 ± 0.01^{ab}	1.19 ± 0.06^{a}	0.09 ± 0.01^{ab}
Oleic acid (C18:1n9c)	5.68 ± 0.20^{bc}	0.77 ± 0.10^{c}	3.48 ± 0.04^{c}	0.43 ± 0.04^{d}	10.23 ± 0.32^{a}	1.31 ± 0.04^{a}	6.31 ± 1.89^{b}	0.96 ± 0.03^{b}	3.44 ± 0.88^{c}	0.27 ± 0.06^{e}
Linoleic acid (C18:2n6c)	16.05 ± 0.02^{d}	2.15 ± 0.25^{a}	17.30 ± 0.24^{c}	2.14 ± 0.20^{a}	19.13 ± 0.16^{b}	2.45 ± 0.10^{a}	18.34 ± 0.65^{b}	2.32 ± 0.32^{a}	20.35 ± 0.49^{a}	1.61 ± 0.08^{b}
Gamma-linolenic acid (C18:3n6)	29.20 ± 0.30^{a}	3.97 ± 0.56^{a}	28.93 ± 0.37^{a}	3.58 ± 0.31^{ab}	22.48 ± 0.42^{c}	2.87 ± 0.05b^{c}	22.23 ± 0.58^{c}	2.81 ± 0.31^{c}	25.96 ± 1.11^{b}	2.05 ± 0.16^{d}
Alfa-linolenic acid (C18:3n3)	0.07 ± 0.02^{b}	0.01 ± 0^{a}	0.09 ± 0.02^{b}	0.01 ± 0^{a}	0.06 ± 0.01^{b}	0.01±0^{a}	0.07 ± 0.01^{b}	0.01 ± 0^{a}	0.67 ± 0.16^{a}	0.06 ± 0.01^{b}
cis-11,14-Eicosadienoic acid (C20:2n6)	0.47 ± 0.03^{b}	0.07 ± 0.01^{bc}	0.45 ± 0.02^{b}	0.06 ± 0.01^{c}	0.42 ± 0.02^{b}	0.05 ± 0.01^{c}	1.44 ± 0.11^{a}	0.21 ± 0.04^{a}	1.36 ± 0.11^{a}	0.11 ± 0.01^{b}
cis-8,11,14-Eicosatrienoic acid (C20:3n6)	0.76 ± 0.04^{b}	0.10 ± 0.01^{b}	0.27 ± 0.02^{c}	0.03 ± 0.01^{b}	1.25 ± 0.11^{b}	0.16 ± 0.01^{b}	7.41 ± 1.33^{a}	0.93 ± 0.13^{a}	1.30 ± 0.09^{b}	0.10 ± 0.01^{b}
Nervonic acid (C24:1n9)	0.06 ± 0.02^{a}	0.01 ± 0^{a}	0.07 ± 0.02 ^{a}	0.01 ± 0^{a}	0.06 ± 0.03 ^{a}	0.01 ± 0^{a}	0.07 ± 0.01 ^{a}	0.01 ± 0^{a}	0.10 ± 0.01^{a}	0.01 ± 0 ^{a}
Arachidonic acid (C20:4n6)	0.15 ± 0.03^{bc}	0.02 ± 0.01^{b}	0.11 ± 0.02^{c}	0.01 ± 0.01^{b}	0.18 ± 0.02^{bc}	0.02 ± 0^{b}	0.34 ± 0.11^{a}	0.04 ± 0.01^{a}	0.22 ± 0.03^{ab}	0.02 ± 0^{b}
cis-13,16-Docosadienoic acid (C22:2)	-	-	-	-	0.15 ± 0.10	0.02 ± 0.02	-	-	-	-

4. Discussion

Culture collections of microalgae are essential resources for the preservation of microalgal biodiversity and allow the availability of strains for education, research and commercial applications; however, the procedures to access and import this material can be long, slow and cumbersome. Every country has its own protocols for importing living biological material such as microalgae strains; besides, some microalgae are potential hydrobiological pests so local environmental regulations usually affect its importation. In Chile, the institution that imports the strains must be authorized to handle this type of material through its incorporation into the National Registry of Aquaculture and the entity that receives the import request and, eventually, authorizes it is the Under Secretary for Fisheries and Aquaculture of Chile (SUBPESCA).

Depending on the country of importation, the strains must sometimes travel long distances from their origin countries, in dark and sealed conditions and without controlled temperature, which is why it is common for the cultures to arrive in suboptimal conditions. Thus, experiments that consider the use of new strains to be acquired from foreign culture collections must always consider the delays in the paperwork of the import procedures as well as the period of recovery and acclimatization of the cultures after their arrival.

In our study, the identification of strains belonging to *Arthrospira*/*Limnospira* or *Spirulina* genus was performed by analyzing the ribosomal ITS sequence. The presence of only one $_{t}$RNA gene ($_{t}$RNA^{Ile}) within the ITS sequence of *Spirulina* and two $_{t}$RNA genes ($_{t}$RNA^{Ile} and $_{t}$RNA^{Ala}) in *Arthrospira* ITS sequence [30, 31], influenced on the separation of both genera in the phylogenetic tree (Figure II.3).

The strains UTEX 1926, UTEX 1928, UTEX 2342, UTEX 2721, CCAP-1475-9, CCM-UdeC 040, CCM-UdeC 136 were confirmed as *Arthrospira*/*Limnospira* and they grouped in one of the two genetic clusters (I or II) (Figure II.3) previously described by Scheldeman et al., [18] and Baurain et al., [31] for *Arthrospira* genus.

Spirulina strains also formed two genetic clusters (A and B) in the phylogenetic tree (Figure II.3). Cluster B included, exclusively, strains of *S. subsalsa*. Our study included sequencing of the ITS region of four *Spirulina* strains: CCAP-1475-2, CCAP-1475-3, UTEX 552 and UTEX 1954; in spite of these strains were originally classified as three different morphospecies (namely *S. major*, *S. versicolor*, *S. subsalsa*), in this study they grouped

together in the same genetic cluster (Figure II.3, cluster A). Based on these findings, the number of species within the genus *Spirulina* should also be revisited.

Strains genetically confirmed as *Arthrospira* (Figure II.3), showed the typical morphological characteristics of the genus: arrangement of multicellular cylindrical trichomes and cells with evident cross walls (Figure II.1 c, e, f, h-k and Figure II.4); which differs from strains of *Spirulina* that present a screw-like trichome and cells with cross walls usually invisible at light microscope [44]. Additionally, the presence of gas vesicles was detected in all *Arthrospira* strains (Figure II.1 c, e, f, h-k and Figure II.4). These structures provide cells with buoyancy and allow mobility through the water column favoring photosynthesis [17, 24]. A morphological characteristic that, at first sight, differentiates the strains belonging to both clusters could be the thickness of the trichome: the strains of cluster I (UTEX 2342, UTEX 2721 and CCAP-1475-9) have thicker trichomes than the strains of cluster II (CCM-UdeC 040, CCM-UdeC 136, UTEX 1926 and UTEX 1928) (Figure II.1 c, e, f, h-k and Figure II.4); however, this difference should be confirmed by morphometric analysis and including more strains from both clusters.

In this study, genetic analysis grouped *Arthrospira*/*Limnospira* strains into two genetic species (Figure II.3), whereas growth and biochemical analyses revealed a remarkable variability among them (Tables II.3 and II.4). Under the same environmental conditions, only genetic attributes of the strains compared can explain phenotypic (i.e. morphological, physiological or biochemical) variations [45-48]. In this work, no correlation between morphology, growth parameters or biochemical composition (Tables II.3 and II.4) and the genetic affiliation of the strains according to their ITS sequences was observed (Figure II.3); nevertheless, differences detected must be due to other genetic attributes, reinforcing thus the importance of checking the intraspecific variability in species of biotechnological interest [49, 50].

In this work, the seven strains genetically confirmed as *Arthrospira*/*Limnospira* (Figure II.3) were cultivated in Zarrouk medium; however, two of them: UTEX 1928 (cluster II) and UTEX 2721 (cluster I) could not be scaled, while strain UTEX 1926 (cluster II) grew poorly (Figure II.3 and Table II.2). In spite of that, strain UTEX 1928 shared morphological attributes with the good growing strain UTEX 2342 (cluster I) (Figure II.4) and also with those of its own cluster (Figure II.1). These results demonstrate how important it is not to extrapolate a priori the properties of a genus/species to all its representatives without having previously checked

the intrinsic attributes of the strain [48, 49, 51].

