COLEGIO DE POSTGRADUADOS

INSTITUCIÓN DE ENSEÑANZA E INVESTIGACIÓN EN CIENCIAS AGRÍCOLAS

CAMPUS MONTECILLO

POSTGRADO EN RECURSOS GENÉTICOS Y PRODUCTIVIDAD

GANADERÍA

DIETAS LÍQUIDAS FERMENTADAS COMO ESTRATEGIAS NUTRICIONALES EN CERDOS

NICOLÁS SALVADOR ESPINOSA GARCÍA

T E S I S

PRESENTADA COMO REQUISITO PARCIAL
PARA OBTENER EL GRADO DE:

DOCTOR EN CIENCIAS

**MONTECILLO, TEXCOCO, ESTADO DE MÉXICO**

2021

La presente tesis titulada: **Dietas líquidas fermentadas como estrategias nutricionales en cerdos**, realizada por el alumno: **Nicolás Salvador Espinosa García**, bajo la dirección del Consejo Particular indicado, fue aprobada por el mismo y aceptada como requisito parcial para obtener el grado de:

DOCTOR EN CIENCIAS
RECURSOS GENETICOS Y PRODUCTIVIDAD
GANADERÍA

CONSEJO PARTICULAR
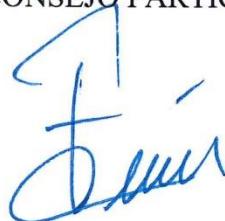
CONSEJERO   _____
DR. JOSÉ LUIS FIGUEROA VELASCO

ASESOR   _____
DR. ERNESTO FAVELA TORRES

ASESOR   _____
DR. JOSÉ ALFREDO MARTÍNEZ AISPURO

ASESORA   _____
DRA. MARÍA TERESA SÁNCHEZ-TORRES ESQUEDA

Montecillo, Texcoco, Estado de México, octubre de 2021.

DIETAS LÍQUIDAS FERMENTADAS COMO ESTREGIAS NUTRICIONALES EN CERDOS

Nicolás Salvador Espinosa García, D.C.
Colegio de Postgraduados, 2021

RESUMEN

Las dietas líquidas fermentadas (DLF) son comunes en la industria porcina Europea desde la prohibición de los antibióticos como promotores del crecimiento (APC) en 2006. Las DLF con bacterias ácido lácticas mejoran la microbiota intestinal e inhiben a los microrganismos patógenos. Este trabajo se dividió en por partes. En el primer estudio, el objetivo fue desarrollar DLF de alta calidad basadas en maíz, el principal cereal de México y del continente Americano para alimentación porcina, con inclusión de *Lactobacillus plantarum* para suplir los APC. En el segundo estudio, el objetivo fue desarrollar DLF de alta calidad basadas en maíz, con la combinación de *Lactobacillus plantarum, Pediococcus pentosaceus* y *Pediococcus acidilacticiti,* y la reducción de la concentración de proteína cruda (PC) en el sustrato. En el primer estudio, se empleó un diseño experimental completamente al azar en cuatro etapas con maíz fermentado como principal fuente de nutrientes. En la primera etapa se determinó el efecto de la relación de agua y alimento (2:1 y 3:1) y la adición de goma xantana (0 y 0.02%); en la segunda etapa se evaluaron tres niveles de proteína (10, 15 y 20%) de origen vegetal y animal; en la tercera etapa se evaluaron dos tiempos de cultivo (12 y 24 h), con adición de melaza (2 y 4%) e inclusión de ácido láctico (0 y 20 mM); finalmente, en la última etapa se evaluaron dos tiempos de cultivo (12 y 24 h), el origen de la proteína (vegetal y animal) y la inclusión de inóculo (240 y 480 µL). El segundo estudio se realizó con un diseño experimental completamente al azar en tres etapas con maíz fermentado como principal fuente de nutrientes. En la primera etapa se determinó el efecto de dos niveles de PC en el sustrato (18 y 20%), adición de inóculos de *Lactobacillus plantarum* (120 y 240 µL)*,* y *Pediococcus pentosaceus* (120 y 240 µL); en la segunda etapa se evaluó el efecto de dos niveles de PC en el sustrato (18 y 20%), adición de inóculos de *Lactobacillus plantarum* (120 y 240 µL)*,* y *Pediococcus acidilacticiti* (120 y 240 µL); finalmente, en la última etapa se determinó el efecto de dos niveles de PC en el sustrato (18 y 20%), adición de inóculos de *Pediococcus pentosaceus* (120 y 240 µL)*,* y *Pediococcus acidilacticiti* (120 y 240 µL). En todas las etapas de ambos estudios, las variables de respuesta fueron: pH, concentraciones de ácido láctico (CAL), acético y etanol. En el primer estudio, el análisis independiente de las variables demostró que la no adición de goma xantana, el incremento de proteína (en particular la de origen animal), la fermentación del cultivo por 24 h y la adición de melaza tienen un efecto positivo sobre la producción de ácido láctico y la disminución del pH. En el segundo estudio, el análisis independiente de las variables demostró que la reducción de los niveles de PC y la combinación de inóculos de BAL con fermentación del cultivo por 24 h tiene un efecto positivo sobre la disminución del pH y la producción de ácido láctico. Las condiciones de cultivo establecidas en las variables evaluadas desarrollaron una DLF de alta calidad con base en maíz para cerdos en el primer estudio. Las condiciones de cultivo desarrollaron una DLF de alta calidad baja en PC con base en maíz para el destete y las otras etapas de alimentación en cerdos en el segundo estudio.

Palabras clave: Ácido láctico, pH, proteína, maíz fermentado.

FERMENTED LIQUID SWINE FEED AS NUTRITONAL STRATEGY

Nicolás Salvador Espinosa García, D.C.
Colegio de Postgraduados, 2021

ABSTRACT

Fermented liquid feed (FLF) is commonly used in the European swine industry since 2006 because of the antibiotics´ ban as growth promoters (AGP). FLF with lactic acid bacteria improves the intestinal microbiota and inhibits pathogenic microorganisms. Two studies were conducted. For the first study, the objective was to develop a high quality FLF based on corn, the main cereal in Mexico and the American continent for swine feed industry. *Lactobacillus plantarum* was included in FLF with the potential to replace AGP. For the second study, the objective was to develop a high quality FLF based on corn, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Pediococcus acidilacticiti* were included with reduction of crude protein concentration. For the first study, a completely randomized experimental design was used in four phases with fermented corn as the main source of nutrients. In the first phase, the effect of the substratum:water ratio (1:2 and 1:3) and the addition of xanthan gum (0 and 0.02%) were determined; In the second phase, three levels of crude protein (10, 15 and 20%) of vegetable and animal origin were evaluated; In the third phase, two fermentation times (12 and 24 h) were evaluated, with addition of molasses (2 and 4%) and inclusion of lactic acid (0 and 20 mM); finally, in the last phase, two fermentation times (12 and 24 h), the origin of the protein (vegetable and animal) and the inclusion of inoculum (240 and 480 µL) were evaluated. For the second study, a completely randomized experimental design with factorial arrangement was used in three phases with fermented corn as the main source of nutrients. In the first phase, the effect of the crude protein concentration (18 and 20%), the inoculum addition of *Lactobacillus plantarum* (120 y 240 µL), and *Pediococcus pentosaceus* (120 and 240 µL) were determined; In the second phase, two levels of protein (18 and 20%), the inoculum addition of *Lactobacillus plantarum* (120 y 240 µL), and *Pediococcus acidilacticiti* (120 and 240 µL) were evaluated; In the third phase, two levels of protein (18 and 20%), the inoculum addition of *Pediococcus pentosaceus* (120 y 240 µL), and *Pediococcus acidilacticiti* (120 and 240 µL) were estimated. In all phases of both studies: pH, lactic acid, acetic acid and ethanol concentrations were measured. In the first study, the independent analysis of the variables showed that the no addition of xanthan gum, the protein increase (particularly; from animal origin), the fermentation time for 24 h and the addition of molasses have a positive effect on the production of lactic acid and the decrease in pH. In the second study, the independent analysis of the variables showed that the crude protein reduction, and the combination of inoculum levels, with the fermentation time for 24 h have a positive effect on the production of lactic acid and the reduction in pH. The fermentation conditions established in the evaluated variables developed a high quality FLF based on corn for pigs in the first study. A high quality FLF based on corn with crude protein reduction and combination of lactic acid bacteria was developed for pigs in the second study.

Keywords: Lactic acid, pH, protein, fermented corn.

AGRADECIMIENTOS

DEDICATORIA

Principalmente, al "Rey de Reyes y Señor de Señores".

No temas, por que yo estoy contigo; no desmayes, por que yo soy tu Dios que te esfuerzo; siempre te ayudaré, siempre te sustentaré, con la diestra de mi justicia. Isaías 41:10

A mis Queridos Padres, Elia y Nicolás, por el amor y apoyo que tienen conmigo.

A mis Hermanas, Bibiana, Norma, Nohemí y Miriam, por sus consejos, cariño, comprensión y ejemplo.

A mis Abuelos, Mercedes[†] y Heriberto[†], por enseñarme que la base del éxito, es el trabajo.

A mi novia Mary Ferrusquia, por todo tu cariño incondicional, te adoro.

A mis sobrinos, Aaron, Rodrigo, Isaias y Leo; mis sobrinas, Briana, Danae y Priscila; mis cuñados Enrique y Alejandro.

A mis amigos del Programa de postgrado en Ganadería del Colegio de Postgraduados. En especial para Ana Luisa Portillo Espinosa y Abigail Rojas Blancas.

A las personas que muestran interés en la investigación pecuaria aplicable.

Sinceramente, N. Salvador Espinosa García

CONTENIDO

LISTA DE CUADROS

LISTA DE FIGURAS

INTRODUCCIÓN GENERAL

Las dietas líquidas fermentadas son una tecnología de alimentación común en la industria porcina Europea, y se basan en los cereales trigo y cebada (Missoten *et al.,* 2010; Plumed-Ferrer y von Wright, 2011; Xu *et al.,* 2019). Las dietas líquidas fermentadas ofrecen varias ventajas como son: reducción del pH gastrointestinal, aumento de bacterias ácido lácticas en la microbiota intestinal, disminución del número de bacterias coliformes (*Salmonella, Shigella, Escherichia coli, etc.)* en el alimento (Canibe *et al.,* 2008; Vieco-Saiz *et al.,* 2019) y la oportunidad de usar subproductos húmedos de bajo costo de la industria alimenticia (Canibe y Jensen, 2012; Shi *et al.,* 2017; Wang *et al.,* 2017; Zhu *et al.,* 2017; Rho *et al.,* 2018).

En la alimentación de cerdos es importante prevenir la aparición de diarreas y mejorar el comportamiento productivo, principalmente durante el destete (Heo *et al.,* 2013; Dong *et al.,* 2014). El destete tiene una alta mortalidad en lechones por causa de la diarrea; la alternativa tradicional para prevenir este problema es el uso de antibiótico como promotor del crecimiento (Dou *et al.,* 2017; He *et al.,* 2020).

El uso de antibiótico como promotor del crecimiento en los alimentos para consumo animal contribuye a la aparición y propagación de la resistencia microbiana; un problema de salud pública mundial (Holmes *et al.,* 2016; Nhung *et al.,* 2016; Vidovic y Vidovic, 2020). Si continua el problema de la resistencia microbiana, se calcula que en el año 2050 contribuiría con 10 millones de muertes por año y un costo aproximado de 100 billones de dólares estadounidenses (Tang *et al.,* 2019). La prohibición por la Unión Europea del antibiótico como promotor del crecimiento en la alimentación porcina comenzó en enero del 2006 por medio de la regulación EC No 1831/2003 (Jensen y Hayes, 2014; Salim *et al.,* 2018; Tang *et al.,* 2019). La restricción de antibiótico como promotor del crecimiento aumenta en el mundo: Estados Unidos y Canadá comenzaron a limitar el uso de antibiótico como promotor del crecimiento en 2017 (FDA, 2012; FDA, 2016; López-Gálvez *et al.,* 2020). Por lo tanto, es necesario desarrollar alternativas alimenticias que reduzcan del uso de antibiótico como promotor del crecimiento en el alimento para cerdos.

Las estrategias alternativas de alimentación para reducir el uso de antibiótico como promotor del crecimiento son varias e incluyen: la adición de lactosa (Jeong *et al.,* 2018; Zhao *et al.,* 2021), ácidos orgánicos (Plumed-Ferrer y von Wright, 2011; Mocherla *et al.,* 2015), extractos herbales, aceites esenciales, zinc, cobre (López-Gálvez *et al.,* 2020), péptidos antimicrobianos (Robinson *et al.,* 2015), trasplante de microbiota intestinal (Hu *et al.,* 2017a; Canibe *et al.,* 2019), prebióticos (Jeong *et al.,* 2018; Yu *et al.,* 2019) y la estrategia más ordinaria: el uso de probióticos (Kim *et al.,* 2019; Guo *et al.,* 2020). No obstante, la alta cantidad de productos comerciales (probióticos, prebióticos o aditivos alimenticios), para observar un beneficio significativo son una limitante en la rentabilidad (Hu *et al.,* 2017b; Nawab *et al.,* 2019).

Las dietas líquidas fermentadas son una alternativa para sustituir el uso de antibiótico como promotor del crecimiento en la alimentación porcina, al aumentar la cantidad de bacterias ácido lácticas, ácidos orgánicos y mejorar el funcionamiento de la microbiota intestinal (Mao *et al.,* 2016; Markowiak y Śliżewska. 2018). No obstante, falta evidencia para demostrar que es posible obtener las dietas líquidas fermentadas de alta calidad con cereales y subproductos diferentes a los que se utilizan en la Unión Europea, con la combinación de bacterias ácido lácticas y con reducción del contenido de proteína cruda. El maíz es el cereal de mayor producción en el mundo; el Continente Americano produce el 49.7% de este cereal (FAO, 2019). Por lo cual, el maíz es el principal cereal en la industria de alimentos para cerdos en el Continente Americano.

