UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE INVESTIGACIONES BIOMÉDICAS, UNAM.

CARACTERIZACIÓN MICROBIOLÓGICA, BIOQUÍMICA Y PROTEÓMICA DEL POZOL

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

JOCELIN MARARI RIZO VILLAGRANA

TUTORA PRINCIPAL DE TESIS: DRA. ROMINA MA. DE LA PAZ RODRÍGUEZ SANOJA
Instituto de Investigaciones Biomédicas, UNAM.
COMITÉ TUTOR: DRA. MARÍA DEL CARMEN WACHER RODARTE
Facultad de Química, UNAM.
COMITÉ TUTOR: DR. SERGIO MANUEL ENCARNACIÓN GUEVARA
Centro de Ciencias Genómica, UNAM.

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P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **08 de febrero de 2021** se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante **RIZO VILLAGRANA JOCELIN MARARI** con número de cuenta **302179295** con la tesis titulada **"Caracterización microbiológica, bioquímica y proteómica del pozol",** bajo la dirección de la **DRA. ROMINA MARÍA DE LA PAZ RODRÍGUEZ SANOJA**, Tutora Principal, quedando integrado de la siguiente manera:

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Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Cd. Universitaria, Cd. Mx., a 28 de abril de 2021

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

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RESUMEN

El pozol es una bebida de origen maya, ácida, refrescante y no alcohólica, elaborada con masa de maíz nixtamalizada y fermentada de forma natural. Mediante diversos estudios microbiológicos se ha demostrado que en este alimento se desarrolla una microbiota muy diversa y abundante, que es capaz de crecer en un sistema donde el almidón es la principal fuente de carbono fermentable y, sin embargo, gran parte de los microorganismos descritos en el sistema no son capaces de utilizar el almidón. Además, diversos estudios han reportado beneficios desde el punto de vista nutrimental, específicamente el incremento en el contenido de nitrógeno de esta bebida durante la fermentación y en consecuencia de proteína cruda, lo que sugiere fijación de nitrógeno atmosférico fenómeno que no ha sido demostrado.

A través de un enfoque multidisciplinario que involucró el análisis microbiológico, químico, bioquímico y metaproteómico, se estudiaron los cambios que se generan durante la fermentación del pozol, se determinaron cuáles son las fuentes de carbono y nitrógeno que se utilizan, y finalmente, se generó un mapa metabólico del sistema de manera integral que permitió elucidar el potencial metabólico de la microbiota.

Se demostró la fijación de nitrógeno por bacterias fijadoras de vida libre a través de la detección de reducción de acetileno directamente en el sustrato, el aislamiento de diazótrofos potenciales y su reinoculación en la masa para establecer fehacientemente que el fenómeno sucede en el sustrato. Las bacterias identificadas pertenecen a la familia *Enterobacteriaceae* y fueron identificadas como *Kosakonia*, *Klebsiella* y *Enterobacter*.

Mediante el análisis metaproteómico, se demostró que el pozol es un ecosistema complejo donde encontramos proteínas de todos los dominios de la vida, incluyendo *Archaea*, cuya presencia no había sido reportada con anterioridad. Al comparar las proteínas a nivel de género se observó que durante las primeras 9 h se dan importantes cambios en la microbiota, que coinciden con la mayor disminución de pH y del contenido de carbohidratos, lo que sugiere que esta es la etapa de mayor actividad metabólica. En este periodo se establecieron las bacterias lácticas como predominantes, con *Streptococcus* como dominante y *Lactobacillus*, *Leuconostoc* y *Enterococcus* como participantes secundarios en este proceso. En el caso de los hongos, las proteínas de las levaduras son las más abundantes, mientras que las proteínas de los hongos filamentosos aumentaron a partir de las 48 h de fermentación. En el sistema de anotación KEGG, se encontraron 1102 proteínas relacionadas a distintas vías metabólicas de carbohidratos, aminoácidos, lípidos y vitaminas. Se identificaron las enzimas necesarias para la utilización de almidón, celulosa y hemicelulosa; además de los transportadores de mono-, di- y oligosacáridos. Finalmente, se identificaron proteínas relacionadas a rutas de producción de aminoácidos y sabores lo que demuestra el potencial funcional de la microbiota del pozol.

ABSTRACT

Pozol is a Mayan refreshing, nonalcoholic acidic beverage made of nixtamalized maize dough naturally fermented. Different microbiological studies has been shown that a diverse and abundant microbiota develops in this food, which is capable of growing in a system where starch is the main fermentable source, however, not all the microorganisms hydrolyze it. Also, in various studies has been reported an increase in nitrogen content and consequently in crude protein, which suggests atmospheric nitrogen fixation; a phenomenon that has not been demonstrated nitrogen and carbon sources that are used were determined. Nitrogen fixation by free-living fixing bacteria was demonstrated through the detection of acetylene reduction directly in the substrate, the isolation of potential diazotrophs and their re-circulation in the dough to establish that the phenomenon occurs in the substrate. The identified bacteria were *Kosakonia*, *Klebsiella* and *Enterobacter*. Pozol was found to be a complex ecosystem where proteins from all domains of life were identified, including Archaea whose presence had not been reported previously. The metaproteomic analysis by fermentation time showed that during the first 9 hours there are important changes in the microbiota, which correlated with the decrease in the pH and carbohydrate content. During this period the maximum metabolic activity occurs, lactic acid bacteria are established as predominant, with *Streptococcus* as dominant and *Lactobacillus*, *Leuconostoc* and *Enterococcus* as secondary participants in this process. In the case of fungi, yeast proteins are the most abundant, while filamentous fungi proteins increase after 48 hours of fermentation. Based on the KEGG annotation system, 1102 proteins were related to different metabolic pathways, including the metabolism of carbohydrates, amino acids, lipids and vitamins. Also, the enzymes for the hydrolysis of starch, cellulose and hemicellulose were identified; in addition to the transporters of mono-, di- and oligosaccharides. Finally, proteins related to the amino acid and flavor and aroma compounds production were identified, demonstrating the functional potential of the pozol microbiota.

INTRODUCCIÓN

Los alimentos fermentados pueden ser definidos como aquellos en los que la actividad enzimática de los microorganismos provoca cambios químicos en los diferentes sustratos que son utilizados para su elaboración. El proceso de fermentación contribuye a la conservación de los alimentos, a enriquecer la dieta con una gran cantidad de alimentos diferentes que poseen sabores, aromas y texturas agradables para el consumidor. Adicionalmente, la fermentación tiene un papel importante en la mejora del valor nutricional y por lo tanto su potencial uso para combatir la desnutrición y problemas de salud tan importantes como la obesidad y en la generación de compuestos bioactivos que fomentan la salud (Steinkraus, 2002).

El estudio de los alimentos fermentados es de suma importancia, sobre todo de aquellos que se elaboran en pequeñas comunidades, ya que se corre el riesgo de que la gran riqueza de ingredientes usados en estos alimentos que existen desaparezca con el tiempo y es claro que la pérdida de la cultura alimentaria tradicional ha resultado en la proliferación y consumo de alimentos hipercalóricos y pobres en nutrientes, que tienen un efecto negativo en la salud.

El pozol es una bebida de origen maya, ácida, refrescante y no alcohólica hecha a base de maíz nixtamalizado, consumido por varios grupos étnicos del sur y sureste de México. Constituye un alimento básico dentro de las poblaciones que lo consumen y tiene importantes usos en la medicina tradicional (Cañas *et al*. 1993).

A diferencia de otros alimentos fermentados a base de maíz, consumidos principalmente en África, el proceso de elaboración del pozol involucra la nixtamalización de los granos de maíz, lo que genera cambios importantes en el grano, entre los que destacan la eliminación de prácticamente todos los carbohidratos solubles, dejando al almidón como el principal sustrato para permitir la fermentación. Diferentes estudios se han enfocado en la búsqueda e identificación de bacterias amilolíticas y de sus enzimas (Nuraida *et al*. 1995; Díaz-Ruiz *et al*. 2003), mostrando que la actividad amilolítica de las bacterias encontradas es menor a la reportada para otras bacterias lácticas aislada de fermentaciones vegetales, por lo que difícilmente la hidrólisis del almidón puede proveer azúcares solubles para el crecimiento de los microorganismos (Díaz-Ruiz *et al*. 2003). En consecuencia, no se entiende que fuente de carbono puede sostener una microbiota tan abundante y diversa.

En este contexto, es necesario replantearse que además del almidón, la celulosa y hemicelulosa procedentes del pericarpio pueden ser fuentes de carbono utilizadas para su crecimiento. Así, se

ha demostrado que *Streptococcus infantarius* ssp. *infantarius* es capaz de crecer en xilano como única fuente de carbono gracias a su actividad xilanolítica (Cooper-Bribiesca *et al*. 2018).

Por otro lado, el incremento de nitrógeno total y la presencia de bacterias proteolíticas a lo largo de la fermentación (Cravioto *et al*. 1955; Ulloa *et al*. 1971; Ramírez, 1987; Loaeza, 1991; Giles, 1995; Rizo, 2015), sugieren que mediante la fijación de nitrógeno atmosférico y/o la hidrólisis de proteínas, los microorganismos obtienen el nitrógeno necesario para la síntesis de diferentes moléculas que permiten su crecimiento. Sin embargo, no existen estudios detallados sobre las bacterias proteolíticas y en su momento, no fue posible continuar con los estudios para demostrar que la fijación de nitrógeno ocurre en el pozol.

Mediante un enfoque multidisciplinario, en este proyecto se realizó el estudio integral de la fermentación del pozol, para determinar la estructura y los cambios de las comunidades microbianas y cómo estas modifican y utilizan el sustrato. Para ello se plantearon dos objetivos: 1) análisis químico y microbiológico del pozol en diferentes etapas de la fermentación y 2) análisis del metaproteoma en diferentes etapas de la fermentación.

En el primer capítulo se plantean los antecedentes con los trabajos que se han realizado hasta el momento en este alimento. El segundo capítulo presenta una revisión acerca de las ciencias ómicas para el estudio de los alimentos fermentados tradicionales, donde se muestran diferentes ejemplos y los principales resultados que se obtienen mediante el uso de estas herramientas. En el tercer capítulo se presenta el artículo, donde se demuestra que en el pozol existen las condiciones necesarias para que se dé la fijación de nitrógeno atmosférico. El cuarto capítulo incluye el artículo en el que se compilan los datos microbiológicos, químicos y metaproteómicos que permiten establecer la composición de la microbiota y sus cambios durante la fermentación del pozol, sus principales actividades enzimáticas y su papel funcional en el alimento. Finalmente, se presenta una discusión general de los resultados obtenidos.

Capítulo primero

Antecedentes

1. Alimentos fermentados

La fermentación es una de las prácticas más antiguas utilizadas para la preparación y conservación de los alimentos, se ha desarrollado en todas las culturas del mundo, pero destaca entre otros procesos, debido al impacto nutricional y a la gran variedad de alimentos que se pueden producir. Su origen está estrechamente relacionado con la evolución humana y es seguramente producto de la necesidad de conservar los alimentos que se recolectaban (frutas, vegetales y cereales), cazaban y pescaban.

Existe una amplia variedad de alimentos fermentados resultado del conocimiento tradicional que se ha ido transmitiendo de generación en generación. Los alimentos fermentados más antiguos que se conocen se elaboran a partir de cereales, frutas, plantas, leche y pescado, y algunos de ellos siguen consumiéndose en la actualidad mediante un proceso de elaboración que ha sufrido pocas modificaciones.

La fermentación de los alimentos ofrece los siguientes beneficios: 1) la conservación del alimento; 2) la eliminación de factores antinutricionales; 3) la mejora de las características organolépticas; 4) el enriquecimiento del sustrato con una gran variedad de metabolitos asociados con propiedades que promueven la salud; 5) el aumento de la digestibilidad del sustrato y; 6) la disminución de los tiempos de cocción (Tamang *et al*. 2016; Marco *et al*. 2017; van Boekel *et al*. 2010; Poutanen, Flander y Katina, 2009; Charalampopoulos *et al*. 2002).

Los alimentos fermentados pueden dividirse en dos tipos dependiendo del proceso de fermentación. Algunos alimentos como el yogurt, vino, cerveza, quesos, entre otros son preparados mediante fermentación controlada, bajo condiciones específicas y con cultivos iniciadores estandarizados, generalmente bacterias ácido lácticas (Leroy y De Vuyst, 2004). Mientras que la gran mayoría de los alimentos fermentados, son preparados en pequeñas comunidades mediante un proceso artesanal, en el cual la inoculación de los microorganismos no es controlada, lo que resulta en una fermentación espontánea en donde participa una microbiota diversa (Vogelmann *et al*. 2009).

Los alimentos fermentados tradicionales han sido preparados por miles de años, y están vinculados a la cultura y tradiciones de los pueblos nativos. Son consumidos como parte esencial de su dieta, forman parte de la medicina tradicional y en algunas culturas constituyen parte de sus

ritos religiosos. Muchos de estos alimentos solo son consumidos en las regiones donde se producen, por lo que el conocimiento sobre la diversidad y el tipo de alimentos fermentados que existen, así como qué microorganismos se desarrollan y sus procesos enzimáticos es limitado (Lacerda y Freitas, 2017; Villéger *et al*. 2017).

En México, hay una gran variedad de alimentos fermentados tradicionales, preparados a partir de sustratos como maíz (pozol, atole agrio, tesgüino, agua agria, entre otros) fruta (tepache y colonche) o plantas (pulque, tuba y taberna). Algunos de estos alimentos se han utilizado desde la antigüedad con fines religiosos y medicinales (Romero-Luna *et al*. 2017).

2. Pozol

2.1 Descripción

El pozol del náhuatl "pozolatl", es una bebida de origen maya, ácida, refrescante y no alcohólica hecha a base de maíz nixtamalizado. Actualmente es consumido cotidianamente en el sur y sureste del país, tanto por mestizos como por varios grupos étnicos, como los chontales y choles de Tabasco; los mayas de Campeche, Yucatán y Quintana Roo; los lacandones, tzotziles o chamulas y mames de Chiapas, y los zapotecos de Oaxaca (Ulloa *et al*. 1987).

Además de ser un alimento esencial en la dieta, los mayas lo utilizaban como ofrenda en sus ceremonias relacionadas con el cultivo y cosecha del maíz y formaba parte de su medicina tradicional. Como bebida se utilizaba para el control de diarreas e infecciones intestinales y como cataplasma (pozol enmohecido) se utilizaba para prevenir o curar infecciones superficiales y heridas (Ulloa *et al*. 1984).

2.2 Etapas de elaboración

Para la elaboración del pozol se puede utilizar granos secos de maíz blanco, amarillo o negro. Generalmente este maíz debe ser maduro al momento de la cosecha ya que el uso de un maíz no maduro (tierno) requiere mayor cantidad de cal para eliminar la cascarilla durante la cocción, lo cual modifica el sabor y resulta desfavorable desde el punto de vista económico. Además, el pozol preparado con maíz tierno se acidifica más rápidamente provocando modificaciones sensoriales en el producto final (Cañas *et al*. 1993).

Las etapas de elaboración del proceso tradicional se describen a continuación (Cañas *et al*. 1993; Ulloa *et al.* 1987; Wacher *et al*. 2000):

Limpieza del maíz: Tiene como finalidad eliminar el material extraño y granos podridos. Para ello, comúnmente se agrega agua al maíz crudo, se deja reposar unos minutos y se elimina todo lo que flota, que es maíz picado y cascarilla.

Nixtamalización: Del náhuatl *nextli* (cenizas) y *tamalli* (masa de maíz cocido) consiste en la cocción de los granos de maíz en cal en una relación aproximada de dos partes de hidróxido de calcio a una porción de maíz. El objetivo de este paso es separar el hollejo (o cascarilla) del grano y termina cuando este se puede eliminar fácilmente al frotar el grano con los dedos. El tiempo de cocción varía entre 60 y 120 minutos.

Lavado: El maíz nixtamalizado es lavado con agua de pozo, río o potable para eliminar el exceso de cal y la cascarilla que se ha desprendido del grano, hasta que el agua de lavado queda libre de turbiedad.

Segunda cocción o reventado: No es llevada a cabo por todos los productores, se ha descrito solo para la preparación de lo que se conoce como pozol “mestizo”. Para ello, el maíz nixtamalizado se cuece en agua hasta reventar el grano, el tiempo de cocción va desde 3 hasta 8 horas. Por lo general esta operación consiste en una ebullición vigorosa durante la primera hora, seguida de un hervor lento el resto del tiempo y tiene como finalidad reducir la cantidad de sedimentos sólidos presentes en la bebida cuando la masa es suspendida en agua.

Remojo: El maíz nixtamalizado se deja remojando toda la noche a temperatura ambiente, normalmente en el agua del último lavado. Tiene como objetivo aumentar la retención de humedad, para evitar que el maíz se seque durante la molienda.

Molienda: Los granos son sometidos a una molienda gruesa para reducir su tamaño. Los indígenas la llevan a cabo siempre en molino de mano, pero también se pueden utilizar molinos comerciales los cuales son de discos de piedra o de metal. La masa resultante de la molienda consiste en fragmentos de germen, residuos de pericarpio y endospermo.

Es importante señalar que los molinos son utilizados durante todo el día para moler lotes de granos destinados a otros productos, como tortillas o masa para tamales, lo que provoca que una cantidad importante de masa se quede en las esquinas del molino. Al final del día, el molino sólo se desmonta para remover los restos de masa, por lo que este paso es considerado como uno de los puntos de inoculación de microorganismos.

Elaboración de la bola y envoltura: La masa se moldea en forma de bolas que pueden pesar desde 15 gramos hasta un 1 kg. Tradicionalmente las bolas de masa eran envueltas en hojas de plátano, para que los indígenas pudieran cargarlas de manera fácil y cómoda durante las jornadas de

trabajo, pero también porque evita la desecación. En la actualidad la masa se cubre con un paño durante el día o se deja en bolsas de plástico si va a ser almacenada por varios días.

Fermentación: Se lleva a cabo a temperatura ambiente y en función del gusto del consumidor puede durar horas o hasta semanas. El resultado de la fermentación es un alimento con características sensoriales únicas, nutritivo ya que en la masa fermentada hay una mayor cantidad de proteína cruda y aminoácidos esenciales y que puede ser conservado por varios días. Para obtener la bebida la masa fermentada se suspende en agua a la cual se le puede adicionar sal, azúcar, canela, miel, cacao, horchata o chiles secos dependiendo del gusto del consumidor.

2.3 El maíz y sus modificaciones durante la elaboración del pozol

El maíz, de la palabra taina *maisi* or *maiji* que significa "lo que sustenta la vida" (Domínguez-Ramírez *et al.* 2017), es una planta de grano grande que se originó a partir de un único evento de domesticación en el sur de México hace unos 9000 años, su antepasado directo es un pasto silvestre conocido como teosinte (*Zea mays*) (Yang *et al*. 2019). Junto con el trigo y el arroz es uno de los cereales más importantes en el mundo, ya que sirve como base para la elaboración de una gran cantidad de alimentos (Paredes *et al*. 2008; Benítez y Perea, 2006).

El grano de maíz está formado por cuatro partes principales que se describen a continuación (Benítez y Perea, 2006):

- Pericarpio: constituye la pared del ovario y se le conoce como "cáscara", se caracteriza por un elevado contenido de fibra cruda (87%), compuesta principalmente por hemicelulosa y celulosa. El resto de la composición química son minerales, proteínas y azúcares solubles en menor proporción.
- Endospermo: es el tejido nutricional del saco embrionario que provee los nutrientes necesarios para la germinación del embrión contenido en la semilla. Esta estructura posee un alto contenido de almidón (87%) y un bajo porcentaje de proteínas (8%).
- Germen o embrión: Es la estructura a partir de la cual se desarrolla la nueva planta. Se caracteriza por un alto contenido de minerales (11%), proteínas (10%) y lípidos (5%).
- Pilorriza o pedicelo: Es la estructura que une el grano con el carozo (comúnmente conocido como olote). Está compuesta principalmente por celulosa, hemicelulosa y otros carbohidratos complejos.

La composición química del maíz depende tanto de la variedad, como del ambiente y las condiciones de siembra (Paredes *et al*. 2008). Como se muestra en la Tabla 1 es un alimento con

bajo contenido de proteínas y alto contenido de carbohidratos, esto lo convierte en una de las principales fuentes de energía para las poblaciones que lo consumen (Badui, 2006).

Tabla 1. Composición química del grano de maíz

	Proteínas	Lípidos	Fibra	Carbohidratos	Humedad	Minerales
g/100g	8-11	2-4	2-13	56-74	11-14	1-3

Datos en base húmeda (Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO), 1993; Koehler y Wieser, 2013; Hamaker *et al*. 2019). Los intervalos engloban los diferentes reportes sobre la composición del maíz.

Las proteínas del maíz se clasifican en cuatro tipos de acuerdo con su solubilidad: albúminas (solubles en agua), globulinas (solubles en soluciones salinas), prolaminas o zeínas (solubles en soluciones alcohólicas) y glutelinas (solubles en soluciones alcalinas o ácidas diluidas). Las glutelinas y prolaminas se encuentran principalmente en el endospermo; ambas proteínas constituyen cerca del 90% de las proteínas del grano de maíz completo. Por otro lado, las albúminas y las globulinas componen casi en su totalidad al germen (Paredes *et al*. 2008; Benítez y Perea, 2006). El grano de maíz también contiene lípidos y se encuentran principalmente en el germen. Los ácidos grasos saturados, como el palmítico y esteárico, se encuentran en baja proporción en comparación con los ácidos grasos no saturados, como el oleico y linoleico, que representan la mayoría de los lípidos contenido en el grano de maíz (Paredes *et al*. 2008).

Con relación a los carbohidratos, el más abundante en el grano maduro es el almidón, que representa en promedio el 72% del peso seco, mientras que los azúcares simples como glucosa, fructosa y sacarosa constituyen entre el 1 y el 3% del peso seco del grano. Además, el grano de maíz contiene polisacáridos estructurales como celulosa, hemicelulosa y lignina, principalmente en el pericarpio; sin embargo, la cantidad de lignina representa una pequeña fracción (menos del 0.1%) en comparación con la celulosa y hemicelulosa que llegan a constituir el 23% y 70%, respectivamente (Koehler y Wieser, 2013; Hamaker *et al*. 2019). La hemicelulosa del pericarpio contiene xilosa (48-50%), arabinosa (33-35%), galactosa (5-11%) y ácido glucurónico (3-6%) que pueden formar la cadena principal y está a su vez puede estar acetilada o tener cadenas laterales con restos de galactosa o xilosa (Saulnier *et al*. 1995; Niño-Media *et al*. 2009; Bento-Silva *et al*. 2018).

Además, el maíz contiene varios compuestos bioactivos, como carotenoides, antocianina y compuestos fenólicos. Los principales carotenoides del maíz son la luteína y la zeaxantina, mientras que la α- y β-criptoxantina, así como el α- y β-caroteno están presentes en menor grado.

Como se muestra en la Tabla 2, el contenido total de estos compuestos bioactivos varía dependiendo del tipo de maíz (Singh *et al*. 2014).

Tabla 2. Contenido de compuestos bioactivos en los diferentes tipos de maíz

Tipos de maíz / mg/100 g de maíz	[a]Compuestos fenólicos totales	[b]Contenido de antocianinas	Carotenoides		Ácido ferúlico
			Luteínas	β-carotenos	
Blanco	260.7	1.33	5.73	4.92	102
Amarillo	285.8	70.2	406	33.6	102
Rojo	243.8	9.75	121.7	20.2	130
Azul	343	36.87	5.17	23.1	152
Morado	465	30.6	-	-	154
Negro	457	76.2	-	-	151

Datos en base seca. [a]Expresado como ácido gálico. [b]Expresado como cianidin-3-glucósido. Información obtenida de Singh *et al*. 2014.

Durante la nixtamalización, la temperatura elevada y el pH alcalino (7.4-12.4) producen diversas transformaciones en el grano de maíz. La cocción alcalina de los granos facilita la solubilización de lípidos y los componentes estructurales del pericarpio (celulosa y hemicelulosa) facilitando su remoción (Sefa-Dedeh *et al.* 2004; Paredes *et al*. 2008; Santiago-Ramos *et al*. 2018). Se dan importantes pérdidas de azúcares simples (glucosa, fructosa, sacarosa y maltosa) asociadas a la cocción de los granos y al proceso de lavado del nixtamal (Santillana, 1995; Díaz, 1996; Ampe *et al*. 1999; Ben Omar y Ampe, 2000). También se ha observado que el tratamiento provoca la liberación de los gránulos de almidón por la desintegración del grano, el cual sufre cambios estructurales, como su hidratación y parcial gelatinización durante el calentamiento y retrogradación, definida como la insolubilización y la precipitación espontánea, principalmente de las moléculas de amilosa, durante el tiempo en que el grano permanece en remojo (Rojas-Molina *et al*. 2007; Santiago-Ramos *et al*. 2018).

Cuando el maíz nixtamalizado se muele pierde su estructura por lo que la masa resultante de la molienda consiste en fragmentos de germen, residuos de pericarpio y endospermo unidos por el almidón parcialmente gelatinizado, y por las proteínas y lípidos emulsificados (Paredes *et al*. 2008). Por otro lado, en la masa fermentada la concentración de nitrógeno proteico total, aminoácidos, como lisina y triptófano y vitaminas, como niacina y riboflavina, es mayor en comparación a los granos de maíz utilizados en su preparación. Sin embargo, debido al

tratamiento de los granos de maíz con agua y calor para obtener el nixtamal, algunos componentes disminuyen, como la tiamina y el fósforo (Tabla 2) (Cravioto *et al*. 1955).

Tabla 2. Análisis del pozol y del maíz utilizado para su preparación.

Constituyentes	Maíz (g/100g de maíz)	Pozol (g/100g de pozol)	Diferencia en porcentaje entre maíz y pozol
Humedad	11.20	61.2	+ 546.42
Cenizas	1.69	1.65	- 2.40
Nitrógeno	1.68	2.43	+ 44.00
Proteína	10.50	15.18	+ 44.00
Extracto etéreo	4.73	8.60	+ 81.00
Fibra cruda	2.55	1.51	- 40.70
	mg/100g de maíz	mg/100g de pozol	
Calcio	28.00	249.00	+ 889.20
Fósforo	438.00	4.52	- 99.00
Tiamina	0.40	0.27	- 32.50
Riboflavina	0.15	0.78	+ 420.00
Niacina	3.26	9.84	+ 201.00
Aminoácidos esenciales			
Arginina	3.08	3.34	+ 8.40
Histidina	2.54	2.07	- 18.51
Lisina	3.05	3.36	+ 29.80
Leucina	12.95	10.02	- 22.70
Isoleucina	5.00	5.16	+ 3.20
Treonina	5.05	5.69	+ 12.66
Valina	4.51	4.53	+ 0.40
Triptófano	0.46	0.71	+ 54.34
Metionina	1.54	1.48	- 0.38
Fenilalanina	5.34	4.30	-19.47

2.4 Microbiota del pozol

Debido a que el pozol constituye un alimento básico en la dieta de las poblaciones rurales del país, se han realizados diversos estudios enfocados en el aislamiento e identificación de los microorganismos que se desarrollan durante la fermentación, como un primer acercamiento para describir el sistema.

La fermentación de este alimento puede considerarse espontánea, ya que no se añade de manera intencional ningún inoculo a la masa, sino que es durante su elaboración, principalmente en la molienda, amasado y moldeado, donde se incorporan de forma natural los microorganismos a la masa (Wacher *et al*. 1993).

La descripción microbiológica se ha llevado a cabo utilizando técnicas dependientes e independientes de cultivo, y muestra que la microbiota del pozol está constituida por bacterias, hongos y levaduras (Wacher *et al*. 2000). Las bacterias constituyen el grupo predominante durante la fermentación y entre ellas, las bacterias acido lácticas son las más abundantes (Nuraida *et al*. 1995; Ampe *et al*. 1999; Ampe *et al*. 1999; Escalante *et al*. 2001; Diaz-Ruiz *et al*. 2003; López, 2005). En la Tabla 3 se enlistan los microorganismos identificados hasta la fecha.

Tabla 3. Microorganismos que participan en la fermentación del pozol.

Hongos filamentosos y levaduras	Bacterias no ácido lácticas	Bacterias ácido lácticas
Alternaria tenius	*Acetobacter aceti*	*Enterococcus sacharolyticus*
Aspergillus flavus	*Acinetobacter* spp.	*Enterococcus sulfureus*
Aureobasidium pullulans	*Aerobacter aerogenes*	*Lactobacillus acidophilus*
Candida guillermondi	*Agrobacterium azotophilum*	*Lactobacillus alimentarius*
Candida krusei	*Alcaligenes branchisepticus*	*Lactobacillus brevis*
Candida parapsilosis	*Bacillus cereus*	*Lactobacillus casei*
Candida tropicalis	*Bacillus lentus*	*Lactobacillus coprophilus*
Cladosporium cladosporioides	*Bacillus subtilis*	*Lactobacillus crispatus*
Cladosporium sp.	*Bacillus minimum*	*Lactobacillus curvatus*
Debaryomyces hansenii	*Bacillus mycoides*	*Lactobacillus delbrueckii*
Epicoccum sp.	*Bacillus subtilis*	*Lactobacillus fermentum*
Fusarium moniliforme	*Bifidobacterium minimum*	*Lactobacillus manihotivorans*
Geotrichum candidum	*Clostridium spp.*	*Lactobacillus paracasei*
Hansenula fabianii	*Escherichia coli*	*Lactobacillus pentosus*
Hansenula pozolis	*Enterobacter aerogenes*	*Lactobacillus plantarum*
Kluyveromyces fragilis	*Enterobacter agglomerans*	*Lactococcus lactis*
Kluyveromyces lactis var. *lactis*	*Escherichia fergusonii*	*Lactococcus raffinolactis*
Monilia sitophila	*Exiguobacterium aurantiacum*	*Lactobacillus reuteri*
Mucor rouxianus	*Klebsiella pneumoniae*	*Leuconostoc argentum*
Paecilomyces fumosoroseus	*Klebsiella* sp.	*Leuconostoc citreum*
Penicillium claviforme	*Kosakonia* sp.	*Leuconostoc mesenteroides*
Penicillium cyclopium	*Paracolobactrum aerogenoides*	*Streptococcus bovis*
Penicillium expansum	*Pseudomonas mexicana*	*Streptococcus equinus*
Penicillium fellutanum	*Serratia marcescens*	*Streptococcus infantarius* subsp. *infantarius*
Penicillium italicum	*Sphingomonas spp.*	*Streptococcus macedonicus*
Penicillium lanosoviride	*Xantomonas spp*	*Weissella cibaria*
Phialophora richardsiae		*Weissella confusa*
Phoma fimeti		*Weissella paramesenteroides*
Phoma glomerata		
Rhizopus nigricans		
Rhodotorula minuta		
Rhodotorula mucilaginosa		
Sacharomyces cerevisiae	.	
Trichoderma viride		
Trichosporum cutaneum		

Recopilado de: Ulloa, 1974; Ulloa y Kurtzman 1975; Nuraida *et al*. 1995; Ampe *et al*. 1999; Ampe *et al*. 1999; Wacher *et al*. 2000; Ben Omar y Ampe, 2000; Escalante *et al*. 2001; Díaz-Ruiz *et al*. 2003; Sainz *et al*. 2005; Olvera *et a*l. 2017; Rizo *et al*. 2020.

Dentro de los estudios microbiológicos realizados en este alimento, destacan por su importancia en la fermentación de la masa de maíz el estudio de microorganismos amilolíticos, ya que, siendo el almidón el principal sustrato para la fermentación, se hipotetiza son ellos los responsables de hidrolizarlo y liberar azúcares solubles para el crecimiento de la microbiota no amilolítica.

En un estudio realizado por Nuraida *et al*. (1995), se analizó la capacidad amilolítica de bacterias lácticas y levaduras. De las UFC (unidades formadoras de colonia) de bacterias acido lácticas aisladas, más del 50% fue capaz de hidrolizar el almidón en la caja Petri en condiciones anaerobias, aunque no se llevó a cabo la identificación, lo cual implica que varios aislados podían tratarse del mismo microorganismo. Las levaduras, mostraron resultados similares, el 50% hidrolizó el almidón produciendo un halo alrededor de la colonia. En este trabajo no se analizaron otro tipo de bacterias u hongos.

Posteriormente, Díaz-Ruiz y colaboradores (2003) encontraron que la cuenta inicial de bacterias lácticas amilolíticas es alta (4.5 log ufc/g de masa seca) y que aumenta hasta 8.4 log ufc/g de masa seca durante las primeras 24 horas y se mantiene constante hasta las 72 horas (8.7 log ufc/g masa seca). De un total de 257 colonias de bacterias acido lácticas capaces de crecer e hidrolizar almidón, se eligieron 40 colonias al azar con diferentes diámetros de zonas de hidrólisis basados, para su posterior caracterización mediante un enfoque taxonómico, fenotípico y molecular. Todas las colonias fueron homofermentativas, ya que solo produjeron ácido láctico a partir de glucosa. Su caracterización molecular y fenotípica dio como resultado la identificación de cuatro especies: *Streptococcus macedonicus, Lactococcus lactis, Enterococcus sulfureus* y *Streptococcus bovis* 25124 (actualmente *S*. *infantarius* ssp. *infantarius, Sii*-25124), la cual resultó ser la especie predominante durante la fermentación.

En este mismo estudio, se analizó el crecimiento en almidón de *Sii*-25124 en medio MRS-almidón. A pesar de su predominancia, esta bacteria se caracterizó por tener un bajo rendimiento de actividad amilolítica relativo a la biomasa [Yamy/x=139 U/g células secas, donde una unidad enzimática se definió como la cantidad de enzima que hidroliza 10 mg de almidón en 30 min] pero, en acuerdo con su predominancia mostró una alta velocidad específica de crecimiento (μ=0.94 h^{-1}) y una conversión eficiente de sustrato en biomasa [0.31 g biomasa (g sutrato^{-1})] cuando se compara con otras bacterias acido lácticas amilolíticas aisladas de alimentos fermentados amiláceos (Díaz-Ruiz *et al*. 2003).

Recientemente se demostró que esta misma cepa (*Sii*-25124) es capaz de crecer en arabinoxilano de maíz (μ= 0.109 h^{-1}) o xilano de madera (μ= 0.0267 h^{-1}) como única fuente de carbono. Se

encontró que *Sii*-25124 tiene las enzimas necesarias para hidrolizar ambos polisacáridos, observándose nuevamente una mayor actividad xilanasa en arabinoxilano (135.8 ± 48.7 IU/mg de proteína) que en xilano de madera (62.5 ± 19.8 IU/mg de proteína). Lo que demuestra la capacidad de la cepa de utilizar otros polisacáridos presentes en la masa de maíz además del almidón (Cooper-Bribiesca *et al*. 2018).

Sin embargo, en la fermentación también se han descrito bacterias lácticas que no pueden crecer en almidón o en xilano. López-Hernández *et al*. (2018) evaluó la capacidad de utilizar xilano como fuente de carbono en varias cepas de *Weissella*, aisladas del pozol. Ninguna de las cepas probadas fue capaz de crecer o metabolizar el xilano, sin embargo, son β-glucosidasa y β-galactosidasa positiva, lo que indica que pueden utilizar como xilobiosa y xilotriosa, por lo que se considera que el xilano es un sustrato importante para la fermentación del pozol (López-Hernández *et al*. 2018).

2.6 Fijación de nitrógeno en el pozol

Como se mencionó anteriormente, el análisis químico de los granos de maíz y de la masa fermentada mostró que durante la fermentación se producen cambios importantes, entre los que destaca por su relevancia en el aporte nutricional del pozol, el incremento de 44.5% en el contenido de nitrógeno total y proteína cruda (Cravioto *et al*. 1955).

En un estudio posterior, Ulloa y colaboradores (1971) sugirieron que el incremento observado era debido a la fijación de nitrógeno atmosférico por los microorganismos que se desarrollan durante la fermentación. La evaluación del contenido de nitrógeno en muestras de masa de maíz sometidas a distintos tratamientos demostró que en todos los casos donde se efectuó una fermentación, hubo incremento en el contenido de nitrógeno total, siendo mayor en la masa de maíz inoculada con pozol natural; sin embargo, este incremento no igualó al observado en el pozol natural (Tabla 4).