In economically important microalgae, intraspecific variability should be considered as a key trait given that it enables genetic improvement by means of simple selection of new strains from nature [45]. In this study, biomass production reached by the strains (Table II.2) was within the range reported for *Arthrospira*/*Limnospira* which goes from 0.34 to 2.31 g L^{-1} [52-55] .

The biochemical composition of strains´ biomass exhibited significant differences, especially for strain UTEX 1926. In spite of this strain exhibited typical *Arthrospira´*s morphological attributes (Figure II.1) and genetically grouped within *Arthrospira´*s cluster II (Figure II.3), it accumulated remarkable lower concentrations of all the metabolites measured (Table II.2), so this strain should probably be discarded for use in productive scaling.

Chlorophyll *a* and total carotenoids reported for *Arthrospira* strains ranges between 1.79 and 20 mg g^{-1} and between 3.5 and 6.36 mg g^{-1}, respectively [27, 56, 57]. Biochemical parameters of two strains belonging to cluster I (i.e. CCAP 1475-9 and UTEX 2342) and two strains of cluster II (i.e. CCM-UdeC 040 and CCM-UdeC 136) were adjusted to these expected values for *Arthrospira*/*Limnospira* strains of commercial interest (Figure II.3 and Table II.2).

Arthrospira is considered the richest source of protein for food and feed. Its biomass may contain up to 70% of good nutritional quality protein, which is much higher than some plant (e.g. soybean 38%) or animal (e.g. eggs 13%) sources [13, 58]. Protein content reported for *Arthrospira*/*Limnospira* biomass ranges from 24 to 71% by dry weight [27, 43, 59, 60]. This wide range is explained because comparisons are made between different strains that are also grown under different culture conditions. In this study, one strain of cluster I (UTEX 2342) and one strain of cluster II (CCM-UdeC 040) accumulated the highest values of protein, which demonstrates that strains with biotechnological potential can be found in either of the two ITS-genetic clusters.

Phycobiliproteins are a group of light-harvesting proteins acting as accessory pigments in cyanobacteria; they include phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE) [61, 62]. Phycocyanin is the most important phycobilin pigment for biotechnological applications of *Arthrospira´*s biomass because it has remarkable antioxidant, anti-inflammatory, antiplatelet, anti-cancer, nephroprotective and hepatoprotective properties [63-65]. Except for strain UTEX 1926, the strains did not exhibited significant differences on

their PC content (Table II.3) but some values obtained were lower to the reported for *Arthrospira* which range between 63.71 and 245 mg g^{-1} [52, 66] .

With respect to the lipids content, values between 1.50 and 16.3% have been reported for *Arthrospira* strains [27, 67, 68]. The wide range of values reported respond to differences in strain identity, culture conditions and phase of the growth curve when the analysis is carried out; besides, different extraction and quantification methods used for lipids quantification by different authors make comparisons even more difficult. In this study lipid content of all the strains was in the lower level of reported values and significant differences were detected among strains (Table II.2).

Regarding fatty acids composition, similar values were obtained for all the strains, included UTEX 1926 (Table II.3), which indicates that this parameter probably behaves like a chemotaxonomic signal for the genus *Arthrospira*/*Limnospira*. Reinforcing this idea, Galloway & Winder [69] compared the effect of phytoplankton composition with that of environmental variables in the fatty acids composition finding that species identity account for 79% of the variation; thus, compared to other biochemical parameters, fatty acids composition would be a more stable parameter at taxonomic group level and less affected by culture conditions.

The most abundant fatty acid detected in the biomass of the *Arthrospira* strains was palmitic acid (Table II.3), which is the most common saturated fatty acid found in the human body and can be provided in the diet or synthesized endogenously via de novo lipogenesis [70]. Palmitic acid proportion reported for *Arthrospira*/*Limnospira* strains fluctuates between 25.41 and 37.06% [67, 68, 71]. Values obtained in this study are within this range.

The main unsaturated fatty acid in *Arthrospira*/*Limnospira* strains was the omega 6 polyunsaturated fatty acid Gamma-linolenic acid (GLA) (Table II.3). GLA (18:3n6) has a recognized therapeutic effect due to its anti-inflammatory, vasodilatory and anti-aggregatory action. The action mechanism of GLA includes its elongation to dihomo-gamma-linolenic acid (DGLA) and reaction with a cyclooxygenase enzyme for producing anti-inflammatory prostaglandins of series 1 (PGE1) and thromboxane A1 (TxA1) [72]. *Arthrospira* is a well-recognized source of GLA [73-76]. Values obtained in this work (Table II.4) were high respect to the previously reported values which range between 4.32 and 37.06% [27,71, 77].

Microalgal cultures are affected by abiotic factors such as irradiance, light quality, temperature and nutrients availability, which regulate biomass composition and overall

productivity [13, 66]. The phase of the growth curve when the biomass harvest is carried out is also important in determining the quality of the biomass obtained from a culture. Recently we compared biochemical composition of strains CCM-UdeC 040 with CCM-UdeC 136, obtaining quite different results from those reported in this study [43]. Differences in the culture medium composition, culture volume, harvesting day and size of initial inoculum could explain these apparent discrepancies since, as mentioned before, culture conditions certainly influence the biochemical composition and productivity of *Arthrospira* [78-82].

Future studies should aim to optimize culture conditions for selected strains of *Arthrospira*/*Limnospira*, as well as culture's time span, considering the metabolites whose production is to be enhanced.

5. Conclusion

Eleven strains originally designated as *Arthrospira*/*Limnospira* or *Spirulina* in three microalgae culture collections were analyzed considering ITS sequence to verify their taxonomic classification. Seven strains were identified as belonging to one of the two clusters previously described for the genus *Arthrospira*/*Limnospira*, while remaining strains were grouped within *Spirulina* genus, which formed two well-supported not previously described clusters. Physiological and biochemical characterization of *Arthrospira*/*Limnospira* strains showed intraspecific variability in growth attributes and biochemical composition. According to our results, four strains of *Arthrospira*/*Limnospira*, currently available in the culture collections CCAP (UK), UTEX (USA) and CCM-UdeC (Chile), would have the appropriated attributes for a commercial scaling, being strains CCM-UdeC 040 and UTEX 2342 the most promising considering, mainly, their higher protein and phycocyanin accumulation capacity. Studies like this one are intended to give an incentive to those who are interested in cultivating *Arthrospira* for commercial purposes by providing practical information on strains that are currently available in culture collections around the world.

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III. CAPÍTULO 3 Obtención y caracterización de hidrolizados proteicos con propiedad antioxidante a partir de un extracto acuoso de la biomasa de la cepa *Arthrospira/Limnospira* UTEX 2342.

Las proteínas provenientes de la dieta constituyen fuentes de aminoácidos necesarios en el crecimiento y el mantenimiento de las células y tejidos del cuerpo. Adicionalmente, los péptidos bioactivos contenidos en estas proteínas, modulan las respuestas fisiológicas e inducen efectos positivos en la salud (Peredo-Lovillo et al., 2022). Diversos estudios han comprobado que una dieta poco saludable constituye uno de los principales factores de riesgo de las enfermedades no transmisibles (ENT) (Mendis et al., 2015). Asimismo, el creciente rechazo hacia la administración de drogas sintéticas (debido a los problemas de toxicidad, eficiencia y resistencia en la población), ha provocado el aumento del interés en alternativas naturales para el tratamiento y prevención de estas enfermedades. Entre estas alternativas, destacan los alimentos que contengan péptidos bioactivos (Daliri et al., 2017).

Los péptidos bioactivos se encuentran inactivos mientras permanecen en su estructura de proteína original y exhiben actividad cuando son escindidos durante el procesamiento de los alimentos, la digestión gastrointestinal o a través de la hidrólisis enzimática (Daliri et al 2018; Lafarga et al., 2021). En la actualidad se han registrado alrededor de 4300 péptidos en la base de datos de péptidos bioactivos BIOPEP-UWM^{TM} (BIOPEP). La mayoría se han obtenido de la leche, productos lácteos, carne, pescado y cereales. Estos se clasifican según la bioactividad que producen: antimicrobiana, antitrombótica, antihipertensiva, opioide, inmunomoduladora, antioxidante, entre otras (Lafarga et al., 2021).