En la presente investigación se realizaron dos estudios. En el primer estudio, el propósito fue desarrollar dietas líquidas fermentadas de alta calidad basadas en maíz para cerdos, con la inclusión de inóculo de *Lactobacillus plantarum.* En el segundo estudio el propósito fue desarrollar dietas líquidas fermentadas de alta calidad basadas en maíz, con diferentes niveles de proteína cruda y con la combinación de inóculos de *Lactobacillus plantarum, Pediococcus pentosaceus* y *Pediococcus acidilacticiti* y con el potencial de sustituir el uso de antibiótico como promotor del crecimiento y reducir así, la resistencia microbiana.

OBJETIVOS E HIPÓTESIS

Objetivos

General

Desarrollar dietas líquidas fermentadas de alta calidad basadas en maíz, el principal cereal de México y del continente Americano para alimentación porcina, con inclusión de *Lactobacillus plantarum* con el potencial para suplir los antibiótico como promotor del crecimiento, y evaluar dietas líquidas fermentadas de alta calidad basadas en maíz, con la combinación de *Lactobacillus plantarum, Pediococcus pentosaceus* y *Pediococcus acidilacticiti,* y la reducción de la concentración de proteína cruda en el sustrato.

Específicos

Determinar la respuesta en maíz fermentado con *Lactobacillus plantarum*, con la inclusión de goma xantana, la relación sustrato:agua, el origen (vegetal o animal), y la cantidad de proteína cruda, sobre el pH final y las concentraciones de ácido láctico, ácido acetico y etanol.

Medir el efecto en maíz fermentado con *Lactobacillus plantarum*, con la inclusión de melaza, ácido láctico, y el tiempo de cultivo (12 y 24 h), sobre el pH final y las concentraciones de ácido láctico, ácido acetico y etanol.

Determinar una estrategia para producir dietas líquidas fermentadas de alta calidad basadas en maíz para cerdos con la combinación de *Lactobacillus plantarum, Pediococcus pentosaceus* y *Pediococcus acidilacticiti,* y con dos niveles de proteína cruda (18 y 20%) en el sustrato.

Hipótesis

Basados en el previo proceso de dietas líquidas fermentadas de alta calidad para cerdos con base en los cereales de importancia europea. Las dietas líquidas fermentadas de alta calidad basadas en maíz, con inclusión de melaza y de *Lactobacillus plantarum* tienen potencial para sustituir los atibioticos como promotores del crecimiento. Se desarrollarán dietas líquidas fermentadas de calidad basadas en maíz, con inclusión de melaza y de *Lactobacillus plantarum* en el primer estudio.

La obtención de una guía derivada del primer estudio permitirá establecer las bases para fermentar y producir dietas líquidas fermentadas de alta calidad basadas en maíz, con la combinación de *Lactobacillus plantarum, Pediococcus pentosaceus* y *Pediococcus acidilacticiti,* y la reducción de la concentración de proteína cruda en el sustrato para el segundo estudio.

REVISIÓN DE LITERATURA

Destete

El destete de la misma manera que el nacimiento son momentos cruciales en la producción de cerdos: el bajo consumo de alimento y la disminución en el crecimiento posterior al destete, son las principales limitaciones en la eficiencia productiva. Los cambios producidos por el destete en la estructura y función intestinal, tales como atrofia de las vellosidades e hiperplasia de las criptas, se asocian generalmente con un comportamiento productivo deficiente, debido a que causan una disminución temporal de la capacidad digestiva y de absorción del intestino delgado (Gao *et al.,* 2019; Vanrolleghem *et al.,* 2019).

Las estrategias de manejo para mejorar el comportamiento productivo en los lechones durante el destete son: 1) cuidar las condiciones e instalaciones para las cerdas gestantes. 2) disminuir el estrés en el manejo de las camadas. 3) asegurar un buen consumo de leche y calostro. 4) promover una interacción temprana entre camadas. 5) emplear preiniciadores en la lactancia. Las estrategias nutricionales para mejorar el comportamiento productivo en los lechones durante el destete son: 1) promover el crecimiento fetal (adición de arginina, ácido fólico, betaína, vitamina B_{12}, carnitina, cromo, y zinc. 2) incrementar la producción de calostro y leche (adición de DL-metionina, arginina, L-carnitina, triptófano, valina y vitamina E) (Blavi *et al.,* 2021).

Incidencia de diarrea al destete en lechones

El destete implica una considerable pérdida económica para la producción de cerdos. Un factor común en las etapas de destete y postdestete es la incidencia de diarreas. Las diarreas requieren de medicamento para su control; el empleo de antibióticos incrementa los costos de producción y contribuye al desarrollo de resistencia microbiana (Gao *et al*., 2019; Wensley *et al*., 2021).

La incidencia de diarreas resulta de una serie de interacciones entre el tipo de alimentación, ambiente y la capacidad inmunológica del lechón. Los lechones que no resisten los desafíos infecciosos, son susceptibles a los trastornos gastrointestinales, que a su vez disminuyen el consumo y retardan el crecimiento e inclusive pueden provocar la muerte (Vanrolleghem *et al.,* 2019; Bonetti *et al.,* 2021).

Tiempo de destete

Las prácticas de manejo para mejorar la producción evolucionaron considerablemente. En granjas comerciales, existe la necesidad de mantener el tamaño de camada en partos posteriores, reducir el intervalo entre el destete y el periodo de apareamiento, así como el intervalo entre partos (Wu *et al.,* 2018). No obstante, en la búsqueda de aumentar el número de lechones y de producción de carne de cerdo en general, la producción intensiva desencadenó una reducción drástica de los días de edad al destete: durante la tercera o cuarta semana de edad. Los lechones se destetan gradualmente alrededor de la semana once y su comportamiento productivo difiere con los lechones destetados de forma precoz, debido a que el sistema digestivo está en desarrollo, lo cual aumenta el número de días necesarios para conseguir el peso a sacrificio (Mou *et al.,* 2019).

Ingredientes alimenticios durante el destete

El programa de alimentación en lechones generalmente se forma de dos etapas: pre y postdestete, su uso es con la finalidad de complementar y sustituir a la leche de cerda, respectivamente. En ambas dietas, los componentes básicos son granos de cereales y pastas de oleaginosas (Huting *et al*., 2021). Sin embargo, desde el punto de vista nutricional, los lechones se alimentan con dietas altamente digestibles y que contengan un alto valor biológico en proteína. Dentro de los ingredientes recomendables destacan los que contienen proteínas de origen animal, es decir: suero de leche, harina de huevo, plasma deshidratado, harina de pescado, etcétera. El empleo de estos ingredientes mejora el comportamiento productivo postdestete de los lechones, pero a su vez aumenta ampliamente el costo en la alimentación (Rist *et al.,* 2013; Balan *et al.,* 2021).

Reducción en el contenido de proteína cruda en dietas para cerdos

En la producción porcina, el contenido de proteína cruda en la dieta se reduce cuando los requerimientos para los aminoácidos esenciales y el nitrógeno total se cumplen; en cerdos la necesidad de proteínas en la dieta es esencialmente para cubrir los requerimientos de aminoácidos (Wang *et al.,* 2018). El contenido limitado de lisina, especialmente por la naturaleza el maíz, en dietas para cerdos con base en maíz y pasta de soya generó que la dieta tradicional de maíz y pasta de soya fuera en altos niveles de proteína cruda para cumplir con los requisitos de lisina en los cerdos (Zhao *et al.,* 2019). Las dietas altas en proteína condujeron a excesos en ambos, aminoácidos no esenciales y excreción de nitrógeno en heces y orina. Esto no sólo reduce la

eficiencia de uso del nitrógeno, pero también genera desventajas; por ejemplo, la fermentación de proteínas en el intestino grueso que daña la salud intestinal (Wang *et al.,* 2018; Wang *et al.,* 2019). Al reducir la proteína cruda en la dieta de 2 a 4% según las recomendaciones de NRC (1998) y cubrir los requerimientos con aminoácidos sintéticos se demostró que se aumenta la eficiencia en el uso de nitrógeno, se mejora la salud intestinal, se reducen los costos de alimentación y la excreción de nitrógeno. Sin perjudicar el crecimiento y rendimiento de los cerdos; por ejemplo, existen estudios de dietas bajas en proteina con la adición de los cuatro aminoácidos sintéticos (L-lisina, DL-metionina, L-treonina y L-triptófano) que en cerdos son los primeros cuatro aminoácidos limitantes para obtener una proporción ideal de proteína, donde el comportamiento fue similar (Zhao *et al.,* 2019). El reciente NRC (2012) para requerimientos de nutrientes en cerdos eliminó las recomendaciones para proteína cruda y lo reemplazó con un requisito de nitrógeno total. Si el requerimiento total de nitrógeno de NRC (2012) se multiplica por el coeficiente de 6.25, el requisito de proteína cruda es 2 a 4% más bajo que el recomendado en NRC anterior (1998) (Wang *et al.,* 2018; Wang *et al.,* 2019). Por lo tanto, la disminución de proteína cruda en dietas para cerdos ahorra ingredientes proteínicos, reduce no sólo la excreción de nitrógeno, sino también los costos de alimentación sin afectar el comportamiento productivo en comparación con las dietas tradicionales.

Epitelio intestinal

El epitelio intestinal es la membrana que recubre el intestino delgado y grueso. También forma parte del sistema inmunológico, que a la vez funciona como barrera y sistema de reconocimiento de primera línea para los agentes patógenos. El intestino de mamíferos se cubre por una capa única de células epiteliales que se renueva cada 4-5 días. Las células epiteliales en el intestino delgado son un tipo de células del borde en cepillo que se empalman entre sí por uniones estrechas para formar una membrana semipermeable (Chen *et al.,* 2018; Nayak *et al.,* 2018).

El epitelio de la mucosa intestinal se forma por diversos tipos de células. Las células absorbentes o enterocitos, las células caliciformes que secretan mucina, las células diferenciadas que son responsables de la renovación y finalmente las células de Paneth que se encargan de producir lizosimas empleadas como mecanismos de defensa antibacteriano (Xiong *et al.,* 2019).

Función metabólica del intestino

La absorción de nutrientes necesarios para el organismo y la protección inmunológica son las principales funciones del intestino delgado, que se divide en tres porciones: duodeno, yeyuno e íleon. Como cualquier tejido, el intestino solicita de nutrientes. El transporte y la absorción de nutrientes que requiere el intestino demanda gran cantidad de energía para el organismo y cuyo primordial combustible para el funcionamiento es la glutamina (Xiong *et al.,* 2019). El intestino requiere de una renovación celular rápida: la renovación de proteína intestinal representa aproximadamente 25% del consumo de proteína. El intestino como centro metabólico, participa en la gluconeogénesis y sintetiza algunos aminoácidos vitales para el cuerpo. Asimismo, el tejido linfoide del intestino es también el mayor órgano inmunológico del organismo (Yang *et al.,* 2019; Ortega *et al.,* 2021).

Vellosidades intestinales

El intestino delgado se compone de cinco capas concéntricas conocidas como: mucosa, submucosa, muscular circular, muscular longitudinal y serosa. La mucosa es la capa más interna, es decir, es la capa que encara el lado luminal del intestino. La mucosa está recubierta con numerosas vellosidades (de 20 a 40 por milímetro cuadrado), y el duodeno es el lugar donde existe el mayor número en comparación con el yeyuno y el íleon. Las vellosidades intestinales son proyecciones digitiformes (en forma de dedo) que tienen entre 0.5 y 1 mm de longitud y están recubiertas por un epitelio columnar. Cada vellosidad se asocia con una delgada extensión de músculo liso que proviene de la capa submucosa y que discurre hasta la punta. El borde libre de las células, que componen el epitelio que recubre a las vellosidades, tiene diminutas microvellosidades recubiertas por glucocálix (una capa rica en carbohidratos) que juntas constituye lo que se denomina el "borde en cepillo". Las vellosidades y las microvellosidades de la mucosa del intestino delgado aumentan el área de superficie (Ensari *et al*., 2018; Bonis *et al*., 2021).

El epitelio que recubre a cada vellosidad tiene distintos tipos de células. Estas, según su función, se clasifican en células de absorción, células caliciformes y células del sistema neuroendocrino difuso. **Los enterocitos** (células de absorción) son los más numerosos y se especializan en la absorción de agua y nutrientes. **Las células caliciformes** son glándulas unicelulares que se encargan de la secreción de mucinógeno, que es la forma deshidratada de la proteína mucina,

componente principal del moco intestinal protector del intestino. **Las células del sistema neuroendocrino difuso** tienen a su cargo la producción de hormonas paracrinas y endocrinas (secretina, colecistoquinina, motilina, péptido inhibidor gástrico, somatostatina, enteroglucagón y neurotensina). Dichas células representan alrededor del 1% del total de las células epiteliales. Finalmente, en las regiones del epitelio cercanas a un nódulo linfoide existen **las células M**, que son células del sistema fagocítico mononuclear encargadas de fagocitar y transportar antígenos desde la luz intestinal hacia los nódulos linfáticos (Ensari *et al*., 2018; Bonis *et al*., 2021).