Tabla 4. Contenido de nitrógeno en masa de maíz sometida a diferentes tratamientos.

Tratamiento[a]	N total mg/mL[b]	N proteico mg/mL[c]	% de aumento con respecto a 1
1. Masa	11.8	73.7	---
2. Masa incubada 5 días a 26°C	14.2	88.7	20.4
3. Masa incubada 10 días a 26°C	14.2	88.7	20.4
4. Masa inoculada con pozol natural	14.7	91.8	24.6
5. Masa inoculada con pozol natural incubada 5 días a 26°C	17.0	106.2	44.1
6. Masa inoculado con pozol natural e incubada 10 días a 26°C	17.0	106.2	44.1
7. Pozol natural[d]	20.0	125.0	69.5

[a] En todos los casos, las muestras para el análisis de nitrógeno fueron secadas de 85-90°C durante 24 horas.
[b] Promedio de los análisis. Determinado por el método de micro-Kjeldahl en muestras de 100 mg (peso seco).
[C] % de nitrógeno x 6.25
[d] Pozol proveniente de Tapachula, Chiapas; las muestras de los tratamientos del 1-6 corresponden a la masa comprada en un molino de maíz en la Ciudad de México.

Además, mediante el análisis microbiológico fue posible el aislamiento e identificación de ocho microorganismos capaces de crecer en medios de cultivo carentes de nitrógeno: *Achromobacter* sp. A y B, *Cladosporium herbarum*, *Candida krusei, Geotrichum candidum*, *Trichosporon cutaneum*, *Paecilomyces fumosoroseus* y *Torulopsis* sp. (Ulloa *et al*. 1971). Sin embargo, la prueba de reducción de acetileno a etileno para demostrar si los microorganismos previamente aislados son capaces de fijar nitrógeno atmosférico, resultó en un solo microorganismo capaz de producir etileno en condiciones aerobias y anaerobias, *Agrobacterium azotophilum* (previamente identificado como *Achromobacter* sp. A) (Taboada *et al*. 1971; Ulloa y Herrera, 1972). La fijación de nitrógeno de *Agrobacterium azotophilum* se vio favorecida por la presencia de isoleucina y D-metionina a concentraciones de 0.002 mM y, ácido glutámico y ácido aspártico a 0.001 mM. Concentraciones mayores de estos aminoácidos y la presencia de sales nitrogenadas o glicina en el medio de cultivo inhiben la fijación (Herrera y Taboada, 1971).

Trabajos posteriores han corroborado que durante la fermentación del pozol hay un incremento en el contenido de nitrógeno entre el 10 y el 35% (Ramírez, 1987; Loaeza, 1991; Giles, 1995; Rizo, 2015). Sin embargo, en ninguno de estos estudios se demostró si el aumento se debe a la fijación de nitrógeno atmosférico en la masa.

Capítulo segundo

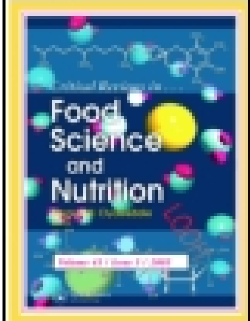
Ómicas para el análisis de los alimentos fermentados

El estudio de las comunidades microbianas en los alimentos fermentados mediante técnicas tradicionales de cultivo ha resultado insuficiente para la descripción e identificación de cada uno de sus miembros, debido principalmente a la necesidad del aislamiento y cultivo de los microoganismos, limitándose al estudio de los que son cultivables bajo condiciones de laboratorio. Además, mediante este enfoque no es posible el estudio de las interacciones microbianas, su distribución espacial, sus actividades y adaptación a los cambios que se generan en su entorno.

El uso de métodos independientes de cultivo ha permitido incrementar el conocimiento de la diversidad microbiana en los alimentos fermentados y también establecer poblaciones activas durante el proceso. Sin embargo, estas técnicas tienen limitaciones asociadas a los métodos de extracción y purificación de ácidos nucleicos de las muestras a estudiar. Hay co-migración de fragmentos de DNA y por lo tanto una subestimación de la diversidad microbiana, lo que limita las inferencias filogenéticas (Díaz-Ruiz y Wacher, 2003). Además, estás técnicas no permiten entender el papel de los microorganismos como parte de un sistema, en el que se generan microambientes con diferentes características y que en conjunto definen al alimento.

El desarrollo de las ciencias ómicas (genómica, transcriptómica, proteómica y metabolómica) permite abordar la complejidad de los alimentos fermentados, ya que se puede establecer la composición y los cambios de la microbiana durante el proceso de fermentación (Ji *et al*. 2017; Jian *et al*. 2017). Se pueden determinar que especies y actividades enzimáticas (papel funcional de la microbiota) son las responsables de generar las características organolépticas de cada alimento fermentado (Xie *et al*. 2020). El estudio de la expresión diferencial de genes y de proteínas permite estudiar cómo cambian y se adaptan las poblaciones microbianas a las condiciones que se generan durante la fermentación (Oosthuizen *et al*. 2002; JiYoung *et al*. 2013). Por último, estos estudios permiten inferir las interacciones de los microorganismos entre sí y con el sustrato (Sieuwerts *et al*. 2008).

El siguiente artículo es una revisión de la aplicación de las técnicas ómicas para el estudio de los alimentos fermentados tradicionales. También se analiza el potencial que estas técnicas ofrecen tanto para el desarrollo y validación del proceso de fermentación, como para el control de calidad de las materias primas y productos finales. Finalmente, se ilustra cómo se procesan los datos ómicos y los desafíos de estos enfoques.



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REVIEW

Omics in traditional vegetable fermented foods and beverages

Jocelin Rizo[a], Daniel Guillén[a], Amelia Farrés[b], Gloria Díaz-Ruiz[b], Sergio Sánchez[a], Carmen Wacher[b], and Romina Rodríguez-Sanoja[a]

[a]Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM), Ciudad de México, México; [b]Departamento de Alimentos y Biotecnología, Facultad de Química, Universidad Nacional Autónoma de México (UNAM), Ciudad de México, México.

ABSTRACT
For a long time, food microbiota has been studied using traditional microbiological techniques. With the arrival of molecular or culture-independent techniques, a strong understanding of microbiota dynamics has been achieved. However, analyzing the functional role of microbial communities is not an easy task. The application of omics sciences to the study of fermented foods would provide the metabolic and functional understanding of the microbial communities and their impact on the fermented product, including the molecules that define its aroma and flavor, as well as its nutritional properties. Until now, most omics studies have focused on commercial fermented products, such as cheese, wine, bread and beer, but traditional fermented foods have been neglected. Therefore, the information that allows to relate the present microbiota in the food and its properties remains limited. In this review, reports on the applications of omics in the study of traditional fermented foods and beverages are reviewed to propose new ways to analyze the fermentation phenomena.

KEYWORDS
Metagenomics; proteomics; metabolomics; foodomics; traditional fermented foods

Introduction

Fermentation is the oldest and most economical method to produce and preserve food. It is a natural way to enhance the food's nutritional value and digestibility. Fermentation eliminates unpleasant tastes, reduces the energy required for cooking and destroys anti-nutritional components, such as phytic acid, tannins and polyphenols (Blandino et al. 2003; Kabak and Dobson 2011).

Fermentation has been performed for centuries with several feedstocks without an understanding of the underlying process. Chemical evidence now enables the oldest beverages to be traced back as far as 7000 B.C. in Henan province in China, where a mixed fermented beverage of rice, honey, and fruit (hawthorn fruit and/or grape) was being produced. Based on oracle inscriptions, during the last Shang Dynasty (1200–1046 B.C.), fermented beverages such as *chang* (herbal wine), *li* (sweet rice or millet beverage) and *jiu* (fermented and filtered rice or millet beverage) were used as an offering for royal ancestors (McGovern et al. 2004). In the period of the Zhou dynasty (1121–256 B.C.), also in China, barley, rice and wheat were used for the preparation of *chu* (moldy starter prepared form soybean). According to "The Book of Documents, *Shu-Ching*," compiled by Confucius (551–479 B.C.), *chu* was essential for making alcoholic beverages (Prakash and Kailasapathy 2010). There are also some historic records that place the fermentation of wine and beer in 5000 B.C. in Iran and Egypt. The origins of the bread-baking process dates from the same period, presumably in Egypt. However, it was not until the 1850s that the process was understood for the first time, when microbiology blossomed as a science and laid the groundwork for comprehending fermentation (Caplice and Fitzgerald 1999).

In Mesoamerica, several species of agave were used to produce alcoholic beverages with high cultural and social relevance. There is archeological evidence of agave spirit production from 400 to 600 A.D. (Zizumbo-Villarreal et al. 2009). In ancient Teotihuacan (150 B.C. to A.D. 650), the analysis of pottery vessels suggests production of *pulque* in this area (Correa-Ascencio et al. 2014).

Different approaches have been applied in the study of fermented foods. Typically, microbiology used culture-dependent techniques, where the isolation of microorganisms is a requirement for subsequent phenotypic and biochemical characterizations. These methods can only detect culturable microorganisms, yet it is an accepted paradigm that these microorganisms represent only a very small fraction of natural microbial diversity (Stackebrandt and Embley 2000).

Sometimes, the growth, survival and activity of a microorganism will be determined by the presence or the interaction with others, and in most cases, it is difficult to reproduce the conditions from which the organisms were isolated (Fleet 1999). As a result, our knowledge of microbial communities is limited. For example, in soil, only 0.1%

CONTACT Romina Rodríguez-Sanoja romina@biomedicas.unam.mx Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM), A.P. 70228, Ciudad Universitaria, Ciudad de México, 04510, México.
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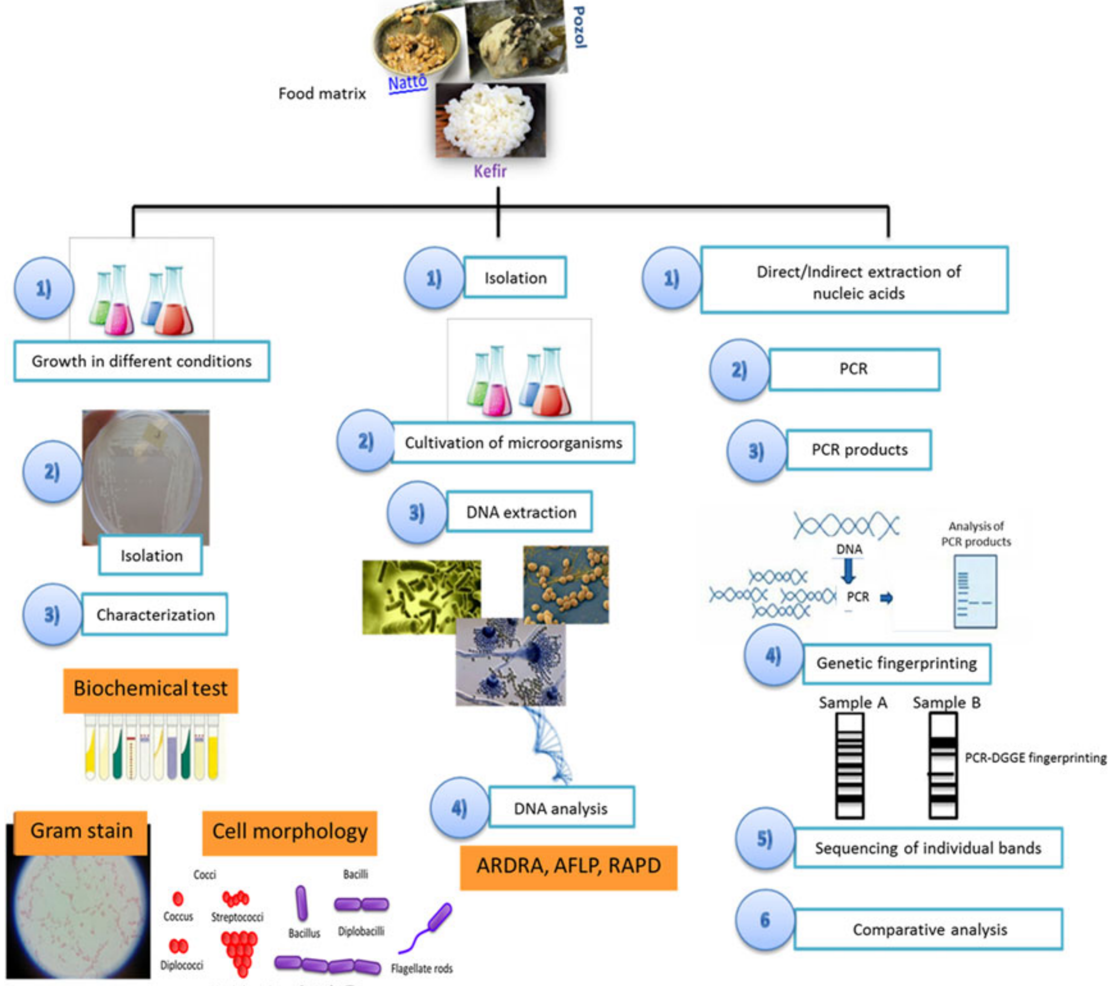

Figure 1. General schematic representation of the different methodologies for the microbial study in fermented foods (Adapted from Díaz-Ruiz and Wacher 2003).

to 1% of bacteria are readily culturable on common media under standard conditions (Torsvik and Øvreås 2002).

Consequently, culture-independent methods (molecular methods) have been developed. Thus, random amplification of polymorphic DNA (RAPD; based on genomic DNA fragment profiles amplified by PCR), amplified fragment length polymorphism (AFLP; based on the selective amplification and separation of genomic restriction fragments), amplified ribosomal DNA restriction analysis (ARDRA) and PCR-DGGE (based on sequence-specific distinctions of 16S rDNA and 26S rDNA amplicons produced by PCR) have been used for microbial identification in fermentations, although metagenomics and massive sequencing are currently preferred. The combination of culture-dependent and molecular methods has enabled the characterization of microbial diversity, spatial distribution and the composition of active microbial populations (Figure 1).

Such studies have already been applied in *pozol*, a fermented maize dough beverage. The results clearly demonstrated that the microbiota in this beverage cannot be effectively studied by only cultivation-dependent methods, since no *Streptococcus* strain was isolated using culture media, but DGGE and rRNA analyses revealed that members of this genus were present and even more, *Streptococcus* represents 25 to 50% of the total active population in pozol (Ampe et al. 1999). Following this discovery, the bacterium could be isolated in a medium that favored its growth (Díaz-Ruiz et al. 2003). Interestingly, in other maize-based fermented foods such as *ogi* and *mawè*, 16S rDNA sequencing revealed that the predominant bacteria are *Lactobacillus plantarum* and *Lactobacillus fermentum,* these differences have been associated with the dough production process (Table 1).

In a yeast diversity study in *ogi*, *mawè*, *gowé* and *tchoukoutou*, DGGE analysis of the D1 region of the 26S rRNA gene (directly extracted from the food matrices) demonstrated the presence of *Dekkera bruxellensis* and *Debaryomyces hansenii,* yeasts not detected with the culture-based approach (Greppi et al. 2013) (Table 1).

Despite the advantages of these “classic molecular” food microbiology techniques, these methods also have inherent biases such as (i) selective extraction of nucleic acids, (ii) selective amplification of 16S rDNA, and (iii) comigration of bands of different sequences in a DGGE analysis to name a few examples (Ampe et al. 1999). Moreover, these techniques do not allow understanding the role of microorganisms as part of a system where abiotic and biotic relations define the fermented food characteristics. This scenario is where

Table 1. Some examples of indigenous fermented food consumed around the world.

Product	Substrate	Region	Use	Main identified microorganisms	Methodological approaches	Reference
Abreh	Sorghum	Sudan	Soft drink	• Bacteria (*Lactobacillus*)	Culture-dependent approach. Isolation of microorganisms	Todorov and Holzapfel (2015)
Atole agrio	Maize	Southeast Mexico	Beverage	• Bacteria (*Leuconostoc*, *Lactococcus*, *Lactobacillus, Pediococcus* and *Weisella*)	Culture-dependent approach. DNA extraction from previously isolated LAB for 16S rRNA gene amplification, 16S-23S rRNA ISR-PCR and RAPD-PCR Culture-independent approach. DNA extraction from the substrate for 16S rRNA gene amplification, sequencing and genus/species identification (rRNA)	Pérez-Cataluña et al. (2018)
Boza	Wheat, millet, barley	Balkans, Asia Minor, Caucasian region	Sour soft drink	• Bacteria (*Enterococcus, Lactobacillus, Leuconostoc, W eisella*) • Yeast (*Saccharomyces, Candida, Issatchenkia*, *Pichia, Rhodotorula* and *Torulaspora*)	Culture-dependent approach. DNA extraction from previously isolated LAB and yeast for 16S and 28S rRNA gene amplification, sequencing and genus/species identification	Botes et al. (2007); Todorov and Holzapfel (2015)
Burukutu	Sorghum and cassava	Nigeria, Benin, Ghana	Alcoholic beverage	• Bacteria (*Escherichia*, *Staphylococcus, Bacillus*, Lactobacillus, *Leuconostoc* and *Acetobacter*) • Yeast (*Saccharomyces, Aspergillus, Rhizopus* and *Candida*)	Culture-dependent approach. Isolation of microorganisms	Anaukwu et al. (2015)
Champuz	Maize or rice	Colombia, Peru	Beverage	Unknown		Wacher (2004)
Cheonggukjang	Soybean	Korea	Condiment	• Bacteria (Lactic acid bacteria and *Bacillus*)	Culture-independent approach. DNA extraction from the substrate for PCR-DGGE analysis and 16S rRNA gene amplification, sequencing and genus/species identification	Nam, Yi, and Lim (2012)
Chicha	Maize	Peru, Colombia	Beverage	• Bacteria (*Enterococcus*, *Lactococcus*, *Streptococcus*, *Weissella*, *Leuconostoc* and *Lactobacillus*) • Yeast (*Saccharomyces)*	Culture-dependent approach. DNA extraction from previously isolated LAB for 16S rRNA gene amplification, 16S-23S rRNA ISR-PCR and RAPD-PCR Culture-independent approach. DNA extraction from the substrate for 16S rRNA gene amplification, sequencing and genus/species identification	Vallejo et al. (2013); Elizaquível et al. (2015)
Dégué	Millet dough	Burkina Faso	Condiment	• Bacteria (*Escherichia*, *Bacillus*, *Lactobacillus* and *Enterococcus*)	Culture-independent approach. DNA extraction from the substrate for TGGE analysis, DNA band sequencing and genus/species identification by 16S rRNA gene	Abriouel et al. (2006)
Gochujang	Soybean, spicy red pepper and cereal (rice)	Korea	Condiment	• Bacteria (*Bacillus*) • Yeast (*Zygosaccharmoyces* and *Candida*) • Molds (*Aspergillus*, *Penicillium* and *Rhizopus*)	Culture-dependent approach. DNA extraction from previously isolated LAB, yeast and molds for 16S, 18S and ITS-5.8S rRNA gene amplification, sequencing and genus/species identification	Shin and Jeong (2015)
Gilaburu	Cranberrybush	Turkish	Beverage	• Bacteria (*Lactobacillus* and *Leuconostoc*)	Culture-dependent approach. DNA extraction from previously isolated LAB for 16S rRNA gene amplification, sequencing and genus/species identification	Sagdic et al. (2014)
Kinema	Soybean	Eastern Himalayas	Consumed as curry with steamed rice	• Bacteria (Bacillus) • Yeast (*Candida* and *Geotrichum*)	Culture-dependent approach. Isolation of microorganisms	Tamang (2015)
Meju	Soybean	Korean	Solid dough used to make doenjang and soy sauce	• Bacteria (*Bacillus*, *Lactobacillus* and *Pediococcus*) • Yeast (*Candida*, *Hansenula* and *Saccharomyces*)	Culture-dependent approach. Isolation and biochemical characterization of microorganisms Culture-independent approach. DNA extraction from the substrate, PCR-DGGE analysis	Shin and Jeong (2015)

(*continued*)

Table 1. Continued.

Product	Substrate	Region	Use	Main identified microorganisms	Methodological approaches	Reference
				• Molds (*Aspergillus, Mucor, Penicillium* and *Rhizopus*)		
Miso	Soybean and rice, soybean and barley or soybean	Japan	Condiment	• Bacteria (*Lactobacillus*) • Yeast (*Torulopsis*) • Molds (*Aspergillus*)	Culture-dependent approach. Isolation of microorganisms	Blandino et al. (2003); Wacher (2004)
Tepache	Pineapple	Mexico	Beverage	• Bacteria (*Bacillus*) • Yeast (*Torulopsis, Saccharomyces and Candida*)	Culture-dependent approach. Isolation of microorganisms	Battcock and Azam-Ali (1998); Blandino et al. (2003); Wacher (2004)
Tesgüino	Maize germ	North Western and North Mexico	Alcoholic beverage	• Yeast (*Candida*, *Saccharomyces* and *Hansenula*)	Culture-dependent approach. Isolation of microorganisms	Ulloa, Herrera, and Lappe (1987)
Thua nao	Soybean	Northern Thailand	Staple food and flavor enhancer	• Bacteria (*Bacillus*)	Culture-dependent approach. Isolation of microorganisms	Chukeatirote (2015)
Tuba	Coconut palm	Western Mexico	Alcoholic beverage	Unknown		Blandino et al. (2003); Wacher (2004)
Poto poto	Maize dough	Congo	Weaning food	• Bacteria (*Lactobacillus*, *Bacillus*)	Culture-independent approach. DNA extraction from the substrate for 16S rRNA gene amplification, sequencing and genus/species identification TGGE analysis and DNA band sequencing	Abriouel et al. (2006)
Pozol	Maize dough	Southeast Mexico	Beverage and weaning food	• Bacteria (*Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, *Weisella*) • Yeast (*Saccharomyces, Candida)* • Molds (*Aspergillus, Penicillum, Rhizopus)*	Culture-dependent approach. Isolation and biochemical characterization of microorganisms. DNA extraction from previously isolated LAB for 16S rRNA gene amplification, sequencing and genus/species identification Culture-independent approach. RNA extraction from the substrate for 16S rRNA hybridization probes. DNA extraction from the substrate for DGGE analysis	Ampe et al. (1999); Ampe, ben Omar, and Guyot (1999); Wacher et al. (2000)
Pulque	several species of *Agave*	Central Mexico	Alcoholic beverage	• Bacteria (*Lactobacillus*, *Leuconostoc*, *Microbacterium*, *Flavobacterium*, *Acetobacter*, *Gluconobacter*, and *Zymomonas*) • Yeast (*Saccharomyces*)	Culture-independent approach. DNA extraction from the substrate for 16S rRNA gene amplification, sequencing and genus/species identification, ARDRA fingerprinting analysis	Escalante et al. (2004); Escalante et al. (2008)

the use of the omic platforms allows us to dissect the complexity of fermented foods establishing the role of microorganisms, what is the metabolic potential of the microbiota and which genes are expressed, which metabolic pathways turn on or off and which are the final products, at the same time that we observe how they adapt to the changing conditions that arise during the process (pH, salt concentrations, the presence of toxic metabolites, etc.), to ultimately ensure the safety, quality, and homogeneity of food and to preserve the unique features that are attributed to native microbiota. In this review, the application of omics in the study of traditional vegetable-based fermented foods and beverages is analyzed. We also illustrate how omics data are processed and the challenges inherent to food matrices when these approaches are used.

Traditional fermented foods

Spontaneous fermentation of different raw materials (such as milk, cereals, legumes, fruits and soybean) is performed in many parts of the world, resulting in a huge variety of traditional products (Vogelmann et al. 2009). Some of these products are used as spices, colorants, beverages, breakfasts or light meals, while others are used as famine foods (Battcock and Azam-Ali 1998). As well as being part of the traditional diet, many of these fermentations are also used by distinct cultures in traditional medicine or as ceremonial elements. Table 1 shows some examples of traditionally fermented vegetable foods and beverages around the world.

Indigenous fermented foods are prepared in small communities in an artisanal way, and virtually none of them has a standardized starter inoculum. Hence, the inoculation of microorganisms occurs through the hands of people involved in their preparation, the raw material, the water, the equipment used in the elaboration or the environment.

In the last 15 years, the number of studies on indigenous fermented beverages and foods around the world has rapidly increased. These studies have developed an overall view of the microbial composition and have proven the importance of fermentation regarding nutritional quality, health benefits, food safety and probiotic effects, among other topics. Some examples of the advances that the omic sciences have allowed on the knowledge of these fermentations are shown in the next sections (Table 2).

Omic sciences

Omics include the study of various molecules involved in the development and maintenance of life on all levels of organization, from the organelle to the ecosystem, including the organismal level. Usually, they are mainly aimed at the detection of genes (genomics), mRNAs (transcriptomics), proteins (proteomics), lipids (lipidomics) and metabolites (metabolomics) in a specific biological sample in a non-targeted and non-biased way (Horgan and Kenny 2011; Lay et al. 2006). The final goal of these studies is to understand the cell as an integrated system that interacts with the environment, rather than as a collection of independent parts (Sánchez et al. 2013).

These sciences are now widely applied to all biological disciplines. In food science, there are two major lines of omics research: from one side foodomics, which studies how food influences health and the development or prevention of diseases and from the other the use of omics to understand the phenomena of fermentation and transformation in foods.

Related to the study of foods fermentation, metagenomic could overcome the limitations due to the presence of culturable and nonculturable organisms, however, all the information cannot be obtained from the study of genes *per se* because proteins, not genes, are responsible for the phenotypes and activity of cells; additionally, proteomics could allow to understand the fermentation process by studying proteins expression as a function of the changing environmental variables. Finally, metabolomic studies could be used to correlate nutritional and sensory qualities, to select taste marker compounds, biomarkers for monitoring the fermentation, etc (Figure 2).

Until now, greater emphasis has been given to the monitoring and control of industrial fermentations, such as cheese, wine and beer, while studies of traditional fermentations, especially vegetable fermentations, are still very scarce, despite their great nutritional and cultural importance.

Metagenomics

Metagenomics is the analysis of the combined genomic DNA from all members of a microbial community (Sabree, Rondon, and Handelsman 2009). In this approach, nucleic acids (DNA or RNA) are directly isolated from a complex sample without culturing the organisms.

Analysis of the total DNA present in the sample, based on random sequencing, is a powerful method that provides a great deal of information about gene function and content, allowing the classification and identification of microbial communities. Additionally, this method makes it possible to characterize the functional potential of the microbiome by reconstructing metabolic pathways. Finally, two or more conditions can be compared to identify differential functional roles, pathway subsystems, microbial dynamics and abundance (Escobar-Zepeda, Vera-Ponce, and Sanchez-Flores 2015).

There are two different metagenomic approaches: (i) amplicon sequencing (target metagenomics) and (ii) shotgun metagenomics. In target metagenomics, specific genes or genomic regions of DNA from communities are amplified, such as the 16S rRNA gene or the large ribosomal subunit (LSU) gene (Kim et al. 2013). In the second approach, the total DNA of the sample is obtained and sequenced.

The transition from genomic to metagenomic studies has been possible due to the quick development of high-throughput technologies, the low cost and rapidly sequencing of genes and genomes, with the creation and exponential growth of genomic databases. In recent years, reported genomes of multiple organism and even entire ecosystems

Table 2. Relevant results in the study of traditional fermented foods through omic science.

Fermented food	Omic approach	Summary of the results	Reference
Kimchi	Metagenomic	• Detection of sources of contamination (inoculation) • Relevant microorganisms finding • Establish of metabolic potential of microorganisms	Jung et al. (2011); Lee, Jung, and Jeon (2015)
Cocoa bean	Metagenomic	• Identification of predominant LAB • Reconstruction of biological pathways based on carbohydrate, amino acid and lipid metabolism genes	Illeghems et al. (2015)
Nuruk	Metagenomic	• Demonstration of the influence of temperature in the predominance of microorganisms	Bal et al. (2016)
Doenjang	Metagenomic	• Relate the type of microorganism and organoleptic profile with the inoculum (traditional vs commercial)	Kim et al. (2016)
Daqu	Metagenomic	• Detection of sources of contamination (inoculation)	Wang, Du, and Xu (2017)
Pozol	Metaproteomic	• The predominance of species, previously identified by molecular methods dependent on culture, was confirmed • Detection of proteins from non-previously found microorganism • Description of the most important metabolic and cellular activities	Cárdenas et al. (2014)
Pu-erh tea	Metaproteomic	• Description of proteins from predominant microorganisms • Important enzymes for substrate utilization and development of antioxidant potential of the tea were described	Zhao et al. (2015)
Sourdough bread for 2500 years	Metaproteomic	• Metaproteomic allows the study of archeological vestiges, particularly in this study the proteins of substrate and starters were identified	Shevchenko et al. (2014)
Hong Qu rice wine	Metaproteomic	• Description of proteins from predominant microorganisms	Xu-Cong et al. (2016)
Soybean fermentation	Metaproteomic	• Description of proteins and peptides with antithrombotic, antioxidant and ACE inhibitor activities	Gibbs et al. (2004)
Rice and rice wine	Metaproteomic	• Description of peptides with antioxidant, hypocholesterolemic, immunomodulatory and ACE inhibitor activities	Han and Xu (2011); Zhang et al. (2010)
Doenjang	Metabolomics	• Description of primary and secondary metabolites during fermentation and their correlation with antioxidant potential • Description of enzymatic activities and their effects in the production of volatile components by the inoculation of various *Aspergillus* species	Lee et al. (2014); Kum et al. (2015)
Meju	Metabolomic	• Description of the metabolites that contribute to taste and nutritional qualities	Kang et al. (2011)
Koji	Metabolomic	• Description of the enzymatic activities and metabolite profiles produced by fermentation	Lee et al. (2016)
Poacai brine	Metabolomic	• Selection of taste markers and effect of LAB in their production	Zhao et al. (2016)
KAMUT® Khorasan flours	Metabolomic	• Relate flour type and organoleptic profile	Balestra et al. (2015)
Fermented tea	Metabolomic	• Description of the effect of fermentation in biochemical profiles, production of volatile compounds and antioxidant potential • Selection of biomarkers for tea classification	Ezgi et al. (2017); Xu et al. (2015); Lee et al. (2011); Cao et al. (2017)
Kimchi	Metatranscriptomic	• Identification of predominant microorganisms • Description of new bacteria not reported previously • Detection of gene expression related to carbohydrate metabolism, vitamin production and lactic acid fermentation	Jung et al. (2013)
Distilled rice liquors	Multi-omic approach (metaproteomic and metagenomic analysis)	• Identification of methanogenic bacteria related to the formation of aroma compounds • Differential expression of the aroma-forming proteins in 300-year and 30-year fermented liquors	Zheng et al. (2015)
Doubanjiang-meju	Multi-omic approach (metagenomic and metabolic analysis)	• Correlation between bacterial community composition and metabolite changes during fermentation	Li et al. (2017)

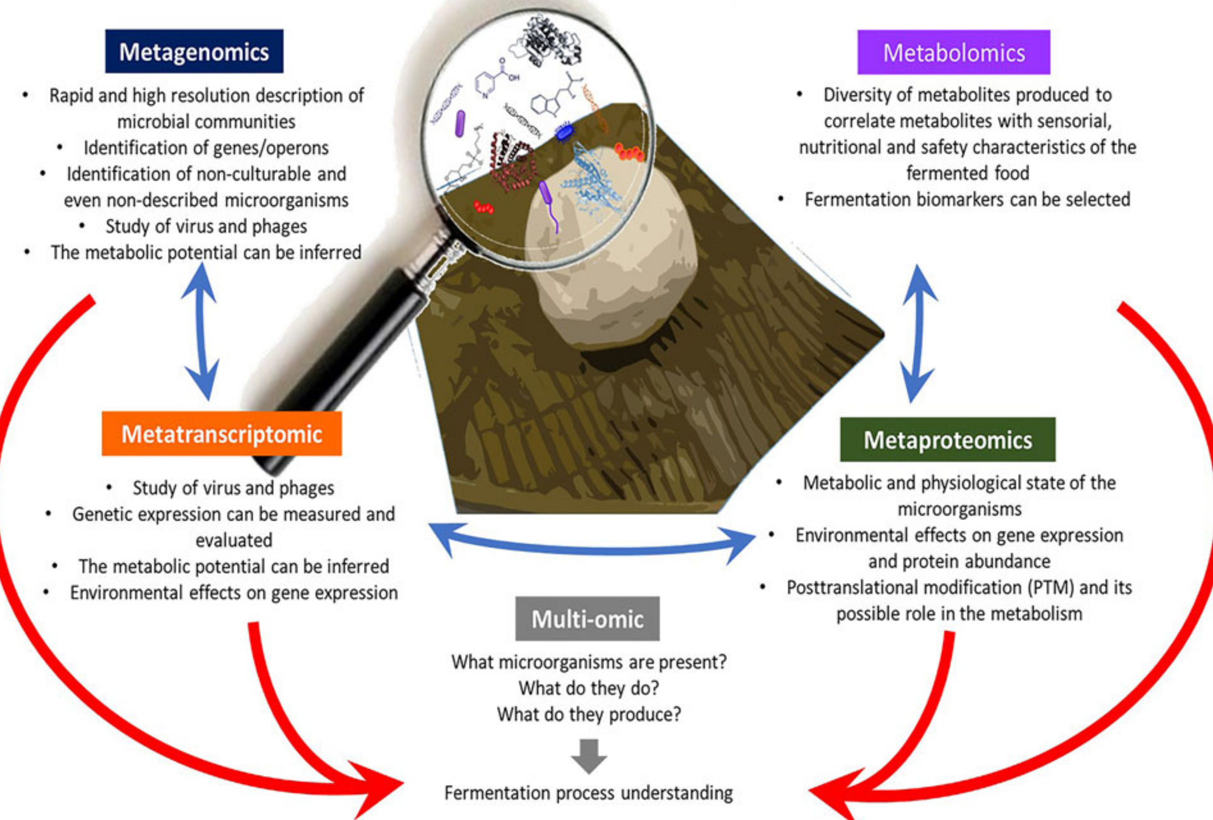

Figure 2. Main advantages of the omic sciences in the study of fermented foods.

has revolutionized the biological sciences, now is possible to obtain extensive information in a single study. However, the correct annotation of the genes still challenge for the correct interpretation of the data in this kind of studies.

The metagenomic approach has been used to investigate the functional and taxonomic features of microbial communities in fermented foods like *kimchi*, a traditional Korean fermented food. *Kimchi* is made with vegetables such as Chinese napa cabbage, cucumber and radish as its major ingredients, and other vegetables as seasonings (red pepper, garlic, leek and ginger), during a 29-day fermentation process. Phylogenetic analysis of bacterial communities by target metagenomics, shows that plant fermentation is governed by three genera. *Leuconostoc* dominates at the beginning stages of fermentation, but as the fermentation progresses, the abundance of *Lactobacillus* and *Weisella* increases. At the end of fermentation, the *Leuconostoc* species dominates again, even at low temperature (4 °C) and high acidity, indicating that fermentation temperature and acidity are not the unique determinants of the microbial populations (Jung et al. 2011).

Additionally, metagenomic sequences have been categorized, and the data sets showed that the *kimchi* microbiome is enriched with genes involved in carbohydrate metabolism, specifically in mono-, di- and oligosaccharide fermentation. Lactate, acetoin and butanodiol metabolism genes were found in the fermentation metabolism subcategory. Finally, H-NMR analysis of various metabolites allowed the detection of carbohydrates, amino acids and free sugars.

Fructose and glucose were detected as the major free sugars; their presence is important not only as carbon sources for the microorganisms but also for the taste of *kimchi*. The level of free sugars (glucose, fructose and sucrose) decreased slowly between days 16 and 23, and after this day, no apparent changes in carbohydrate levels was observed. The fermentation products lactate, acetate and ethanol were detected (Jung et al. 2011), what corroborates the lactic metabolism suggested by the metagenome.

Lee, Jung, and Jeon (2015) searched for the source of contamination in the raw material used to prepare *kimchi* and evaluated the successions of lactic acid bacteria (LAB) during fermentation in five *kimchi* samples. They reported that the main sources of inoculation were garlic and ginger, both of which provided an important bacterial content, especially LAB. Contrary to Jung et al. (2011), they observed that *Lactobacillus* and *Weisella* genera were the main players during fermentation and identified pH as the determinant factor for their survival and succession, indicating that these strains have different acid tolerances.