La obtención de péptidos mediante hidrólisis enzimática se produce bajo condiciones controladas (pH, temperatura, concentración del sustrato, etc.) y mediante el empleo de una o múltiples enzimas. Este método de modificación de proteínas es el más utilizado en las industrias alimentaria y farmacéutica, puesto que no deja residuos de los solventes orgánicos utilizados en el proceso (Hou et al., 2017; Abuine et al., 2019). En este proceso se utilizan proteasas que son extraídas de tejidos vegetales (ficina, papaína, bromelina); animales (pepsina, quimotripsina, tripsina) y de células microbianas (proteinasa K, pronasa, colagenasa, alcalasa, flavourenzima) (Jakubczyk et al., 2020). Dado que las enzimas proteolíticas tienen posiciones específicas de clivaje, los hidrolizados proteicos obtenidos con diversas enzimas muestran diferentes secuencias aminoacídicas y longitudes de péptidos; factores estrechamente relacionados con sus actividades biológicas (Hou et al

2017; Jakubczyk et al., 2020). La efectividad de estos péptidos se debe, en gran medida, a que son absorbidos en el intestino y entran al torrente sanguíneo, lo que asegura su biodisponibilidad in vivo y el efecto fisiológico en los órganos diana. Generalmente, los péptidos pequeños (di-tri péptidos) son absorbidos más eficientemente que los péptidos grandes, ya que estos últimos son más propensos a la hidrólisis por peptidasas de los enterocitos (Singh et al., 2021; Wang et al., 2021). Con el fin de obtener fragmentos de menor tamaño se utiliza la combinación de enzimas proteolíticas, conocida como hidrólisis secuencial (Singh et al., 2014).

Las microalgas son materias primas ricas en proteínas, por lo que constituyen una potencial fuente de péptidos bioactivos (Lafarga et al., 2021), lo que unido a la tendencia actual de disminución del consumo de proteínas de origen animal, representa una excelente oportunidad para su empleo como alternativa vegana o vegetariana. La cianobacteria *Arthrospira* contiene un alto contenido de proteínas (45-77%) además de otros nutrientes profilácticos y farmacéuticos que incluyen vitaminas del complejo B, minerales, acido γ-linolénico, vitamina E, carotenoides, ficocianina, entre otros (Furmaniak et al., 2017,Torres-Tiji et al., 2020). Además de su alto valor nutricional, *Arthrospira* posee numerosas bioactividades y carece de toxinas, por lo que se ha clasificado como GRAS (Marles et al., 2011; Fujisawa et al., 2010; Cheevadhanarak et al., 2012), lo cual representa una ventaja como fuente de proteínas para la generación de péptidos bioactivos, puesto que los consumidores la consideran como una materia prima saludable, segura, nutritiva y sustentable (Lafarga et al., 2021).

La cepa *Arthrospira/Limnospira* UTEX 2342 fue previamente identificada como una cepa capaz de producir significativamente más proteínas que otras cepas del mismo género. En este trabajo se analizó el potencial de esta cepa para la obtención de hidrolizados proteicos, con propiedad antioxidante, a partir de la hidrólisis de su extracto acuoso con proteasas comerciales.

1. Materiales y Métodos

1.1. Condiciones de cultivo y preparación del extracto acuoso

La cepa *Arthrospira*/*Limnospira* UTEX 2342 se mantuvo y se cultivó en medio Zarrouk. Se escaló a un volumen de 100 mL y posteriormente a 500 mL en matraces Erlenmeyer. El cultivo experimental se inició a una densidad celular de 400 µg clorofila *a* L^{-1} en un volumen de 15 L, se mantuvo a 21 ± 1°C, una irradiancia de 100 µmol m^{-2} s^{-1} y un fotoperíodo de 12:12 (luz/noche). El cultivo se aireó continuamente con aire filtrado a través de un poro de 0,22 µm. A partir de la biomasa obtenida del cultivo de la cepa UTEX 2342, se preparó el extracto acuoso siguiendo la metodología presentada en el Capítulo 1 de la tesis.

1.2. Hidrólisis enzimática del extracto acuoso y caracterización de hidrolizados de proteínas

La hidrólisis enzimática del extracto acuoso se realizó en las instalaciones del Centro de Investigación de Proteínas Vegetales (CIProVe) perteneciente a la Facultad de Ciencias Exactas de la Universidad Nacional de la Plata, Argentina. La hidrólisis enzimática del extracto acuoso se realizó utilizando proteasas comerciales en orden secuencial siguiendo la metodología del Capítulo 1 y presentada en la siguiente Figura III.1.

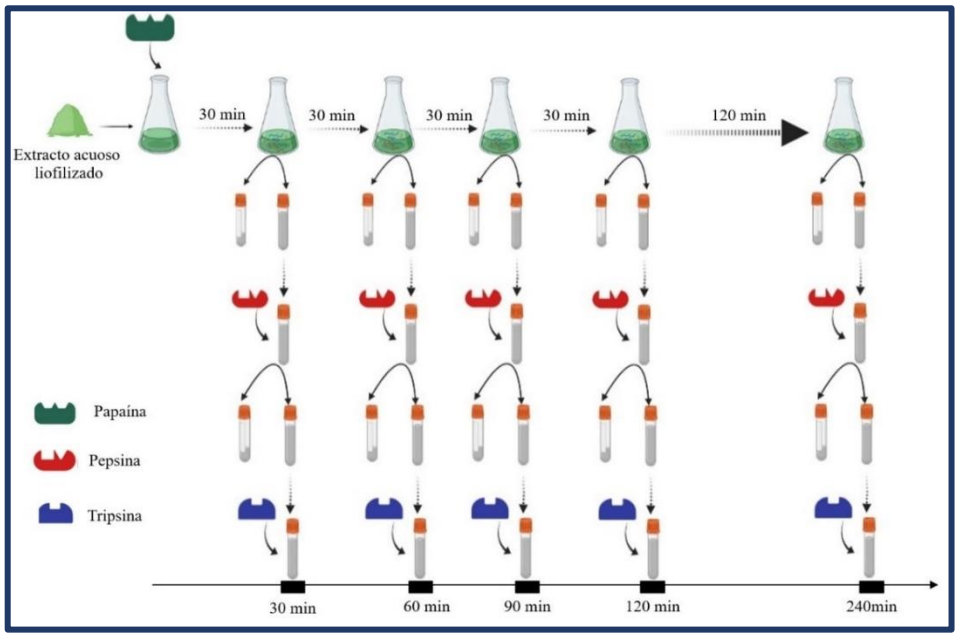

Figura III.1. Esquema general de la metodología empleada en la generación de hidrolizados proteicos a partir del extracto acuoso de la cepa UTEX 2342. Los hidrolizados se obtuvieron mediante hidrólisis secuencial con tres enzimas proteolíticas: papaína, pepsina y tripsina. Además, se evaluaron cinco tiempos de incubación con cada enzima: 30, 60, 90, 120 y 240 minutos. El esquema se dibujó a través de la plataforma BioRender.com.

1.2.1. Determinación del grado de hidrólisis

El grado de hidrólisis (GH) se estimó a través del método del ácido o-ftalaldehído (OPA) siguiendo la metodología de Church et al. (1983) y se utilizó la ecuación propuesta por Spellman et al. (2003).

1.2.2. Cromatografía líquida de proteínas a alta velocidad (FPLC)

El perfil proteico del extracto acuoso y de los hidrolizados se analizó a través de cromatografía de exclusión molecular por tamaño en un sistema FPLC (SEC-FPLC) siguiendo la metodología propuesta en el Capítulo 1.

1.2.3. Evaluación de la actividad antioxidante de los hidrolizados

La actividad antioxidante mediante el método ABTS se realizó siguiendo el protocolo de Cotabarren et al. (2019).

1.3. Análisis estadístico

Los análisis estadísticos de los datos obtenidos se realizaron en el programa R versión 3.2.3. La evaluación de las premisas de normalidad y homogeneidad de varianzas se realizó mediante la prueba Kolmogórov-Smirnov y la prueba Levene, respectivamente. Se realizó una Análisis de Varianza (ANOVA) Multifactorial para evaluar el grado de hidrólisis de los extractos acuosos con proteasas (considerando el origen de los hidrolizados, las proteasas y los tiempos de incubación con las proteasas). Mientras que las diferencias significativas entre medias se evaluaron a través de las pruebas de Dunn y Tukey. Todos los análisis se realizaron con nivel de significación de $\alpha = 0.05$ y utilizando réplicas técnicas.

