UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS BIOMÉDICAS

INSTITUTO DE ECOLOGÍA

Ecología microbiana de biorreactores productores de hidrógeno

TESIS QUE PARA OPTAR POR EL GRADO DE DOCTOR EN CIENCIAS

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CIUDAD UNIVERSITARIA, CIUDAD DE MÉXICO. SEPTIEMBRE DE 2022

Agradecimientos institucionales

Al Programa de Doctorado en Ciencias Biomédicas, al Instituto de Ecología, al Laboratorio Nacional de Ciencias de la Sostenibilidad (LANCIS) y a la Universidad Nacional Autónoma de México (UNAM).

Al Consejo Nacional de Ciencia y Tecnología (CONACyT): CVU: 598321; Número de becario: 594615.

A mi comité tutor:
Dra. Ana E. Escalante Hernández (IE-UNAM).
Dra. Idania Valdez-Vazquez (II-UNAM).
Dr. Miguel Ángel Cevallos Gaos (CCG-UNAM).

A la Dra. Morena Avitia Cao Romero del Laboratorio de Ecogenómica de Sistemas (LANCIS, UNAM) por su ayuda técnica en el trabajo de biología molecular.

Al Laboratorio de Investigación en Procesos Avanzados de Tratamiento de Aguas (LIPATA), Instituto de Ingeniería, UNAM. En especial a la Dra. Idania Valdez Vazquez y a la Dra. Marisol Pérez Rángel.

Al Laboratorio de Ingeniería Ambiental (LIA), Instituto de Ingeniería, UNAM. En especial al Dr. Adalberto Noyola Robles y a la Mtra. Margarita Cisneros Ortiz.

Al jurado de examen doctoral:

Presidente: Dra. Valeria Francisca Eugenia Leopoldina De María Guadalupe Souza Saldivar
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Resumen

La fermentación obscura es un proceso donde una comunidad microbiana convierte materia orgánica en biogás rico en hidrógeno. Esta tecnología tiene un gran potencial para la biorremediación de aguas residuales y la disminución del uso de combustibles fósiles y ha recibido mucha atención debido a las cualidades del hidrógeno como combustible limpio y eficiente. Sin embargo, el proceso enfrenta algunos retos como la inestabilidad de los biorreactores y el bajo rendimiento. La fermentación obscura deriva de la digestión anaerobia, proceso por el que se produce metano a partir de hidrógeno y ácidos grasos volátiles resultado de la fermentación de materia orgánica. Para evitar el consumo de hidrógeno, se inhiben a las arqueas metanógenas (las principales consumidoras del hidrógeno) por medio de pretratamientos al inóculo. Debido a los pretratamientos, la estructura de las comunidades cambia drásticamente perdiéndose una gran parte de la diversidad y de las interacciones bióticas que ocurren en las comunidades de origen. Las consecuencias de esta pérdida de diversidad e interacciones no están bien estudiadas y sus consecuencias ecológicas se desconocen.

Un paso importante para mejorar el entendimiento de comunidades naturales y el diseño de comunidades en biotecnología es entender los mecanismos ecológicos que dirigen sus dinámicas poblacionales y su comportamiento funcional. Con este objetivo en mente, en esta tesis evaluamos el papel de la diversidad y de las interacciones entre bacterias en la producción de hidrógeno en biorreactores. De esta forma, primero se realizó una revisión y metaanálisis acerca de la composición microbiana, parámetros de cultivo y rendimiento de los biorreactores en experimentos publicados de fermentación oscura y de digestión anaerobia (Capítulo I). Encontramos que no sólo la diversidad de las especies, sino también las interacciones bióticas entre ellas dan lugar a las propiedades observadas a nivel de comunidad (como la estabilidad). En el Capítulo II proponemos un marco de trabajo para el estudio ecológico de comunidades microbianas con énfasis en comunidades usadas para propósitos biotecnológicos. En este marco de trabajo resaltamos la importancia del diseño experimental y de la sistematización de la investigación de la ecología de comunidades microbianas. Finalmente, en el Capítulo III, ponemos en práctica, de forma experimental, el marco de trabajo propuesto en el Capítulo II para analizar el efecto que tiene la pérdida de diversidad en la estabilidad y el funcionamiento de los reactores productores de hidrógeno. Encontramos que la relación entre la diversidad microbiana y la función de los reactores es multinivel y tiene efectos distintos para las distintas propiedades medibles a nivel comunidad. Los resultados que aquí se presentan proveen nuevos panoramas para la integración de la teoría ecológica en el estudio de las comunidades microbianas y pretenden acercarnos al objetivo de entender, predecir y mejorar el uso las comunidades microbianas bajo diversos escenarios que van desde el conocimiento básico hasta aplicaciones en sostenibilidad.

Abstract

Dark fermentation is a process where a microbial community converts organic matter into hydrogen-rich biogas. This technology has great potential for the bioremediation of wastewater and the reduction of fossil fuels use. It has received much attention due to the qualities of hydrogen as a clean and efficient fuel. However, the process faces some challenges such as bioreactor instability and poor performance. Dark fermentation derives from anaerobic digestion, a process by which methane is produced from hydrogen and volatile fatty acids resulting from the fermentation of organic matter. To avoid the consumption of hydrogen, the methanogenic archaea (the main hydrogen consumers) are inhibited by inoculum pretreatment. Due to the pretreatments, the structure of the communities changes drastically, losing a large part of the diversity and the biotic interactions that occur in the original communities. The consequences of this loss of diversity and interactions are not well studied, and their ecological consequences are unknown.

An important step in improving the understanding of natural communities and community design in biotechnology is to understand the ecological mechanisms that drive their population dynamics and functional behavior. With this goal in mind, in this thesis we evaluated the role of diversity and interactions between bacteria in hydrogen production in bioreactors. In this way, a review and meta-analysis about the microbial composition, culture parameters and performance of the bioreactors in published dark fermentation experiments was first carried out (Chapter I). We found that not only the diversity of the species, but also the biotic interactions between them give rise to the properties observed at the community level (such as stability). In Chapter II we propose a framework for the ecological study of microbial communities with an emphasis on communities used for biotechnological purposes. In this framework we highlight the importance of experimental design and systematization of research on the ecology of microbial communities. Finally, in Chapter III, we experimentally implemented the framework proposed in Chapter II to analyze the effect of diversity loss on the stability and performance of hydrogen-producing reactors. We found that the relationship between microbial diversity and bioreactor function has different effects for different properties measurable at the community level. The results presented here provide new scenarios for the integration of ecological theory in the study of microbial communities and aim to get us closer to the goal of understanding, predicting and improving the use of microbial communities under various scenarios ranging from basic knowledge to sustainability applications.

Contenido

Prólogo

Los microorganismos presentan una enorme diversidad taxonómica y funcional que solamente se ha descrito parcialmente. La ecología microbiana estudia esta diversidad en el contexto de interacciones, tanto bióticas como abióticas, buscando entender la relación entre la diversidad y funciones microbianas. El metabolismo de los microorganismos es indispensable para todos los ecosistemas del planeta ya sea como parte de los ciclos biogeoquímicos, en el tracto digestivo de un mamífero o en el funcionamiento de un biorreactor. Sin embargo, los microorganismos normalmente existen en comunidades o grupos de distintas especies que son muy complejas y en donde cada especie interactúa de formas diferentes con el resto. Incluso las comunidades más pequeñas son difíciles de predecir debido a la complejidad de sus interacciones. Queda mucho por entender acerca de las interacciones microbianas, y sus consecuencias, en contextos comunitarios y ambientales.

Esta investigación doctoral se enfocó en integrar y generar información acerca de cómo ocurren las interacciones microbianas y cuál es su efecto en funciones de interés, a nivel comunidad. Para esto se utilizaron como modelo de estudio consorcios productores de hidrógeno al ser un tipo de comunidad de relativamente baja diversidad y cuyo funcionamiento puede medirse fácilmente. Para cumplir los objetivos de investigación se utilizaron aproximaciones tanto bioinformáticas como experimentales. Esta tesis está conformada por una introducción general que describe el marco conceptual bajo el que se desarrolló el trabajo de investigación. Se presentan tres artículos científicos a manera de capítulos. El **Capítulo I** es un metaanálisis en el que se reunió información de cientos de experimentos publicados acerca de consorcios microbianos productores de hidrógeno y de fermentación obscura. Este metaanálisis se realizó con el objetivo de generar hipótesis acerca de los microorganismos involucrados en el funcionamiento de los reactores, de sus interacciones bióticas y de los factores ambientales que podrían influir tanto en la composición como en el establecimiento de estas interacciones. El **Capítulo II** es una propuesta de marco de trabajo para la generación de hipótesis sobre la relación entre la composición microbiana e interacciones con el funcionamiento de biorreactores productores de hidrógeno. En este capítulo postulamos el valor de la generación de hipótesis con soporte estadístico para su posterior verificación experimental y aporte al entendimiento de los mecanismos involucrados en la relación diversidad-función. El **Capítulo III** presenta los resultados de un experimento en el que se usaron 12 réplicas de biorreactores a escala laboratorio en dos condiciones de cultivo para analizar el efecto de los niveles de diversidad en las interacciones microbianas y en el funcionamiento de los biorreactores. Finalmente, se presentan las conclusiones generales y perspectivas de este trabajo.

Introducción general

1. Ecología microbiana

El desarrollo de los primeros microscopios y las observaciones de Antoine van Leeuwenhoek, Robert Hooke y Athanasius Kircher en el siglo XVII confirmaron la existencia de seres vivos microscópicos (Wainwright, 2003). Estos descubrimientos dieron origen a la microbiología, que durante los primeros dos siglos de su existencia se enfocó, principalmente, en la importancia médica de los microorganismos. Hacia finales del siglo XIX, Sergei Winogradsky realizó estudios sobre las bacterias que participan en el ciclo del nitrógeno y Martinus Beijerinck investigó las bacterias anaerobias participantes del ciclo del azufre (Burton, 2011; Dworkin, 2012). El nuevo enfoque de Winogradsky y Beijerinck de estudiar los microbios en su contexto ambiental y comunitario dio inicio a la ecología microbiana (Bertrand et al., 2015). Más adelante, a mediados del siglo XX, Robert Hungate y Tomotari Mitsuoka realizaron estudios pioneros acerca de la ecología del microbioma intestinal de rumiantes y humanos (Hungate, 1960; Mitsuoka, 2014). Estos estudios establecieron las bases técnicas de cultivo para describir morfológica, taxonómica y funcionalmente a distintos grupos bacterianos.

Hoy en día sabemos que las bacterias han colonizado todos los ecosistemas de la Tierra. Estos microorganismos son muy diversos taxonómica y funcionalmente y son indispensables en el mantenimiento de la vida en el planeta. Las bacterias son responsables de llevar a cabo gran parte de los ciclos biogeoquímicos del planeta que permiten el flujo de nutrientes por los ecosistemas, de mantener la salud del agua y la productividad del suelo (Falkowski et al., 2008; Singh, 2015). A su vez, la diversidad funcional microbiana tiene el potencial de ser utilizada por los humanos para la solución de problemas ambientales y de salud. Las bacterias forman comunidades complejas donde millones de células interactúan y funcionan de formas diferentes, lo que las vuelve difíciles de predecir y controlar.

La ecología microbiana estudia la diversidad de microorganismos y aspira a entender el complejo funcionamiento de sus comunidades. Esta disciplina estudia cómo las poblaciones de especies microbianas se ensamblan para formar comunidades y cómo interactúan entre ellas y con el medio ambiente (Madigan et al., 2015) . Específicamente, la ecología microbiana estudia las siguientes características de las comunidades microbianas (Barton, Larry and Northup, Diana, 2011; Bertrand et al., 2015):

1 El origen de las especies de microorganismos.

2 La diversidad taxonómica y funcional de los microorganismos.
3 El rol de cada especie microbiana en una comunidad.
4 Las interacciones que ocurren entre microorganismos y entre microorganismos y macroorganismos.
5 Las interacciones entre microorganismos y su ambiente.
6 La evolución de las poblaciones microbianas.
7 La capacidad comunitaria de transformar compuestos a manera de servicios ecosistémicos y biotecnológicos.

El desarrollo de técnicas moleculares independientes del cultivo (aunado al renovado interés en técnicas dependientes de cultivo) ha permitido obtener información a gran escala acerca de la diversidad y función microbianas (Gray and Head, 2008; Lagier et al., 2015). Actualmente, la ecología microbiana enfrenta el desafío de descifrar patrones y generar teorías generalizables a partir de esta gran cantidad de información con el fin de entender cómo la diversidad y función microbianas surgen y responden a perturbaciones (Costello et al., 2012).

2. Complejidad en comunidades microbianas

2.1. Relación entre diversidad y función

Todas las comunidades microbianas tienen una estructura característica de número y abundancia de especies que se conoce como *diversidad taxonómica* (Barton, Larry and Northup, Diana, 2011). La diversidad taxonómica de una comunidad microbiana es resultado de las capacidades metabólicas de las especies, de las condiciones ambientales (como pH, nutrientes y humedad) y de procesos ecológicos y evolutivos (p. ej. migraciones, adaptaciones e interacciones bióticas; Bertrand et al., 2015; Ladau & Eloe-Fadrosh, 2019). Por otra parte, cada especie contribuye, con sus capacidades metabólicas, a la diversidad funcional de la comunidad. La diversidad funcional se refiere a la variedad de capacidades metabólicas (o *funciones*) que tiene un grupo de organismos que coexisten en un lugar y momento determinados (Klassen, 2018). Esta diversidad funcional es considerada como el enlace fundamental entre la diversidad taxonómica y los servicios ecosistémicos (es decir, los beneficios que reciben los seres humanos de las comunidades microbianas; Escalas et al., 2019). Por ejemplo, las bacterias pueden ayudar al aprovechamiento de nutrientes y prevención de enfermedades cuando forman parte de los microbiomas de macroorganismos, en la agricultura mantienen la productividad del suelo y permiten la biorremediación de suelo y agua contaminados (Elena et al., 2016; McKenney et al., 2018; Ladau and Eloe-Fadrosh, 2019; de Corato, 2020). Sin embargo, la relación entre la

diversidad taxonómica de una comunidad y su función es compleja y depende de las características de las especies involucradas, de las condiciones ambientales y de sus interacciones.

Tanto la estructura taxonómica como el perfil funcional de una comunidad microbiana son difíciles de predecir debido a que son propiedades que no pueden ser inferidas, solamente, como resultado de sus partes individuales (Escalante et al., 2015; Madsen et al., 2018). Estas propiedades (conocidas como propiedades emergentes) determinan propiedades ecológicas de las comunidades como la resiliencia, coexistencia de especies, capacidades metabólicas y la autoorganización espacial (Berg et al., 2022). Además, las comunidades microbianas son susceptibles a cambios en las condiciones ambientales (i.e. cambio climático, contaminación y disponibilidad de recursos) que pueden cambiar su composición y función (Allison and Martiny, 2009). En principio, existe evidencia de que algunas funciones son altamente dependientes de la diversidad microbiana (Morris et al., 2020). Por ejemplo, durante la descomposición de materia orgánica en el suelo, la degradación de sustratos complejos (como la lignina) está asociada a unos cuantos grupos bacterianos (p. ej. Bacterias de las familias Comamonadaceae y Caulobacteraceae; Wilhelm et al., 2019). También, durante el ciclo del nitrógeno, la oxidación anaerobia del amonio está asociada a grupos bacterianos reducidos como el género *Brocadia* (Wang et al., 2019). Sin embargo, es importante reconocer que se ha observado que, en algunos casos, la variedad taxonómica puede cambiar constantemente mientras que las funciones del ecosistema se mantienen estables. Este fenómeno puede deberse a que diversos procesos (como transferencia horizontal o evolución convergente) resultan en redundancia funcional (dónde diferentes grupos taxonómicos, filogenéticamente distantes, poseen las mismas capacidades metabólicas; Louca et al., 2018). Más aún, los microorganismos que se encuentran en proporciones bajas pueden tener impactos muy importantes en la función de toda la comunidad como los productores de metano en el tracto digestivo de los rumiantes y los fijadores de nitrógeno en el océano (Shade et al., 2014). Finalmente, un nivel más de complejidad en la relación entre la diversidad taxonómica y la función es la constante retroalimentación que existe entre ellas. Aunque en primera instancia la presencia de ciertas especies en una comunidad da lugar a un grupo determinado de funciones, estas funciones cambian las condiciones ambientales afectando el metabolismo y crecimiento del resto de los microorganismos de la comunidad (Fiegna et al., 2015; Wang et al., 2017; Netzker et al., 2018). Estos efectos que tienen los microorganismos entre ellos es posible estudiarlos desde un contexto de interacciones ecológicas que son parte de los mecanismos más importantes que subyacen la relación diversidad-función. Así, entender las funciones microbianas desde un contexto ecológico nos puede dar acceso a un manejo preciso de las comunidades microbianos en escenarios de cambio climático y biotecnológicos (Jobard et al., 2014).

2.2. Interacciones ecológicas en comunidades microbianas

Las bacterias no viven de forma aislada, en cambio, forman parte de comunidades donde se llevan a cabo complejas interacciones ecológicas. Georgy Gause y otros ecólogos realizaron estudios de laboratorio clásicos con levaduras y ciliados que demostraron por primera vez la existencia de interacciones ecológicas entre especies microbianas (Gause, 1932; Vandermeer, 1969). En estos experimentos se preguntaban si dos organismos con un nicho similar que colonizan un mismo ambiente coexistirían o alguno se extinguiría. Los experimentos de Gause llevaron a la adopción del principio de exclusión competitiva que explica que dos organismos con un nicho similar no podrían coexistir debido a la fuerte competencia entre especies por espacio y nutrientes (Hardin, 1960). El principio de exclusión competitiva se volvió un paradigma, bajo el cual, se estudió el ensamblaje de comunidades tanto de macroorganismos como de microorganismos (Cavender-Bares et al., 2009). Estos estudios sentaron las bases para la actual investigación de las interacciones ecológicas entre microorganismos. Sin embargo, en la naturaleza es común la coexistencia de especies, ¿cómo se pueden generar diferentes especies y, por lo tanto, interacciones entre especies, si una especie lleva a todas las demás especies emergentes a la extinción?

Las interacciones que ocurren entre pares de especies bacterianas se pueden clasificar en un eje continuo que va desde las interacciones negativas hasta las positivas. Las interacciones negativas incluyen la competencia, el amensalismo y el parasitismo (Ghoul and Mitri, 2016). La competencia, además, se dividide en explotación e interferencia (Birch, 1957). Mientras que la explotación ocurre por similitud de los recursos utilizados, la interferencia se refiere a formas directas de inhibición. Por otra parte, las interacciones positivas incluyen el comensalismo y el mutualismo (Goldford et al., 2018). De forma similar, formas de cooperación (procesos eco-evolutivos donde le selecciona la inversión en una interacción positiva; West et al., 2007b) que originalmente se habían observado en macroorganismos se han comprobado experimentalmente en microorganismos; por ejemplo, la discriminación por parentesco (interacciones mediadas por la similitud del genotipo; Liu et al., 2021) o la división del trabajo (cuando organismos cooperadores se especializan en realizar tareas complementarias; West and Cooper, 2016). El signo y la fuerza de las interacciones entre los microorganismos tienen consecuencias importantes para el surgimiento de las propiedades emergentes de las comunidades, ayudando a determinar lo que define a una comunidad, en contraste a sólo ser una colección aleatoria de especies (Gorter et al., 2020; Ratzke et al., 2020). A pesar de su importancia, conocemos poco de cómo surgen las interacciones ecológicas entre bacterias y aún no podemos predecir su impacto en la composición y función de las comunidades.

Una consideración importante es que las interacciones ecológicas pueden ser incidentales o haberse desarrollado a través de procesos de evolución y adaptación (Tan et al., 2015). Dependiendo de su historia evolutiva, su impacto en otros organismos puede variar, así como el efecto de la pérdida de diversidad de las comunidades en los organismos que participan en las interacciones. Las interacciones incidentales se refieren a las que suceden como resultado del metabolismo de los organismos y que no reciben una inversión metabólica ni han sido resultado de adaptaciones a la interacción (Germerodt et al., 2016). Por ejemplo, en un momento particular, una especie puede estar realizando la degradación de un carbohidrato complejo por medio de una enzima extracelular o produciendo ácidos grasos como desecho. Los subproductos como azucares simples o ácidos grasos quedan disponibles en el medio y pueden ser aprovechados por organismos diferentes a los que realizan la función. Se ha propuesto que las interacciones incidentales pueden dar lugar a interacciones más complejas por medio de adaptaciones específicas (Gorter et al., 2020). Por ejemplo, si la liberación de enzimas extracelulares, al ser un proceso costoso, es aprovechado por organismos ajenos al productor puede convertirse en un tipo de parasitismo y, en el organismo productor de la enzima, pueden seleccionarse adaptaciones que eviten que las enzimas puedan ser aprovechados por no productores. De esta manera, pueden surgir funciones como la producción de sustancias inhibidoras como bacteriocinas o la formación de biopelículas que doten de estructura espacial a las comunidades que eviten la fuga de funciones costosas (Riley and Wertz, 2002; West et al., 2007a). La *Hipótesis de la Reina Roja* se propuso para explicar cómo las especies se enfrascan en carreras armamentistas donde las especies deben adaptarse y evolucionar, no sólo para tener una ventaja reproductiva, sino también para sobrevivir porque los organismos que compiten también están evolucionando (Bonachela et al., 2017). En contraste, la *Hipótesis de la Reina Negra* se propuso para explicar el surgimiento de las relaciones cooperativas en bacterias que interactúan por periodos prolongados (Morris et al., 2012). Esta hipótesis propone que, a partir de interacciones incidentales por medio de funciones que se hacen disponibles al medio extracelular, dos poblaciones de bacterias pueden perder la capacidad de realizar las funciones que reciben de las demás dando lugar a interdependencias metabólicas y coexistencia (Morris, 2015). La *Hipótesis de la Reina Negra* ha sido observada experimentalmente, lo que ha demostrado que las interacciones pueden surgir y cambiar en tiempos cortos que pueden ser relevantes, por ejemplo, en la operación de biorreactores (Jeffrey Morris et al., 2014). Por las razones descritas aquí, el consenso general es que las interacciones bióticas tienen un impacto profundo en las dinámicas de ensamblado, diversidad y función de las comunidades microbianas (Cordero and Datta, 2016; Ghosh et al., 2016; Madsen et al., 2018). Sin embargo, debido a las características intrínsecas de los organismos microbianos (es decir, su pequeño tamaño y sus interacciones mediadas químicamente) el estudio de sus interacciones es complicado cuando se

estudian comunidades completas (Schmidt et al., 2015; Franzosa et al., 2019). Para entender estos mecanismos es posible utilizar técnicas moleculares, microbiológicas, bioinformáticas y estadísticas, que, en conjunto con diseños experimentales sólidos, permiten generar hipótesis cada vez más precisas acerca del comportamiento de las comunidades microbianas. Recientemente, se han desarrollado nuevas aproximaciones, tanto experimentales como bioinformáticas, para generar hipótesis para el origen de las interacciones microbianas y su efecto en las dinámicas poblaciones y funcionales (Bauer and Thiele, 2018; Nai and Meyer, 2018; Amor and Bello, 2019).

2.3. Aproximaciones al estudio de la relación diversidad-función y de las interacciones ecológicas

Gracias al desarrollo de las tecnologías de secuenciación masiva y de análisis de parámetros fisicoquímicos se puede generar una gran cantidad de información acerca de la composición y función de las comunidades microbianas. Para esto, la secuenciación del gen 16S rRNA (que codifica para el RNA ribosomal 16S que forma parte de la subunidad menor de los ribosomas procariotas) es el estándar usado en los estudios de ecología microbiana para determinar la abundancia e identidad de los microrganismos presentes en una comunidad (Case et al., 2007). Por otra parte, determinar parámetros fisicoquímicos del medio, así como la concentración de metabolitos de interés para cada comunidad sirven como medidas representativas de la función microbiana. En consecuencia, se generan amplios conjuntos de datos multidimensionales compuestos por múltiples variables (p. ej. Abundancia de especies, genes, concentración de metabolitos, proteínas o ácidos grasos) que conllevan una alta dificultad de análisis e interpretación. Con estos objetivos en mente, se han implementado diferentes análisis estadísticos para la inferencia de procesos ecológicos a partir de la información de las comunidades microbianas. Entre estas aproximaciones se encuentran los análisis de correlación y de ordenación que, en conjunto con sus respectivas representaciones gráficas (p. ej. Mapas de calor, redes o gráficas de ordenación), permiten detectar patrones de coocurrencia entre diferentes especies o establecer la relación entre especies y parámetros funcionales. Estos análisis son implementados en paquetes informáticos muy usados para el estudio de comunidades microbianas como QIIME2 (Bolyen et al., 2019) y MicrobiomeAnalyst (Dhariwal et al., 2017). No obstante, no existe una *receta* de análisis estadísticos que sea útil para todos las comunidades y experimentos ya que cada análisis tiene requerimientos y ventajas y desventajas que deben tomarse en cuenta.

En el caso de los análisis de correlaciones, en estos se calculan coeficientes de correlación como el de Pearson o el de Spearman entre los datos de abundancia de las distintas bacterias presentes (p. ej. obtenidas a través del conteo del gen 16sRNA) o entre los datos de abundancia y datos funcionales (para tratar de inferir posibles interacciones ecológicas o relaciones entre diversidad

y función respectivamente; Vanwonterghem et al., 2014; Hugerth and Andersson, 2017). A partir de matrices de correlación de abundancia de bacterias, es posible inferir redes ecológicas dónde los nodos representan las bacterias y los vértices las interacciones negativas o positivas inferidas (Weiss et al., 2016). Este tipo de análisis y algunas variaciones que involucran el uso conjunto de otras medidas de distancia o similitud están disponibles en paquetes informáticos como CoNet (Faust and Raes, 2016) o MENAP (Deng et al., 2012). Las ventajas de estos análisis es su fácil implementación (ya que múltiples paquetes informáticos son capaces de realizarlos), fácil visualización de los resultados por medio de mapas de calor y el uso conjunto de coeficientes y medidas de distancia y similitud para mejorar las inferencias producidas (Faust et al., 2012; Weiss et al., 2016; Wang et al., 2017). Sin embargo, para los análisis de correlación se han observado desventajas como falta de sensibilidad y precisión ya que tienden a generarse falsos positivos (Weiss et al., 2016; Mainali et al., 2017; Carr et al., 2019) y que la interpretación de la relación causa-efecto muchas veces se realiza sin bases mecanísticas sólidas (Prosser, 2020).

Por otra parte, los análisis de ordenación son un conjunto de análisis que resumen y presentan datos multivariados para mostrar, gráficamente, las diferencias entre muestras en menos dimensiones que el conjunto de datos original (Syms, 2008). Esta reducción de dimensionalidad va acoplada con una interpretación sencilla a través de una gráfica de ordenación o *biplot* (como se les conoce en inglés a estas gráficas), donde los objetos cercanos tienen valores similares de variables y, los lejanos, valores diferentes (Paliy and Shankar, 2016). Existen dos tipos de ordenaciones, sin restricción y restringidas. En las ordenaciones sin restricción los análisis se llevan a cabo con una sola tabla de datos y tienen el objetivo de identificar patrones entre muestras e identificar variables responsables de esos patrones (Syms, 2008). En las ordenaciones con restricción se usan dos o más tablas de datos (p. ej. Una tabla de abundancia de especies y una tabla de variables ambientales) y sólo la variación en la tabla de especies que puede ser explicada por la tabla de variables es analizada y mostrada con el objetivo de identificar las relaciones entre ambas (Ramette, 2007). Aunque estos análisis tienen un uso extendido en ecología microbiana debido a su gran utilidad, cuentan con desventajas como el requerimiento de un número de muestra alto (Forcino et al., 2015) y que existen una gran cantidad de variantes de análisis de ordenación que complica elegir el análisis adecuado para el tipo de datos a analizar (Buttigieg and Ramette, 2014). Cada tipo de análisis de ordenación tiene distintas bases estadísticas y la selección de cada una depende de la estructura de los datos que se van a analizar.

Es importante mencionar que el desarrollo de nuevos análisis para estudiar la relación diversidad-función en comunidades microbianas es un área de constante desarrollo. En este sentido, se han desarrollado otras aproximaciones como análisis de especies indicadoras y diversas implementaciones de modelos estadísticos y matemáticos para identificar asociaciones entre bacterias (De Cáceres et al., 2010; Griffith et al., 2016; Shaw et al., 2016). Más recientemente, el modelado metabólico ha surgido como una opción para integrar la información genómica y metabólica de los organismos con sus procesos ecológicos permitiendo la inferencia de sus funciones e interacciones bióticas (Bauer et al., 2017; Bauer and Thiele, 2018; Jansma and Aidy, 2020). Las distintas aproximaciones descritas aquí han sido utilizadas con éxito en distintos escenarios en los que han demostrado su utilidad para ayudar a entender las comunidades microbianas muy diferentes.

Por ejemplo, usando análisis estadísticos, se ha determinado que el pH del suelo es uno de los principales determinantes en la composición y diversidad microbianas a nivel global (Lauber et al., 2009), se han identificado los microorganismos que pueden realizar la degradación de moléculas complejas como la celulosa (Jiang et al., 2015), los plásticos (Oberbeckmann et al., 2016), las proteínas (Kato et al., 2008) o los lípidos (Nobu et al., 2015). En cuanto a las interacciones ecológicas entre microorganismos, se ha identificado que las bacterias patógenas en el microbioma de la placa dental presentan coocurrencias que pueden indicar complementariedad (Faust et al., 2012) y también se han identificado bacterias asociadas a resistencia a patógenos en la piel de anfibios (Rebollar et al., 2016). Estos estudios representan un primer paso para la generación de hipótesis que luego podrán guiar la realización de experimentos para resolver preguntas específicas. Una vez que se han identificado interacciones potencialmente relevantes para un sistema de estudio (p. ej. usando métodos estadísticos) es posible plantear un estudio experimental de dichas interacciones. Para este objetivo es posible usar aproximaciones dependientes de cultivo donde se aíslan los organismos de una comunidad o se usan cepas de colección para realizar co-cultivos o construir comunidades sintéticas controladas (Abreu and Taga, 2016). De esta manera se han estudiado las interacciones cooperativas en el tracto digestivo de los seres humanos (Vrancken et al., 2019), las interacciones antagonistas en comunidades bacterianas acuáticas (Aguirre-von-Wobeser et al., 2014) y las interacciones que son importantes en el ensamblaje de comunidades de suelo (Friedman et al., 2017). Además, el uso de técnicas moleculares como análisis de isótopos e hibridación fluorescente in-situ ayudan a descifrar los metabolitos involucrados y a descifrar la estructura espacial en las comunidades (D'Souza et al., 2018).

3. El caso de los consorcios microbianos productores de hidrógeno

Una aproximación útil para estudiar la relación diversidad-función es el uso de consorcios microbianos con una complejidad reducida que permitan generar y probar hipótesis ecológicas. Los consorcios utilizados para la producción de biocombustibles y consorcios sintéticos se han utilizado para probar hipótesis ecológicas y proponer teorías ecológicas generalizables (De Roy et al., 2014; Vanwonterghem et al., 2014). En particular, los consorcios microbianos productores de hidrógeno representan un buen sistema para estudiar hipótesis ecológicas ya que son sistemas poco diversos con funciones medibles bien estudiadas (Wang et al., 2020). La producción biológica de hidrógeno por medio de consorcios microbianos se lleva a cabo por un proceso conocido como fermentación obscura dónde se lleva a cabo la fermentación de materia orgánica dando lugar a hidrógeno, ácidos grasos volátiles y alcoholes. Los consorcios que participan en la fermentación obscura provienen de los consorcios de digestión anaerobia (un proceso por el cual se genera metano) pero son pretratados agresivamente para seleccionar sólo a los organismos con capacidad para la producción de hidrógeno (Wang and Yin, 2017). La digestión anaerobia es un proceso robusto que se usa con éxito para el tratamiento de aguas residuales y la producción de biogás rico en metano. En contraste, la fermentación obscura es un proceso inestable y poco robusto (Castelló et al., 2020). Se desconoce cómo los pretratamientos agresivos y la pérdida de diversidad en los consorcios de fermentación obscura impactan su estructura, interacciones y, estos a su vez, su funcionamiento. Aunque se ha estudiado la composición microbiana de consorcios microbianos de fermentación obscura, se ha dejado de lado el estudio de mecanismos (como las interacciones bióticas) que impactan en su dinámica y su función.

El proceso de digestión anaerobia comprende cuatro etapas: hidrólisis, acidogénesis, acetogénesis y metanogénesis (Figura 1). La hidrólisis hace metabólicamente disponibles los polímeros que forman la materia orgánica al transformarlos en monómeros por medio de la liberación de enzimas extracelulares (p. ej. Celulasas, celobiasas, xilanasas, lipasas y proteasas; Weiland, 2010) y es, por lo tanto, considerado un paso limitante en la digestión anaerobia (Vavilin et al., 2008). Durante la acidogénesis, los monómeros son fermentados para producir ácidos grasos volátiles de distintas longitudes, alcoholes, CO_2 y H_2 (Chandra et al., 2012). Los ácidos grasos y el hidrógeno son intermediarios en la producción del metano (Figura 1), por lo que este paso es esencial en la producción de substratos para el último paso de la digestión anaerobia. La acetogénesis comprende la formación de acetato a partir de varios substratos y rutas, por ejemplo: los ácidos propiónico y butírico y el etanol son convertidos en acetato mientras se produce hidrógeno (Weiland, 2010), las azúcares son transformadas solamente en acetato (homoacetogénesis; Müller, 2003) y el CO_2 es reducido usando hidrógeno (Chandra et al., 2012). En la digestión anaerobia, la acetogénesis también

es un paso limitante pues el acetato es el principal substrato para la producción de metano (Chandra et al., 2012). La metanogénesis es la producción de metano por parte de arqueas metanógenas. Este proceso puede ser de tres tipos (Chandra et al., 2012): acetoclástica (acetato como substrato), hidrogenotrófica (CO_2 y H_2 como substrato) y metilotrófica (metanol como substrato). La metanogénesis tiene un papel muy importante en la digestión anaerobia pues consume el H_2 que inhibe el metabolismo de las bacterias productoras de hidrógeno y de las acetógenas (Weiland, 2010). Cada etapa puede ser realizada por grupos de organismos diferentes o un grupo puede participar en más de una etapa. Sin embargo, los pretratamientos son aplicados para eliminar las especies bacterianas que consumen hidrógeno para la producción de metano y de acetato.