Recently, metagenomic sequence data from a sample of cocoa bean after 30 h of fermentation was used by Illeghems et al. (2015) to investigate the metabolic pathways of the different bacterial members present in the sample. Meta-pathways were reconstructed based on carbohydrate, amino acid and lipid metabolism genes (Figure 3).

In this fermentation, *L. plantarum* and *L. fermentum* were the dominant LAB; all genes involved in heterolactic fermentation and multiple genes associated with pyruvate

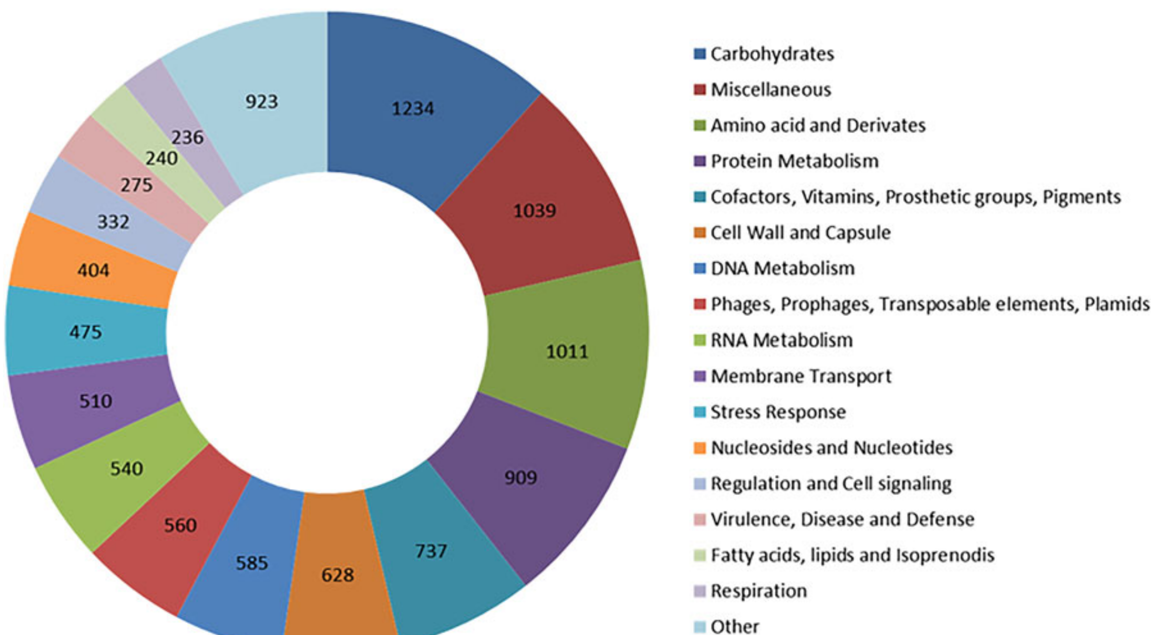

Figure 3. COG from metagenomic data from a sample of cocoa bean fermentation. The data were collected and adapted from Illeghems, Weckx, and Vuyst (2015).

and citrate metabolism were found. The sucrose transport system and enzymes involved in sucrose assimilation were detected, indicating the use of this carbohydrate as an energy source during fermentation. The authors also found the genes that suggest that pyruvate is metabolized by three different mechanisms in which the final products (acetaldehyde, acetoin and diacetyl) give honey like and buttery flavors to fermented cocoa bean.

Concerning amino acid metabolism, only a few genes could be associated with LAB (i.e., oligopeptide and amino acid transport systems and peptidases), indicating that amino acid degradation for flavor production is not a key metabolic pathway during cocoa bean fermentation. Interestingly, the authors observed that the *Enterobacteriaceae* family is involved in the methylglyoxal detoxification pathway, pectinolysis and bacteriocin production. Therefore, this family could play a more important functional role during fermentation than previously assumed. Although the analysis was done in a single sample, the results in this study provided a better knowledge of the functional role of the ecosystem in cocoa bean fermentation. Moreover, the essential roles of LAB during fermentation were confirmed.

In general, traditional fermented food are made under ancestral and empirical knowledge, making difficult the development of appropriate technologies for its large-scale production. Some alternatives have been adopted, such as the use of starter cultures naturally obtained, or starter cultures of defined microorganism inoculated on substrates, the production of food processing enzymes and the stabilization or manipulation of spontaneous fermentations (Achi 2005). An example of how fermentation conditions can be manipulated is *nuruk*, traditionally used in Korea for the elaboration of alcoholic beverages *Makgeolli* and *Takju* (Bal et al. 2014).

Nuruk is prepared in a traditional way from unsterile wheat dough, fermented spontaneously in a temperature range of 30–45 °C, this range of temperatures may favor the selection of certain microbial communities over others. A recent metagenomic study demonstrated that the temperature is a selective parameter for the microbial development. When *nuruk* is incubated at 36 °C, *Mucorales* sp. as the predominant species, followed by *Aspergillus cibarius*, *Aspergillus candidus*, *Rhizomucor pusillus* and *Lichtheimia* sp., whereas an initial fermentation step at 45 °C for ten days followed by twenty days at 35 °C favors *Rhizomucor pusillus* predominance, with *Mucorales* sp. and *Thermoascus crustaceus* present in lower numbers. Remarkably, the authors found some species at day zero associated with plants, *Nectriaceae* sp., *Monographella nivalis*, *Tremellomycetes* sp., *Glomeromycetes* sp. and *Ustilaginaceae*, however, these species are reduced or eliminated during the fermentation thereby they are not necessary for the process (Bal et al. 2016)

The use of standardized starter cultures inoculated on defined substrates is now applied to the commercial production of fermented foods produced at their origin, only traditionally. *Doenjang*, a Korean fermented bean paste, is produced in a traditional or commercial way. In the first one, *doenjang* is primarily made with *meju*, a natural fermented soybean, in the commercial, the fermented soybean paste is made using wheat *koji* inoculated with *A. oryzae.* Metagenomic analysis in the traditional and commercial *doenjang*, clearly demonstrated differences in microbial composition. In commercial *doenjang* the diversity at family level was lower, only *Bacillaceae*, *Enterococcaceae*, *Staphylococcaceae* and *Cyanobacteria* families were present, while in traditional *doenjang* seven more families were identified, *Thermoactinomycetaceae*, *Lactobacillaceae*, *Leuconostocaceae*, *Brucellaceae*, *Enterobacteriaceae*, *Halomonadaceae* and *Corynebacteriaceae*. The study also demonstrated that the

communities found in *doenjang* are closely related to the sensory characteristics, while traditional *doenjang* exhibited a fish sauce flavor, *meju*, bitterness, sourness, and saltiness, the modified samples had sweetness, umami and an alcohol odor (Kim et al. 2016).

In some fermented foods there is more than one source of inoculation, as a result, the origins of microbes in traditional fermented foods or beverages remain poorly characterized, which complicates more the control of the fermentation process. The elaboration of Chinese strong-flavor liquor is a special solid-state fermentation, the process includes *Daqu* making (crushed wheat, mixing with water, shaped into blocks, spontaneously fermented by 30 days and stored for 3–6 months), mixing the *Daqu* with steamed rice husks and transferring into fermentation pit for about 40–45 days to finally distilling the fermented grain. The fermenting pit is constructed by digging a rectangular cave under the ground, and the inside walls of pit are covered with pit mud (Hu et al. 2016).

Pit mud together with *Daqu* acts as an inoculum and habitat of important microbes for the breakdown of macromolecules into small peptides and monosaccharides to produce characteristic flavor components of the liquor. Wang, Du, and Xu (2017) study through a metagenomic analysis *Daqu*, pit mud and fermented grains samples to elucidate the dynamics of fermentation. They found that the principal source of strict aerobes and facultative aerobes is *Daqu*, on the other hand, pit mud was the sustained-release source of anaerobes. In the first 3 days of fermentation a total of 123 genera were found in fermented grains and *Daqu*, mainly strict aerobes (e.g., *Staphylococcus, Thermoactinomyces, Gluconobacter* and *Acetobacter*) and some facultative aerobes (e.g., *Bacillus, Lactobacillus, Leuconostoc, Lactococcus, Pediococcus* and *Weissella*). Finally, when fermented for 45 days, 14 other genera were found shared by pit mud and fermented grains. The results indicated that *Daqu* had stronger influence on the prokaryotic communities in fermented grain at the prophase of fermentation (3 days), but pit mud influenced the fermented grain continuously during the whole fermentation process (Wang, Du, and Xu 2017).

Metaproteomics

The DNA within each cell contains the genetic map of the whole organism, however, the cellular phenotype can only be detected with proteomics. Each of these proteins is responsible for carrying out one or more specific functions within the cell. The different patterns of gene expression under different conditions explain the changes in cellular function throughout the life of the cells and, clearly, during the development of fermentations. Furthermore, once a protein is produced, it can be further modified in post-translational modifications, which affect the function that the protein performs in the cell or environment. Together, these various possibilities constitute the proteome—the entire set of proteins made by a cell or, in the case of multicellular organisms, such as humans, by a cell type or tissue in the body—that is many times larger and much more complex than its corresponding genome (Chandramouli and Qian 2009; Merril and Mazza 2006; Pischetsrieder and Baeuerlein 2009).

Although genomics shows the potential of a cell or organism to face the environment, proteomics offers a precise picture of what is occurring at a certain time in the organism, including which genes are expressed and which modifications the resulting proteins may have, depending on environmental variables.

In the last years, the improvements in protein separation techniques, including two-dimensional polyacrylamide gel electrophoresis, liquid chromatography and isotope-coded affinity tag (ICAT), together with progress in mass spectrometry and database searches, have enabled the study of proteins in complex matrices such as foods (Elviri and Mattarozzi 2012). In food science, this approach has been used to reveal composition, to specify biomarkers for the identification of quality and authenticity, to identify food allergens, to show the effects of processing and storage on proteins and to find new bioactive compounds (Pischetsrieder and Baeuerlein 2009).

However, to address the proteomic study of food fermentation, several factors should be considered. First, fermented food matrices, especially traditional ones, contain several microbial species that express numerous proteins, some with specific enzymatic activities responsible for the final properties of texture, taste and flavor in fermented foods. Additionally, the expression and activity of proteins varies as a result of external factor changes, such as various stresses, metabolic states, and interactions with other organisms or chemical substances; consequently, the proteome is a complex dynamic system. Additionally, the concentration range of different proteins may vary dramatically; most biological samples contain certain highly abundant proteins, which are often not interesting for proteomic analysis, whereas the microorganism's proteins of high importance may be several orders of magnitude less abundant (Elviri and Mattarozzi 2012). Finally, in most fermented foods, the raw material used for their elaboration contains components, including their own proteins that interfere with detection of the microbial proteins.

For these reasons, studies in the fermented foods field are still scarce, and many are focused on the development of protein extraction methods since it is a critical step for further protein analysis and identification. For example, in three Korean fermented soybean products, three extraction methods were compared to determine the profile changes of soluble proteins by image analysis of the protein spots in 2-D gel electrophoresis at different fermentation times (0 to 120 days). Most of the proteins decreased as the fermentation proceeded. However, the identification of fluctuating proteins (proteins that increase or decrease) is limited by the lack of a soy protein database (Lim 2008).

Cárdenas et al. (2014) developed a methodology for protein recovery from *pozol*. The high starch concentration (75%) and low protein content of the maize dough hindered the extraction. Nevertheless, the extraction methodology allowed obtaining and characterizing the metaproteome of a

15-day fermented pozol. Numerous bacterial and fungal proteins were identified; LAB proteins predominated, mainly from the *Lactobacillus* genus, while fungi were mainly represented by *Aspergillus*. Prevalent proteins belonged to carbohydrate metabolism and energy production pathways.

More recently, Zhao et al. (2015) studied the microbial proteins of *Pu-erh* tea fermented for 21 days; this Chinese tea is produced by a natural fermentation of green tea leaves. To conduct the metaproteomic analysis, four extraction methods were tested, and the proteins were analyzed in SDS-PAGE. The LC-MS/MS analysis revealed that *Pseud*omonas and *Lactococcus* were the dominant bacterial genera, whereas *Aspergillus* was the dominant fungus and produced most of the identified proteins (50.45%). Many of the extracellular microbial enzymes could degrade the plant cell wall, allowing the decomposition of the tea leaves. Moreover, several catalases and peroxidases were also identified; these enzymes may catalyze the oxidization of catechins, which have been identified as potent antioxidants that contribute to the color and taste of black tea.

Remarkably, Shevchenko et al. (2014) identified the composition of a sourdough bread dating back approximately 2500 years from the Subeixi cemetery in China. Metaproteomic analysis allowed to identify 106 proteins that according to their origin were divided in: 1) plant proteins, the most abundant; 2) yeast of Saccharomycetaceae family; 3) lactic acid bacteria, specifically the *Lactobacilli* and Leuconostocaceae genus (*W. cibaria*, *L. pseudomesenteroides*) and 4) human proteins. A few peptides from fermented milk (alpha-casein 1) were also detected. 2500 years later, the authors proposed that the fermentation could be started with fermented flour mixed with water and/or milk.

Given that there is a relationship between fungal, yeast and bacterial activities during the fermentation process, the selection of suitable and beneficial microbial starter strains is an important step during the elaboration of traditional fermented foods. In *Hong Qu* rice wine, ten samples of traditional fermentation starters from different geographical areas of the Fujian province in China were analyzed by MALDI-TOF mass spectrometry fingerprinting. This new proteomic fingerprinting identification method demonstrated to be a faster and more accurate method to identify the bacterial isolates than molecular biology method based on 16S rRNA gene sequencing (Xu-Cong et al. 2016).

Six different genera, *Bacillus, Staphylococcus, Leuconostoc, Pediococcus, Lactobacillus* and *Lactococcus*, were identified. *B. amyloliquefaciens* was predominant, followed by *B. megaterium*, *L. lactis* subsp. *lactis* and *B. subtilis*. These bacteria secrete a wide range of hydrolytic enzymes, contributing to the final flavor of *Hong Qu*.

Fermented food products could be considered "functional foods" as they have beneficial effects on human health beyond their nutritional effects. Their consumption has been associated with good health, given that they can contain probiotics, prebiotics or both. Moreover, proteins and peptides from food have been found to have several biological activities (Hartmann and Meisel 2007; Stanton et al. 2005).

Studies on the production of active peptides derived from fermented animal products are numerous. However, recent investigations have focused on the possibility of obtaining bioactive peptides from vegetables (cereals, legumes, tubers, seeds and others) and their fermented products. For example, in soybean fermentation, the release of many small peptides has been observed from the hydrolysis of soybean proteins by microbial proteases (Sanjukta and Rai 2016). In soy products, fermentation with *Bacillus* and *Rhizopus* strains allows the production of peptides with antithrombotic and antioxidant activities, as well as angiotensin-converting-enzyme inhibitor (ACE inhibitor) activity (Gibbs et al. 2004). Similarly, in rice and rice wine, antioxidant peptides and peptides with hypocholesterolemic, immunomodulatory and angiotensin-converting-enzyme inhibitor activity (ACE inhibitor) have been reported (Han and Xu 2011; Zhang et al. 2010).

Recently, a proteolytic *B. subtilis* was used in a submerged fermentation to produce bioactive peptides from tomato seed proteins. ACE inhibitor and antioxidant activities were identified regardless of whether the protein had previously been fermented or not. However, both activities were significantly higher in the fermented samples, showing more than 4- and 2.5-fold increases, respectively. The authors proposed that ACE inhibitor sequences are probably encrypted in tomato seed proteins and released during fermentation by employing a suitable proteolytic system (Moayedi et al. 2017).

The functional components present in food play an important role in health conservation. Evidence to date suggests that fermentation is a way to improve the bioavailability of bioactive peptides and that proteomic and peptidomic analysis will increase our knowledge in this area.

Metabolomics

Metabolomics is an emerging science that addresses the analysis of endogenous and exogenous small molecules, such as peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, fatty acids, and phenolic compounds. It focuses on the type of metabolites that the cell produces, and unlike genomics, it reflects the cell's biochemical state (Mozzi et al. 2013).

Two types of approaches are used in metabolic studies: untargeted and targeted analyses. In the first approach, the overall metabolite profile is evaluated without knowledge of what kind of compound(s) is responsible for changes in the system. The idea is to get a pattern of fingerprints through the detection of many groups of metabolites. On the other hand, in targeted metabolomic analysis, a specific group of metabolites is identified and quantified in a sample under certain conditions (Cevallos-Cevallos and Reyes-de-Corcuera 2012; Patti, Yanez, and Siuzdak 2012).

Metabolic studies have been performed (mainly in fermented soy foods) in which the primary metabolites, such as proteins, amino acids, organic acids and carbohydrates, have been analyzed (Table 3). These components are important because they add the sensory qualities to food,

Table 3. Metabolomics applied to different fermented foods.

Fermented food	Microorganisms	Technique	Most important metabolites	Type of metabolite	Changes (if is reported)	Sensorial properties	Reference
Chenggukjang (fermented soybean paste)	*Bacillus* strains	GC/TOF-MS and PCA analysis	Alanine, glycine and lysine	Amino acids (umami taste)	Concentration increases significantly between 24 and 48 hours	Sweet and umami taste were the predominate	Baek et al. (2010)
			Glutamic acid, asparagine, phenylalanine, tyrosine		Produced during fermentation		
			Fructose, glucose, sucrose, melibiose and maltose	Carbohydrates (distinct tastes and aromas)	Concentration increases within first 12 hours		
			Arabinose, ribose, xylose, mannose		Concentration increases during fermentation		
			Oxalic acid, succinic acid and malonic acid	Organic acids (resulting in different pH levels, tastes and aroma)	Not found in the unfermented *Cheonggukjang* but present during fermentation		
Doenjang (fermented soybean paste)	*Bacillus*, *Rhizopus*, *Mucor* and *Aspergillus*	GC–MS and PCA	Glycine, alanine, serine, threonine, glutamic acid and asparagine	Amino acid (responsible for the sweet and umami taste)	Concentration increases between 140 and 160 days	Sweet and umami taste	Namgung et al. (2010)
			Lactic acid, succinic acid, mandelic acid, propionic acid and glutaric acid	Organic acids	Concentration increases rapidly with the time of fermentation		
			Glucose	Carbohydrates	Concentration is reduced after 20 days		
			Fructose, Galactose		Insignificant amounts after 100 days		
Fermented soymilk	*Bifidobacteria* and *Streptococci*	H NMR and PCA	Phenylalanine	Amino acid	Not reported	Not reported	Yang et al. (2009)
			Citric acid, Lactic acid, Malic acid, Oxalacetic acid, Succinic acid	Organic acids			

Amino acid, organic acid and carbohydrates analyzed.
H NMR, proton-nuclear magnetic resonance; GC/TOF-MS, gas chromatography/time of flight-mass spectrometry; PCA, principal component analysis.

yielding a sweet taste. In contrast, secondary metabolites, such as isoflavones, saponins, phytic acid and tocopherols, contribute to the antioxidant properties of soybean-based foods (Lee et al. 2014).

As previously mentioned, Korean meal *doenjang* is produced in 2 steps: first, soybeans are steamed, dried and fermented to make *meju*. In the second step, salt water is added to the *meju*, and the mixture is fermented again. The mixture is separated into the solid portion, *doenjang* (soy paste), and the liquid portion, *kanjang* (soy sauce). The final characteristics of the product change in accordance with the metabolites present, which depend on the physiochemical or enzymatic activities occurring during each step of the fabrication. For that reason, Lee et al. (2014) investigated not only the primary metabolite changes during industrial processing but also the secondary metabolite changes.

In the primary metabolite profiles, the authors found malonic acid, succinic acid, malic acid, citric acid, γ-aminobutyric acid, sucrose, maltose, melibiose and raffinose as the major metabolites related to the initial fermentation (soybean to meju fermentation steps), while most amino acids, monosaccharides and fatty acids were associated with the brining and *doenjang* aging steps. The major secondary metabolites during the processing of *doenjang* were isoflavones and soya saponin. These results showed a strong correlation of metabolite production with *doenjang*'s antioxidant potential.

To understand which metabolites produced during *meju* fermentation contribute to the taste and the nutritional qualities of *doenjang* and *kanjang*, Kang et al. (2011) analyzed *meju* samples taken at different fermentation times in an UPLC-quadrupole-time of flight mass spectrometry. The authors observed that the soy protein in *meju* was degraded during fermentation, which resulted in increases in the total contents of free amino acids and peptides. It was clear that various metabolites (amino acids, nucleosides, urea cycle intermediates) were altered with the increase of fermentation time, compared with non-fermented soy. In this analysis, the authors identified that threonine and proline were responsible for sweet taste; phenylalanine, tyrosine, leucine, isoleucine, valine and methionine were responsible for bitter taste and glutamic acid for umami flavor (Table 3). With these results, the authors found that it was possible to use certain biomarkers to monitor the fermentation because several metabolites were positively correlated with the nutritional and sensory qualities.

To understand the relationship between the enzymatic activities and the formation of volatile components in *doenjang* fermentation, Kum et al. (2015) determined the effect of three species of *Aspergillus* on metabolite formation during an eight-week fermentation. Regardless the inoculated *Aspergillus*, ten different classes of compounds were found and classified as acids, alcohols, carbonyls, furan derivatives, hydrocarbons, phenols, esters, sulfur-containing compounds and miscellaneous groups. Among these, long-chain fatty acid ethyl esters tended to increase with fermentation time, and their production was strongly correlated with lipase activity, imparting fatty and oily odor notes.

Alpha-amylase activity was associated with an early stage of fermentation, with high levels of enzyme activity in the samples inoculated with *A. oryzae*. This activity slowly decreased as the microorganism consumed the substrate and affected the formation of metabolites derived directly and/or indirectly from sugar-degradation products, such as 2-methylpyrazine, 3-hydroxy-2-methylpyran-4-one, and furan-2-carbaldehyde.

On the other hand, protease activity increased during fermentation, affecting metabolites such as ethyl 3-phenylpropanoate, 2-phenylethyl acetate, (E)-2-phenylbut-2-enal, and 2-phenylethanol, derived from phenylalanine. Among these compounds, the formation of two ethyl esters (ethyl 3-phenylpropanoate and 2-phenylethyl acetate) could be influenced by the actions of both protease and esterase. An important correlation between lipase activity and the production of methyl 2-methylprop-2-enoate and pentyl propanoate during fermentation was also observed. The former is known to contribute to sweet, fruity, and apple-like odor notes, and the latter imparts sweet and mixed fruit, tropical odor notes that could be formed by the esterification of pentanol and propionic acid (Kum et al. 2015).

In the same way, *A. oryzae* and *B. amyloliquefaciens Koji* fermentation were studied. The metabolic profiles could distinguish between the rice *Koji* fermentation based on the species used for the fermentation. The *A. oryzae* fermentation had relatively high contents of carbohydrate metabolism intermediates, such as sugars and sugar alcohols, organic acids, phenolic acids, and lipid metabolism intermediates, such as fatty acids. Instead, relatively high abundances of flavonoids, lysophospholipids and amino acids, leading to higher antioxidant activity were found in samples of *B. amyloliquefaciens* fermentations (Lee et al. 2016).

A metabolomic approach has also been used to select taste marker compounds. In poacai brine, a Chinese sauerkraut, Zhao et al. (2016) established the relationship between lactic acid bacteria and some taste features. For instance, glucose, fructose, and sucrose lead to a difference in sweetness, and lactic acid content affects the sourness. Glutamic acid content provides an umami taste, and in this fermented food, it is probably generated by *L. plantarum* and *L. buchneri*. Other amino acids, such as proline, valine, leucine, isoleucine, and phenylalanine, contributed to the bitter taste. However, it is very interesting that the LAB used in the study have incomplete pathways for amino acid production. This observation indicates that metabolic complementation among the different genera in the fermentation process may play an important role in the flavor formation pathways.

The baking industry is continuously seeking to optimize their production technology because the consumer prefers products made with traditional sourdough, which is more healthy, natural and tasty than other options. Bread makers must adapt their products to market challenges by either completely or partially replacing wheat flour with other flour types. In this context, durum wheat and KAMUT® Khorasan flours are particularly interesting for use in bread doughs. To predict the flavoring impact of wheat and KAMUT® Khorasan flours on fermented dough, Balestra

et al. (2015) evaluated the volatile metabolites in different dough samples. With this approach, the authors were able to distinguish between the types of dough and discriminate between different grain samples and different types of fermentation. Additionally, it was possible to distinguish sensorial characteristics in each group, which were principally associated with sulfur compounds, sulfur organic compounds, and methane-aliphatic compounds. In general, a higher concentration of volatile metabolites was observed in KAMUT® Khorasan dough than in other types of doughs.

Tea is a popular nonalcoholic beverage, there are four categories to classify the tea according to their degree of fermentation and manufacturing process: non-fermented (green tea), semi-fermented (oolong tea), fully fermented (black tea) and post-fermented (dark tea). In semi-fermented and black teas, the "fermentation" process refers to natural browning reactions induced by tea leaves oxidative enzymes while in dark tea the fermentation occurs under natural or controlled conditions in which multiple fungi species are involved. Different from other teas, in the post-fermented teas the ingredients can be affected by environmental microorganisms, temperature and humidity resulting in the production of a variety of flavor compounds (Hai-Peng et al. 2013).

Studies of the chemical constituents of tea have progressed in the last years, demonstrating that tea contains large amounts of polyphenols and phenolic acids which contributes to many biological functions (Ito et al. 2008; Plumb et al. 1998).

However, is still unknown if the fermentation process contributes to the formation of new molecules. Recently, a comparative metabolomic study showed that while in unfermented teas L-theanine, catechins and catechins derivatives are abundant, in fermented teas catechins are oxidized into theaflavin, which increases its antioxidant potential (Ananingsih et al. 2013; Ezgi et al. 2017). In fermented teas, gallic acid, manghaslin, theaflavin, 3,7-dimethyluric acid, fuzhuanin C and caffeine are abundant, suggesting that fungi has a significant influence on the biochemical profiles during the fermentation process (Xu et al. 2015; Lee et al. 2011).

Further investigation in different dark tea samples revealed that some compounds can be used as markers for the classification of the teas, since differences of volatile compounds may be related to its own manufacturing process. Authors also found that volatile components are also markers for maturity of tea leaves, variation of plants and predominance of fungi involved in fermentation (Cao et al. 2017).

Combinatorial analysis (multi-omic approach)

Most of omics studies in fermented food only apply one of the available techniques, generating a partial picture of the fermentation. Probably due to the cost of experiments or the need for multidisciplinary teams to recover and analyze the data. There are only few examples that combine different omics approaches for studying traditional fermented food.

The traditional food that best exemplifies the potential of the multi-omic approach is *kimchi*. Different aspects of the microbial community have been addressed using metagenomic, metabolomics and metatranscriptomics data. The combinational analysis of the microbiota and metabolites enables a better understanding of the relationship between the food matrix and its microbiota as never before.

The metagenomic approach was previously described in this article (see Metagenomic section). This technique has allowed to describe the source of contamination in the raw material, the microbial diversity, composition and evolution during fermentation. The analysis of different group of genes has demonstrated the presence of many phage DNA sequences indicating infection by bacteriophages during fermentation. Also, it has been demonstrated that carbohydrate fermentation seems to be a key category of genes that enable survival in this ecosystem (Jung et al. 2011; Lee, Jung, and Jeon 2015).

The metabolomic analysis has allowed to identify the metabolites produced during the fermentation. Fructose, glucose and sucrose were detected as the major free sugars at the beginning of fermentation; metabolites important as carbon source and as a part of the taste in *kimchi*. The organic acids (lactate, acetate and succinate) and bioactive substances such as GABA (gamma-aminobutyric acid) and mannitol are produced during long-term storage. These compounds were correlated with the decreased of free sugars (Jung et al. 2011; Jeong et al. 2013).

Finally, the metatranscriptomic approach has been used to evaluate the gene expression and the active populations during *kimchi* fermentation. The samples were selected according to the changes observed in pyrosequencing community analysis (Jung et al. 2011), leaving five *kimchi* samples. The total mRNA sequencing reads were matched to the completed genomes of six chosen reference LAB strains. The relative gene expression of *L. mesenteroides* was high during the early-stage fermentation and decreased gradually as the fermentation progressed, while *L. sakei* and *W. koreensis* were most active during the development of fermentation. The presence of *L. mesenteroides* and *L. sakei* has been previously described (Lee, Jung, and Jeon 2015; Jung et al. 2011) whereas the role of *Weissella* were unknown. Metatranscriptomic data showed the increase of expression levels in *W. koreensis* in a low pH *kimchi*. The acid tolerance of this specie correlates with the high expression rate of stress resistance genes, especially pH resistance ones. The functional annotation of mRNA reads showed that the *Leuconostoc* species have several copies of mannitol dehydrogenase encoding genes which indicates that these species are responsible for mannitol production. Contrary to previous studies, this work demonstrated that homo- and heterofermentative pathways were active and all genes coding for both pathways were expressed during the process. The approach allowed to reveal some interesting features, such as the presence of genes to produce vitamins (folate and riboflavin) and the absence of amino acid decarboxylase genes responsible for biogenic amine production, factors important for flavor and human nutrition (Jung et al. 2013).

By combining quantitative metaproteomic and metagenomic analysis Zheng et al. (2015) compared two distilled rice liquors subsequently fermented for 30 and 300 years to correlate protein function and aroma compounds. Authors found 59 proteins related to aroma-forming highly expressed in the 300 years liquor. Both metaproteomic and metagenomic analysis demonstrated the presence of methanogenic bacteria, *Clostridium* and *Methanobacterium*, which clearly are related to the formation of aroma compounds like butyric, caproic and acetic acid and therefore the production of high quality liquor. This result indirectly indicates that more compounds would be formed if the fermentation process occurs for longer periods (Zheng et al. 2015).

Recently, the study of bacterial community succession (metagenomic study) and metabolite changes (metabolomic study) was done in *doubanjiang-meju*, an important ingredient to produce traditional Chinese fermented food, such as *doubanjiang* (also called *Pixian-douban*), broad bean sauce, broad bean paste, etc. The results showed a strong correlation between bacterial community composition and metabolite changes during fermentation. It is clear that the decrease of pH stimulates changes in the prokaryotic community structure since some strains are not acid tolerant. The abundance of some bacteria increased rapidly at the beginning of fermentation, for example, *Staphylococcus*, unclassified bacteria and unclassified Leuconostocaceae, while others decreased up to the 14 days and then were relatively stable until the end of fermentation, *Tetragenococcus* increased, followed by *Acinetobacter*, *Lactobacillus* and *Pseudomonas*. On the other hand, the authors found an important association between three bacterial populations and the metabolites. *Pseudomonas* was significantly correlated with two carbonic compounds (glucose and arabinose) and eight nitrogen compounds (glutamate, alanine, homoserine, guanidoacetate, aspartate, glycine, phenylalanine and pyroglutamate), while *Streptococcus* was significantly correlated with six carbonic metabolites (glycerol, arabinose, acetate, ribose, fructose and lactate) and eight nitrogenous metabolites (glutamate, alanine, valine, threonine, guanidoacetate, aspartate, glycine and phenylalanine). Finally, *Tetragenococcus* was significantly correlated with guanidoacetate, phenylalanine and chromaticity. Altogether, the results allowed to elucidate the fermentation dynamic from the microbiological and metabolic points of view (Li et al. 2017).

The depth that allows the simultaneous analysis of several omics is promising, the metabolic and functional understanding of the microbial communities and their impact on the fermented product, including the molecules that define its aroma and flavor, as well as its nutritional properties suggest that it will be possible to control fermentation to obtain safer products of constant quality.

Foodomics

Currently, food safety is a major concern in the global food industry. The industrialization and mass production of some products, the globalization of food products, the variations in food consumption patterns, and the differences in food preparation habits are some factors that contribute to the increase in various food safety issues. The main challenges in food safety include the emergence of new food pathogens, the adulteration of food materials, the presence of contaminants, and the unknown effects of the consumption of genetically modified foods (Pinu 2016). Clearly, these concerns also impact fermented foods, especially since quality and hygiene are not controlled as they are in commercial foods.

Currently, there is a worldwide tendency to value traditional fermented foods for their functional properties. Several groups are attempting to produce standardized inocula and to generate processes for commercial production. Omics could participate in these efforts through a new concept called "foodomics." The application of techniques from these fields is gaining importance in food technology, both for process development and validation as well as quality control of the raw materials and final products. Foodomics has been mainly applied in the detection of allergenic proteins and chemical contaminants in food, the identification of biomarkers to confirm the quality of foodstuffs and the creation of novel strategies for pathogenic microorganism detection.

In the food industry, the presence of food spoilage microorganisms (bacteria, yeast and molds) cause undesirable variation in organoleptic characteristics (flavor, smell and taste) and directly affect food quality and safety. Moreover, the consumption of foods and/or beverages contaminated by pathogenic microorganisms is an important public health problem with high incidence because of the existence of many vulnerable groups, poor quality processing that leads to incomplete elimination of food pathogens, and the production of toxic compounds. The presence of spoilage microorganisms is a direct indicator of the hygienic and sanitary quality of the food. However, these disadvantages in fresh food must be seen differently in fermented foods, where it is first necessary to establish who the members of the bacterial communities are, as well as their functional participation in order to define afterwards who the unwanted microorganisms are. Facing a lack of knowledge of traditional fermentations, we present some alternatives that could be applied in the control process of traditional fermentations.

Proteomic tools can be used for the characterization, identification and quantification of pathogens and toxins. The main applications of proteomics in food safety have been conducted with common food pathogens. These studies have enabled the identification of proteins involved in pathogenicity and those that contribute to virulence, the definition of pathogen protein expression under different conditions and the determination of potential virulence factors in pathogens (Kalb et al. 2015; Quiblier et al. 2013; Srajer et al. 2013; Yang et al. 2012; Ye et al. 2016).

Another advance in modern food safety research is the use of whole-genome sequencing (WGS) as a tool for the detection of foodborne pathogens. The major advantage in this approach is the possibility of evaluating some specific regions (virulence genes) of a particular microorganism to determine its potential pathogenicity, identify to which genus, species and/or strain belongs and understand how the microbes grow and survive in the niche (Abee et al.

2004). This kind of analysis have shown high discrimination power among food-borne pathogens and non-pathogens (Kim et al. 2008).

When food is contaminated, the metabolic process of the undesirable microorganisms leads to food spoilage (Wang et al. 2016). The use of microbial volatile organic compounds as indicators of microbial contamination in foodstuffs is another interesting alternative in food safety research. The approach has already been used in dairy products, meat and fish products, fruit and fruit preserves and beverages to compare volatile compound production under different conditions, to identify the volatile compounds in contaminated foods and to compare the metabolic profiles of different samples. The information could also be used for the discrimination of bacteria.

Food allergy is a specific form of intolerance to a food component that activates the immune system in predisposed individuals. Food allergies affect 2–3% of adults and at least 8% of children worldwide, and the ingestion of food allergens can induce minor to severe reactions (Sancho and Mills 2010). The foods that cause the most severe reactions are cereals (due to high gluten content), eggs, fish, milk, soy, nuts and some dried fruits. At least 70 foods have been linked to food allergies.

Information on the effects of fermentation on food allergens is limited. For example, it has been demonstrated that soy protein can retain some of its allergenicity throughout the fermentation process (Hefle et al. 2005). The fermentation process itself does not degrade the major allergenic soy proteins (β-conglycinin, glycinin), while the use of enzymes results in the breakdown of soy allergen structures into smaller peptides (Meinlschmidt et al. 2016). In contrast, the antigenicity of whey proteins was reduced by more than 99% after lactic acid fermentation (Jedrychowski 1999). However, these studies remain few, and most of them have been conducted by immunoassay. Consequently, the implementation of foodomics in this field is necessary to accelerate our knowledge of the effects of fermentation on food allergens.