2. Resultados y Discusión

La Figura III.2 muestra las actividades realizadas a partir de la cepa UTEX 2342, las que incluyeron el escalado y cultivo en volúmenes de 15 litros; cosecha del cultivo y preparación del extracto acuoso a partir de la biomasa liofilizada; hidrólisis secuencial con tres proteasas del extracto acuoso liofilizado y la caracterización de los hidrolizados resultantes.

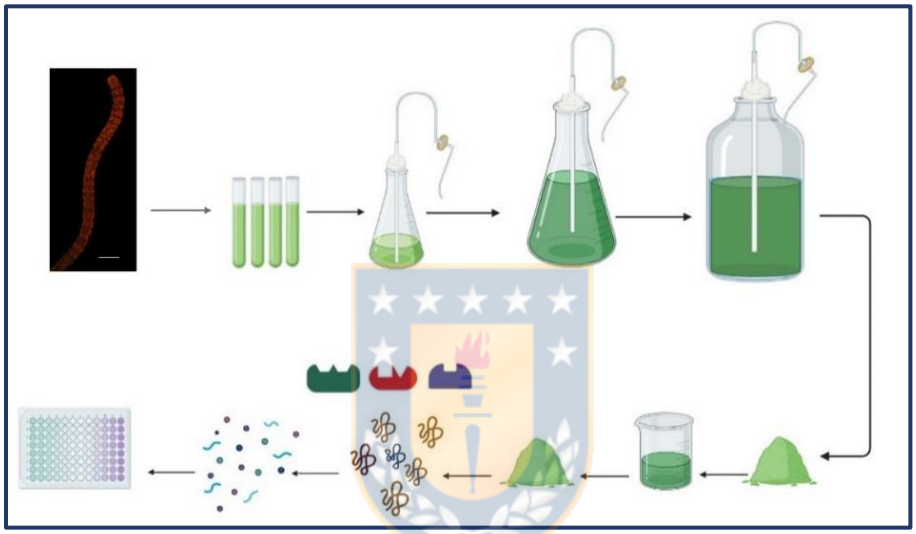

Figura III.2. Actividades realizadas a partir del extracto acuoso de la cepa UTEX 2342: escalado del cultivo hasta volumen de 15 L, cosecha y liofilización de la biomasa, hidrólisis secuencial del extracto acuoso con papaína, pepsina y tripsina y la caracterización de los hidrolizados proteicos. Dibujo de la plataforma BioRender.com.

2.1. Determinación del grado de hidrólisis

El extracto acuoso de la biomasa de la cepa UTEX 2342 se hidrolizó utilizando papaína, pepsina y tripsina en orden secuencial. El grado de hidrólisis (GH) se determinó durante 240 min utilizando el método OPA (Figura III.3). La generación de péptidos con papaína durante 30 minutos de reacción fue elevada (21,09%) (Figura III.3), sin embargo, no se detectaron diferencias entre los tiempos de incubación 30, 60 y 90 min ($p \geq 0,05$).

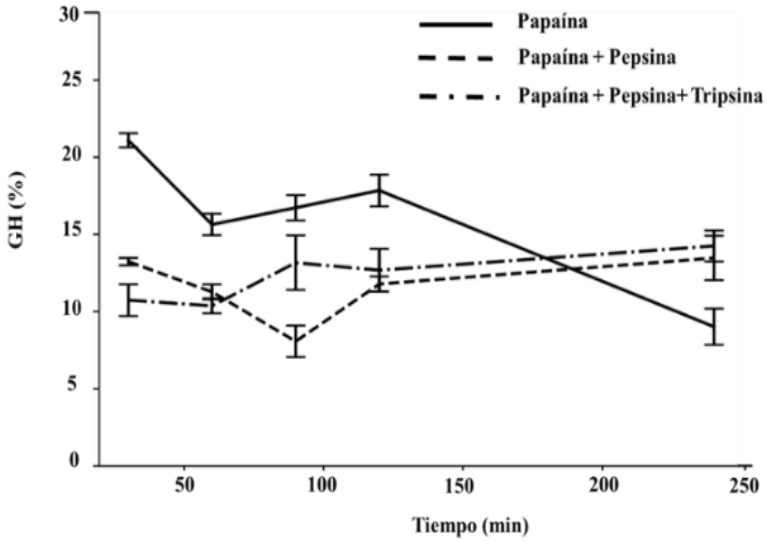

Figura III.3. Grado de hidrólisis (GH) de los hidrolizados obtenidos a través de la hidrólisis secuencial con papaína, pepsina y tripsina del extracto acuoso de la biomasa de UTEX 2342, medido por OPA**.**

Los valores de grado de hidrólisis registrados fueron similares a los obtenidos a partir de la hidrólisis del extracto acuoso de las cepas CCM-UdeC 040 y CCM-UdeC 136 (Capítulo 1). Adicionalmente, los valores fueron similares a los obtenidos de la hidrólisis de ficocianina de *A. platensis* con papaína por tres horas (28.4%) (Tong et al., 2020), aunque superiores a los registrados en la hidrólisis de *Arthrospira* con papaína durante prolongados tiempos de incubación: 8 y 10 horas (11,12% y 18,3%, respectivamente) (Wang & Zhang, 2016; Fan et al., 2018).

En nuestro estudio se registró la disminución de la producción de péptidos con el aumento de los tiempos de incubación con papaína: a los 240 minutos de reacción se obtuvo un valor de GH correspondiente a la mitad del obtenido a los 30 minutos (Figura III.3). Este resultado difiere del comportamiento observado durante el procesamiento de los hidrolizados de las cepas CCM-UdeC 040 y CCM-UdeC 136, donde la incubación de la muestra de CCM-UdeC 040 con papaína por 240 minutos arrojó similares resultados a los obtenidos a 30, 60 y 90 minutos de incubación, mientras que en el caso CCM-UdeC 136, este tratamiento fue el más eficiente (37,48%). Similarmente, Tong et al., (2020) detectaron que el GH de la proteólisis de *Arthrospira* con papaína aumentó gradualmente después de los 30 minutos

de reacción.

La combinación de papaína y posteriormente pepsina fue menos eficiente que la hidrólisis sólo con papaína (Figura III.3). No se detectaron diferencias entre los tiempos de incubación 30, 60, 120 y 240 min ($p \geq 0,05$), mientras que la hidrólisis secuencial por 90 minutos produjo el valor de GH más bajo (8,07%) y por lo tanto, menor generación de péptidos. Este resultado difiere del obtenido en el Capítulo 1, ya que en el caso del extracto acuoso de la cepa CCM-UdeC 040, la combinación de papaína más pepsina fue eficiente después de 120 minutos de reacción (20,29 %), detectándose diferencias entre los tiempos 30 y 90, 120, 240 minutos. Mientras que la hidrólisis del extracto acuoso de la cepa CCM-UdeC 136 con la combinación papaína y pepsina por 30 minutos fue más eficiente (34,98%) que con 120 min (25,72%) y el resto de los tiempos de incubación generaron valores similares a los obtenidos a los 30 min.

La hidrólisis secuencial de UTEX 2342 con papaína, pepsina y tripsina produjo valores similares de GH para todos los tiempos de incubación, alrededor del 12% (Figura III.3). Sin embargo, la combinación de estas tres enzimas durante 90 min de incubación, produjo la mayor generación de péptidos a partir del extracto acuoso de CCM-UdeC 040 (38,54%)(Capítulo 1), mientras que la hidrólisis de CCM-UdeC 136 con la combinación de las tres proteasas generó similares resultados que los obtenidos con dos enzimas, por lo que es probable que la última combinación de enzimas no sea necesaria.

Los resultados de la hidrólisis del extracto acuoso de la cepa de *Arthrospira/Limnospira* más promisoria, UTEX 2342, muestran que la hidrólisis sólo con papaína es más eficiente que con las combinaciones de tripsina y pepsina. La enzima papaína hidroliza los enlaces que involucran aminoácidos básicos y aminoácidos de cadena lateral larga e hidrofóbica (por ejemplo, leucina, glicina, arginina, lisina, fenilalanina). La enzima pepsina actúa sobre los enlaces adyacentes a los residuos de L-aminoácidos aromáticos o dicarboxílicos (alanina, valina, leucina, isoleucina, fenilalanina, triptófano, tirosina) y la enzima tripsina hidroliza los enlaces que involucran a los grupos carboxilo de L-arginina o lisina (Hou et al., 2017; Hudson, 1992). Considerando esta información y los resultados obtenidos del GH (Figura III.3), se podría hipotetizar que el extracto acuoso de UTEX 2342 contiene un alto contenido de aminoácidos básicos y aminoácidos portadores de cadenas laterales hidrófobas largas, ya que se obtuvo un mayor grado de hidrólisis con papaína como proteasa.