Las vellosidades intestinales son proyecciones que salen de la pared del intestino delgado. Su función es aumentar el área de absorción, lo cual permite ampliar el paso de nutrientes en el lumen. En la superficie de las vellosidades se hallan también enzimas que sirven para digestión del alimento. Existen varias causas que provocan la atrofia de vellosidades (ayuno prolongado, enfermedad, destete) y generan una mala absorción de nutrientes (Walton *et al.,* 2018). Durante el destete, la atrofia de vellosidades aumenta la pérdida de células y existe un bajo índice de renovación; esto se asocia con una ampliación de criptas profundas, mientras se observa una reducción en la altura de vellosidades. La relación entre la profundidad de la cripta y la altura de vellosidad, manifiesta de mejor forma el efecto negativo del destete en el funcionamiento del intestino delgado (Ensari *et al.,* 2018; Bonis *et al.,* 2021).

Componentes de la barrera intestinal

La barrera intestinal se compone de células epiteliales, uniones estrechas y espacio lateral intercelular. Las uniones estrechas polarizan a la célula en las regiones apicales y basolaterales, las cuales regulan la difusión pasiva de los solutos y de las macromoléculas. De hecho, la barrera intestinal sirve como la primera línea de defensa contra un ambiente hostil dentro del lumen intestinal. Los componentes naturales de defensa en la mucosa intestinal, son mecanismos que reducen la capacidad del patógeno y de sus toxinas para invadir la mucosa, al asegurar la reparación rápida de defectos en la capa epitelial (Moeser *et al.,* 2017; Weström *et al.,* 2020).
La regulación de la difusión pasiva a través de la barrera intestinal se centra en la capacidad de las uniones estrechas en permitir el paso de los solutos selectos que son beneficiosos al organismo, mientras que previenen el paso de antígenos, toxinas bacterianas y patógenos. Una vez que se interrumpe la barrera epitelial, los mecanismos epiteliales de la reparación forman rápidamente

una capa monomolecular epitelial continua para prevenir la absorción de las toxinas bacterianas (Moeser *et al*., 2017; Weström *et al*., 2020).

Deformación del epitelio intestinal

El epitelio intestinal tiene por lo menos dos mecanismos disponibles de respuesta a deformación. El primero implica el alto estímulo de la renovación y algunos aspectos de la diferenciación celular, además de la movilidad de inhibición de la célula; y el segundo es la movilidad de la célula mientras que impide la renovación y el mantenimiento funcional fisiológico de la mucosa intestinal (Hosoe *et al.,* 2019). Otros mecanismos importantes de deformación, son las fuerzas físicas complejas que la mucosa intestinal experimenta, tales como: peristaltismo, movilidad de las vellosidades e interacción entre el contenido del lumen y el intestino, lo cual es dañino para la mucosa y afecta su funcionamiento (Montoro-Huguet *et al.,* 2021).

Mecanismos de reparación de la barrera intestinal

Después de lesión aguda de la mucosa, se llevan a cabo tres eventos locales: a) la restauración continua y normal de la permeabilidad del epitelio; a) la contracción de vellosidades, que reduce la superficie total y el área desnuda para su pronta reparación; y c) la migración de células epiteliales para sellar la membrana basal expuesta, con el propósito de cerrar los espacios intercelulares epiteliales y las uniones estrechas (Luissint *et al.,* 2016).

Estos eventos se inician en pocos minutos después de la lesión y están regulados a nivel local por los mediadores derivados de una red compleja de nervios, fibroblastos, células endoteliales y la matriz extracelular subyacente en la lámina propia. Dichos eventos se llevan a cabo aproximadamente en las 24 h posteriores al daño. No obstante, una mayor generación de células en la cripta, reemplazará las células perdidas y restaurará la arquitectura de las vellosidades, así como la función de digestión y absorción de nutrientes (Martini *et al.,* 2017).

Uso de antibiótico como promotor de crecimiento en dietas para cerdos

En la producción animal intensiva las dosis subterapéuticas de antibióticos como promotores del crecimiento se emplearon durante muchos años en dietas para animales. Su uso incluye los siguientes modos de acción: 1) reducción del número de enterobacterias intestinales e incremento de la población benéfica, principalmente en la microbiota intestinal; 2) disminución de la competencia por los nutrientes entre el huésped y los microrganismos; 3) reducción de la

descarboxilación microbiana de aminoácidos para formar aminas; y 4) mejorar la absorción de nutrientes en el intestino delgado. No obstante, la adición de antibióticos en la nutrición animal desarrolla la propagación de la resistencia microbiana, una importante amenaza para la salud pública mundial (Walia *et al*., 2019; López-Gálvez *et al*., 2020).

La preocupación por la salud humana y animal por la inclusión de antibiótico como promotor del crecimiento en los alimentos para animales, produjeron la prohibición total del antibiótico en dosis subterapéuticas en los alimentos para animales en la Unión Europea en 2006 (Reglamento (EC) No 1831/2003). Estados Unidos y Canadá también limitaron el uso de antibiótico como promotor del crecimiento en los alimentos para animales, en enero de 2017, al entrar en vigor un nuevo reglamento que exige una prescripción veterinaria para los aditivos terapéuticos (FDA, 2012). A pesar de la prohibición del uso de antibiótico como promotor del crecimiento en las principales economías, el consumo de antibióticos en los animales aumenta en el mundo. El consumo mundial de antimicrobianos en la producción de alimentos para animales se estimó en 63,151 ($\pm$ 1,560) toneladas en 2010 y se espera que aumente en un 67% en 2030. Dentro de la producción animal, la porcicultura es el sector que presenta un uso excesivo de antibióticos (Van Boeckel *et al*., 2015). Aunque el antibiótico como promotor del crecimiento se utiliza en toda la fase de crecimiento, su principal uso es en lechones destetados. El destete tiene una alta mortalidad en lechones por causa de la diarrea (Dou *et al*., 2017; He *et al*., 2020). Por lo tanto, la industria de alimentos balanceados para cerdos usa antibióticos en dosis subterapéuticas para prevenir y tratar la diarrea postdestete (Suresh *et al*., 2018; López-Gálvez *et al*., 2020).

Restricción del uso de antibióticos

En noviembre de 2017, la Organización Mundial de la Salud publicó la nueva guía sobre el uso de antibióticos en alimentos para animales en producción. El documento exhortó a los países miembros a reducir el uso de toda clase de antibióticos vitales para la salud humana en animales y reiteró la visión de la Organización Mundial de la Salud para que los antibióticos no se empleen como promotores del crecimiento. Una recomendación clave se refirió al uso de antibióticos con fines profilácticos. Es decir, los animales sanos sólo reciben antibióticos para prevenir una enfermedad si dicha enfermedad se diagnosticó en otros animales del mismo grupo (Aidara-Kane *et al*., 2018; Burki, 2018).

La guía de la Organización Mundial de la Salud se basó en una revisión sobre los efectos de reducir el uso de antibióticos en animales. El metanálisis de 81 estudios encontró que restringir el uso de antibióticos en la población animal resultó en una disminución del 10-15% en la resistencia microbiana en los animales. También hubo una disminución del 24% en la resistencia microbiana en humanos, principalmente entre aquellos en contacto directo con animales, aunque este resultado se basó en sólo 13 estudios. El gobierno del Reino Unido formuló un informe sobre la resistencia microbiana con 139 artículos académicos, donde tres cuartas partes de los artículos concluyeron que existía un vínculo entre el consumo animal de antibióticos y los niveles de resistencia microbiana en humanos. Asimismo, según la guía de la Organización Mundial de la Salud, las concentraciones bajas durante períodos prolongados es absolutamente la peor forma de usar antibióticos (Aidara-Kane *et al*., 2018; Burki, 2018).

En resumen, la guía de la Organización Mundial de la Salud recomienda una reducción general del uso de antibióticos en animales productores de alimentos, incluida la restricción completa del uso de antibióticos de importancia en salud humana. Además, recomienda varias formas de reducir la necesidad de antibióticos en la producción animal; por ejemplo: mejorar la higiene, incrementar el uso de vacunas, reducir el tamaño de los grupos de animales en producción y hacer conciencia, pues muchos productores desconocen los riesgos asociados con el uso excesivo de antibióticos (Aidara-Kane *et al*., 2018; Burki, 2018).

Tipos de restricción de antibióticos

El uso de antibiótico es una parte indispensable de la producción animal para el tratamiento y control de enfermedades, así como promotor del crecimiento. El uso frecuente de antibióticos en la producción animal origina residuos en productos de origen animal, lo que es un problema potencial para la salud humana (Nhung *et al*., 2016; Vidovic y Vidovic, 2020). La presencia de residuos de antibióticos en alimentos de origen animal induce problemas de salud graves como son las reacciones alérgicas y la resistencia microbiana. La resistencia microbiana es una amenaza para la salud pública mundial, ya que se asocia con un aumento en la morbilidad y mortalidad (Hosain *et al*., 2021). La resistencia microbiana contribuirá para el año 2050 con 10 millones de muertes por año y un costo aproximado de 100 billones de dólares estadounidenses (Tang *et al*., 2019). Por lo tanto, es importante conocer las definiciones de los términos utilizados en el esquema de clasificación de las restricciones (Cuadro 1) y la clasificación de las restricciones del uso de

antibiótico en la producción animal (Cuadro 2). Finalmente, la restricción amplia en el uso de antibiótico es la manera más eficaz para reducir la resistencia microbiana, en comparación con las restricciones especificas a un antibiótico o a una clase de antibióticos (Salim *et al*., 2018; Tang *et al*., 2019; Hosain *et al*., 2021).

Cuadro 1. Definiciones de los términos utilizados en el esquema de clasificación de las restricciones (Tang *et al*., 2019)

Terminología	Definición
Antibiótico como promotor del crecimiento	Administración de antibiótico en dosis subterapéuticas, para estimular el crecimiento y la eficiencia alimenticia en los animales.
Uso de antibióticos no terapéuticos	Administración de antibióticos a animales sin enfermedades infecciosas identificables. Incluye el uso de antibióticos para la promoción del crecimiento, la profilaxis y la metafilaxis de enfermedades.
Metafilaxis	Tratamiento de un grupo de animales sin evidencia de enfermedad, pero que probablemente estén en una fase de incubación, por estar en contacto con animales clínicamente enfermos.
Profilaxis	Administración de antibióticos en animales con riesgo de enfermedad infecciosa (pero sin enfermedad actual o detectada en el grupo). Se utiliza cuando las condiciones ambientales o los cambios presagian un mayor riesgo de infección; por ejemplo, el transporte de animales y el confinamiento de animales en espacios pequeños.
Uso terapéutico del antibiótico	Administración de antibióticos sólo para tratar animales con evidencia clínica de enfermedad infecciosa.

Alternativas al uso de antibióticos

Existen varios aditivos que son efectivos en regular la microbiota intestinal y que mejoran el crecimiento y comportamiento de los cerdos que se alimentan sin el uso de antibiótico como promotor del crecimiento. Por ejemplo, los acidificantes reducen el pH gastrointestinal y limitan la cantidad de enterobacterias en la microbiota intestinal, lo que mejora la digestibilidad de nutrientes. La inclusión de zinc y cobre dietético además de su efecto benéfico en el aporte nutricional como minerales, mejoran la microbiota intestinal por causa de su propiedad antimicrobiana.

Cuadro 2. Clasificación de las restricciones del uso de antibiótico en la producción animal (Tang *et al*., 2019)

Categoría	Descripción
Restricción completa	Restricción total al uso de todos los antibióticos.
Restricción de una sola clase de antibiótico	Restricción del uso de una clase de antibiótico, para todas las indicaciones de uso.
Restricción de antibiótico único	Restricción del uso de un antibiótico especifico, para todas las indicaciones de uso.
Restricción total de antibiótico al uso no terapéutico	Restricción del uso de antibióticos para todas las indicaciones no terapéuticas, incluido la promoción del crecimiento, profilaxis y metafilaxis (se permite sólo el tratamiento de animales enfermos).
Restricción como promotor de crecimiento y profilaxis	Restricción del uso de antibióticos para las indicaciones no terapéuticas. Promotor del crecimiento y profilaxis (tratamiento y metafilaxis están permitidos).
Restricción como promotor de crecimiento	Restricción del uso de antibióticos sólo como promotor del crecimiento (tratamiento, metafilaxis y profilaxis se permiten).
Otra / indeterminada	Incapacidad para determinar el tipo de restricción en una de las categorías anteriores.

Los prebióticos incrementan la población de microrganismos benéficos en el tracto gastrointestinal, pues sirven como sustratos para microorganismos específicos que mejoran la microbiota intestinal. El uso de nucleótidos y extractos herbales también mejoran la microbiota intestinal que resulta en un mejor comportamiento productivo en cerdos (Liu *et al*., 2018; Walia *et al*., 2019). Finalmente, la estrategia más ordinaria para sustituir el uso de antibiótico como promotor de crecimiento es el uso de probióticos (Kim *et al*., 2019; Guo *et al*., 2020). No obstante, la alta cantidad de productos comerciales, probióticos, prebióticos o aditivos alimenticios, para observar un beneficio significativo son una limitante en la rentabilidad (Nawab *et al*., 2019), por lo cual existe un alto número de aditivos alimenticios con el potencial uso en dietas para cerdos. El principal desafío con todos estos aditivos alimenticios, es que sus resultados en el comportamiento productivo sean no sólo significativamente buenos, sino consistentes. La inconsistencia en la efectividad de cada aditivo se debe a que dependen de las características de la

dieta y el estatus de salud de los animales. Por lo tanto, no es posible recomendar un aditivo específico para todas las diferentes dietas en cerdos, pues los resultados serían similares a no adicionar antibiótico como promotor del crecimiento. Sin embargo, lo que es realmente posible, es recomendar un aditivo concreto para las características delimitadas de una dieta y el estatus de salud especifico de los animales (Liu *et al*., 2018; Walia *et al*., 2019).