Another important area to study is the presence of antinutritional compounds, as well as chemical contaminants, which are found in many vegetal foods. Legumes and cereal grains contain some natural toxicants including tannins, phytic acid, protease and trypsin inhibitors, saponins, metal chelates, cyanogens, isoflavonoids, phytoalexins, flatus factors, hemagglutinins and polyphenols (Gilani et al. 2005; Gilani et al. 2012; Humer and Schedle 2016). Fermentation with lactic acid bacteria and other microorganisms, such as fungi of the genus *Aspergillus*, reduces the content of tannins, phytates and polyphenols in many traditional fermentations and improves the nutritional value and organoleptic qualities of foods; however, there are no control processes to avoid variations in quality in different fermentations (Difo et al. 2015; Roger, Léopold, and Funtong 2015).

Finally, peptidomic studies are emerging as an important component of foodomics studies. The aim of these studies is to identify whether the products of protein degradation have positive (health-promoting activity) or undesirable (allergy, intolerance, toxicity) effects on human health. Additionally, the information generated in peptidomic studies could provide information on the authenticity, allergenicity and origins of the fermented food products (Jiménez-Martínez and Gutiérrez-López 2012; Menschaert et al. 2010).

Conclusions

Our understanding of complex fermentations will largely depend on the application of omics technologies. Omic sciences or "next generation" methods for the study of fermented foods would allow the determination and quantification of microbial composition, the observation of changes associated with their development and the identification of metabolites that they produce. The data obtained through these studies must be integrated to understand how the final properties of the food are achieved; beyond this, it is desirable to develop a technology that enables the reproducible production of fermented foods without losing the unique characteristics of the traditional products that they are derived from.

However, meta-omics science still has to address several critical points. Perhaps the most important point is related to the currently low number of microorganisms with sequenced and correctly annotated genomes in the databases that are used as a reference for identifying genes and proteins. Therefore, in proteomics studies, a significant number of "identified" proteins are likely to be false positives since their identification depends on databases that are not necessarily biocurated or curated databases that still have a limited number of entries. Meanwhile, for metabolomics, the youngest field in the omics family, the major challenge is the development of standardized data repositories to reflect the diverse and sensitive nature of the metabolome.

The application of the omics to traditional fermentations will provide knowledge of the microbiota that constitute them and how their metabolism impacts the organoleptic characteristics of the food, as well as the information regarding the health benefits of these foods, while highlighting the problems associated with a lack of processes because of the traditional methods of production. Therefore, omics may positively impact the development and/or optimization of food products with major potential benefits.

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ORCID

Sergio Sánchez http://orcid.org/0000-0002-6350-5052

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Capítulo tercero

Fijación de nitrógeno en el pozol

Los cereales constituyen un elemento básico para la elaboración de diferentes alimentos para consumo humano. Son una fuente importante de carbohidratos, proteínas, lípidos, vitaminas y minerales, sin embargo, contienen cantidades importantes de factores antinutricionales y son deficientes en aminoácidos esenciales como lisina, triptófano y treonina (Blandino *et al*. 2003). La fermentación de los cereales es un proceso económico que además de permitir la eliminación de factores antinutricionales (inhibidor de proteasa, ácido fítico, estrógenos), ayuda a conservarlos para la posterior preparación de diversos alimentos, los cuales se caracterizan por una mejora en su digestibilidad, sus propiedades sensoriales y más importante aún su valor nutricional, producto de la actividad metabólica de los microorganismos que se desarrollan durante la fermentación (Steinkraus, 2002).

El pozol es una bebida preparada con masa de maíz nixtamalizada y fermentada, se consume en las ciudades de los estados del sureste de México como bebida refrescante a cualquier hora del día, mientras que para algunas poblaciones rurales el pozol es la base de la dieta diaria (Ulloa, 1984; Wacher *et al*. 2000). Este alimento destaca por una mayor concentración en algunos aminoácidos esenciales, vitaminas, nitrógeno total y proteína en comparación con el maíz que se utiliza para su elaboración (Cravioto *et al*. 1955). Considerando que trabajos previos han relacionado el aumento en la concentración de nitrógeno total como resultado de la fijación de nitrógeno atmosférico, se decidió evaluar si este es el proceso por el cual se da dicho incremento.

Los resultados que demuestran la fijación de nitrógeno en el pozol y a las bacterias responsables de dicho fenómeno se presentan en el siguiente artículo. Mediante su análisis químico, se corroboró que durante las primeras 9 horas de fermentación se dan incrementos significativos en el contenido de nitrógeno y proteína cruda. El aislamiento de bacterias en dos medios mínimos sin fuente de nitrógeno en las distintas muestras permitió seleccionar un total de 28 aislados, a los cuales se les cuantifico la actividad nitrogenasa mediante el ensayo de reducción de acetileno (ARA). Las bacterias positivas para esta actividad fueron agrupadas de acuerdo con sus características macro y microscópicas y se identificaron mediante 16S RNAr como *Kosakonia*, *Klebsiella* y *Enterobacter*. Por último, se demostró que, para aumentar el contenido de nitrógeno fijado, es necesario promover el crecimiento de bacterias diazotróficas, lo que demuestra su importancia en los alimentos fermentados tradicionales.

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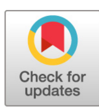

Nitrogen Fixation in Pozol, a Traditional Fermented Beverage

Jocelin Rizo,[a] Marco A. Rogel,[b] Daniel Guillén,[a] Carmen Wacher,[c] Esperanza Martinez-Romero,[b] Sergio Encarnación,[b] Sergio Sánchez,[a] Romina Rodríguez-Sanoja[a]

[a]Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico
[b]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico
[c]Facultad de Química, Universidad Nacional Autónoma de México, Mexico City, Mexico

ABSTRACT Traditional fermentations have been widely studied from the microbiological point of view, but little is known from the functional perspective. In this work, nitrogen fixation by free-living nitrogen-fixing bacteria was conclusively demonstrated in pozol, a traditional Mayan beverage prepared with nixtamalized and fermented maize dough. Three aspects of nitrogen fixation were investigated to ensure that fixation actually happens in the dough: (i) the detection of acetylene reduction activity directly in the substrate, (ii) the presence of potential diazotrophs, and (iii) an *in situ* increase in acetylene reduction by inoculation with one of the microorganisms isolated from the dough. Three genera were identified by sequencing the 16S rRNA and *nifH* genes as *Kosakonia*, *Klebsiella*, and *Enterobacter*, and their ability to fix nitrogen was confirmed.

IMPORTANCE Nitrogen-fixing bacteria are found in different niches, as symbionts in plants, in the intestinal microbiome of several insects, and as free-living microorganisms. Their use in agriculture for plant growth promotion via biological nitrogen fixation has been extensively reported. This work demonstrates the ecological and functional importance that these bacteria can have in food fermentations, reevaluating the presence of these genera as an element that enriches the nutritional value of the dough.

KEYWORDS nitrogen fixation, diazotrophs, traditional fermentations, food fermentation, pozol

Fermentation is the oldest and most economical method to produce and preserve food. The great variety of fermented foods is the result of traditional knowledge and experiences transmitted from generation to generation, as well as of cultural practices, ethnic preferences, geographical location, raw materials, etc.

Fermentation technology has an important role in food conservation, especially in areas with limited resources where preservation techniques, such as cold storage, cannot be used. For foodstuffs produced in this way, the presence of antimicrobial compounds (bacteriocins, organic acids, ethanol, etc.) reduces the risk of contamination and the proliferation of pathogens and spoilage microorganisms (1, 2). Other advantages of fermented foods include the removal of antinutritional compounds and digestibility improvement. Some microorganisms may degrade or reduce antinutritional compounds (cyanide, glycoside linamarin, phytic acid, tannins, and polyphenols), while others may produce enzymes to hydrolyze polysaccharides to simple carbohydrates (3–5). Moreover, fermentation plays a decisive role in human nutrition since during fermentation, a range of metabolites associated with health-promoting properties are produced: bioactive peptides, exopolysaccharides with significant antioxidant and free radical scavenging properties, folate production, improvement in the bioavailability of minerals, and fiber solubilization (6–8).

In cereal-based foods, the nutritional value is strongly influenced by the protein

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Address correspondence to Romina Rodríguez-Sanoja, romina@biomedicas.unam.mx.

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TABLE 1 Proximal analysis of pozol at different fermentation times

Fermentation time (h)	Content in pozol (mg/g of dry dough)[a]						Nitrogen/ash ratio (mg/mg)
	Total carbohydrate	Crude fiber	Lipids	Ash	Nitrogen	Protein	
0	845 ± 9.9	24 ± 4.1	46 ± 4.2	13 ± 0.2	11.477 ± 0.2 A	71.7326 ± 1.2 A	0.88
9	839 ± 8	12 ± 0.5	57 ± 1.2	13 ± 4	12.56366 ± 0.0 B	78.5229 ± 0.3 B	0.97
24	841 ± 3	9 ± 1	64 ± 0.3	13 ± 0.9	12.4410 ± 0.1 B	77.75650 ± 0.5 B	0.96
48	832 ± 7	8 ± 1	68 ± 0.3	13 ± 0.2	12.525 ± 0.0 B	78.2828 ± 0.0 B	0.96

[a]The results are expressed as the mean of three replicates ± standard deviation. Differences were evaluated by ANOVA followed by Tukey's multicomparison test ($P < 0.001$). Different uppercase letters indicate statistically significant differences between samples.

content as well as by the protein composition of the cereal; both characteristics can be improved by the fermentation process (9–11). Pozol is a refreshing, nonalcoholic acidic Mayan beverage made of fermented nixtamal (alkaline cooking of maize kernels, generating a nonsticky dough) (12–14). Pozol has been consumed since pre-Columbian times as a food or refreshment at any hour of the day and represents a staple food, especially for low-income persons. Ethnological studies have reported health benefits related to pozol consumption, such as the control of diarrhea and the reduction of fever (13).

A wide variety of microorganisms have already been described and isolated from this spontaneous fermentation; these microorganisms include fungi, yeasts, lactic acid bacteria (LAB), and non-lactic acid bacteria (non-LAB) (15–21). However, little is known about the nutritional value of pozol, and the only available study dates from the 1950s, showing that the fermentation process increases the content of some essential amino acids, vitamins, and protein (22), which are enrichments widely reported in other fermented foods (2, 23–27). Years later, Ulloa and collaborators (28) isolated several bacteria and fungi from pozol that were able to grow in nitrogen-free medium; therefore, the authors suggested that the protein increase could be due to nitrogen fixation during fermentation. From the isolated microorganisms, just one bacterium, identified as *Agrobacterium azotophilum*, was capable of reducing acetylene (29, 30). However, subsequent studies questioned the identity of the bacterium (31), and no other attempt to identify the phenomenon was made. What has been repeatedly reported is the nitrogen content increase in the fermented dough (32–34).

Despite the large number of niches where nitrogen-fixing bacteria are found (35–40), to our knowledge, there are no publications that demonstrate nitrogen fixation directly in a fermented food. Here, we explored the nitrogen fixation process during pozol fermentation through the acetylene reduction test; at the same time we isolated and identified the bacteria responsible for the phenomenon.

RESULTS

Pozol nitrogen fixation. Pozol is an extraordinarily complex fermentation product in which a wide variety of microorganisms develop in great abundance. In an attempt to understand the dynamic of the system, first, a proximal analysis of the samples obtained at times 0, 9, 24 and 48 h of fermentation was performed since these times represent the period when most people consume it. The proximate analysis of pozol samples is presented in Table 1. During fermentation, total carbohydrates remain constant while fiber is consumed, and lipids increase. These modifications can be explained in terms of carbon metabolism in the fermentation. However, the nitrogen content showed an increased from 11.4 to 12.5 mg/g of dry pozol in the first 9 h of fermentation; this increment is not usually observed in food fermentations, and it may be attributed to nitrogen fixation.

Since in the fermentation process some percentage of carbon can be lost as CO_2, we propose a simple mathematical framework that couples the nitrogen inputs with the carbon outputs through respiration to eliminate the bias that this loss could have on the increase in nitrogen observed. In this framework, the carbon content is considered the sum of carbohydrates, lipids, and fiber concentration in the different pozol samples. The initial carbon concentration was 915 mg/g dry pozol, and at 9 h of

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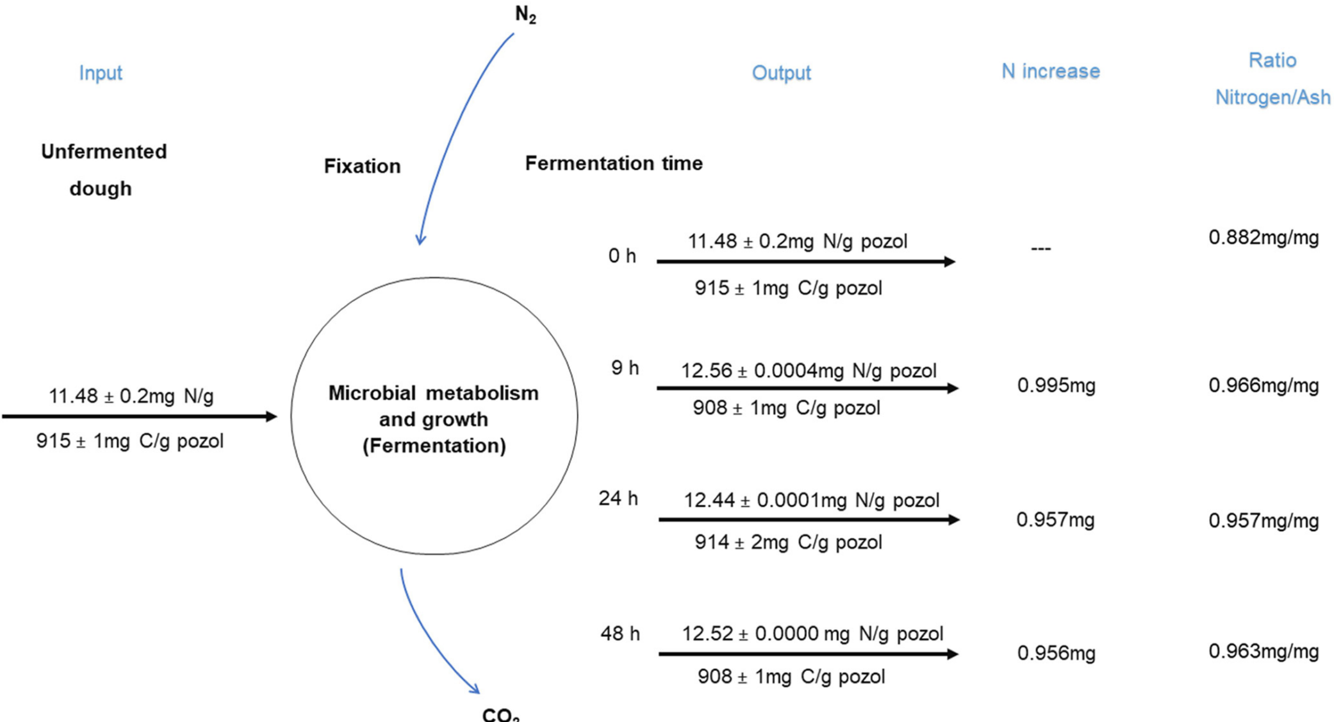

FIG 1 Balance of carbon and nitrogen during pozol fermentation. Carbon and nitrogen concentrations are given per gram of dry dough (Table 1). The calculation of the nitrogen increase was made relative to the concentrations in the unfermented dough. The results obtained for nitrogen take into account the carbon decrease, probably as CO_2 (see text). The last column shows the nitrogen concentration normalized as a function of ash.

fermentation, a reduction of 1.4% was observed. Therefore, changes in the nitrogen and carbon content were considered to determine the amount of fixed nitrogen (Nfx) defined by the following equation: Nfx $= N_f - N_i(C_i/C_f)$, where N_f is the final nitrogen concentration at a given fermentation time x, N_i is the initial nitrogen concentration in the unfermented dough, and C_i/C_f is the carbon concentration ratio between the unfermented and fermented doughs at fermentation time x. Figure 1 shows the general balance of carbon and nitrogen during the pozol fermentation. Input refers to the concentration of elements in pozol in the unfermented dough, and output refers to the concentrations of N and C at different fermentation times. Even considering the decrease in the mass of pozol during fermentation due to the loss of CO_2 or considering the concentration of nitrogen as a function of ash concentration (minerals), we found an increase in the nitrogen of 8 to 9% at 9 h, which remained constant until the end of the fermentation.

To determine if the increase in nitrogen concentration is the product of biological fixation, the nitrogenase activity was evaluated by an acetylene reduction assay (ARA) directly in the pozol dough inoculated in semisolid nitrogen-free minimal medium MMK or MMp/299 (see Materials and Methods). Interestingly, acetylene reduction was found from the beginning of the fermentation in both media, indicating the presence of active nitrogen-fixing bacteria in the dough even after nixtamalization. Regardless of the medium used, the maximum ethylene reduction was obtained in the samples with 24 h of fermentation; however, at 48 h, the activity dropped drastically (Fig. 2).

Nitrogen-fixing bacteria. (i) Isolation and identification of nitrogen-fixing bacteria. Nitrogen-fixing bacteria were isolated from all fermentation times using solid agar medium without a nitrogen source. Based on colony characteristics, 28 CFU (bacterial isolates) were selected, and all of them were analyzed for nitrogenase activity by ARA.

Some strains once isolated had low activity; for example, strain 16 produced only 0.16 nmol C_2H_4 h^{-1}; nonetheless these strains were considered positive since there was

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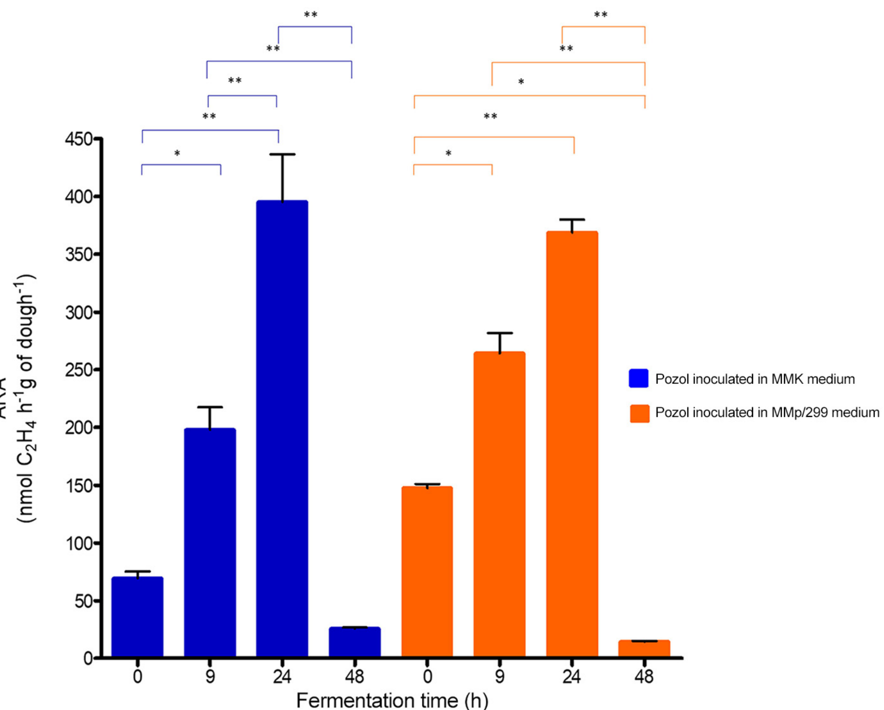

FIG 2 Nitrogen-fixing activity in pozol samples. Ethylene was produced in samples at different fermentation times on semisolid media. The results are expressed as the means of three replicates ± standard deviations. Differences were evaluated by ANOVA followed by Tukey's multicomparison test (*, $P < 0.05$; **, $P < 0.001$).

the formation of the characteristic ethylene peak, while in the negative controls and in two isolated bacteria, no ethylene was observed (data not shown). Interestingly, almost all isolates appeared to have a high capacity to fix nitrogen since they showed greater acetylene-reducing activity than that of the positive control, *Klebsiella variicola* ATCC BAA-830. Especially six isolates, numbers 2, 3, 4, 8, 10, and 21, showed more than 10-times-higher activity than that of the positive control (Fig. 3).

From the 26 positive ARA isolates, 25 presented similar microscopic characteristics, with Gram-negative, rod-shaped bacteria and the presence of exopolysaccharide (Table 2).

(ii) 16S rRNA-based identification. Based on the acetylene reduction ability, 16 different isolates were selected for molecular identification by the 16S rRNA gene sequence. PCR amplification of genomic DNA led to the generation of partial 16S rRNA gene fragments of approximately 1,100 bp. The sequences were analyzed with the online blastn program using the NCBI database for 16S rRNA sequences from *Bacteria* and *Archaea*. The isolated bacteria have similarities with three members of the *Enterobacteriaceae* family (*Klebsiella*, *Kosakonia*, and *Enterobacter*). The bacterium with colony characteristics different from the others presented the highest identity, 99.90%, with *Kosakonia oryzendophytica* and a slightly lower identity with *Enterobacter* sp. (99.88%). Two additional colonies were identified as *Kosakonia*, with 99.80% and 99.70% identities. The rest of the isolates had greater than 99% identity with members of the genus *Klebsiella* (Table 3).

The phylogenetic relationship between isolated organisms was established by comparing the obtained 16S rRNA gene sequences with the corresponding reference sequences from different strains of *Klebsiella pneumoniae*, *Klebsiella quasipneumoniae*, *Klebsiella variicola*, *Klebsiella oxytoca*, *Klebsiella michiganensis*, *Klebsiella aerogenes*, *Enterobacter cloacae*, *Enterobacter kobei*, *Enterobacter hormaechei*, *Enterobacter soli*, *Enterobacter asburiae*, *Kosakonia pseudosacchari*, *Kosakonia sacchari*, *Kosakonia radicincitans*, *Kosakonia arachidis*, *Kosakonia oryzae*, and *Kosakonia cowanii*.

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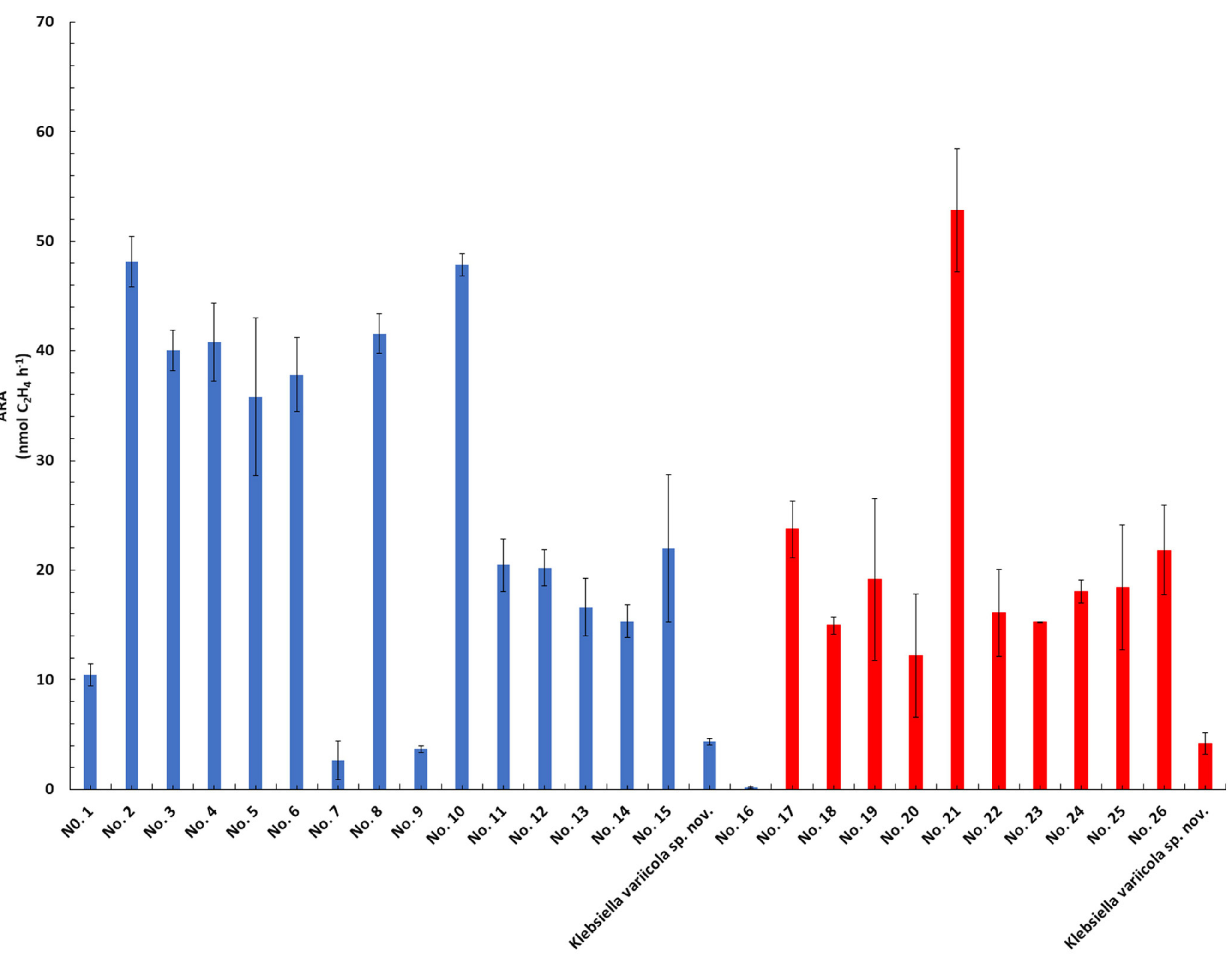

FIG 3 Acetylene reduction assay in different bacteria isolated from pozol (blue, bacteria isolated from pozol inoculated on semisolid MMp/299 medium; red, bacteria isolated from pozol inoculated in semisolid MMK medium). *Klebsiella variicola* ATCC BAA-830 was used as the positive control. The results are expressed as the means of three replicates ± standard deviations.

Based on these 16S rRNA gene sequences, bacterial isolates 5, 9, 21, and 23 grouped as expected with the cluster formed by *Klebsiella variicola* strain F2R9. Isolate number 7 grouped with *Klebsiella variicola* strain LX3, suggesting that the correct identification of the strain is *Klebsiella variicola* and not *Klebsiella pneumoniae*, as suggested by the highest similarity in the blast. The strains identified as *Klebsiella quasipneumoniae* clustered together (isolates 12, 13, 22, 25, and 26). The 16S rRNA gene sequence data of the reference strains *K. pneumoniae* strain ATCC 13883, *K. pneumoniae* strain DSM 30104, and *K. quasipneumoniae* subsp. *quasipneumoniae* strain 01A030 were phylogenetically intermixed and included bacterial isolates 3, 10, and 24. Finally, bacterial isolates 2, 11, and 17 formed a group with the *Kosakonia* genus (Fig. 4).

(iii) *nifH*-based identification. To support the identification of some strains or to group some other isolates that did not form a specific clade with any of the sequences of the reference strains, the *nifH* gene sequence was used. The sequences were analyzed in NCBI with the online blastn program in the database of the nucleotide collection (nr/nt). Table 3 shows the results of the identification; again, the genera *Klebsiella*, *Enterobacter*, and *Kosakonia* were identified.

For the phylogenetic analysis, the sequences of dinitrogenase reductases deposited in NCBI of the *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Klebsiella variicola*, *Kosakonia sacchari*, *Kosakonia oryzae*, *Enterobacter oryziphilus*, *E. sacchari*, *E. oryzendophyticus*, *E.*

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TABLE 2 Morphological characteristics of the isolated colonies

Number of strains	*Colony shape*	*Colony size (cm)*	*Stereo microscope images (10X)*
1 (No.11)	Color: Yellow Form: Irregular Margin: Undulate Elevation: Convex Texture Dry	0.1	
25 (No.1-No.10 and No.12-No.26)	Color: Yellow or bluish Form: Circular Margin: Entire Elevation: Raised Texture: Mucoid	0.3-0.4	

cloacae, and *Enterobacter* sp. were used. *Enterobacter* and *Kosakonia* strains were intermixed and formed cluster A and B. In clusters C, D, E, and F, all *Klebsiella* sequences were grouped. Supported by the high bootstrap value, strain 11 was closely related to *Enterobacter oryzendophyticus*. Cluster C contained the isolates 3, 13, 22, and 25; the reference strains *Klebsiella variicola and Klebsiella pneumoniae* formed clusters D, E, and F. Strain 21 formed a well-defined clade with *Klebsiella variicola* DX120E that was recovered in 86% of the bootstrap samples. Although strain 10 was identified as *Klebsiella pneumoniae*, in the phylogenetic analysis, it formed an outgroup (Fig. 5).

Nitrogen-fixing ability in pozol. To finally demonstrate nitrogen fixation directly in the pozol, the homogenized dough was directly transferred to vials containing only semisolid agar. As shown in Fig. 6, acetylene reduction was evident at all fermentation times although the maximum reduction was observed at 9 h of fermentation. Additionally, to determine whether the isolated bacteria can fix nitrogen in pozol, the microbe with the apparently highest nitrogenase activity, *Klebsiella variicola* (isolate 21), was inoculated directly into the homogenized dough and transferred to semisolid agar; *K. variicola* effectively increased the ethylene concentration in the samples with zero and 9 h of fermentation, reaching maximum activity at 9 h (Fig. 6).

DISCUSSION

Nitrogen fixation in pozol. Cereal-based foods are an essential component of the human diet. They are an important source of carbohydrates, lipids, fiber, vitamins, and minerals. However, the presence of antinutritional factors, poor digestibility, and deficiency in some basic compounds (essential amino acids) make them unattractive for consumption and even more so if their nutritional value is compared with that of milk, milk products, or animal-derived foods (41, 42). Cereal fermentation plays a key role in the production of cereal-based foods since it is a simple and economical process to improve sensory properties, functional qualities, and nutritional value, with a final positive effect on human health (42).

Since the nutritional value of cereal-based food is mainly determined by protein content, some authors have proposed its supplementation with protein of vegetable origin, with protein concentrates, or with other foods, by controlled fermentation with specific microorganisms or with the use of genetic engineering strategies (41, 43–46). One important condition is that these approaches should not affect the nutritional value of the food. In this study, we demonstrate the potential of fermentation to increase the content of nitrogen and protein in vegetal fermentation since the results showed that in pozol, the fermentation process produces an increase of 8 to 9% in nitrogen concentration after only 9 h of fermentation (Fig. 1; Table 1). This value is

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TABLE 3 Identification of bacteria based on the 16S rRNA and dinitrogenase reductase genes[a]

		16S rRNA gene-based identification			*nifH* gene-based identification		
Medium and isolate no.	Fermentation time (h)	Organism	Total score	Identity (%)	Organism	Total score	Identity (%)
MMp/299							
2	0	*Kosakonia radicincitans* DSM 16656	1,495	99.63	*Kosakonia oryzae* R5-395	553	98.11
		Kosakonia oryzae Ola51	1,489	99.51			
3	0	*Klebsiella pneumoniae* DSM 30104	1,500	99.76	*Klebsiella* sp. strain CRPV0611a	571	98.47
5	9	*Klebsiella variicola* F2R9	1,513	99.88			
7	24	*Klebsiella pneumoniae* DSM 30104	1,465	99.02			
		Klebsiella variicola F2R9	1,454	98.78			
9	48	*Klebsiella variicola* F2R9	1,495	99.63			
10	48	*Klebsiella pneumoniae* DSM 30104	1,498	99.76	*Klebsiella* sp. strain CRLIQ728	569	99.37
		Klebsiella pneumoniae subsp. *rhinoscleromatis* R-70	1,838	99.80	*Klebsiella pneumoniae* NG14	564	99.36
11	48	*Kosakonia oryzendophytica* REICA 082	1,500	99.76	*Enterobacter* sp. strain BKA4	510	95.62
		Enterobacter cloacae DSM30054, NBRC 13535, 279-56, and subsp. *dissolvens* LMG2683	1,469	99.02	*Enterobacter oryzendophyticus* REICA_082	462	94.95
12	48	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* 07A044	1,480	99.75			
13	48	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* strain 07A044	1,504	99.76	*Klebsiella* sp. strain CRPV0611a	569	99.37
					Klebsiella quasipneumoniae subsp. *similipneumoniae* ATCC 700603	564	99.36
MMK							
17	0	*Kosakonia radicincitans* DSM 16656	1,506	98.8	*Klebsiella pneumoniae*	468	100
					Kosakonia radicincitans DSM 16656	468	98.08
					Kosakonia oryzae R5-395	446	98.80
21	24	*Klebsiella variicola* F2R9	1,463	99.50	*Klebsiella variicola* AJ29, E57-7, WCHKP19, GJ3, GJ2, GJ1, DX120E	490	100
22	24	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* strain 07A044	1,506	99.76	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* ATCC 700603	490	100
23	24	*Klebsiella variicola* F2R9	1,511	100	*Klebsiella variicola* gene for dinitrogenase reductase, partial CDS, strain NGB-FR96	490	100
24	48	*Klebsiella pneumoniae* strain DSM 30104	1,498	99.76			
		Klebsiella pneumoniae subsp. *rhinoscleromatis* R-70	1,820	99.31			
25	48	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* 07A044	1,827	98.46	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* ATCC 700603	499	100
26	48	*Klebsiella quasipneumoniae* subsp. *similipneumoniae* 07A044	1,493	99.51			

[a]Total score is the sum of alignment scores of all segments from the same database sequence that match the query sequence (calculated over all segments).

similar to that found by Loaeza (33) in pozol fermented for 9 to 10 days but much lower than values reported in other pozol studies, which reported increases in the nitrogen concentration between 20 and 40% (22, 28, 32, 34, 47).

The effect of fermentation on the protein and nitrogen content in cereal fermented foods is variable. For example, in togwa (maize and sorghum, cassava, or millet gruel), only a slight increase in the total protein content was reported when the sorghum flour was supplemented with malt, but no significant differences were observed in native togwa or togwa fermented with starter cultures (48). For lohoh (fermented millet for bread), no apparent changes in the protein content were observed during the first 24 h of fermentation, but after this period, a significant increase was reported (49). In

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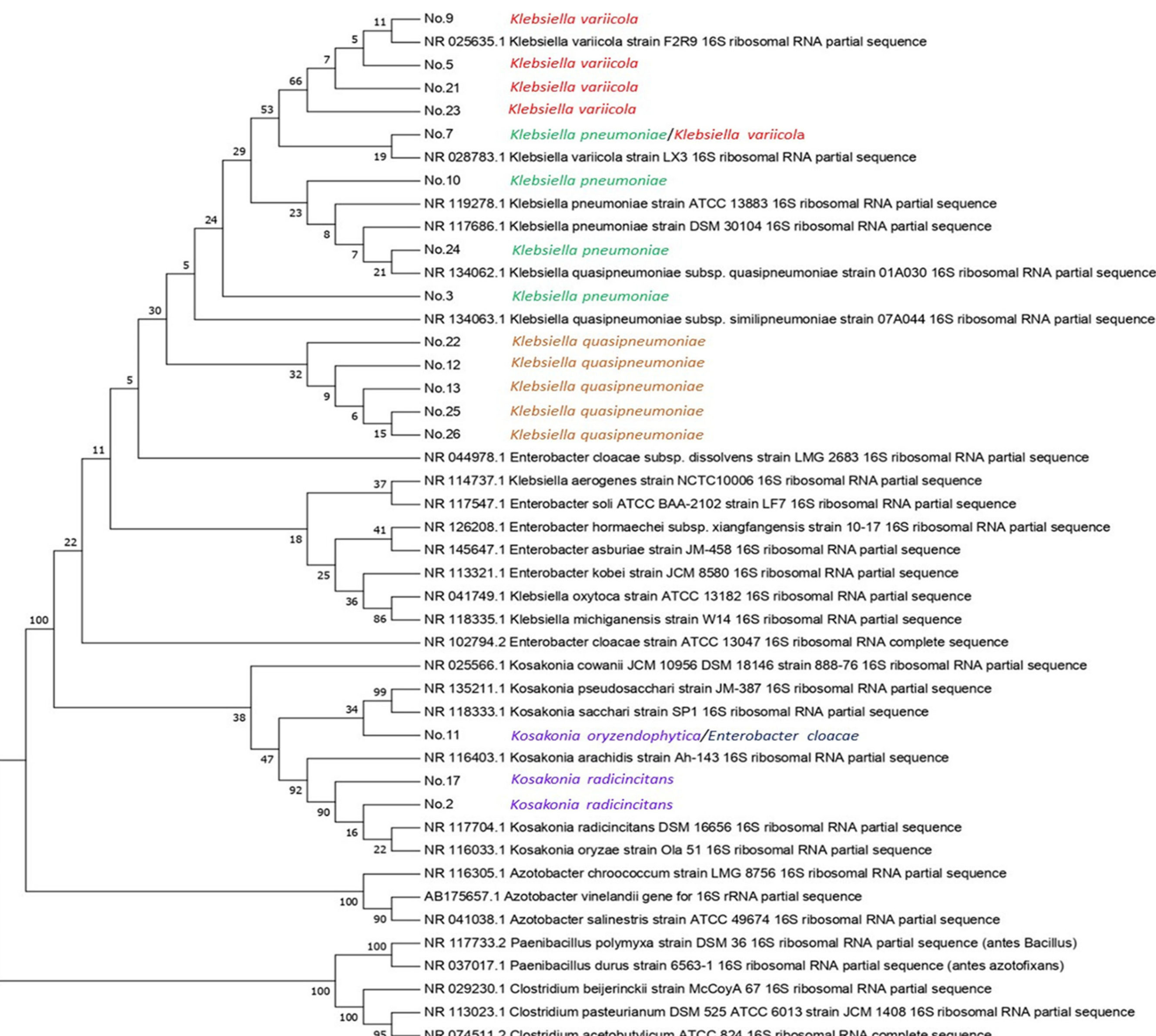

FIG 4 Phylogenetic 16S rRNA analysis of nitrogen-fixing bacteria isolated from pozol. The final data set consisted of 730 positions. The analysis involved 45 nucleotide sequences. Study isolates are highlighted in color.

contrast, fermentation decreased the crude protein content in traditionally fermented fufu (cassava and green plantain dough) and in cassava roots fermented with two strains of *Lactobacillus plantarum* (50). For ogi (maize, sorghum, or millet pudding), the increase in the protein content depends on the malting levels and not on the fermentation process (51). In kinema (soybean food), kombucha (fermented black or green tea), and amahewu (maize gruel), the same tendency observed in pozol of increased protein as a result of fermentation has been reported (52–54).