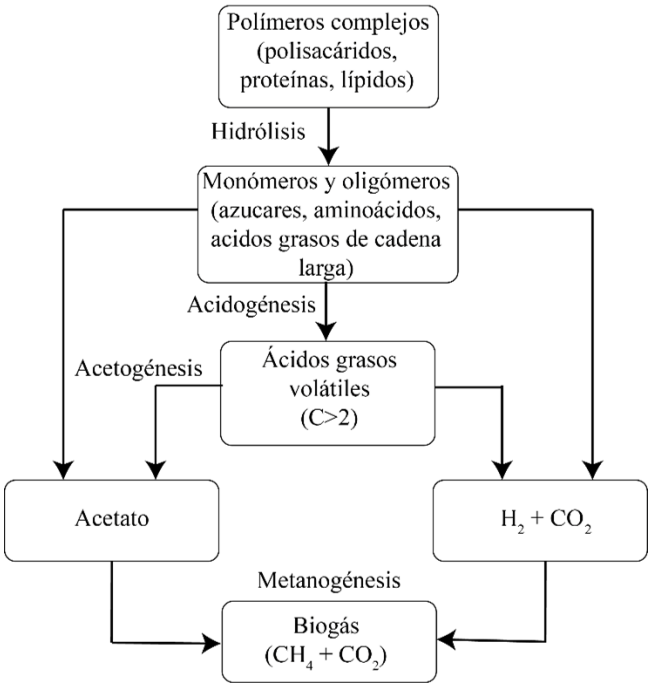

Figura 1. Etapas de la digestión anaerobia. (Modificada de Chandra *et al*., 2012).

Aun cuando se ha investigado extensamente el efecto de los parámetros de cultivo en la producción de hidrógeno se conoce poco acerca de la ecología de los consorcios que habitan los reactores. Sin embargo, recientemente se han hecho algunos esfuerzos pioneros para dar un enfoque ecológico a las funciones de las bacterias que conforman los consorcios microbianos productores de hidrógeno. Por ejemplo, se sabe que especies del género *Bacillus* participan en hidrólisis de sustratos complejos que hacen disponibles azúcares simples a productores de hidrógeno (Cabrol et al., 2017). Por otra parte, especies del género *Klebsiella* consumen oxígeno permitiendo el crecimiento de los productores de hidrógeno que son estrictamente anaerobios (p.ej. del género *Clostridium*) (Vasconcelos et al., 2016). También se ha identificado la formación de estructura espacial (por

ejemplo, por bacterias del género *Prevotella*) y la regulación del pH (gracias a bacterias el género *Megasphaera*) como parte de las interacciones positivas que tienen bacterias no productoras de hidrógeno (Cabrol et al., 2017). Por otro lado, se han identificado interacciones que perjudican la producción de hidrógeno, como la producción de bacteriocinas (un tipo de competición por interferencia) o el consumo de hidrógeno (un tipo de comensalismo) (Castelló et al., 2020). También se ha reconocido a la diversidad como una propiedad global de las comunidades con un impacto en la función. Por ejemplo, una alta diversidad puede mejorar la estabilidad por medio de redundancia funcional, incrementando la productividad por medio de la complementariedad de funciones o incrementando la posibilidad de la ocurrencia de cepas productoras de hidrógeno resistentes a inhibidores (Jobard et al., 2014; Castelló et al., 2020). Es importante destacar que apenas se ha establecido la función de una pequeña parte de los microorganismos de los consorcios de fermentación obscura y que el efecto de los pretratamientos y las condiciones de cultivo en las interacciones ecológicas no se ha estudiado. También, la mayoría de las inferencias que se han realizado carecen de comprobación experimental explícita. Debemos mejorar la integración del estudio ecológico de los consorcios que habitan los reactores al estudio de sus parámetros y rendimiento. De esta manera, podremos entender las interacciones que explican su ensamble y funciones, lo que nos acercará a un mejor manejo con fines industriales.

Capítulo 1: Estudio comparativo de la estructura de comunidades de digestión anaerobia y de fermentación obscura.

Prefacio

El número, identidad, abundancia e interacciones de las especies que conforman las comunidades microbianas tienen un fuerte impacto en cómo éstas se comportan y funcionan. Por ejemplo, se sabe que el número de especies en una comunidad puede determinar si es robusta a invasiones o a la pérdida de especies y que las interacciones bióticas influyen su dinámica de abundancia y función. Por esta razón, el estudio de la relación diversidad-función en comunidades microbianas es indispensable para poder controlar y predecir el comportamiento de los sistemas microbianos. Para entender las diferencias en estabilidad y rendimiento entre biorreactores de fermentación obscura y de digestión anaerobia, en este capítulo utilizamos la información publicada de la composición microbiana de más de 200 experimentos para comparar la estructura ecológica de los consorcios microbianos asociados a ambos tipos de fermentación. También, recopilamos información de las condiciones de cultivo (pH, temperatura), del origen del inóculo y de la función de los reactores (producción de biogás). Con esta información y, por medio de aproximaciones estadísticas (análisis multivariados y de redes de coocurrencia), evaluamos la asociación entre las características de cultivo y la función de los biorreactores con la composición microbiana. Por otra parte, realizamos la inferencia de interacciones por medio de la construcción de redes de coocurrencias para comparar la estructura ecológica de los dos tipos de consorcios (digestión anaerobia y fermentación obscura).

En resumen, los resultados muestran una codependencia entre la comunidad microbiana, las condiciones operacionales y la función de los reactores (rendimiento de hidrógeno). Se observó una relación positiva entre la riqueza de especies y el rendimiento de hidrógeno. Algunos grupos bacterianos (p. ej. Tissierellaceae, Oxalobacteraceae y Tepidimicrobium), además de la familia Clostridiaceae, se relacionaron positivamente con la producción de hidrógeno. Más aún, el análisis de redes encontró que las comunidades de digestión anaerobia son más robustas que las de fermentación obscura, posiblemente, debido a la pérdida de diversidad y de interacciones resultado de los pretratamientos y de las condiciones de cultivo en el caso de las segundas. Nuestros resultados apoyan la hipótesis de que una mayor diversidad tiene efectos benéficos para la estabilidad y función de los consorcios microbianos. Causas de esto pueden ser la diversidad metabólica y la redundancia funcional. Por otra parte, el mayor número de las interacciones positivas en los consorcios

microbianos de digestión anaerobia pueden también pueden contribuir a su mayor estabilidad. Detrás de estas interacciones inferidas pueden estar comportamientos como facilitación o cooperación debido a la degradación de sustratos complejos, eliminación de toxinas o formación de biopelículas. Proponemos que la investigación futura en ecología microbiana debe considerar los procesos ecológicos y evolutivos para entender las causas de la estabilidad a largo plazo de los consorcios microbianos. En particular, se deben investigar experimentalmente las relaciones entre condiciones ambientales, composición microbiana, interacciones bióticas y función.

INTERNATIONAL JOURNAL OF HYDROGEN ENERGY 41 (2016) 17297–17308

Available online at www.sciencedirect.com

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Ecological perspectives of hydrogen fermentation by microbial consortia: What we have learned and the way forward

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ARTICLE INFO

Article history:
Received 4 May 2016
Received in revised form
1 August 2016
Accepted 4 August 2016
Available online 26 August 2016

Keywords:
Biohydrogen
Anaerobic digestion
Ecological networks
Microbial interactions
Microbial diversity

ABSTRACT

Research on hydrogen production by dark fermentation has improved the management and performance of bioreactors, although yield and long-term stability challenges still exist. To understand the causes of these challenges we propose to investigate the ecological properties of microbial consortia residing in hydrogen-producing bioreactors. In this study, we analyzed scientific literature (until 2016) on hydrogen fermentation and investigated the relationships between bioreactor operational conditions and microbial composition, as well as co-occurrences of microbial groups through multivariate and network analyses, respectively. The results of the analyses highlight ecological aspects that may need to be considered when aiming for increased performance and stability of bioreactors, such as: (i) the relationship between key parameters and their influence on microbial diversity (e.g. positive relationship between richness with H_2 yield and higher diversity of certain inocula), (ii) the positive relationship of the presence of specific microbial families and genera (often overlooked) with increased H_2 yield (e.g. Tissierellaceae, Oxalobacteraceae and Tepidimicrobium), (iii) the importance of specific groups and their interactions (e.g. potential cooperative interactions between H_2 producers, biofilm producers and facultative anaerobic bacteria) and (iv) the importance of such interactions on the systemic properties of the consortia (e.g. increased network robustness with higher diversity). Beyond the ecological implications of our analyses, we also discuss the limitations of current methods for characterizing microbial communities and the potential for the application of modern methodologies such as high throughput sequencing and proteomics to re-evaluate the diversity and functional information thus far published in helping to disentangle the ecological phenomena that occur within hydrogenogenic consortia.

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http://dx.doi.org/10.1016/j.ijhydene.2016.08.027

Introduction

The urgency to find energy production alternatives to fossil fuels has driven the development of different processes with less polluting consequences. Hydrogen is a high-energy carrier that can be used for electricity generation and as a source of energy for transportation, domestic or industrial settings, while its combustion results mainly in water [1]. Hydrogen plays a central role in reducing contaminant emissions from vehicles and industries to help reach the "carbon zero" goals established by several countries. In this sense, the European Union (EU) and specific countries, such as the USA, Norway and Germany, have dedicated specialized budgets to research projects related to hydrogen production and its application with the goal of introducing hydrogen into their energy systems (e.g., in the case of EU ensuring full access to hydrogen refueling points by 2025) [2,3]. Currently, hydrogen is mainly produced by high-energy demand processes such as water electrolysis and methane reforming [4]. Dark fermentation, a low-energy demand process for hydrogen production, has been investigated in recent decades as a promising alternative to produce hydrogen on a large scale. It comprises the conversion, by microorganisms, of renewable carbohydrate-rich substrates from industrial or household sources [1,5]. Dark fermentation is a promising avenue for hydrogen production because it has high production rates compared to other biotechnological options [5], is easy to operate, has low energy requirements and has the possibility to be integrated with other technologies such as photo-fermentation and microbial fuel cells [6].

Dark fermentation arises from gaseous anaerobic metabolism, which is a complex process resulting from microbial activity, under anaerobic conditions, that leads to the formation of organic acids, methane and hydrogen. This is a process that occurs naturally in lake sediments and soils [7], and consists of four phases (hydrolysis, acidogenesis, acetogenesis and methanogenesis) [8]. H_2 is produced during acidogenesis and is then consumed to produce acetate and methane during acetogenesis and methanogenesis, respectively [9]. Because anaerobic communities (e.g., anaerobic digestion reactors, compost) have been the most used inocula for hydrogen fermentation bioreactors [10], several strategies have been followed to prevent H_2 consumption. The oldest and most widely used method is the inoculum pretreatment (i.e., heat shocks as they allow hydrogen-producing spore-forming bacteria to survive while methanogenic archaea are deactivated) [8]. Other methods include pH control (because methanogenic microorganisms can only thrive in a reduced range of pH, namely 6.8–8.2) [11] and decreasing hydraulic retention time (as methanogenic archaea have slower growth rates than hydrogen producers they are washed out of bioreactors) [11]. These methods eliminate methanogenic populations and, consequently, the process arrests at the acidogenesis phases where hydrogen is produced, along with CO_2, volatile fatty acids and alcohols [8]. Moreover, molecular characterization of consortia, mainly by means of Denaturing Gradient Gel Electrophoresis (PCR-DGGE), have confirmed that overall diversity is reduced mainly to Firmicutes species (hydrogen-producing and spore-forming bacteria such as *Clostridium* species due to their ability to resist pretreatment conditions and bioreactors culture conditions) [5,12–14]. Still, low productivity and stability have precluded large-scale implementation of hydrogen fermentation bioreactors. On one hand, productivity stands on thermodynamic bases, with H_2 partial pressure and temperature playing an important role in the metabolic pathways that microorganisms use for obtaining energy. High H_2 partial pressures and low temperatures make unfavorable the catabolic reactions that give rise to H_2 production, thus, directing metabolic activity to ethanol or lactate production [15–17]. On the other hand, stability, meaning the capability of bioreactors to operate for long periods, has proved to be an obstacle [13,18,19], except in a few cases [20]. Because significant diversity is lost after pretreatments of inocula and due to culture conditions, some attributes related to their ecological structure might also be lost. The loss of these attributes is possibly related to the low stability of hydrogen-producing consortia, as opposed to the high stability and yield that characterize anaerobic digestion reactors [21]. For this reason, it is necessary to develop methods that are useful, not only for promoting rapid growth of hydrogen-producers, but also, that allow to maintain hydrogen production for extended periods. To overcome these obstacles in biohydrogen production, it is critical to investigate the microbial component of bioreactors considering ecological aspects of the consortia such as diversity, potential interactions and systemic properties of their organization [22].

Specifically, it is crucial to understand how ecological attributes of microbial consortia are related to stability and yield, and also how they are affected by the pretreatments and culture conditions. Some of these ecological attributes include functional redundancy and its relationship with diversity [23] and the biotic interactions between the community members [24,25]. The ecological attributes of any biological system, particularly microbial consortia, are at the foundation of population dynamics with direct impact in the evolutionary processes that can lead to variations in a reactor's performance [26]. Current knowledge about the ecological attributes of hydrogen-producing reactors is limited and mainly focused on some interactions associated with specific processes such as granule formation (e.g., *Streptococcus* species) [27], anoxic conditions maintenance (e.g., *Klebsiella* species) [27], substrate hydrolysis (e.g., *Pseudomonas* and *Bifidobacterium* species) [28] and inhibitions by bacteriocins (e.g., *Lactobacillus*) [29]. It is important to mention here that, given the great interest in biohydrogen, there is abundant literature that reports the diversity of hydrogen producing bioreactors. Nonetheless, little attention has been given to analyze this information with an ecological perspective, which has limited the conclusions and progress in managing microbial consortia and reactors. On the one hand, multivariate statistical analyses might help to comprehend the complex influence of culture conditions on the microbial component of hydrogen production [30]. Currently, our understanding is limited to the control of specific populations and operational parameters. For example, high temperature has proven to increase hydrogen production by enrichment of high-yield thermophilic organisms [31–33], while pH limits methanogenic growth, influences metabolic pathways in species of microbial consortia and selects for distinct bacteria even at the species level [11,34–36]. On the

other hand, ecological networks can be inferred and analyzed to examine their systemic properties. Topological measures (e.g., connectivity or robustness) can be calculated and related to biological properties of the actual communities [37–39]. Overall, knowledge of the ecological properties of hydrogen producing consortia (diversity and interactions) can provide a systemic understanding on their functioning and help toward a better and sustained performance.

In this work, we reviewed scientific publications corresponding to 111 hydrogen fermentation and 122 anaerobic digestion experimental settings. This information was used to construct databases on composition and operational conditions for multivariate and network analyses. The main goal was to investigate the relationships between culture conditions of bioreactors, biological factors (i.e., diversity, co-occurrence, and network properties) and H_2 yield. Specific goals included the following: (i) investigating specific microbial groups related to certain operational conditions and variations in H_2 yield; (ii) determining the structure of hydrogen-producing and anaerobic consortia in terms of their ecological co-occurrence networks, and (iii) finding structural changes related to their stability.

Methods

Literature search

A key word search was conducted in SCOPUS using "dark fermentation" and "microbial" for hydrogen-producing experiments and "anaerobic digestion" and "microbial" for anaerobic digestion experiments (publications until 2016 were included). Since physical interaction is assumed among microorganisms (co-occurrence), only data from experiments performed in a single bioreactor were used.

Data extracted from the literature

Attributes registered from the hydrogen-producing reactor experiments included the following factors: H_2 yield, temperature, pH, pretreatment, inoculum origin, substrate, and prokaryotic species richness and identities. From anaerobic digestion experiments, we only recorded species identities. The information was registered at the time of maximum yield or production (either H_2 or methane) based on the assumption of equivalent ecological status; thus, the potential coexistence of the recorded members of the communities was possible. In 51 of the experiments, the H_2 yield data were transformed to mol H_2/mol $hexose_{consumed}$ for the multivariate analysis.

Microbial composition data

Microbial composition was analyzed in terms of presence/absence data based on 16S rDNA gene sequence differences as the standard marker for species identity in prokaryotes [40]. Importantly, most of the experiments used PCR-DGGE for analyzing microbial communities for which band sequencing was not always performed (also, when bands were excised and sequenced, generally, only a subset of the total bands were chosen). When reported sequences were of at least 1000 bp, their *GenBank* accession number was recorded. For sequences below 1000 bp, their identity was recorded according to sequence similarity with their closest relative (at least 97%, 95% and 90% similarity to 16S rDNA sequence for species, genera and family identity, respectively). For experiments where no sequences were available, the reported species identities were recorded and, in the case of unidentified OTUs (Operational Taxonomic Units) without reported sequences, they were only counted as part of total richness. The available sequences were downloaded using their accession numbers and grouped according to genera identities using the *UCLUST* script [41] in *QIIME* [42] (95% similarity cut). The statistical analyses were performed at genus and family levels, although ecological patterns were only found and reported at the family level.

Statistical analyses

To visualize and identify patterns of environmental variables (culture and operation parameters of the reactors) and their correspondence with microbial composition, a multivariate ordination approach was followed. First, culture conditions parameters and H_2 yield were standardized via a z-score transformation [30]. Then, three Canonical Correspondence Analyses (CCAs) were performed to model the OTUs (family level; removing unique families) response to environmental parameters, inoculum source, pretreatment, and substrate complexity (simple for defined substrates using a single carbohydrate as carbon source or complex for undefined substrates, including waste water and organic residues). These analyses were performed using the *vegan* package [43] implemented in *R* software [44]. Additionally, an *indicator species analysis* was carried out using community composition and H_2 yield. Each experiment, was categorized according to H_2 yield using 3 quantiles (0.02–1.02, 1.19–2.12 and 2.2–3.2 mol H_2/mol $hexose_{consumed}$ for low, medium and high H_2 yield). The *indicspecies* package for R [45] was used for this analysis.

Network analysis

To investigate the systemic properties of the studied microbial communities, co-occurrence networks were reconstructed using the complex networks reconstruction algorithm, *Sets2-Networks* [46]. Only experiments where two or more families were reported were used (hydrogen fermentation: 106 experiments; anaerobic digestion: 122 experiments). Correlations with probability values of 0.70 or higher were considered statistically significant as described by Clark et al. [46]. Networks were topologically analyzed using *Cytoscape* [47] and connectance was measured as an important topological parameter for ecological networks [48]. Modularity analyses were performed using the community detection algorithm by Blondel et al. [49] implemented in *Gephi* [50]. Finally, robustness analyses were performed consisting of measuring their robustness coefficient [39] when nodes were removed at random or by decreasing degree and their average vulnerability [37].

Results and discussion

Richness and temperature are positively correlated with H_2 production

Three CCAs allowed investigation of the relationship between culture conditions, microbial diversity and H_2 yield (Fig. 1, see Tables S1 and S2). The first CCA included inocula origin, the second included the type of pretreatment applied to the inocula and the third included the medium complexity.

Our results show that species richness and temperature are positively related to H_2 yield. In this sense, CCAs showed a positive relationship between H_2 yield and richness (experiments with six or more species tended to have a higher H_2 yield in comparison to those with only two to three species; 1.94 vs 1.77 mol H_2/mol hexose$_{consumed}$). This observation underpins previous studies documenting that more diverse communities (in terms of number of species) are also more diverse in terms of metabolic pathways that can lead to a wider range of usable substrates [23,51]. In addition, a hydrogen-producing consortium with more species might also increase functional redundancy and stability in the face of environmental perturbations or invasions [25,52].

In the case of temperature, the CCA modeling microbial diversity and pretreatments shows a positive relationship between the temperature and H_2 yield (Fig. 1B; likewise, those bioreactors operated at high temperatures, from 50 to 70 °C, were associated with medium-to-high H_2 yield). This result validates the multivariate analyses because it agrees with the fact that high temperatures favor H_2 production and prevent external species invasion such as by pathogens [53,54]. Interestingly, pH was also positively related to H_2 yield, although, accounting for the range analyzed in our experiments (4–7), this might be an effect caused by pH inhibition because H_2 production falls with decreasing pH due to increased metabolic costs being totally inhibited at approximately pH 4 [55,56]. Furthermore, optimal pH values have been reported between 5.5 and 7 [15].

Regarding the complexity of substrate, Fig. 1C shows that experiments where simpler media are used are prone to have an increased H_2 yield. Since these defined, simple media are generally sterile, invasions and competition might be reduced. On the other side, complex media, like manure, sewage or waste water might carry invasive species that outgrow hydrogen producers [57]. Unexpectedly, simple-media experiments tended to show a higher richness than complex media experiments. Further research is needed to investigate this relationship explicitly, but some potential explanations for this pattern include the complex interaction between inoculum sources (with varying initial diversity; [58]), pretreatments (that drastically reduce potential diversity),

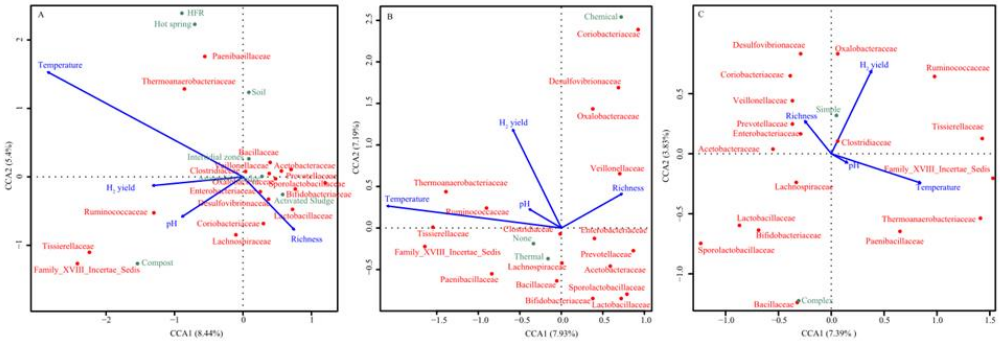

Fig. 1 – Canonical Correspondence Analyses (CCAs). (A) CCA modeling OTU (family level) response to environmental parameters of hydrogen-producing bioreactors and inoculum origin (Compost, Hot Springs, Hydrogen Fermentation Reactor [HRF], Soil, Anaerobic and Activated Sludge). The first axis (CCA1) accounted for 8.44% of the total variation, mainly involving temperature. The second axis (CCA2) accounted for 5.4% of the total variation, mainly involving hydrogen yield. Significance of independent variables (p-values): model: 0.107; hydrogen yield: 0.018; temperature: 0.002; pH: 0.228; richness: 0.512; inoculum source: 0.639. (B) CCA modeling OTUs (family level) response to environmental parameters of hydrogen-producing bioreactors and pretreatments (Thermal, Chemical and None). The first axis (CCA1) accounted for 7.93% of the total variation, mainly involving temperature. The second axis (CCA2) accounted for 7.19% of the total variation, mainly involving hydrogen yield. Significance of independent variables (p-values): model: 0.001; Hydrogen yield: 0.008; Temperature: 0.001; pH: 0.184; Richness: 0.434; Pretreatment: 0.11. (C) CCA modeling OTU (family level) response to environmental parameters of hydrogen-producing bioreactors and substrate complexity (*Simple* for defined media with single carbohydrates as carbon source and *Complex* for undefined medium, like wastewater or organic wastes). The first axis (CCA1) accounted for 7.39% of the total variation, mainly involving temperature. The second axis (CCA2) accounted for 3.83% of the total variation, mainly involving hydrogen yield. Significance of independent variables (p-values): model: 0.004; hydrogen yield: 0.025; temperature: 0.001; pH: 0.206 richness: 0.495; substrate complexity: 0.449. Text corresponds to family names for the prokaryotes found in the experiments included in the analysis (51), and vectors show scaled environmental variables.

culture conditions (representing a strong selective force) and substrate complexity. Examples of the complexity of interactions among different ecological parameters can be found in literature from microbial experimental evolution looking explicitly at the resulting diversity when imposing different selection regimes, substrates and environmental conditions. In particular, we would like to point at a couple of studies where simple or single carbon sources (substrates) have been used in culture conditions and have resulted in the emergence or stable coexistence of more that one strain or bacterial "type" due to the appearance or *de novo* interactions by cross-feeding strategies [22,58–60]. In the light of this knowledge, the complexity of the ecological systems that represent bioreactors, and that the analyzed experiments where not designed to control for diversity of substrates and diversity of microbial communities it is thus difficult to simply expect a direct relationship between these two parameters.

Some families are related to variations in hydrogen production

Some families, such as Ruminococcaceae, Tissierellaceae, Oxalobacteraceae and Clostridiales Family XVIII, were associated with high H_2 yield in the CCAs, although it was not possible to establish a statistical relationship through the "*indicator species analysis*". The positive correlation of Ruminococcaceae members with high H_2 yield can be explained by experimental evidence that associates some of its members with ruminal biohydrogenation [61] as they are H_2 producers and important substrate hydrolyzers [27]. Moreover, *Tepidimicrobium*, a Tissierellaceae family member, may contribute to complex substrate degradation because they are xylanolytic bacteria [62]. Furthermore, some Oxalobacteraceae members (genus: *Janthinobacterium*) are hydrogen producers [63], and in the Clostridiales family XVIII, genera such as *Anaerovorax* degrade putrescine (protein degradation product), producing hydrogen [64]. Other families associated with high H_2 production include Thermoanaerobacteraceae (a widely known thermophilic high-yield hydrogen-producer family) [19], Desulfovibrionaceae (some members are H_2 producers) [65] and Lachnospiraceae (butyric acid producers commonly found in rumen) [66].

Families associated with low H_2 yield included Sporolactobacillaceae, Bacillaceae, Lactobacillaceae, Enterobacteriaceae, Paenibacillaceae and Bifidobacteriaceae (see Fig. 1). The first three families were also associated with low H_2 yield in the "*indicator species analysis*" (p-value < 0.05). The negative correlation of the presence of Lactobacillaceae, Sporolactobacillaceae and Bifidobacteriaceae families with H_2 yield can be explained by their metabolic capabilities of lactate and alcohol production, processes that in turn reduce hydrogen production [67]. Furthermore, these and the Paenibacillaceae families are also reported to produce bacteriocins that are proven to have a negative effect on *Clostridium* species, which are well-recognized H_2 producers [27,29,68]. Because Bacillaceae and Enterobacteriaceae members are facultative anaerobes, they are thought to enhance H_2 production by consuming O_2 in mixed cultures [27,69], although they also produce H_2 via pyruvate formate-lyase (with a maximum H_2 yield of just 2 mol of H_2 per mol of glucose) [70], acting as substrate competitors decreasing H_2 production. Additionally, it might be possible that hydrogen consumers or lactate producers take advantage of these conditions and outgrow hydrogen producers as previously reported [18].

Interestingly, Clostridiaceae was not associated with any of the axis, indicating that this family is prevalent in these experiments although not all of its members produce H_2. Because the main pretreatment used was heat-shock, Clostridiaceae dominance was expected [71]. These results reinforce the idea that H_2 production can be achieved even when members of Clostridiaceae are not present [72].

Inocula and pretreatments affect microbial composition and H_2 production

The CCA including the origin of inoculum shows that the compost inocula was associated with the highest H_2 yield (Fig. 1A). This result is in agreement with previous studies that reported that these communities are highly hydrolytic and acidogenic, especially when degrading lignocellulosic substrates [73]. Along with compost, rumen and manure communities were included in this category; these communities can carry adaptations (e.g., enzymes) for complex substrate degradation, potentially increasing H_2 production [74]. Meanwhile, activated sludge inocula was associated with the highest richness that agrees with their high diversity [75]. Despite this result, higher richness does not translate into high H_2 yield, probably because drastic selective and culture conditions eliminate a large part of this diversity as previously reported [76].

From the pretreatments conditions analyzed, we observed that samples with no pretreatment were related to intermediate H_2 yield and to some unwanted species such as those in Paenibacillaceae and Sporolactobacillaceae families, as expected [27]. Thermal shock pretreatment is associated with the Clostridiaceae family, as mentioned above [71,77]. However, thermal shock pretreatment is negatively related to H_2 yield, perhaps because substrate competitors such as Bacillaceae are capable of forming spores [77]. Finally, chemical pretreatments (e.g., pH, chloroform) were associated with high H_2 yield as previously reported [78,79].

It is important to note that the number of experiments included (51) might not be representative of all the diversity present in hydrogenogenic consortia, so caution should be taken when making further generalizations. Hereof, standardization of the methods and units used in the experiments is essential to make wide-scale comparisons and is the first step for identifying patterns that can help to better understand microbial communities. Nonetheless, these findings show that further research of specific groups correlated with H_2 yield is essential to characterize their metabolic capabilities, interactions and culture requirements toward increasing H_2 yield. Finally, it is worth noting that some inocula may not include the specific groups that have the best response and performance in terms of H_2 yield, which should be considered.

Anaerobic digestion consortia are more robust than H_2 producing consortia

To investigate potential interactions among members and systemic properties of hydrogen-producing and

methanogenic microbial consortia, network analyses were performed. In general, the topological attributes of both networks (i.e., centrality, connectance and density; Table 1) indicate that anaerobic digestion network is less central and more densely connected than the hydrogen fermentation network (Figs. 2 and 3; data from Tables S3 and S4; significant edges in Tables S5 and S6). In both networks, Clostridiaceae is the most important node (in terms of its betweenness centrality) reflecting the importance of this family in the energy flux of anaerobic communities. Although the anaerobic digestion network has a higher connectance than the hydrogen fermentation network, both have low connectance even when compared against macroorganism networks [48]. This could be because networks were inferred at the family level using DGGE data and not by massive sequencing, i.e., diversity was underestimated and pattern detection power at the finer scale was lost.

Particular differences between topological attributes of both networks indicate that the anaerobic digestion network is more robust than the hydrogen fermentation network. In fact, the robustness analysis mathematically confirmed expectations about the topological attributes and their relationship with robustness. This analysis indicates that the anaerobic digestion network has a higher robustness coefficient either when nodes are removed randomly (84.78% ± 19.74 vs 89.47% ± 6.98) or by degree (19.62% vs 33.6%). Moreover, the anaerobic digestion network has a lower average vulnerability than the hydrogen fermentation network ($1.35*10^{-16}$ vs $1.92*10^{-17}$). The intersection network reveals that only 16 nodes and 14 edges (out of 38 nodes and 117 edges of the anaerobic digestion network) are conserved between both networks (Fig. 4), which reveals that after the pretreatments, not only is the diversity greatly reduced, but also that few of the original interactions are conserved. These topological attributes can give us insights into the ecological mechanisms that allow anaerobic digestion systems to stably operate for extended periods [80].

One possible ecological explanation for bioreactor stability derived from the topological network analysis is that, by being densely connected, the microbial communities of these reactors are robust against random or directed species losses [81] as well as against species invasions [82]. However, the evidence for the latter is mixed [83,84]. As the diagram in Fig. 5 shows, only a small part of the original diversity of the anaerobic digestion consortia remains in the hydrogenogenic consortia, which may mean that richness is a determining factor in the stability of ecological communities (e.g., resilience and resistance), for example, by enhancing functional redundancy [25,51,85]. When taking into account the substrate complexity, Fig. 5 shows that, independently of the substrate type, anaerobic digestion experiments have a higher richness. Although in dark fermentation experiments the majority of families are shared among simple and complex substrate experiments, some families are present only in complex substrate experiments. The diversity-invasibility relationship hypothesizes that high diversity increases resource competition, which results in decreased niche opportunities hindering alien invasions [86]. Since low diversity and high resource availability characterize hydrogen fermentation communities, invasion might be facilitated by collapsing the bioreactor's function as found in other communities [87]. Furthermore, the lack of natural competitors might facilitate invasion success [88], although it is not known how the same applies for microbial communities. Overall, our results indicate that potentially cooperative associations among members of the anaerobic digestion microbial communities are lost in hydrogen fermentation bioreactors, which negatively affects diversity and stability, precluding their long-term operation.

Further exploration of the ecological organization of microbial consortia was achieved by a modularity analysis of anaerobic and hydrogen fermentation networks. Both networks are decomposed into two modules, which correspond with ecological associations that can be exploited to increase the system stability and H_2 production (Figs. 2 and 3). The anaerobic digestion network modules indicate that they have a complex structure matching previously known biological information because the two inferred modules broadly represent the process division into the acidogenic and the methanogenic phases. As such, the first module comprises function-related families such as Clostridiaceae (hydrolyzer and H_2 producers) [5,23], Enterobacteriaceae (which aid in maintaining anaerobic conditions in bioreactors and produce H_2) [89], Streptococcaceae (which aid in granulation or biofilm formation) [5,90], Bacillaceae and Bifidobacteriaceae (substrate hydrolysers; the earlier is also a H_2 producer) [5,91]. The second module includes methanogenic archaea along with hydrolyzers and H_2 producers (e.g., Ruminococcaceae) [27,61] and syntrophic bacteria (e.g., Syntrophobacteraceae) [92]. The coexistence of several archaea groups agrees with the niche separation between them due to their metabolic diversity [9]. In contrast, the hydrogen network's division into two modules has no biological correspondence other than the previously mentioned disruption of ecological interactions and roughly corresponding to a divided acidogenic phase. Still, the earlier mentioned functions should be tracked to particular strains that can provide these functions without precluding H_2 production so that consortia design can be achieved successfully.

It is relevant to discuss the validity and caveats that the use of PCR-DGGE data in diversity analyses, such as the presented

Table 1 – Topological attributes of the resulting networks at the family level.