Dietas líquidas fermentadas para cerdos

El uso de la fermentación para mejorar o conservar los alimentos no es algo nuevo. Durante mucho tiempo, ambos, alimentos y bebidas fermentadas, contribuyeron de manera importante en la dieta de humanos y animales (Missotten *et al*., 2015). La mayoría de los alimentos fermentados tienen la ventaja de inhibir el crecimiento de microorganismos; como resultado, los alimentos fermentados tienen una mayor vida de anaquel en comparación con el producto original (Canibe y Jensen, 2012). Esta acción inhibitoria es una de las características para el uso de dietas líquidas fermentadas en cerdos.

El naciente interés en el uso de dietas líquidas fermentadas como estrategias de alimentación para mejorar el comportamiento productivo en lechones y cerdos en finalización, surgió debido al anuncio por parte de la Unión Europea de prohibir el uso de antibióticos en dosis subterapéuticas para promover el crecimiento en los alimentos para cerdos (Niba *et al*., 2009; Plumed-Ferrer y Von Wright, 2009). Por definición una dieta líquida fermentada es una mezcla de alimento y agua, en relación desde 1:1 hasta 1:4, que fue fermentada durante un tiempo suficiente (generalmente largo) para poseer las condiciones estables, usualmente conocida como fermentación espontánea (sin inóculo) (Brooks, 2008). No obstante, existe otra forma de fermentación, que es la fermentación controlada (con inóculo), en la cual se reduce el tiempo para obtener las condiciones óptimas de fermentación; en este caso, se logra al mezclar el sustrato en fermentación y los inóculos de bacterias ácido lácticas (Missotten *et al*., 2015).

Dietas líquidas fermentadas de calidad y sus características deseadas

En el momento en el que el agua y el alimento se mezclan, existe una alta posibilidad de que la fermentación (espontánea) inicie. De acuerdo con Canibe y Jensen (2003) la fase inicial de la fermentación se caracteriza por bajos niveles de bacterias ácido lácticas, levaduras y ácido láctico; pero con un pH alto y una alta concentración de microrganismos coliformes. No obstante, dentro

de la fase inicial existe una segunda fase, la cual se distingue por los altos niveles de ácido láctico, bacterias ácido lácticas, levaduras; con un bajo pH y una reducción en el número microorganismos patógenos (Brooks, 2008). Brooks (2008) dividió la fase inicial; es decir, la fase uno donde existe un pH alto que permite el aumento de bacterias coliformes, y la fase dos, en la cual la reducción en el pH genera el aumento de bacterias ácido lácticas e inhibe la multiplicación de microorganismos patógenos, debido a la mayor producción de ácidos orgánicos (particularmente ácido láctico). Finalmente, en la fase de estabilidad o fase tres, la población de bacterias ácido lácticas y el pH son estables; sin embargo, en esta misma fase la concentración de levaduras en el alimento continúa en aumento. Ambas fases, inicial y estable, se muestran en la Figura 1.
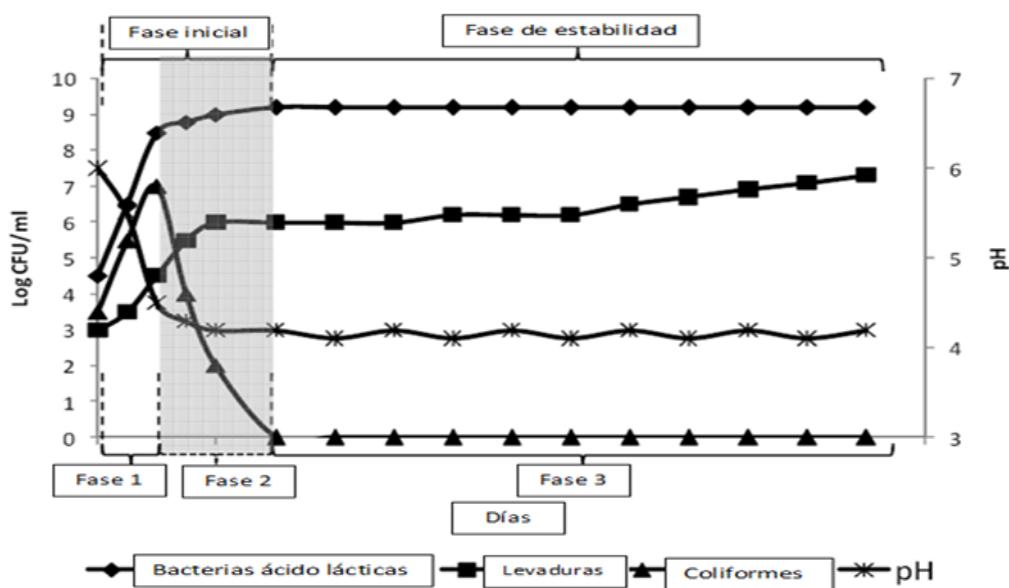
Figura 1. Fases de la fermentación láctica (Brooks, 2008)

Van Winsen *et al.* (2000) señalaron que la concentración de ácido láctico es el principal responsable del efecto antimicrobiano en las dietas líquidas fermentadas. Por lo cual es importante señalar que el uso de inóculos es la mejor opción para obtener las dietas líquidas fermentadas de calidad, con una alta concentración de ácido láctico y una disminución de microorganismos patógenos en el alimento (Vieco-Saiz *et al*., 2019). Van Winsen *et al.* (2001) señalaron que las características deseables en una dieta líquida fermentada son: pH menor a 4.5; concentración de bacterias ácido lácticas mayor de 9 log10 unidades formadoras de colonia/mL; concentración de ácido láctico mayor de 150 mM y concentración inferior de 40 y 0.8 mM de ácido acético y etanol en la dietas líquidas fermentadas.

Asimismo, Beal *et al.* (2002) observaron que para prevenir el crecimiento de *Salmonela spp.*, las DLF necesitan al menos 75 mM de ácido láctico. Además, Brooks *et al.* (2008) reportaron que para disminuir la concentración de enterobacterias, la concentración de ácido láctico debe ser superior de 100 mM. Por lo tanto, esta alta concentración de ácido láctico tiene un efecto benéfico en el comportamiento productivo; es decir, aumenta no sólo el consumo diario de alimento, sino también la ganancia diaria de peso y mejora la eficiencia alimenticia (Xu *et al.*, 2019). Finalmente, ambas formas D y L de ácido láctico son metabolizadas por los animales; aunque, la forma D en el ácido láctico, se metaboliza con una velocidad más lenta que la forma L (Everts *et al.*, 2000).

Efecto del pH en las dietas líquidas fermentadas sobre el tracto gastrointestinal

El uso de la dieta líquida fermentada para la alimentación de lechones, a diferencia de la dieta seca y líquida sin fermentar, frecuentemente reduce el pH gástrico (Yuan *et al.*, 2017). Por otra parte, el pH en el intestino delgado en alimentación con DLF es comúnmente más alto que en los animales alimentados con dieta seca o dieta líquida (Bunte *et al.*, 2020), lo cual se relaciona con un aumento en la secreción de jugo pancreático, estimulado principalmente por el pH bajo y la alta concentración de ácido láctico en las dietas líquidas fermentadas (Plumed-Ferrer y Von Wright, 2009; Pearnil *et al.*, 2020).

Mocherla *et al.* (2015) reportaron una mejora en el crecimiento de los cerdos alimentados con dietas que contenían ácidos orgánicos, lo cual está directamente relacionado con pH gástrico más bajo, que permite no sólo una mejor actividad proteolítica en el estómago, sino también una reducción en la concentración y crecimiento de microorganismos patógenos del alimento. Esta situación es similar para los cerdos cuando son alimentados con dietas líquidas fermentadas; por ejemplo, el estómago es una barrera importante contra los microrganismos coliformes especialmente en lechones destetados, los cuales normalmente no son capaces de producir suficiente acido gástrico (HCl) (Missotten *et al.*, 2015).

Efecto de la dieta líquida fermentada sobre la población microbiana en el tracto gastrointestinal

La alimentación con dietas líquidas fermentadas influye en la población microbiana en el tracto gastrointestinal. Canibe y Jensen (2003) no observaron diferencias entre los distintos tipos de alimentación con bacterias ácido lácticas que crecen a una temperatura de 37°C en la parte distal del intestino delgado. No obstante, a una temperatura de incubación de 20°C la proporción de

bacterias ácido lácticas con disponibilidad para crecer fue significativamente más alta, en cerdos alimentados con dietas líquidas fermentadas y en comparación con ambas formas, dietas secas y dietas líquidas. Asimismo, estos autores también observaron que la concentración de levaduras fue casi similar en todos los segmentos del tracto gastrointestinal; no obstante, fueron significativamente mayores en los animales alimentados con dietas líquidas fermentadas. En contraste, todos los segmentos del tracto gastrointestinal de los animales alimentados con dietas líquidas fermentadas mostraron menor población de bacterias coliformes. Moran (2001) reportó que los lechones alimentados con dietas líquidas fermentadas presentaron una mejor relación de las poblaciones de bacterias ácido lácticas y bacterias coliformes en los segmentos del tracto gastrointestinal a favor de bacterias ácido lácticas. No obstante, en ambos casos para los animales alimentados con otras formas; es decir, dieta seca y dieta líquida, la relación fue mayor para las bacterias coliformes.

Efecto de la dieta líquida fermentada sobre las vellosidades intestinales

La transición de una dieta líquida y frecuente como la leche, a una sólida y con menor reiteración, reduce el consumo de alimento; al tener menor ingesta de nutrientes, la renovación del epitelo intestinal y el crecimiento de las vellosidades se reduce, lo cual afecta el crecimiento en los lechones destetados (Brooks y Tsourgiannis, 2003). La reducción en la altura de las vellosidades intestinales se asocia con la menor capacidad de absorción y digestión de los nutrientes (Montagne *et al*., 2007). La alimentación líquida, con o sin fermentación, mejorará el consumo de alimento, pues exite una interaccion entre el consumo de alimento y el consumo de agua (Missotten *et al*., 2015). La alimentación líquida para los lechones durante el destete reduce la transición en la forma de alimentación; es decir, el cambio de dieta líquida a líquida es menor comparado con dieta líquida a sólida, pues se cubren las dos necesidades, sed y hambre en los animales (Brooks, 2008). La dieta líquida como forma de alimentación para los lechones en el destete mejora la arquitectura de las vellosidades intestinales en comparación con las dietas secas (Hurst, 2002). Scholten *et al.* (2002) reportaron que lechones alimentados con dietas líquidas fermentadas, con 45% de trigo fermentado, tenían una mejor composición en la arquitectura de las vellosidades intestinales, en comparación con los lechones alimentados sólo con dieta líquida. En el primer segmento del intestino delgado los lechones presentaron no sólo una mayor altura de vellosidades, sino también una mejor relación entre la altura de vellosidad y la profundidad de la cripta.

Fermentación espontánea en comparación con fermentación controlada

El desarrollo de dietas líquidas fermentadas ocurre mediante dos procesos: fermentación espontánea y fermentación controlada. La fermentación espontánea no es recomendable; por ejemplo, Beal *et al.* (2005) fermentaron 56 tipos de trigo y 44 de cebada, pero sólo pocas muestras alcanzaron los 100 mM de ácido láctico necesarios para eliminar *Salmonella spp.* (20% después de 72 horas de fermentación a 30°C). La fermentación espontánea resulta en una mayor concentración de ácido acético y aminas biogénicas, productos no deseados pues reducen la palatabilidad y el consumo (Niven *et al*., 2006). Por lo tanto, la fermentación espontánea no es un método adecuado para obtener un producto palatable y de calidad.

La mejor forma de obtener un fermentado de calidad, es el uso de inóculos bacterianos. La fermentación controlada con inóculos de BAL que produzcan altas concentraciones de ácido láctico ayuda a que las dietas líquidas fermentadas sean homogéneas y de calidad (Brooks *et al*., 2003). Existen varias cepas de bacterias ácido lácticas empleadas para producir dietas líquidas fermentadas de calidad; *Lactobacillus plantarum* es una bacteria de uso frecuente en la producción de dietas líquidas fermentadas (Niven *et al*., 2006; Missotten *et al*., 2007). Otra bacteria de uso frecuente en la creación de inóculos para dietas líquidas fermentadas es *Pediococcus spp*. (Beal *et al*., 2002; Niven *et al*., 2006; Missotten *et al*., 2007).

Importancia de las bacterias ácido lácticas en la nutrición de cerdos

La fermentación de alimentos se practicó mucho antes de que se descubrieran las bacterias ácido lácticas. Los romanos describieron que los productos lácteos fermentados se utilizaron en el tratamiento de infecciones gastrointestinales desde el año 76 a.C. En 1907, el científico ruso Elie Metchnikoff propuso el concepto de probióticos, y se planteó que el consumo de leche fermentada eliminaría el crecimiento de bacterias proteolíticas en el intestino, al mejorar así la vida del huésped. Según la OMS y la FAO, los probióticos se definen como "microorganismos vivos" que, cuando se administran en cantidades adecuadas, confieren un beneficio para la salud del huésped (Yang *et al.,* 2015; Nawab *et al*., 2019).

Los probióticos administrados en los alimentos necesitan sobrevivir a las condiciones intestinales, resistir los jugos gástricos y a las sales biliares, para crecer y colonizar el intestino. Un buen probiótico cumplirá con los siguientes requisitos: 1) capacidad para adherirse a las células epiteliales; 2) disminuir la adherencia patógena en el epitelio intestinal; 3) capacidad para

multiplicarse y producir ácidos, peróxido de hidrógeno y bacteriocinas, sustancias que limitan el crecimiento de patógenos; 4) ser seguro, no invasivo, no cancerígeno y no patógeno; y 5) capacidad de establecimiento para formar una microbiota intestinal benéfica (Yang *et al*., 2015; Guo *et al*., 2020).