2.2. Cromatografía de exclusión por tamaño

Si se considera aplicar nuestros resultados en la industria alimentaria, cosmética o nutraceútica, es importante considerar sus requerimientos. El empleo de bajas temperaturas y cortos tiempos de hidrólisis, constituyen dos de las características más deseadas en la generación de péptidos en la industria alimentaria (Pasupuleti & Braun, 2010). Teniendo en cuenta esta premisa y que la incubación con papaína durante 30 minutos produjo el mayor porcentaje de hidrólisis, se decidió continuar con la caracterización de estos hidrolizados.

Los hidrolizados obtenidos con papaína durante 30 minutos y la muestra control se sometieron a cromatografía de exclusión por tamaño (SEC de sus siglas en inglés) (Figura III.4).

Los hidrolizados obtenidos y la muestra control sometidos a SEC mostraron diferencias entre los patrones de elución medidos a las absorbancias 280 nm y 215 nm. La señal detectada a 280 nm refleja que la reacción con papaína condujo a la hidrólisis completa de la fracción 1, correspondiente a proteínas de alto peso molecular, como se puede detectar en el patrón H1-280 (correspondiente a la fracción C1-280 hidrolizada). El perfil reveló una ligera disminución de la fracción 2 en la muestra hidrolizada en comparación con el control; mientras que la intensidad de la señal de la fracciones 3 y 4 de los hidrolizados fue superior a la del control; lo que refleja un incremento en la concentración de péptidos de bajo peso molecular como resultado de la elevada actividad proteolítica de las fracciones anteriores.

Sin embargo, si analizamos la señal detectada a 215 nm se detectan resultados contradictorios (Figura III.4), ya que las señales emitidas por las fracciones 3 y 4 mostraron altas intensidades. Estas fracciones, según el principio explicado anteriormente, corresponderían a proteínas de bajo peso molecular que eluyen con un mayor volumen. Esta aparente hidrólisis de la muestra control podría ser resultado de la presencia de enzimas proteolíticas en la muestra proteica, sin embargo el grado de hidrólisis (a través del método OPA) no respalda esta aseveración. Por lo que este resultado podría deberse a potenciales problemas o limitaciones de la técnica de separación empleada. Idealmente no debería existir interacción entre el soluto y la fase estacionaria (Štulík et al., 2003), ya que se supone que la fase estacionaria es “inerte” frente a los componentes de la mezcla a separar. Sin embargo, las interacciones (no-específicas) (hidrofóbicas o de van der Waals, enlaces de hidrógeno, iónicas, etc.) entre las proteínas y la fase estacionaria (resina)

ocurren y afectan los tiempos de retención, la resolución cromatográfica, la forma del peak de la señal de elución y el nivel de detección de las proteínas (Shire, 2015; Lambrecht et al., 2015). En este sentido, las altas señales detectadas a mayores volúmenes de elución podrían ser resultado de interacciones entre las proteínas del extracto sin hidrolizar y la fase estacionaria (resina Sephacryl), lo que provocaría el aumento en el tiempo de retención de esta muestra en la columna, visualizándose como un retardo en la señal de elución. Similar comportamiento en el patrón de elución medido a 215 nm fue detectado para la muestra control (extracto acuoso de la biomasa) de la cepa CCM-UdeC 040 (Capítulo 1; Figura 1.3). Es probable que las proteínas presentes en la muestra control de UTEX 2342 y de CCM-UdeC 040 presenten un alto nivel de desnaturalización, debido a que se sometieron a las condiciones de hidrólisis (pero en ausencia de papaína) que conllevaron significativos cambios de temperatura, agitación, formación de hielo (como resultado de congelamiento y descongelamiento), cambios en las fuerzas iónicas; factores que afectan la estabilidad de conformación de las proteínas (Štulík et al., 2003). La pérdida de la estructura secundaria y terciaria (desnaturalización) provoca el desplegamiento de las proteínas y contribuye al potencial incremento de la afinidad de estas por las resinas de la fase estacionaria (Lambrecht et al 2015). Además, en ocasiones los cambios conformacionales inducen interacciones proteína-proteína que conllevan a la agregación de estas (Shire, 2015), lo que puede afectar el tamaño, los enlaces intermoleculares, morfología e hidrofobicidad de las proteínas y por tanto su posterior separación por exclusión molecular (Fekete et al., 2014).

Para la eliminación de estos efectos no deseados durante la separación de proteínas (interacciones proteínas-fase estacionaria) se puede modificar la fase estacionaria y la fase móvil, por ejemplo a través de cambios en el pH, fuerza iónica y la concentración de un modificador orgánico. La disminución del pH en la fase móvil elimina la ionización de los solutos ácidos (en la superficie de la fase estacionaria y dentro de la fase móvil) y por lo tanto suprime sus interacciones electrostáticas, mientras que potencia la ionización de los solutos básicos. La adición de sales disminuye las interacciones iónicas dado que reduce la repulsión entre las proteínas cargadas negativamente y la columna, sin embargo el aumento en la fuerza iónica puede conducir a interacciones hidrofóbicas (Lambrecht et al., 2015). Adicionalmente, se recomienda el uso de modificadores orgánicos (metanol, etanol, acetonitrilo) para reducir las interacciones iónicas e hidrofóbicas (Štulík et al., 2003; Fekete

et al., 2014; Lambrecht et al., 2015). Para evitar los efectos indeseados durante la separación de la proteína, se recomienda la utilización de pH cercano al punto isoeléctrico de la proteína, esta a su vez debe ser soluble y estable en el buffer (fase móvil), para ello se aconseja utilizar una solución buffer con una fuerza iónica de 50–200 mM (Fekete et al., 2014).

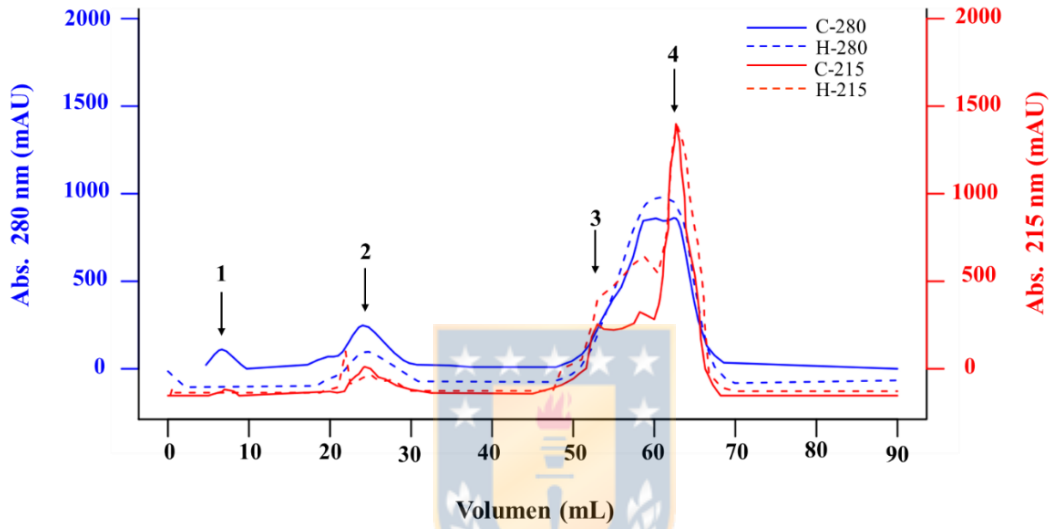

Figura III.4. Perfil de elución de cromatografía de exclusión por tamaño de los hidrolizados obtenidos con papaína durante 30 min de incubación. Las flechas indican las fracciones colectadas. C-280, C-215: Control para hidrólisis del extracto acuoso de UTEX 2342 con papaína medida a 280 nm y 215 nm respectivamente; H-280, H-215 Hidrolizados de UTEX 2342 medidos a 280 nm y 215 nm.