The decrease in protein content has been related to protein utilization by microorganisms during fermentation due to their metabolic activities (55). While the increase in the crude protein content has been attributed to a concentration effect by the loss of dry matter, mainly carbohydrates and lipids (48), or to the production of volatile compounds that produced a loss of weight (56), some authors have proposed that during fermentation, the microorganisms could synthesize proteins from metabolic intermediates, which may explain the increase in protein content (57, 58).

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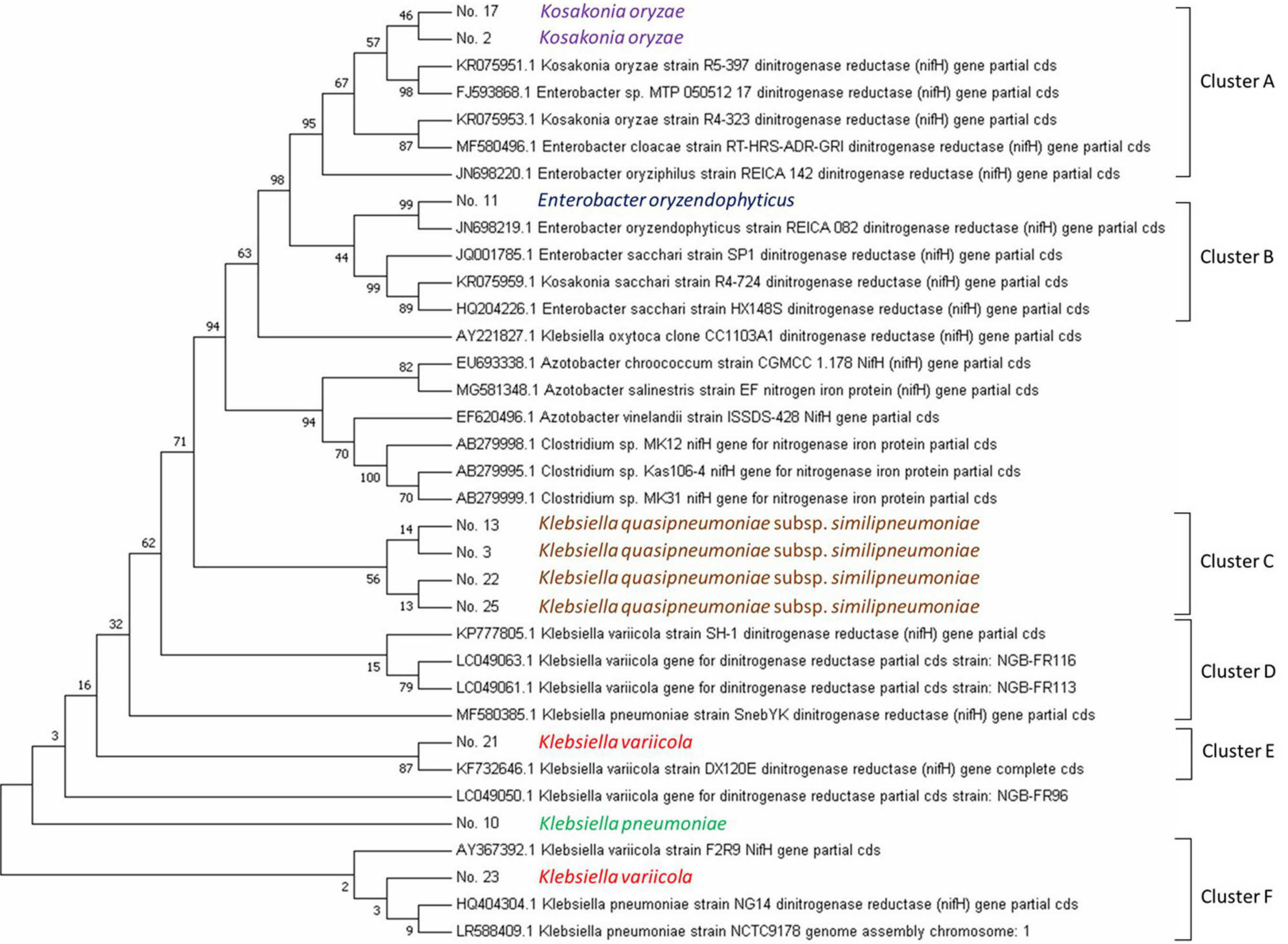

FIG 5 Phylogenetic *nifH* gene analysis of nitrogen-fixing bacteria isolated from pozol. The final data set consisted of a total of 231 positions. The analysis involved 35 nucleotide sequences. Study isolates are highlighted in color.

To demonstrate that the fermentation process produces the observed increases and to minimize the effects of weight change, all determinations were calculated in dry weight; in addition, to ponder the net nitrogen increase, the nitrogen concentration was related to the loss of carbon in the system, and also the nitrogen/ash ratio was obtained (Fig. 1; Table 1) (59). Both approaches confirm that the nitrogen content increases with the fermentation process, which points to nitrogen fixation as a possible mechanism.

To conclusively demonstrate the phenomenon, the ARA was performed. In a first approach, the pozol samples were directly inoculated in defined medium without a nitrogen source to promote the growth and activity of nitrogen-fixing bacteria (Fig. 2). Nitrogenase activity was positive for all the samples tested, proving the presence of diazotrophs in the dough. From this, 26 nitrogen-fixing bacteria were isolated with different levels of nitrogenase activity.

It is important to highlight that biological nitrogen fixation (BNF) can be an economical and simple method that has the potential to improve the nutritional value of fermented cereal-based foods. However, to increase the protein content via nitrogen fixation, it would be advisable to promote the growth of diazotrophic bacteria. We demonstrated that the inoculation of *Klebsiella variicola* (isolate 21) in the dough results in a high ethylene concentration during the first 9 h of pozol fermentation (Fig. 6). These results suggested that pozol dough is an environment with the necessary conditions for atmospheric nitrogen fixation.

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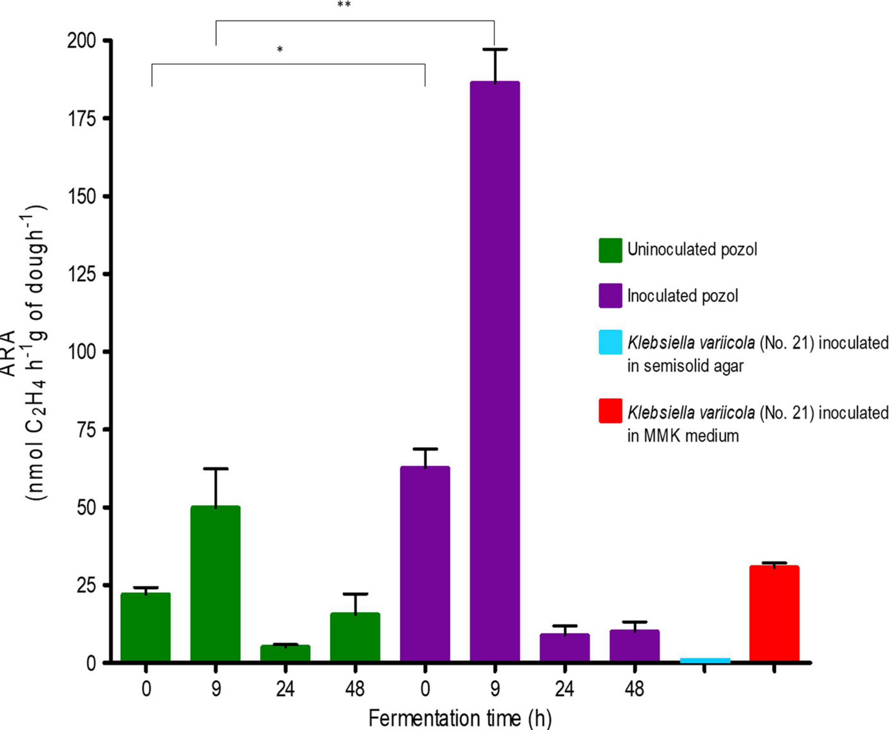

FIG 6 Nitrogen-fixing activity in inoculated pozol samples. Ethylene was produced in the samples at different fermentation times. Inocula consisted of 10^7 CFU/g of dough. Values represent means ± standard deviations from three replicates. Differences were evaluated by ANOVA followed by Tukey's multicomparison test (*, $P < 0.05$; **, $P < 0.001$).

The results at 24 and 48 h showed a significant decrease in the activity of nitrogenase, which could be related to the decrease in the concentration of enterobacteria as a result of the decrease in pH that occurs in the dough during fermentation (17, 18, 60). Furthermore, it is known that nitrogen fixation is a highly regulated, oxygen-sensitive, energy-dependent process and is inhibited by some amino acids (35, 61).

Nitrogen-fixing bacteria in pozol. As previously mentioned, the bacteria isolated from pozol were identified as *Klebsiella variicola*, *Klebsiella pneumoniae*, *Klebsiella quasipneumoniae*, *Kosakonia oryzae*, *Kosakonia radicincitans*, and *Kosakonia oryzendophytica/Enterobacter cloacae* (Table 3). The phylogenetic analysis of 16S rRNA gene sequence data allowed us to form a defined clade for *Klebsiella variicola*, *Klebsiella pneumoniae*, and *Klebsiella quasipneumoniae* as well as *Kosakonia*. However, the *Enterobacter* genus resulted in a heterogeneous group, making it difficult to cluster it. However, it is important to consider that in closely related species, such as the *Enterobacteriaceae* family, the use of 16S rRNA gene sequences in bacterial detection or identification is not enough (62–67).

To overcome this problem, housekeeping gene sequencing has been used in phylogenetic studies and in enterobacterial species identification (68). For example, *rpoB*, *gyrA*, *mdh*, *infB*, *phoE*, and *nifH* genes allowed the identification of new *Klebsiella* species, such as *Klebsiella variicola*, from clinical and plant-associated isolates (69). In this study, the *nifH* gene was selected to support the identification of some strains. The results confirmed the identification of the genera *Klebsiella*, *Enterobacter*, and *Kosakonia*. With this approach, the species level was corroborated for isolates 2, 10, 21, 22, 23, and 25, while for isolate 11, only genus identification was possible (Table 3). The phylogenetic tree again showed that all species of the *Klebsiella* genus analyzed form a clade, while those of *Enterobacter* and *Kosakonia* genera form the mixed clades A and B. The use of other housekeeping genes for the new classification of some *Enterobacter* species has resulted in the same observation: a group composed of these two genera separated from other genera of the family *Enterobacteriaceae* (66, 67).

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The nitrogen-fixing activity of free-living diazotrophs of the genera *Klebsiella*, *Enterobacter*, and *Kosakonia* has been widely documented. *Enterobacter* and *Klebsiella* increased the nitrogen content via BNF and promoted the growth and plant uptake of nitrogen in sugarcane plants (70–72). *Klebsiella* spp. and *Enterobacter* species isolated from fruit flies were able to grow in nitrogen-free medium and reduce acetylene to ethylene (35). *Kosakonia radicincitans* fixed atmospheric nitrogen and increased nitrogen uptake in young tomato plants and maize, showing higher nitrogenase activity than that of *Azotobacter vinelandii* under nitrogen-limited conditions (73, 74).

Although the changes in the composition of nitrogen-fixing bacteria during pozol fermentation were not evaluated, we observed that the bacteria isolated varied as a function of time. *Kosakonia* was isolated only in the unfermented dough (0 h), *Enterobacter* was isolated at only 48 h of fermentation, and *Klebsiella* strains were isolated at all fermentation times, which indicates that this bacterium is responsible for the fixation that we observe in the dough. The prevalence of enterobacteria in pozol fermentation has been studied by Giles (60), demonstrating that the *Klebsiella pneumoniae* isolated from pozol is capable of surviving under acidic conditions and with a concentration of lactic acid of 2.1 g/100 g, in contrast to the clinically isolated *Klebsiella pneumoniae* that, under the same conditions, is undetectable.

The relevance of enterobacteria in traditionally fermented food was previously demonstrated in tempeh, where the inoculation of *Klebsiella pneumoniae* and *Citrobacter freundii* increased the vitamin B_{12} content. The authors of that work also demonstrated that the two strains lacked genes that encode three different types of enterotoxins (75). Recently, metagenomic sequence data from a 30-h sample of cocoa bean fermentation demonstrated that the *Enterobacteriaceae* family is involved in the methylglyoxal detoxification pathway, pectinolysis, and bacteriocin production, implying a more important functional role during fermentation than previously assumed (76).

Our results confirmed an active nitrogen fixation process in pozol, representing the occurrence of this phenomenon directly in a fermented food. The BNF in pozol was supported by the ARA directly in the dough, as well as in the isolated bacteria, and by detection of the *nifH* gene in the tested strains. The importance of the existence of this phenomenon in food is accentuated if we consider that it improves the nutritional value of fermented beverages and foods.

Molecular methods resulted in identification of the genera *Enterobacter*, *Klebsiella*, and *Kosakonia*, the last being a genus not previously reported in pozol fermentation; *Klebsiella* was the most abundantly isolated bacterium at all fermentation times. However, we suggest a deeper analysis with other genes for corroboration and identification of the species, as well as to establish their safety.

Usually, the presence of enterobacteria is an indicator of poor quality and safety in food and beverages. However, although considered undesirable microorganisms, their potential as microorganisms that contribute to improving the quality of the substrate should be reconsidered, especially if the absence of virulence factors and lack of enterotoxin production are demonstrated.

MATERIALS AND METHODS

Sample description. Freshly ground nixtamal dough samples were acquired from two producers at the Pino Suárez market in Tabasco, Mexico. Samples were mixed and shaped into 300-g balls, wrapped in banana leaves, and incubated in triplicate at 37°C. Sampling was performed at 0, 9, 24, and 48 h, with all practices performed under aseptic conditions.

Proximate composition of pozol. For this analysis, dough samples (10 g) were frozen and ground in a mortar with solid CO_2 until a homogenous powder was obtained. Ash, nitrogen, and crude protein ($N \times 6.25$ [88]) contents were determined according to official method 992.23 of the Association of Official Analytical Chemists (AOAC) International (77) at the chemical analysis laboratory of the Department of Animal Nutrition and Biochemistry of the Faculty of Veterinary Medicine and Zootechnic of the Universidad Nacional Autónoma de México (UNAM). All determinations were performed in triplicate and analyzed with a one-way analysis of variance (ANOVA) using GraphPad Prism, version 4, software. The significant differences were estimated with a Tukey *post hoc* test.

Isolation of nitrogen-fixing bacteria. For the isolation, pozol samples were serially diluted in 0.9% (wt/vol) NaCl, and the homogenate (100 μl) was streaked onto nitrogen-free MMK plates (2.2 g/liter

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TABLE 4 Primers used in the amplification of the 16S rRNA and *nifH* genes

Gene	Primer		Sequence position (nt)[a]	Sequence	Product (bp)	Reference
	Direction	Name				
16S rRNA gene	Forward	8F or pA	9	AGAGTTTGATCCTGGCTCAG	1,100	87
	Reverse	16R1093	1109	GTTGCGCTCGTTGCGGGACT		
nifH	Forward	Polf	115	TGCGAYCCSAARGCBGACTC	360	69
	Reverse	Polr	457	ATSGCCATCATYTCRCCGGA		

[a]Sequence position is relative to that in *Escherichia coli* or *A. vinelandii* for the 16S rRNA gene or *nifH* coding sequence, respectively. nt, nucleotide.

$Na_2HPO_4 \cdot H_2O$, 0.425 g/liter $Na_2H_2PO_4 \cdot H_2O$, 0.435 g/liter $MgSO_4 \cdot 7H_2O$, 1 g/liter sucrose, and 15 g/liter agar) and MMp/299 plates (3.8 g/liter K_2HPO_4, 3 g/liter KH_2PO_4, 0.1 g/liter $MgSO_4 \cdot 7H_2O$, 0.005 g/liter $Fe(C_6H_5O_7)$, 0.1 g/liter $CaCl_2$, 1 g/liter sucrose, and 15 g/liter agar) and incubated at 29°C until colony growth was observed. The culture was used to inoculate the corresponding semisolid medium, and the ARA was performed. The positive colonies were characterized by Gram staining and their morphology. Pure cultures were stored in 20% glycerol as a cryoprotectant at −70°C until analysis. Bacterial isolates were deposited at the Institute of Biomedical Research (IIBM)-UNAM Culture Collection (World Data Centre for Microorganisms 48 [WDCM48]).

ARA. Acetylene reduction assays (ARAs) were performed in 10-ml vials containing 5 ml of semisolid MMK or MMp/299 medium. The glass tubes were sealed with airtight rubber, and 0.6 ml of acetylene gas was injected by removing an equal volume of air from the tube. Ethylene production was detected in duplicate at 24 h in a gas chromatograph equipped with a flame ionization detector and a capillary column. The ethylene concentration in the headspace was measured by injecting 0.4 ml of the gas into a Bruker gas chromatograph, model GC 450, with a flame ionization detector (FID) and a Porapak-N column. The detector temperature was maintained at 105°C, and the injector and column temperature were maintained at 95°C. Nitrogen served as the carrier gas, with a flow rate of 35 ml min^{-1}. The chromatograms were used to integrate the areas of the curves of acetylene (C_2H_2) and ethylene (C_2H_4) to estimate C_2H_4 production (78).

Test vials with equivalent volumes of uninoculated broth served as negative controls. *Klebsiella variicola* sp. nov. ATCC BAA-830, from the Center for Genomic Science collection (CCG, UNAM), was used as a positive control.

For ARA determination directly in the pozol samples, 150 g of the dough was homogenized in 100 ml of 0.9% (wt/vol) NaCl, and 100 μl of the solution was inoculated in semisolid MMK and MMp/299 medium. Data were analyzed with one-way ANOVA using GraphPad Prism, version 4, software. The significant differences were estimated with a Tukey *post hoc* test.

Pozol inoculation experiment. To verify that previously isolated bacteria can fix nitrogen in the pozol dough, isolated bacteria were grown overnight in Luria-Bertani agar at 29°C. Bacteria at 10^7 CFU were inoculated aseptically in 1 g of pozol and homogenized by agitation. Then, 100 μl of suspension was added to vials containing only semisolid agar.

Two controls were used in this experiment. In the first control, the uninoculated pozol was resuspended and added to the vials with semisolid agar. In the second control, the isolated bacteria were directly inoculated (10^7 CFU) in semisolid MMK and semisolid agar. Finally, the ARA was performed 24 h later. Two biological replicates were performed for each experimental and control group.

DNA extraction. Overnight cultures from the positive colonies were harvested by centrifugation (10 min, 10,000 $\times$ *g*, 4°C) and washed twice in sterile Milli-Q water. The cells were suspended in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA), treated with lysozyme (100 mg ml^{-1}) for 30 min at 37°C, and centrifuged (10 min, 10,000 $\times$ *g*, 15°C). The resulting pellet was suspended in TE buffer, and the cell suspension was treated with TEN buffer (0.1 M NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA, pH 8) and 20% (wt/vol) SDS for 30 min at 37°C. Cellular debris was eliminated by adding 5 M NaCl for 2 h on ice followed by centrifugation (20 min, 10,000 $\times$ *g*, 15°C) (79). The extracted DNA was purified by the phenol-chloroform method as described by Green and Sambrook (80).

Amplification of 16S rRNA and *nifH* gene fragments. Amplification of the 16S rRNA gene fragment was performed with the universal primers shown in Table 4 in a 25-μl reaction volume containing 50 ng of template DNA with 12.5 pmol of each primer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 2.5 mM $MgCl_2$, and 1.25 U of *Taq* DNA polymerase (Thermo Scientific). The PCR was conducted using the following conditions: preheating at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min.

Nitrogenase amplification primers are shown in Table 4. All reactions were performed in a 25-μl reaction volume containing 10 ng of DNA with 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1 U *Taq* DNA polymerase (Thermo Scientific). The reaction mixture was incubated for 2 min at 94°C and then subjected to 30 cycles of denaturation at 94°C for 4 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min.

Amplified products were analyzed by electrophoresis in a 1% agarose gel.

Sequence analysis. 16S rRNA and *nifH* gene fragments were purified with a Wizard PCR Preps DNA purification system (Promega) according to the manufacturer's instructions. The purified PCR products were sequenced at the Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud, UNAM. The quality of the sequences was verified with the Chromas (version 2.6.6) program and then analyzed for bacterial identification in the NCBI (National Center for Biotechnology

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Information) database using the online BLAST program (81). The sequences were aligned using the MUSCLE program (multiple sequence comparison by log expectation) (82), edited to uniform length, and manually corrected.

For the phylogenetic relationships of the obtained 16S rRNA gene sequences, the reference sequences (RefSeq NCBI Database) from different strains of *Klebsiella pneumoniae* (ATCC 13883 and DSM30104), *Klebsiella quasipneumoniae* 01A030 and 07A044, *Klebsiella variicola* F2R9 and LX3, *Klebsiella oxytoca* ATCC 13182, *Klebsiella michiganensis* W14, *Klebsiella aerogenes* NCTC10006, *Enterobacter cloacae* LMG2683 and ATCC 13047, *E. kobei* JCM8580, *E. hormaechei* 10–17, *E. soli* ATCC BAA-2102, *E. asburiae* JM-458, *Kosakonia pseudosacchari* JM-387, *Kosakonia sacchari* SP1, *Kosakonia radicincitans*, *Kosakonia arachidis* Ah143, *Kosakonia oryzae* Ola51, and *Kosakonia cowanii* JCM10956 were used. The 16S rRNA gene sequences of *A. vinelandii* AB175657.1, *Azotobacter chroococcum* LMG8756, *Azotobacter salinestris* ATCC 49674, *Paenibacillus polymyxa* DSM36, *Paenibacillus durus* 6563-1, *Clostridium pasteurianum* ATCC 6013, and *Clostridium acetobutylicum* ATCC 824 were used as outgroups.

For the phylogenetic analysis of dinitrogenase reductase, the sequences deposited in NCBI of *Klebsiella oxytoca* CC1103A1, *Klebsiella pneumoniae* SnebYK, NG14, and NCTC9178, *Klebsiella variicola* SH-1, NGB-FR116, NGB-FR113, DX120E, NGB-FR96 and F2R9, *Kosakonia sacchari* R4-724, *Kosakonia oryzae* strain R4-323 and R5-397, *Enterobacter oryziphilus* REICA_142, *E. sacchari* SP1, and HX148S, *E. oryzendophyticus* REICA 082, *E. cloacae* RT-HRS-ADR-GRI, and *Enterobacter* sp. strain MTP 050512 17 were used. *Clostridium* sp. strain MK12, *Clostridium* sp. strain Kas106-4, *Clostridium* sp. strain MK31, *A. chroococcum* CGMCC, *A. vinelandii* ISSDS-428, and *A. salinestris* EF strains were used as outgroups.

Sequences were aligned with MEGA, version 7 (83), and the phylogenetic tree was constructed using the neighbor-joining method (84); evolutionary distances were computed using the maximum composite likelihood method (85) and are presented in units of the number of base substitutions per site. Statistical significance was evaluated by bootstrap analysis with 1,000 repeats (86).

Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (86).

The rate variation among sites was modeled with a gamma distribution (shape parameter of 1). All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

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Capítulo cuarto

Estudio metaproteómico del pozol

A pesar de las limitaciones de las técnicas tradicionales y moleculares utilizadas en el estudio de los alimentos fermentados, ha sido posible establecer las características fisiológicas, morfológicas y metabólicas de los distintos miembros de la comunidad microbiana, y determinar su composición y diversidad. Sin embargo, se han realizado enormes esfuerzos para cambiar la perspectiva de la taxonomía a la función y proporcionar una comprensión más profunda de los mecanismos moleculares en los alimentos fermentados a través de enfoques ómicos (Chen *et al*. 2017). La metaproteómica es una herramienta poderosa para estudiar las funciones de los ecosistemas microbianos, ya que permite descubrir y describir qué proteínas se expresan bajo ciertas condiciones, su distribución temporal cuando se comparan distintas muestras y permite definir la función de los genes a nivel de proteína, lo que proporciona nuevos conocimientos sobre el papel de la diversidad microbiana y su función en los alimentos fermentados (Zhao *et al*. 2015; Ji *et al*. 2017; Jian *et al*. 2017; Xie *et al*. 2019).

Para elucidar la composición microbiana, sus cambios en las distintas etapas de la fermentación y su posible función en este alimento, se realizó un estudio metaproteómico del pozol en diferentes tiempos de fermentación. Para determinar la composición y la dinámica de fermentación, se estimó la abundancia relativa de las proteínas identificadas. Los resultados mostraron que la mayoría de las proteínas pertenecen al filo de los *Firmicutes* y *Proteobacteria*. Las proteínas más abundantes fueron de BAL, con *Streptococcus* como el género predominante en todos los tiempos y *Lactobacillus*, *Leuconostoc* y *Enterococcus* como participantes secundarios en este proceso. En los hongos, se encontraron un menor número de géneros distribuidos en 5 filos distintos. Las proteínas de levaduras se identificaron en todos los tiempos, mientras que las proteínas de los hongos filamentosos aumentaron al final de la fermentación. Para evaluar el posible papel de los microorganismos duranta la fermentación, las proteínas se analizaron con la herramienta en línea KEGG. Se encontró que hay enzimas implicadas en la producción de intermediarios esenciales para la síntesis de diferentes compuestos como: ácidos orgánicos, acetoína, butanodiol, ácidos grasos y aminoácidos, los cuales están relacionados con las características organolépticas y nutricionales del alimento. Finalmente, se encontraron enzimas para la hidrólisis de diferentes polisacáridos, lo que implica que además del almidón, la celulosa y hemicelulosa son utilizadas como fuente de carbono.

Metaproteomic insights into de microbial community in pozol

Jocelin Rizo[1,2], Daniel Guillén[1], Gloria Díaz-Ruiz[3], Carme Wacher[3], Sergio Encarnación[4], Sergio Sánchez[1] and Romina Rodríguez-Sanoja[1*]

[1]Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México City, México.

[2]Programa de Doctorado en Ciencias Biológicas, Universidad Nacional Autónoma de México, México City, México.

[3]Departamento de Alimentos y Biotecnología, Facultad de Química, Universidad Nacional Autónoma de México, México City, México.

[4]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México.

*** Correspondence:**
Corresponding Author
romina@biomedicas.unam.mx

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Abstract

Pozol is an acidic, refreshing, and nonalcoholic traditional Mayan beverage made with nixtamalized corn dough that is fermented spontaneously. The extensive analysis of the microbiology, biochemistry and metaproteomics of pozol allowed the construction of a comprehensive image of the fermentation system. The main changes in both the substrate and the microbiota occurred in the first 9 hours of fermentation. The increase in microorganisms correlated with the drop in pH and with the decrease in the contents of carbohydrates, lipids, and nitrogen, which shows that this stage has the highest metabolic activity. Bacteria were mainly represented by lactic acid bacteria, and among them, the genus Streptococcus was overwhelmingly the most abundant. Yeasts were present in all the analyzed samples, while filamentous fungi increased up to 48 h. The metaproteomic approach allowed us to identify several previously unknown enzyme complexes in the system. Additionally, enzymes for hydrolysis of starch, hemicellulose and cellulose were found, indicating that all these substrates can be used as a carbon source by the microbiota. Finally, enzymes related to the production of essential intermediates involved in the synthesis of organic acids, acetoin, butanediol, fatty acids and amino acids important for the

35 generation of compounds that contribute to the organoleptic characteristics of pozol, were
36 found.

37 1 Introduction

38 Pozol is a traditional refreshing, acidic and nonalcoholic beverage made from fermented
39 nixtamalized maize dough. It is consumed in Southeastern Mexico, in the Mayan region,
40 not only as an important part of the diet but also as a culturally important mainstay in
41 traditional medicine and ceremonies (1, 2). Pozol preparation begins with the careful
42 selection of maize grains to remove any contaminants. Then, the grains are cooked in water
43 with lime by a process known as nixtamalization; the kernels are subsequently washed to
44 remove the lime. The cooked grains are coarsely ground, and the resulting heterogeneous
45 dough is kneaded and molded to form a compact ball that is wrapped in banana plant leaves
46 and left at ambient temperature to allow spontaneous fermentation from just a few hours to
47 several days. The fermented dough is then suspended in water and consumed daily as a
48 refreshing beverage (1, 3). After nixtamalization, the soluble sugars present in maize
49 drastically decrease, so it is logical to think that the main carbon source used during
50 fermentation is starch.

51 More than 40 different species of bacteria, fungi and yeast have been reported from pozol
52 (4, 5, 6, 7). Most microbiological studies have focused on the isolation and identification of
53 bacteria, mainly lactic acid bacteria (LAB), since LAB are the predominant group during
54 fermentation and because of their relevance as probiotics in fermented foods and in the
55 industry as starter cultures (4, 5, 6, 7, 8, 9). In contrast, other bacteria, fungi and yeast have
56 been poorly studied, and the ecological niche of microorganisms in pozol fermentation has
57 not been reported, generating a partial understanding of the fermentation mechanism.

58 Elucidation of the role and behavior of microorganisms in different microbial ecosystems
59 can be addressed with different nonexclusive approaches that target different types of
60 molecules. A powerful strategy to understand the functions of fermentation microbiota is
61 protein study by metaproteomic techniques, which aim to identify “metagenome”
62 expression patterns under different conditions and allow an accurate picture of the
63 metabolic processes that actually occur in microorganisms (10).

64 Metaproteomics has been successfully applied in different food fermentations to describe
65 not only the microbial composition and succession but also, its role in the process and the
66 relationship of microorganisms with flavor development (11, 12, 13, 14). In this study, a
67 metaproteomic approach was used for a detailed analysis of the structure of the microbial
68 community, temporal changes and the functional role of the microbiota during pozol
69 fermentation.

70 2 Material and methods

71 2.1 Samples

72 Freshly ground nixtamal dough samples were acquired from two different producers at Pino
73 Suárez market in Tabasco, México. The samples were mixed until homogeneous, and 300 g
74 balls were wrapped in sanitized banana leaves and incubated at 37 °C. Triplicate

75 fermentations were performed, and samples were taken for analysis at 0, 9, 24 and 48 h.
76 Collected samples were stored at −80 °C for further metaproteomic analyses.
77 Microbiological and chemical studies were carried out immediately after the fermentation
78 time had elapsed.

79 **2.2. Enumeration of microorganisms**

80 Serial dilutions of 25 g homogenized dough sample in 225 mL of 0.1% (w/v) peptone water
81 (Bacteriological peptone, Oxoid L37) were used for microbial enumerations with the
82 following media: plate count agar (Oxoid CM 325) for total aerobic mesophilic bacteria,
83 MRS agar (BD Difco) for LAB, MRS starch (containing 1% soluble starch instead of
84 glucose) for amylolytic lactic acid bacteria (ALAB), violet red bile glucose agar for
85 enterobacteria (Oxoid CM 485) and potato dextrose agar (Oxoid CM 139) acidified to pH
86 3.5 for fungi (3, 4, 7).

87 **2.3. Analytical methods**

88 **pH.** For this purpose, dough samples (1 g) were homogenized in a mortar and mixed with 5
89 mL distilled water. The pH was determined using an OakTon pH meter with a reference
90 glass electrode.

91 **Proximate composition of pozol**. Dough samples (10 g) were pulverized in a mortar with
92 solid CO_2 until powder was obtained. Lipid, fiber and nitrogen-free extract (NFE) contents
93 were determined as described previously (15). Total soluble carbohydrates (those
94 solubilized by dissolving the pozol powder in water) were determined by the phenol-
95 sulfuric acid method (16). All determinations were performed in triplicate and analyzed
96 with one-way analysis variance (ANOVA) using GraphPad Prism 4 software. Significant
97 differences were estimated with the post hoc Tukey test.

98 **2.4. Protein extraction and quantification**

99 Protein extraction was performed in triplicate for each fermentation time using a method
100 that combines the methodological approaches as previously reported (17, 18). First, a
101 representative pozol sample containing inner, middle and external portions of the dough
102 was ground with solid CO_2 until a powder was obtained. Then, 1 g of sample was
103 mechanically mixed at room temperature for two hours with 10 mL of 0.1 M citrate-
104 phosphate buffer pH 5 with protease inhibitor 1:100 (Sigma Protease Inhibitor cocktail for
105 general use). The samples were centrifuged (800 x g for 10 min) at 4 °C, and the
106 supernatant was collected. Two more extractions were performed on the obtained pellet
107 with 10 mL of buffer and mechanical agitation for 30 min. The resulting supernatant was
108 cloth filtered and centrifuged (13,200 x g for 10 min) at 4 °C. The pellet was used for
109 protein extraction as described previously (18); briefly, the pellet was treated with 1.0 mL
110 of buffer ASB-14 (7 M urea, 2 M thiourea, 2% CHAPS (w/v), 2% ASB-14 (w/v), and 30
111 mM buffer Tris-HCl pH 7) and glass beads (Sigma 710-1180 µm) were added to the pellet.
112 The samples were vortexed vigorously for 5 min each 20 min for 1 hour at room
113 temperature and then centrifuged three times for 10 min at 13,200 x g and 4 °C. The

114 clarified supernatant from each centrifugation was recovered. Three extractions were made
115 per fermentation time.

116 After the extraction, the protein concentration was quantified using the Bio-Rad Protein
117 Assay method (Bio-Rad), and bovine serum albumin (BSA) was used as the standard. To
118 avoid any interference related to the extraction buffer, samples were precipitated with 10%
119 (w/v) trichloroacetic acid (TCA) (19), solubilized in 50 µl of 6 M urea and finally dissolved
120 in 750 µl of distilled water.

121 **2.5. 1D-GE, trypsin digestion and LC–MS/MS**

122 Protein from the supernatant (50 µg) was precipitated with 10% (w/v) TCA and collected
123 by centrifugation (13 200 x g, 20 min, 4 °C). The pellets were resuspended in 10 µL of 6 M
124 urea and 10 µL of 2x Laemmli buffer and heated 5 min at 96 °C for. The samples were
125 resolved on SDS-PAGE 10% acrylamide using 10 mA per gel until the dye migrated off the
126 gel. The gel was stained with Coomassie brilliant blue, and each lane was divided into 6
127 pieces and sent to Institut de Recherches Cliniques de Montréal (IRCM) for sequencing.