Durante las últimas décadas, se utilizaron antibióticos en el alimento para promover el crecimiento en lechones al destete y prevenir las diarreas. Sin embargo, existe una creciente preocupación en relación con el desarrollo de bacterias resistentes a los antibióticos y con el potencial de que estas afecten la salud humana. Por lo tanto, existe un interés continuo en disminuir o eliminar la inclusión de antibióticos para promover el crecimiento en alimento para cerdos en el mundo. Como resultado, la demanda en el mercado internacional de carne de cerdo producida sin antibióticos aumentará y es necesario desarrollar estrategias alternativas de alimentación en cerdos (Dou *et al*., 2017; He *et al*., 2020; López-Gálvez *et al.,* 2020).

Las bacterias ácido lácticas son aditivos alimenticios naturales, económicos y seguros que no ponen en peligro el ambiente al no producir residuos o resistencia microbiana. Las bacterias ácido lácticas son útiles en la producción porcina al mejorar el bienestar animal y disminuir los problemas de salud intestinal. Estos beneficios permiten a los productores diferenciar la productividad, estar de acuerdo con las nuevas regulaciones alimentarias y satisfacer las demandas de los consumidores de carne segura. Las bacterias ácido lácticas no sólo producen ácidos orgánicos, sino también otras sustancias que inhiben el crecimiento de microorganismos patógenos. Sin embargo, no todas las bacterias ácido lácticas reducen en la misma forma a los microrganismos patógenos en el alimento para cerdos. Por lo tanto, es importante la selección cuidadosa de las bacterias ácido lácticas administradas y que las condiciones sean adecuadas (Yang *et al*., 2015; Dowarah *et al*., 2018).

Metabolismo de fermentación en bacterias ácido lácticas

Las bacterias ácido lácticas comprenden un grupo heterogéneo de microorganismos con la característica metabólica de la producción de ácido láctico como producto final de la fermentación. Las bacterias ácido lácticas son organismos anaerobios facultativos Gram (+), generalmente inmóviles, no esporulantes, catalasas negativas, tolerantes a los ácidos. A excepción de unas pocas especies, las bacterias ácido lácticas son organismos no patógenos y se reconocen como microorganismos seguros (GRAS, por sus siglas en inglés). Desde un punto de vista práctico, de

tecnología alimentaria, los siguientes se consideran los principales géneros de bacterias ácido lácticas: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, y *Weissella*. La clasificación de bacterias ácido lácticas en diferentes géneros se basa principalmente en la morfología, el modo de fermentación de la glucosa, el crecimiento a diferentes temperaturas, la configuración del ácido láctico producido, la capacidad de crecer a altas concentraciones de salinidad y la tolerancia ácida o alcalina (Blajman *et al.,* 2018; Abedi *et al.,* 2020).

Una característica importante utilizada en la diferenciación de los géneros de bacterias ácido lácticas es el modo de fermentación de la glucosa en condiciones estándar. Las bacterias ácido lácticas obligatoriamente homofermentativas (grupo 1) son capaces de fermentar hexosas casi únicamente a ácido láctico por la vía de Embden-Meyerhof-Parnas mientras que las pentosas y el gluconato no se fermentan ya que carecen de fosfocetolasa. Las bacterias ácido lácticas heterofermentativas facultativas (grupo 2) degradan las hexosas a ácido láctico por la vía Embden-Meyerhof-Parnas y también son capaces de degradar las pentosas y, a menudo, el gluconato, ya que poseen tanto aldolasa como fosfocetolasa. Finalmente, las bacterias ácido lácticas obligatoriamente heterofermentativa (grupo 3) degradan hexosas por la vía del fosfogluconato que producen lactato, etanol o ácido acético y dióxido de carbono. Varias cepas de los grupos 1 y 2 y algunas del grupo 3 se utilizan en alimentos fermentados, pero el grupo 3 se asocia comúnmente con la descomposición de los alimentos (Blajman *et al.,* 2018; Abedi *et al.,* 2020).

Las bacterias ácido lácticas homofermentativas tienen la enzima aldolasa y convierten la glucosa casi exclusivamente en ácido láctico. Las bacterias ácido lácticas homofermentativas producen 2 moles de ácido láctico como producto final por cada mol de glucosa consumida y el rendimiento en la producción de ácido láctico es de más del 85%. Las bacterias ácido lácticas homofermentativas incluyen los géneros: *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus* y algunos *Lactobacillus*. Las bacterias ácido lácticas heterofermentativas fermentan 1 mol de glucosa para formar, 1 mol de ácido láctico, 1 mol de etanol y 1 mol de CO_2. El rendimiento en la producción de ácido láctico es de alrededor del 50%. Este grupo tiene la enzima fosfocetolasa, pero carece de la aldolasa y la hexosa isomerasa; por lo cual, en lugar de seguir la vía Embden-Meyerhof-Parnas, utilizan las vías de la hexosa monofosfato o la de las pentosas (Blajman *et al.,* 2018; Enan *et al.,* 2018).

Bacterias ácido lácticas con crecimiento en almidón

Es posible producir ácido láctico a partir de desechos de plantas azucareras (melaza y jugo de remolacha azucarera), biomasas almidonadas, así como de los disacáridos (lactosa y sacarosa), monosacáridos hexosas (glucosa, fructosa y galactosa) y pentosas (xilosa y arabinosa). Es decir, las bacterias ácido lácticas fermentan estos azúcares a través de glucólisis o la vía de las pentosas. Existe un gran interés en introducir materiales ricos en almidón como sustratos para la producción de ácido láctico, debido a su abundancia, bajo precio y por ser derivados de fuentes renovables. Las bacterias ácido lácticas amilolíticas como son: *Lactobacillus plantarum, Lactobacillus fermentum*, *Lactobacillus manihotivorans*, *Lactobacillus amylophilus* y *Lactobacillus amylovorus,* fermentan ingredientes ricos en almidón. Las bacterias ácido lácticas amilolíticas debido a la producción de α-amilasas transforman directamente el almidón en ácido láctico (Reddy *et al.,* 2008; Abedi *et al.,* 2020).

En la fermentación láctica a escala comercial, la adición de glucosa es una alternativa cara. La conversión directa con almidón en ácido láctico por parte de bacterias ácido lácticas amilolíticas elimina el proceso de dos pasos, y lo hace económico. Hasta el año 2021 se reportan muy pocas bacterias ácido lácticas amilolíticas con alto potencial para producir ácido láctico en altas concentraciones. La mayoría de las bacterias ácido lácticas amilolíticas se utilizan en la fermentación de alimentos. Las bacterias ácido lácticas amilolíticas también se usan en alimentos fermentados a base de cereales como el pan de centeno europeo, el pan salado asiático y la producción de bebidas no alcohólicas. No obstante, pocas bacterias ácido lácticas amilolíticas se utilizan para la producción de ácido láctico en la fermentación de almidón en un sólo paso. El uso de una fuente de azúcares más barata, como el almidón, la materia prima disponible más abundante en la tierra junto a la celulosa, en combinación con bacterias ácido lácticas amilolíticas disminuye el costo del proceso de fermentación en general. Como resultado, las bacterias ácido lácticas amilolíticas tienen el potencial para desarrollar una dieta líquida fermentada de calidad y económica para la alimentación de cerdos con base en cereales u otros subproductos abundantes en almidón (Reddy *et al.,* 2008; Abedi *et al.,* 2020).

Bacterias ácido lácticas con alta producción de ácido láctico

El ácido láctico se deriva del metabolismo de carbohidratos y aminoácidos, y es un componente natural de muchos alimentos. El ácido láctico existe en dos formas, L (+) - y D (-). El lactato

producido por las células de los mamíferos es L-lactato y este es el principal isómero en la sangre. El D-lactato normalmente está presente en concentraciones muy bajas, principalmente debido a la fermentación microbiana en el intestino. En dietas líquidas fermentadas el contenido de ácido láctico puede llegar hasta el 10% de la materia seca. Cuando la producción de D-lactato excede la capacidad del cuerpo para su metabolismo y excreción, D-lactato se acumula en la sangre para causar acidosis metabólica y rigidez muscular. Dietas con 10% de ácido láctico no tuvieron efectos negativos sobre la salud y desempeño de cerdos. No obstante, en cerdos la forma D del ácido láctico se metaboliza por igual, aunque a un ritmo más lento, en comparación con la forma L. Por lo cual, es importante seleccionar las bacterias ácido lácticas en dietas líquidas fermentadas para cerdos con mayor producción de ácido láctico en la forma L (Everts *et al*., 2000; EFSA, 2017).

Bacterias ácido lácticas con alta adhesión a la vellosidad intestinal

La adhesión de las bacterias ácido lácticas a la superficie de la mucosa intestinal se considera uno de los sucesos primordiales en la colonización exitosa del tracto gastrointestinal del hospedero. Existe un interés en determinar cómo las bacterias ácido lácticas colonizan y coexisten con su huésped, lo que depende de las interacciones entre el huésped y las bacterias ácido lácticas. Las bacterias ácido lácticas presentan varias propiedades adhesivas en la mucina. Las bacterias ácido lácticas se adhieren al entorno intestinal del hospedero, el cual está en constante cambio. La adhesión de las bacterias ácido lácticas les permite colonizar el tracto gastrointestinal del hospedero y evita el crecimiento de otras bacterias. Actualmente, los hallazgos sobre adhesión intestinal de bacterias ácido lácticas se identifican a nivel de cepas específicas. De tal manera, es importante seleccionar las bacterias ácido lácticas con alta adhesión intestinal y el potencial para desarrollar una dieta líquida fermentada de calidad para la alimentación de cerdos (Nishiyama *et al.,* 2016; Yadav *et al.,* 2017; Monteagudo-Mera *et al.,* 2019).

Sustancias antimicrobianas que producen las bacterias ácido lácticas

Las bacterias ácido lácticas producen sustancias antimicrobianas como: peróxido de hidrógeno, dióxido de carbono, diacetil, ácido láctico y otros componentes orgánicos. Pero las bacterias ácido lácticas también producen acetaldehído, etanol y bacteriocinas. Todas estas sustancias tienen una importancia antimicrobiana (Khalid, 2011; Enan *et al*., 2018).

Ácido láctico: es el principal componente antimicrobiano que producen las bacterias ácido lácticas. El ácido láctico es el mayor ácido orgánico que originan las bacterias ácido lácticas en la

fermentación. La actividad antimicrobiana del ácido láctico ocurre a través del cambio en las funciones de la membrana celular, debido al desenso en el pH. En condiciones de un pH bajo, se inhibe el transporte activo en las células; este efecto disminuye el pH intracelular, reduce la actividad metabólica y como resultado se limita el crecimiento de enterobacterias: por ejemplo, *Escherichia coli* inhibe su crecimiento a un pH de alrededor de 5.1. **Diacetilo:** es un componente aromático que se produce por cepas dentro de todos los géneros de bacterias ácido lácticas por fermentación de citrato. El diacetil se produce por bacterias ácido lácticas heterofermentativas como subproducto. El diacetil es un producto de alto valor y se usa ampliamente en la industria láctea como un compuesto de sabor preferido. El diacetil también tiene propiedades antimicrobianas. El diacetil reacciona con la proteína de unión a arginina de las bacterias gram negativas e interfiere con la función de este aminoácido. **Peróxido de hidrógeno:** las bacterias ácido lácticas producen peróxido de hidrógeno a partir del lactato. El efecto antimicrobiano del H_2O_2 resulta de la oxidación de los grupos sulfhidrilo que provoca la desnaturalización de varias enzimas y de la peroxidación de los lípidos de la membrana, lo que aumenta la permeabilidad de la membrana. La mayoría de las bacterias indeseables (*Pseudomonas spp.*) son sensibles al H_2O_2. **Dióxido de carbono:** se produce principalmente por bacterias ácido lácticas heterofermentativas. El CO_2 juega un papel en la creación de un ambiente anaeróbico e inhibe las descarboxilaciones enzimáticas, y la acumulación de CO_2 en la bicapa lipídica de la membrana causa una disfunción en la permeabilidad. El CO_2 inhibe el crecimiento especialmente de las bacterias gram negativas. **Reuterina:** es un compuesto antimicrobiano producido, en condiciones anaeróbicas y con glicerol, por un número amplio de *Lactobacillus*. La reuterina limita el crecimiento de *Aspergillus* y *Fusarium* lo que previene la formación de micotoxinas en alimentos fermentados. Finalmente, la reuterina limita el crecimiento de enterobacterias como son: *Salmonella*, *Shigella*, *Clostridium*, *Listeria,* etc. (Khalid, 2011; Enan *et al*., 2018).

Importancia de las bacteriocinas que producen las bacterias ácido lácticas

Las bacteriocinas son péptidos sintetizados ribosómicamente, que ejercen su actividad antimicrobiana contra cepas de la misma especie que la productora de bacteriocina (limitadas ocasiones), o contra cepas de especies diferentes (mayoría de las ocasiones). Se estima que entre el 30 y el 99% de todas las bacterias ácido lácticas producen bacteriocinas. La producción de bacteriocinas por bacterias ácido lácticas es muy significativa desde el punto de vista de sus

aplicaciones potenciales en los sistemas alimentarios. Las bacteriocinas también desempeñan un papel en la señalización celular. Los microorganismos que producen bacteriocinas poseen mecanismos de inmunidad para conferir autoprotección. La resistencia microbiana por el uso de antibióticos y la preocupación de los consumidores sobre los riesgos para la salud asociados con los conservadores químicos en los alimentos, aumentan el interés por las bacteriocinas (Ha *et al.,* 2016; Chikindas *et al.,* 2017).