2.3. Evaluación de la actividad antioxidante de los hidrolizados proteicos

La mayoría de los estudios realizados sobre péptidos derivados de *Arthrospira* se han centrado en la generación de péptidos antiinflamatorios, antitumorales, antidiabéticos, antiobesidad y antihipertensivos (Aiello et al., 2019; Anekthanakul et al., 2019; Fan et al., 2018; Hu et al., 2019; Vo & Kim, 2013; Wang & Zhang, 2016). Sin embargo, en el momento en que se inició nuestro estudio, la generación de hidrolizados proteicos de *Arthrospira* con propiedades antioxidantes estaba poco explorada. En los últimos años, ha aumentado el interés en estos compuestos, lo que se traduce en un incremento de las publicaciones en el tema (Costa et al., 2019; López-Rodríguez et al., 2021; Pereira et al., 2019; Wang et al.,

2021; Zeng et al., 2020). Motivados por la búsqueda de nuevas fuentes de compuestos antioxidantes, evaluamos si el proceso de hidrólisis tenía efecto sobre la capacidad antioxidante del extracto acuoso de la biomasa de la cepa UTEX 2342. Para ello se empleó el ensayo de captación de radicales $ABTS^{+}$, el cual es un método *in vitro* rápido, sencillo y utilizado en una amplia gama de pH y en solventes acuosos u orgánicos. Además es considerado como un buen método predictor de la actividad ORAC (Awika et al., 2003).

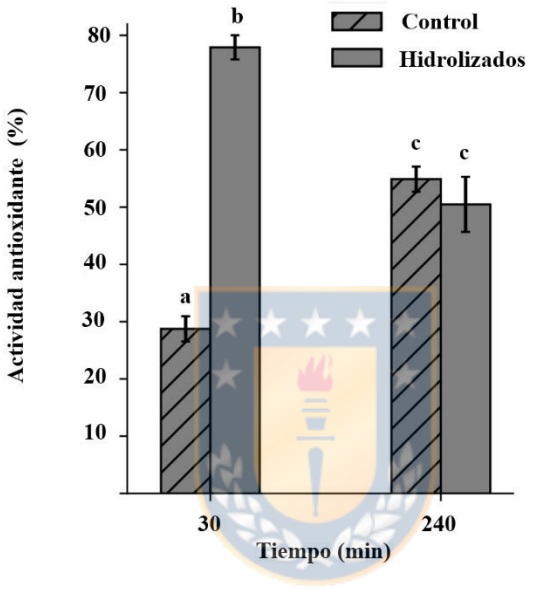

Figura III.5. Actividad antioxidante de los hidrolizados del extracto acuoso tratado con papaína evaluada a través del método $ABTS^{\cdot+}$. Los datos representan las medias y desviaciones estándar. Las diferentes letras indican diferencias estadísticas detectadas mediante la Prueba de Dunn con $p < 0,05$.

Las diferencias en la capacidad antioxidante evaluada a través del ensayo ABTS entre los hidrolizados con papaína y el control fueron estadísticamente significativas ($p < 0,05$) (Figura III.4.), lo que refleja que la actividad proteolítica es responsable, en gran medida, de la producción de péptidos antioxidantes. Además, los hidrolizados del extracto acuoso de UTEX 2342 con papaína mostraron una captación de radicales de 77,90%, similar a la actividad detectada por los hidrolizados de la cepa CCM-UdeC 136 (75,91%), pero ligeramente inferior a la inducida por los hidrolizados de CCM-UdeC 040 (87,47%)(Capítulo 1). Adicionalmente, la muestra hidrolizada de la cepa UTEX 2342 durante 30 min alcanzó mayor captación de radicales que los hidrolizados obtenidos con 240 min ($p < 0,05$), lo cual

puede deberse a la menor producción de péptidos a 240 min (Figura III.3).

Al parecer, los cambios de pH y temperatura afectaron tanto la actividad antioxidante del extracto acuoso (control) como la de los hidrolizados obtenidos después de 240 minutos de reacción, ya que se detectaron diferencias entre los controles de los tiempos 30 minutos y 240 minutos, así como entre los hidrolizados (Figura III.5). Por lo que trabajos futuros deberán analizar la influencia del pH y la temperatura en la actividad antioxidante de los extractos acuosos crudos y de los péptidos resultantes de su hidrólisis.

Los resultados obtenidos apoyan la elección de 30 min como tiempo de incubación eficiente durante la hidrólisis con papaína para obtener péptidos con actividad antioxidante a partir de un extracto proteico de la cepa UTEX 2342 .

Los péptidos antioxidantes generalmente tienen de tres a seis aminoácidos, poseen baja masa molecular (<1 kDa) y están compuestos por aminoácidos hidrofóbicos y con residuos aromáticos como triptófano, fenilalanina, metionina, histidina, prolina, glicina, lisina, valina, isoleucina, tirosina (Elias et al., 2008; Piovesana et al., 2018; Jakubczyk et al., 2020; Wang et al., 2021). Los aminoácidos con residuos aromáticos son capaces de donar protones a los radicales deficientes en electrones, lo que mejora las propiedades de captación de radicales de los residuos de aminoácidos. Además, el grupo tiol (SH) de la cisteína interactúa directamente con los radicales (Qian et al., 2008). Sin embargo, es importante destacar que la actividad antioxidante neta es debida a los efectos integradores de estas acciones, más que a los efectos individuales de los péptidos (Chen et al., 1998).

Estudios futuros deberán considerar el análisis de la bioactividad de los hidrolizados obtenidos con las restantes combinaciones de enzimas y tiempos de incubación, puesto que hidrolizados proteicos con bajo grado de hidrólisis han mostrado actividad biológica. Muestra de ello es el trabajo de Cotabarren et al. (2019), en el cual los hidrolizados del expeller de chía con papaína por 40 minutos produjeron un grado de hidrólisis bajo (14,3%), pero con elevada actividad antioxidante. Similarmente, la investigación de Fan et al. (2018) arrojó que la hidrólisis del extracto acuoso de *A. platensis* con pepsina registró un grado de hidrólisis de sólo 3,6% pero los hidrolizados resultantes inhibieron, significativamente, la vialidad de las células pre-adipocitos (3T3-L1) así como la actividad de la lipasa pancreática, mostrando una fuerte actividad antiobesidad**.** En este sentido muchos estudios han mostrado que no existe relación lineal entre el grado de hidrólisis de las proteínas y la actividad biológica de los péptidos resultantes de la hidrólisis (Wiriyaphan, 2015).

Entre las proteínas que pudiesen estar presentes en el extracto acuoso de UTEX 2342 y ser responsables de la actividad antioxidante detectada, se encuentra la enzima ribulosa-1,5-bisfosfato carboxilasa/oxigenasa (Rubisco). Esta proteína es considerada como la más abundante del planeta y representa alrededor del 50% de las proteínas solubles en las plantas (Andersson & Backlund, 2008). Tiene un peso molecular de 560 KDa y una significativa proporción de aminoácidos hidrofóbicos, responsables de varias bioactividades: actividad antihipertensiva, opioide, antibacteriana, antioxidante y estimulante de la ingesta de alimentos (Udenigwe et al., 2017). Paralelamente, en el extracto proteico de *Arthrospira platensis* se han detectado como principales proteínas: la subunidad β de C-PC (21.4 kDa); subunidad β de APC (17.5 kDa); subunidad α de C-PC (17 kDa) y la subunidad α de APC (15.7 kDa) (Wang et al., 2021), las cuales podrían potencialmente estar presentes en nuestro extracto y ser las responsables de la actividad antioxidante detectada.

Es importante destacar que se debe continuar con la caracterización del extracto acuoso de la cepa UTEX 2342, realizar su análisis bioquímico, analizar su actividad antioxidante utilizando otros ensayos y su potencial citotoxicidad sobre líneas celulares, con el fin de evaluar su inocuidad y sus potenciales aplicaciones en la industria alimentaria. Así también, extender la caracterización y análisis de los hidrolizados generados con las proteasas comerciales a través de diferentes métodos como: la electroforesis en gel de poliacrilamida con SDS (SDS-PAGE), que permite la separación de polipéptidos y proteínas basado en la masa molecular y el punto isoeléctrico (Moche et al., 2013; Wu et al., 2021) y por ende la caracterización del proteoma de la cepa; la cromatografía de exclusión molecular por tamaño FPLC (SEC-FPLC), que permite evaluar la ocurrencia de hidrólisis y proporciona información sobre el perfil de masas moleculares de los hidrolizados en función de las condiciones de hidrólisis (enzima, duración de la reacción y temperatura). Paralelamente, debe evaluarse la actividad antioxidante de los hidrolizados y de cada una de las fracciones colectadas después de la cromatografía de exclusión molecular por tamaño FPLC (SEC-FPLC) y caracterizar la fracción bioactiva a través de espectrometría de masas MALDI-TOF. Esto permitiría identificar el peso molecular de los péptidos responsables de la actividad antioxidante (con mayor resolución que la electroforesis SDS-PAGE) y el análisis de la secuencia aminoacídica de los péptidos. La identificación de la secuencia aminoacídica permite el análisis de la estructura primaria de los péptidos bioactivos, así como entender la relación entre la estructura y función de estos. Esta información es vital para la síntesis de

estos péptidos bioactivos y su posterior aplicación en la industria farmacéutica.