Attribute	H_2 fermentation network	Anaerobic digestion network
Average degree	3.586	6.158
Centrality	0.937	0.652
Avg. path length	1.872	2.037
Density	0.128	0.166
Connectance	0.062	0.081
Modularity	0.545 (2 modules)	0.434 (2 modules)
Clustering coefficient	0.445	0.609
Heterogeneity	1.430	1.015
Diameter	2	4
Nodes	29	38
Shared nodes	16	
Edges	52	117

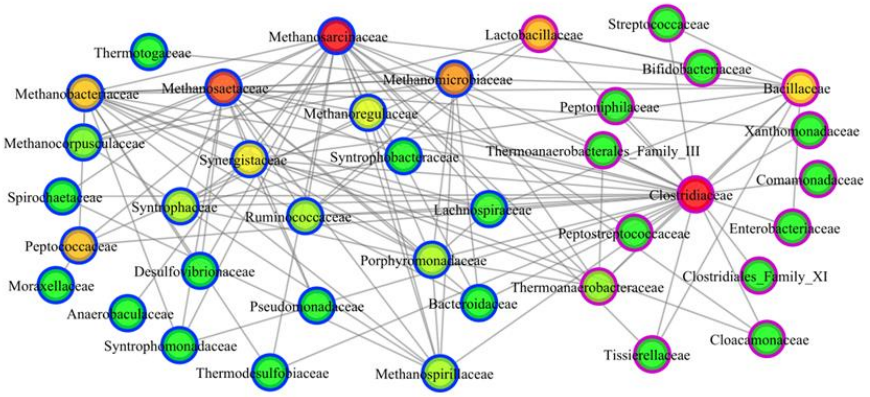

Fig. 2 – Co-occurrence network of the anaerobic digestion consortia at the family level. Node color accounts for betweenness centrality of the nodes from low (green) to high values (red). Node edge color shows membership to a module. Experiments included = 103. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

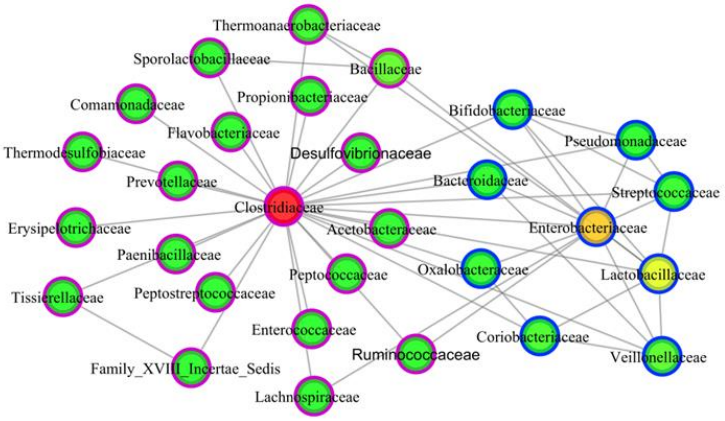

Fig. 3 – Co-occurrence network of the hydrogen-producing consortia at the family level. Color accounts for betweenness centrality of the nodes from low (green) to high values (red). Node edge color shows membership to a module. Experiments included = 122. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

here, may impose to our conclusions and the perspectives for future research with these considerations. Although DGGEs have been reported as highly reproducible and congruent with Next Generation Sequencing (NGS) methods (also known as high throughput sequencing) in terms of profiling complex communities, it is true that diversity in DGGE profiles is not completely captured (only numerically dominant taxonomic groups are consistently found in both methodological approaches) [93–96], it has also been noted that protocol-specific variations in DGGEs can lead to significant difference in banding patterns. For example, amplicon choice (specific genes and gene regions), double band patterns due to changes in DNA structure, polymerase errors and preferential band sequencing influence the quality of the data and, can impact negatively the comparability of experiments [97–100]. Thus, it is undeniable that limitations exist in regard to the type of data collected (i.e. DGGE derived), and diversity estimates here presented should be taken cautiously by themselves, nonetheless and given the comparative nature of the analyses here presented, it is valid to say that the findings regarding statistical relationships between diversity and operational parameters as well as H_2 yield may hold even with more accurate methodologies of community composition analysis. Nonetheless it is worth mentioning that modern technologies for microbial composition characterization such as NGS, proteomics and metabolomics will help to re-evaluate the diversity

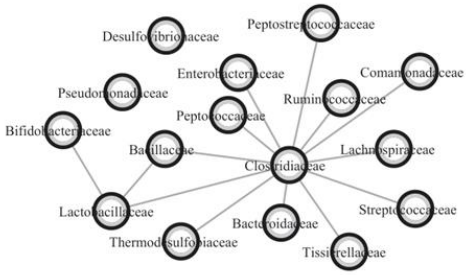

Fig. 4 – Intersection network between hydrogen-producing and anaerobic digestion consortia.

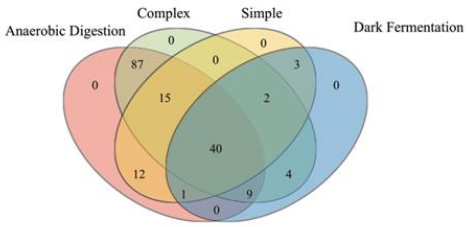

Fig. 5 – Venn diagram depicting the distribution of OTUs between anaerobic digestion and hydrogen or dark fermentation consortia and between simple and complex-substrate experiments.

and functional information so far published and to further disentangle the ecological phenomena that occur within hydrogenogenic consortia beyond statistical relationships of parameters.

In summary, results from the multivariate analyses show a codependency between operational conditions, community structure and bioreactor functioning (H_2 yield). Although these results should be taken with caution due to the methodological bias for identifying species, a clear, positive relationship is observed between temperature, pH and richness with high H_2 yield. Some families and species highlighted here, (besides from the highly studied Clostridiaceae), are frequently ignored or overlooked in the design and enrichment of consortia (and even in the isolation of hydrogen-producing strains). These species might be good candidates, *per se* or in association, for enhancing hydrogen production via other functions and interactions. Moreover, the network analyses confirmed the hypothesis that hydrogen fermentation consortia are less robust than anaerobic digestion consortia, possibly due to the loss of diversity and interactions by aggressive inocula pretreatments and culture conditions.

Conclusions

As future research aims to design high-yield and long-term stable consortia, ecological and evolutionary processes should be considered. As shown by our results, some overlooked aspects that need to be included in future research are: the relationship between microbial diversity and stability (including metabolic diversity and functional redundancy) and the amount, type and members of microbial interactions (e.g. facilitation and mutual cooperation between hydrogen producers and biofilm producers or facultative anaerobes). Also, this work highlights the limitations of currently used methods for exploring microbial diversity (i.e. DGGE). Finer molecular approaches (e.g., massive sequencing or transcriptomics) can provide more detailed and species-level information about microbial composition in terms of identity, abundance and functional role in bioreactors. Future work is necessary to experimentally investigate the relationships found in this study and to apply them effectively to overcome the productivity and yield problems. For example, questions that derive from our work include: (1) how to increase microbial diversity while maintaining high yield? (2) how to prevent or restore the ecological interactions and processes lost in hydrogen producing consortia? (3) what conditions give rise to cooperative interactions between microorganisms present in this consortia? Nonetheless, the information presented here can direct future research to focus on specific microbial groups and their interactions. This research can transition from in silico modeling of bioreactors to specific experiments, including synthetic biology design, considering both evolutionary and ecological dynamics.

Acknowledgments

This work was supported by Consejo Nacional de Ciencia y Tencología (CONACyT) [grant 168921 to IVV]. This work constitutes part of the doctoral research of the first author who received scholarship from the Consejo Nacional de Ciencia y Tecnología (CONACyT, grant no. 594615) and thanks to the Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijhydene.2016.08.027.

REFERENCES

[1] Momirlan M, Veziroglu T. The properties of hydrogen as fuel tomorrow in sustainable energy system for a cleaner planet. Int J Hydrogen Energy 2005;30:795–802. http://dx.doi.org/10.1016/j.ijhydene.2004.10.011.

[2] Salvi BL, Subramanian KA. Sustainable development of road transportation sector using hydrogen energy system. Renew Sustain Energy Rev 2015;51:1132–55. http://dx.doi.org/10.1016/j.rser.2015.07.030.

[3] Sgobbi A, Nijs W, De Miglio R, Chiodi A, Gargiulo M, Thiel C. How far away is hydrogen? Its role in the medium and long-term decarbonisation of the European energy system. Int J Hydrogen Energy 2015;41:19–35. http://dx.doi.org/10.1016/j.ijhydene.2015.09.004.

[4] Salvi BL, Subramanian KA, Panwar NL. Alternative fuels for transportation vehicles: a technical review. Renew Sustain Energy Rev 2013;25:404–19. http://dx.doi.org/10.1016/j.rser.2013.04.017.
[5] Show KY, Lee DJ, Tay JH, Lin CY, Chang JS. Biohydrogen production: current perspectives and the way forward. Int J Hydrogen Energy 2012;37:15616–31. http://dx.doi.org/10.1016/j.ijhydene.2012.04.109.
[6] Kumar G, Bakonyi P, Kobayashi T, Xu K-Q, Sivagurunathan P, Kim S-H, et al. Enhancement of biofuel production via microbial augmentation: the case of dark fermentative hydrogen. Renew Sustain Energy Rev 2016;57:879–91. http://dx.doi.org/10.1016/j.rser.2015.12.107.
[7] Koyama T. Gaseous metabolism in lake sediments and paddy soils and the production of atmospheric methane and hydrogen. J Geophys Res 1963;68:3971–3. http://dx.doi.org/10.1029/JZ068i013p03971.
[8] Valdez-Vazquez I, Poggi-Varaldo HM. Hydrogen production by fermentative consortia. Renew Sustain Energy Rev 2009;13:1000–13. http://dx.doi.org/10.1016/j.rser.2008.03.003.
[9] Costa KC, Leigh JA. Metabolic versatility in methanogens. Curr Opin Biotechnol 2014;29:70–5. http://dx.doi.org/10.1016/j.copbio.2014.02.012.
[10] Ghimire A, Frunzo L, Pirozzi F, Trably E, Escudie R, Lens PNL, et al. A review on dark fermentative biohydrogen production from organic biomass: process parameters and use of by-products. Appl Energy 2015;144:73–95. http://dx.doi.org/10.1016/j.apenergy.2015.01.045.
[11] Liu D, Zeng RJ, Angelidaki I. Effects of pH and hydraulic retention time on hydrogen production versus methanogenesis during anaerobic fermentation of organic household solid waste under extreme-thermophilic. Biotechnol Bioeng 2008;100:1108–14. http://dx.doi.org/10.1002/bit.21834.
[12] Cisneros-Pérez C, Carrillo-Reyes J, Celis LB, Alatriste-Mondragón F, Etchebehere C, Razo-Flores E. Inoculum pretreatment promotes differences in hydrogen production performance in EGSB reactors. Int J Hydrogen Energy 2015;40:6329–39. http://dx.doi.org/10.1016/j.ijhydene.2015.03.048.
[13] Jo JH, Jeon CO, Lee DS, Park JM. Process stability and microbial community structure in anaerobic hydrogen-producing microflora from food waste containing kimchi. J Biotechnol 2007;131:300–8. http://dx.doi.org/10.1016/j.jbiotec.2007.07.492.
[14] Marino-Marmolejo EN, Corbalá-Robles L, Cortez-Aguilar RC, Contreras-Ramos SM, Bolaños-Rosales RE, Davila-Vazquez G. Tequila vinasses acidogenesis in a UASB reactor with *Clostridium* predominance. Springerplus 2015;4:419. http://dx.doi.org/10.1186/s40064-015-1193-2.
[15] Guo XM, Trably E, Latrille E, Carrre H, Steyer JP. Hydrogen production from agricultural waste by dark fermentation: a review. Int J Hydrogen Energy 2010;35:10660–73. http://dx.doi.org/10.1016/j.ijhydene.2010.03.008.
[16] Pawar SS, Van Niel EWJ. Thermophilic biohydrogen production: how far are we? Appl Microbiol Biotechnol 2013;97:7999–8009. http://dx.doi.org/10.1007/s00253-013-5141-1.
[17] Lee H, Salerno MB, Rittmann BE. Thermodynamic evaluation on H_2 production in glucose fermentation. Environ Sci Technol 2008;42:2401–7.
[18] Koskinen P, Kaksonnen A, Puhakka J. The relationship between instability of H_2 production and compositions of bacterial communities within a dark fermentation fluidised bed bioreactor. Biotechnol Bioeng 2007;97:742–58. http://dx.doi.org/10.1002/bit.
[19] Urbaniec K, Bakker RR. Biomass residues as raw material for dark hydrogen fermentation - a review. Int J Hydrogen Energy 2015;40:3648–58. http://dx.doi.org/10.1016/j.ijhydene.2015.01.073.
[20] Carrillo-Reyes J, Trably E, Bernet N, Latrille E, Razo-Flores E. High robustness of a simplified microbial consortium producing hydrogen in long term operation of a biofilm fermentative reactor. Int J Hydrogen Energy 2016;41:1–10. http://dx.doi.org/10.1016/j.ijhydene.2015.11.131.
[21] Werner JJ, Knights D, Garcia ML, Scalfone NB, Smith S, Yarasheski K, et al. Bacterial community structures are unique and resilient in full-scale bioenergy systems. Proc Natl Acad Sci U S A 2011;108:4158–63. http://dx.doi.org/10.1073/pnas.1015676108.
[22] Escalante AE, Rebolleda-Gómez M, Benítez M, Travisano M. Ecological perspectives on synthetic biology: insights from microbial population biology. Front Microbiol 2015;6:1–10. http://dx.doi.org/10.3389/fmicb.2015.00143.
[23] Tracy BP, Jones SW, Fast AG, Indurthi DC, Papoutsakis ET. Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Curr Opin Biotechnol 2012;23:364–81. http://dx.doi.org/10.1016/j.copbio.2011.10.008.
[24] Proulx S, Promislow D, Phillips P. Network thinking in ecology and evolution. Trends Ecol Evol 2005;20:345–53. http://dx.doi.org/10.1016/j.tree.2005.04.004.
[25] Shade A, Peter H, Allison SD, Baho DL, Berga M, Bürgmann H, et al. Fundamentals of microbial community resistance and resilience. Front Microbiol 2012;3:1–19. http://dx.doi.org/10.3389/fmicb.2012.00417.
[26] Goldman RP, Brown SP. Making sense of microbial consortia using ecology and evolution. Trends Biotechnol 2009;27:3–4. http://dx.doi.org/10.1016/j.tibtech.2008.10.003.
[27] Hung C-H, Chang Y-T, Chang Y-J. Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative biohydrogen production systems – a review. Bioresour Technol 2011;102:8437–44. http://dx.doi.org/10.1016/j.biortech.2011.02.084.
[28] Weiland P. Biogas production: current state and perspectives. Appl Microbiol Biotechnol 2010;85:849–60. http://dx.doi.org/10.1007/s00253-009-2246-7.
[29] Thuault D, Beliard E, Le Guern J, Bourgeois CM. Inhibition of *Clostridium tyrobutyricum* by bacteriocin-like substances produced by lactic acid bacteria. J Dairy Sci 1991;74:1145–50. http://dx.doi.org/10.3168/jds.S0022-0302(91)78266-0.
[30] Ramette A. Multivariate analyses in microbial ecology. FEMS Microbiol Ecol 2007;62:142–60. http://dx.doi.org/10.1111/j.1574-6941.2007.00375.x.
[31] Kargi F, Eren NS, Ozmihci S. Bio-hydrogen production from cheese whey powder (CWP) solution: comparison of thermophilic and mesophilic dark fermentations. Int J Hydrogen Energy 2012;37:8338–42. http://dx.doi.org/10.1016/j.ijhydene.2012.02.162.
[32] Rittmann S, Herwig C. A comprehensive and quantitative review of dark fermentative biohydrogen production. Microb Cell Fact 2012;11:115. http://dx.doi.org/10.1186/1475-2859-11-115.
[33] Zhao C, O-Thong S, Karakashev D, Angelidaki I, Lu W, Wang H. High yield simultaneous hydrogen and ethanol production under extreme-thermophilic (70°C) mixed culture environment. Int J Hydrogen Energy 2009;34:5657–65. http://dx.doi.org/10.1016/j.ijhydene.2009.05.057.
[34] Chen WH, Sung S, Chen SY. Biological hydrogen production in an anaerobic sequencing batch reactor: pH and cyclic duration effects. Int J Hydrogen Energy 2009;34:227–34. http://dx.doi.org/10.1016/j.ijhydene.2008.09.061.
[35] Hao LP, Lü F, Li L, Shao LM, He PJ. Shift of pathways during initiation of thermophilic methanogenesis at different

initial pH. Bioresour Technol 2012;126:418–24. http://dx.doi.org/10.1016/j.biortech.2011.12.072.
[36] Song J, An D, Ren N, Zhang Y, Chen Y. Effects of pH and ORP on microbial ecology and kinetics for hydrogen production in continuously dark fermentation. Bioresour Technol 2011;102:10875–80. http://dx.doi.org/10.1016/j.biortech.2011.09.024.
[37] Costa LDF, Rodrigues FA, Travieso G, Villas Boas PR. Characterization of complex networks: a survey of measurements. Adv Phys 2007;56:167–242. http://dx.doi.org/10.1080/00018730601170527.
[38] Newmann MEJ. The structure and function of complex networks. SIAM Rev 2003;45:167–256.
[39] Piraveenan M, Uddin S, Chung KSK. Measuring topological robustness of networks under sustained targeted attacks. Proc 2012 IEEE/ACM Int Conf Adv Soc Networks Anal Mining, ASONAM 2012 2012:38–45. http://dx.doi.org/10.1109/ASONAM.2012.17.
[40] Stackebrandt E, Goebel BM. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 1994;44:846–9. http://dx.doi.org/10.1099/00207713-44-4-846.
[41] Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26:2460–1. http://dx.doi.org/10.1093/bioinformatics/btq461.
[42] Caporaso J, Kuczynski J, Stombaugh J. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335–6. http://dx.doi.org/10.1038/nmeth0510-335.
[43] Oksanen J, Blanchet G, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. Vegan: community ecology package. 2013.
[44] R Core Team. R: a language and environment for statistical computing. 2013.
[45] De Cáceres M, Legendre P. Associations between species and groups of sites: indices and statistical inference. Ecology 2009;90:3566–74.
[46] Clark NR, Dannenfelser R, Tan CM, Komosinski ME, Ma'ayan A. Sets2Networks: network inference from repeated observations of sets. BMC Syst Biol 2012;6:89. http://dx.doi.org/10.1186/1752-0509-6-89.
[47] Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13:2498–504. http://dx.doi.org/10.1101/gr.1239303.
[48] Dunne J, Williams RJ, Martinez ND. Food-web structure and network theory: the role of connectance and size. Proc Natl Acad Sci U S A 2002;99:12917–22. http://dx.doi.org/10.1073/pnas.192407699.
[49] Blondel V, Guillaume J-L, Renaud L, Lefebvre E. Fast unfolding of communities in large networks. J Stat Mech Theory Exp 2008;2008:P10008.
[50] Bastian M, Heymann S, Jacomy M. Gephi: an open source software for exploring and manipulating networks. ICWSM 2009:361–2.
[51] Vanwonterghem I, Jensen PD, Dennis PG, Hugenholtz P, Rabaey K, Tyson GW. Deterministic processes guide long-term synchronised population dynamics in replicate anaerobic digesters. ISME J 2014;8:1–14. http://dx.doi.org/10.1038/ismej.2014.50.
[52] Vanwonterghem I, Jensen PD, Ho DP, Batstone DJ, Tyson GW. Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Curr Opin Biotechnol 2014;27:55–64. http://dx.doi.org/10.1016/j.copbio.2013.11.004.
[53] Lu J, Gavala HN, Skiadas IV, Mladenovska Z, Ahring BK. Improving anaerobic sewage sludge digestion by implementation of a hyper-thermophilic prehydrolysis step. J Environ Manag 2008;88:881–9. http://dx.doi.org/10.1016/j.jenvman.2007.04.020.
[54] Valdez-Vazquez I, Ríos-Leal E, Esparza-García F, Cecchi F, Poggi-Varaldo HM. Semi-continuous solid substrate anaerobic reactors for H_2 production from organic waste: mesophilic versus thermophilic regime. Int J Hydrogen Energy 2005;30:1383–91. http://dx.doi.org/10.1016/j.ijhydene.2004.09.016.
[55] Cavinato C, Giuliano A, Bolzonella D, Pavan P, Cecchi F. Bio-hythane production from food waste by dark fermentation coupled with anaerobic digestion process: a long-term pilot scale experience. Int J Hydrogen Energy 2012;37:11549–55. http://dx.doi.org/10.1016/j.ijhydene.2012.03.065.
[56] Gadhamshetty V, Arudchelvam Y, Nirmalakhandan N, Johnson DC. Modeling dark fermentation for biohydrogen production: ADM1-based model vs. Gompertz model. Int J Hydrogen Energy 2010;35:479–90. http://dx.doi.org/10.1016/j.ijhydene.2009.11.007.
[57] Vasconcelos EAF, Leitão RC, Santaella ST. Factors that affect bacterial ecology in hydrogen-producing anaerobic reactors. BioEnergy Res 2016. http://dx.doi.org/10.1007/s12155-016-9753-z.
[58] Moura P, Valdez-Vazquez I, Saratele GD, Saratale RG, Silva C, Ortigueira J. Dark fermentative hydrogen production: from concepts to a sustainable production. In: Darvishi F, Hiligsmann IS, editors. Microbial Fuels: Technologies and Applications. CRC Press; 2017 [in press].
[59] Rosenzweig RF, Sharp RR, Treves DS, Adams J. Microbial evolution in a simple unstructured environment: genetic differentiation in *Escherichia coli*. Genetics 1994;137:903–17.
[60] Morris JJ, Papoulis SE, Lenski RE. Coexistence of evolving bacteria stabilized by a shared black queen function. Evol (N Y) 2014:2960–71. http://dx.doi.org/10.1111/evo.12485.
[61] Huws SA, Kim EJ, Lee MRF, Scott MB, Tweed JKS, Pinloche E, et al. As yet uncultured bacteria phylogenetically classified as *Prevotella*, Lachnospiraceae incertae sedis and unclassified Bacteroidales, Clostridiales and Ruminococcaceae may play a predominant role in ruminal biohydrogenation. Environ Microbiol 2011;13:1500–12. http://dx.doi.org/10.1111/j.1462-2920.2011.02452.x.
[62] Niu L, Song L, Liu X, Dong X. *Tepidimicrobium xylanilyticum* sp. nov., an anaerobic xylanolytic bacterium, and emended description of the genus *Tepidimicrobium*. Int J Syst Evol Microbiol 2009;59:2698–701. http://dx.doi.org/10.1099/ijs.0.005124-0.
[63] Ning Y, Wang S, Jin D, Harada H, Shi X. Formation of hydrogen-producing granules and microbial community analysis in a UASB reactor. Renew Energy 2013;53:12–7. http://dx.doi.org/10.1016/j.renene.2012.10.051.
[64] Matthies C, Evers S, Ludwig W, Schink B. *Anaerovorax odorimutans* gen. nov., sp. nov., a putrescine-fermenting, strictly anaerobic bacterium. Int J Syst Evol Microbiol 2000;50:1591–4. http://dx.doi.org/10.1099/00207713-50-4-1591.
[65] Venkata Mohan S, Raghavulu SV, Goud RK, Srikanth S, Babu VL, Sarma PN. Microbial diversity analysis of long term operated biofilm configured anaerobic reactor producing biohydrogen from wastewater under diverse conditions. Int J Hydrogen Energy 2010;35:12208–15. http://dx.doi.org/10.1016/j.ijhydene.2010.08.008.
[66] Meehan CJ, Beiko RG. A phylogenomic view of ecological specialization in the lachnospiraceae, a family of digestive tract-associated bacteria. Genome Biol Evol 2014;6:703–13. http://dx.doi.org/10.1093/gbe/evu050.
[67] Sikora A, Błaszczyk M, Jurkowski M, Zielenkiewicz U. Lactic acid bacteria in hydrogen-producing consortia: on purpose or by coincidence? In: Kongo M, editor. Lact. Acid Bact. - R D

Food, Heal. Livest. Purp.. 1st ed. InTech; 2013. http://dx.doi.org/10.5772/50364.
[68] Girardin H, Albagnac C, Dargaignaratz C, Nguyen-The C, Carlin F. Antimicrobial activity of foodborne *Paenibacillus* and *Bacillus* spp. against *Clostridium botulinum*. J Food Prot 2002;65:806–13.
[69] Yokoi H, Tokushige T, Hirose J, Hayashi S, Takasaki Y. H2 production from starch by a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes*. Biotechnol Lett 1998;20:143–7. http://dx.doi.org/10.1023/A:1005372323248.
[70] Manish S, Banerjee R. Comparison of biohydrogen production processes. Int J Hydrogen Energy 2008;33:279–86. http://dx.doi.org/10.1016/j.ijhydene.2007.07.026.
[71] Kannaiah Goud R, Sarkar O, Venkata Mohan S. Regulation of biohydrogen production by heat-shock pretreatment facilitates selective enrichment of *Clostridium sp*. Int J Hydrogen Energy 2013;39:7572–86. http://dx.doi.org/10.1016/j.ijhydene.2013.10.046.
[72] Valdez-Vazquez I, Pérez-Rangel M, Tapia A, Buitrón G, Molina C, Hernández G, et al. Hydrogen and butanol production from native wheat straw by synthetic microbial consortia integrated by species of *Enterococcus* and *Clostridium*. Fuel 2015;159:214–22. http://dx.doi.org/10.1016/j.fuel.2015.06.052.
[73] Yue ZB, Li WW, Yu HQ. Application of rumen microorganisms for anaerobic bioconversion of lignocellulosic biomass. Bioresour Technol 2013;128:738–44. http://dx.doi.org/10.1016/j.biortech.2012.11.073.
[74] Hess M, Sczyrba A, Egan R, Kim T-W, Chokhawala H, Schroth G, et al. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science 2011;331:463–7. http://dx.doi.org/10.1126/science.1200387.
[75] Zhang T, Shao M-F, Ye L. 454 pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. ISME J 2012;6:1137–47. http://dx.doi.org/10.1038/ismej.2011.188.
[76] Baghchehsaraee B, Nakhla G, Karamanev D, Margaritis A. Fermentative hydrogen production by diverse microflora. Int J Hydrogen Energy 2010;35:5021–7. http://dx.doi.org/10.1016/j.ijhydene.2009.08.072.
[77] Wong YM, Wu TY, Juan JC. A review of sustainable hydrogen production using seed sludge via dark fermentation. Renew Sustain Energy Rev 2014;34:471–82. http://dx.doi.org/10.1016/j.rser.2014.03.008.
[78] Ning Y-Y, Jin D-W, Sheng G-P, Harada H, Shi X-Y. Evaluation of the stability of hydrogen production and microbial diversity by anaerobic sludge with chloroform treatment. Renew Energy 2012;38:253–7. http://dx.doi.org/10.1016/j.renene.2011.07.038.
[79] Zhu H, Béland M. Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. Int J Hydrogen Energy 2006;31:1980–8. http://dx.doi.org/10.1016/j.ijhydene.2006.01.019.
[80] Liu FH, Wang SB, Zhang JS, Zhang J, Yan X, Zhou HK, et al. The structure of the bacterial and archaeal community in a biogas digester as revealed by denaturing gradient gel electrophoresis and 16S rDNA sequencing analysis. J Appl Microbiol 2009;106:952–66. http://dx.doi.org/10.1111/j.1365-2672.2008.04064.x.
[81] Dunne J. The network structure of food webs. In: Pascual M, Dunne J, editors. Ecol. Networks Link. Struct. to Dyn. Food Webs. 1st ed. Santa Fe Institute Studies on the Sciences of Complexity; 2005. p. 27–92.
[82] Romanuk TN, Zhou Y, Brose U, Berlow EL, Williams RJ, Martinez ND. Predicting invasion success in complex ecological networks. Philos Trans R Soc B Biol Sci 2009;364:1743–54. http://dx.doi.org/10.1098/rstb.2008.0286.
[83] Heleno R, Devoto M, Pocock M. Connectance of species interaction networks and conservation value: is it any good to be well connected? Ecol Indic 2012;14:7–10. http://dx.doi.org/10.1016/j.ecolind.2011.06.032.
[84] Lurgi M, Galiana N, López BC, Joppa LN, Montoya JM. Network complexity and species traits mediate the effects of biological invasions on dynamic food webs. Front Ecol Evol 2014;2:1–11. http://dx.doi.org/10.3389/fevo.2014.00036.
[85] Rodrigues LR, Duncan AB, Clemente SH, Moya-Laraño J, Magalhães S. Integrating competition for food, hosts, or mates via experimental evolution. Trends Ecol Evol 2015;31:158–70. http://dx.doi.org/10.1016/j.tree.2015.12.011.
[86] Mallon CA, Elsas JD, Salles JF. Microbial invasions: the process, patterns, and mechanisms. Trends Microbiol 2015;23:719–29. http://dx.doi.org/10.1016/j.tim.2015.07.013.
[87] Mallon C, Poly F, Le Roux X, Marring I, van Elsas JD, Salles JF. Resource pulses can alleviate the biodiversity – invasion relationship in soil microbial communities. Ecology 2015;96:915–26. http://dx.doi.org/10.1890/14-1001.1.
[88] Shea K, Chesson P. Community ecology theory as a framework for biological invasions. Trends Ecol Evol 2002;17:170–6. http://dx.doi.org/10.1016/s0169-5347(02)02495-3.
[89] Hung C-H, Cheng C-H, Guan D-W, Wang S-T, Hsu S-C, Liang C-M, et al. Interactions between *Clostridium sp*. and other facultative anaerobes in a self-formed granular sludge hydrogen-producing bioreactor. Int J Hydrogen Energy 2011;36:8704–11. http://dx.doi.org/10.1016/j.ijhydene.2010.06.010.
[90] Hung CH, Lee KS, Cheng LH, Huang YH, Lin PJ, Chang JS. Quantitative analysis of a high-rate hydrogen-producing microbial community in anaerobic agitated granular sludge bed bioreactors using glucose as substrate. Appl Microbiol Biotechnol 2007;75:693–701. http://dx.doi.org/10.1007/s00253-007-0854-7.
[91] Cheng CH, Hsu SC, Wu CH, Chang PW, Lin CY, Hung CH. Quantitative analysis of microorganism composition in a pilot-scale fermentative biohydrogen production system. Int J Hydrogen Energy 2011;36:14153–61. http://dx.doi.org/10.1016/j.ijhydene.2011.05.023.
[92] Morris BEL, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. FEMS Microbiol Rev 2013;37:384–406. http://dx.doi.org/10.1111/1574-6976.12019.
[93] Ercolini D. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. J Microbiol Methods 2004;56:297–314. http://dx.doi.org/10.1016/j.mimet.2003.11.006.
[94] Leite AMO, Mayo B, Rachid CTCC, Peixoto RS, Silva JT, Paschoalin VMF, et al. Assessment of the microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. Food Microbiol 2012;31:215–21. http://dx.doi.org/10.1016/j.fm.2012.03.011.
[95] Samarajeewa AD, Hammad A, Masson L, Khan IUH, Scroggins R, Beaudette LA. Comparative assessment of next-generation sequencing, denaturing gradient gel electrophoresis, clonal restriction fragment length polymorphism and cloning-sequencing as methods for characterizing commercial microbial consortia. J Microbiol Methods 2015;108:103–11. http://dx.doi.org/10.1016/j.mimet.2014.11.013.
[96] Cleary DFR, Smalla K, Mendonça-Hagler LCS, Gomes NCM. Assessment of variation in bacterial composition among microhabitats in a mangrove environment using DGGE fingerprints and barcoded pyrosequencing.

PLoS One 2012;7:1–8. http://dx.doi.org/10.1371/journal.pone.0029380.

[97] Piterina AV, Pembroke JT. Use of PCR-DGGE based molecular methods to analyse microbial community diversity and stability during the thermophilic stages of an ATAD wastewater sludge treatment process as an aid to performance monitoring. ISRN Biotechnol 2013;2013:1–13. http://dx.doi.org/10.5402/2013/162645.

[98] Hong H, Pruden A, Reardon KF. Comparison of CE-SSCP and DGGE for monitoring a complex microbial community remediating mine drainage. J Microbiol Methods 2007;69:52–64. http://dx.doi.org/10.1016/j.mimet.2006.11.016.

[99] Speksnijder AGCL, Kowalchuk GA, Jong SDE, Kline E, Stephen JR, Laanbroek HJ, et al. Microvariation artificats introduced by PCR and Cloning of closely related 16S rRNA gene sequences. Appl Environ Microbiol 2001;67:469–72. http://dx.doi.org/10.1128/AEM.67.1.469.

[100] Neilson JW, Jordan FL, Maier RM. Analysis of artifacts suggests DGGE should not be used for quantitative diversity analysis. J Microbiol Methods 2014;92:256–63. http://dx.doi.org/10.1016/j.mimet.2012.12.021.Analysis.

Capítulo 2: Propuesta de marco de trabajo para el estudio de consorcios productores de hidrógeno con un enfoque ecológico.

Prefacio

Para poder predecir el funcionamiento de comunidades microbianas y su respuesta ante perturbaciones (p. Ej. Cambio climático) es indispensable entender los mecanismos que subyacen sus dinámicas poblacionales. En el caso de la fermentación obscura, su escalamiento depende de la optimización de la estabilidad funcional de los consorcios asociados a los biorreactores. Sin embargo, hasta ahora, la investigación de los consorcios de fermentación obscura se ha realizado, principalmente, sólo desde el enfoque de la ingeniería de los biorreactores.

El proceso de la investigación científica no es lineal. En cambio, puede verse como un proceso iterativo en el que nuevas ideas y experimentos se basan en información generada previamente. En ese contexto, la investigación sobre ecología de sistemas microbianos puede verse beneficiada de una sistematización y estandarización de los análisis usados. Esta aproximación puede proveer de profundidad y detalle al entendimiento mecanismos básicos como las interacciones y la relación diversidad-función. Los resultados de estudios sistemáticos permiten generar nuevas hipótesis a probar experimentalmente y avanzar de manera sostenida hacia la comprensión de mecanismos subyacentes a los patrones observados. En este capítulo se propone un marco de trabajo para el estudio integral de consorcios microbianos usando como modelo los consorcios de fermentación obscura. Nuestro enfoque permite el análisis del comportamiento general de la función y diversidad microbianas de los reactores como primer paso. Posteriormente, nuestro enfoque se orienta en inferir la relación entre la diversidad y la función del sistema.

Usando un experimento típico de fermentación obscura como caso de estudio, mostramos la importancia de construir hipótesis mecanísticas usando evidencias de distintas fuentes. Por una parte, proponemos que el uso de análisis correlativos y multivariados reducen la complejidad de la información y permiten la detección de organismos y patrones relevantes. El uso de análisis que incluyan la biología de los organismos, como la modelación metabólica, son propuestos como complemento a los análisis correlativos y permiten generar hipótesis acerca de los mecanismos y consecuencias de las interacciones microbianas. Adicionalmente, nuestro marco de trabajo hace hincapié en llevar a cabo diseños experimentales que incluyan contrastes y replicación, lo que aporta robustez estadística a las observaciones y conclusiones. Estos diseños permiten evaluar de forma rigurosa el efecto de las condiciones de cultivo, contrastar con experimentos control el efecto de los

tratamientos aplicados y llevar a cabo la modelación estadística de los procesos biológicos. Finalmente, propone el uso de herramientas computacionales con base estadística para el estudio de los mecanismos ecológicos y evolutivos (como las interacciones bióticas) que producen las dinámicas observadas, lo que permite la construcción de nuevas hipótesis y el diseño de experimentos que lleven a avances sólidos en el conocimiento de mecanismos subyacentes. También se ejemplifica la utilidad que los resultados obtenidos por este marco de trabajo en la planeación de nuevos experimentos por medio de la retroalimentación entre estudios. Los resultados obtenidos por medio de nuestro marco de trabajo son utilizados como caso de estudio para demostrar la utilidad de la sistematización y robustez experimental en la investigación científica circular necesaria para descifrar los complejos mecanismos que gobiernan las comunidades microbianas.

INTERNATIONAL JOURNAL OF HYDROGEN ENERGY 45 (2020) 31706–31718

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/he

A framework for integrating functional and microbial data: The case of dark fermentation H2 production

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HIGHLIGHTS

- Metabolic and microbial data integration reveals bioreactors ecological mechanisms.
- Bioreactors ecological basis can be investigated by statistical modeling approach.
- Antagonistic interactions due to culture conditions harm bioreactors performance.
- Informed hypotheses narrow the study of microbially-mediated bioreactors function.