Las bacteriocinas se producen de forma natural, por lo que los consumidores las aceptan fácilmente. Las bacteriocinas suelen clasificarse al combinar varios criterios. Los principales son: la familia de bacterias productoras, el peso molecular y finalmente sus homologías de secuencia de aminoácidos y / o organización de grupos de genes. Basado en un enfoque reciente, las bacteriocinas que producen las bacterias ácido lácticas se clasifican en dos clases principales: las bacteriocinas o lantibióticos que contienen lantionina (clase I) y los antimicrobianos de péptidos lineales (clase II). Las bacteriocinas se consideran como aditivos alimenticios seguros en el tracto gastrointestinal después de consumirse. Es decir, se degradan por algunas proteasas, por lo que es posible que sean inofensivas y digeridas (Ha *et al.,* 2016; Chikindas *et al.,* 2017).

CAPÍTULO I. PRODUCTION OF HIGH-QUALITY FERMENTED LIQUID SWINE FEED BASED ON CORN AND INOCULATED WITH *Lactobacillus plantarum*

1.1. ABSTRACT

Background: Fermented liquid feed (FLF) is commonly used in the European swine industry since 2006 because of the antibiotics´ ban of antibiotics as growth promoters (AGP). FLF with lactic acid bacteria improves the intestinal microbiota and inhibits pathogenic microorganisms. The objective of this study was to develop a high-quality FLF based on corn, the main cereal in Mexico and the American continent for the swine feed industry. *Lactobacillus plantarum* was included in FLF with the potential to replace AGP. **Methods:** A completely randomized experimental design was conducted in four phases with fermented corn as the main source of nutrients. In all phases: pH, lactic acid, acetic acid, and ethanol concentrations were measured. In the first phase, the effect of the substrate: water ratio (1:2 and 1:3) and the addition of xanthan gum (0 and 0.02%) were determined; in the second phase, three levels of protein (10, 15, and 20%) of vegetable and animal origin were evaluated; in the third phase, two fermentation times (12 and 24 h) were evaluated, with the addition of molasses (2 and 4%) and lactic acid (0 and 20 mM). Finally, in the last phase, two fermentation times (12 and 24 h), the origin of the protein (vegetable and animal), and the inoculum size (240 and 480 µL) were evaluated. **Results:** The independent analysis of the variables showed that the absence of xanthan gum, the protein increase (particularly; animal origin), the 24 h fermentation time, and the addition of molasses have a positive effect on the production of lactic acid and the decrease in pH. **Conclusions:** The selected experimental design allows developing a high-quality fermented liquid feed for pigs based on corn. Keywords: Lactic acid, pH, protein, fermented corn.

1.2. BACKGROUND

Fermented liquid feed (FLF) is a common feeding technology in the European swine industry and it is based on wheat and barley cereals [1, 2, 3]. Fermented liquid feed offers several advantages such as reduction in gastrointestinal pH, increase in lactic acid bacteria (LAB) for intestinal microbiota, food reduction in the number of coliform bacteria (*Salmonella*, *Shigella*, *Escherichia coli*, etc.) [4, 5], and a great potential opportunity to use low-cost wet by-products from the food industry [6 - 11].

There are both feeding strategies in pigs with and without the use of antibiotics. Both feeding strategies are used to prevent diarrhea illness and improving growth performance; especially during weaning [12, 13, 14]. Feeding strategies without the use of antibiotics are very diverse, so they include: lactose [15, 16, 17], organic acids [2, 18], herbal extracts, essential oils, zinc, copper [19], antimicrobial peptides [20], intestinal microbiota transplantation [21, 22, 23], prebiotics [16, 24], and the most common strategy: the use of probiotics [25, 26, 27]. Probiotics are defined as live microorganisms that, when consuming in adequate quantities, develop a benefit in the intestinal microbiota to the host [28, 29]. However, the great variety of commercial products such as; probiotics, prebiotics, and feed additives, to obtain significant benefits present economic limitations for the swine industry [30, 31, 32]. Since weaning diarrhea causes both high mortality and morbidity in piglets, the most traditional alternative for farmers is using antibiotic as growth promoter not only to reduce economic losses but also to minimize a high risk for swine production [33 - 36].

The use of antibiotic as growth promoter in the animal feed industry contributes to the increasing and spreading of microbial resistance [37, 38]. Microbial resistance is an international public health risk [39, 40]. It would contribute to 10 million deaths by year in 2050, an annual reduction from 2 to 3.5% in the world gross domestic product with an approximate cost of 100 billion US dollars [41]. The European Union's ban on antibiotic as growth promoter in the swine feed industry began in january 2006 through regulation EC No 1831/2003 [41, 42, 43]. The restriction of antibiotic as growth promoter increased in the world. The USA and Canada began limiting the use of antibiotic as growth promoter in 2017, so they were using new regulations requiring veterinary prescriptions to administer antibiotic as growth promoter in animal feed [19, 44, 45]. Therefore, it is necessary to develop new feed alternatives that contribute by reducing the use of antibiotic as growth promoter in feed for pigs.

Fermented liquid feed is an alternative to replace antibiotic as growth promoter in feed for pigs; they increase the amount of lactic acid bacteria and organic acids improving the intestinal microbiota [46, 47, 48]. However, there is not enough evidence to prove that it is possible to obtain high-quality fermented liquid feed with both viability and profitability in pig farms, by using cereals and by-products different from those used in the European Union [49, 50]. Corn is the cereal with the highest production in the world; the American Continent produces up to 49.7% of this cereal. That's why corn is the most important cereal in the swine feed industry in the American

continent [51]. According to Missotten *et al.* [76] the high-quality fermented liquid feed have the following features: first, a pH lower than 4.5; second, a lactic acid concentration of at least 150 mM; third, an acetic acid concentration lower than 40 mM; and fourth, an ethanol concentration of less than 0.8 mM.

This study aimed to develop a high-quality fermented liquid feed based on corn for pigs and inoculated with *Lactobacillus plantarum* and potential to replace antibiotics as growth promoters and reduce microbial resistance.

1.3. MATERIALS AND METHODS

Microorganism: *Lactobacillus plantarum* strain was obtained from the lactic acid bacteria collection of the Solid-state fermentation pilot plant from the "Universidad Autónoma Metropolitana-Unidad Iztapalapa, México." The strain was preserved in tubes with cryoprotective beads (Technical Services Consultant LTD, England), in glycerol 20% (v v^{-1}) at -20 ° C and lyophilized.

Inoculum preparation and experimental units

For inoculum production, a bead with the conserved strain was placed in a 50 mL test tube with 30 mL of the Man, Rogosa, and Sharpe (MRS) broth containing (g L^{-1}) [Glucose 20, peptone 10, meat extract 8, sodium acetate 5, yeast extract 4, dipotassium phosphate 2, Tween-80, ammonium citrate 2, magnesium sulfate 2 and manganese sulfate 0.05] (Difco, Nueva Jersey, EUA). Everything was previously sterilized at 120 °C for 15 min. Cultures were incubated at 35 ˚C for 10 h.

Lactic fermentation

Falcon® 50 mL conical tubes were used as experimental units. In each tube, 10 g of the substrate and 20 mL of distilled water at 90 °C were added (substrate: water ratio 1: 2). Each tube was mixed with a vortex for 1 min and it was cooled for one hour at environmental temperature to be immediately inoculated. Substrate composition, inoculum size, and lactic fermentation conditions are described further in this section. The incubation temperature was 30 °C in all phases.

Yellow cornflour (origin, USA; Productores Agropecuarios Tepexpan S.A. de C.V.), meat meal (origin, Mexico; Productores Agropecuarios Tepexpan S.A. de C.V.), soybean meal (origin USA; Productores Agropecuarios Tepexpan S.A. de C.V.), soy protein isolate (origin USA; Amfher Foods S.A. de C.V.), and molasses (origin, Mexico; Productores Agropecuarios Tepexpan S.A. de

C.V.) were used as substrates. The additives xanthan gum (origin USA; Amfher Foods S.A. de C.V.) and lactic acid (origin USA; Amfher Foods S.A. de C.V.) were added in different steps of this study.

Experimental design. A completely randomized experimental design with factorial arrangements, and distributed in four phases were used:

Phase I: Twenty Falcon® plastic tubes of 50 mL with 10 g of cornflour were used. Tubes were distributed in four treatments (Treatment 1: with 20 mL distilled water and without xanthan gum; Treatment 2: with 20 mL distilled water and 0.002 g of xanthan gum; Treatment 3: with 30 mL distilled water and without xanthan gum; Treatment 4: with 30 mL distilled water and 0.002 g of xanthan gum. In all treatments, 100 μL of *Lactobacillus plantarum* inoculum were added and incubated for 30 h at 30 °C. A completely randomized experimental design with a 2×2 factorial arrangement and five repetitions for treatment was used.

Phase II: Twenty-four Falcon® plastic tubes of 50 mL with 10 g of a mix of cornflour and protein ingredient, with 20 mL of distilled water were used. Tubes were divided into two groups: First, the protein content of: 10, 15 and, 20% of crude protein. Protein levels were calculated with the nutritional composition of all ingredients, cornflour, soybean meal, and meat meal, according to the NRC tables [52]. Second, protein origin: soybean meal and meat meal from vegetable, and animal origin respectively. All tubes were distributed in six treatments (Treatment 1:10% of crude protein level with vegetable origin; Treatment 2:15% of crude protein level with vegetable origin; Treatment 3:20% of crude protein level with vegetable origin; Treatment 4:10% of crude protein level with animal origin; Treatment 5:15% of crude protein level with animal origin; Treatment 6:20% of crude protein level with animal origin). In all treatments, 100 μL of *Lactobacillus plantarum* inoculum were added and incubated for 20 h at 30 °C. A completely randomized experimental design with a 3×2 factorial arrangement and four repetitions for treatment was used.

Phase III: Thirty-two Falcon® plastic tubes of 50 mL with 10 g of a mix of cornflour and meat meal (20% of crude protein), with 20 mL of distilled water were used. Tubes were divided into three groups: First, fermentation time at two levels: 12 and 24 h. Second, molasses addition at two levels: 2 and 4%. Third, lactic acid addition at two levels: 0 and 20 mM. All tubes were distributed in eight treatments (Treatment 1: Fermentation time of 12 h, molasses addition level of 2% and no addition of lactic acid; Treatment 2: Fermentation time of 12 h, molasses addition level of 2% and lactic acid addition of 20 mM; Treatment 3: Fermentation time of 12 h, molasses addition level of

4% and no addition of lactic acid; Treatment 4: Fermentation time of 12 h, molasses addition level of 4% and lactic acid addition of 20 mM; Treatment 5: Fermentation time of 24 h, molasses addition level of 2% and no addition of lactic acid; Treatment 6: Fermentation time of 24 h, molasses addition level of 2% and lactic acid addition of 20 mM; Treatment 7: Fermentation time of 24 h, molasses addition level of 4% and no addition of lactic acid; Treatment 8: Fermentation time of 24 h, molasses addition level of 4% and lactic acid addition of 20 mM). In all treatments, 200 µL of *Lactobacillus plantarum* inoculum were added and incubated at 30 °C. A completely randomized experimental design with a $2 \times 2 \times 2$ factorial arrangement and four repetitions for treatment was used.

Phase IV: Thirty-two Falcon® plastic tubes of 50 mL with 10 g of a mix of cornflour and protein ingredient (20% of crude protein), with 20 mL of distilled water were used. Tubes were divided into three groups: First, fermentation time at two levels: 12 and 24 h. Second, the origin of protein ingredient: a mix of cornflour, soybean meal and, soy protein isolate as an ingredient of vegetable origin, and a mix of cornflour and meat meal as an ingredient of animal origin. Third, inoculum addition of *Lactobacillus plantarum* at two levels: 240 and 480 µL. All tubes were distributed in eight treatments (Treatment 1: Fermentation time of 12 h, protein ingredient of vegetable origin and inoculum addition with 240 µL; Treatment 2: Fermentation time of 12 h, protein ingredient of vegetable origin and inoculum addition with 480 µL; Treatment 3: Fermentation time of 12 h, protein ingredient of animal origin and inoculum addition with 240 µL; Treatment 4: Fermentation time of 12 h, protein ingredient of animal origin and inoculum addition with 480 µL; Treatment 5: Fermentation time of 24 h, protein ingredient of vegetable origin and inoculum addition with 240 µL; Treatment 6: Fermentation time of 24 h, protein ingredient of vegetable origin and inoculum addition with 480 µL; Treatment 7: Fermentation time of 24 h, protein ingredient of animal origin and inoculum addition with 240 µL; Treatment 8: Fermentation time of 24 h, protein ingredient of animal origin and inoculum addition with 480 µL). All treatments were incubated at 30 ° C. A completely randomized experimental design with a $2 \times 2 \times 2$ factorial arrangement and four repetitions for treatment was used.

Measurement of response variables. At the end of fermentation time, the fermented substrate was homogenized in a vortex and two fractions were taken: 3 g of fermented substrate were used to measure pH (Conductronic pH120); 3 g of fermented substrate were centrifuged at 5,000 rpm for 15 min and the supernatant was used for lactic acid, acetic acid and ethanol analysis with an HPLC

Perkin Elmer LC250, SHIMADZU, by using the isocratic method described by Diano *et al.* [53]. An Aminex HPX-87H column was used at 60 °C, with a mobile phase of H_2SO_4 at 5 mM and a flow of 0.6 mL min^{-1}. A refractive index detector (LC-30, SHIMADZU) was used.

Statistic analysis. The experimental design used in each of the four phases met the statistical assumptions: normality (Shapiro's test), variances homogeneity (Bartlett's test), and independence. The last was completed when assigning the experimental units for the different treatments by using randomization with R program version 3.5.1. [54]. The statistical analysis of variance for four stages was performed by employing GLM procedure with the statistical program, SAS (2012) version 9.4. Any difference was considered significant with a P-value lower than 0.05, both significant factors and interactions were compared with Tukey's test.