A partir de los resultados de nuestro trabajo, se puede proponer a la cepa *Arthrospira/Limnospira* UTEX 2342 como fuente de proteínas y de compuestos con actividad antioxidante, los cuales pueden ser utilizadas como ingredientes de alimentos funcionales, nutracéuticos y cosméticos.

3. Conclusión

En el capítulo anterior obtuvimos que la cepa de *Arthrospira/Limnospira* UTEX 2342 resultó ser la cepa con mayor contenido de proteínas, lo que en principio representaría una potencial fuente de péptidos con actividad biológica. Animados por esta premisa y siguiendo la metodología del Capítulo 1, se realizó la hidrólisis secuencial del extracto acuoso de UTEX 2342 utilizando proteasas comerciales. La hidrólisis del extracto con papaína por 30 minutos generó la mayor proporción de péptidos, con resultados similares a los obtenidos en el Capítulo 1 (para las cepas CCM-UdeC 040 y CCM-UdeC 136); sin embargo, el aumento en el tiempo de incubación, afectó negativamente la generación de péptidos. Teniendo en cuenta que el empleo de bajas temperaturas y cortos tiempos de hidrólisis son las características más buscadas en la generación de péptidos en la industria alimentaria, se analizaron los hidrolizados obtenidos a través de la hidrólisis con papaína por 30 minutos. Estos hidrolizados mostraron significativa actividad antioxidante y superior a las proteínas parentales sin hidrolizar. A partir de nuestro trabajo se propone a la cepa UTEX 2342 como fuente de péptidos bioactivos, considerando su elevado contenido de proteínas, la significativa actividad antioxidante de los hidrolizados derivados de su biomasa y su disponibilidad en una colección de cultivo de microalgas ampliamente conocida.

IV. Discusión general

Las colecciones de cultivos de microalgas son repositorios biológicos donde se mantiene y preserva el material biológico viable para propósitos de conservación, investigación, educación y biotecnología (Boundy-Mills et al., 2015; Lourenço, 2020). Los depositantes de las cepas de microalgas, así como los encargados de las colecciones de cultivo (privadas o públicas), realizan significativos esfuerzos para mantener cultivos puros, estables e identificados; sin embargo, muchas cepas disponibles no están correctamente identificadas (Day et al., 1999, Friedl & Lorenz, 2012). La ausencia de frecuentes controles de verificación de identidad conduce a que las cepas se desvíen de su material original y que representen una falsa referencia (Stackebrandt, 2010). La correcta identificación de las cepas constituye una de las principales preocupaciones de las colecciones de cultivos, ya que se estima que cerca del 50% de las cepas no se corresponden con los taxones a los que están asignadas (Komárek & Anagnostidis, 1989).

Aunque existen evidencias fenotípicas, ecológicas y genéticas que demuestran que *Arthrospira* y *Spirulina* son dos géneros distintos (Ballot et al., 2004; Li et al., 2001; Manen & Falquet, 2002; Scheldeman et al., 1999; Vonshak & Tomaselli, 2000), en la actualidad se continua comercializando a *Arthrospira* como suplemento alimenticio bajo la denominación de "*Spirulina"* (Furmaniak et al., 2017; Zhang et al., 2020). Como hemos destacado anteriormente, este error taxonómico representa un peligro, debido a la potencial presencia de cianotoxinas en *Spirulina* (Gantar et al., 2009). En este sentido, se debe alertar sobre este error taxonómico, así como delimitar e identificar las cepas de *Arthrospira* que están disponibles en las colecciones de cultivos de microalgas y que potencialmente pueden ser utilizadas con fines de alimentación. En esta delimitación es importante la utilización de marcadores estables y confiables, como son los marcadores genéticos de secuencias variables no codificantes, como es la región espaciadora interna transcrita entre los genes del ARNr de 16 y 23 S (Papapanagiotou & Gkelis, 2019). Estas secuencias varían en longitud, estructura secundaria y presencia/ausencia de genes de ARN_t (Iteman et al., 2000), lo que ha permitido la detección e identificación de las dos especies (monofiléticas) de *Arthrospira* (Baurain et al., 2002; Dadheech et al., 2010; Scheldeman et al., 1999).

Precisamente, nuestro estudio se enfocó en la delimitación e identificación de cepas del género *Arthrospira* depositadas en tres colecciones de cultivo: CCAP, UTEX y CCM-UdeC.

En un primer acercamiento al objetivo general del trabajo, se analizaron dos cepas depositadas en la Colección de Cultivo de Microalgas de la Universidad de Concepción (CCM-UdeC) y catalogadas como *Arthrospira*: CCM-UdeC 040 y CCM-UdeC 136. Ambas cepas mostraron diferencias morfológicas y bioquímicas entre sí. Sin embargo, a partir del análisis genético de su secuencia ITS, se confirmó que ambas pertenecen a la misma especie genética del género *Arthrospira,* lo que evidencia la variabilidad intraespecífica existente en el género. Además, a partir de sus biomasas se generaron extractos acuosos que evidenciaron significativa actividad antioxidante, comparable a la de frutos como morera negra, arándanos, maqui entre otros (Garzón et al., 2020; Quispe-Fuentes et al., 2018; Sritalahareuthai et al., 2020; Suttisansanee et al., 2020). La actividad antioxidante difirió entre ambas cepas: CCM-UdeC 040 mostró mayor bioactividad, lo que podría estar asociado a un mayor contenido de proteínas y ficocianina en el extracto (Tabla I.3).

Los extractos acuosos carecieron de toxicidad (Tabla I.3), lo que evidencia su inocuidad y su potencial aplicación en la industria alimentaria. Además, la hidrólisis secuencial de los extractos acuosos de estas cepas con proteasas comerciales como papaína, tripsina y pepsina, potenció la actividad antioxidante de los mismos (Figura I.4). Los hidrolizados resultantes y su efecto producido fue diferente para cada cepa: los péptidos generados a partir de la hidrólisis de CCM-UdeC 040 con papaína, exhibieron mayor actividad antioxidante que los péptidos generados a partir de CCM-UdeC 136 (Figura I.4). La reacción de hidrólisis con papaína por 30 minutos generó péptidos con bioactividad, lo que es importante si se proyecta nuestra investigación con fines biotecnológicos: esta enzima es de origen vegetal y el tiempo utilizado fue corto; lo que contribuye a la disminución de los costos de producción. A partir de los resultados obtenidos en el Capítulo 1, se propone la utilización de dos cepas de *Arthrospira* (verificado por análisis genético) en la industria alimentaria o nutraceútica, debido a su inocuidad, composición química y el valor añadido que representa la actividad antioxidante de los extractos acuosos, la cual es a su vez potenciada después de la hidrólisis con papaína.

En general, la correcta identificación de las cepas de *Arthrospira* es crucial, puesto que es una de las microalgas más utilizada para fines comerciales (Mitra & Mishra, 2019; Sathasivam et al., 2019). *Arthrospira* es una cianobacteria comestible, con alto valor nutricional y aplicaciones farmacéuticas, por lo que es reconocido por la FDA como GRAS y distinguido como uno de los mejores superalimentos (Marles et al., 2011; Furmaniak et al.,

2017). Además, el desarrollo biotecnológico comienza con la búsqueda de recursos biológicos que puedan ser explotables y la consecuente selección de la mejor opción (Bull et al., 1992) y para ello es importante la caracterización de la diversidad genética (Day et al., 1999; Stackebrandt, 2010), en este caso del género *Arthrospira*.