ARTICLE INFO

Article history:
Received 19 June 2020
Received in revised form
10 August 2020
Accepted 21 August 2020
Available online 17 September 2020

Keywords:
Modeling
Microbial ecology
Microbial interactions
Microbial diversity
Ecological networks

ABSTRACT

Research in dark fermentation has emphasized an engineering view of bioreactors overlooking its integration with the ecological dynamics of the process. Ecological dynamics driven by microbial interactions impact bioreactors performance and are critical to understanding the mechanistic bases of dark fermentation. We propose an analytical framework consisting of "*description*", "*association*" and "*modeling*" phases or inquiry levels to elucidate potential ecological interactions in bioreactor-associated microbial consortia. Each phase deepens into obtaining mechanistic insights of the microbial behavior in bioreactors. We present a study case to illustrate each step of the framework, their limitations and opportunities. We show that analytical frameworks integrating metabolic data and microbial diversity give insights into the ecological processes underlying the performance of dark fermentation bioreactors and can guide future research through informed hypotheses.

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https://doi.org/10.1016/j.ijhydene.2020.08.189

Introduction

Increased greenhouse gases emissions and dependency on fossil fuels motivate research on sustainable and clean energy production based on renewable resources [1]. Dark fermentation by microbial consortia is a promising avenue for transforming residual carbohydrate-rich biomass into low-contaminant hydrogen, volatile fatty acids, and alcohols [2], and it has advantages over other strategies of biological hydrogen production (e.g. photofermentation, microbial fuel cells) that require specialized equipment and substrates. However, the performance and stability of dark fermentation remain as issues and continue to be investigated to successfully scale up the process [3,4].

While few studies have incorporated the ecological aspect of dark fermentation (i.e. [5,6]), most research has emphasized the role of operating conditions on the performance of bioreactors mostly overlooking the underlying ecological dynamics of the microbial consortia. Despite this, studying the species richness and abundance of microbial consortia associated with dark fermentation and their relationship to bioreactors performance has provided a broad catalog of the most common species, their potential metabolism and functions [7]. The few studies that have investigated microbial interactions in hydrogen-producing bioreactors have provided hypotheses regarding the role of some bacterial groups in critical functions such as hydrogen production, oxygen consumption, or granule formation [6,8–11]. With this basic knowledge, some efforts have been made to integrate data qualitatively [12] and quantitatively [13], but the lack of statistically sound experimental designs and an integrated analytical approach for studying microbial interactions have hindered the emergence of clear patterns or informed hypotheses in terms of the diversity-function relationship and microbial interactions.

Statistical modeling has been considered a powerful approach to gain a mechanistic understanding of complex microbial systems (as the ones bioreactors represent [14,15]). The strength of statistical modeling relies heavily on the replication of experiments that allow data collection for analytical integration regarding microbial diversity, metabolites production-consumption, and biotic interactions [16,17]. Two exemplary cases that have followed this approach include the study of the human gut and the frog skin microbiomes. In particular, given the information provided by statistical modeling of complex microbial communities, it has been possible to identify the microbial community structure characteristic of healthy and sick individuals and the species of symbiotic bacteria that might prevent or treat diseases in the skin of frogs and the human gut microbiome [18–20]. Moreover, statistical modeling approaches coupled with the power of high-throughput sequencing and genomics represent an unprecedented opportunity to advance in the understanding of the mechanistic basis of the functioning of microbial consortia, from bioreactors to microbiomes.

In the present work, we propose an analytical framework to investigate the microbial ecology of consortia in dark fermentation reactors through the statistical modeling of multivariate data (i.e. microbial diversity, metabolite composition, reactor performance) (Fig. 1). In particular, the framework intends for and in-depth understanding of the ecological basis of dark fermentation bioreactors through a sequence of questions with increasing depth of inquiry: (1) How microbial populations diversity change during bioreactor operation? (level 1: *description*) (2) How microbial and metabolite dynamics are related during bioreactor operation? (level 2: *association*) and (3) Given bioreactor culture conditions, which interactions among existing microbial groups can be inferred? (level 3: *modeling*). To illustrate the framework, and as a proof of concept, we present as a study case a hydrogen-producing bioreactor operated during 45 days in which data of performance, microbial diversity, and metabolite production/concentration were analyzed. We aim to contribute to bringing attention to the importance of experimental design and ecology-driven analyses to advance in the understanding of dark fermentation consortia. From our study case, which represents a standard bioreactor setting, we exemplify the potential of our framework and discuss the limitations of current experimental settings and analyses. For instance, we describe how interactions between bacteria could occur with a potential effect on performance, given the operation conditions of the analyzed bioreactor. Also, we make evident how lack of replicates, controls, and limited sampling points negatively affect the robustness of conclusions. Our results also provide hypotheses on the performance to be specifically tested in further experiments, illustrating the power of an analytical framework that can systematically narrow down the study of the ecological basis of bioreactors performance.

A framework for integrating functional and microbial data

The proposed framework allows for the analytical integration of operating parameters (performance) and microbial data at three inquiry/analytical levels depending on the objectives of the experiment and data availability. The framework is thought in the context of the complex interactions of microbial communities which difficult a predictive control of bioreactors. The framework inquiry levels are built upon statistical analyses, some of which, are commonly carried out in microbial ecology research, but are presented here in a streamlined fashion to facilitate its application and the identification of patterns from multivariate data. Using this approach, it is possible not only to build a catalog of microbial diversity but also to identify candidate microbial groups related to particular functions, their interactions, and their impact on the performance of bioreactors. Beyond the conceptual presentation of the framework, we also describe the complementary analytical methodologies of the different inquiry levels of the framework, their requirements, and applications in helping to disentangle ecological relationships in dark fermentation consortia. We also identify the limitations and possible adjustments of experimental designs that can improve research on dark fermentation microbial consortia. Finally, it is important to notice that this framework is thought of as a roadmap of an iterative process in which models are refined through experimental validation.

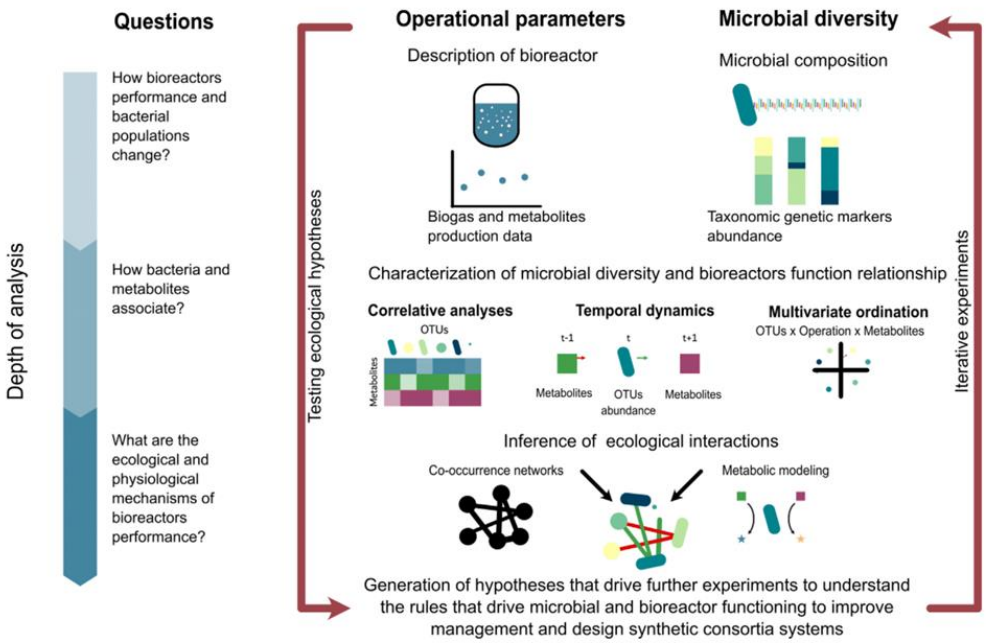

Fig. 1 – An analytical framework for studying ecological interactions in dark fermentation bioreactors. To improve bioreactors control, it is important to investigate the composition, function, and interactions of the microbial populations involved in the fermentation processes. For that purpose, this framework aims to organize conceptually the questions and their reach (left-hand side), depending on the type of information and analyses conducted (right-hand side). In particular, we envision at least three stages or depth of the approach: "*description*", "*association*" and "*modeling*". Overall, the approach is intended to deepen the knowledge about the relationship between microorganisms and bioreactors operating conditions to determine the interactions occurring within it. In each stage, the depth of analysis is increased so that each level of analysis contributes to complementary evidence. The first stage (description) seeks to answer how the operating parameters and microbial populations change. In this stage, changes in key metabolites and species can be identified and followed in the subsequent steps. The second stage (association) responds to how bacterial populations and bioreactors performance relate to each other. For achieving this, statistical analyses are carried out to get specific evidence of biotic interactions. First, it is necessary to correlate the concentration of metabolites to bacterial abundance. Second, by incorporating temporal dynamics, cross-feeding, or inhibition by metabolites are investigated. Additionally, multivariate analyses can be performed when the sample size is enough. In the third stage (modeling), statistical and metabolic modeling come into hand. Using statistical tools like correlation and similarity indices co-presence and co-exclusions between bacteria can be found. These analyses can be performed in software such as CoNet [21] or MENAP [22]. Statistical models of interactions can be revised using flux balance analyses (FBA). FBA takes as input information from the chemical composition of the culture medium and the chemical reactions that bacteria can perform as described in their genomes to predict which species can help or inhibit the growth of others [23]. FBA methodologies are implemented in software like MMinte [24] or BacArena [25]. We propose that through this approach it is possible to integrate all lines of evidence and generate hypotheses that can be iteratively tested in new experiments.

The process of developing mechanistic models of dark fermentation should involve the collection of detailed microbial and function data. Importantly, these models should link biological and engineering information for a comprehensive or system-level analysis. To conduct comprehensive analyses that lead to robust conclusions, reproducibility, and predictability, adjustments to experimental designs may be needed. One major and critical adjustment of experimental designs for microbial ecology research in bioreactors is replication and hypothesis-driven investigation of microbial communities. The benefits of replicated experimental design have been deeply discussed in microbial ecology research

[26–28] but it has not been implemented successfully in bioprocess research except for few cases [29,30]. For example, replicates are of utmost importance as it is not possible to capture the biological complexity of microbial consortia and their response to perturbations in a single trial [31]. In turn, not replicating makes it impossible to distinguish if the observed responses are due to physiological processes or culture conditions. Likewise, replicated controls are indispensable to determine the effect of treatments on microbial diversity and function. These considerations will enable to step up from observational and catalog studies to predictive models.

Level 1: Description of microbial diversity and bioreactor performance

Given the variation of microbial consortia composition (diversity) and bioreactors performance, it is common to present a relatively simple summary of the functional and microbial diversity dynamics. Microbial diversity descriptive analyses are commonly presented in dark fermentation studies [32,33]. Bar graphs are the most frequently used visualization tool since they allow immediate identification of the differences in the relative abundance of microbial groups across samples [34]. To complement bar graphs, heatmaps built with standardized abundance data can be presented. Heatmaps are graphical representations of relative abundance tables and allow to observe changes in composition regardless of the overall abundance of taxonomic groups [35]. Additionally, heatmaps allow for clustering visualization in dendrograms in which rows and columns are reordered as the result of hierarchical clustering analysis. The dendrogram arrangement displays clusters of species (rows) with similar abundance and samples (columns) with similar bacterial composition [34]. Also, dendrograms show the hierarchical relationship between species and samples (reflecting the beta diversity, or differences in composition across samples) [36]. It is noteworthy that no function-diversity relationships should be interpreted from these graphs alone since they give no statistical support. Bar graphs and heatmaps can be constructed in QIIME 2 [37], R, Python, or using web platforms such as MicrobiomeAnalyst [38] or VAMPS [36]. Further analyses for revealing associations between the culture environment and microbial diversity can be explored with other approaches, some of which are described in the following sections.

Level 2: Association of microbial diversity with bioreactor performance

In microbial consortia, metabolites are consumed and produced by microbial groups simultaneously as the basis for their ecological interactions [39,40]. Thus, identifying the bacteria that are involved in the production/consumption of certain metabolites is ecologically meaningful. Information on metabolite dynamics, in parallel with microbial diversity data, can help to determine which bacterial groups and their metabolism may impact bioreactors performance. Nonetheless, the analytical coupling of metabolite-microbial diversity data should take into account at least three biological aspects of microbial physiology: (1) transformation of metabolites is not linear and cross-feeding and feedbacks occur [41,42]; (2) several species might participate in more than one function (or metabolite transformation [39]); and (3) even when microbial species are closely related or from well-known microbial groups, they might contribute differently to bioreactors performance due to variations in response to conditions or metabolic capabilities [12].

Inference of diversity-function relationships

By using microbial abundance and metabolites concentration data it is possible to make statistical inferences about which microbial groups participate in the transformations of which metabolites. The main idea is to statistically relate changes in microbial species abundance with changes in metabolites concentration. Additionally, it is also relevant to observe patterns at a larger scale (i.e. sub-groups of species).

To determine associations between the microbial composition and bioreactor performance (metabolic data), correlative analyses (e.g. Spearman or Pearson correlations) have frequently been used in natural, medical and biotechnological systems [43–46]. As with diversity data, heatmaps are usually useful to summarize and visualize the correlations between the microbial abundance and the concentration of the metabolites [34]. Another common approach to investigate the relationship between microbial groups and function is ecological ordination. In ecological ordination, all variables (metabolites) are reduced to new synthetic axes in which objects (species) are displayed [47]. The reduction of variables and plotting of species result in ordination plots that allow to identify if species have a positive or negative relationship with metabolites. Several ordination analyses are available depending on the type of data (mainly species abundance and metabolites concentration tables). The most common are the principal components analysis (PCA), the redundancy analysis (RDA), the non-metric multidimensional scaling (NMDS), and the canonical correspondence analysis (CCA) [47,48]. These analyses can be carried out in R using the vegan package [49] or using the MicrobiomeAnalyst [38] and VAMPS [36] web platforms. Extensive reviews of these analyses have been published previously [47,48], and the decision on the best one depends on the characteristics of the study and type of data. For example, PCAs are typically performed with microbial abundance data only to observe similarity between sample composition [50]. In contrast, CCAs can help to include operational parameters (even categorical) to further relate diversity with bioreactors function [50].

Considering that microbial communities are dynamic and exhibit complex responses to their environment [51] it can be ecologically informative to analyze temporal changes of the correlations between microbial diversity and metabolites composition. To this end, temporal correlations are calculated using data values with temporal lags. This method can help to infer the pathways of metabolites transformations. These correlations are calculated between the different bacterial abundance and metabolites concentrations at different temporal lags (e.g. metabolites concentration at day t-1 with bacterial abundance at day t). Python packages pandas v0.23.4 [52] and sklearn v0.19.2 [53]. Finally, from the correlation matrix between metabolites at time t-1 vs. microbes at time t and microbes at time t vs. metabolites at time t+1, a temporal

correlation network can be generated using the appropriate software such as Cytoscape v3.6 [54].

Level 3: Inference of ecological interactions

In the final inquiry level of the framework, analyses reach the deepest level to generate mechanistic hypothesis of microbial interactions. In this level, the goal is to generate an ecological network hypothesis of the biotic interactions between the microorganisms in the bioreactor. The networks represent hypotheses of the potential interactions, whether positive or negative and can be analyzed mathematically to identify properties of their constituents and the global structure of the microbial consortia [55]. Once analyzed, ecological networks can help to visualize potential mechanics of microbial consortia behavior by bringing attention to key species or interactions with possible consequences on performance. Further, the described interactions are then candidates to be tested in experiments that investigate their effect on performance and their response to culture conditions.

Co-occurrence networks

The most accessible method for reconstructing ecological networks is through the correlation of microbial abundance data [56]. The results are co-occurrence/co-exclusion networks in which nodes represent species and edges the correlation between their abundance as a proxy of potential interactions [57]. Edges can signify positive interactions if species tend to occur simultaneously or negative interactions if species tend to exclude themselves across samples [57]. The main advantage of inferring co-occurrence networks is that the only requirement is the abundance data of consensus 16s rRNA gene sequences for each microbial species. Most commonly, Spearman, Pearson, or Kendall correlations are used followed by calculation of p-values [56]. Other common filters to narrow down the statistically valid correlations is to determine a minimum value of the correlation index and only keeping interactions supported by two or three distinct correlation methods [58]. The CoNet plugin v1.1.1 [21] for Cytoscape v3.6 [54] allows to streamline the process of inferring co-occurrence networks using several correlation indices and allowing to filter valid correlations by p-value calculation and randomization routines. As with other correlation-based methods described before, the sample size must be as large as possible (especially with increasing diversity), replication is one way to increase sample size, as this reduces the false positive rate and increases analyses robustness [21].

Metabolic modeling

Metabolic modeling consists of the mathematical interpretation of the bacterial-mediated biochemical transformations required for the uptake and excretion of nutrients and metabolites obtained from annotated bacterial genomes [59]. Metabolic models include all the known chemical reactions a bacterium is capable to perform and the associated genes (thus called genome-scale models [15]). With the information of the species genomes, two individual genomic models can be coupled to infer potential interactions of species based on the products and substrates (i.e. pairwise metabolic models) [23,24]. Metabolic modeling can be incorporated into the network inference process by using only statistically significant abundance correlations in the generation of pairwise models. The MMinte software [24] can carry out the identification of the metabolic models by using 16sRNA gene sequences of the identified bacteria (e.g. obtained from level 1) and downloading the metabolic models of their closets relatives from the ModelSeed database [60]. Then, the same software can carry out the coupling process by the calculation of the pairwise models from a user-given list of significant co-occurrences and evaluation of the potential interactions using flux balance analysis. The output from MMinte is a list of potential interactions that can be plotted using Cytoscape software [54]. Other options for performing metabolic modeling of microbial interactions include The Microbiome Modeling Toolbox [61] BacArena [25], and COMETS [62] which can also take into account spatial and temporal scales.

Results and discussion

Case of study

The experimental data sets employed in this study are from Muñoz-Páez et al. [63]. Briefly, experiments were performed in an anaerobic sequential bioreactor operated during 45 days at 35 °C with controlled pH at 5.5. The substrate and inoculum consisted of an acid-dilute lignocellulosic hydrolyzate and a heat-treated anaerobic granular sludge, respectively. Phases of hydrogen production were delimited by periods of increasing substrate concentrations until hydrogen production fell (Fig. 2). An overview of metabolites produced during bioreactors operations is presented in Fig. S1. Detailed information about the experiments and operating conditions is available in Muñoz-Páez et al. [63].

***Level 1**: Description of microbial diversity* and bioreactor performance

The first inquiry level of the proposed analytical framework includes the description of microbial diversity and the performance of hydrogen-producing bioreactors. As we previously mentioned, bar graphs are the most frequently used visualization tool of microbial diversity, since they allow immediate identification of the differences in the relative abundance of microbial groups across samples. However, bar graphs are not well suited to observe changes in fine detail. For instance, bar graphs tend to be cluttered when several species are present and only changes in highly abundant species are evident [34]. Sub-dominant species can be keystone species in the sense that they contribute to nutrient cycling and increasing carbon flow, thus of relevance in the functioning of consortia [17,64,65]. In our study case, we used a complementary heatmap of standardized species abundances to inspect patterns of changes in all the identified bacterial species, independently of their relative abundance.

The implementation of both bar and heatmap graphs as diversity analyses and visualization tools in our study case is presented in Fig. 3. From the bar graphs, it can be observed that microbial composition was highly variable in the course of the experiment (Fig. 3a). Besides, with the heatmap analysis, it is possible to appreciate more detail regarding diversity

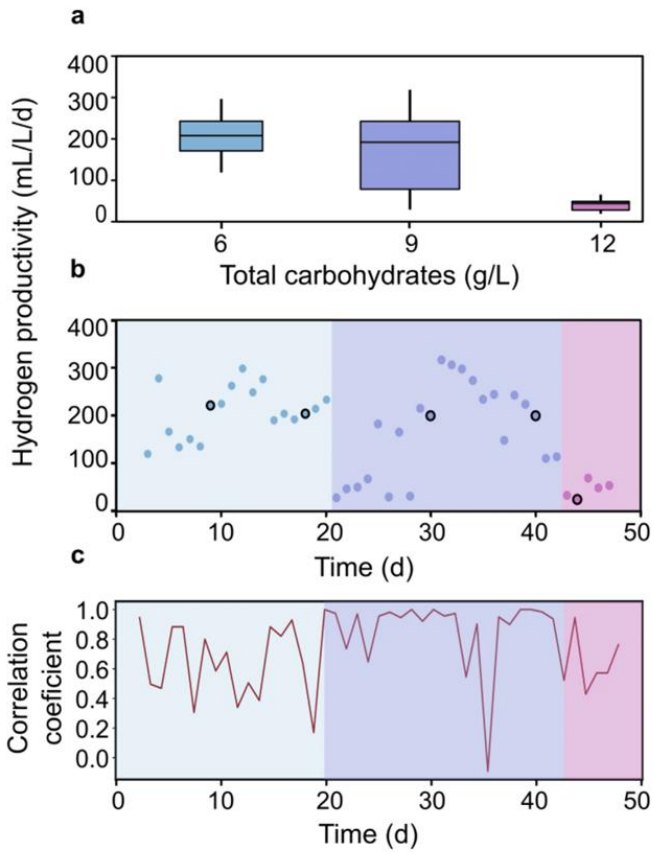

Fig. 2 – Bioreactor performance metrics throughout three operation phases (phase 1, from days 1–19 = 6.3 g L^{-1}; phase 2, from days 20–42 = 9 g L^{-1}; and phase 3 from days 43–49 = 11.4 g L^{-1}) a. Mean hydrogen productivity per productivity phase; b. Daily hydrogen productivity, black circles represent microbial diversity sampling time points; c. Spearman correlation coefficients of metabolites concentrations between day t and day t+1 during the experiment. Higher correlation coefficients indicate similar metabolites between days, while lower correlation coefficients indicate sudden shifts in metabolites composition.

information. For instance, at least two clusters of bacterial species were observed in the analysis, based on species abundance changes along five sampling points (Fig. 3b). These clusters represent subsets of the bacterial diversity that coexisted in a given time with potential positive interactions within each cluster, and potential negative interactions between clusters. In this regard, heatmaps can guide the interpretation of further analyses by looking more closely into the clusters. For instance, in our study case, two clusters of bacterial species are formed and persist for the duration of the experiment with distinctive abundance patterns. During the first 28 days of the experiment, a cluster composed of several *Clostridium* species (*e.g. Clostridium oryzae* and *Clostridium autoethanogenum*), *Ethalonigenens spp.*, *Pseudomonas panacis*, and *Lactobacillus buchneri* were most abundant. After the first 28 days of the experiment, the other cluster, composed of species such as *Mitsuokella multacida*, *Clostridium beijerinckii*, and *Yokenella regensburgei* increased their abundance. Although the composition of clusters from this analysis can be taken into account in further analysis, caution should be taken in the ecological interpretation of the clusters given that, in this analysis, the clustering of species has no statistical support.

If analyses are not continued from this point, the result is a catalog of microbial diversity that cannot fully support ecological hypothesis [66]. Proper ecological interpretation requires the integration of metabolic data and microbial composition since microbial diversity and function are the results of complex interactions among bacteria and with their environment [67]. Finally, the main caveat to be noticed here is that lack of replication of the experiment poses limitations

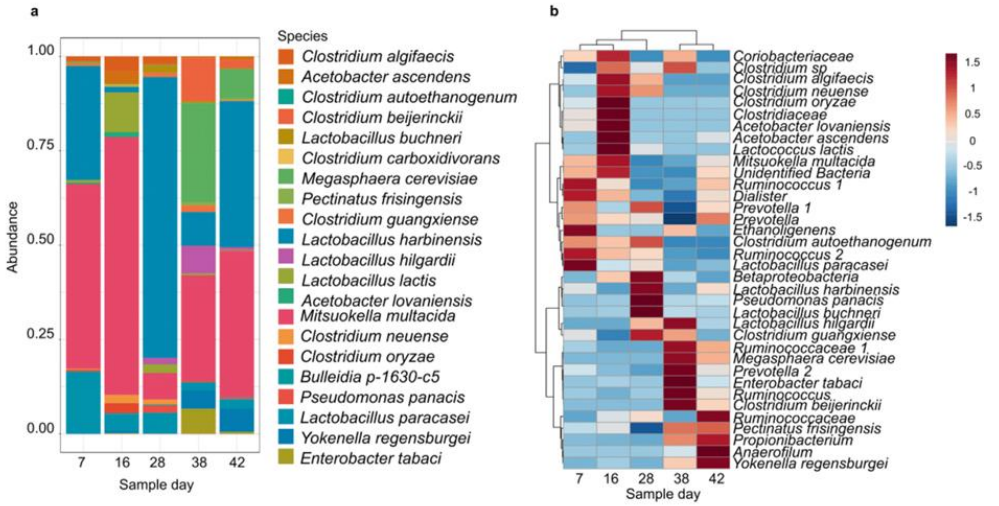

Fig. 3 – Bacterial OTU diversity and composition at species level (97% 16sRNA gene similarity) per sampling point (days 9, 18, 30, 41, and 44). a. Relative abundance of OTUs identified at species level at different sampling points. b. Clustered heat map of bacterial OTUs relative abundance (i.e. normalized) per sampling point.

to the robustness of statements regarding actual differences across samples throughout the experiment.

Level 2: Association of microbial diversity with bioreactor performance

The integrated analysis of microbial and metabolite data aims to understand how bacterial composition relates to bioreactors performance or function ("*association*" in Fig. 1). It has been largely known that variations in the microbial composition of communities affect the function of ecosystems [68]. Despite cutting edge advances in dark fermentation research and some progress in the exploration of the microbial ecology of bioreactors performance (i.e. [32,69,70]), limited understanding still exists on the microbial consortia structure and its relationship with performance. As mentioned earlier, longitudinal studies where temporal scale is considered will allow to follow up on the transformations metabolites undergo and the associated microorganisms to such transformations.

Avoiding a "black box" approach when studying bioreactors will contribute to improve bioreactors management. For example, it is necessary to understand which organisms participate as primary degraders of complex substrates, which use smaller hydrolyzed compounds, and which might help to recycle scarce nutrients. In our study case, we identified at least two clusters of bacteria based on correlation patterns with metabolites (Fig. 4a). Notably, bacteria of these clusters correlate, in opposite directions, with hydrogen production. The first cluster includes bacteria like *P. panacis*, *L. buchneri*, *C. oryzae*, and *C. autoethanogenum*, and the second cluster includes bacteria like *C. beijerinckii and Y. regensburgei*, which coincides with the clustering based on relative abundance similarity of the samples (Fig. 3b). Notably, this associations were similar in lagged correlations (e.g. *C. autoethanogenum positive with hydrogen and butyric acid and Y. regensburgei negative with hydrogen and butyric acid*). Two, not mutually exclusive processes could have contributed to the observed clustering of bacterial species that would require further investigation. One possible explanation could be differential responses to the changes in culture conditions (Fig. 2). Another, reason could be that interactions between members of each cluster might have contributed to distinct growth or inhibition of the bacteria. Evidence of the second potential explanation is that species of one the first cluster positively correlate with hydrogen, butyric acid, and propionic acid representing a H_2 producing subgroup [71]. In this first cluster, *Clostridium* spp. are common hydrogen producers [72], but bacteria like *Lactobacillus* have a less clear role. *Lactobacillus* might be competitive [5] or even cooperative [73] to hydrogen producers. In contrast, bacteria of the second cluster positively correlate with carbohydrate and acetate consumption which might indicate a competitive advantage in nutrient uptake.

In the experiment presented here as an example, correlative analyses serve as a first exploration of the microbial diversity-function relationship. Although no consensus exists when determining the minimum sample size for statistical tests, in this and similar experiments, the robustness of the analyses can be compromised if no replication is considered [28]. We acknowledge that logistical and financial reasons might prohibit the implementation of replicates in bioreactor research. Yet, it is important to consider the use of all the possible replicates per treatment to assess the statistical significance of correlation and ordination analyses (and their complementary ANOVAs or PERMANOVAs) which are negatively affected by low sample sizes [21,74,75]. Lastly, neither of

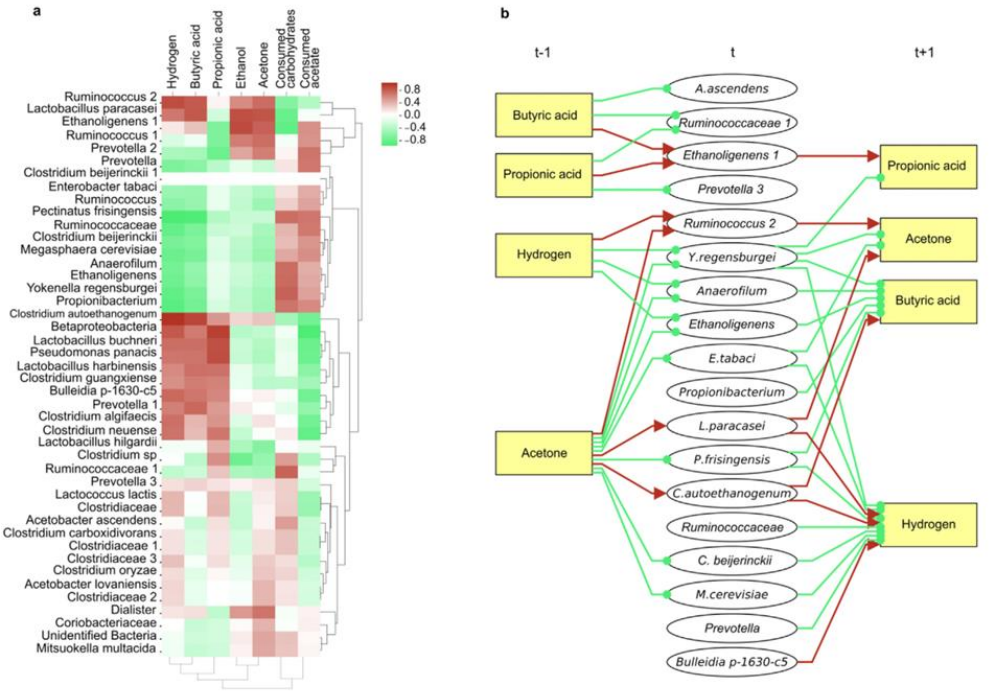

Fig. 4 – Correlations between microbial composition and reactor metabolites a. Correlations between OTUs abundance and metabolites concentration at the same sampling time. b. Temporal correlations between microbial abundance and metabolites concentration before (t-1) and after (t+1) sampling for diversity analysis (blue lines with open squares indicate negative correlations; pink lines with open arrows indicate positive correlations). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

the described methods explain causality nor the mechanism underling the phenomena, but they serve as a guide to detecting potentially relevant interactions [66]. Follow-up experiments in the same system (i.e. culture conditions, substrates, and inocula) must be performed to test the generated hypotheses. Additional metabolites (non-performance related such as bacteriocines) and molecular techniques (as proteomics) can provide direct information on the underlying metabolic mechanisms of interactions, as has been done in anaerobic reactor research [43].

Level 3: Inference of ecological interactions

To date, approaches in dark fermentation research tend to focus on bacteria as standalone entities ignoring the interactions that occur among different microbial groups. When reconstructing ecological networks, species co-occurrence or mutual exclusions, which are statistical inferences, could be the result of actual ecological interactions, either positive or negative respectively [57]. Nonetheless, further integration of information is needed to support ecological hypotheses from network inferences, such as metabolic data that can help to identify potential metabolic pathways involved [76]. Genomic-derived metabolic modeling can add information to further support detailed hypotheses of microbial interactions, the involved metabolites, ecological functions, and niche of key bacteria in microbial consortia [76]. Statistical support and multivariate information help in formulating data-informed hypotheses to be tested and refined in an iterative process embedded in the presented framework.

In the "modeling" step of our proposed analytical framework, microbial and metabolite data allow inference of ecological interactions through network analyses and metabolic modeling. In the bioreactor described here, the inferred network showed that negative (or antagonistic, competitive) interactions are more prevalent than cooperative interactions, as has been observed in other systems where competition is prevalent due to nutrient or spatial limitation [23,77] which might be also the case for bioreactors fed with easily fermentable substrates [78]. Some of the inferred relationships between bacteria based only in their abundance and

Fig. 5 – Metabolic modeling network inference of ecological interactions at a species level. Inference is based on species pairs growth in a hypothetical culture medium with composition akin to the bioreactor's experimental conditions (Supplementary text 1). Colors of edges represent the types of interactions as follows: yellow, parasitism; red, competition and green, commensalism. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

metabolite concentrations are coherent with the results of the modeled ecological network. For example, *C. autoethanogenum* and *C. beijerinckii* were found to have a potentially competitive interaction (Fig. 5). This competitive behavior might be the result of their overlap in metabolic capabilities, as has been already observed for different *Clostridium* species and their overlap in certain energy sources [79] and metabolically similar bacteria [80]. Likewise, the predicted negative relationship between *C. autoethanogenum* and *Y. regensburgei* could be subject to further investigation as no previous reports on it have been made. In contrast, *L. buchneri* and *P. panacis* were found to potentially engage in a cooperative interaction (Fig. 5), with previous information on the coexistence of these genera [13]. Furthermore, the commensal interactions detected by the ecological model (Fig. 5) tend to occur between bacteria that are in the same cluster of bacterial-metabolite correlations (*i.e. P. panacis* and *L. buchneri*; Fig. 4a), while parasitic (negative) interactions occur between bacteria in different clusters. It is not clear what would be the functional consequences at the bioreactor scale of cooperative interactions between non-hydrogen producers on bioreactors performance.

Network inference and metabolic modeling are complementary analyses, allowing us to have ecological hypotheses of the observed patterns of co-occurrence and co-exclusion. Thus, to couple metabolic modeling (or other metabolic inferences) to network analysis is strongly advised, since correlative analyses (network inference) are limited in reflecting actual biological interactions [81–84]. Additionally, since statistical methods are the first step in network reconstruction, a replicated experimental design indispensable to obtain trustworthy results [21]. An extensive review of additional methods for ecological networks reconstruction and their requirements can be consulted elsewhere [85]. Independently of the method used for networks reconstruction, an important point to consider is that biological information to generate mechanistic models used in interactions description is still scarce and an interdisciplinary effort between experiments and models is required [15]. Strain-level information has been suggested as necessary given the genotypic and phenotypic heterogeneity in same-species bacterial populations [86–89].

Conclusions

Studying bioreactors from an ecological point of view is key to understand the microbially mediated processes, thus necessary to improve their control and performance. To gain mechanistic insights into the ecological interactions that are the basis of bioreactors behavior it is necessary to integrate the data of their performance with their associated microbial diversity. Here, we described a conceptual and analytical framework to investigate the ecological basis of dark fermentation bioreactors as tightly interconnected populations of bacteria. This framework allows to identify species, and their interactions, that might represent examples of ecological dynamics with impact in bioreactors function. The presented framework relies heavily on statistical analysis and modeling, which requires to design experiments with appropriate replication and controls for robust ecological inferences. Finally, the conceptual basis of the framework emphasizes the value of generating statistically sound hypotheses and test them experimentally in an iterative and robust process to advance into mechanistic knowledge on the ecological basis of dark fermentation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Marcelo Navarro-Díaz is a doctoral student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and was supported by CONACYT (scholarship no. 598321). Partial financial support of this work was received from the "Fondo de Sustentabilidad Energética SENER-CONACYT (Mexico)", Gaseous Biofuels Cluster, No. Grant: 247006.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijhydene.2020.08.189.