128 **2.5.1. Protein digestion with trypsin**

129 Both digestion and LC-MS/MS analysis were performed as previously described (18). Gel
130 pieces were washed with water for 5 min and destained twice with destaining buffer (100
131 mM sodium thiosulfate, 30 mM potassium ferricyanide) for 15 min. An additional wash of
132 5 min was performed after destaining with a buffer of ammonium bicarbonate (50 mM).
133 Gel pieces were then dehydrated with acetonitrile. Proteins were reduced with reduction
134 buffer (10 mM dithiothreitol, 100 mM ammonium bicarbonate) for 30 min at 40 °C and
135 then alkylated with alkylation buffer (55 mM iodoacetamide, 100 mM ammonium
136 bicarbonate) for 20 min at 40 °C. Gel pieces were dehydrated and washed at 40 °C with
137 acetonitrile (ACN) for 5 min before discarding all the reagents. Gel pieces were dried for 5
138 min at 40 °C and then rehydrated at 4 °C for 40 min with trypsin solution (6 ng/µL trypsin
139 sequencing grade from Promega and 25 mM ammonium bicarbonate). The trypsin
140 concentration was kept low to reduce signal suppression effects and background originating
141 from autolysis products when performing LC–MS/MS analysis. Protein digestion was
142 performed at 58 °C for 1 h and stopped with 15 µL of 1% formic acid/2% acetonitrile. The
143 supernatant was transferred to a 96-well plate, and peptide extraction was performed with
144 two 30-min extraction steps at room temperature using extraction buffer (1% formic
145 acid/50% ACN). All peptide extracts were pooled into 96-well plates and then thoroughly
146 dried in a vacuum centrifuge. The plates were sealed and stored at – 20 °C until LC–
147 MS/MS analysis.

148 **2.5.2. LC–MS/MS**

149 Prior to LC–MS/MS, peptide extracts were resolubilized under agitation for 15 min in 12
150 µL of 0.2% formic acid and then centrifuged at 2000 x g for 1 min. The LC column was a
151 C18 reversed-phase column packed with a high-pressure packing cell. A 100-mm-long 75
152 µm i.d. fused silica capillary was packed with a C18 Jupiter 5 µm 300 Å reverse-phase
153 material (Phenomenex). This column was installed on the nanoLC-2D system (Eksigent)
154 and coupled to the LTQ Orbitrap Velos (Thermo Fisher Scientific). The buffers used for

155 chromatography were 0.2% formic acid (buffer A) and 100% acetonitrile/0.2% formic acid
156 (buffer B). During the first 12 min, 5 µL of sample was loaded on the column with a flow
157 of 650 nL/min, and subsequently, the gradient went from 2 to 80% buffer B in 20 min and
158 then returned to 2% buffer B for 10 min. LC–MS/MS data acquisition was accomplished
159 using an eleven-scan event cycle comprised of a full scan MS for scan event one acquired
160 in the Orbitrap, which enables high resolution/high mass accuracy analysis. The mass
161 resolution for MS was set to 60,000 (at m/z 400) and used to trigger the ten additional
162 MS/MS events acquired in parallel in the linear ion trap for the top three most intense ions.
163 The mass over charge ratio range was from 380 to 2000 for MS scanning with a target
164 value of 1,000,000 charges and from ~ 1/3 of the parent m/z ratio to 2000 for MS/MS
165 scanning with a target value of 10,000 charges.

166 The data-dependent scan events used a maximum ion fill time of 100 ms and 1 microscan
167 to increase the duty cycle for ion detection. Target ions already selected for MS/MS were
168 dynamically excluded for 15 s. Nanospray, capillary and tube lens voltages were set to 0.9–
169 1.6 kV, 5 and 100 V, respectively. Capillary temperature was set to 225 °C. MS/MS
170 conditions were as follows: normalized collision energy, 35 V; activation q, 0.25; and
171 activation time, 30 ms.

172 **2.5.3. Database searching**

173 Raw MS files were analyzed by Mascot (Matrix Science, London, UK; version 2.5.1) and
174 X! Tandem (The GPM, thegpm.org; version 2007.01.01.1) or by MaxQuant (version
175 1.6.2.10) with the default parameters (20).

176 Tandem mass spectra were first analyzed by Mascot version 2.5.1. Charge state
177 deconvolution and deisotoping were not performed. Mascot was set up to search the
178 UniProt sprot database (selected for bacteria, unknown version, 329683 entries and for
179 fungi, unknown version, 31499 entries), UniProt_Maize_txid4577_20160927 database
180 (unknown version, 160789 entries) and UniProt_Archaea_txid2157_20160921 database
181 (unknown version, 1351908 entries) with trypsin as the digestion enzyme. O+18 of
182 pyrrolysine and carbamidomethyl of cysteine were specified in Mascot as fixed
183 modifications. Oxidation of methionine was specified in Mascot as a variable modification.

184 For the analysis in MaxQuant software, the identification of individual peptides was
185 conducted in protein groups. The MaxQuant searches were executed against the CAZy
186 database (CAZyDB.07312019.fa). The following search parameters were used: enzymatic
187 cleavage rule of Trypsin/P and a maximum of two missed cleavage sites,
188 carbamidomethylation on cysteine as fixed modification, whereas protein N-terminal
189 acetylation and methionine oxidation were defined as variable modifications for the
190 database searches. All entries were filtered using a false positive rate of 1%, and all false
191 positives were removed.

192 For all analyses, the first search peptide tolerance was 20 ppm, and the main search peptide
193 tolerance was 4.5 ppm. A fragment ion mass tolerance of 0.60 Da and a parent ion
194 tolerance of 10 ppm were established.

2.6. Protein identification criteria

Scaffold_4.8.6 (Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications for Mascot and X! Tandem results. For the validation of the identified proteins, the FDR was adjusted to obtain probabilities greater than 95% for the peptides and 99% for the proteins. Both probabilities were assigned by the Protein Prophet algorithm (21). All identification and search criteria are specified for each database in the supplementary materials. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy parsimony principles (Institut de Recherches Cliniques of Montreal, IRCM, Quebec, Canada). For the description of the microbial diversity, proteins that contained at least two identified peptides were considered, while for the functional classification, proteins that contained at least a unique peptide were accepted. In both cases, only the proteins that were identified in at least two of the three experimental replicates were considered, and false positives were manually removed.

3 Results

3.1 Enumeration of microorganisms

To correlate the metaproteomic data with the microbiota, the main microbial groups were quantified. We observed that just after nixtamalization, the bacterial counts for all groups analyzed were on the order of 10^6 CFU, except for Enterobacteriaceae, which were at a concentration of 10^4 CFU. Aerobic mesophiles began fermentation with a concentration of 10^6 CFU · g of dough^{-1}, increasing after 24 h of fermentation to 10^9 CFU · g^{-1}. Among them, LAB, the predominant group during fermentation, started with a count of 10^6 CFU · g^{-1} and increased after 24 h of fermentation to 10^{10} CFU · g^{-1}. This increase in bacterial count clearly coincided with the drop in pH by almost 3 units, from 7.75 to 4.83 (Figure 1).

Since starch is the main carbohydrate in nixtamal and LAB the most abundant microorganisms, previous studies considered that ALAB should play a relevant role during fermentation, providing glucose and malto-oligosaccharides for the nonamylolytic microbial community (4, 9). ALAB started fermentation at a concentration of 10^6 CFU · g^{-1}, with an increase during the first 9 h of two logarithmic units (Figure 1).

Enterobacteriaceae are consistently found in traditional fermented foods. Their presence is generally an indicator of food quality and safety, and they are considered undesirable microorganisms. The microbial analysis revealed an initial count of 10^4 CFU · g^{-1}; during the first 9 h, the concentration increased to 10^6 CFU · g^{-1}, from which point the Enterobacteriaceae arrested their growth and began to decline slowly to 10^5 CFU · g^{-1} after 48 h. On the other hand, as the pH value of pozol became more acidic, the fungal concentration increased, with a maximum of 10^6 CFU · g^{-1} at 48 h (Figure 1).

At the same time, the carbohydrate content decreased, with the reduction in fiber content being the most obvious and interesting change since it involves the consumption of polysaccharides such as cellulose and hemicellulose (Table 1).

3.2 Metaproteomic analysis

235 **3.2.1. Microbial community in pozol fermentation**

236 The metaproteomic analysis at different fermentation times allowed the identification of
237 3215 proteins. The protein biodiversity analysis showed that eukaryotic, bacterial and
238 archaeal domains were present during pozol fermentation, with abundances of 69.9%,
239 28.5% and 1.6%, respectively. The protein analysis showed that the highest number of
240 identified proteins belonged to maize (1037 proteins).

241 To estimate the relative abundance of the microorganisms during the fermentation, the total
242 number of identified proteins with two unique peptides was considered. In bacteria, a total
243 of 704 proteins were identified, distributed in 8 different phyla and 71 different genera
244 (Figure 2). The phylum classification showed that *Firmicutes* (70.61%) and *Proteobacteria*
245 (22.62%) were predominant. Other phyla accounted for less than 2% of the relative
246 abundance and included *Actinobacteria*, *Chloroflexi*, *Spirochaetes*, *Planctomycetes* and
247 *Bacteroidetes*. The *Firmicutes* phylum was mainly represented by proteins of LAB
248 (64.63%). *Streptococcus* was the prevalent genus in this community, with 47.87%,
249 followed by the *Lactobacillus* genus (7.1%). Additionally, proteins of *Leuconostoc*
250 (4.69%), *Enterococcus* (3.41%) and *Lactococcus* (1.56%) were identified but in minor
251 abundance (Figure 2).

252 In the *Proteobacteria* phylum, proteins from enterobacteria were identified, with the
253 *Escherichia* and *Citrobacter* genera as the most abundant (2.84% and 2.41%, respectively).
254 Furthermore, proteins from nitrogen-fixing bacteria such as *Klebsiella* and *Enterobacter*
255 were found. Other genera represented less than 1% of the metaproteome, i.e.,
256 *Actinobacillus*, *Staphylococcus*, and *Rhizobium*, among others.

257 A lower number of fungal proteins were identified (245); these proteins were distributed in
258 5 phyla and 28 genera. Most of the proteins belong to the phylum *Ascomycota*, with the
259 most abundant genera being *Neurospora* (23.36%), *Schizosaccharomyces* (20.90%) and
260 *Saccharomyces* (17.21%). *Aspergillus* and *Candida* proteins represented 6.96% and 4.09%
261 of the relative abundance, respectively. Other identified proteins belong to the genera
262 *Ashbya*, *Kluyveromyces*, *Puccinia* and *Penicillium* (Figure 3).

263 Metaproteomic analysis allowed the identification of bacterial and fungal proteins whose
264 genera have not been previously described in pozol, such as *Actinobacillus*,
265 *Corynebacterium*, *Gloeobacter*, *Nitrobacter*, *Pantoea*, *Ajellomyces*, *Puccinia*, *Fusarium*,
266 *Schizosaccharomyces* and *Yarrowia*, which represented less than 1% of the metaproteome.

267 **3.2.2. Dynamic changes during pozol fermentation**

268 The genus-level classification of the bacterial and fungal protein sequences showed the
269 microbiota dynamics in pozol at different fermentation times. In the unfermented dough, a
270 lower number of proteins were found, these proteins basically belong to environmental
271 microorganisms, especially aquatic bacteria and cyanobacteria. Proteins were also found
272 from endosymbionts and plant pathogens, soil bacteria, and proteins from animal
273 commensals and pathogens to a lesser extent. Throughout fermentation, some of the
274 detected proteins persisted, and the vast majority disappeared, indicating that the most

275 abundant microorganisms at the beginning of fermentation were unable to grow in the
276 dough. One exception to this was proteins of the genus *Escherichia*, a ubiquitous
277 enterobacteria whose proteins were maintained during all the sampling times (Figure 4A).

278 Remarkably, during the first 9 h of fermentation, the proteins of LAB increased rapidly,
279 reaching 64.63% of the total bacterial metaproteome. *Streptococcus* was the dominant
280 genus, representing 45% and 48% at 9 h and 24 h, respectively. In the 9 h fermented
281 sample, *Leuconostoc* (11%), *Citrobacter* (5%) and *Acinetobacter* (3%) were also present,
282 but as fermentation progressed, *Acinetobacter* proteins were enriched, while *Citrobacter*
283 and *Leuconostoc* proteins decreased to 3%. At this point, *Lactobacillus* proteins began to
284 increase, reaching a relative abundance of 6%. At 48 h of fermentation, 79% of the
285 identified proteins were from LAB, with absolute dominance of *Streptococcus* proteins,
286 which reached 56% of the identified proteins. Proteins from the *Lactobacillus*,
287 *Enterococcus* and *Lactococcus* genera were also detected, with relative abundances of 14%,
288 4% and 2%, respectively, while *Leuconostoc* remained at 3%. *Enterobacteriaceae* proteins
289 were identified at all fermentation times, but as fermentation progressed, their presence was
290 reduced and, in the end, these proteins represented less than 3% of the metaproteome
291 (Figure 4A).

292 Fungi are known to grow in pozol as well; however, they have not been practically studied.
293 In this work, we observed that the predominant fungal genus was *Schizosaccharomyces*
294 during the first 9 h, followed by *Saccharomyces*, which represented 21% of the
295 metaproteome at 24 h of fermentation. The *Candida* genus was detected after 9 h, but the
296 sample fermented for 48 h contained mostly proteins of the genus *Neurospora*, these
297 proteins accounted for 44%; *Schizosaccharomyces* and *Saccharomyces* were reduced to less
298 than 10% at this time (Figure 4B).

299 **3.2.3. Functional classification of the metaproteome**

300 Based on the KEGG Orthology Annotation System, a total of 491 proteins for bacteria and
301 287 proteins for fungi could be annotated to a wide spectrum of metabolic pathways
302 (Figure 5A and 5B).

303 For bacteria, proteins associated with amino acid metabolism (20.16%), biosynthesis of
304 secondary metabolites (14.05%), genetic information processing (15.27%) and
305 carbohydrate metabolism (15.27%) were the most abundant (Figure 5A). For fungi, five
306 main processes were found: carbohydrate metabolism (21.95%), genetic information
307 processing (19.86%), amino acid metabolism (15.68%), biosynthesis of secondary
308 metabolites (14.63%) and biosynthesis of antibiotics (10.10%) (Figure 5B). In both cases,
309 proteins associated with energy metabolism, metabolism of cofactors and vitamins,
310 nucleotide metabolism and lipid metabolism were found but in minor abundance. The most
311 relevant differences were observed in proteins related to transport systems and drug
312 resistance present only in bacteria, while proteins for xenobiotic biodegradation and
313 metabolism were present only in fungi.

314 **3.2.4. Meta-analysis of the metaproteome**

315 The meta-pathways of pozol fermentation were reconstructed based on the metaproteomic
316 data for bacteria, fungi and maize. For each protein, a KO identifier was assigned for its
317 integration into a metabolic pathway. A total of 1102 identifiers were analyzed, resulting in
318 the partial or total reconstruction of different metabolic pathways related to central carbon
319 metabolism, amino acid metabolism, lipid metabolism, synthesis of cofactors and vitamins
320 and energy metabolism (Figure 6).

321 In the metabolic pathways related to carbohydrate central metabolism, proteins for
322 glycolysis, gluconeogenesis, pyruvate oxidation, Krebs cycle and the pentose phosphate
323 pathway were identified. For amino acid metabolism, different proteins were grouped for
324 biosynthesis and degradation. However, the analysis showed that only in some cases was
325 possible to completely reconstruct the pathways. In lipid metabolism, proteins for fatty acid
326 degradation, fatty acid biosynthesis, long-chain fatty acid elongation, biosynthesis of
327 unsaturated fatty acids, cholesterol and biosynthesis of other lipids (lactosylceramide,
328 triacylglycerol and jasmonic acid) were retrieved. Regarding the biosynthesis of cofactors
329 and vitamins, only a few proteins were identified, and in none of the cases the complete
330 pathways were found.

331 As expected, proteins related to carbon fixation were found in processes exclusive to plants,
332 such as photosynthesis, to produce energy in the form of ATP and NADPH. Other carbon
333 fixation pathways were the reductive citric acid cycle and the reductive acetyl-CoA
334 pathway.

335 As shown in Figure 6, the microbial community activities and raw material (maize)
336 enzymes may display metabolic complementation and contribute to the same biosynthetic
337 pathway. However, some metabolic processes were exclusively related to maize or bacteria
338 and fungi. With the metaproteome data, reconstruction of the entire metabolic map of pozol
339 was performed.

340 **3.2.4.1. Carbohydrate metabolism**

341 **Pyruvate metabolism**. Pyruvate, a key intermediate for flavor compounds production,
342 could be produced in pozol through glycolysis. Pyruvate can be further metabolized in
343 pozol by different mechanisms. First, the enzymes related to the production of acetyl-CoA,
344 which is then metabolized into acetate and ethanol, were retrieved. Second, the reduction of
345 pyruvate to lactate by lactate dehydrogenase was evidenced. Third, pyruvate decarboxylase,
346 which is necessary for acetaldehyde production, was found. Fourth, the analysis showed the
347 presence of the enzyme formate acetyltransferase, which catalyzes the reversible
348 conversion of pyruvate and coenzyme-A into formate and acetyl-CoA. Finally, pyruvate
349 was found to be metabolized to acetoin, diacetyl and 2,3-butanediol via butanoate
350 metabolism (Supplementary Figure 1).

351 Additionally, the acetyl-CoA from pyruvate and oxaloacetate (produced from aspartate)
352 could be condensed to produce citrate. Although we could not find a transport system for
353 this molecule, the analysis allowed us to identify the enzymes required for the oxidation,
354 decarboxylation and dehydration reactions to form succinate, fumarate and malate.

355 **Polysaccharide degradation**. Several polysaccharides, such as starch, cellulose and
356 hemicellulose, were present in the dough and can be hydrolyzed by the microbiota during
357 fermentation, providing monosaccharides, disaccharides and oligosaccharides, which serve
358 as the most important carbon sources for microbial growth. Thus, a more in-depth analysis
359 was carried out in the search for the enzymes that participate in the metabolism of these
360 carbohydrates (Figure 7A, 7B and 7C; Supplementary Table 1).

361 Analysis with the CAZy (Carbohydrate Active Enzymes) database allowed the
362 identification of proteins from plants, fungi and bacteria. Most of the proteins were related
363 to bacteria, and the predominant activities were related to starch degradation, while the
364 number of identified proteins was similar for hemicellulose and cellulose. In the case of
365 fungi, fewer proteins were identified, and the majority were involved in cellulose
366 degradation. For plants, two different proteins were identified for starch degradation and
367 one for cellulose degradation.

368 Analysis with the UniProt database allowed the identification of enzymes for starch,
369 cellulose and hemicellulose degradation, all of which were associated with fungi.
370 Additionally, other glycosyl hydrolases involved in the utilization of other carbohydrates
371 were detected (data not shown).

372 Transporters for some products of polysaccharide degradation, such as glucose, mannose,
373 galactose, cellobiose and some oligosaccharides (maltooligosaccharides,
374 arabinooligosaccharide and maltodextrin), were also found.

375 **3.2.4.2. Amino acid and protein metabolism**

376 Amino acids can be produced from precursors by their metabolism or by the action of
377 different proteases for protein degradation and recycling. In the metaproteomic analysis, the
378 results showed that amino acid biosynthesis occurs by the three mechanisms.

379 For glutamate, glutamate dehydrogenase was identified as involved in the reductive
380 amination of the intermediary α-ketoglutarate to glutamate. This amino acid is subsequently
381 metabolized to arginine, glutamine and proline. Arginine biosynthetic enzymes from
382 ornithine were identified. The enzyme glutamine synthetase responsible for glutamate and
383 ammonia condensation to form glutamine was also found. The pathway for proline
384 biosynthesis was incomplete; however, proline can be obtained from protein degradation by
385 the action of proline iminopeptidase (Supplementary Figure 2).

386 The proteins involved in the metabolism of oxaloacetate for aspartate and asparagine
387 synthesis were identified. Although methionine and threonine are also synthesized from this
388 precursor, the analysis showed that if these amino acids are produced during fermentation,
389 they are produced from homocysteine and homoserine, respectively (Supplementary Figure
390 2).

391 Finally, for the amino acids serine, lysine, tyrosine, histidine, leucine, isoleucine and valine,
392 the proteins for the synthesis of their respective precursors were detected. Nevertheless, in
393 some cases, not all the enzymes necessary to complete the metabolic pathways were
394 identified, or the pathways were truncated in the final step (Supplementary Figure 2).

395 Concerning amino acid degradation, aminotransferases for the transamination reaction were
396 identified; however, no proteins for the subsequent conversion of α-keto acids were
397 retrieved, indicating that amino acid degradation for flavor production is not a key
398 metabolic pathway during pozol fermentation.

399 Regarding protein degradation, different proteases, aminopeptidases, and
400 carboxypeptidases were identified, so this can be an alternative pathway to obtain amino
401 acids as well as for the release of peptides of different sizes. Finally, several amino acid,
402 peptide, dipeptide and oligopeptide transport proteins were also detected.

403 4 Discussion

404 4.1 Microbial community dynamics in pozol fermentation

405 The microbial community in traditional fermented foods has been studied with the use of
406 culture-dependent and culture-independent strategies. Different methods based on DNA
407 analysis (RAPD, AFLP, ARDRA, DGGE) have been commonly used; however, these
408 approaches have inherent biases and do not allow an understanding of the role of
409 microorganisms in fermentation. With the introduction of omics techniques, it is easier to
410 dissect the complexity of microbial-rich environments such as fermentation since microbial
411 community dynamics and functionality can be established. Metaproteomics can suggest the
412 presence of microbial species by assigning identified proteins. This approach allows the
413 study of microbial activity within the system and simultaneously provides functional
414 knowledge about the community under analysis.

415 The microbiology of pozol has been extensively described (3, 4, 5, 6, 7, 8, 9, 15, 22, 23, 24,
416 25, 26, 27) and some of these studies have focused on the description of the dynamics of
417 the microbial community, their spatial distribution and the possible relationship between
418 the chemical changes during fermentation and microbiota development. However, the role
419 of microorganisms during fermentation, their enzymatic activities and therefore their
420 metabolic potential and how they contend with this particular environment are still
421 unknown. In this study, a combination of chemical, culture and metaproteomic analyses
422 was employed to investigate pozol fermentation.

423 Pozol metaproteomic analysis revealed that this fermented food is a complex ecosystem
424 where bacteria, fungi and archaea are found. The most abundant proteins were identified
425 from the substrate, i.e. more than 60.5% of the identified proteins were from plants; there
426 was an important representation of bacterial and fungal proteins, which accounted for
427 28.5% and 9.4% of the metaproteome, and only 1.6% of the metaproteome was associated
428 with archaea.

429 The analysis by fermentation time showed that the unfermented pozol was composed of
430 heterogeneous microbiota. At this point, the identified bacteria were related to the
431 environment, the substrate or the material used for pozol production, such as water, soil or
432 skin. However, these species were reduced or eliminated during fermentation,
433 consequently, it is very likely that they do not participate in the process.

434 As fermentation took place, a core of microorganisms was established, composed mainly of
435 LAB, whose proteins were identified in high abundance throughout the fermentation. LAB
436 are present in fermented foods prepared from different raw materials, such as milks,
437 cereals, vegetables and animal sources. LAB metabolic activity is closely related to
438 improving the taste, aroma, texture, shelf life, and nutritional value of fermented foods. The
439 production of organic acids (lactic, acetic and formic) by LAB not only impacts the
440 characteristic flavors of the food but also restricts the growth and survival of pathogenic
441 microorganisms by decreasing the pH (28, 29, 30). During pozol fermentation, the drastic
442 reduction in pH of 7.75 to 4.83 was associated with the increase in LAB concentration and
443 the number of proteins identified for this group.

444 After 9 h of fermentation, bacteria of the genus *Streptococcus* dominated the fermentation,
445 in concordance with previous microbiological studies (5, 6, 7, 9). *Streptococcus spp.* are
446 widely distributed bacteria in food fermentations, and they have great importance in
447 medicine and industry (31, 32, 33, 34). Several streptococci are commensal in mammals;
448 however, some can also cause illnesses ranging from mild to acute. In industry,
449 Streptococcus thermophilus is widely used as a yogurt and cheese starter.

450 Since 2003, Diaz-Ruiz and coworkers (9) described several amylolytic streptococci in
451 pozol, being *S. infantarius* subsp. *infantarius*, the most amylolytic. More recently, Cooper
452 et al. (26) established that this same microorganism could grow in corn arabinoxylan.
453 Clearly, the diversity of enzymes that these bacteria express and their adaptability for
454 surviving and developing in extreme pH conditions, both alkaline and acidic (27), allows
455 them to dominate during the early fermentation stages of this dough and persist during the
456 entire fermentation process even when other LAB become more abundant.

457 Other proteins of lactic acid bacteria identified in the metaproteome belonged to
458 *Lactobacillus*, *Leuconostoc* and *Enterococcus*, bacteria previously described as present in
459 pozol (4, 5, 6, 7, 8, 9, 35). Their presence during fermentation is determined by their
460 capacity to degrade some of the polysaccharides present in the dough. The low abundance
461 of *Enterococcus* during fermentation, compared to the *Lactobacillus* and *Leuconostoc*
462 genera, could be related to their low amylolytic activity (36) and to the fact that some
463 *Lactobacillus* and *Leuconostoc* species can use starch and xylan as the only carbon sources
464 for growth (4, 37, 38). Contrary to what was found in pozol, in other amylaceous fermented
465 foods prepared from cassava, maize and other cereals, *Lactobacillus* is the predominant
466 amylolytic genus in fermentation (39, 40, 41) absence one of the most amylolytic LAB
467 genera, in pozol and the relatively low abundance of this genus in pozol might be the result
468 of the conditions generated by the nixtamalization process (9).

469 Other important changes in the microbial composition were observed in the enterobacteria
470 group. Both the plate count and the metaproteomic analysis showed that this group
471 decreased throughout fermentation but was still present even at low pH. Diverse studies
472 have reported the presence of enterobacteria in different stages of the fermentation process
473 (3, 5, 6, 35, 42), and the characterization of these microorganisms has allowed the
474 identification of several serotypes of *Escherichia coli*, *Escherichia fergusonii*, *Enterobacter*
475 *cloacae*, *Enterobacter aerogenes*, *Enterobacter sakazakii*, *Enterobacter* spp., *Klebsiella*
476 *pneumoniae*, *Klebsiella* spp., *Klebsiella variicola* and *Kosakonia* spp. (1, 15, 43, 44). Their

477 presence even at high organic acid concentrations has been attributed to the existence of
478 microenvironments within the dough, to the acid tolerance of some strains or to the use of
479 organic acids by other microorganisms for their growth, decreasing the local concentration
480 (6, 7, 44, 45). Despite their negative reputation, some of these bacteria can directly fix
481 atmospheric nitrogen in the dough, which allows for its nutritional enrichment (15).

482 Throughout fermentation, fungal proteins were identified, mainly represented by yeast
483 proteins, both in the unfermented dough and in the different fermented samples, with an
484 increase in the number of identified proteins of filamentous fungi at the end of
485 fermentation. The presence of yeasts since the first hours of fermentation is related to their
486 short reproductive cycles, while filamentous fungi developed when the surface dried and
487 pH decreased, while yeasts continued to grow (46, 47).

488 Proteins of the filamentous fungi *Ajellomyces*, *Cercospora* and *Fusarium* were identified
489 only in the unfermented dough, which could indicate that these species do not survive the
490 changes that are generated during fermentation or that the substrate does not have the
491 necessary conditions for their development. In the fermented samples, the predominant
492 genera were *Schizosaccharomyces*, *Saccharomyces* and *Neurospora*.

493 Proteins of other genera, such as *Aspergillus*, *Penicillium*, *Puccinia*, *Kluyveromyces* and
494 *Komagataella*, showed fluctuating changes throughout fermentation. The development of
495 this group was associated with the production of organic acids by LAB. It has been
496 suggested that the proliferation of fungi in foods is stimulated by the acidic environment
497 that LAB generate by their metabolism, and in turn, fungi favor the growth of other
498 bacteria, providing growth factors such as vitamins and soluble nitrogen compounds (48).

499 These genera have been reported in other fermented foods based on wheat or rice flour or
500 wheat or barley grains as well as in traditional alcoholic beverages and fermented milk
501 products (49, 50, 51). Although fungi in fermented foods can be considered harmful due to
502 the production of enterotoxins, some of them may have an important role during the
503 fermentation process due to the production of enzymes for the degradation of complex
504 polysaccharides and antinutritional factors. Additionally, the metabolism of these
505 organisms can result in an improvement in the organoleptic characteristics and nutritional
506 value through the production of characteristic flavors due to the degradation of fatty acids
507 and the production of vitamins, amino acids and folic acid (52, 53, 54, 55, 56).

508 **4.2 Carbohydrate and lipid metabolism**

509 **4.2.1 Degradation of polysaccharides in pozol**

510 Pozol is characterized by a low concentration of soluble carbohydrates and a high starch
511 content that must support an abundant microbiota. ALAB have been considered the most
512 relevant group during fermentation to provide glucose and maltooligosaccharides for the
513 nonamylolytic microbial community. Although ALAB are present at high concentrations
514 (10^8 CFU · g of dry weight $^{-1}$), its low amylolytic activity does not explain the rich
515 microbial diversity and the high abundance of nonamylolytic microorganisms (9). In

516 addition, it should be considered that in nixtamal dough, alternative carbon sources,
517 hemicellulose and cellulose, are available for fermentation.

518 For starch hydrolysis, two proteins from maize were identified: a β-amylase that produces
519 maltose and an α-glucosidase that produces glucose from starch and maltose. These
520 enzymes could play an important role in pozol, mainly in the first stage of fermentation,
521 releasing simple sugars that can be used for the initial growth of microorganisms. In
522 bacteria, multiple amylolytic enzymes, exoamylases (α-amylase) and endoamylases (α-
523 glucosidase), and debranching enzymes (pullulanase), were identified. In combination,
524 these enzymes allow the depolymerization of starch, demonstrating the importance of this
525 group during fermentation (Figure 7 A). Previous studies demonstrated the presence of
526 amylolytic bacteria and yeast (4, 9); however, no yeast amylase was identified in the
527 metaproteome. Characterization of the most amylolytic LAB species, *Streptococcus*
528 *infantarius* subsp. *infantarius*, produced two amylases, an amylopululanase with
529 debranching capacity and an α-amylase (9, 57).

530 On the other hand, the pericarp is a source of crude fiber and consequently an important
531 source of carbon. The fiber in maize kernels has been reported to contain approximately
532 40% arabinoxylan and 20% cellulose (58). However, their degradation requires the action
533 of several enzymes for the hydrolysis of the backbone and the different residues that they
534 may contain.

535 Corn arabinoxylan is formed by a main chain of d-xylopyranose linked in β- (1,4) with side
536 chains of α-1-arabinofuranose. A high degree of structural heterogeneity is given by other
537 sugars in the branches, including galactose, glucuronic acid and xylose (59). Metaproteome
538 analysis showed that in pozol, bacteria and fungi might degrade hemicellulose into xylose,
539 xylobiose and xylo-oligosaccharides, since the enzymes for the degradation of the main
540 chain and the different substitutions were identified (Figure 7B). Previous studies have
541 demonstrated that bacteria isolated from pozol are able to grow and metabolize xylan and
542 arabinoxylan (26).

543 Cellulose degradation requires three classes of enzymes: β-1,4-endoglucanases (EGL),
544 exoglucanases/cellobiohydrolases (CBH), and β-glucosidase (BGL). Endoglucanases from
545 bacteria and plants were found in the metaproteome, and this enzyme is necessary to
546 initiate the hydrolysis of the polysaccharide, reducing the degree of polymerization (60). In
547 addition, enzymes for the production of cellobiose, glucose and cello-oligosaccharides from
548 cellulose were identified (Figure 7C).

549 Together, the metaproteome data and chemical analysis demonstrate that during
550 fermentation, starch, hemicellulose and cellulose are fermentable carbon sources. Changes
551 in the carbohydrate content can be associated with the enzymatic activities identified for the
552 degradation of the different polysaccharides. During the first 9 h of fermentation, the crude
553 fiber concentration significantly decreased, indicating that in this period, the microbiota
554 consumes polysaccharides such as cellulose and hemicellulose for the release of simple
555 carbohydrates, which in fact increased (Table 1). Finally, the analysis of proteins suggests a
556 possible synergism between bacteria, fungi and plants for the degradation of the different
557 substrates.

558 **4.2.2 Lipid metabolism**

559 During carbohydrate catabolism through glycolysis, acetyl-CoA is produced, and its main
560 function is energy production in the TCA cycle and as an intermediate in fatty acid, leucine
561 and lysine biosynthesis.

562 Fatty acid synthesis starts with acetyl-CoA carboxylation to malonyl CoA, in the pozol
563 metaproteome, acetyl-CoA carboxylase was present. The set of enzymes for the subsequent
564 series of condensation, reduction and dehydration reactions included fatty acid synthase
565 (FAS) enzymes. In the analysis, two different types of FASs were identified, type I and
566 type II. FAS type I multienzymes are found in eukaryotic organisms (animals and fungi)
567 and in a few bacteria, and the FAS type II system occurs in archaea, prokaryotic organisms
568 and plastids of plants [61]. This set of enzymes allows the production of fatty acids with
569 different chain lengths and the generation of unsaturated or saturated fatty acids [62, 63].
570 These molecules have fundamental roles as the main constituent of cellular membranes,
571 where they form the precursors of phospholipids, sphingolipids and sterols as secondary
572 metabolites and signaling molecules, representing a suitable compound for energy and
573 carbon storage. Consistent with the presence of enzymes for fatty acid biosynthesis, an
574 increase in lipid concentration in the dough was observed (Table 1).

575 Additionally, enzymes for fatty acid degradation to produce acetyl-CoA were found.
576 Acetyl-CoA is oxidized for energy production in the TCA cycle and can be used for
577 conversion to succinate in the glyoxylate cycle for subsequent carbohydrate synthesis.
578 These processes allow microorganisms to synthetize complex molecules that require and
579 utilize simple carbon compounds in the absence of available carbohydrates such as glucose
580 during growth.

581 **4.3 Metabolic potential of pozol microbiota**

582 Fermented foods result from chemical changes in the substrate due to the metabolism of the
583 microorganisms that develop during the process. These microorganisms can modify the raw
584 material in different ways, improving the organoleptic and nutritional properties of the final
585 product. Moreover, during fermentation, microorganisms associated with fermented food or
586 beverages develop multiple functional properties that can stimulate consumer health (64,
587 65).

588 **4.3.1 Flavor development**

589 As shown in Figure 6, pyruvate metabolism may contribute to the organoleptic
590 characteristics of pozol through three metabolic mechanisms. First, pyruvate is converted to
591 lactate by lactate dehydrogenase. Second, acetyl-CoA is produced and then metabolized to
592 acetate and ethanol. Third, pyruvate is metabolized by acetolactate decarboxylase and by
593 diacetyl reductase to acetoin and 2,3-butanediol. The results imply that in fermentation,
594 homofermentative and heterofermentative metabolism occurs. A previous study by DGGE
595 analysis showed that fermentation can be divided into two stages; in the initial stage,
596 homofermentative bacteria such as *Streptococcus* and *Enterococcus* are present; in the
597 second stage (24 to 48 h), in addition to homofermentative bacteria, heterofermentative

598 LAB, including *L. fermentum* and *Leuconostoc* species, develop and reach maximum levels
599 at 48 h. These bacterial activities result in the production of lactate as the major
600 fermentation product, with acetate and ethanol at lower concentrations (7). Although
601 acetoin and butanediol have not been reported in pozol, metaproteomic analysis identified
602 the enzymes involved in their biosynthesis. In other fermented foods, acetoin and
603 butanediol production is mainly associated with LAB metabolism, where these compounds
604 can make a great contribution to flavor (66, 67, 68). These results demonstrate the
605 metabolic potential of microbiota to produce a wide range of compounds important for food
606 safety, sensory attributes and the presence of enzymes that may have important
607 biotechnological applications.