1.4. RESULTS

Phase I. Effect of water content and xanthan gum addition

The xanthan gum addition (Table 3) did not affect the final pH (P > 0.05), or acetic acid and ethanol production. The xanthan gum addition negatively affected (P = 0.02) lactic acid production, where the best treatment was without the addition of xanthan gum.

The substrate: water ratio (Table 3) for fermented corn did not affect (P > 0.05) the individual response of the final pH. The production of lactic acid, acetic acid and, ethanol were similar (P > 0.05) either in individual response to substrate: water ratio or in the effect of the interaction between the substrate: water ratio and xanthan gum addition. Therefore, for the following assays the substrate: water ratio of 1:2 without xanthan gum addition was selected. Under these conditions, lactic acid production was maximized (Figure 2).

Table 3. Effect of the substrate:water ratio and / or xanthan gum addition in fermented corn with *Lactobacillus plantarum* on the final pH and the concentration of lactic acid, acetic acid and ethanol.

W1X1	W1X2	W2X1	W2X2	Probability		
				W	X	W*X
Final pH						
3.65 ± 0.01	3.64 ± 0.02	3.52 ± 0.02	3.64 ± 0.03	0.076	0.139	0.069
Lactic acid (mM)						
100.78 ± 8.59	87.84 ± 8.07	76.46 ± 6.92	59.94 ± 6.41	0.164	0.021	0.861
Acetic acid (mM)						
3.12 ± 0.31	2.76 ± 0.23	2.73 ± 0.32	3.04 ± 0.24	0.893	0.968	0.437
Ethanol (mM)						
0.69 ± 0.11	0.34 ± 0.13	0.87 ± 0.12	0.86 ± 0.13	0.052	0.307	0.312

Data are means and standard error of each treatment; W = effect of substrate:water ratio; W1 = substrate:water ratio (1:2); W2 = substrate:water ratio (1:3); X = effect of xanthan gum addition; X1 = xanthan gum addition of 0.0 g; X2 = xanthan gum addition of 0.002 g; W*X = Effect of the interaction between the substrate:water ratio and xanthan gum addition.
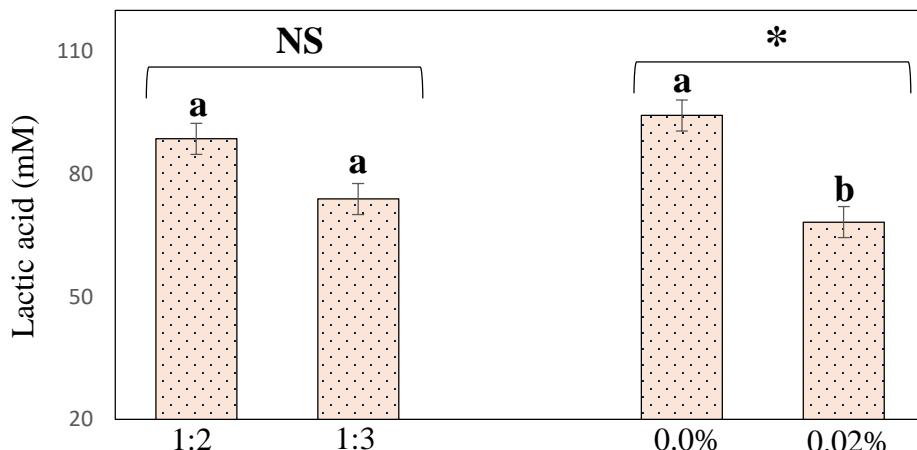
Figure 2. Lactic acid concentration in response to the xanthan gum addition (0.0 and 0.02%) and the substrate:water ratio (1: 2 and 1: 3) in fermented corn with *Lactobacillus plantarum*. [ab]Values without similar letters are significantly different (P < 0.05), NS (no significant; P > 0.05), *(P < 0.05).

Phase II. Effect of crude protein concentration and protein origin on fermented corn

The final pH of the fermented substrate (Table 4) was reduced (P = 0.001) with the increase of the protein concentration, where the best level (Figure 3) was with 20% of crude protein. Similarly, lactic acid production was increased (P = 0.001) with the increase of the protein concentration in the fermented substrate; the level with the best response (Figure 4) was with 20% of crude protein. The meat meal addition as an animal origin protein ingredient was the best option (Figure 3) not only for the final pH, but also for the lactic acid production (Figure 4). The acetic acid and ethanol production (Table 4) were similar (P > 0.05) and independent of the crude protein concentration or the origin of the protein ingredient, so it was the same result as an individual or an interaction effect.

Table 4. Effect of crude protein concentration and vegetable or animal protein origin in fermented corn with *Lactobacillus plantarum* on the final pH and the concentration of lactic acid, acetic acid and ethanol.

P1O1	P2O1	P3O1	P1O2	P2O2	P3O2	Probability		
						P	O	P*O
Final pH								
3.72 ± 0.04	3.87 ± 0.04	3.86 ± 0.04	4.00 ± 0.04	4.03 ± 0.04	4.14 ± 0.04	0.001	0.005	0.908
Lactic acid (mM)								
45.6 ± 10	81.8 ± 11	97.7 ± 10	129.1 ± 10	145.9 ± 15	183.2 ± 16	0.001	0.008	0.994
Acetic acid (mM)								
ND	ND	ND	ND	ND	ND	ND	ND	ND
Ethanol (mM)								
ND	ND	ND	ND	ND	ND	ND	ND	ND

Data are means and standard error of each treatment; P = effect of crude protein concentration; P1 = crude protein concentration of 10%; P2 = crude protein concentration of 15%; P3 = crude protein concentration of 20%; O = effect of vegetable or animal protein origin; O1 = vegetable protein origin O2 = animal protein origin; P*O = effect of the interaction between crude protein concentration and vegetable or animal protein origin; ND = not detectable.
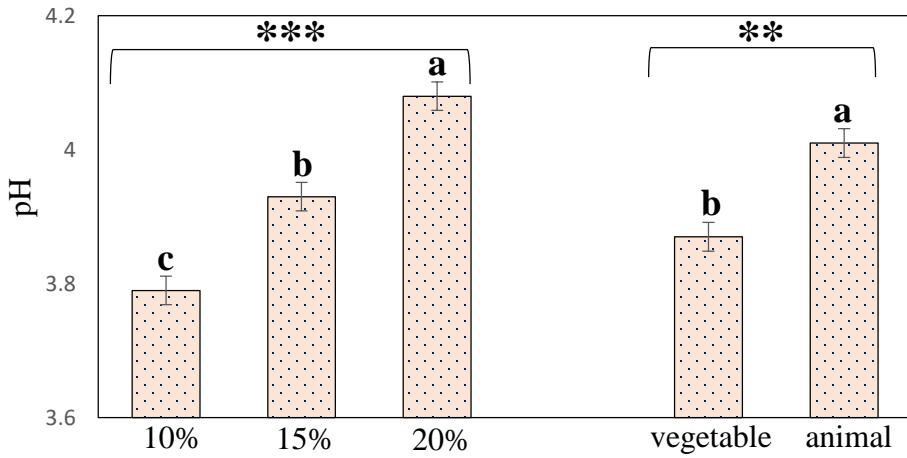
Figure 3. Final pH in response to crude protein concentration (10, 15 and 20%) and protein origin (vegetable or animal) in fermented corn with *Lactobacillus plantarum*. [ab]Values without similar letters are significantly different (P < 0.05), ** (P < 0.01), *(P < 0.001).**
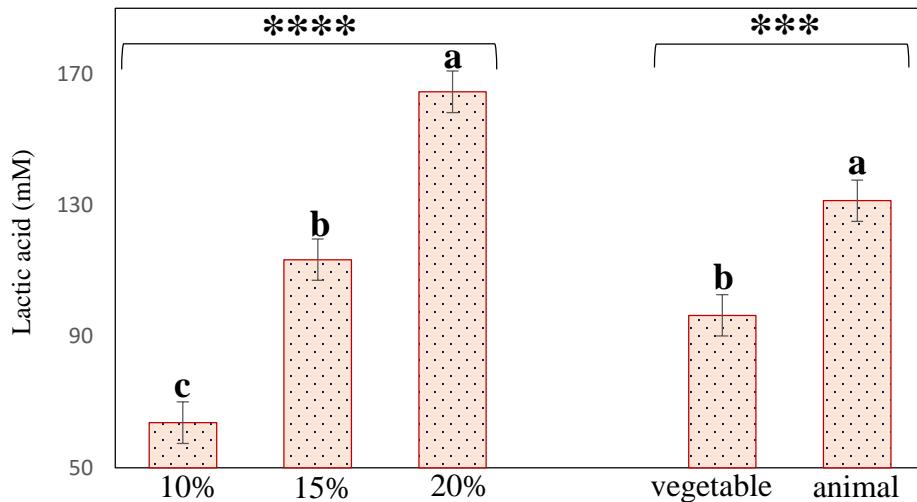
Figure 4. Lactic acid concentration in response to crude protein level (10, 15 and 20%) and protein origin (vegetable or animal) in fermented corn with *Lactobacillus plantarum*. [ab]Values without similar letters are significantly different (P < 0.05), *(P < 0.001), ****(P < 0.0001).**

To obtain a high-quality fermented liquid feed based on corn; for the following assays, the crude protein level of 20% and meat meal addition as animal origin protein were selected. Under these conditions, lactic acid production was maximized (Figure 4).

Phase III. Effect of fermentation time and the addition of molasses and lactic acid

The final pH of the fermented substrate (Table 7) decreased (P = 0.001) as a function of both factors, fermentation time and molasses addition; where the best fermentation time and level of

molasses addition were with 24 h and 4% respectively (Figure 5). Likewise, the lactic acid production (Table 7) increased (P = 0.001) as a response to these two factors, resulting in the most productive lactic acid concentration with 4% of molasses addition and fermentation time with 24 h (Figure 6).
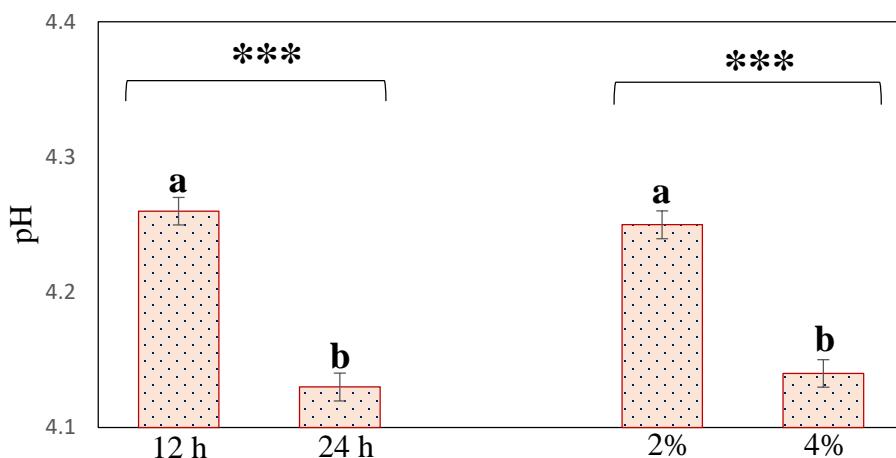
Figure 5. Final pH in response to fermentation time (12 and 24 h) and molasses addition (2 and 4%) in fermented corn with *Lactobacillus plantarum*. [ab]Values without similar letters are significantly different (P < 0.05), *(P < 0.001).**
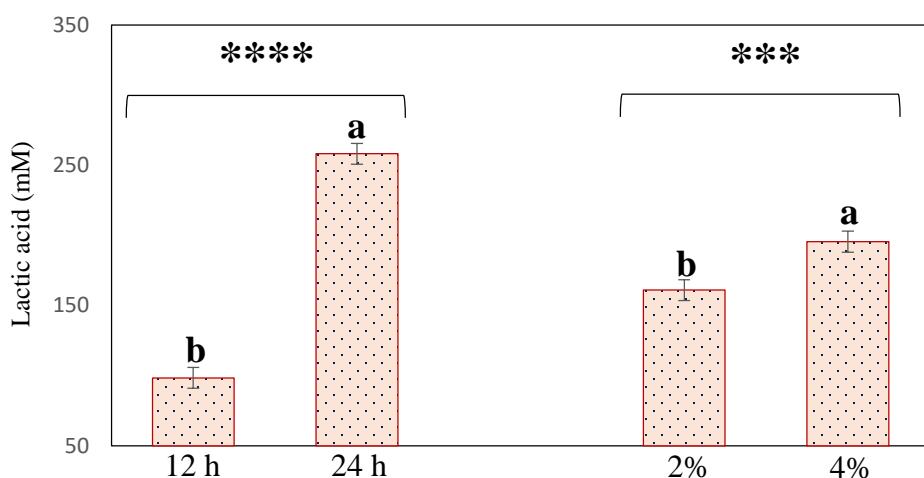
Figure 6. Lactic acid concentration in response to fermentation time (12 and 24 h) and molasses addition (2 and 4%) in fermented corn with *Lactobacillus plantarum*. [ab]Values without similar letters are significantly different (P < 0.05), *(P < 0.001), ****(P < 0.0001).**

The response of lactic acid addition wasn´t different (P > 0.05) neither in the final pH nor lactic acid, acetic acid and ethanol production. However, the analysis of variance (Table 7) showed an

interaction (P = 0.027) among the fermentation time, lactic acid, and molasses inclusion for the final lactic acid concentration. The best combination (Figure 7) was the addition of molasses (4%) and lactic acid (20 mM), during fermentation for 24 h.
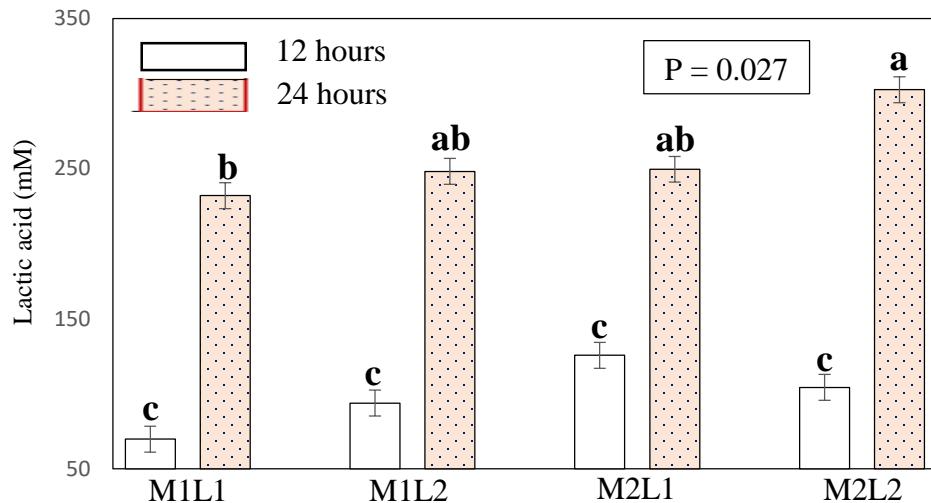
Figure 7. Lactic acid concentration in response to fermentation time (12 and 24 h), molasses (M1=2 and M2=4%) and lactic acid addition (L1=0 and L2=20 mM) in fermented corn with *Lactobacillus plantarum.* [ab]**Values without similar letters are significantly different (P < 0.05).**

The addition of molasses reduces the final pH and increase the lactic acid concentration at the end of the fermentation allowing to obtain a high quality fermented liquid feed (Figures 5 and 6) based on corn; however, the addition of lactic acid did not improve the final pH and lactic acid content in the fermented corn substrate. The 24 h fermentation time reduced the final pH and increased the final lactic acid concentration in the corn-based diet (Figures 5 and 6). This approach based on four combinations allows obtaining a high of quality fermented liquid feed for pigs based on corn (Table 5).