REFERENCES

[1] Sharma S, Ghoshal SK. Hydrogen the future transportation fuel: from production to applications. Renew Sustain Energy Rev 2015;43:1151–8. https://doi.org/10.1016/j.rser.2014.11.093.

[2] Ghimire A, Frunzo L, Pirozzi F, Trably E, Escudie R, Lens PNL, et al. A review on dark fermentative biohydrogen production from organic biomass: process parameters and use of by-products. Appl Energy 2015;144:73–95. https://doi.org/10.1016/j.apenergy.2015.01.045.

[3] Elbeshbishy E, Dhar BR, Nakhla G, Lee HS. A critical review on inhibition of dark biohydrogen fermentation. Renew Sustain Energy Rev 2017. https://doi.org/10.1016/j.rser.2017.05.075.

[4] Castelló E, Nunes Ferraz-Junior AD, Andreani C, del P Anzola-Rojas M, Borzacconi L, Buitrón G, et al. Stability problems in the hydrogen production by dark fermentation: possible causes and solutions. Renew Sustain Energy Rev 2020;119. https://doi.org/10.1016/j.rser.2019.109602.

[5] Park JH, Kim DH, Kim SH, Yoon JJ, Park HD. Effect of substrate concentration on the competition between *Clostridium* and *Lactobacillus* during biohydrogen production. Int J Hydrogen Energy 2017;43:11460–9. https://doi.org/10.1016/j.ijhydene.2017.08.150.

[6] Santiago SG, Trably E, Latrille E, Buitrón G, Moreno-Andrade I. The hydraulic retention time influences the abundance of *Enterobacter*, *Clostridium* and *Lactobacillus* during the hydrogen production from food waste. Lett Appl Microbiol 2019;69:138–47. https://doi.org/10.1111/lam.13191.

[7] Wang S, Zhang T, Bao M, Su H, Xu P. Microbial production of hydrogen by mixed culture technologies: a review. Biotechnol J 2020;15:1–8. https://doi.org/10.1002/biot.201900297.

[8] Hernández C, Alamilla-Ortiz ZL, Escalante AE, Navarro-Díaz M, Carrillo-Reyes J, Moreno-Andrade I, et al. Heat-shock treatment applied to inocula for H 2 production decreases microbial diversities, interspecific interactions and performance using cellulose as substrate. Int J Hydrogen Energy 2019. https://doi.org/10.1016/j.ijhydene.2019.03.124.

[9] Hung CH, Lee KS, Cheng LH, Huang YH, Lin PJ, Chang JS. Quantitative analysis of a high-rate hydrogen-producing microbial community in anaerobic agitated granular sludge bed bioreactors using glucose as substrate. Appl Microbiol Biotechnol 2007;75:693–701. https://doi.org/10.1007/s00253-007-0854-7.

[10] Arimi MM, Knodel J, Kiprop A, Namango SS, Zhang Y, Geissen SU. Strategies for improvement of biohydrogen production from organic-rich wastewater: a review. Biomass Bioenergy 2015;75:101–18. https://doi.org/10.1016/j.biombioe.2015.02.011.

[11] Bundhoo MAZ, Mohee R, Hassan MA. Effects of pre-treatment technologies on dark fermentative biohydrogen production: a review. J Environ Manag 2015;157:20–48. https://doi.org/10.1016/j.jenvman.2015.04.006.

[12] Cabrol L, Marone A, Tapia-Venegas E, Steyer JP, Ruiz-Filippi G, Trably E. Microbial ecology of fermentative hydrogen producing bioprocesses: useful insights for driving the ecosystem function. FEMS Microbiol Rev 2017;41:158–81. https://doi.org/10.1093/femsre/fuw043.

[13] Navarro-Díaz M, Valdez-Vazquez I, Escalante AE. Ecological perspectives of hydrogen fermentation by microbial consortia: what we have learned and the way forward. Int J Hydrogen Energy 2016;1. https://doi.org/10.1016/j.ijhydene.2016.08.027. 0–11.

[14] O'Malley MA, Travisano M, Velicer GJ, Bolker JA. How do microbial populations and communities function as model systems? Q Rev Biol 2015;90:269–93. https://doi.org/10.1086/682588.

[15] Zaccaria M, Dedrick S, Momeni B. Modeling microbial communities: a call for collaboration between experimentalists and theorists. Processes 2017;5:1–19. https://doi.org/10.3390/pr5040053.

[16] Röttjers L, Faust K. Can we predict keystones? Nat Rev Microbiol 2019;17:193. https://doi.org/10.1038/s41579-018-0132-y.

[17] Banerjee S, Schlaeppi K, van der Heijden MGA. Keystone taxa as drivers of microbiome structure and functioning. Nat Rev Microbiol 2018;16:567–76. https://doi.org/10.1038/s41579-018-0024-1.

[18] Rebollar EA, Antwis RE, Becker MH, Belden LK, Bletz MC, Brucker RM, et al. Using "omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. Front Microbiol 2016;7:68. https://doi.org/10.3389/fmicb.2016.00068.

[19] Vrancken G, Gregory AC, Huys GRB, Faust K, Raes J. Synthetic ecology of the human gut microbiota. Nat Rev Microbiol 2019;17:754–63. https://doi.org/10.1038/s41579-019-0264-8.

[20] Rebollar EA, Bridges T, Hughey MC, Medina D, Belden LK, Harris RN. Integrating the role of antifungal bacteria into skin symbiotic communities of three Neotropical frog species. ISME J 2019;13:1763–75. https://doi.org/10.1038/s41396-019-0388-x.

[21] Faust K, Raes J. CoNet app: inference of biological association networks using Cytoscape. F1000Research 2016;5:1519. https://doi.org/10.12688/f1000research.9050.2.

[22] Deng Y, Jiang YH, Yang Y, He Z, Luo F, Zhou J. Molecular ecological network analyses. BMC Bioinf 2012;13. https://doi.org/10.1186/1471-2105-13-113.

[23] Bauer E, Thiele I. From network analysis to functional metabolic modeling of the human gut microbiota. mSystems 2018. https://doi.org/10.1128/mSystems.00209-17.

[24] Mendes-Soares H, Mundy M, Soares LM, Chia N. MMinte: an application for predicting metabolic interactions among the microbial species in a community. BMC Bioinf 2016;17:1–10. https://doi.org/10.1186/s12859-016-1230-3.

[25] Bauer E, Zimmermann J, Baldini F, Thiele I, Kaleta C. BacArena: individual-based metabolic modeling of heterogeneous microbes in complex communities. PLoS

Comput Biol 2017;13. https://doi.org/10.1371/journal.pcbi.1005544. e1005544.
[26] Celiker H, Gore J. Clustering in community structure across replicate ecosystems following a long-term bacterial evolution experiment. Nat Commun 2014;5:1–8. https://doi.org/10.1038/ncomms5643.
[27] Hekstra DR, Leibler S. Contingency and statistical laws in replicate microbial closed ecosystems. Cell 2012;149:1164–73. https://doi.org/10.1016/j.cell.2012.03.040.
[28] Knight R, Jansson J, Field D, Fierer N, Desai N, Fuhrman JA, et al. Unlocking the potential of metagenomics through replicated experimental design. Nat Biotechnol 2012;30:513–20. https://doi.org/10.1038/nbt.2235.
[29] Vanwonterghem I, Jensen PD, Dennis PG, Hugenholtz P, Rabaey K, Tyson GW. Deterministic processes guide long-term synchronised population dynamics in replicate anaerobic digesters. ISME J 2014;8:2015–28. https://doi.org/10.1038/ismej.2014.50.
[30] Liébana R, Modin O, Persson F, Szabó E, Hermansson M, Wilén BM. Combined deterministic and stochastic processes control microbial succession in replicate granular biofilm reactors. Environ Sci Technol 2019;53:4912–21. https://doi.org/10.1021/acs.est.8b06669.
[31] Prosser JI. Replicate or lie. Environ Microbiol 2010;12:1806–10. https://doi.org/10.1111/j.1462-2920.2010.02201.x.
[32] Im S, Lee MK, Yun YM, Cho SK, Kim DH. Effect of storage time and temperature on hydrogen fermentation of food waste. Int J Hydrogen Energy 2020;45:3769–75. https://doi.org/10.1016/j.ijhydene.2019.06.215.
[33] Castelló E, Braga L, Fuentes L, Etchebehere C. Possible causes for the instability in the H2 production from cheese whey in a CSTR. Int J Hydrogen Energy 2018;43:2654–65. https://doi.org/10.1016/j.ijhydene.2017.12.104.
[34] Sudarikov K, Tyakht A, Alexeev D. Methods for the metagenomic data visualization and analysis. Curr Issues Mol Biol 2017;24:37–58. https://doi.org/10.21775/cimb.024.037.
[35] Hugerth LW, Andersson AF. Analysing microbial community composition through amplicon sequencing: from sampling to hypothesis testing. Front Microbiol 2017;8:1–22. https://doi.org/10.3389/fmicb.2017.01561.
[36] Huse SM, Mark Welch DB, Voorhis A, Shipunova A, Morrison HG, Eren AM, et al. VAMPS: a website for visualization and analysis of microbial population structures. BMC Bioinf 2014;15. https://doi.org/10.1186/1471-2105-15-41.
[37] Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 2019;37:852–7. https://doi.org/10.1038/s41587-019-0209-9.
[38] Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nat Protoc 2020;15:799–821. https://doi.org/10.1038/s41596-019-0264-1.
[39] Louca S, Polz MF, Mazel F, Albright MBN, Huber JA, O'Connor MI, et al. Function and functional redundancy in microbial systems. Nat Ecol Evol 2018;2. https://doi.org/10.1038/s41559-018-0519-1.
[40] Flemming H-C, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol 2016;14:563–75. https://doi.org/10.1038/nrmicro.2016.94.
[41] Estrela S, Brown SP. Metabolic and demographic feedbacks shape the emergent spatial structure and function of microbial communities. PLoS Comput Biol 2013;9. https://doi.org/10.1371/journal.pcbi.1003398.
[42] Cavaliere M, Feng S, Soyer OS, Jiménez JI. Cooperation in microbial communities and their biotechnological applications. Environ Microbiol 2017;19:2949–63. https://doi.org/10.1111/1462-2920.13767.
[43] Vanwonterghem I, Jensen PD, Ho DP, Batstone DJ, Tyson GW. Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Curr Opin Biotechnol 2014;27:55–64. https://doi.org/10.1016/j.copbio.2013.11.004.
[44] Shu D, He Y, Yue H, Wang Q. Microbial structures and community functions of anaerobic sludge in six full-scale wastewater treatment plants as revealed by 454 high-throughput pyrosequencing. Bioresour Technol 2015;186:163–72. https://doi.org/10.1016/j.biortech.2015.03.072.
[45] Weiss S, Van Treuren W, Lozupone C, Faust K, Friedman J, Deng Y, et al. Correlation detection strategies in microbial data sets vary widely in sensitivity and precision. ISME J 2016;10:1669–81. https://doi.org/10.1038/ismej.2015.235.
[46] Galand PE, Pereira O, Hochart C, Auguet JC, Debroas D. A strong link between marine microbial community composition and function challenges the idea of functional redundancy. ISME J 2018;12:2470–8. https://doi.org/10.1038/s41396-018-0158-1.
[47] Paliy O, Shankar V. Application of multivariate statistical techniques in microbial ecology. Mol Ecol 2016;25:1032–57. https://doi.org/10.1111/mec.13536.
[48] Buttigieg PL, Ramette A. A guide to statistical analysis in microbial ecology: a community-focused, living review of multivariate data analyses. FEMS Microbiol Ecol 2014;90:543–50. https://doi.org/10.1111/1574-6941.12437.
[49] Oksanen J, Blanchet F, Kindt R, Legendre P. Vegan: community ecology package. R package version 2.0-10. 2013.
[50] Ramette A. Multivariate analyses in microbial ecology. FEMS Microbiol Ecol 2007;62:142–60. https://doi.org/10.1111/j.1574-6941.2007.00375.x.
[51] Gonze D, Coyte KZ, Lahti L, Faust K. Microbial communities as dynamical systems. Curr Opin Microbiol 2018;44:41–9. https://doi.org/10.1016/j.mib.2018.07.004.
[52] McKinney W. Data structures for statistical computing in Python. In: Proc 9th Python sci conf (SCIPY 2010); 2010. p. 51–6. https://doi.org/10.1016/S0168-0102(02)00204-3. 1697900.
[53] Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, et al. Scikit-learn: machine learning in Python. J Mach Learing Res 2011;12:2825–30. https://doi.org/10.1007/s13398-014-0173-7.2.
[54] Shannon P, Markiel A, Owen Ozier, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003:2498–504. https://doi.org/10.1101/gr.1239303.metabolite.
[55] Röttjers L, Faust K. From hairballs to hypotheses–biological insights from microbial networks. FEMS Microbiol Rev 2018;42:761–80. https://doi.org/10.1093/femsre/fuy030.
[56] Faust K, Raes J. Microbial interactions: from networks to models. Nat Rev Microbiol 2012;10:538–50. https://doi.org/10.1038/nrmicro2832.
[57] Faust K, Sathirapongsasuti JF, Izard J, Segate N, Gevers D, Raes J, et al. Microbial co-occurrence relationships in the human microbiome. PLoS Comput Biol 2012;8. https://doi.org/10.1371/journal.pcbi.1002606.
[58] Wang H, Wei Z, Mei L, Gu J, Yin S, Faust K, et al. Combined use of network inference tools identifies ecologically meaningful bacterial associations in a paddy soil. Soil Biol Biochem 2017;105:227–35. https://doi.org/10.1016/j.soilbio.2016.11.029.

[59] Edwards JS, Covert M, Palsson B. Metabolic modelling of microbes: the flux-balance approach. Environ Microbiol 2002;4:133–40. https://doi.org/10.1046/j.1462-2920.2002.00282.x.
[60] Henry CS, DeJongh M, Best AA, Frybarger PM, Linsay B, Stevens RL. High-throughput generation, optimization and analysis of genome-scale metabolic models. Nat Biotechnol 2010;28:977–82. https://doi.org/10.1038/nbt.1672.
[61] Baldini F, Heinken A, Heirendt L, Magnusdottir S, Fleming RMT, Thiele I. The microbiome modeling toolbox: from microbial interactions to personalized microbial communities. Bioinformatics 2019;35:2332–4. https://doi.org/10.1093/bioinformatics/bty941.
[62] Harcombe WR, Riehl WJ, Dukovski I, Granger BR, Betts A, Lang AH, et al. Metabolic resource allocation in individual microbes determines ecosystem interactions and spatial dynamics. Cell Rep 2014;7:1104–15. https://doi.org/10.1016/j.celrep.2014.03.070.
[63] Muñoz-Páez KM, Alvarado-Michi EL, Moreno-Andrade I, Buitrón G, Valdez-Vazquez I. Comparison of suspended and granular cell anaerobic bioreactors for hydrogen production from acid agave bagasse hydrolyzates. Int J Hydrogen Energy 2020;45:275–85. https://doi.org/10.1016/j.ijhydene.2019.10.232.
[64] Rafrafi Y, Trably E, Hamelin J, Latrille E, Meynial-Salles I, Benomar S, et al. Sub-dominant bacteria as keystone species in microbial communities producing bio-hydrogen. Int J Hydrogen Energy 2013;38:4975–85. https://doi.org/10.1016/j.ijhydene.2013.02.008.
[65] Lynch MDJ, Neufeld JD. Ecology and exploration of the rare biosphere. Nat Rev Microbiol 2015;13:217–29. https://doi.org/10.1038/nrmicro3400.
[66] Prosser JI. Putting science back into microbial ecology: a question of approach. Philos Trans R Soc B Biol Sci 2020;375. https://doi.org/10.1098/rstb.2019.0240.
[67] Freilich MA, Wieters E, Broitman BR, Marquet PA, Navarrete SA. Species co-occurrence networks: can they reveal trophic and non-trophic interactions in ecological communities? Ecology 2018;99:690–9. https://doi.org/10.1002/ecy.2142.
[68] Heather E, Reed JBHM. Testing the functional significance of microbial composition in natural communities. FEMS Microbiol Ecol 2007;90:441–51. https://doi.org/10.1111/j.1574-6941.2007.00386.x.
[69] Toledo-Alarcón J, Cabrol L, Jeison D, Trably E, Steyer JP, Tapia-Venegas E. Impact of the microbial inoculum source on pre-treatment efficiency for fermentative H2 production from glycerol. Int J Hydrogen Energy 2020;45:1597–607. https://doi.org/10.1016/j.ijhydene.2019.11.113.
[70] Li H, Song W, Cheng J, Ding L, Zhou J, Li YY. Effects of harvest month on biochemical composition of alligator weed for biohydrogen and biomethane cogeneration: identifying critical variations in microbial communities. Int J Hydrogen Energy 2020;45:4161–73. https://doi.org/10.1016/j.ijhydene.2019.11.208.
[71] Palomo-Briones R, Razo-Flores E, Bernet N, Trably E. Dark-fermentative biohydrogen pathways and microbial networks in continuous stirred tank reactors: novel insights on their control. Appl Energy 2017;198:77–87. https://doi.org/10.1016/j.apenergy.2017.04.051.
[72] Valdez-Vazquez I, Poggi-Varaldo HM. Hydrogen production by fermentative consortia. Renew Sustain Energy Rev 2009;13:1000–13. https://doi.org/10.1016/j.rser.2008.03.003.
[73] García-Depraect O, Valdez-Vázquez I, Rene ER, Gómez-Romero J, López-López A, León-Becerril E. Lactate- and acetate-based biohydrogen production through dark co-fermentation of tequila vinasse and nixtamalization wastewater: metabolic and microbial community dynamics. Bioresour Technol 2019;282:236–44. https://doi.org/10.1016/j.biortech.2019.02.100.
[74] Penton CR, Gupta VVSR, Yu J, Tiedje JM. Size matters: assessing optimum soil sample size for fungal and bacterial community structure analyses using high throughput sequencing of rRNA gene amplicons. Front Microbiol 2016;7:1–11. https://doi.org/10.3389/fmicb.2016.00824.
[75] Forcino FL, Leighton LR, Twerdy P, Cahill JF. Reexamining sample size requirements for multivariate, abundance-based community research: when resources are limited, the research does not have to be. PloS One 2015;10:1–18. https://doi.org/10.1371/journal.pone.0128379.
[76] Muller EEL, Faust K, Widder S, Herold M, Martínez Arbas S, Wilmes P. Using metabolic networks to resolve ecological properties of microbiomes. Curr Opin Struct Biol 2018;8:73–80. https://doi.org/10.1016/j.coisb.2017.12.004.
[77] Feng K, Zhang Z, Cai W, Liu W, Xu M, Yin H, et al. Biodiversity and species competition regulate the resilience of microbial biofilm community. Mol Ecol 2017;26:6170–82. https://doi.org/10.1111/mec.14356.
[78] Chung WSF, Walker AW, Vermeiren J, Sheridan PO, Bosscher D, Garcia-Campayo V, et al. Impact of carbohydrate substrate complexity on the diversity of the human colonic microbiota. FEMS Microbiol Ecol 2018;95:1–13. https://doi.org/10.1093/femsec/fiy201.
[79] Laanbroek HJ, Smit AJ, Nulend GK, Veldkamp H. Competition for L-glutamate between specialised and versatile *Clostridium* species. Arch Microbiol 1979;120:61–6. https://doi.org/10.1007/BF00413275.
[80] Russel J, Røder HL, Madsen JS, Burmølle M, Sørensen SJ. Antagonism correlates with metabolic similarity in diverse bacteria. Proc Natl Acad Sci U. S.A. 2017. https://doi.org/10.1073/pnas.1706016114.
[81] Antoniewicz MR. A guide to deciphering microbial interactions and metabolic fluxes in microbiome communities. Curr Opin Biotechnol 2020;64:230–7. https://doi.org/10.1016/j.copbio.2020.07.001.
[82] Hirano H, Takemoto K. Difficulty in inferring microbial community structure based on co-occurrence network approaches. BMC Bioinf 2019;20:1–14. https://doi.org/10.1186/s12859-019-2915-1.
[83] Mainali KP, Bewick S, Thielen P, Mehoke T, Breitwieser FP, Paudel S, et al. Statistical analysis of co-occurrence patterns in microbial presence-absence datasets. PloS One 2017;12:1–21. https://doi.org/10.1371/journal.pone.0187132.
[84] Carr A, Diener C, Baliga NS, Gibbons SM. Use and abuse of correlation analyses in microbial ecology. ISME J 2019;13:2647–55. https://doi.org/10.1038/s41396-019-0459-z.
[85] Layeghifard M, Hwang DM, Guttman DS. Disentangling interactions in the microbiome: a network perspective. Trends Microbiol 2017. https://doi.org/10.1016/j.tim.2016.11.008.
[86] Larkin AA, Martiny AC. Microdiversity shapes the traits, niche space, and biogeography of microbial taxa. Environ Microbiol Rep 2017;9:55–70. https://doi.org/10.1111/1758-2229.12523.
[87] Philippot L, Andersson SGE, Battin TJ, Prosser JI, Schimel JP, Whitman WB, et al. The ecological coherence of high bacterial taxonomic ranks. Nat Rev Microbiol 2010;8:523–9. https://doi.org/10.1038/nrmicro2367.
[88] García-García N, Tamames J, Linz AM, Pedrós-Alió C, Puente-Sánchez F. Microdiversity ensures the maintenance of functional microbial communities under changing

environmental conditions. ISME J 2019;13:2969–83. https://doi.org/10.1038/s41396-019-0487-8.
[89] Jaspers Elke, Overmann Jorg. Ecological significance of microdiversity: identical 16S rRNA gene sequences can be found in bacteria with highly divergent genomes and ecophysiologies. Appl Environ Microbiol Aug 2004;70(8):4831–9. https://doi.org/10.1128/AEM.70.8.4831.

Capítulo 3: Comparación de estructura ecológica de consorcios productores de hidrógeno.

Prefacio

Como se ha explicado en los capítulos anteriores, la diversidad de las comunidades microbianas tiene un gran impacto en su función y estabilidad. Esta diversidad puede variar por razones como perturbaciones ambientales o por razones intrínsecas a las poblaciones y comunidades, como interacciones bióticas o adaptaciones al medio ambiente. En cualquier caso, la diversidad de un consorcio puede conferir robustez por medio de redundancia funcional, influir en la capacidad de resistir invasiones de especies y aumentar la diversidad metabólica del consorcio. Por estas razones y para tener la posibilidad de intervenir para mejorar las respuestas funcionales y ante perturbaciones o invasiones, es necesario analizar las consecuencias de las variaciones en la diversidad en consorcios microbianos.

En el caso particular de los consorcios de fermentación obscura, los pretratamientos y las condiciones de cultivo representan perturbaciones ambientales que tienen el potencial de afectar la diversidad. Esta pérdida de diversidad puede afectar sus dinámicas ecológicas y evolutivas con consecuencias funcionales no estudiadas hasta ahora. En este capítulo presentamos los resultados de un experimento en el que se exploraron las consecuencias que la pérdida de diversidad tiene en la estabilidad y la función de consorcios microbianos de fermentación obscura. Para esto se establecieron biorreactores productores de hidrógeno bajo dos tratamientos a partir de un inóculo común procedente de gránulos de digestión anaerobia. Como se mencionó en el capítulo anterior, consideramos que incluir replicación en el diseño experimental es indispensable para obtener resultados y conclusiones robustos. Debido a esto, utilizamos 12 biorreactores réplica para cada uno de los tratamientos. En el primer tratamiento los biorreactores se inocularon con gránulos de digestión anaerobia bajo condiciones operacionales típicas para la producción de hidrógeno. En el segundo tratamiento el inóculo fue pretratado térmicamente previo al establecimiento de los reactores, los cuales, se mantuvieron bajo las mismas condiciones que los anteriores. Una vez estabilizada la producción de biogás, se realizó una invasión controlada para determinar la invasibilidad de ambos tipos de comunidades.

Se analizó la composición taxonómica (diversidad del gen 16S rRNA) y función de la comunidad microbiana (producción de biogás y ácidos grasos volátiles) a lo largo del tiempo, estableciendo

relaciones estadísticas entre los grupos bacterianos y la producción de metabolitos. Además, se estudió la estructura de diversidad de dichas comunidades al inferir las posibles interacciones ecológicas. Los resultados muestran que los biorreactores tuvieron dinámicas similares entre las réplicas de cada tratamiento, aunque los distintos tratamientos fueron notablemente distintos en sus dinámicas. De forma general, se observó que en ambos tratamientos la producción de biogás aumentó de forma constante hasta su estabilización. En los biorreactores sin pretratamiento (que mostraron mayor diversidad), la producción de biogás aumentó a medida que la composición microbiana cambió hacia el predominio de bacterias productoras de hidrógeno. En este escenario, los procesos deterministas como las interacciones bióticas y la fisiología de las bacterias involucradas parecen haber jugado un papel importante en el ensamblaje y funcionamiento de las comunidades. Además, los biorreactores no pretratados mostraron menos susceptibilidad a la invasión (con el invasor estableciéndose solo en una réplica en los biorreactores no pretratados frente a 6 réplicas invadidas en los reactores pretratados), pero el efecto observado fue mayor ya que las bacterias invasoras lograron establecerse y convertirse en una especie dominante. Este estudio provee una base para el estudio de los mecanismos que dan lugar a la compleja relación entre la diversidad microbiana y la función de comunidades microbianas productoras de hidrógeno. Se han identificado relaciones potenciales entre bacterias y funciones particulares, interacciones ecológicas entre las bacterias de las comunidades con potencial de afectar la función y el efecto de la diversidad en la invasión de los reactores. La investigación futura puede usar esta información para diseñar experimentos que prueben estos resultados y, eventualmente, incorporarlos al diseño de comunidades microbianas productoras de hidrógeno y al manejo de sus condiciones de cultivo.

Variations in microbial diversity affect the stability and function of dark fermentation bioreactors

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Abstract

The relationship between the taxonomic diversity and the function of microbial communities is complex. Specifically, the ecological mechanisms that drive the dynamics of microbial populations and the consequences of these dynamics on functional traits have remained elusive. Among the simplest but natural microbial communities are dark fermentation consortia, a subset of the more diverse and complex microbial communities, anaerobic digestion communities. Dark fermentation consortia have been of interest as they produce biofuels such as hydrogen and different alcohols that can be used as fossil fuels alternatives. However, these hydrogen-producing communities have unresolved instability and low yield issues. We have previously proposed that instability and low yields in dark fermentation communities could be due to reduced diversity that results from aggressive pretreatments of original anaerobic digestion communities. In this work, we used dark fermentation communities to examine experimentally the effect of diversity reduction in functional traits, including stability and microbial interactions. We established two types of treatment, (i) maintaining strict culture conditions that are known to induce hydrogen production and ii) applying a heat-shock treatment known for selecting

hydrogen-producing bacteria, which resulted in two types of communities, high and low diversity. Each treatment consisted of 12 replicates that were transferred to fresh medium daily (during 27 days for the non-treated bioreactors and 60 days for the heat-shock treated bioreactors) Microbial communities of the two treatments were characterized in their function as well as resistance to invasion. Microbial composition was characterized by culture-independent 16S rRNA gene amplicon sequencing. We analyzed microbial community composition and function through time, establishing statistical relationships between bacterial groups and metabolite production. Also, we inferred the potential ecological interactions that might have been established. Results show that the replicate bioreactors for each treatment predictably shifted to a similar composition and increased and stable biogas production. The non-treated bioreactors showed less susceptibility to the invasion (with the invasive bacteria establishing only in one replicate in the non-treated bioreactors vs 6 invaded replicates of the low diversity treatment). However, the effect observed in the non-treated bioreactor replicate where the invader bacteria established was more drastic since the invasive bacteria managed to become dominant.

Introduction

The high taxonomic and metabolic diversity of bacterial groups give rise to complex functional behaviors in microbial communities where even thousands of different bacteria might coexist (Locey and Lennon, 2016; Louca et al., 2018). The relationship between the microbial composition, diversity and their functional outcomes can be difficult to predict and control since these result from complex metabolic and environmental interactions (Escalante et al., 2015; Hays et al., 2015). Also, microbial communities are susceptible to perturbations (i.e. climate change, pollution and nutrient availability) which might change their composition and functioning (Allison and Martiny, 2009). Moreover, the mechanisms behind such changes have been observed to be case-specific (Shade, 2017). It has been suggested that the increase in diversity components (i.e. richness and evenness) leads to higher stability and functional resilience due to functional redundancy, niche complementation and

differential response traits against perturbations, but mixed evidence has been found (Shade et al., 2012). In bioreactors, such as those from dark fermentation and anaerobic digestion, it has been found that consortia with higher alfa-diversity were more resilient to environmental pH disturbances (Feng et al., 2017), while evenness has been found to increase stability by allowing the community more capacity to use a varied array of metabolic pathways (Werner et al., 2011). Similarly, in soil bacterial communities increased diversity was related to increased stability against heat perturbations (Tardy et al., 2014; Xu et al., 2021). In contrast, Wertz et al. found no effect on stability against heat disturbances of decreasing bacterial diversity in bacterial communities of soil (Wertz et al., 2007) and Glasl et al. found that in marine sponges-associated bacterial communities varying levels of microbial diversity did not affect the stability against salinity disturbances (Glasl et al., 2018). Further, not only the identity and abundance of species are important for the stability and function of bacterial communities. For example, biotic interactions are important to determine the assembly and composition of bacterial communities (Pérez-Gutiérrez et al., 2013; Datta et al., 2016; Friedman et al., 2017; Meroz et al., 2021). Besides, these interactions are important in determining the function, stability and invasibility of communities (Ghosh et al., 2016; Kinnunen et al., 2016; Madsen et al., 2018; Ratzke et al., 2020). Since microbial communities carry out important processes like nutrient cycling, substrate degradation and metabolites production, understanding the mechanisms that drive the diversity-function relationship can help in the management of microbial communities, for example, in biotechnological o bioremediation settings (Johns et al., 2016).

Using microbial consortia with reduced complexity can be the first step to understanding the mechanisms that link diversity and function. Simplified microbial consortia (like those used for biofuels production or artificially-assembled consortia) have been used to test ecological hypotheses and propose generalizable ecological theories since they offer controlled environments and easily measurable functions (De Roy et al., 2014; Stenuit and Agathos, 2015; Cairns et al., 2018). Due to the intrinsic characteristics of microbial organisms (i.e. their small size and chemically-mediated

interactions; (Schmidt et al., 2015) the exploration of ecological mechanisms (e.g. biotic interactions) in microbial communities is frequently performed via statistical inference. In this line, to advance the knowledge regarding the precise ecological dynamics of microbial consortia, specifically designed experiments and systematized research can be performed (Navarro-Díaz et al., 2020). Experimental controls, contrasting experimental settings and replication are useful to determine the impact of culture conditions, statistical detection of patterns and robust interpretation of results. In turn, results obtained in this manner can narrow the scope of hypotheses directing further experiments.

Hydrogen-producing microbial consortia represent a good system to study ecological hypotheses since they are low-diverse systems with well-studied measurable functions (Wang et al., 2020). Hydrogen-producing (also known as dark fermentation consortia) are microbial consortia derived from anaerobic digestion communities in which hydrogen-producing bacteria are selected by using aggressive pretreatments and strictly controlling culture conditions (Wang and Yin, 2017). In hydrogen-producing reactors, instability and low yield are still unresolved issues and proposed causes underlie ecological processes like biodiversity loss and competitive interactions (Castelló et al., 2020). It has been known that certain species present in hydrogen-producing consortia have a certain impact on the community function. For instance, *Clostridium* species have been attributed with hydrogen production and degradation of complex substrates and *Bacillus* species with eliminating toxic oxygen (Cabrol et al., 2017). Other bacteria like *Lactobacillus* species have more intriguing roles. Initially depicted as inhibitors of hydrogen producers by the production of bacteriocins, recently, they have been regarded as aiding hydrogen production and participants in detoxification processes (Sikora et al., 2013; Muñoz-Páez et al., 2018). Importantly, most of these functions have not been studied in an ecological context that can provide insights into population dynamics and community-level properties.

In this work, we studied the effect that changes in diversity had on the function and the stability of microbial consortia. For this, we used hydrogen-producing consortia as a model system.

Carrying out two commonly used strategies for achieving hydrogen production, we established two sets of microbial consortia with two levels of diversity from the same inoculum. The first strategy consisted of applying an aggressive heat-shock pretreatment to the inoculum while the second consisted of maintaining specific culture conditions previously reported to allow for hydrogen production. We hypothesize that since diversity determines the function and stability of microbial consortia, each of the two sets of hydrogen-producing bioreactors will not only have varying degrees of diversity but also their long-term behavior and resistance to perturbations.

Methods

Experimental design

To investigate if microbial diversity affects the ecological robustness of dark fermentation microbial consortia, we used lab-scale bioreactors inoculated with anaerobic digestion granules and applied two different treatments to control for levels of diversity that permitted to evaluate the different functional outcomes. The experiment was conducted in two sequential stages, in the first stage we intended to specifically evaluate the effect of diversity on the functional stability of the bioreactors; in the second stage, we challenged the bioreactors with a controlled invasion of *Lactobacillus plantarum* culture. For the first stage, we artificially reduced the diversity of the original inoculum with a heat-shock treatment (Valdez-Vazquez and Poggi-Varaldo, 2009) to further compare the microbial community dynamics and performance of the heat-shock treated microbial communities against the non-treated communities. Twelve replicates per treatment (heat-shock treated and non-treated) were maintained with daily transfers into fresh medium and until biogas production stability was achieved. After biogas production stabilized, for each of the two "diversity treatments" we chose the six replicates with less variance in biogas production to start the second stage of the experiment. The six chosen replicates per diversity treatment were inoculated with a strain of *L. plantarum* that was isolated from a hydrogen-producing reactor anaerobic bioreactor (Pérez-Rangel et al., 2021), the other six were used as non-invasion controls (Figure 1). The second stage (invasion) of the experiment

was terminated when biogas production stabilized in the invaded bioreactors. The functioning or performance of the bioreactors throughout the experiments was determined based on three parameters: daily biogas production, volatile fatty acids (VFAs) concentration and pH. Microbial diversity and composition were determined by culture-independent 16S rDNA amplicon high throughput sequencing.