608 **4.3.2 Nutritional improvement**

609 Fermentation provides an easy and economical method to enrich the different substrates
610 used for production. During fermentation, different molecules (essential amino acids,
611 vitamins, bioactive peptides, etc.) can be synthesized by microorganisms. The metabolism
612 of microorganism may increase mineral bioavailability, improve protein digestibility, and
613 the degradation of complex macromolecules into simple biomolecules occurs (69, 70).

614 In pozol, the fermentation of maize dough produces changes in the amino acid and vitamin
615 concentrations. Amino acids such as histidine, leucine, methionine and phenylalanine
616 decreased in the fermented dough; in contrast, the content of other amino acids (arginine,
617 lysine, isoleucine, threonine and tryptophan) increased during fermentation (71). In the
618 metaproteome, several enzymes for amino acid metabolism were grouped in the general
619 classification, and a detailed analysis showed that the synthesis of glutamate, glutamine,
620 serine, arginine, aspartate and asparagine is possible since all the enzymes involved in their
621 respective metabolic pathways were identified. For the rest of the amino acids, none or only
622 some of the required enzymes for their biosynthesis were identified. However, protein
623 degradation through the action of different proteases could release different amino acids, as
624 in the case of proline, which could explain the increments in the essential amino acid
625 concentration. Regarding vitamins, the same study showed that the raw material is enriched
626 during fermentation with riboflavin and niacin, but in the metaproteomic data, fewer
627 proteins were identified for the metabolism of vitamins, being impossible to complete the
628 respective pathways.

629 **5 Conclusions**

630 The simultaneous analysis of the microbiology, biochemistry and metaproteomics of pozol
631 allowed the construction of a comprehensive image of the fermentation system. Bacteria,
632 fungi, yeasts and archaea were found to participate in the pozol fermentation process, in
633 addition to the enzymatic systems of the substrate itself, being bacteria the most represented
634 group throughout fermentation.

635 The main changes both in the substrate and the microbiota occur in the first 9 hours of
636 fermentation, and the logarithmic increase in microorganisms is correlated with the
637 decrease in pH and in the content of carbohydrates and fiber, which indicates that this stage
638 has the highest metabolic activity. Many environmental microorganisms related to corn and

water were found for the first time; however, most of them disappeared or drastically decreased over fermentation.

Bacteria were mostly represented by LAB, being the genus Streptococcus by far, the most abundant. All these bacteria can be found in the environment, but their most important habitat is in mammals, which indicates that although spontaneous fermentation occurs, human participation in the nixtamalization and production process is decisive in the microbiota that develops. Yeasts were present in all the analyzed samples, with an increase in filamentous fungi at the end of fermentation.

The metaproteomic approach allowed the identification of several unknown proteins in the system so far. Systems for the hydrolysis of starch, hemicellulose and cellulose were also found, indicating that all these substrates can be used as a carbon source by the microbiota. It is striking that nonfungal enzymes were found for starch degradation, and the enzymes from corn and bacteria were responsible for the hydrolysis of this substrate. For hemicellulose and cellulose, both bacteria and fungi were involved, always with a predominance of bacterial enzymes. This diverse repertoire of enzymes found will allow new biochemical and structural studies.

The metabolic process revealed the synthesis of various fermentation products, such as organic acids, acetoin, butanediol and important intermediates, involved in the synthesis of fatty acids and amino acids and the generation of compounds that contribute to the organoleptic characteristics of pozol.

6 Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

7 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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917 Table 1. Chemical composition of pozol at different fermentation times.

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Fermentation time (h)	Content in pozol (mg/g of dry dough)			
	Nitrogen-free extract	Total soluble carbohydrates	Crude fiber	Lipids
0	845±9.9A	12.22 ± 0.01A	24 ± 0.41A	46 ± 0.42A
9	839±8A	11.67± 0.21AB	12 ± 0.05B	57 ± 0.12AB
24	841±3A	12.66± 0.05B	9.0 ± 1B	64 ± 0.03B
48	832±7A	12.96± 0.09C	8.0 ± 1B	68 ± 0.03C

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920 [a]The results are expressed as the mean of three replicates ± standard deviation. Differences
921 were evaluated by ANOVA followed by Tukey's multicomparison test ($p<0.05$). Different
922 uppercase letters indicate statistically significant differences between samples.

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937 Figure captions

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939 Figure 1. Microbial and chemical changes during pozol fermentation. Concentration of
940 microorganisms and pH values. The results are expressed as the mean of three replicates
941 ±standard deviation.

942 Figure 2. Donut plots representing the bacterial population in all pozol fermentation
943 process. The inner and outer circles show the percentage distribution of the proteins at
944 phylum and genus level, respectively.

945 Figure 3. Donut plots representing the fungi population in all pozol fermentation process.
946 The inner and outer circles show the percentage distribution of the proteins at phylum and
947 genus level, respectively.

948 Figure 4. Abundance by gender and fermentation time of the proteins identified in the pozol
949 metaproteome. Proteins were identified from 2 unique peptides in MS/MS analysis. **(A)**
950 Bacteria proteins. **(B)** Fungal proteins.

951 Figure 5. General functional classification base on KEGG annotations of metaproteome
952 data. **(A)** Bacteria proteins. **(B)** Fungi proteins.

953 Figure 6. KEGG atlas of the metabolic pathways found in the pozol metaproteome. The
954 proteins identified only in bacteria and fungi are shown in blue. Proteins identified only in
955 plants are shown in pink. The cyan lines indicate common proteins.

956 Figure 7. Schematic representation of the different polysaccharides presents in maize dough
957 and the enzymes implicated in their degradation. **(A)** Identified proteins for starch
958 hydrolysis; **(B)** Identified proteins for cellulose hydrolysis; **(C)** Identified proteins for
959 arabinoxylan.

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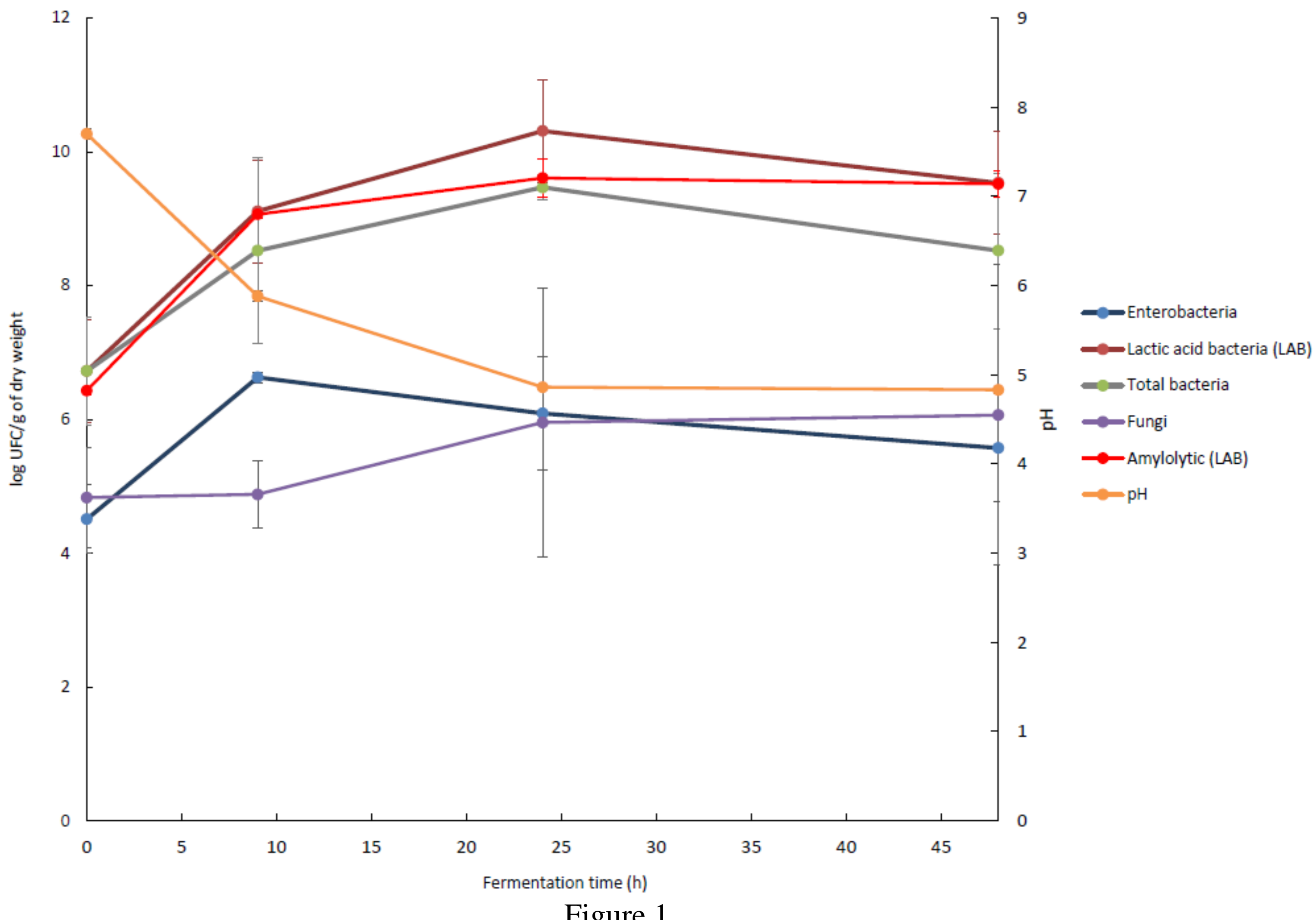

973 Figure 1.

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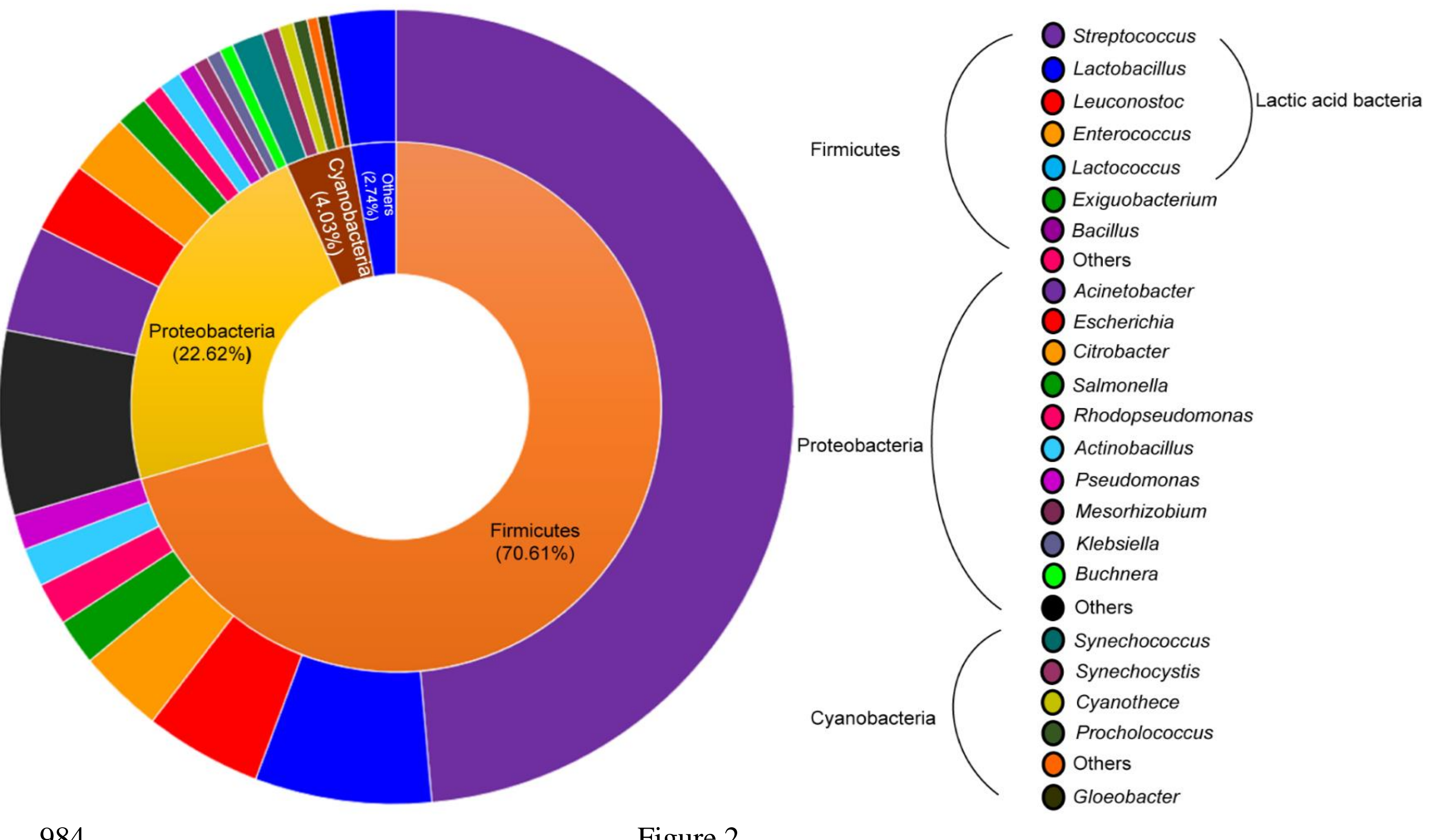

984 Figure 2.

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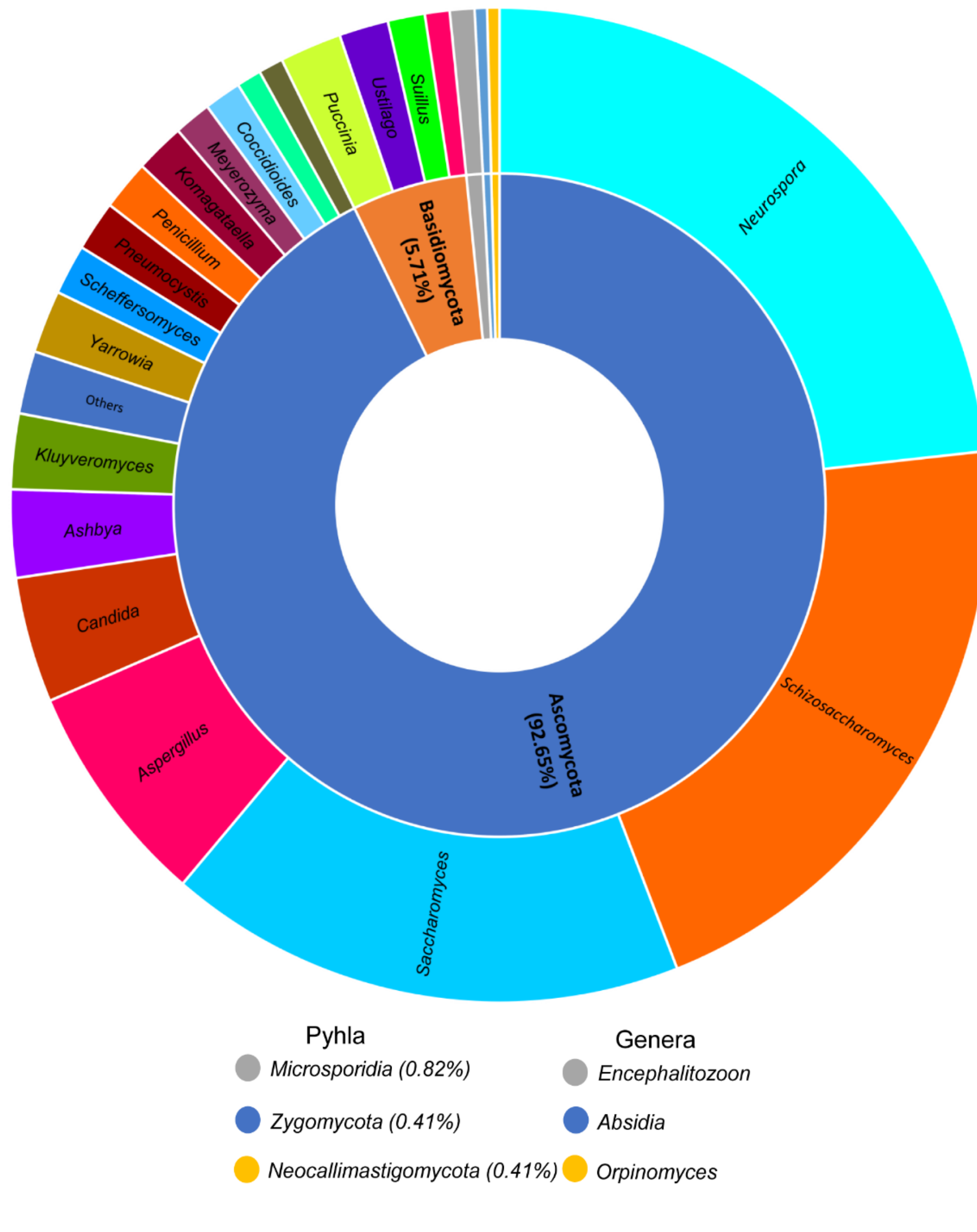

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996 Figure 3.

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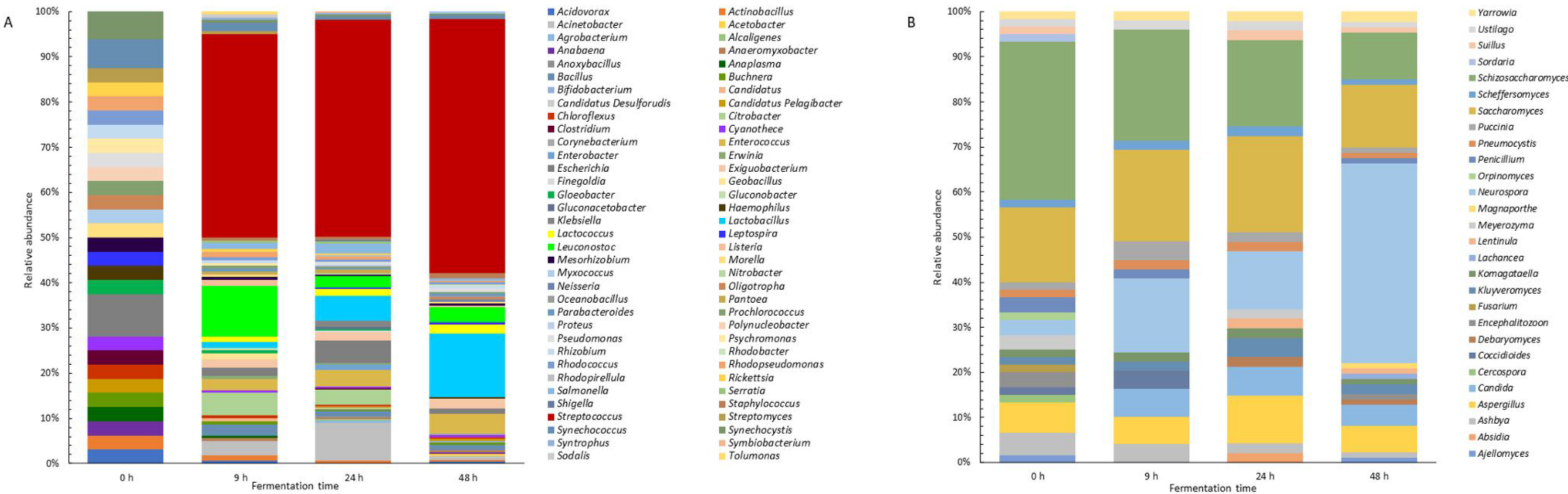

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Figure 4.

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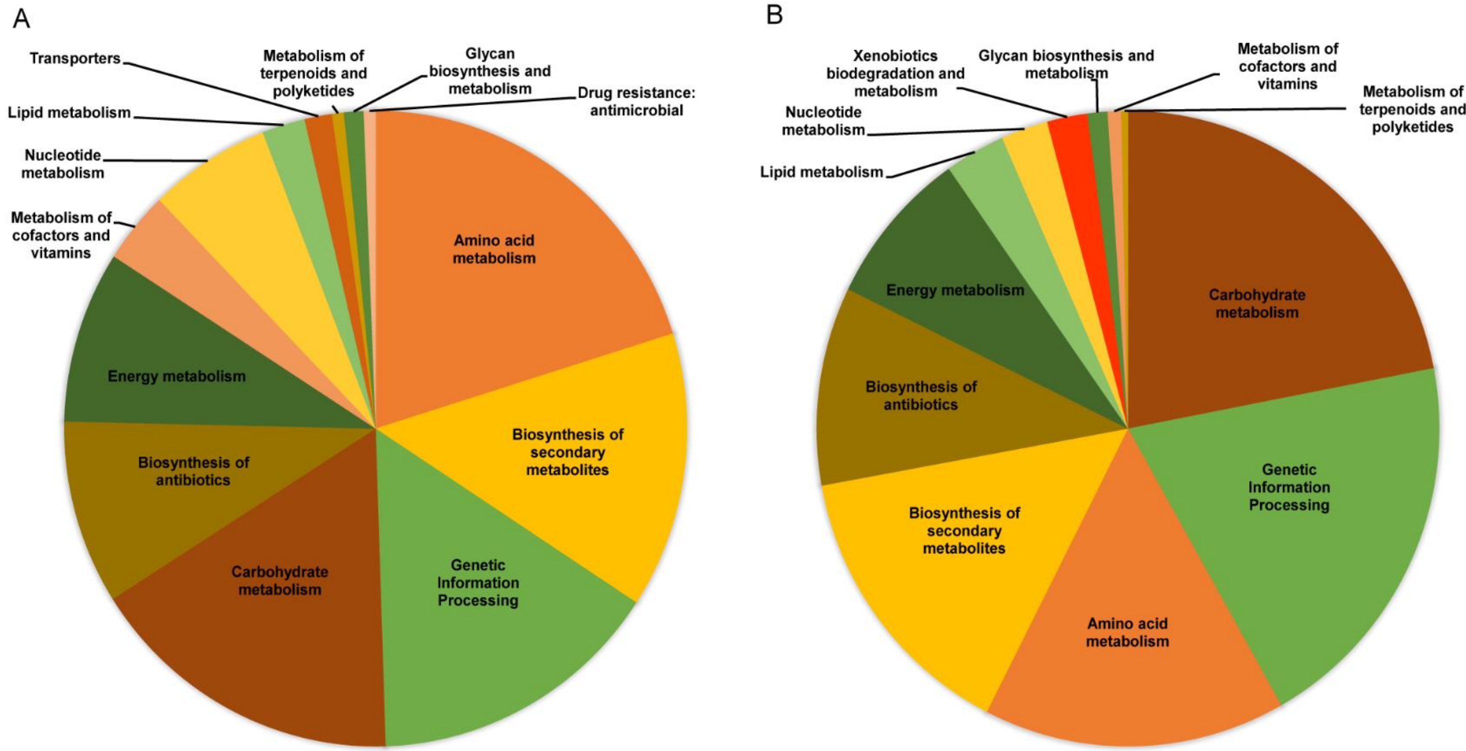

1004 Figure 5.

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1006 Figure 6.

A

Identified proteins in bacteria

Identified proteins in plants

A

C

C

B

A

C

Limit dextrin

Linear oligosaccharides

Maltotriose

Panose

Maltose

Glucose

(A) Endoamylase — α-amylase

(B) Exoamylase — β-amylase; Glucoamylase

(C) Debranching — Isoamylase; Pullulanase I; Pullulanase II; Pullulan hydrolase

B

Glucose

Cellobiose

β-G

CB

CPH

NR

R

CH

Identified proteins in bacteria

Identified proteins in fungi

C Cellulase

β-G β-glucosidase

CPH Cellobiose phosphorylase

CB Cellulose 1,4-beta-cellobiosidase

CH β-1,4-cellobiohydrolase

Glucose NR, nonreducing cellulose end

Cellobiose R, reducing cellulose end

Cello-oligomers

C

β-XYL

β-XYL

XYN

AXE

FE

α-GLU

α-GAL

Identified proteins in bacteria

Identified proteins in fungi

Xylan

Xylan-oligosaccharides

Arabinose

Ferulic acid

D-glucoronic acid

D-galactose

Acetyl group

XYN Endo-1,4-β-xylanase

β-XYL Exo-1,4-β-xylosidase

FE Feruloyl esterase

AXE Acetylxylan esterase

α-GLU α-D-glucoronidase

α-GAL α-galactosidase

1007 Figure 7.

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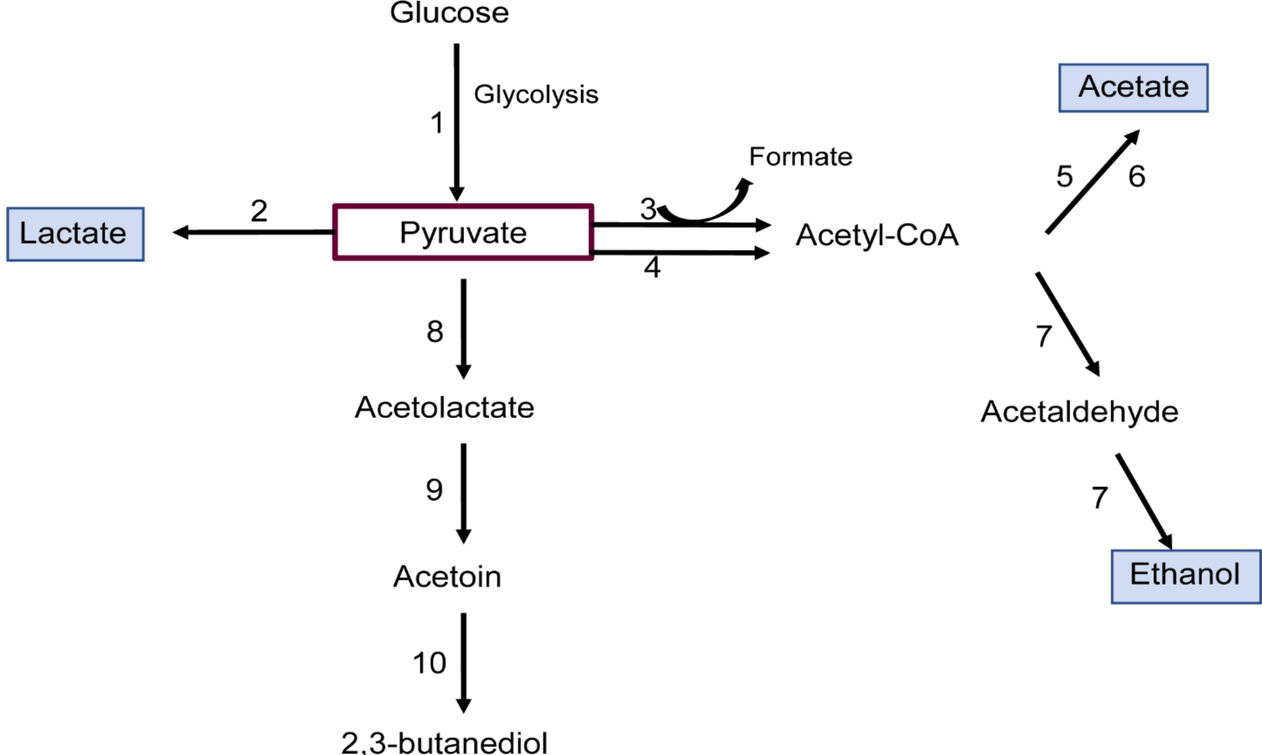

1009 **Supplementary Figure 1.** Identified enzymes related to the flavor and odors production in pozol
1010 fermentation. 1) Oxaloacetate decarboxylase. 2) Lactate dehydrogenase. 3) Pyruvate formate-lyase.
1011 4) Pyruvate dehydrogenase. 5) Phosphotransacetylase. 6) Acetate kinase. 7) Alcohol dehydrogenase.
1012 8) Acetolactate synthase. 9) Acetolactate decarboxylase. 10) Diacetyl reductase.

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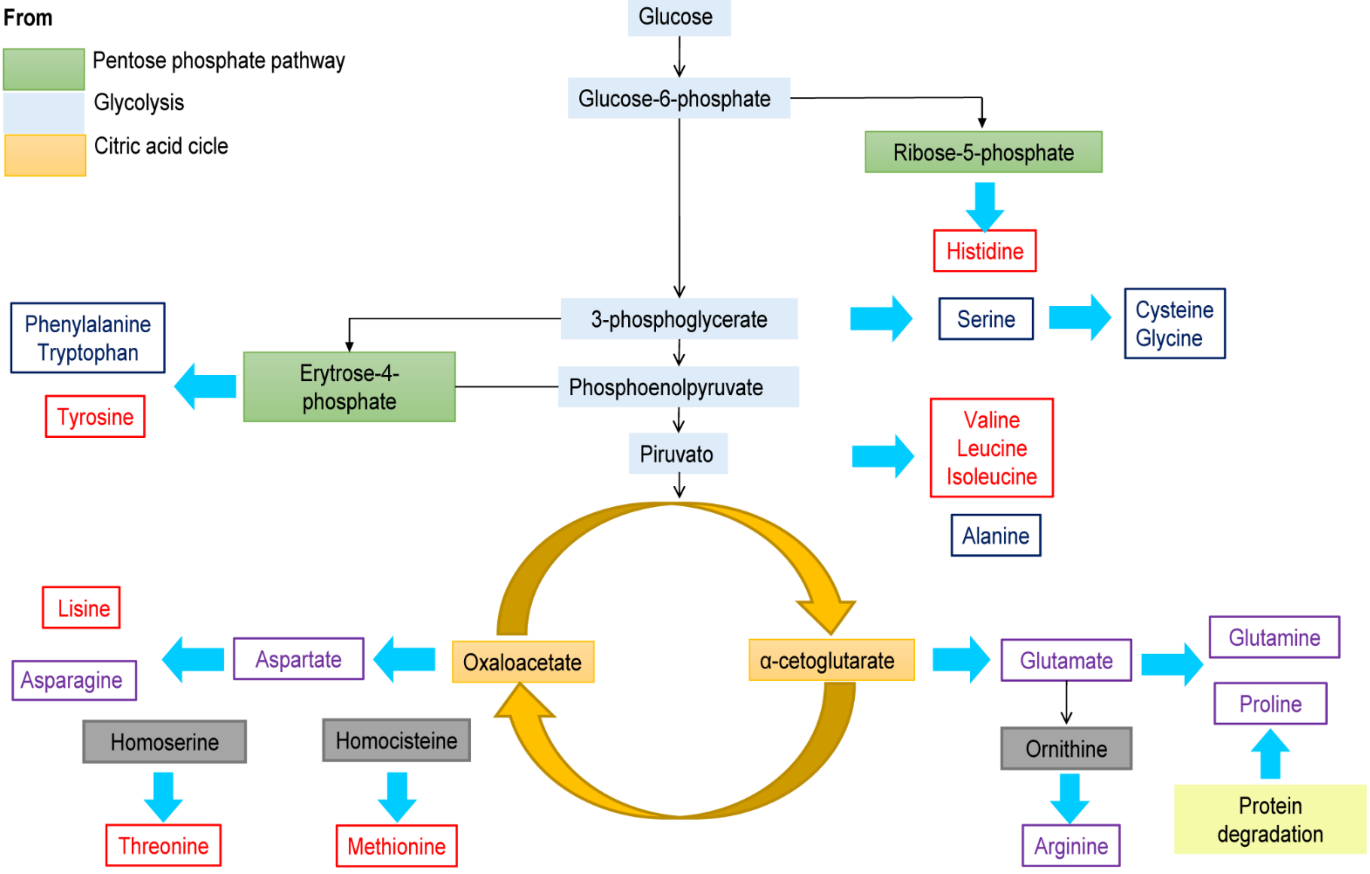

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1017 **Supplementary Figure 2.** Amino acid biosynthesis overview. The metabolic pathway and their
1018 respective intermediates for amino acids biosynthesis are shown. In the purple square the amino acids
1019 that can be produced in the pozol fermentation are shown. In the red square the amino acids for
1020 which some of the proteins were identified are shown. In the blue square the amino acids for which
1021 the enzymes for their biosynthesis were not identified are shown.

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1029 **Supplementary Table 1**. Identified enzymes in the metaproteome for the degradation of the different
1030 polysaccharides.

Substrate	Conventional name	E.C. number	Database/organism
Starch	Alpha-1,4-glucosidase	3.2.1.20	CAZy/Bacteria CAZy/Plants
	Alpha amylase	3.2.1.1	CAZy/Bacteria
	Glucan 1,4-α-glucosidase	3.2.1.3	
	Isoamylase	3.2.1.68	
	Amylopullulanase	3.2.1.1/41	
	Pullulanase	3.2.1.41	
	Neopullulanase	3.2.1.135	
	Beta-amylase	3.2.1.2	CAZy/Plants
Cellulose	Beta-glucosidase	3.2.1.21	CAZy/Bacteria CAZy/Fungi UniProt/ Fungi
	Beta-1,4-endoglucanase	3.2.1.4	CAZy/Bacteria UniProt/ Fungi CAZy/Plants
	Cellobiose phosphorylase	3.4.1.20	CAZy/Bacteria
	Cellulose 1,4-beta-cellobiosidase	3.2.1.91	
Hemi cellulose	Enzymes involved in the degradation of the backbone		
	Xylan 1,4-beta-xylosidase	3.2.1.37	CAZy/Bacteria UniProt/ Fungi
	Endo-1,4-beta-xylanase	3.2.1.8	CAZy/Bacteria CAZy/Fungi
	Xyloglucan exo-beta-1,4-glucanase	3.2.1.155	CAZy/Bacteria
	Accessory, side-group-removing enzymes		
	Alpha-L-arabinofuranosidase	3.2.1.55	CAZy/Bacteria CAZy/Fungi UniProt/ Fungi
	Feruloyl esterase	3.1.1.73	CAZy/Bacteria UniProt/ Fungi
	Xylan alpha-1,2- glucuronosidase	3.2.1.131	CAZy/Bacteria
	Alpha-galactosidase	3.2.1.22	
	Acetylxylan esterase	3.1.1.72	
	Alpha-fucosidase	3.2.1.51	

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DISCUSIÓN GENERAL

En los últimos años ha habido un incremento en la apreciación de los alimentos tradicionales fermentados, las principales razones incluyen su mejora en el valor nutricional y por lo tanto su potencial uso para combatir la desnutrición, su uso para el tratamiento de distintas enfermedades gastrointestinales y por los diferentes efectos benéficos a la salud que se les han atribuido, tanto por la presencia de bacterias probióticas como por la de moléculas bioactivas producto del metabolismo microbiano (Tamang *et al*. 2016; Angmo *et al*. 2016; Rezac *et al*. 2018; Şanlier *et al*. 2019). Actualmente, los estudios enfocados en los alimentos fermentados han tomado relevancia, evidenciando la gran diversidad que existe en el mundo y el poco conocimiento que se tiene sobre algunos de ellos.

Pocos son los alimentos que se han investigado a fondo y en su mayoría los estudios se han centrado en la descripción de los microorganismos que se desarrollan durante la fermentación, dejando de lado el papel que estos tienen desde el punto de vista ecológico y funcional del alimento.

Para entender cómo el crecimiento y los cambios de los microorganismos durante el proceso de fermentación pueden dar como resultado variaciones en la composición química, en las propiedades organolépticas, en la producción de metabolitos y en la mejora de las propiedades nutrimentales, es necesario su estudio integral, lo que se ha logrado combinando la microbiología clásica con las diferentes herramientas ómicas.

Para el estudio de la fermentación del pozol, se emplearon distintos enfoques que permitieron determinar la composición de la microbiota, sus cambios en las distintas etapas de la fermentación, las fuentes de carbono y nitrógeno que son utilizadas por los microorganismos para su crecimiento, los posibles cambios que se generan por su metabolismo y así poder hacer una reconstrucción parcial o total del mapa metabólico del sistema.

- Composición y cambios de la microbiota en la fermentación del pozol

En los alimentos fermentados tradicionales la presencia de los diferentes microorganismos está determinada por el tipo de sustrato utilizado durante su elaboración y por su inoculación en el proceso, resultando en una microbiota que es característica de cada alimento y que deber ser capaz de adaptarse a las condiciones que se generan a lo largo de la fermentación, por lo que durante el proceso se dan cambios dinámicos de la población microbiana. En algunos alimentos, alteraciones menores de la diversidad o el número de especies pueden dar como resultado

productos alimenticios significativamente diferentes y variaciones en la calidad y características de este (Labib *et al*. 2018). Por lo tanto, resulta importante establecer la composición y los cambios de los microorganismos durante la fermentación y determinar su papel funcional.