Considerando que una gran parte de las cepas depositadas en las colecciones de cultivos de microalgas se encuentran erróneamente clasificadas y que en la actualidad se continúa comercializando a *Arthrospira* bajo la denominación de "*Spirulina",* en esta tesis se adquirieron todas las cepas de *Arthrospira/Limnospira* y *Spirulina* disponibles en tres colecciones de cultivo (dos internacionales y una chilena). De las 11 cepas estudiadas a través del análisis de la secuencia ITS, siete se agruparon en el clúster genético de *Arthrospira/Limnospira* y cuatro en el clúster de *Spirulina*. El análisis permitió establecer que cuatro cepas que estaban clasificadas como *Spirulina* en su colección original (UTEX), se encuentran genética, morfológica y bioquímicamente relacionadas con el clúster de *Arthrospira* y separados del género *Spirulina* (Figuras II.1; II.3 y Tablas II.3; II.4). Es importante destacar que las cepas de *Spirulina* formaron a su vez dos clústers genéticos (A y B), no descritos previamente en la literatura para este marcador genético (ITS), lo que evidencia la necesidad de estudiar y caracterizar en más detalle al género *Spirulina* "sensu stricto".

Si bien el análisis genético agrupó a las cepas de *Arthrospira*/*Limnospira* en dos especies genéticas, el análisis fisiológico y bioquímico reveló significativa variabilidad entre estas. Además, no se logró establecer una relación entre la morfología, los paramétros de crecimiento o la composición bioquímica, con la agrupación de las cepas según su secuencia ITS; lo que evidencia la existencia de variabilidad intraespecífica.

Cabe destacar que en los capítulos 1 y 2 se analizaron las cepas CCM-UdeC 040 y CCM-UdeC 136, obteniéndose diferentes perfiles bioquímicos entre ambos estudios. Resulta interesante que el contenido de carotenoides, APC, PC y PE fue superior en el primer estudio; mientras que el contenido de proteínas fue menor. Sin embargo, en ambos estudios se detectaron niveles similares de clorofila *a*, lípidos y ácidos grasos (entre ellos ácido palmítico y GLA). Si bien en ambos estudios se analizaron las mismas cepas, las condiciones experimentales empleadas fueron diferentes: concentración inicial de inóculo de las cepas, composición del medio de cultivo, irradiancia, período de cultivo y sistema de cultivo. Diversos estudios han mostrado que estos factores influyen en la composición

bioquímica y productividad de *Arthrospira* (Pelizer et al., 2003; Rodrigues et al., 2011; Bezerra et al., 2011; Pelizer et al., 2015; Chaiklahan et al., 2022), lo cual explica las diferencias detectadas entre ambos estudios. Cabe mencionar que la composición de ácidos grasos fue similar en ambos estudios, apoyando la idea de que éste representaría un parámetro estable en *Arthrospira/Limnospira* (Galloway & Winder, 2015).

La caracterización de las cepas de *Arthrospira/Limnospira* analizadas en el Capítulo 2 permitió identificar que la cepa UTEX 2342 destacó por su rápido crecimiento y elevado contenido de proteínas (62.25%). Mientras que las cepas CCM-UdeC 040 y CCM-UdeC 136 acumularon altos contenidos de ácido gamma-linolénico, mayor incluso que otras cepas de *Arthrospira* (Aouir et al., 2017; Casazza et al., 2020).

El alto contenido de proteínas registrado en la cepa UTEX 2342 motivó el estudio de los hidrolizados proteicos obtenidos a partir de su biomasa. La hidrólisis del extracto acuoso de esta cepa potenció la actividad antioxidante del extracto (Figura III.5) y mostró diferencias con respecto a los resultados obtenidos previamente para las cepas CCM-UdeC 040 y CCM-UdeC 136. El grado de hidrólisis y por tanto la generación de péptidos, fue diferente en las tres cepas; así como la actividad antioxidante obtenida a través de la hidrólisis con papaína. Estas diferencias probablemente están dadas por el material de inicio de la hidrólisis (Chalamaiah et al., 2012), es decir, las diferencias en la composición de los extractos proteicos de las cepas CCM-UdeC 040, CCM-UdeC 136 y UTEX 2342. Dado que la hidrólisis generada a través de diferentes proteasas producen diversas funcionalidades (Wang et al., 2021), los trabajos posteriores debieran considerar el uso de otras proteasas y/o tiempos de incubación, así como la evaluación de otras bioactividades (además de la actividad antioxidante).

Considerando que las características más comunes de los péptidos bioactivos antioxidantes son: bajo peso molecular (<1 kDa) y presencia de aminoácidos hidrofóbicos y aromáticos en su composición (Piovesana et al., 2018; Jakubczyk et al., 2020), es probable que los péptidos generados a partir del extracto acuoso con papaína posean también estas características, lo que explicaría la actividad antioxidante mostrada.

Generalmente las enzimas que se utilizan en la generación de péptidos a partir de *Arthrospira* son: pepsina, tripsina, quimiotripsina y alcalasa (Lafarga et al., 2021); por lo que, a partir de los resultados de nuestro trabajo, destacamos la importancia y aplicabilidad de la enzima papaína como proteasa. Esta enzima es de origen vegetal y tiene categoría

GRAS. Debido a que los péptidos bioactivos preparados a través de una hidrólisis enzimática utilizando enzimas GRAS, son también considerados como GRAS (Singh et al., 2014), la posibilidad de utilizar los hidrolizados obtenidos en nuestro trabajo en la industria alimentaria es mayor. Además, el empleo de proteasas de origen animal, microbiano o recombinante es altamente cuestionado, por razones éticas, religiosas, restricciones regulatorias y hábitos alimentarios de las personas (vegetarianos, veganos) (Feijoo-Siota & Villa, 2011; Mazorra-Manzano et al., 2018).

Las cepas CCM-UdeC 040, CCM-UdeC 136 y UTEX 2342, disponibles en las Colecciones CCM-UdeC de Chile y UTEX de Estados Unidos, respectivamente, producen biomasas e hidrolizados proteicos que pudieran incorporarse como ingredientes en el desarrollo de alimentos funcionales y nutracéuticos, como potenciales terapias para múltiples enfermedades crónicas, no transmisibles (Wang et al., 2021). Sin embargo, es importante destacar que, si bien las colecciones de cultivos de microalgas permiten la preservación de la biodiversidad y el acceso a las cepas, la adquisición y la utilización de estas no es inmediata, sino que depende de los requerimientos de importación del país de destino. En este sentido, es crucial conocer las leyes y procedimientos establecidos para la adquisición de los permisos de importación en el territorio nacional, puesto que permite el desarrollo del proyecto de investigación de manera legal y sin atrasos.

En nuestro caso, se realizó la solicitud de un permiso legal y oficial de importación de las cepas a la Subsecretaría de Pesca y Acuicultura (SUBPESCA) de Chile. El proceso involucró la inscripción de nuestro laboratorio en la institución de sanidad correspondiente: el Registro Nacional de Acuicultura (RNA) perteneciente al Servicio Nacional de Pesca y Acuicultura (SERNAPESCA). El proceso de solicitud del permiso de importación de las cepas y consecuente otorgamiento, demoró más de siete meses, lo que conllevó a atrasos en el cronograma de actividades en la etapa inicial de esta investigación. Adicionalmente, las cepas importadas recorrieron un largo trayecto, desde Texas y Escocia a Chile, lo que provocó que varias de ellas arribaran en pésimas condiciones, dificultando, y en algunos casos impidiendo, la incorporación de algunas de las cepas adquiridas en el estudio comparativo.

V. Conclusiones generales

1. El análisis de la secuencia de la región ITS del ADNr permitió confirmar y/o corregir la clasificación taxonómica, a nivel de género, de las cepas de *Spirulina* y *Arthrospira/Limnospira* depositadas actualmente en las Colecciones de Cultivo de Microalgas: UTEX (USA), CCAP (Escocia) y CCM-UdeC (Chile).
2. Las cepas de *Spirulina* depositadas en las Colecciones de Cultivo de Microalgas: UTEX, CCAP y CCM-UdeC, formaron dos clústers genéticos no descritos en estudios previos.
3. Todas las cepas de *Arthrospira/Limnospira* depositadas en las Colecciones de Cultivo de Microalgas: UTEX, CCAP y CCM-UdeC, se agruparon en una de las dos especies filogenéticas descritas para el género *Arthrospira/Limnospira*.
4. Se detectó variabilidad en el crecimiento, composición bioquímica de su biomasa y capacidad antioxidante de extractos proteicos e hidrolizados dentro y entre las especies filogenéticas de *Arthrospira/Limnospira.*

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