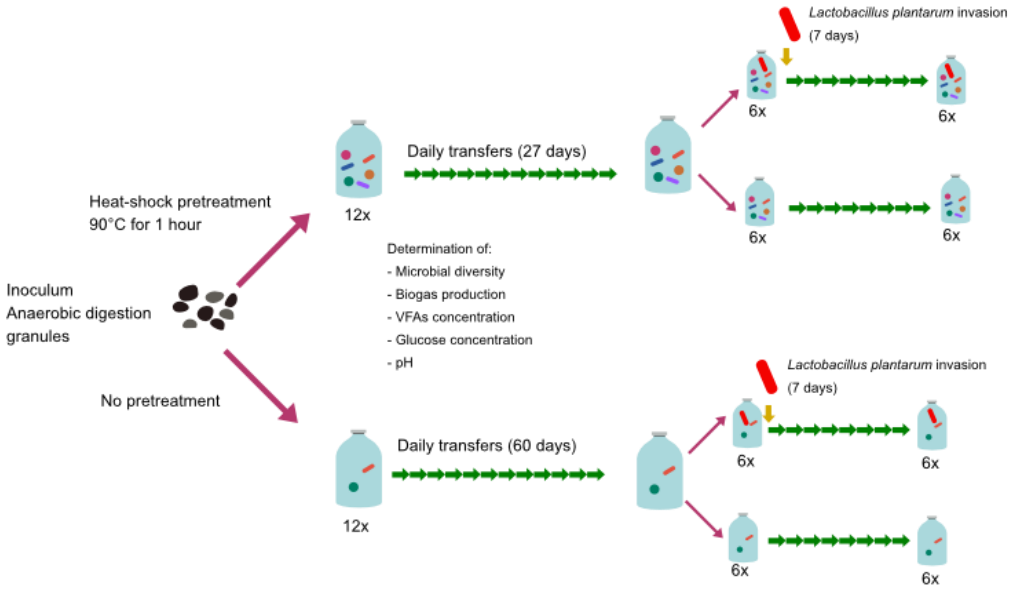

Figure 1. Experimental design. Starting from a single inoculum, we followed two experimental treatments to artificially modify microbial diversity. Each treatment consisted of 12 replicates derived from a single inoculum. To test the stability of reactors in each treatment, we periodically measured the performance of bioreactors until biogas production stabilized. After biogas production stabilization, we challenged the reactors with a controlled invasion and determined the success of the invasive bacteria based on their abundance in the reactors. In the invasion experiment, 6 bioreactors per treatment were selected and inoculated with *Lactobacillus plantarum* simulating a biological invasion, while the remaining 6 bioreactors per treatment were used as controls. After biogas production in invaded bioreactors stabilized, the experiment was terminated. All the data on the diversity and performance of the bioreactors were subject to statistical analyses for formal comparisons of the experimental treatments.

Bioreactors' setup

To compare the effect of diversity on the function and stability of bioreactors, we artificially reduced the diversity of the original inoculum with a heat-shock treatment. The original microbial inoculum consisted of anaerobic digestion granules obtained from a brewery wastewater treatment plant. Before the experiment, 15g of the inoculum was homogenized in 20mL of 1:1 PBS/glycerol solution and frozen at –80°C until use. From this inoculum, the two diversity treatments bioreactors were established as overnight cultures using 20 mL of homogenized inoculum and 40 mL of fresh medium at 37°C. For the low diversity treatment, a heat-shock pretreatment of 90°C for 1h was applied after the overnight culture. Since, after the heat-shock pretreatment, no growth was achieved under aerobic conditions, strictly anaerobic conditions had to be used in this treatment by adding 0.5 g/L of cysteine to the culture medium. Once initial cultures were established for both treatments, they were divided into 12 replicates per treatment. To partition each reactor, 20 mL of the culture were transferred into a new serum bottle with 40 mL of fresh medium. Then, 20mL of the culture in original bioreactor was kept and also 40mL of fresh medium was added. This partition step was repeated 4 times and 12 random replicates were used for each treatment. From that point, every 24 hours, 20mL of the culture of each replicate culture was reinoculated into 40mL of fresh medium. After each transfer, pH was adjusted to 6. The medium had the following composition per liter: glucose (5 g/L), urea (0.65 g/L), K_2HPO_4 (0.25 g/L), $MgCl_2{\cdot}6H_2O$ (0.376 g/L), $FeSO_4{\cdot}\ 7H_2O$ (0.1 g/L), $CoCl_2{\cdot}6H_2O$ (0.0025 g/L), $MnCl_2{\cdot}4H_2O$ (0.0025 g/L), KI (0.0025 g/L), $NiCl_2{\cdot}6H_2O$ (0.0005 g/L), $ZnCl_2$ (0.0005 mg/L) and yeast extract (0.5 g/L); modified from (Mizuno et al., 2000). For all the cultures we used 100 mL serum bottles as lab-scale semi-continuous bioreactors with a working volume of 60mL. Bioreactors were incubated at 37°C and shaken at 50 rpm. Transfers were performed until biogas production stabilized.

Invasion test

After the initial stabilization period, each of the 6 most similar replicates in terms of biogas production for each treatment was divided again and one of the derived bioreactors for each replicate was

inoculated with a strain of *Lactobacillus plantarum* previously isolated from a hydrogen-producing bioreactor (Pérez-Rangel et al., 2021). The strain of *L. plantarum* was grown overnight in LB medium (Sigma Aldrich, USA) and then acclimatized for 3 days in the same medium and conditions that were used in our bioreactors. The invasion was performed at a 10% ratio. For this, we followed the same protocol for daily transfers, but instead of just 40mL of fresh medium, we used 34 mL of fresh medium and 6 mL of the overnight culture of *L. plantarum*.

Bioreactors´ performance characterization

To characterize the performance of the bioreactors, we measured three parameters: daily biogas production, volatile fatty acids (VFAs) concentration and pH. Daily biogas production was measured in an inverted graduated cylinder using the water displacement technique and, to ensure an exact measurement, we prevented CO_2 absorption by using a 5N HCL solution (pH < 2; (Boshagh and Rostami, 2020). Volatile fatty acids (VFAS) concentration was determined using an SRI 8610-00 gas chromatograph equipped with a Porapak Q column and using Helium at a 30mL/min flow rate as carrying gas. Temperatures for the injector, column and oven were 150C°, 50C° and 50C° respectively.

Characterization of microbial diversity

At the same time points in which chemical analyses were performed, we obtained samples for microbial composition analyses. Samples were stored at -80°C until processing. DNA was extracted using the Quick-DNA Fungal/Bacterial DNA Microprep Kit (Zymo Research, USA) following the manufacturer's instructions. Sequencing library preparation was performed targeting the V4 region of the 16S SSU rRNA gene using primers 515F: GTGYCAGCMGCCGCGGTAA (Parada et al., 2016) and 806R: GGACTACNVGGGTWTCTAAT (Apprill et al., 2015). Amplifications were performed in 25 µL reactions with the Platinum Hot Start PCR Master Mix (Invitrogen, USA) 0.5 µL of 10 mM primers, and 1 µL of DNA template. Negative controls were included for each PCR reaction to ensure that no contamination occurred. Later, amplification products were visualized using electrophoresis gels. Finally, the

sequencing reaction was performed using the Miseq 300PE platform at the Macrogen facilities (Korea) using Reagent V3 and Phix at 30% and with a red length of 250PE.

16S rRNA sequence data processing

De-multiplexing, filtering, and chimera check. Raw sequences (10,540,264) were demultiplexed using the *demuxbyname* script from the BBTools suite (Bushnell B., sourceforge.net/projects/bbmap/). Demultiplexed sequences were next processed using QIIME 2 v2021.2 (Bolyen et al., 2019). Sequence quality control (denoising and chimera check) and inference of amplicon sequence variants (ASVs) was performed using the *dada2 denoise-paired* command. We modified the *trim left f*, *trun len f*, *trim left r*, and *trun len r* parameters to remove 30bp from both extremes of the reverse and forward sequences. After quality control, 7,893,591 (74.89%) sequences were retained. The raw data (paired-end files) were deposited in the NCBI sequence read archive (SRA) with the accession number: PRJNA814689.

ASV assignment. After quality filtering, samples were rarefied to the average of reads per sample (27,082 reads) using the *qiime feature-table rarefy* command with the *--p-with-replacement* argument in QIIME 2 v 2021.2. Taxonomy was assigned to 115 ASVs using BLAST (Altschul et al., 1990) against the NCBI's 16S ribosomal RNA refseq database (O'Leary et al., 2016). Finally, to improve pattern recognition and reduce technical variability, ASVs with less than 20 reads in 20% of the samples were filtered out (retaining 24 ASVs) of the following analyses based on the filtering criteria published previously (filtering ASVs with less m = 20 counts in at least k = 20 samples, where m and k were selected as 0.1% of the minimum sample library size which was ~20,000) (Cao et al., 2021).

Statistical analyses

To assess the variability of microbial function over time, for each treatment, we plotted the 12 bioreactors function data (biogas production and AGVs). Similarly, to assess the variation of the average microbial abundance over time we used stacked bar plots of the average abundance of each

ASV for the 12 replicates. To investigate how low-abundance ASVs behaved over time the average abundance was plotted in a heatmap, normalizing ASVs abundance across samples. To analyze the relationship between microbial composition and bioreactors we followed two approaches. First, we performed Spearman correlations between ASVs abundance counts and metabolites concentrations and biogas production obtained using the *cor* function in the *stats* R package v4.0.5. We represented the correlations as heatmaps for visual interpretation. Then, we used the *cca* function in the vegan R package v.2.5-7 (Oksanen et al., 2013) to perform a canonical correspondence analysis (CCA) using the abundance counts of ASVs and normalized metabolites concentrations and biogas production. All analyses were performed using R v.4.0.5 (R Core Team, 2016). Finally, to infer the potential interactions occurring between the members of the hydrogen-producing consortia, we used the Lotka-Volterra-based network inference approach implemented in MetaMIS v1.02 (Shaw et al., 2016). For each sampling time point, the average of the non-normalized microbial abundance of the 12 replicates was computed and used to infer ecological networks. The networks were visualized using Cytoscape 3.0 (Shannon et al., 2003).

Results

Bioreactor's function

For both treatments (the treated and the non-treated treatments), hydrogen production showed increased production and stability over time being the main difference between treatments that the low diversity treatment showed a higher biogas production overall (Figure 2). For the non-treated bioreactors, initially, hydrogen production was approximately 250 mL/L, and at the end of the experiment, it reached 800 mL/L. Also, biogas production stabilized towards the end of the experiment, such that all replicates showed similar productivity both on contiguous days and between replicates. For the treated bioreactors, high and stable biogas production was quickly achieved and showed a smaller increase since the 12 replicates produced around 1500 mL/L of biogas stabilizing at the end of the experiment around 1700mL/L. Importantly, intra-replicate variation was higher in the

non-treated than in the treated bioreactors (Figure 2). In both treatments, the concentration of VFAs remained mostly stable over time (Figure 3). The prevalence of acetic and butyric acids indicates that hydrogen production was occurring although gas composition was not measured. Overall, all replicates from both treatments stabilized in terms of biogas production several weeks after the start of the experiment and reached different production peaks depending on the treatment. In the non-treated bioreactors, counterintuitively, biogas production and concentration of VFAs did not show any parallel pattern of change. In this sense, biogas production showed a steady increase while VFAs did not show the same pattern of change. In the treated bioreactors, VFAs and biogas showed the same pattern of stable production.

The effect of the invasion by *L. plantarum* on the function of the bioreactors was different in each of the treatments. In both experiments, invasion induced a reduction in biogas production observed 24 hours after the inoculation (Figure 2). After the initial reduction, biogas production increased again to the levels observed before the invasion. However, in the non-treated bioreactors, one replicate did not recover from invasion and stopped producing biogas. Also, for the heat-treated bioreactors, biogas production recovery was faster than in the non-treated consortia. The concentration of the analyzed VFAs was not seemingly affected by the invasion in either treatment (Figure 3).

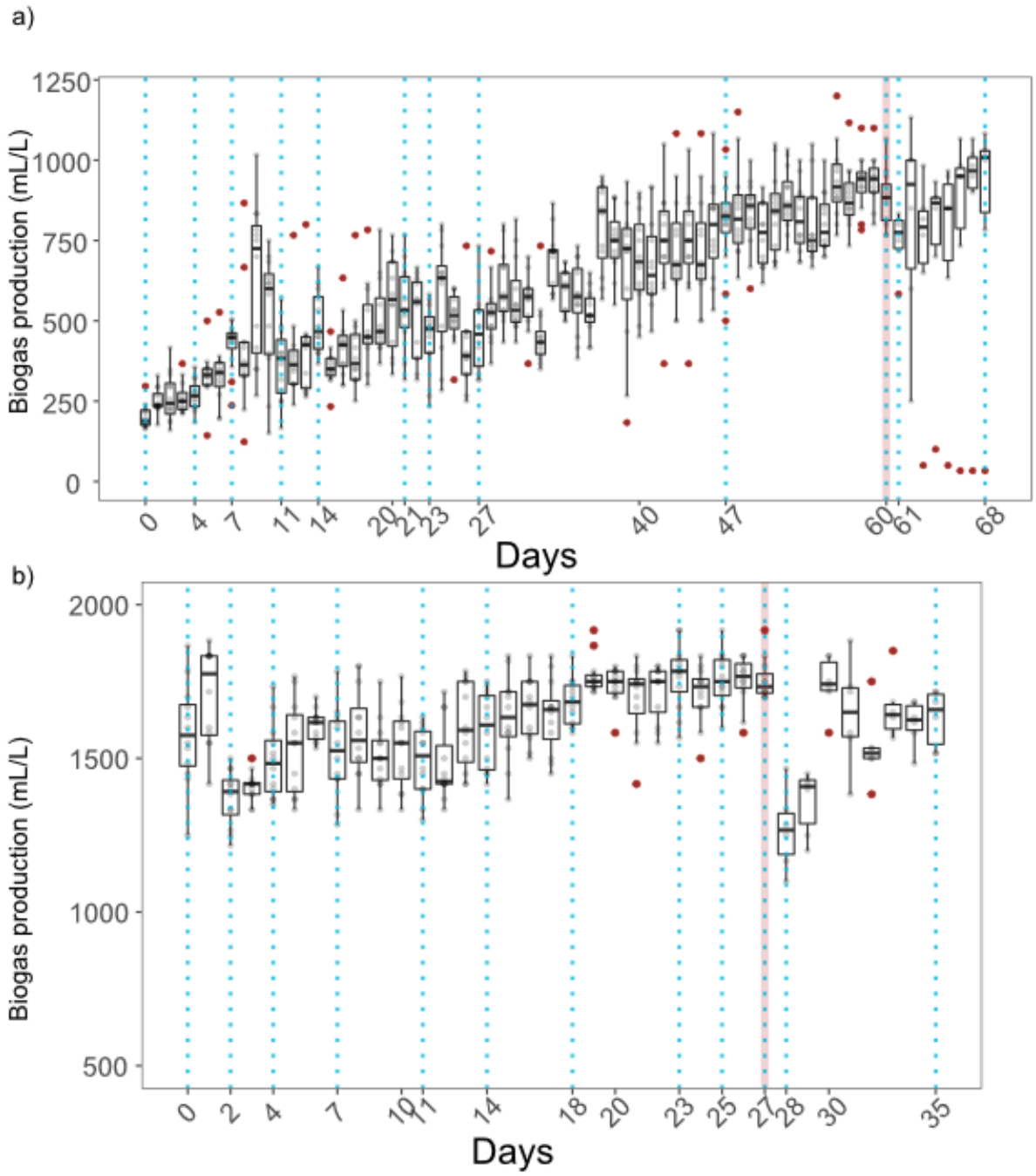

Figure 2. Biogas production over time in the non-treated (panel a) and heat-shock treated (panel b) bioreactors. Dotted vertical lines represent the sampling days for microbial and VFAs characterization. The red vertical line represents the day where the invasion with *L. plantarum* occurred. 12 replicates were measured per day until the invasion day where only the 6 invaded samples are shown.

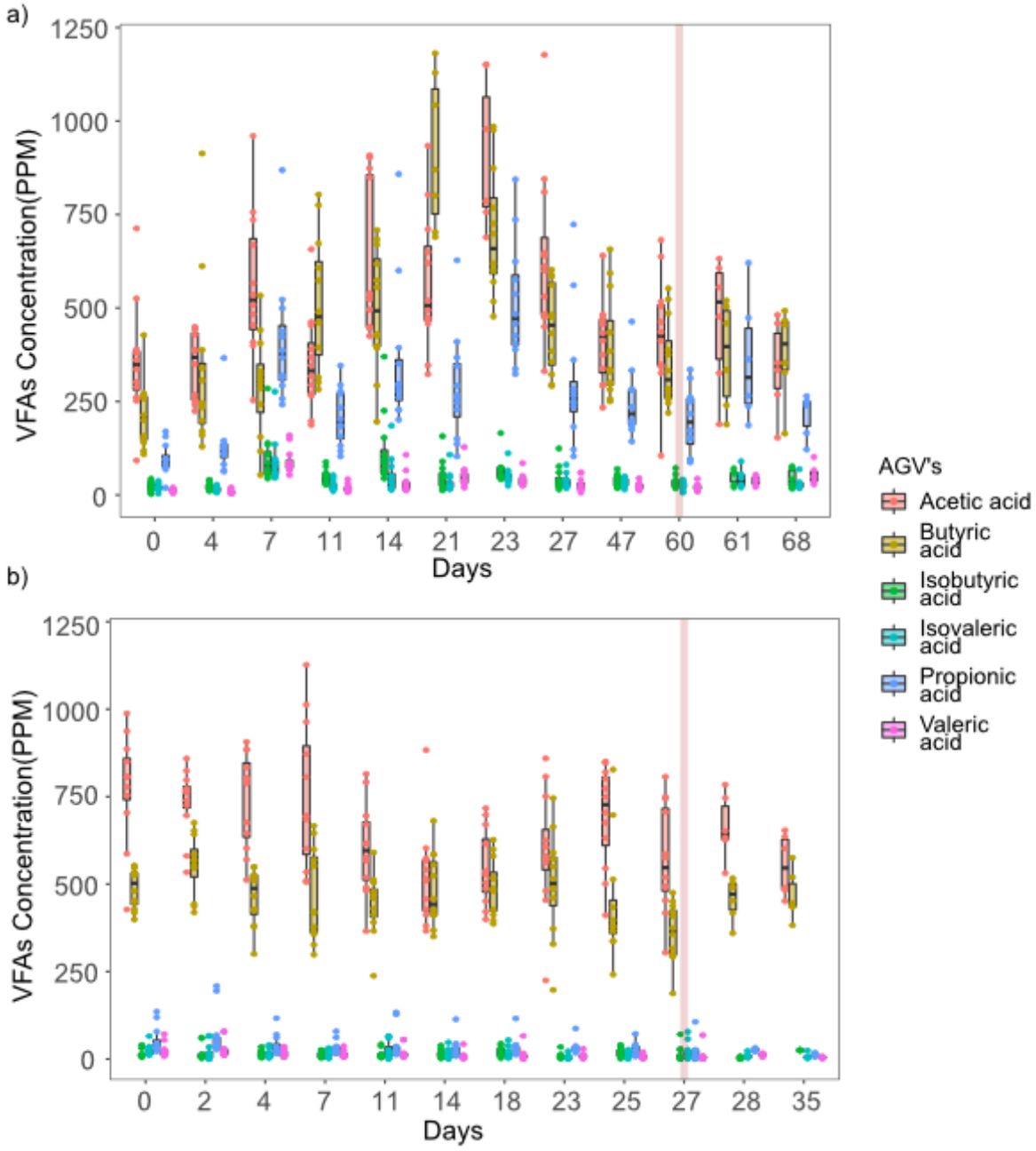

Figure 3. Volatile fatty acids production over time in the non-treated (panel a) and heat-shock treated (panel b) bioreactors. The red vertical line represents the day where the invasion with *L. plantarum* occurred. 12 replicates were measured per day until the invasion day where only the 6 invaded samples are shown.

Microbial composition

In the non-treated bioreactors, the microbial consortia were composed of several bacterial ASVs that

showed a distinctive pattern of change during the experiment. Initially, the bacterial composition was highly dominated by *Citrobacter amalonaticus, Clostridium butyricum, and Enterococcus xiangfangensis* (Figure 4a). Towards the end of the experiment, the abundance of *C. amalonaticus* and *C. butyricum* decreased and *Ethanoligenens harbinense, Enterococcus olivae, E. xiangfangensis* and *Clostridium pasteurianum* became dominant. Aside from these highly abundant ASVs, 15 other ASVs comprised about a fifth of the total bacterial abundance. These, from now on called rare ASVs, showed independent dynamics from the highly abundant species (Figure 4a and Figure 5). In particular, the abundance of rare ASVs remained stable during the 60 days of the experiment. In contrast, after the heat-shock treatment, only two ASVs were present (*C. guangxiense* and *C. pasteurianum*; Figure 2b), and their abundance remained stable throughout the experiment being *C. guangxiense* the dominant ASV.

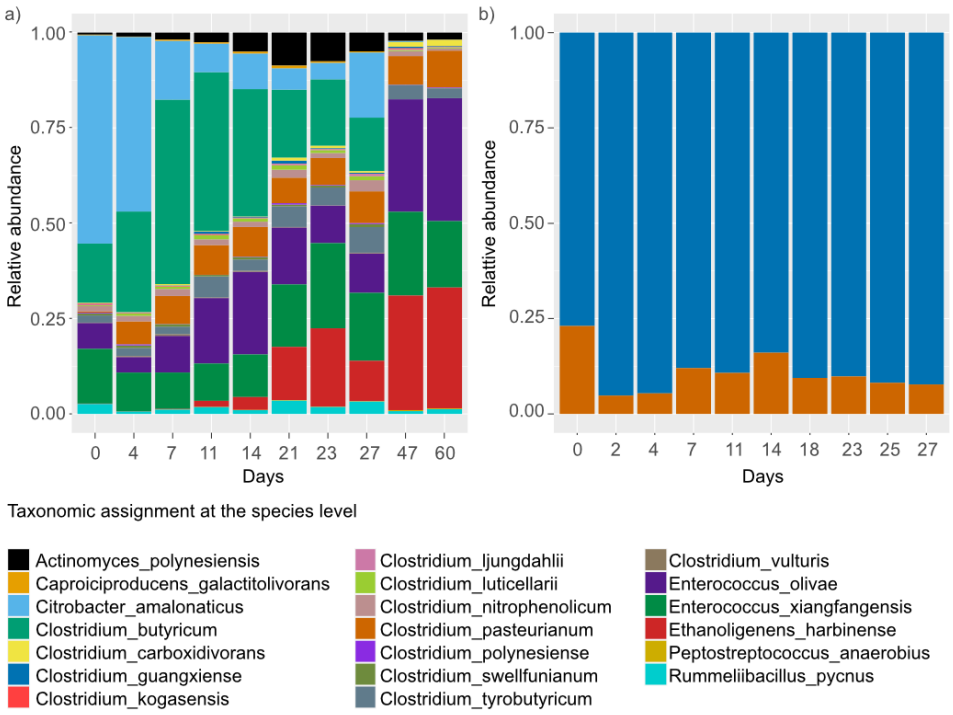

Figure 4. Microbial diversity composition per treatment and over time. Mean relative abundance (N=12) of different amplicon sequence variants (ASVs) in (a) the non-treated and (b) heat-shock treates bioreactors.

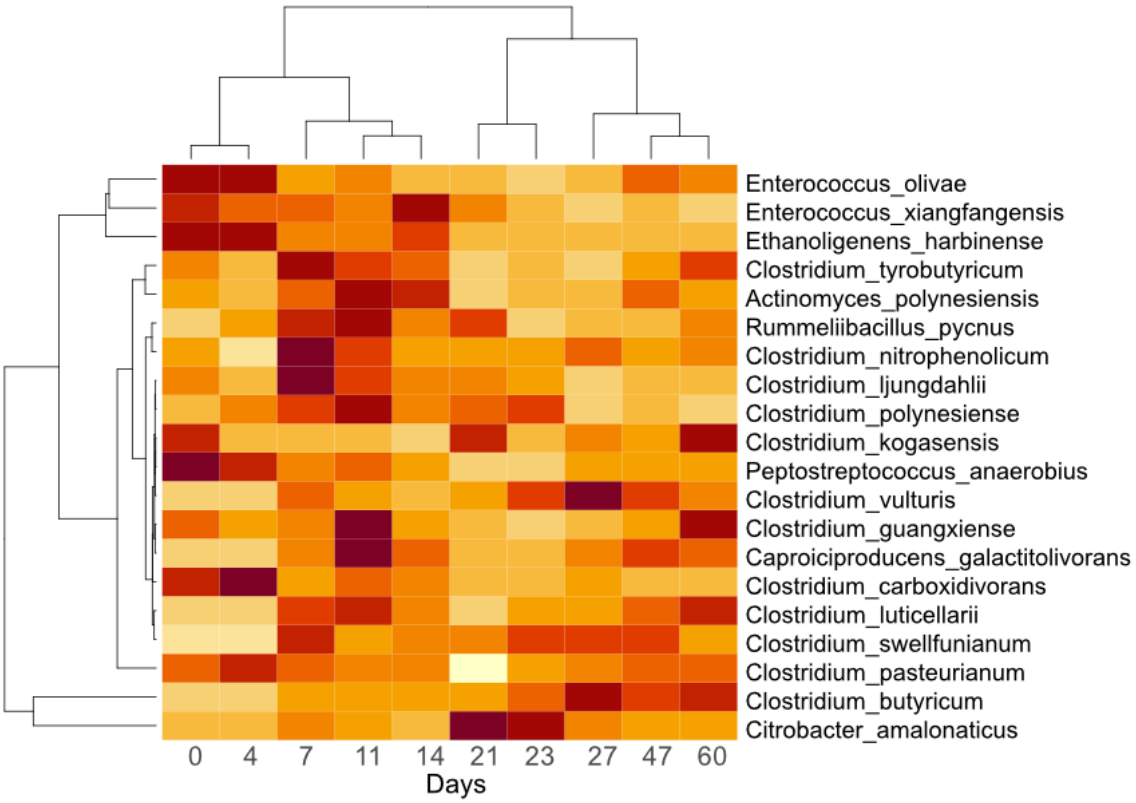

Figure 5. Heatmap showing the variability of the relative bacterial abundance over time in the non-treated bioreactors. Abundance is standardized per ASV to reflect variability between times for each ASV. Each day represents the average abundance per ASV (n = 12).

Relationship between microbial diversity and function of bioreactors

Since only two species survived in the heat-treated bioreactors and little variation was observed in their function, all correlation and multivariate analyses were performed only in the communities of non-treated bioreactors. It was found that hydrogen production was positively related to the abundance of certain ASVs. For instance, *E. harbinense*, *C. pasterianum E. olivae, Clostridum carboxidorans* and *Peptostreptococcus anaerobius* are positively correlated with biogas production while *C. amalonaticus*, *C. butyricum, Clostridium swellfunianum*, *Clostridium vulturis*, *Rummelibacillus pycnus* and *Clostridium nitrophenolicum* were negatively correlated (CCA in Figure 6 and Pearson correlations in Figure 7). The bacteria that had negative correlations with biogas production tended to present positive correlations with, either butyric acid and/or propionic acid. For instance, ASVs like

R. pycnus were positively related to butyric acid, while *Clostridium luticellarii* was positively related to, both, butyric and propionic acids. The rest of the analyzed volatile acids (isobutyric acid, isovaleric acid and valeric acid) and microbial ASVs show no clear relationship to hydrogen production. The CCA (Figure 6), also shows that the structure of the microbial composition of samples in all replicates followed similar composition and function during the experiment. Thus, samples from earlier times in the experiment showed similar microbial structure and functional performance to samples from the end of the experiment.

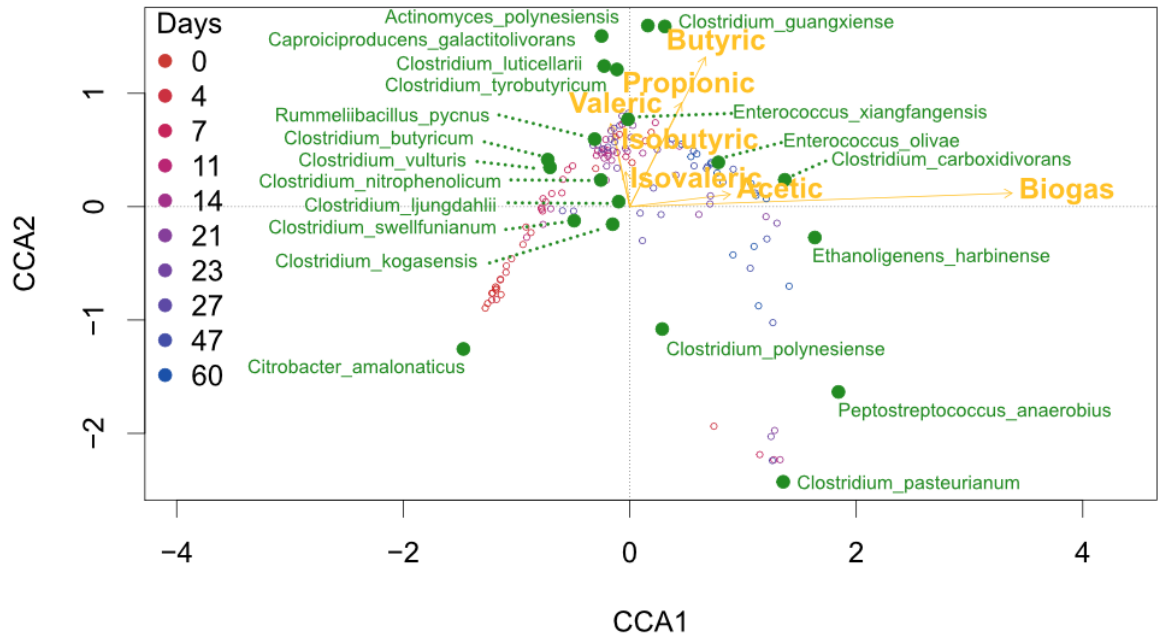

Figure 6. Canonical correspondence analysis (CCA) biplot (n = 120, 12 per day). CCA representing the relationship between bioreactors function (biogas and volatile fatty acids) and microbial composition in the non-treated bioreactors. Yellow points represent ASVs, vectors represent metabolites and hollow points represent samples colored by the time of the experiment when they were taken. Eigenvalues: axis 1 = 0.324, axis 2 = 0.111. Percentage of variance explained: 15.54 (axis 1), 5.33 (axis 2). Cumulative percentage explained: 20.88.

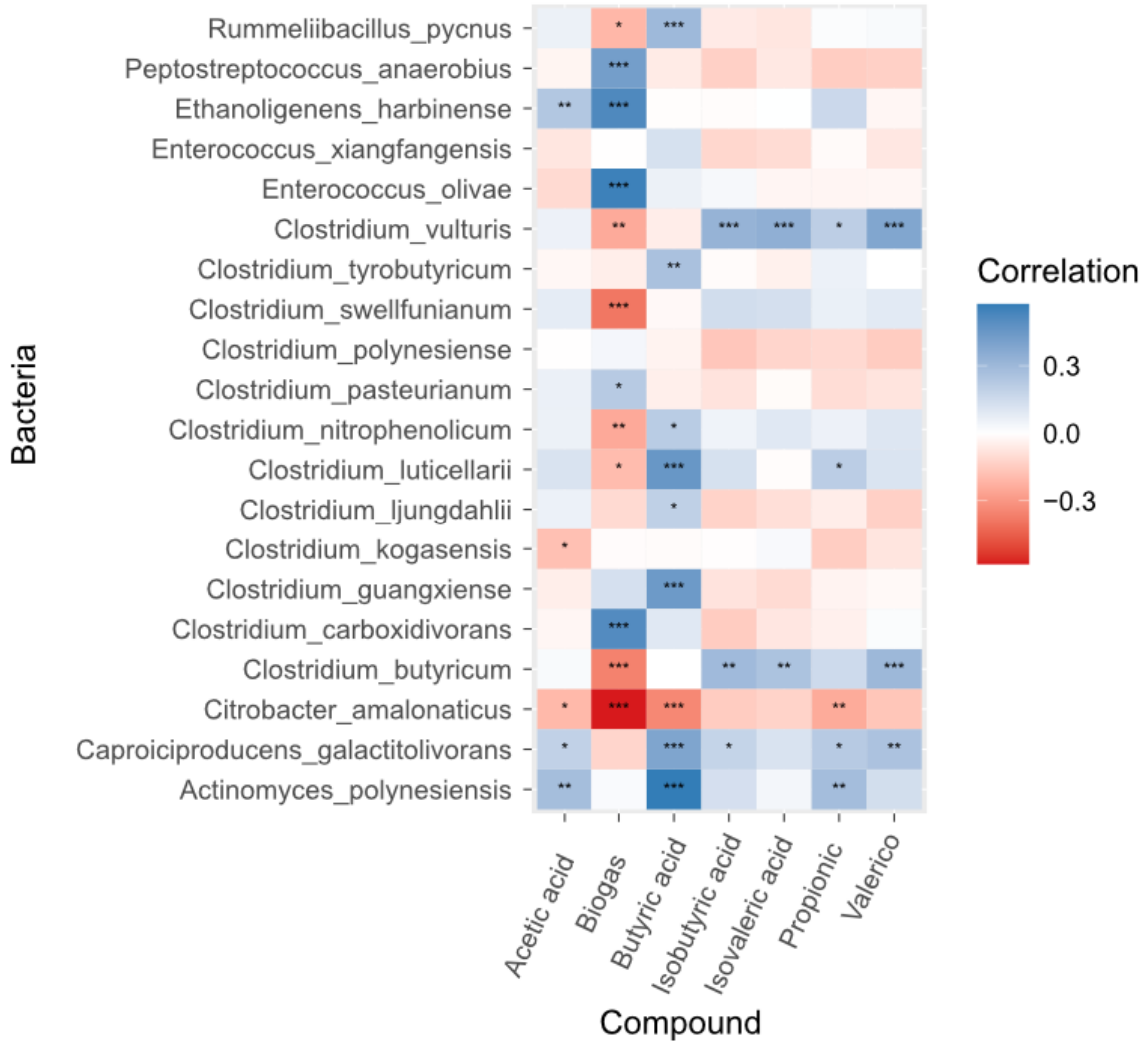

Figure 7. Pearson correlations between microbial abundance, biogas production and volatile fatty acids concentration in the non-treated bioreactors (n= 120). Red shades represent negative correlations and blue shades represent positive correlations. Stars inside each cell represent the significance of the correlation ("***" = 0.001; "**" = 0.01; "*" = 0.05).

Potential ecological interactions

The inferred interactions tended to be potentially beneficial for bacteria that were positively related to biogas (such as *E. harbinense* and *E. olivae*) and potentially negative for bacteria that were negatively related to biogas production (like *C. amanoliticus* and *C. butyricum*; Figure 8). For example, it was inferred that *E. harbinense* and *E. olivae* were benefited by *C. pasteurianum.* On the other side, *C. amanoliticus* was negatively affected by *C. pasteurianum* and *R. pycnus, and C. butyricum* was

affected by *E. xiangfangensis*. Meanwhile, *C. nitrophenolicum* was a low-abundant bacteria with several interactions that could have modified the consortia composition. For instance, *C. nitrophenolicum* benefited *E. harbinense* but negatively affected by C*. amanoliticus and C. butyricum*. In turn, *C. nitrophenolicum* was benefited by *E. xiangfangensis* and *Clostridium tyrobutyricum*.