Para el pozol, diversos estudios microbiológicos han demostrado los cambios y la distribución espacial de los principales grupos microbianos durante la fermentación y se han aislado e identificado un gran número de microorganismos (Nuraida *et al*. 1995; Ampe, Ben Omar y Guyot, 1999; Ampe *et al*. 1999; Wacher *et al*. 2000; Ben Omar y Ampe, 2000; Escalante *et al*. 2001; Díaz-Ruiz *et al*. 2003; Sainz *et al*. 2005; Wacher-Rodarte *et al*. 2015; Olvera *et al*. 2017; López-Hernández *et al*. 2018; Cooper-Bribiesca *et al*. 2019; Rizo *et al*. 2020; Domínguez-Ramírez *et al*. 2020). Sin embargo, los pocos estudios que han determinado la dinámica de la comunidad microbiana durante la fermentación se enfocaron en las bacterias, principalmente en las lácticas.

Mediante el estudio metaproteómico se encontró que este alimento fermentado es un ecosistema complejo, donde no solo encontramos proteínas del sustrato, sino también proteínas de bacterias, hongos y arqueas. Evidentemente, las proteínas identificadas en mayor proporción fueron del sustrato, 69.9% del metaproteoma corresponde a maíz, con un importante número de proteínas de bacterias y hongos, las cuales representaron el 28.5% del metaproteoma y un menor porcentaje (1.6%) asociado a arqueas.

El estudio por tiempo de fermentación mostró qué en la masa no fermentada hay proteínas de bacterias, hongos y levaduras relacionadas con el ambiente, lo que sugiere que la nixtamalización no es un proceso esterilizante. Sin embargo, muchas de estas especies se reducen o eliminan durante la fermentación, indicando que algunas no sobreviven a los cambios que se generan o el sustrato no cuenta con las condiciones necesarias para su desarrollo.

A medida que la masa se fermenta, se establece un núcleo de microorganismos, compuesto principalmente por bacterias ácido lácticas (BAL), de las cuales se identificaron proteínas en alta abundancia durante todo el proceso de fermentación. La mejora de las características como el sabor, aroma, textura, vida útil y valor nutricional en los alimentos fermentados se ha atribuido a sus actividades metabólicas. Estas producen diferentes ácidos orgánicos que imparten sabores característicos a los alimentos y provocan una disminución de pH, lo que restringe el crecimiento y la supervivencia de microorganismos patógenos (Blandino *et al*. 2003; Oyewole, 1997; Nout y Rombouts, 1992). Durante la fermentación del pozol, la drástica disminución de pH correlacionó con el aumento en la concentración de BAL y con el número de proteínas identificadas para este grupo.

Dentro de las BAL el género predominante fue *Streptococcus*, que al final de la fermentación representó el 56% del metaproteoma. Estudios previos demostraron que *Streptococcus infantarius* subsp. i*nfantarius* tiene características únicas, como una alta velocidad específica de crecimiento, una conversión eficiente de sustrato a biomasa, tiene actividad amilolítica, es capaz de crecer en arabinoxilano de maíz y sobrevive en condiciones extremas de pH (ácidos y alcalinas), lo que le da ventajas competitivas sobre otras bacterias y explica su abundancia durante la fermentación (Díaz-Ruíz *et al*. 2003; Cooper-Bribiesca *et al*. 2018; Domínguez-Ramírez *et al*. 2020).

Otras proteínas identificadas de BAL corresponden a los géneros *Leuconostoc*, *Lactobacillus* y *Enterococcus,* géneros previamente descritos en el pozol (Wacher, 1995; Nuraida *et al*. 1995; Ampe *et al.* 1999; Ampe *et al*. 1999; Ben Omar y Ampe, 2002; Escalante *et al*. 2001; Díaz-Ruiz *et al.* 2003). Su presencia en la fermentación está determinada por su capacidad para degradar algunos de los polisacáridos presentes en la masa. La baja abundancia de *Enterococcus* en comparación con los géneros *Lactobacillus* y *Leuconostoc*, podría estar relacionada con su baja actividad amilolítica (Díaz, 2003) y a que algunas especies de *Lactobacillus* y *Leuconostoc* pueden utilizar almidón y xilano como única fuente de carbono para crecer (Nuraida *et al*. 2001; Flores, 1995; Flores, 2007).

Otros cambios importantes en la composición microbiana corresponden al grupo de las enterobacterias. Tanto la cuenta en placa como el análisis metaproteómico, mostraron que están presentes desde el inicio de la fermentación y que a medida que la masa se va acidificando, su concentración disminuye considerablemente, pero no desaparecen. Diferentes estudios han reportado la presencia de enterobacterias en diferentes etapas del proceso de fermentación (Wacher, 1995; Ampe *et al*. 1999; Ampe *et al*. 1999; Wacher *et al*. 2000; Morales, 2011), su caracterización ha permitido identificar varias especies, *Escherichia coli*, *Escherichia fergusonii*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Enterobacter sakazakii*, *Enterobacter* spp., *Klebsiella pneumoniae*, *Klebsiella* sp., *Klebsiella variicola* y *Kosakonia* sp. (Ulloa *et al*. 1987; Sainz, 1998; Sainz *et al*. 2001; Rizo *et al*. 2020).

Su presencia a pesar de la alta concentración de ácidos orgánicos puede deberse a la generación de microambientes dentro de la masa, a la tolerancia de algunas cepas o porque otros microorganismos utilizan los ácidos orgánicos para su crecimiento, disminuyendo localmente su concentración (Ampe et al. 1999; Ben Omar y Ampe, 2000; Giles, 2012). El estudio de la resistencia ácida de diferentes cepas de *E. coli* mostró un cambio en los perfiles de las proteínas de la membrana externa (Omps), particularmente la proteína OmpX se expresó cuando las cepas fueron

sometidas a condiciones de estrés en presencia de ácidos orgánicos, por lo que esta proteína puede estar involucrada en el mantenimiento de la homeostasis en condiciones de estrés (Sainz *et al*. 2005).

La presencia de estas bacterias se considera usualmente un indicador de higiene deficiente, sin embargo, en este trabajo demostramos su importancia en el enriquecimiento proteico del alimento vía la fijación de nitrógeno.

Por otro lado, a lo largo de toda la fermentación se encontraron proteínas de hongos, representadas por levaduras durante todo el proceso y con un incremento en el número de proteínas identificadas para hongos filamentosos en la muestra fermentada por 48 h.

Las proteínas de los hongos filamentosos asociados a suelo y plantas como *Ajellomyces*, *Cercospora* y *Fusarium* solo se identificaron en la masa no fermentada. En las muestras fermentadas se encontró que los géneros *Schizosaccharomyces*, *Saccharomyces* y *Neurospora* fueron los predominantes. El desarrollo de este grupo coincidió con la disminución de pH asociada a la producción de ácidos orgánicos. Se ha sugerido que la proliferación de hongos en los alimentos es estimulada por las BAL y a su vez los hongos favorecen el crecimiento de otras bacterias, aportando factores de crecimiento como vitaminas y compuestos nitrogenados solubles (Nout, 1991).

Estos géneros han sido reportados en otros alimentos fermentados a base de harina de trigo o arroz, de granos de trigo o cebada, en bebidas alcohólicas tradicionales y en productos fermentados de leche (Kanwar *et al.* 2007; Lv *et al*. 2013; Watanabe *et al*. 2008). Si bien los hongos en los alimentos fermentados pueden considerarse dañinos por la producción de enterotoxinas, estos pueden tener un papel relevante durante el proceso, debido a la producción de enzimas para la degradación de polisacáridos complejos y factores antinutricionales. Además, el metabolismo de estos organismos puede resultar en una mejora en las características organolépticas, mediante la producción de sabores característicos por la degradación de ácidos grasos y en un aumento en el valor nutricional del alimento, por la producción de vitaminas, aminoácidos y ácido fólico (Venkatasubbaiah *et al. 1984*; Sunesen y Stahnke, 2003; Venkatasubbaiah *et al.* 1985; Kanekar y Joshi, 1993; Chen *et al. 2014*).

A pesar de que la fermentación del pozol se lleva a cabo por una microbiota natural que puede proceder de diferentes fuentes como los utensilios usados en la preparación, las manos de los productores, el suelo, aire y el agua, se observa que en términos generales el comportamiento de los grupos microbianos es el mismo. Lo anterior implican que, lo que determina la microbiota del

pozol es la composición química del alimento y los factores involucrados en dicho proceso (Ulloa, 1974).

- Metabolismo de carbohidratos

El pozol se caracteriza por tener una baja concentración de carbohidratos solubles, un alto contenido de almidón y presencia de celulosa y hemicelulosa del pericarpio, por lo que estos polisacáridos representan las principales fuentes de carbono, que en conjunto deben sostener una abundante y compleja microbiota.

En los datos metaproteómicos se identificaron diferentes enzimas implicadas en la hidrólisis de almidón, celulosa y hemicelulosa, fuentes de carbono presentes en la masa nixtamalizada. Para el almidón, se identificaron dos proteínas en la base de datos de plantas, una β-amilasa que produce maltosa y una α-glucosidasa que produce glucosa a partir de almidón y maltosa. Estas enzimas podrían jugar un papel importante, principalmente en la primera etapa de fermentación, liberando azúcares simples que pueden ser utilizados para el crecimiento de microorganismos que se encuentran en la materia prima. En bacterias, se identificaron múltiples enzimas amilolíticas, α-amilasas, α-glucosidasas y enzimas desramificantes (pululanasas), juntas pueden despolimerizar al almidón en oligosacáridos y azúcares más pequeños (glucosa y maltosa). Trabajos previos han reportado la presencia de bacterias lácticas capaces de crecer e hidrolizar almidón en distintas etapas de la fermentación (Nuraida *et al.* 1995; Ampe *et al*. 2000; Díaz-Ruiz *et al*. 2003). La caracterización de algunas bacterias resultó en la identificación de *Streptococcus macedonicus*, *Lactococcus lactis*, *Enterococcus sulfureus* y *Streptococcus infantarius* subsp. i*nfantarius*, siendo esta última la especie predominante durante la fermentación. Se sabe que la actividad de estas bacterias está asociada a la célula y que sus amilasas son de alto peso molecular (Díaz, 2003). Específicamente, *Streptococcus infantarius* subsp. *infantarius* produce dos amilasas, una amilopululanasa con capacidad desramificante y una α-amilasa (Díaz-Ruiz *et al*. 2003; Rodríguez, 2018). Con relación a los hongos y levaduras, los estudios han resultado variables en cuanto a si este grupo es capaz de degradar el almidón.

Nuraida y colaboradores (1995) reportaron que el 50% de las levaduras aisladas son capaces de hidrolizar almidón tanto en medio sólido como líquido. También se ha demostrado que los hongos *Phoma glomerata* y *Cladosporium* sp. tienen actividad amilolítica, alcanzado la máxima actividad a las 72 h, sin embargo, los valores obtenidos muestran que su capacidad amilolítica es baja en comparación con el grupo de las bacterias (Rivera, 2001). Por otro lado, Díaz-Ruiz (2003) encontró

que, aunque hay crecimiento de hongos y levaduras en un medio con almidón como única fuente de carbono, no fue posible cuantificarlos como amilolíticos ya que no hubo formación de halo de hidrólisis que indique la degradación de almidón, resultados que coindice con lo reportado por Ampe *et al*. (1999). En el metaproteoma no se identificaron enzimas amilolíticas de hongos y levaduras.

La hidrólisis de celulosa requiere tres clases de enzimas, β-1,4-endoglucanasas (EGL), exoglucanasas/celobiohidrolasas (CBH) y β-glucosidasa (BGL). Las endoglucanasas producen por cortes al azar una disminución en su grado de polimerización, liberando glucosa, celobiosa y celotriosa. Las BGL pueden hidrolizar la celobiosa previamente liberada para forma glucosa (Bajpai, 1996). En este trabajo se identificaron endoglucanasas de bacterias y plantas, lo que permite la hidrólisis inicial de la celulosa (Houfani *et al*. 2020). Además, se identificaron enzimas para la producción de celobiosa, glucosa y celo-oligómeros a partir de este sustrato. De manera inexplicable no se identificaron enzimas celulolíticas de hongos

Por otro lado, la degradación de la hemicelulosa imponer varios retos, ya que es un polímero de alto peso molecular y es muy variable en su estructura, por lo que su degradación requiere la acción de varias enzimas que deben actuar de forma sinérgica para una eficiente degradación del polímero. Los componentes de la cadena principal pueden ser xilano, galactomanano o xiloglucano, los cuales son degradados por enzimas específicas: la β-1,4-endoxilanasa y β-1,4-xilosidasa hidrolizan el xilano; β-1,4-endoglucanasa y β-1,4-glucosidasa para xiloglucano y; β-1,4-endomananasa y β-1,4-manosidasa para (galacto-) manano (van den Brink y de Vries, 2011). Además, la completa degradación de la hemicelulosa requiere la presencia de enzimas que actúen sobre los distintos tipos de decoraciones, para finalmente liberar azucares simples y xilooligosacáridos. En los datos metaproteómicos se identificaron proteínas de bacterias y hongos que actúan sobre el esqueleto principal de la cadena y las diferentes sustituciones, lo que permite la completa hidrólisis de este polisacárido y la liberación de xilosa, xilobiosa y xilooligosacáridos.

La evaluación de la capacidad de degradar xilano por bacterias previamente aisladas del pozol demostró que, de las 93 cepas probadas, el 60% puede hidrolizar este carbohidrato, es decir, se observó la presencia de un halo de hidrólisis en la placa cuando fue revelada con rojo congo. La posterior identificación de estas bacterias resultó en: *Leuconostoc mesenteroides*, *Leuconostoc citreum*, *Lactobacillus plantarum*, *L. pentosus*, *L. coprophilus*, *L. fermentum*, *L. acidophilus*, *L. curvatus*, *L. brevis*, *L. lactis* y *Lactococcus raffinolactis* (Flores, 1995; Flores, 2007). Recientemente, se demostró que *Streptococcus infantarius* subsp. i*nfantarius* es capaz de degradar xilano de maíz

o madera y que varias cepas de *Weissella* solo pueden utilizar xilooligosacáridos como fuente de carbono para su crecimiento (Cooper-Bribiesca *et al*. 2018; López-Hernández *et al*. 2018), lo que indica que el crecimiento de *Weissella* en la fermentación depende de la capacidad de otros microoganismos para degradar la hemicelulosa. Además, se debe considerar que muchas de las proteínas identificadas para la degradación de los polisacáridos no pertenecen al grupo de las bacterias lácticas, por lo que es necesario enfocar los estudios a la descripción e identificación de otras bacterias, que podrían jugar un papel importante en la liberación de azucares simples para el resto de la microbiota no amilolítica. En el grupo de trabajo se han aislado bacterias y hongos utilizando medios con almidón, hemicelulosa y xilosa como única fuente de carbono en distintas etapas de la fermentación. Los resultados demostraron que tanto las bacterias como los hongos son capaces de crecer y degradar estos polisacáridos (Martínez, 2019). Actualmente se tiene un banco de microorganismos positivos para estas actividades y se está trabajando en su caracterización e identificación.

En correlación con los resultados anteriores, el análisis químico mostró una disminución importante en el contenido de fibra, carbohidratos que se puede asociar a las actividades enzimáticas de las proteínas identificadas en el estudio metaproteómico. Con estos datos se puede sugerir que durante las primeras 9 h de fermentación la microbiota consume además del almidón, polisacáridos como la celulosa y hemicelulosa, lo que libera azucares solubles, que de hecho aumentaron significativamente. Después de este periodo se observó una disminución importante de estos carbohidratos, lo que implica su utilización y explicaría el aumento en las cuentas de microorganismos.

Finalmente, el análisis de las proteínas sugiere un posible sinergismo entre bacterias, hongos y maíz para la degradación de los diferentes sustratos.

- Metabolismo de nitrógeno

El nitrógeno junto con el carbono, oxígeno e hidrógeno es un elemento esencial para el crecimiento de los microorganismos. Forma parte de moléculas necesarias para la actividad biológica como proteínas, ácidos nucleicos, coenzimas y fosfolípidos. En este trabajo se demostró que, en la fermentación del pozol los microorganismos pueden utilizar nitrógeno orgánico e inorgánico para la síntesis de aminoácidos (Figura 1).

Por un lado, la presencia de bacterias y hogos proteolíticos en el sistema (Loaeza, 1991; Cruz, en proceso) y la identificación de diferentes proteasas, aminopeptidasas y carboxipeptidasas en el

metaproteoma, permite establecer que una de las vías utilizada por los microorganismos para la obtención de nitrógeno es la degradación de proteínas. Este proceso depende tanto de la actividad de los microorganismos como de las enzimas del maíz, que en conjunto hidrolizan las proteínas del sistema para la generación de péptidos y oligopéptidos de diferentes tamaños. Posteriormente, los péptidos generados pueden ser degradados completamente para la liberación de aminoácidos que pueden ser utilizados tanto por bacterias como hongos para su crecimiento (Figura 1).

Por otro lado, se demostró que la fijación de nitrógeno permite la asimilación de nitrógeno atmosférico a amoníaco. En este proceso, el amoníaco se ioniza rápidamente a amonio para su posterior incorporación a glutamato a través de la ruta glutamina sintetasa-glutamato sintasa (Figura 1).

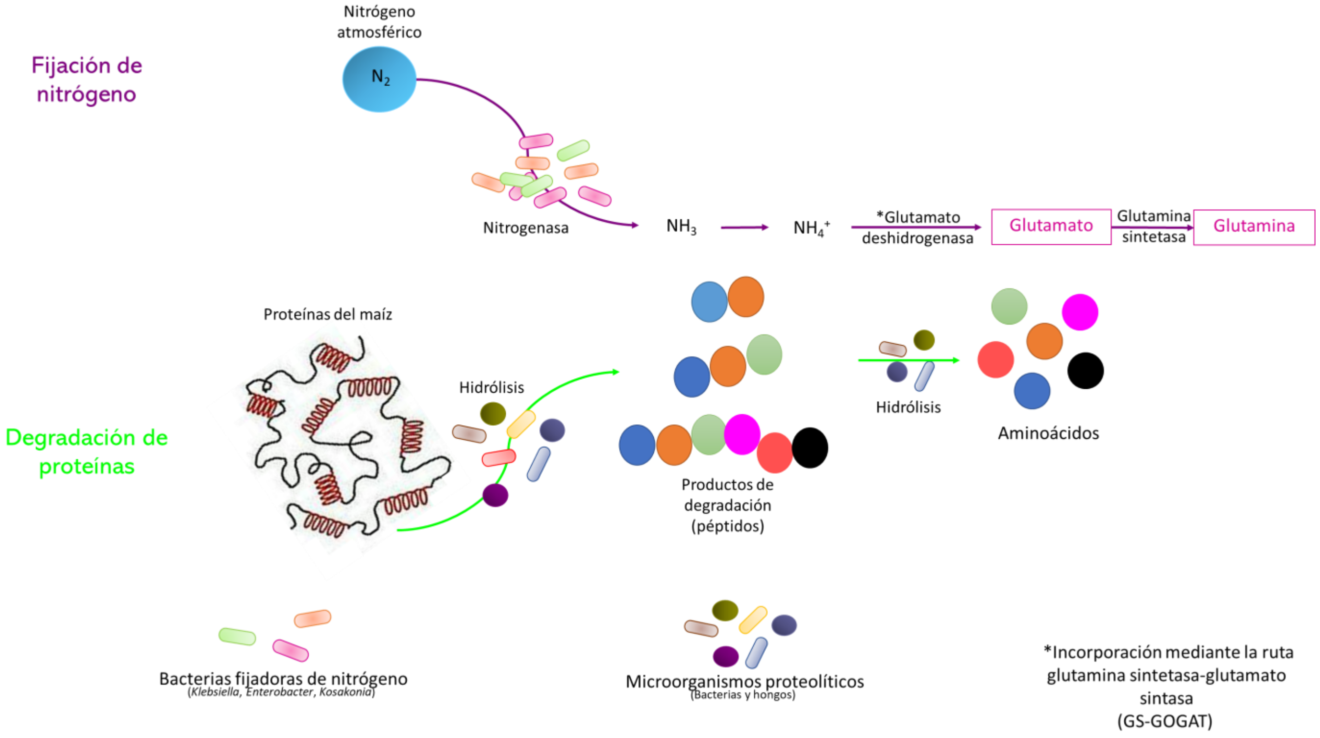

Figura 1. Esquema del metabolismo de nitrógeno. Se representa el metabolismo de nitrógeno inorgánico que corresponde al proceso de fijación de nitrógeno y su posterior asimilación para la síntesis de aminoácidos. El metabolismo de nitrógeno orgánico corresponde a la degradación de las proteínas del sustrato para producir aminoácidos.

Para demostrar que la fijación de nitrógeno es un proceso que ocurre en el pozol, se realizaron diferentes estrategias. Primero se determinó la composición química de la masa de maíz nixtamalizada en las distintas etapas de la fermentación. Los datos mostraron que hay un aumento significativo en el contenido de nitrógeno y proteína, comportamiento que no ha sido reportado en otros alimentos fermentados y que puede estar relacionado con el fenómeno de fijación. Sin embargo, se debe tomar en cuenta que las determinaciones se hacen en función del peso, por lo que es necesario descartar que el incremento observado se deba a pérdidas de materia orgánica, principalmente por metabolismo aerobio. Para ello se consideró que los microorganismos emplean en mínima proporción residuos inorgánicos, por lo que se determinó la relación nitrógeno/ceniza. Además, se hizo un balance de materia relacionando la concentración de nitrógeno con la pérdida de carbono en el sistema y así poder calcular el nitrógeno neto fijado. Con ambas aproximaciones se demostró que en todos los tiempos analizados hay un aumento significativo en el contenido de nitrógeno respecto a la muestra sin fermentar.

Estudios previos reportaron incrementos mayores en el contenido de nitrógeno (21-68%) a los que observamos en este trabajo (Cravioto *et al*. 1955; Ulloa *et al*. 1971; Ramírez, 1987; Giles, 1995; Rizo, 2015), sin embargo, se debe considerar que en algunos de estos trabajos no se reportan duplicados biológicos o los respectivos análisis estadísticos, por lo que los incrementos observados pueden deberse a errores en las técnicas utilizadas. Además, estos aumentos se dan en etapas tardías de la fermentación, a partir de las 120 h, mientras que aquí se encontró en las primeras 9 h, lo que podría explicar las diferencias en el nitrógeno fijado.

Como segunda estrategia se decidió evaluar la actividad nitrogenasa con la prueba de reducción de acetileno a etileno (ARA por sus singlas en inglés) directamente sobre la masa de maíz; para promover el crecimiento y actividad de las bacterias fijadoras de nitrógeno, se inocularon directamente las muestras de pozol en dos medios definidos carentes de nitrógeno. Las bacterias aisladas fueron resembradas para comprobar la fijación e identificadas por secuenciación de los genes *nifH* y el gen ribosomal 16S, todas resultaron enterobacterias pertenecientes a los géneros *Enterobacter*, *Klebsiella* y *Koskonia*.

Otra estrategia consistió en colocar la masa de pozol en agar suave y, finalmente las distintas muestras de pozol se inocularon con el aislado *Klebsiella variicola* (aislado 21) y se colocaron en agar suave. En todas las condiciones se observó actividad nitrogenasa positiva. Los resultados demuestran que en el pozol existen las condiciones necesarias para que se dé la fijación de nitrógeno, fenómeno que tiene implicaciones importantes no solo desde el punto de vista

microbiológico sino también desde el punto de vista nutricional, ya que es un método viable, económico y simple para mejor el contenido de proteína en alimentos fermentados a base de cereales.

- Mapa metabólico y análisis funcional de la microbiota

El proceso de fermentación de los alimentos varía en función de la materia prima que se utiliza para su elaboración, de las condiciones que se generan durante el proceso y del tipo de microorganismos que son capaces de sobrevivir y desarrollarse. Así, cada alimento fermentado posee características organolépticas y nutricionales únicas, determinadas por una mezcla de compuestos que son el resultado del metabolismo microbiano de carbohidratos, proteínas, aminoácidos, lípidos y ácidos grasos. Para elucidar el impacto de la microbiota en la fermentación del pozol, se realizó el análisis de los datos metaproteómicos en la base de datos KEGG, para la reconstrucción de las vías metabólicas e identificar las relacionadas con la mejora del sustrato.

Como era de esperarse, se encontraron las rutas implicadas en el metabolismo central de carbono para la obtención de energía, para la producción de intermediarios requeridos en diferentes rutas biosintéticas (síntesis de aminoácidos, ácidos nucleicos, lípidos, vitaminas y cofactores) y para la síntesis de pared celular que permiten el crecimiento de los microorganismos. Además, se encontraron proteínas involucradas en el procesamiento de información genética para el mantenimiento de funciones básicas importantes en la vida, incluida la replicación y reparación del ADN, la transcripción y la traducción. Su abundancia puede estar relacionada con los altos niveles de expresión y con las condiciones drásticas que prevalecen y que pueden aumentar el daño del ADN y la degradación proteínas, por lo que estas proteínas podrían ayudar a aliviar el estrés inducido por el pH ácido (Lin, *et al*. 2016).

Dentro del metabolismo central de carbono, se encontraron proteínas implicadas en el metabolismo de piruvato para la síntesis de lactato, acetato y etanol, lo que implica que en la fermentación se desarrollan especies de BAL homo y heterofermentativas. Un estudio previo utilizando la técnica de electroforesis en gel con gradiente desnaturalizante (DGGE por sus siglas en inglés) mostró que la fermentación se puede dividir en dos etapas; en la etapa inicial se presentan bacterias homofermentativas como *L. plantarum*, *L. casei* y *L. delbrueckii*, en la segunda etapa (24 a 48 h) se desarrollan BAL heterofermentativas, que incluyen a *L. fermentum* y especies de *Leuconostoc* y *Weissella*. La actividad de estas bacterias da como resultado la producción de lactato como principal producto de fermentación, acetato y etanol en menor concentración (Ampe

et al. 1999). Aunque en pozol no se ha estudiado la producción de acetoína y butanodiol, en el análisis metaproteómico se identificaron las proteínas involucradas en su síntesis. Se sabe que, en otros alimentos fermentados, la síntesis de estos dos compuestos está asociada principalmente al metabolismo de las BAL y, aunque no representan un producto principal de la fermentación, son importantes para la mejorar de las características organolépticas (Güzel-Seydim *et al*. 1999; Jung *et al*. 2011; Wu *et al.* 2015).

Por último, se ha reportado que en el pozol hay un incremento de aminoácidos esenciales y vitaminas (Cravioto *et al*. 1995), lo que contribuye de manera importante en el aumento del valor nutricional de este alimento. El análisis de los datos metaproteómicos permitió establecer que los diferentes aminoácidos se pueden producir mediante tres mecanismos: 1) a través de precursores de otras vías metabólicas, como el glutamato y aspartato; 2) por la acción de diferentes proteasas para la degradación y el reciclaje de proteínas, como la prolina y; 3) por su metabolismo, como la asparagina y glutamina (Figura 2).

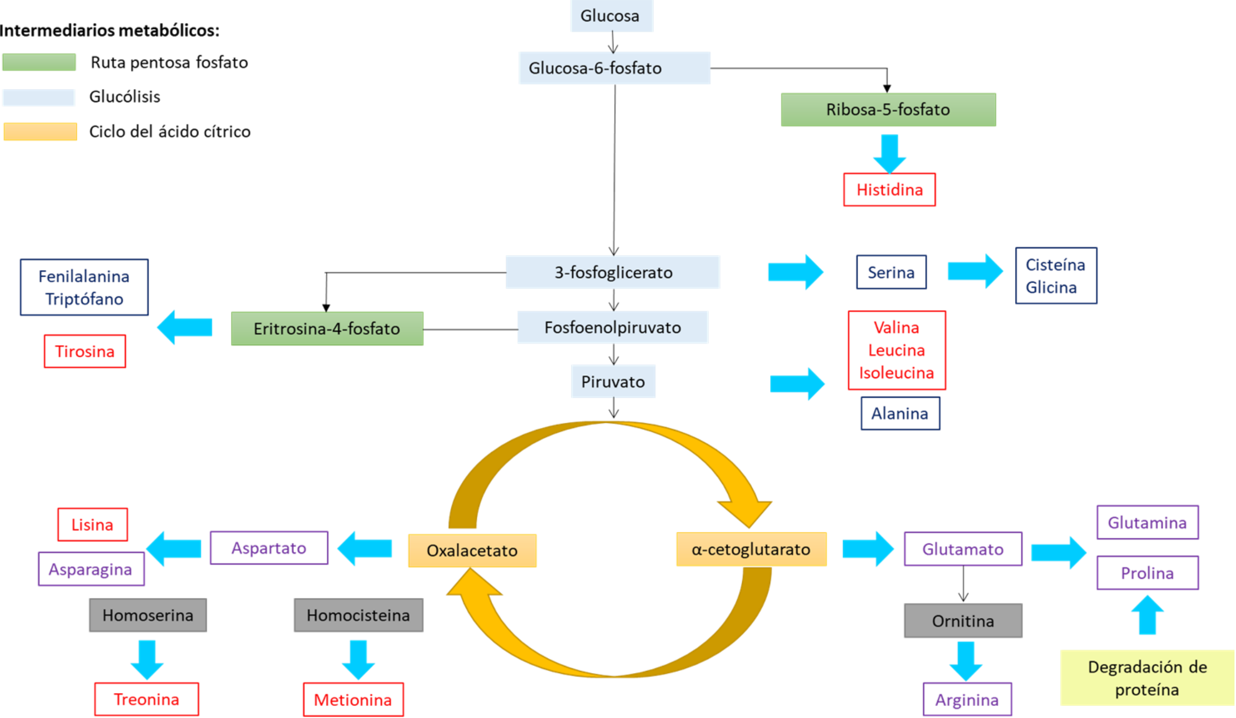

Figura 2. Descripción general de la biosíntesis de aminoácidos. Se muestran la vía metabólica y sus respectivos intermediarios para la biosíntesis de aminoácidos. En el cuadro violeta se muestran los aminoácidos que se pueden producir en la fermentación del pozol. En el cuadro rojo se muestran los aminoácidos para los que se identificaron algunas de las proteínas involucradas en su síntesis. En el cuadro azul se muestran los aminoácidos para los que no se identificaron las enzimas para su biosíntesis.

Respecto al metabolismo de vitaminas se encontraron varias proteínas para la biosíntesis de biotina, tiamina, riboflavina, entre otras, sin embargo, en ninguno de los casos se logró completar las respectivas vías.

Los resultados demuestran el potencial metabólico de la microbiota para la producción de una amplia gama de compuestos importantes para la seguridad alimentaria y atributos sensoriales como ácidos orgánicos, y la presencia de enzimas que pueden tener aplicaciones biotecnológicas importantes como las amilasas, xilanasas, proteasas, entre otras.

- Limitantes de los estudios metaproteómicos

A pesar de que la metaproteómica es una herramienta poderosa para el estudio de las comunidades microbianas en sistemas complejos (suelo, agua, alimentos fermentado, cuerpo humano, etc.), es importante tomar en cuenta que estos estudios a menudo se ven sesgados hacia la identificación de las proteínas más abundantes y de los microorganismos predominantes (Mueller y Pan, 2013). Además, en la identificación de proteínas por espectrometría de masas, un paso fundamental es la correcta asignación de los espectros a las secuencias de péptidos, la cual está determinada por la calidad de los espectros obtenidos, por la presencia de péptidos homólogos y por la posibilidad de que los péptidos puedan ser ionizados eficientemente, condición que depende de las características fisicoquímicas del péptido y que en ocasiones causa fragmentación atípica (Nesvizhskii *et al.* 2007; Nesvizhskii *et al*. 2006). Otra consideración importante es que la interpretación taxonómica y funcional de los resultados puede ser ambigua, debido a que péptidos idénticos que pertenecen a proteínas homólogas provocan una identificación de proteínas redundante (Herbst *et al.* 2016).

Por otro lado, el análisis de los datos metaproteómicos requiere el desarrollo de algoritmos más eficientes y equipo computacional, que permita hacer el análisis de un gran número de datos cuando se requiere hacer la búsqueda en bases de datos de referencias completas, que puede tener más de siete millones de entradas y provocar fallas debido a las limitaciones del software o hardware (Heyer *et al*. 2017). Por último, se debe tomar en cuenta que no es posible la identificación de todas las proteínas, ya que en algunas bases de datos faltan entradas que corresponde a organismos que aún no han sido secuenciados, como consecuencia de esto en ocasiones los estudios metaproteómicos son insuficientes para la descripción taxonómica y funcional completa de las comunidades microbianas.

CONCLUSIONES

El análisis integral microbiológico, bioquímico y metaproteómico del pozol permitió la construcción de una imagen completa del sistema de fermentación. Se encontró que en el proceso de fermentación intervienen bacterias, hongos, levaduras y arqueas, además de los sistemas enzimáticos del propio sustrato, el maíz. El grupo de las bacterias es el mayormente representado a lo largo del proceso, mientras que las proteínas de hongos filamentosos y levaduras se encontraron en menor abundancia.

Los principales cambios tanto en el sustrato como en la microbiota se dan en las primeras 9 horas de fermentación y se correlaciona con el aumento logarítmico de los microorganismos, con la caída del pH, con la disminución del contenido de carbohidratos, lípidos y nitrógeno, lo que muestra esta etapa como la de mayor actividad metabólica. Se encontraron por primera vez muchos microorganismos ambientales, relacionados con el maíz y con el agua, sin embargo, la mayor parte de ellos desaparecen o disminuyen drásticamente, por lo que se puede considerar que su presencia no determina las características del alimento.

Las bacterias se encuentran representadas en su mayoría por bacterias lácticas y dentro de estas el género *Streptococcus* es de manera contundente el más abundante, todas estas bacterias pueden encontrarse en el medio ambiente pero su hábitat más importante son los mamíferos, lo que indica que si bien se trata de una fermentación espontanea, la participación humana a nivel del proceso de nixtamalización y de inoculación es determinante en la microbiota que se desarrolla. Con relación a las levaduras, se observó que están presentes en todas las muestras analizadas, con un incremento de hongos filamentosos al final de la fermentación.

El enfoque metaproteómico permitió encontrar diversos complejos enzimáticos desconocidos en el sistema hasta el momento. Se encontraron también los sistemas para la hidrólisis del almidón, la hemicelulosa y la celulosa por lo que todos estos sustratos pueden ser utilizados como fuente de carbono por la microbiota. Resulta interesante que en la degradación del almidón no se encontraron sistemas enzimáticos de hongos y sean las enzimas propias del maíz y de las bacterias las que se encarguen de la hidrólisis del sustrato. En el caso de hemicelulosa y celulosa intervienen tanto bacterias como hongos, con predominancia siempre de las enzimas bacterianas. Este diverso repertorio de enzimas encontradas permitirá nuevos estudios bioquímicos y estructurales.

El metabolismo encontrado permite la síntesis de diversos productos de fermentación como ácidos orgánicos, acetoina, butanediol e intermediarios importantes involucrados en la síntesis de ácidos grasos y aminoácidos. Compuestos que contribuyen con las características organolépticas y nutricionales del pozol. Otra característica distintiva e importante de esta fermentación fue la presencia de enterobacterias diazótrofas capaces de fijar nitrógeno atmosférico en la masa. La fijación biológica de nitrógeno permite el incremento del valor nutricional del pozol, lo que demuestra que la fermentación del pozol puede transformar un alimento relativamente pobre, en un producto más rico, lo que abre la posibilidad de estudiar este proceso como un método viable para ser utilizado en otros alimentos bajos en contenido proteínico.

PROSPECTIVAS

- Análisis de las bacterias fijadoras de nitrógeno para descartar patogenicidad.

- Análisis del proteoma de las bacterias fijadoras de nitrógeno identificadas para la búsqueda de las enzimas implicadas en el proceso de fijación de nitrógeno atmosférico.

- Análisis del perfil de aminoácidos en la masa del pozol fermentada a distintos tiempos.

- Análisis proteómico de las bacterias y hongos más abundantes para determinar su potencial metabólico.

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