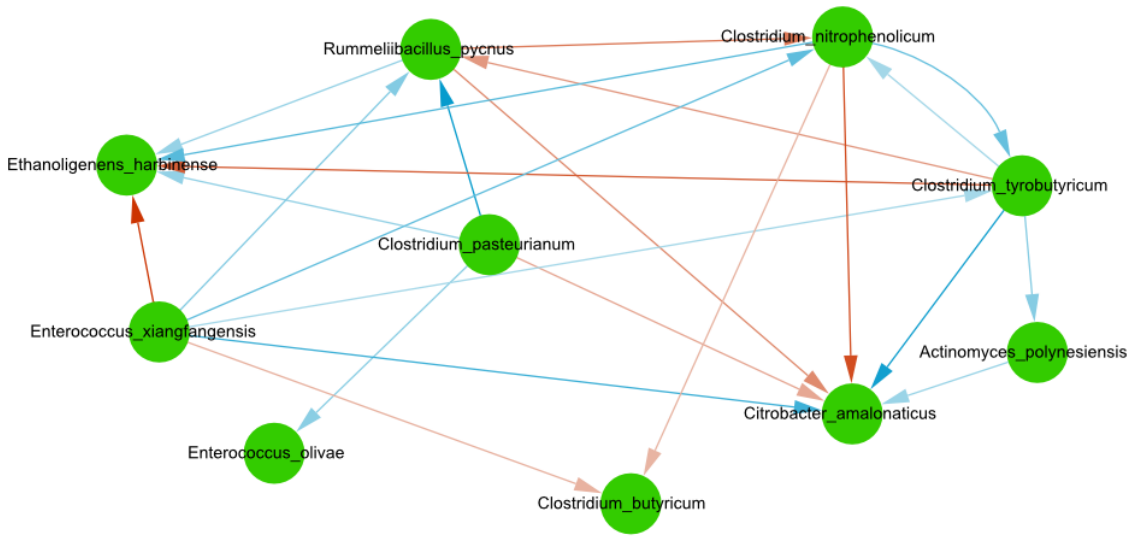

Figure 8. Interactions inferred in the non-treated bioreactors. Nodes denote bacterial species, blue edges denote positive interactions, red edges denote negative interactions, the direction of the arrows represents the direction of the interaction, and the intensity of the color of edges represents the strength of the interaction.

Robustness to invasion

Although both types of consortia were resistant to invasion by *L. plantarum*, each treatment showed different responses to invasion. Figure 7 shows the microbial composition of the bioreactors after the invasion of *L. plantarum* was performed. In the non-treated biroreactors, after 24 hours of the invasion, *L. plantarum* was present in all the bioreactors where it was inoculated. After one week of inoculation, *L. plantarum* was detectable in only one of the bioreactors. Importantly, in the bioreactor

where *L*. *plantarum* was still present, it became the dominant organism causing the collapse of hydrogen production. In the heat-shock treated bioreactors, an initial presence of *L*. *plantarum* was observed 24 hours after the inoculation. Contrarily to the non-treated bioreactors, *L. plantarum* never became dominant in any of the bioreactors, rather, it persisted in small abundances after one week. At the end of the experiment, all the bioreactors where *L*. *plantarum* did not establish kept a microbial composition like that observed before the inoculation.

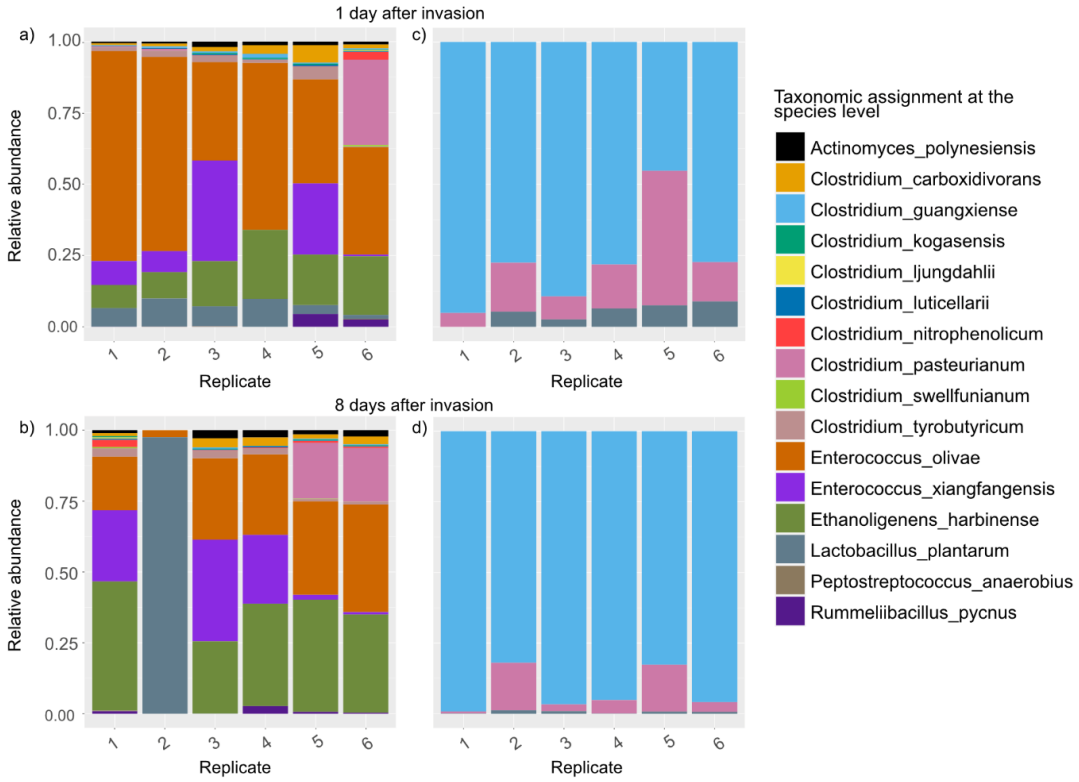

Figure 9. Microbial composition after 6 replicates were divided and inoculated with *L*. *plantarum*. Panels a and b correspond to the non-treated bioreactors, 1 day and 7 days after the inoculation respectively. Panels c and d correspond to the heat-shock treated diversity bioreactors, 1 day and 7 days after the inoculation respectively.

Discussion

In microbial communities, diversity and function are tightly related although the relationship is not straightforward (Louca et al., 2018; Escalas et al., 2019). Further, microbial composition and function are dependent on, not only the culture conditions but also the biological characteristics of the involved

microorganisms. In hydrogen-producing bioreactors, microbial diversity is harnessed to achieve a particular function derived from it. These microbial communities are subjected to a varied set of culture conditions to achieve hydrogen production that have distinctive impacts on their diversity (from mild to drastic microbial diversity reduction by aggressive pretreatments). However, this diversity reduction alters the structure, function and stability of the microbial communities with unknown ecological consequences (Philippot et al., 2013; Wagg et al., 2014; Hernández et al., 2019). In this work, we aimed to analyze the effect of reducing the diversity of microbial consortia on ecological processes (like biotic interactions and robustness).

Treatments on inoculum predictably affected the diversity, structure and function

To determine how variation in microbial diversity affected function, we periodically tracked the biogas and volatile fatty acid production in both, the non-treated and heat-shock treated bioreactors. Overall, the results show that the bioreactors of both treatments showed distinctive patterns of composition and functional stability (Figures 2 and 3).

Both treatments showed a slow but steady increase in biogas production with a decreased variability at the end of the experiment. In the high diversity treatment, such a pattern was more noticeable. This pattern of ascending biogas production has been observed previously (Kim and Shin, 2008; Kannaiah Goud et al., 2014). The most obvious cause is a shift in microbial composition either to an increased abundance of hydrogen-producing bacteria (Castelló et al., 2018; Palomo-Briones et al., 2018; Yang and Wang, 2019). Also, the higher stability and biogas production in the pretreated treatment are in accordance with the previous observation that pretreated communities have been observed to have better long-term stability by selective enrichment of *Clostridum* species (Singh and Wahid, 2015; Cabrol et al., 2017).

To investigate the microbial composition associated with the observed patterns in the biogas production, we performed the culture-independent molecular characterization of the microbial diversity in ten time points in the 12 replicates for each treatment. Figure 4 shows that in the non-treated bioreactors a significant shift of the dominant bacteria occurred. Also, the CCA (Figure 6) showed that there was a shift in microbial composition where a subsample of ASVs (including *E. olivae*, *E. harbinense* and *C. pasteurianum* and *C. carboxidivorans*) increased in abundance late in the experiment and were positively related with biogas production. The presumed positive relationship of *E. Harbinense, E. olivae*, *C. pasteurianum* and *C. carboxidivorans* with hydrogen production was confirmed in the correlation analysis (Figure 7). *E. harbinense and C. pasteurianum* are known hydrogen producers (Wang et al., 2009; Masset et al., 2012; Srivastava et al., 2017) and *Enterococcus* species are frequently present in hydrogen-producing consortia and some are hydrogen producers (Pachapur et al., 2015; Braga et al., 2018; Yin and Wang, 2019). *E. olivae* has been reported to produce gas on glucose (Lucena-Padrós et al., 2014) although this gas was not identified so, although possible, it is not clear if *E. olivae* contributed to hydrogen production. On the other side, *C. carboxidivorans* is an homoacetogen capable of growing by consuming H_2 and CO_2 (Fuentes et al., 2021). The positive relationship between *C. carboxidivorans* and hydrogen could be the result of its increased growth due to hydrogen availability.

In the heat-treated bioreactors, microbial richness was severely reduced consisting only of *C. guangxiense* (which dominated the bioreactors) and *C. pasteurianum*. Although our case is an extreme example of reduced microbial diversity, other experiments also show very few ASVs (e.g. (Muñoz-Páez et al., 2019)). It is worth noticing that the stability observed might be deceiving since anaerobic conditions and sterility might difficult scaling the process and currently represent important challenges to the industrial application of dark fermentation (Cabrol et al., 2017) and have been discussed earlier as being the result of selective pretreatments (Hawkes et al., 2002). Our observations tell us that the loss of diversity possibly affected ecological processes in the bioreactors which resulted

in different behaviors between treatments. Resolving these ecological processes might help to explain why microbial composition changed in the non-treated bioreactors through time or why in the heat-shock treated bioreactors both ASVs remained with a stable abundance.

Stability in diversity treatments was the result of distinctive ecological processes

The impact of ecological microbial interactions on composition and function has been studied in several systems. For example, cooperative and competitive interactions have been reported to affect the assemblage of communities in natural and synthetic consortia (Foster and Bell, 2012; Cordero and Datta, 2016). Further, biotic interactions have been shown to influence the function of microbial systems, for example by affecting their growth (Pekkonen et al., 2013), productivity (Fiegna et al., 2015) and resilience (Feng et al., 2017). Also, from an evolutionary point of view, interactions have been reported to evolve in time frames relevant to the operation time of bioreactors (Rosenzweig et al., 1994; Harcombe, 2010; Poltak and Cooper, 2011; Jeffrey Morris et al., 2014). Thus, studying the biotic interactions in microbial communities can help us deduce which organisms had a role in shaping the abundance and function in microbial systems like bioreactors.

In both treatments, we could observe that ecological interactions and derived ecological processes can influence the observed dynamics in contrasting ways. In the non-treated bioreactors, more ASVs meant more chances that interactions with strong effects on composition and function occurred for a longer time. For instance, in the non-treated bioreactors, negative (e.g. *C. pasteurianum* towards *E. harbinense* and *E. olivae*) and positive interactions (e.g. *Clostridium tyrobutyricum* and *Enterococcus xiangfangensis* towards directed towards *E. Harbinense*) may be responsible for the increase of abundance *E. harbinense* and *E. olivae* in the last part of the experiment. Interestingly, *Enterococcus* ASVs frequently coexist with *Clostridium* and *Ethanoligenens* ASVs in various environments that range from human hosts to bioreactors (Wang et al., 2009; Pachapur et al., 2015; Valdez-Vazquez et al., 2015; Braga et al., 2018). As such, our data indicates that the variable roles

reported for lactic acid bacteria (like *Enterococcus*) in hydrogen-producing consortia (Sikora et al., 2013) are ASVs-specific. For example, in our experiment other enterococci (*E. xiangfangensis*) had a negative relationship with, both, *E. harbinense* and *C. butyricum*. Lastly, low-abundant bacteria (like *C. nitrophenolicum*, *Rummeliibacillus pycnus* and *Clostridium tyrobutyricum*) were involved in interactions that could favor the change in the overall composition. Low-abundance bacteria have been observed to be able to affect the whole structure of microbial communities either by acting as keystone species, being highly active, or by acting as reservoir against environmental perturbations and should not be overlooked (Rafrafi et al., 2013; Shade et al., 2014; Lynch and Neufeld, 2015).

Contrarily, in the case of the heat-shock treated bioreactors fewer ASVs led the consortia to reach stability more quickly. The compositional and functional stability was presumably the result of the two ASVs showing a mostly neutral interaction. We deduce this neutrality since the two ASVs coexisted without many changes in abundance during the whole experiment. Also, based on the interactions inferred for the non-treated bioreactors, no significant interaction was found between *C. pasteurianum* and *C. guangxiense*. This agrees with observations in other communities, both natural and artificial, where several species of *Clostridium* can coexist (Graf et al., 2015; Thi Hoang et al., 2018). Since not every *Clostridium* (or for that matter any bacterial group) contributes equally to function, our results draw attention to fully understanding the reasons behind the presence of certain species in microbial consortia. In the case of the heat-shock treated bioreactors, the presence of *C. pasteurianum* and *C. guangxiense* might be the result of deterministic processes like different spore germination times and growth rates that have been noted to be important in community assembly (Hawkes et al., 2002; Elke Jaspers and Jorg Overmann, 2014). Also, stochastic processes like drift could have been important as has been noted earlier for the assembly of microbial communities in bioreactors (Liébana et al., 2019). As mentioned before, the drastic reduction in ASVs in the heat-shock treated bioreactors might give the impression of increased stability in the bioreactors. However, in addition to the fact that strict anaerobic conditions had to be used, the diminished diversity can

lead to less long-term instability, due to, for example, a lack of functional redundancy in case of perturbations (Louca et al., 2018).

Invasion by competitive species in non-sterilized substrates is one of the reasons for bioreactor's instability (Castelló et al., 2018). Several factors have been shown to influence the resistance to invasions that microbial communities. For example, diversity has been positively linked with a reduced probability of invasion while stochastic processes have been linked to invasion success (Mallon et al., 2015). In this line, the microbial communities of the two treatments showed different responses to invasion (the ratio of invasion establishment and the effect the invasion had) that could be related to ecological processes specific to each of them. In the non-treated bioreactors, only in one replicate, the invader became dominant while in the rest of the replicates no presence of the invader was observed after one week. Contrastingly, in the heat-shock treated bioreactors, *L. plantarum* established in at least 4 of them (although in low abundances) but never dominated. In the heat-shock treated bioreactors *L. plantarum* might have managed to exploit an available niche which is an important factor regarding establishment of invaders (Kinnunen et al., 2016). In the non-treated bioreactors, since the establishment of *L. plantarum* occurred only once, it is possible that random processes like drift contributed to invasion success in the particular bioreactor where it established (Kinnunen et al., 2016). Additionally, the high effect of *L. plantarum* in the community might be explained by similarity in fitness with the resident community which might have increased the invasion effect (Li et al., 2019). Thus, our results show that the relationship between invasion success and microbial diversity is complex even in simple communities. Further studies have to be performed using complex substrates (like agro-industrial effluents) and varying conditions like those intended to be used in industrial-scale bioreactors.

Conclusions

Despite a long line of evidence showing that diversity has a strong influence on the function and stability of bacterial communities, it is not clear how ecological mechanisms (from which function and stability are dependent) are related to diversity and how they are affected when diversity changes. The results in this work show that the establishment and medium-term behavior of bacterial communities are the outcomes of the interplay between the number of ASVs, biotic interactions and culture conditions. In particular, we found that: (1) higher diversity slowed the stabilization of microbial abundance and dynamics but was ultimately predictable as biotic interactions were important for the assembly of microbial consortia, (2) loss of diversity made stabilization faster but less resilient against adverse culture conditions (aerobic conditions) and (3) higher diversity made invasion success less probable. Further studies investigating the molecular basis of interactions and confirmatory experiments on the nature of the interactions and their effect could help identify mechanisms and processes that caused changes in function and invasion behaviors.

Finally, due to the importance of controlling diversity in biotechnological settings, this study provides a baseline to incorporate ecological theory and experimental designs into the study of biotechnological settings like bioreactors. Further, this type of study might increase the knowledge of the relationship between function and diversity which in turn will help improve the yield and stability of hydrogen-producing bioreactors.

Acknowledgements

We thank Marisol Perez Rangel for technical assistance during bioreactors establishment and for providing the strain *Lactobacillus plantarum* used in this work. The authors acknowledge Adalberto Noyola Robles and Margarita Cisneros Ortiz for her support in the laboratory work at the Laboratorio de Ingeniería Ambiental, Instituto de Ingeniería, UNAM. Marcelo Navarro-Díaz is a doctoral student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM), and was supported by Consejo Nacional de Ciencia y Tecnología (CONACyT), scholarship no.

594615. Financial support of this work was received from the “Fondo de Sustentabilidad Energética SENER-CONACYT (Mexico)”, Bioalcohols Cluster (Grant No. 249564).

References

Allison, S. D., and Martiny, J. B. H. (2009). Resistance, resilience, and redundancy in microbial communities. *Light Evol.* 2, 149–166. doi:10.17226/12501.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–10. doi:10.1016/S0022-2836(05)80360-2.

Apprill, A., Mcnally, S., Parsons, R., and Weber, L. (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat. Microb. Ecol.* 75, 129–137. doi:10.3354/ame01753.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi:10.1038/s41587-019-0209-9.

Boshagh, F., and Rostami, K. (2020). A review of measurement methods of biological hydrogen. *Int. J. Hydrogen Energy* 45, 24424–24452. doi:10.1016/j.ijhydene.2020.06.079.

Braga, J. K., Motteran, F., Sakamoto, I. K., and Varesche, M. B. A. (2018). Bacterial and archaeal community structure involved in biofuels production using hydrothermal- and enzymatic-pretreated sugarcane bagasse for an improvement in hydrogen and methane production. *Sustain. Energy Fuels* 2, 2644–2660. doi:10.1039/c8se00312b.

Cabrol, L., Marone, A., Tapia-Venegas, E., Steyer, J. P., Ruiz-Filippi, G., and Trably, E. (2017). Microbial ecology of fermentative hydrogen producing bioprocesses: Useful insights for driving the ecosystem function. *FEMS Microbiol. Rev.* 41, 158–181. doi:10.1093/femsre/fuw043.

Cairns, J., Jokela, R., Hultman, J., Tamminen, M., Virta, M., and Hiltunen, T. (2018). Construction and characterization of synthetic bacterial community for experimental ecology and evolution. *Front. Genet.* 9. doi:10.3389/fgene.2018.00312.

Cao, Q., Sun, X., Rajesh, K., Chalasani, N., Gelow, K., Katz, B., et al. (2021). Effects of Rare Microbiome Taxa Filtering on Statistical Analysis. *Front. Microbiol.* 11, 1–15. doi:10.3389/fmicb.2020.607325.

Castelló, E., Braga, L., Fuentes, L., and Etchebehere, C. (2018). Possible causes for the instability in the H2 production from cheese whey in a CSTR. *Int. J. Hydrogen Energy* 43, 2654–2665. doi:10.1016/j.ijhydene.2017.12.104.

Castelló, E., Nunes Ferraz-Junior, A. D., Andreani, C., Anzola-Rojas, M. del P., Borzacconi, L., Buitrón, G., et al. (2020). Stability problems in the hydrogen production by dark fermentation: Possible causes and solutions. *Renew. Sustain. Energy Rev.* 119. doi:10.1016/j.rser.2019.109602.

Cordero, O. X., and Datta, M. S. (2016). Microbial interactions and community assembly at microscales. *Curr. Opin. Microbiol.* 31, 227–234. doi:10.1016/j.mib.2016.03.015.

Datta, M. S., Sliwerska, E., Gore, J., Polz, M. F., and Cordero, O. X. (2016). Microbial interactions lead to rapid micro-scale successions on model marine particles. *Nat. Commun.* 7, 11965. doi:10.1038/ncomms11965.

De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., and Boon, N. (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environ. Microbiol.* 16, 1472–1481. doi:10.1111/1462-2920.12343.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* 28, 350–356. doi:10.1021/ac60111a017.

Elke Jaspers and Jorg Overmann (2014). Ecological Significance of Microdiversity: Identical 16S rRNA Gene Sequences Can Be Found in Bacteria with Highly Divergent Genomes and Ecophysiologies. *Inst. fur Chemie und Biol. des Meeres, Univ. Oldenburg, D-26111 Oldenburg, Ger.* 70, 62. doi:10.1128/AEM.70.8.4831.

Escalante, A. E., Rebolleda-Gómez, M., Benítez, M., and Travisano, M. (2015). Ecological perspectives on synthetic biology: Insights from microbial population biology. *Front. Microbiol.* 6, 1–10.

doi:10.3389/fmicb.2015.00143.

Escalas, A., Hale, L., Voordeckers, J. W., Yang, Y., Firestone, M. K., Alvarez-Cohen, L., et al. (2019). Microbial functional diversity: From concepts to applications. *Ecol. Evol.* 9, 12000–12016. doi:10.1002/ece3.5670.

Feng, K., Zhang, Z., Cai, W., Liu, W., Xu, M., Yin, H., et al. (2017). Biodiversity and species competition regulate the resilience of microbial biofilm community. *Mol. Ecol.* 26, 6170–6182. doi:10.1111/mec.14356.

Fiegna, F., Moreno-Letelier, A., Bell, T., and Barraclough, T. G. (2015). Evolution of species interactions determines microbial community productivity in new environments. *ISME J.* 9, 1235–1245. doi:10.1038/ismej.2014.215.

Foster, K. R., and Bell, T. (2012). Competition, not cooperation, dominates interactions among culturable microbial species. *Curr. Biol.* 22, 1845–1850. doi:10.1016/j.cub.2012.08.005.

Friedman, J., Higgins, L. M., and Gore, J. (2017). Community structure follows simple assembly rules in microbial microcosms. *Nat. Ecol. Evol.* 1, 1–7. doi:10.1038/s41559-017-0109.

Fuentes, L., Palomo-Briones, R., de Jesús Montoya-Rosales, J., Braga, L., Castelló, E., Vesga, A., et al. (2021). Knowing the enemy: homoacetogens in hydrogen production reactors. *Appl. Microbiol. Biotechnol.* 105, 8989–9002. doi:10.1007/s00253-021-11656-6.

Ghosh, S., Chowdhury, R., and Bhattacharya, P. (2016). Mixed consortia in bioprocesses: role of microbial interactions. *Appl. Microbiol. Biotechnol.* 100, 4283–4295. doi:10.1007/s00253-016-7448-1.

Glasl, B., Smith, C. E., Bourne, D. G., and Webster, N. S. (2018). Exploring the diversity-stability paradigm using sponge microbial communities. *Sci. Rep.* 8, 1–9. doi:10.1038/s41598-018-26641-9.

Graf, D., Di Cagno, R., Fåk, F., Flint, H. J., Nyman, M., Saarela, M., et al. (2015). Contribution of diet to the composition of the human gut microbiota. *Microb. Ecol. Heal. Dis.* 26. doi:10.3402/mehd.v26.26164.

Harcombe, W. (2010). Novel cooperation experimentally evolved between species. *Evolution (N. Y).* 64, 2166–2172. doi:10.1111/j.1558-5646.2010.00959.x.

Hawkes, F. R., Dinsdale, R., Hawkes, D. L., and Hussy, I. (2002). Sustainable fermentative hydrogen production: Challenges for process optimisation. *Int. J. Hydrogen Energy* 27, 1339–1347. doi:10.1016/S0360-3199(02)00090-3.

Hays, S. G., Patrick, W. G., Ziesack, M., Oxman, N., and Silver, P. A. (2015). Better together: Engineering and application of microbial symbioses. *Curr. Opin. Biotechnol.* 36, 40–49. doi:10.1016/j.copbio.2015.08.008.

Hernández, C., Alamilla-Ortiz, Z. L., Escalante, A. E., Navarro-Díaz, M., Carrillo-Reyes, J., Moreno-Andrade, I., et al. (2019). Heat-shock treatment applied to inocula for H2 production decreases microbial diversities, interspecific interactions and performance using cellulose as substrate. *Int. J. Hydrogen Energy* 44, 13126–13134. doi:10.1016/j.ijhydene.2019.03.124.

Jeffrey Morris, J., Papoulis, S. E., and Lenski, R. E. (2014). Coexistence of evolving bacteria stabilized by a shared Black Queen function. *Evolution (N. Y).* 68, 2960–2971. doi:10.1111/evo.12485.

Johns, N. I., Blazejewski, T., Gomes, A. L. C., and Wang, H. H. (2016). Principles for designing synthetic microbial communities. *Curr. Opin. Microbiol.* 31, 146–153. doi:10.1016/j.mib.2016.03.010.

Kannaiah Goud, R., Sarkar, O., and Venkata Mohan, S. (2014). Regulation of biohydrogen production by heat-shock pretreatment facilitates selective enrichment of Clostridium sp. *Int. J. Hydrogen Energy* 39, 7572–7586. doi:10.1016/j.ijhydene.2013.10.046.

Kim, S. H., and Shin, H. S. (2008). Effects of base-pretreatment on continuous enriched culture for hydrogen production from food waste. *Int. J. Hydrogen Energy* 33, 5266–5274. doi:10.1016/j.ijhydene.2008.05.010.

Kinnunen, M., Dechesne, A., Proctor, C., Hammes, F., Johnson, D., Quintela-Baluja, M., et al. (2016). A conceptual framework for invasion in microbial communities. *ISME J.* 10, 2773–2779. doi:10.1038/ismej.2016.75.

Li, S. peng, Tan, J., Yang, X., Ma, C., and Jiang, L. (2019). Niche and fitness differences determine invasion success and impact in laboratory bacterial communities. *ISME J.* 13, 402–412. doi:10.1038/s41396-018-0283-x.

Liébana, R., Modin, O., Persson, F., Szabó, E., Hermansson, M., and Wilén, B. M. (2019). Combined Deterministic and Stochastic Processes Control Microbial Succession in Replicate Granular Biofilm Reactors. *Environ. Sci. Technol.* 53, 4912–4921. doi:10.1021/acs.est.8b06669.

Locey, K. J., and Lennon, J. T. (2016). Scaling laws predict global microbial diversity. *Proc. Natl. Acad. Sci. U. S. A.* 113, 5970–5975. doi:10.1073/pnas.1521291113.

Louca, S., Polz, M. F., Mazel, F., Albright, M. B. N., Huber, J. A., O'Connor, M. I., et al. (2018). Function and functional redundancy in microbial systems. *Nat. Ecol. Evol.* 2. doi:10.1038/s41559-018-0519-1.

Lucena-Padrós, H., González, J. M., Caballero-Guerrero, B., Ruiz-Barba, J. L., and Maldonado-Barragán, A. (2014). Enterococcus olivae sp. nov., isolated from Spanish-style green-olive fermentations. *Int. J. Syst. Evol. Microbiol.* 64, 2534–2539. doi:10.1099/ijs.0.062208-0.

Lynch, M. D. J., and Neufeld, J. D. (2015). Ecology and exploration of the rare biosphere. *Nat. Rev. Microbiol.* 13, 217–229. doi:10.1038/nrmicro3400.

Madsen, J. S., Sørensen, S. J., and Burmølle, M. (2018). Bacterial social interactions and the emergence of community-intrinsic properties. *Curr. Opin. Microbiol.* 42, 104–109. doi:10.1016/j.mib.2017.11.018.

Mallon, C. A., Van Elsas, J. D., and Salles, J. F. (2015). Microbial invasions: The process, patterns, and mechanisms. *Trends Microbiol.* 23, 719–729. doi:10.1016/j.tim.2015.07.013.

Masset, J., Calusinska, M., Hamilton, C., Hiligsmann, S., Joris, B., Wilmotte, A., et al. (2012). Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures of Clostridium spp. *Biotechnol. Biofuels* 5, 1. doi:10.1186/1754-6834-5-35.

Meroz, N., Tovi, N., Sorokin, Y., and Friedman, J. (2021). Community composition of microbial microcosms follows simple assembly rules at evolutionary timescales. *Nat. Commun.* 12, 1–9.

doi:10.1038/s41467-021-23247-0.

Mizuno, O., Dinsdale, R., Hawkes, F. R., Hawkes, D. L., and Noike, T. (2000). Enhancement of hydrogen production from glucose by nitrogen gas sparging. *Bioresour. Technol.* 73, 59–65. doi:10.1016/S0960-8524(99)00130-3.

Muñoz-Páez, K. M., Alvarado-Michi, E. L., Buitrón, G., and Valdez-Vazquez, I. (2018). Distinct effects of furfural, hydroxymethylfurfural and its mixtures on dark fermentation hydrogen production and microbial structure of a mixed culture. *Int. J. Hydrogen Energy*, 1–9. doi:10.1016/j.ijhydene.2018.04.139.

Muñoz-Páez, K. M., Alvarado-Michi, E. L., Buitrón, G., and Valdez-Vazquez, I. (2019). Distinct effects of furfural, hydroxymethylfurfural and its mixtures on dark fermentation hydrogen production and microbial structure of a mixed culture. *Int. J. Hydrogen Energy* 4, 2289–2297. doi:10.1016/j.ijhydene.2018.04.139.

Navarro-Díaz, M., Martinez-Sanchez, M. E., Valdez-Vazquez, I., and Escalante, A. E. (2020). A framework for integrating functional and microbial data: The case of dark fermentation H2 production. *Int. J. Hydrogen Energy* 45, 31706–31718. doi:10.1016/j.ijhydene.2020.08.189.

O'Leary, N. A., Wright, M. W., Brister, J. R., Ciufo, S., Haddad, D., McVeigh, R., et al. (2016). Reference sequence (RefSeq) database at NCBI: Current status, taxonomic expansion, and functional annotation. *Nucleic Acids Res.* 44, D733–D745. doi:10.1093/nar/gkv1189.

Oksanen, J., Blanchet, F., Kindt, R., and Legendre, P. (2013). vegan: Community Ecology Package. R package version 2.0-10.

Pachapur, V. L., Sarma, S. J., Kaur Brar, S., Le Bihan, Y., Soccol, C. R., Buelna, G., et al. (2015). Co-culture strategies for increased biohydrogen production. *Int. J. ENERGY Res.* 39, 1479–1504. doi:10.1002/er.

Palomo-Briones, R., Trably, E., López-Lozano, N. E., Celis, L. B., Méndez-Acosta, H. O., Bernet, N., et al. (2018). Hydrogen metabolic patterns driven by Clostridium-Streptococcus community shifts in a continuous stirred tank reactor. *Appl. Microbiol. Biotechnol.* doi:10.1007/s00253-018-8737-

7.

Parada, A. E., Needham, D. M., and Fuhrman, J. A. (2016). Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ. Microbiol.* 18, 1403–1414. doi:10.1111/1462-2920.13023.

Pekkonen, M., Ketola, T., and Laakso, J. T. (2013). Resource Availability and Competition Shape the Evolution of Survival and Growth Ability in a Bacterial Community. *PLoS One* 8, 1–12. doi:10.1371/journal.pone.0076471.

Pérez-Gutiérrez, R.-A., López-Ramírez, V., Islas, Á., Alcaraz, L. D., Hernández-González, I., Olivera, B. C. L., et al. (2013). Antagonism influences assembly of a Bacillus guild in a local community and is depicted as a food-chain network. *ISME J.* 7, 487–497. doi:10.1038/ismej.2012.119.

Pérez-Rangel, M., Barboza-Corona, J. E., Navarro-Díaz, M., Escalante, A. E., and Valdez-Vazquez, I. (2021). The duo Clostridium and Lactobacillus linked to hydrogen production from a lignocellulosic substrate. *Water Sci. Technol.* 83, 3033–3040. doi:10.2166/wst.2021.186.

Philippot, L., Spor, A., Hénault, C., Bru, D., Bizouard, F., Jones, C. M., et al. (2013). Loss in microbial diversity affects nitrogen cycling in soil. *ISME J.* 7, 1609–1619. doi:10.1038/ismej.2013.34.

Poltak, S. R., and Cooper, V. S. (2011). Ecological succession in long-term experimentally evolved biofilms produces synergistic communities. *ISME J.* doi:10.1038/ismej.2010.136.

R Core Team (2016). R: A Language and Environment for Statistical Computing. Available at: http://www.r-project.org/.

Rafrafi, Y., Trably, E., Hamelin, J., Latrille, E., Meynial-Salles, I., Benomar, S., et al. (2013). Sub-dominant bacteria as keystone species in microbial communities producing bio-hydrogen. *Int. J. Hydrogen Energy* 38, 4975–4985. doi:10.1016/j.ijhydene.2013.02.008.

Ratzke, C., Barrere, J., and Gore, J. (2020). Strength of species interactions determines biodiversity and stability in microbial communities. *Nat. Ecol. Evol.* 4, 376–383. doi:10.1038/s41559-020-1099-4.

Rosenzweig, R. F., Sharp, R. R., Treves, D. S., and Adams, J. (1994). Microbial evolution in a simple

unstructured environment: Genetic differentiation in Escherichia coli. *Genetics* 137, 903–917. doi:10.1093/genetics/137.4.903.

Schmidt, R., Cordovez, V., de Boer, W., Raaijmakers, J., and Garbeva, P. (2015). Volatile affairs in microbial interactions. *ISME J.* 9, 2329–2335. doi:10.1038/ismej.2015.42.

Shade, A. (2017). Diversity is the question, not the answer. *ISME J.* 11, 1–6. doi:10.1038/ismej.2016.118.

Shade, A., Jones, S. E., Gregory Caporaso, J., Handelsman, J., Knight, R., Fierer, N., et al. (2014). Conditionally rare taxa disproportionately contribute to temporal changes in microbial diversity. *MBio* 5, 1–9. doi:10.1128/mBio.01371-14.

Shade, A., Peter, H., Allison, S. D., Baho, D. L., Berga, M., B??rgmann, H., et al. (2012). Fundamentals of microbial community resistance and resilience. *Front. Microbiol.* 3, 1–19. doi:10.3389/fmicb.2012.00417.

Shannon, P., Markiel, A., Owen Ozier, 2, Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.*, 2498–2504. doi:10.1101/gr.1239303.metabolite.

Shaw, G. T. W., Pao, Y. Y., and Wang, D. (2016). MetaMIS: A metagenomic microbial interaction simulator based on microbial community profiles. *BMC Bioinformatics* 17, 1–12. doi:10.1186/s12859-016-1359-0.

Sikora, A., Błaszczyk, M., Jurkowski, M., and Zielenkiewicz, U. (2013). Lactic acid bacteria in hydrogen-producing consortia: on purpose or by coincidence? *Lact. Acid Bact. Food, Heal. Livest. Purp.*, 487–514. doi:10.5772/50364.

Singh, L., and Wahid, Z. A. (2015). Methods for enhancing bio-hydrogen production from biological process: A review. *J. Ind. Eng. Chem.* 21, 70–80. doi:10.1016/j.jiec.2014.05.035.

Srivastava, N., Srivastava, M., Kushwaha, D., Gupta, V. K., Manikanta, A., Ramteke, P. W., et al. (2017). Efficient dark fermentative hydrogen production from enzyme hydrolyzed rice straw by Clostridium pasteurianum (MTCC116). *Bioresour. Technol.* 238, 552–558.

doi:10.1016/j.biortech.2017.04.077.

Stenuit, B., and Agathos, S. N. (2015). Deciphering microbial community robustness through synthetic ecology and molecular systems synecology. *Curr. Opin. Biotechnol.* 33, 305–317. doi:10.1016/j.copbio.2015.03.012.

Tardy, V., Mathieu, O., Lévêque, J., Terrat, S., Chabbi, A., Lemanceau, P., et al. (2014). Stability of soil microbial structure and activity depends on microbial diversity. *Environ. Microbiol. Rep.* 6, 173–183. doi:10.1111/1758-2229.12126.

Thi Hoang, V., Huong Hoang, D., Duc Pham, N., My Tran, H., Thi Viet Bui, H., and Anh Ngo, T. (2018). Hydrogen production by newly isolated Clostridium species from cow rumen in pure- and co-cultures on a broad range of carbon sources. *AIMS Energy* 6, 846–865. doi:10.3934/energy.2018.5.846.

Valdez-Vazquez, I., Pérez-Rangel, M., Tapia, A., Buitrón, G., Molina, C., Hernández, G., et al. (2015). Hydrogen and butanol production from native wheat straw by synthetic microbial consortia integrated by species of Enterococcus and Clostridium. *Fuel* 159, 214–222. doi:10.1016/j.fuel.2015.06.052.

Valdez-Vazquez, I., and Poggi-Varaldo, H. M. (2009). Hydrogen production by fermentative consortia. *Renew. Sustain. Energy Rev.* 13, 1000–1013. doi:10.1016/j.rser.2008.03.003.

Wagg, C., Bender, S. F., Widmer, F., and Van Der Heijden, M. G. A. (2014). Soil biodiversity and soil community composition determine ecosystem multifunctionality. *Proc. Natl. Acad. Sci. U. S. A.* 111, 5266–5270. doi:10.1073/pnas.1320054111.

Wang, A., Gao, L., Ren, N., Xu, J., and Liu, C. (2009). Bio-hydrogen production from cellulose by sequential co-culture of cellulosic hydrogen bacteria of Enterococcus gallinarum G1 and Ethanoigenens harbinense B49. *Biotechnol. Lett.* 31, 1321–1326. doi:10.1007/s10529-009-0028-z.

Wang, J., and Yin, Y. (2017). Principle and application of different pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures. *Int. J. Hydrogen Energy* 42, 4804–

4823. doi:10.1016/j.ijhydene.2017.01.135.

Wang, S., Zhang, T., Bao, M., Su, H., and Xu, P. (2020). Microbial Production of Hydrogen by Mixed Culture Technologies: A Review. *Biotechnol. J.* 15, 1–8. doi:10.1002/biot.201900297.

Werner, J. J., Knights, D., Garcia, M. L., Scalfone, N. B., Smith, S., Yarasheski, K., et al. (2011). Bacterial community structures are unique and resilient in full-scale bioenergy systems. *Proc. Natl. Acad. Sci.* 108, 4158–4163. doi:10.1073/pnas.1015676108.

Wertz, S., Degrange, V., Prosser, J. I., Poly, F., Commeaux, C., Guillaumaud, N., et al. (2007). Decline of soil microbial diversity does not influence the resistance and resilience of key soil microbial functional groups following a model disturbance. *Environ. Microbiol.* 9, 2211–2219. doi:10.1111/j.1462-2920.2007.01335.x.

Xu, M., Li, X., Kuyper, T. W., Xu, M., Li, X., and Zhang, J. (2021). High microbial diversity stabilizes the responses of soil organic carbon decomposition to warming in the subsoil on the Tibetan Plateau. *Glob. Chang. Biol.* 27, 2061–2075. doi:10.1111/gcb.15553.

Yang, G., and Wang, J. (2019). Changes in microbial community structure during dark fermentative hydrogen production. *Int. J. Hydrogen Energy* 44, 25542–25550. doi:10.1016/j.ijhydene.2019.08.039.

Yin, Y., and Wang, J. (2019). Optimization of fermentative hydrogen production by Enterococcus faecium INET2 using response surface methodology. *Int. J. Hydrogen Energy* 44, 1483–1491. doi:10.1016/j.ijhydene.2018.11.154.

Conclusiones generales

El trabajo realizado durante esta investigación doctoral integró teoría ecológica a la investigación de la ingeniería de biorreactores productores de hidrógeno. Se realizaron aportes en el conocimiento de las comunidades productoras de hidrógeno que intentan, al mismo tiempo, ser generalizables para otras comunidades microbianas. Reconociendo a las comunidades microbianas como sistemas complejos, investigamos el papel que la diversidad, las condiciones de cultivo, la fisiología y las interacciones bióticas tienen en las propiedades a nivel comunidad. Adicionalmente, se espera inspirar a investigadores de los ramos de la biotecnología e ingeniería a considerar las ideas y teorías utilizadas.

El metaanálisis que presentamos en el Capítulo I nos permitió conocer el estado del conocimiento de la diversidad y la ecología de las comunidades de fermentación obscura y detectar áreas dónde hacía falta profundizar la investigación. Este trabajo fue fundamental para confirmar la idea que originó toda la investigación doctoral. En pocas palabras, los resultados de este metaanálisis demostraron la importancia de entender estas comunidades desde el enfoque ecológico. En particular, nos mostró la complejidad de las comunidades microbianas de producción de hidrógeno a pesar de su baja diversidad. Por una parte, dejo evidencia que la estabilidad de las comunidades era resultado tanto de las especies presentes como de sus interacciones. Por otra parte, nos mostró que, al estudiar las interacciones ecológicas en comunidades productoras de hidrógeno, es necesario enfocarnos en todos los grupos y no sólo en los que aparentemente son los más importantes funcionalmente. Grupos menos conocidos y frecuentemente pasados por alto mostraron ser importantes para determinar la abundancia y dinámicas poblacionales de las comunidades. A pesar de las limitaciones de nuestra aproximación, este trabajo fue la base para continuar explorando las numerosas avenidas por las cuales la integración de la investigación ecológica en procesos biotecnológicos puede mejorar su control y rendimiento.

Tomando como base lo aprendido en el Capítulo I, en el Capítulo II se realizó una propuesta de marco de trabajo para el estudio de comunidades productoras de hidrógeno. Después de revisar cientos de experimentos de biorreactores productores de hidrógeno para el metaanálisis del Capítulo I, nos fue posible identificar algunas áreas que podrían ser mejoradas para obtener resultados de mayor impacto y con mayor potencial de aplicarse en escenarios industriales. A pesar de que estos experimentos eran de gran calidad e interés, algunas características surgían continuamente que impedían obtener resultados generalizables. Específicamente, detectamos la falta de réplicas, de diseños experimentales contrastantes y de un bajo aprovechamiento de los resultados en la

generación de nuevas hipótesis aplicables a experimentos posteriores. Es importante mencionar que utilizamos un experimento típico de fermentación obscura como caso de estudio para mostrar la utilidad del marco de trabajo, pero las ideas y metodologías propuestas son aplicables para una gran diversidad de comunidades microbianas. Así, este trabajo tiene un gran potencial para generar un cambio de aproximación en el estudio ecológico de las comunidades microbianas productoras de hidrógeno (y otras comunidades similares) al promover la integración de la metodología usada en estudios ecológicos de alto impacto con el estudio de los parámetros operacionales y de rendimiento de biorreactores.

El trabajo que se describe en el Capítulo III representa la culminación de los esfuerzos realizados en esta investigación doctoral para integrar la ecología microbiana con la ingeniería de biorreactores. Estudiamos un parámetro ecológico de gran relevancia y con una extensa historia dentro de la ecología microbiana, la diversidad taxonómica. Demostramos cómo, al seguir los principios del marco de trabajo que propusimos en el Capítulo II, es posible comenzar a desentrañar los mecanismos ecológicos que subyacen el comportamiento de los reactores. Fue posible observar la predictibilidad de los procesos que ocurren en las comunidades microbianas en su dinámica taxonómica y funcional. En particular, se mostró que la pérdida de la diversidad taxonómica tuvo un efecto importante para determinar la estabilidad y función a mediano plazo de las comunidades. El hallazgo principal fue que el efecto se da a varios niveles ya que la alta diversidad promueve que inicialmente las múltiples interacciones bióticas retrasen la estabilización de la comunidad, pero que esta alta diversidad promueve una resistencia ante perturbaciones ambientales que son relevantes de forma industrial. Por otra parte, la alta diversidad parece disminuir la probabilidad de invasión, pero no necesariamente el efecto una vez que una especie invasora se establece. Finalmente, es importante mencionar que de nuestros resultados surgieron nuevas preguntas más específicas que no eran consideradas antes de realizar los experimentos y análisis de este trabajo. Esto demuestra que al realizar un experimento con esas características de diseño es posible planear futuras direcciones de investigación en un sistema de estudio de forma precisa. A continuación, se presentan las perspectivas y las preguntas que surgen de nuestros resultados para la investigación futura, tanto en el estudio de comunidades microbianas productoras, como en cuanto a las preguntas de ecología microbiana de forma general.

Perspectivas

Esta investigación doctoral representó, en varios aspectos, uno de los esfuerzos pioneros del estudio ecológico de las comunidades microbianas productoras de hidrógeno. Por esta razón, aunque se respondieron preguntas concretas, muchas nuevas preguntas surgieron de los resultados. A continuación, se presentan algunas propuestas de investigación a futuro que nacen de estas preguntas.

Capítulo I: Este capítulo nos mostró la importancia de realizar la revisión sistemática y cuantitativa de la información disponible acerca de fenómenos biológicos de interés. También hizo patente la necesidad de transitar de una caracterización, a manera de catálogo de la diversidad microbiana, hacia aproximaciones dedicadas a la prueba de hipótesis ecológicas y evolutivas. En este sentido será importante seguir integrando y generando hipótesis ecológicas al estudio de los biorreactores productores de hidrógeno. Finalmente, y debido a que se genera nueva información por las decenas de experimentos publicados cada año, es muy relevante repetir la revisión cuantitativa de esta información de forma periódica para formalizar los avances en el conocimiento y seguir detectando áreas específicas dónde hace falta realizar investigación.

Capítulo II: Al proponer un marco de trabajo, como lo hicimos en el Capítulo II, se tiene que reconocer que las metodologías propuestas tienen ciertas limitaciones. Actualmente, algunas de las limitaciones de estas metodologías (que son aplicables para cualquier investigación en ecología microbiana) tienen que ver con el poco conocimiento que tenemos de muchas especies microbianas. Por esta razón, es necesario seguir, al menos, dos avenidas que permitan realizar modelos más precisos de las comunidades microbianas. Por una parte, realizar el aislamiento y caracterización de cepas bacterianas es muy importante para conocer el metabolismo, interacciones y funciones de los organismos que se identifiquen en comunidades microbianas. Por otra parte, el mejoramiento de los métodos estadísticos y computacionales es importante para mejorar la identificación de especies e interacciones relevantes y mejorar el modelado de las comunidades.

Capítulo III: Los resultados de este capítulo confirmaron que los procesos ecológicos que ocurren en las comunidades microbianas son altamente complejos. En particular, observamos que el efecto de las variaciones de diversidad en el funcionamiento de las comunidades no es fácil de predecir. En este caso, realizar experimentos con una combinación de metodologías tanto microbiológicas, moleculares y bioinformáticas ayudarán a confirmar las observaciones realizadas aquí. En particular, es necesario confirmar el papel de los grupos bacterianos y de sus interacciones.

También es importante utilizar condiciones experimentales más cercanas a las condiciones industriales que se planean usar para determinar cómo afectan los componentes de la diversidad (como la riqueza y la abundancia de especies) y la estabilidad y probabilidad de invasión de las comunidades. Esta información nos permitirá mejorar el diseño y control de consorcios microbianos en ambientes biotecnológicos y entender la respuesta de las comunidades naturales ante escenarios de perturbación.

Referencias

Abreu, N. A., and Taga, M. E. (2016). Decoding molecular interactions in microbial communitiesa. *FEMS Microbiol. Rev.* 40, 648–663. doi:10.1093/femsre/fuw019.

Aguirre-von-Wobeser, E., Soberón-Chávez, G., Eguiarte, L. E., Ponce-Soto, G. Y., Vázquez-Rosas-Landa, M., and Souza, V. (2014). Two-role model of an interaction network of free-living γ-proteobacteria from an oligotrophic environment. *Environ. Microbiol.* 16, 1366–1377. doi:10.1111/1462-2920.12305.

Allison, S. D., and Martiny, J. B. H. (2009). Resistance, resilience, and redundancy in microbial communities. *Light Evol.* 2, 149–166. doi:10.17226/12501.

Amor, D. R., and Bello, M. D. (2019). Bottom-up approaches to synthetic cooperation in microbial communities. *Life* 9. doi:10.3390/life9010022.

Barton, Larry, L., and Northup, Diana, E. (2011). *Microbial Ecology*.

Bauer, E., and Thiele, I. (2018). From Network Analysis to Functional Metabolic Modeling of the Human Gut Microbiota. *mSystems*. doi:10.1128/mSystems.00209-17.

Bauer, E., Zimmermann, J., Baldini, F., Thiele, I., and Kaleta, C. (2017). BacArena: Individual-based metabolic modeling of heterogeneous microbes in complex communities. *PLoS Comput. Biol.* 13, e1005544. doi:10.1371/journal.pcbi.1005544.

Berg, N. I. Van Den, Machado, D., Santos, S., Rocha, I., Chacón, J., Harcombe, W., et al. (2022). Ecological modelling approaches for predicting emergent properties in microbial communities. *Nat. Ecol. Evol.* doi:10.1038/s41559-022-01746-7.

Bertrand, J.-C., Caumette, P., Lebaron, P., Matheron, R., Normand, P., and Sime-Ngando, T. (2015). *Environmental Microbiology: Fundamentals and Applications Microbial Ecology*.

Birch, L. C. (1957). The Meanings of Competition. *Am. Nat.* 91, 5–18.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi:10.1038/s41587-019-0209-9.

Bonachela, J. A., Wortel, M. T., and Stenseth, N. C. (2017). Eco-evolutionary Red Queen dynamics regulate biodiversity in a metabolite-driven microbial system. *Sci. Rep.* 7, 1–9. doi:10.1038/s41598-017-17774-4.

Burton, A. (2011). It's life, Martinus, but not as we know it. *Front. Ecol. Environ.* 9, 84. doi:https://doi.org/10.1890/1540-9295-9.1.84.

Buttigieg, P. L., and Ramette, A. (2014). A guide to statistical analysis in microbial ecology: A community-focused, living review of multivariate data analyses. *FEMS Microbiol. Ecol.* 90, 543–550. doi:10.1111/1574-6941.12437.

Cabrol, L., Marone, A., Tapia-Venegas, E., Steyer, J. P., Ruiz-Filippi, G., and Trably, E. (2017). Microbial ecology of fermentative hydrogen producing bioprocesses: Useful insights for driving the ecosystem function. *FEMS Microbiol. Rev.* 41, 158–181. doi:10.1093/femsre/fuw043.

Carr, A., Diener, C., Baliga, N. S., and Gibbons, S. M. (2019). Use and abuse of correlation analyses in microbial ecology. *ISME J.* 13, 2647–2655. doi:10.1038/s41396-019-0459-z.

Case, R. J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W. F., and Kjelleberg, S. (2007). Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. *Appl. Environ. Microbiol.* 73, 278–288. doi:10.1128/AEM.01177-06.

Castelló, E., Nunes Ferraz-Junior, A. D., Andreani, C., Anzola-Rojas, M. del P., Borzacconi, L., Buitrón, G., et al. (2020). Stability problems in the hydrogen production by dark fermentation: Possible causes and solutions. *Renew. Sustain. Energy Rev.* 119. doi:10.1016/j.rser.2019.109602.

Cavender-Bares, J., Kozak, K. H., Fine, P. V. A., and Kembel, S. W. (2009). The merging of community ecology and phylogenetic biology. *Ecol. Lett.* 12, 693–715. doi:10.1111/j.1461-0248.2009.01314.x.

Chandra, R., Takeuchi, H., and Hasegawa, T. (2012). Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production. *Renew. Sustain. Energy Rev.* 16, 1462–1476. doi:10.1016/j.rser.2011.11.035.

Cordero, O. X., and Datta, M. S. (2016). Microbial interactions and community assembly at microscales. *Curr. Opin. Microbiol.* 31, 227–234. doi:10.1016/j.mib.2016.03.015.

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M., and Relman, D. A. (2012). The application of ecological theory toward an understanding of the human microbiome. *Science (80-.).* 336, 1255–1262. doi:10.1126/science.1224203.

D'Souza, G., Shitut, S., Preussger, D., Yousif, G., Waschina, S., and Kost, C. (2018). Ecology and evolution of metabolic cross-feeding interactions in bacteria. *Nat. Prod. Rep.* 35, 455–488. doi:10.1039/c8np00009c.

De Cáceres, M., Legendre, P., and Moretti, M. (2010). Improving indicator species analysis by combining groups of sites. *Oikos* 119, 1674–1684. doi:10.1111/j.1600-0706.2010.18334.x.

de Corato, U. (2020). Towards new soil management strategies for improving soil quality and ecosystem services in sustainable agriculture: Editorial overview. *Sustain.* 12, 1–5. doi:10.3390/su12229398.

De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., and Boon, N. (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environ. Microbiol.* 16, 1472–1481. doi:10.1111/1462-2920.12343.

Deng, Y., Jiang, Y. H., Yang, Y., He, Z., Luo, F., and Zhou, J. (2012). Molecular ecological network

analyses. *BMC Bioinformatics* 13. doi:10.1186/1471-2105-13-113.

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., and Xia, J. (2017). MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* 45, W180–W188. doi:10.1093/nar/gkx295.

Dworkin, M. (2012). Sergei Winogradsky: A founder of modern microbiology and the first microbial ecologist. *FEMS Microbiol. Rev.* 36, 364–379. doi:10.1111/j.1574-6976.2011.00299.x.

Elena, C., Stefania, P., Alessandra, C., Collins, O., Marina, R., and Lorenzo, B. (2016). Ecosystem services and bioremediation of polluted areas. *Ecol. Eng.* 87, 139–149. doi:10.1016/j.ecoleng.2015.09.045.

Escalante, A. E., Rebolleda-Gómez, M., Benítez, M., and Travisano, M. (2015). Ecological perspectives on synthetic biology: Insights from microbial population biology. *Front. Microbiol.* 6, 1–10. doi:10.3389/fmicb.2015.00143.

Escalas, A., Hale, L., Voordeckers, J. W., Yang, Y., Firestone, M. K., Alvarez-Cohen, L., et al. (2019). Microbial functional diversity: From concepts to applications. *Ecol. Evol.* 9, 12000–12016. doi:10.1002/ece3.5670.

Falkowski, P. G., Fenchel, T., and Delong, E. F. (2008). The microbial engines that drive earth's biogeochemical cycles. *Science (80-.).* 320, 1034–1039. doi:10.1126/science.1153213.

Faust, K., and Raes, J. (2016). CoNet app: inference of biological association networks using Cytoscape. *F1000Research* 5, 1519. doi:10.12688/f1000research.9050.2.

Faust, K., Sathirapongsasuti, J. F., Izard, J., Segate, N., Gevers, D., Raes, J., et al. (2012). Microbial Co-occurrence Relationships in the Human Microbiome. *PLoS Comput. Biol.* 8. doi:10.1371/journal.pcbi.1002606.

Fiegna, F., Moreno-Letelier, A., Bell, T., and Barraclough, T. G. (2015). Evolution of species interactions determines microbial community productivity in new environments. *ISME J.* 9, 1235–1245. doi:10.1038/ismej.2014.215.

Forcino, F. L., Leighton, L. R., Twerdy, P., and Cahill, J. F. (2015). Reexamining sample size requirements for multivariate, abundance-based community research: When resources are limited, the research does not have to be. *PLoS One* 10, 1–18. doi:10.1371/journal.pone.0128379.

Franzosa, E. A., Sirota-Madi, A., Avila-Pacheco, J., Fornelos, N., Haiser, H. J., Reinker, S., et al. (2019). Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat. Microbiol.* 4, 293–305. doi:10.1038/s41564-018-0306-4.

Friedman, J., Higgins, L. M., and Gore, J. (2017). Community structure follows simple assembly rules in microbial microcosms. *Nat. Ecol. Evol.* 1, 1–7. doi:10.1038/s41559-017-0109.

Gause, G. F. (1932). Experimental Studies on the Struggle for Existence. *J. Exp. Biol.* 9, 389–402. doi:10.1242/jeb.9.4.389.

Germerodt, S., Bohl, K., Lück, A., Pande, S., Schröter, A., Kaleta, C., et al. (2016). Pervasive Selection for Cooperative Cross-Feeding in Bacterial Communities. *PLoS Comput. Biol.* 12, 1–21. doi:10.1371/journal.pcbi.1004986.

Ghosh, S., Chowdhury, R., and Bhattacharya, P. (2016). Mixed consortia in bioprocesses: role of microbial interactions. *Appl. Microbiol. Biotechnol.* 100, 4283–4295. doi:10.1007/s00253-016-7448-1.

Ghoul, M., and Mitri, S. (2016). The Ecology and Evolution of Microbial Competition. *Trends Microbiol.* 24, 833–845. doi:10.1016/j.tim.2016.06.011.

Goldford, J. E., Lu, N., Bajić, D., Estrela, S., Tikhonov, M., Sanchez-Gorostiaga, A., et al. (2018). Emergent simplicity in microbial community assembly. *Science (80-.).* 361, 469–474. doi:10.1126/science.aat1168.

Gorter, F. A., Manhart, M., and Ackermann, M. (2020). Understanding the evolution of interspecies interactions in microbial communities. *Philos. Trans. R. Soc. B Biol. Sci.* 375. doi:10.1098/rstb.2019.0256.

Gray, N. D., and Head, I. M. (2008). Microbial Ecology. *Science (80-.).* 201, 520–520. doi:10.1126/science.201.4355.520.

Griffith, D. M., Veech, J. A., and Marsh, C. J. (2016). Cooccur: Probabilistic species co-occurrence analysis in R. *J. Stat. Softw.* 69, 1–17. doi:10.18637/jss.v069.c02.

Hardin, G. (1960). The competitive exclusion principle. *Science (80-.).* 131, 1292–1297. doi:10.1126/science.131.3409.1292.

Hugerth, L. W., and Andersson, A. F. (2017). Analysing microbial community composition through amplicon sequencing: From sampling to hypothesis testing. *Front. Microbiol.* 8, 1–22. doi:10.3389/fmicb.2017.01561.

Hungate, R. (1960). Symposium: selected topics in microbial ecology. I. Microbial ecology of the rumen. *Bacteriol. Rev.* 24, 353–364. doi:https://doi.org/10.1128/br.24.4.353-364.

Jansma, J., and Aidy, S. El (2020). Understanding the host-microbe interactions using metabolic modeling. *bioRxiv*, 1–14. doi:10.1101/2020.06.12.147918.

Jeffrey Morris, J., Papoulis, S. E., and Lenski, R. E. (2014). Coexistence of evolving bacteria stabilized by a shared Black Queen function. *Evolution (N. Y).* 68, 2960–2971. doi:10.1111/evo.12485.

Jiang, H., Gadow, S. I., Tanaka, Y., Cheng, J., and Li, Y. Y. (2015). Improved cellulose conversion to bio-hydrogen with thermophilic bacteria and characterization of microbial community in continuous bioreactor. *Biomass and Bioenergy* 75, 57–64. doi:10.1016/j.biombioe.2015.02.010.

Jobard, M., Pessiot, J., Nouaille, R., and Sime-Ngando, T. (2014). Microbial diversity supporting dark fermentation of waste. *Trends Biotechnol.* 32, 549–550. doi:10.1016/j.tibtech.2014.09.005.

Kato, S., Haruta, S., Cui, Z. J., Ishii, M., and Igarashi, Y. (2008). Network relationships of bacteria in a stable mixed culture. *Microb. Ecol.* 56, 403–411. doi:10.1007/s00248-007-9357-4.

Klassen, J. L. (2018). Defining microbiome function. *Nat. Microbiol.* 3, 864–869. doi:10.1038/s41564-018-0189-4.

Ladau, J., and Eloe-Fadrosh, E. A. (2019). Spatial, Temporal, and Phylogenetic Scales of Microbial Ecology. *Trends Microbiol.* 27, 662–669. doi:10.1016/j.tim.2019.03.003.

Lagier, J. C., Hugon, P., Khelaifia, S., Fournier, P. E., La Scola, B., and Raoult, D. (2015). The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. *Clin. Microbiol. Rev.* 28, 237–264. doi:10.1128/CMR.00014-14.

Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl. Environ. Microbiol.* 75, 5111–5120. doi:10.1128/AEM.00335-09.

Liu, M., West, S. A., and Cooper, G. A. (2021). Relatedness and the evolution of mechanisms to divide labor in microorganisms. *Ecol. Evol.* 11, 14475–14489. doi:10.1002/ece3.8067.

Louca, S., Polz, M. F., Mazel, F., Albright, M. B. N., Huber, J. A., O'Connor, M. I., et al. (2018). Function and functional redundancy in microbial systems. *Nat. Ecol. Evol.* 2. doi:10.1038/s41559-018-0519-1.

Madigan, M. T., Martinko, J. M., Bender, K. S., Buckley, D. H., and Stahl, D. A. (2015). *Brock biology of microorganisms*.

Madsen, J. S., Sørensen, S. J., and Burmølle, M. (2018). Bacterial social interactions and the emergence of community-intrinsic properties. *Curr. Opin. Microbiol.* 42, 104–109. doi:10.1016/j.mib.2017.11.018.

Mainali, K. P., Bewick, S., Thielen, P., Mehoke, T., Breitwieser, F. P., Paudel, S., et al. (2017). Statistical analysis of co-occurrence patterns in microbial presence-absence datasets. *PLoS One* 12, 1–21. doi:10.1371/journal.pone.0187132.

McKenney, E. A., Koelle, K., Dunn, R. R., and Yoder, A. D. (2018). The ecosystem services of animal microbiomes. *Mol. Ecol.* 27, 2164–2172. doi:10.1111/mec.14532.

Mitsuoka, T. (2014). Establishment of intestinal bacteriology. *Biosci. Microbiota, Food Heal.* 33, 99–116. doi:10.12938/bmfh.33.99.

Morris, A. H., Meyer, K. M., and Bohannan, B. J. M. (2020). Linking microbial communities to ecosystem functions: What we can learn from genotype-phenotype mapping in organisms. *Philos. Trans. R. Soc. B Biol. Sci.* 375. doi:10.1101/740373.

Morris, J. J. (2015). Black Queen evolution: The role of leakiness in structuring microbial communities. *Trends Genet.* 31, 475–482. doi:10.1016/j.tig.2015.05.004.

Morris, J. J., Lenski, R. E., and Zinser, E. R. (2012). The black queen hypothesis: Evolution of dependencies through adaptive gene loss. *MBio* 3, 1–7. doi:10.1128/mBio.00036-12.

Müller, V. (2003). Energy Conservation in Acetogenic Bacteria. *Appl. Environ. Microbiol.* 69, 6345–6353. doi:10.1128/AEM.69.11.6345-6353.2003.

Nai, C., and Meyer, V. (2018). From Axenic to Mixed Cultures: Technological Advances Accelerating a Paradigm Shift in Microbiology. *Trends Microbiol.* 26, 538–554. doi:10.1016/j.tim.2017.11.004.

Netzker, T., Flak, M., Krespach, M. K., Stroe, M. C., Weber, J., Schroeckh, V., et al. (2018). Microbial interactions trigger the production of antibiotics. *Curr. Opin. Microbiol.* 45, 117–123. doi:10.1016/j.mib.2018.04.002.

Nobu, M. K., Narihiro, T., Rinke, C., Kamagata, Y., Tringe, S. G., Woyke, T., et al. (2015). Microbial dark matter ecogenomics reveals complex synergistic networks in a methanogenic bioreactor. *ISME J.* 9, 1710–1722. doi:10.1038/ismej.2014.256.

Oberbeckmann, S., Osborn, A. M., and Duhaime, M. B. (2016). Microbes on a bottle: Substrate, season and geography influence community composition of microbes colonizing marine plastic debris. *PLoS One* 11, 1–24. doi:10.1371/journal.pone.0159289.

Paliy, O., and Shankar, V. (2016). Application of multivariate statistical techniques in microbial ecology. *Mol. Ecol.* 25, 1032–1057. doi:10.1111/mec.13536.

Prosser, J. I. (2020). Putting science back into microbial ecology: A question of approach. *Philos. Trans. R. Soc. B Biol. Sci.* 375. doi:10.1098/rstb.2019.0240.

Ramette, A. (2007). Multivariate analyses in microbial ecology. *FEMS Microbiol. Ecol.* 62, 142–160. doi:10.1111/j.1574-6941.2007.00375.x.

Ratzke, C., Barrere, J., and Gore, J. (2020). Strength of species interactions determines biodiversity and stability in microbial communities. *Nat. Ecol. Evol.* 4, 376–383. doi:10.1038/s41559-020-1099-4.

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., et al. (2016). Using “omics” and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.* 7, 68. doi:10.3389/fmicb.2016.00068.

Riley, M. A., and Wertz, J. E. (2002). Bacteriocins: Evolution, ecology, and application. *Annu. Rev. Microbiol.* 56, 117–137. doi:10.1146/annurev.micro.56.012302.161024.

Schmidt, R., Cordovez, V., de Boer, W., Raaijmakers, J., and Garbeva, P. (2015). Volatile affairs in microbial interactions. *ISME J.* 9, 2329–2335. doi:10.1038/ismej.2015.42.

Shade, A., Jones, S. E., Gregory Caporaso, J., Handelsman, J., Knight, R., Fierer, N., et al. (2014). Conditionally rare taxa disproportionately contribute to temporal changes in microbial diversity. *MBio* 5, 1–9. doi:10.1128/mBio.01371-14.

Shaw, G. T. W., Pao, Y. Y., and Wang, D. (2016). MetaMIS: A metagenomic microbial interaction simulator based on microbial community profiles. *BMC Bioinformatics* 17, 1–12. doi:10.1186/s12859-016-1359-0.

Singh, J. S. (2015). Microbes Play Major Roles in the Ecosystem Services. *Clim. Chang. Environ. Sustain.* 3, 163. doi:10.5958/2320-642x.2015.00018.6.

Syms, C. (2008). "Ordination," in *Encyclopedia of Ecology*, eds. S. E. Jørgensen and B. D. Fath (Academic Press), 2572–2581. doi:https://doi.org/10.1016/B978-008045405-4.00524-3.

Tan, J., Zuniga, C., and Zengler, K. (2015). Unraveling interactions in microbial communities - from co-cultures to microbiomes. *J. Microbiol.* 53, 295–305. doi:10.1007/s12275-015-5060-1.

Vandermeer, J. H. (1969). The Competitive Structure of Communities: An Experimental Approach with Protozoa. *Ecology* 50, 362–371. doi:10.2307/1933884.

Vanwonterghem, I., Jensen, P. D., Ho, D. P., Batstone, D. J., and Tyson, G. W. (2014). Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. *Curr. Opin. Biotechnol.* 27, 55–64. doi:10.1016/j.copbio.2013.11.004.

Vasconcelos, E. A. F., Leitão, R. C., and Santaella, S. T. (2016). Factors that affect bacterial ecology in hydrogen-producing anaerobic reactors. *Bioenergy Res.* 9, 1260–1271. doi:10.1007/s12155-016-9753-z.

Vavilin, V. A., Fernandez, B., Palatsi, J., and Flotats, X. (2008). Hydrolysis kinetics in anaerobic degradation of particulate organic material: An overview. *Waste Manag.* 28, 939–951. doi:10.1016/j.wasman.2007.03.028.

Vrancken, G., Gregory, A. C., Huys, G. R. B., Faust, K., and Raes, J. (2019). Synthetic ecology of the human gut microbiota. *Nat. Rev. Microbiol.* 17, 754–763. doi:10.1038/s41579-019-0264-8.

Wainwright, M. (2003). An alternative view of the early history of microbiology. *Adv. Appl. Microbiol.* 52, 333–355. doi:10.1016/S0065-2164(03)01013-X.

Wang, H., Wei, Z., Mei, L., Gu, J., Yin, S., Faust, K., et al. (2017). Combined use of network inference tools identifies ecologically meaningful bacterial associations in a paddy soil. *Soil Biol. Biochem.* 105, 227–235. doi:10.1016/j.soilbio.2016.11.029.

Wang, J., and Yin, Y. (2017). Principle and application of different pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures. *Int. J. Hydrogen Energy* 42, 4804–4823. doi:10.1016/j.ijhydene.2017.01.135.

Wang, S., Zhang, T., Bao, M., Su, H., and Xu, P. (2020). Microbial Production of Hydrogen by Mixed

Culture Technologies: A Review. *Biotechnol. J.* 15, 1–8. doi:10.1002/biot.201900297.

Wang, Y., Xu, L., Wang, S., Ye, F., and Zhu, G. (2019). Global Distribution of Anaerobic Ammonia Oxidation (Anammox) Bacteria – Field Surveys in Wetland, Dryland, Groundwater Aquifer and Snow. *Front. Microbiol.* 10, 1–12. doi:10.3389/fmicb.2019.02583.

Weiland, P. (2010). Biogas production: Current state and perspectives. *Appl. Microbiol. Biotechnol.* 85, 849–860. doi:10.1007/s00253-009-2246-7.

Weiss, S., Van Treuren, W., Lozupone, C., Faust, K., Friedman, J., Deng, Y., et al. (2016). Correlation detection strategies in microbial data sets vary widely in sensitivity and precision. *ISME J.* 10, 1669–1681. doi:10.1038/ismej.2015.235.

West, S. A., and Cooper, G. A. (2016). Division of labour in microorganisms: an evolutionary perspective. *Nat. Rev. Microbiol.* 14, 716–723. doi:10.1038/nrmicro.2016.111.

West, S. A., Diggle, S. P., Buckling, A., Gardner, A., and Griffin, A. S. (2007a). The Social Lives of Microbes. *Annu. Rev. Ecol. Evol. Syst.* 38, 53–77. doi:10.1146/annurev.ecolsys.38.091206.095740.

West, S. A., Griffin, A. S., and Gardner, A. (2007b). Evolutionary Explanations for Cooperation. *Curr. Biol.* 17, 661–672. doi:10.1016/j.cub.2007.06.004.

Wilhelm, R. C., Singh, R., Eltis, L. D., and Mohn, W. W. (2019). Bacterial contributions to delignification and lignocellulose degradation in forest soils with metagenomic and quantitative stable isotope probing. *ISME J.* 13, 413–429. doi:10.1038/s41396-018-0279-6.