Table 5. Quality treatments with molasses and / or lactic acid addition in fermented corn with *Lactobacillus plantarum*.

Variable	Quality values	Combinations with 24 hours of fermentation			
		M1L1	M1L2	M2L1	M2L2
Final pH	4.5	4.11	4.18	4.16	4.08
Lactic acid (mM)	150	232.08	248.20	249.60	302.68
Acetic acid (mM)	40	ND	ND	ND	ND
Ethanol (mM)	0.8	ND	ND	ND	ND

M1 = molasses addition of 2%; M2 = molasses addition of 4%; L1 = lactic acid addition of 0 mM; L2 = lactic acid addition of 20 mM. ND = not detectable.

Phase IV. Effect of fermentation time, inoculum size and, protein origin

The final pH of the fermented substrate (Table 8) was affected (P = 0.001) as a function of the fermentation time, or the protein origin; where the best fermentation time for final pH was 24 h and the vegetable origin protein reduced the final pH (Figure 8). Lactic acid production (Table 8) was increased (P = 0.001) by both factors, fermentation time and protein origin in the substrate; as a result, the best time was with 24 h of fermentation and the most important protein ingredient was the animal origin (Figure 9).
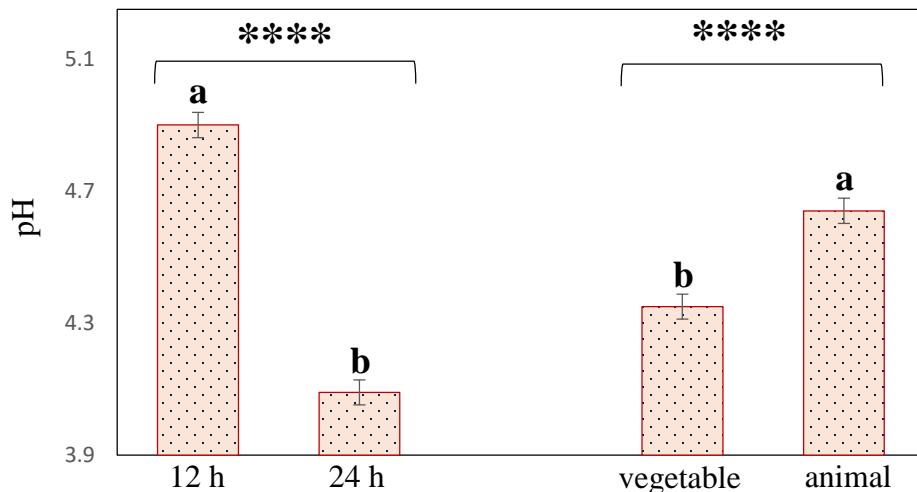
Figure 8. Final pH in response to fermentation time (12 and 24 h) and vegetable or animal protein origin in fermented corn with *Lactobacillus plantarum*. [ab]Values without similar letters are significantly different (P < 0.05), **(P < 0.0001).**
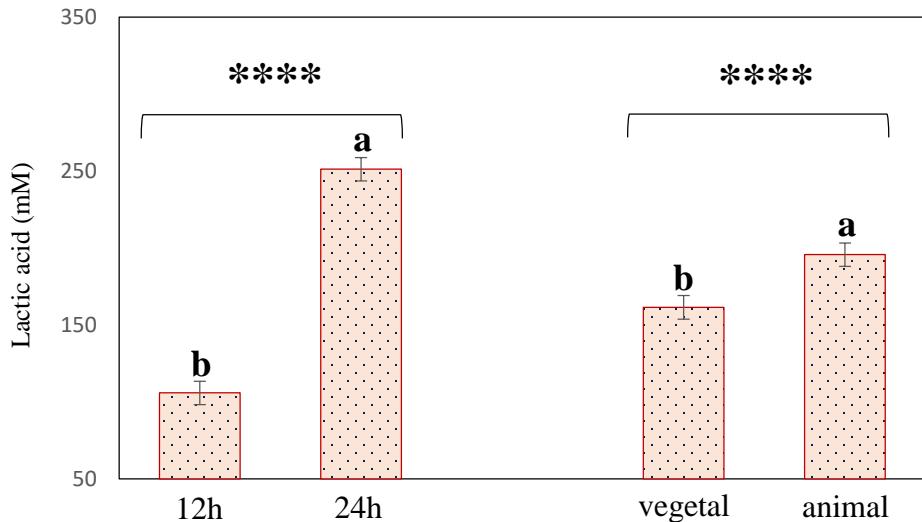
Figure 9. Lactic acid concentration in response to fermentation time (12 and 24 h) and protein origin (vegetable or animal) in fermented corn with *Lactobacillus plantarum*. [ab]**Values without similar letters are significantly different (P < 0.05), ****(P < 0.0001).**

The individual response of inoculum size addition was similar (P > 0.05) for the final pH, acetic acid and, ethanol concentrations. However, ANOVA (Table 8) showed that there were two interactions; the first one was in the final pH (P = 0.001) among the fermentation time, protein origin source and, the inoculum size. The second interaction was for the lactic acid production (P = 0.027) among those three factors. In this phase, the best combinations to obtain a low final pH (Figure 10) were with vegetable origin protein, 24 h of fermentation and, the inoculum of *Lactobacillus plantarum* in both levels 240 or 480 µL. Likewise, the best combination for lactic acid concentration (Figure 11) was with animal origin protein, 24 h of fermentation and, the inoculum level 240 µL.
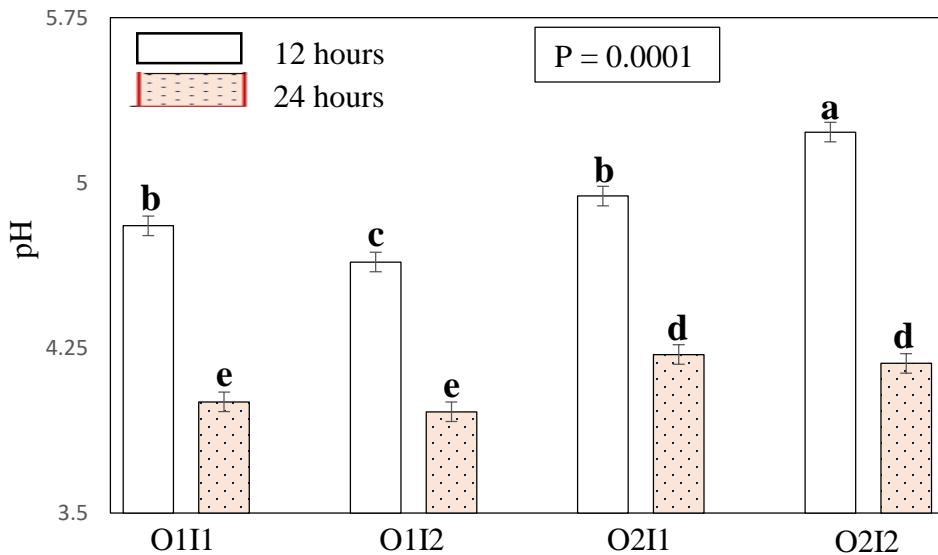
Figure 10. Final pH in response to fermentation time (12 and 24 h), protein origin (O1=vegetable and O2=animal), and inoculum size (I1=240 and I2= 480 μL) in fermented corn with *Lactobacillus plantarum*. [ab]Values without similar letters are significantly different (P < 0.05).
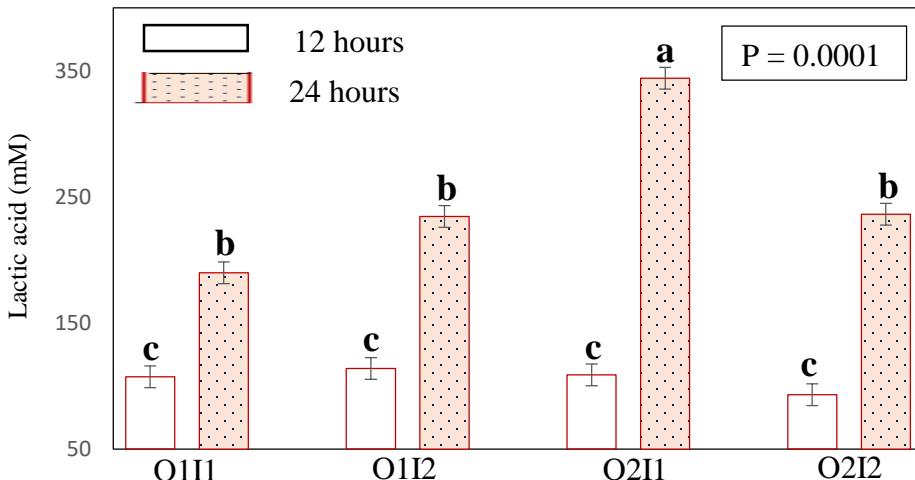
Figure 11. Lactic acid concentration in response to fermentation time (12 and 24 h), protein origin (O1=vegetable and O2=animal), and inoculum size (I1=240 and I2= 480 μL) in fermented corn with *Lactobacillus plantarum*. [ab]Values without similar letters are significantly different (P < 0.05).

High-quality fermented liquid feed based on corn was possible in phase IV. Four combinations were developed for high-quality fermented liquid feed based on corn with 24 h of fermentation (Table 6), two combinations were for vegetable origin protein, and the other two for animal origin protein.

Table 6. Quality treatments with two protein origins and / or two levels of inoculum in fermented corn with *Lactobacillus plantarum*.

Variable	Quality values	Combinations with 24 hours of fermentation			
		O1I1	O1I2	O2I1	O2I2
Final pH	4.5	4.01	3.96	4.22	4.18
Lactic acid (mM)	150	189.91	234.51	344.01	236.27
Acetic acid (mM)	40	6.11	ND	ND	5.80
Ethanol (mM)	0.8	ND	ND	ND	ND

O1 = vegetable protein origin; O2 = animal protein origin; I1 = inoculum size of 240 µl; I2 = inoculum size of 480 µl. ND = not detectable.

1.5. DISCUSSION

The use of fermented liquid feed in pigs is particularly beneficial for weaned piglets and it has two main advantages. First, animals acquire food and water simultaneously; which reduces both the time for animals when finding nutrients [6, 55] and the drastic change during the transition from milk to a traditional dry diet during weaning [1, 56, 57, 58]. Second, fermented liquid feed reduces the pH in the diet, therefore the animal's stomach improves its role as the first defense against pathogenic microorganisms [47, 56, 59]. However, the use of fermented liquid feed has shown different results; the animals expressed an improvement in daily weight gain, but a poorer feed efficiency [60, 61]. Sedimentation of solids has a detrimental effect producing a higher water intake and lower nutrients consumption. As a result, piglets had a very poor growth performance [62]. Sedimentation in fermented liquid feed could be avoided by the addition of 0.2 g kg^{-1} of xanthan gum. Xanthan gum addition at the beginning of fermentation (Figure 1) had a negative effect on lactic acid production; therefore, it should be added at the end of fermentation.

For fermented liquid feed the substrate: water ratio fluctuated between 1:1.5 to 1:4. Hurst *et al.* [63] showed that there was a linear improvement in both feed digestibility and energy when increasing the substrate:water ratio from 1:1.5 to 1:3; in other words, liquid food improved animal intake. After weaning, 50% of piglets consume its first meal within the first 24 hours, and after 48 hours 10% of animals. Piglets lost weight during the first week after weaning, so animals needed one or two weeks to recover that weight [18]. In phase I, the two substrate:water ratios produced

a similar response either for the final pH or for lactic acid concentration. That's why it was decided to work with the substrate: water ratio of 1:2 in the following phases.

There were many studies that highlight the importance of fermented protein in pig nutrition [64, 65]. The phase II results that lowered the pH and increased the lactic acid concentration in fermented corn were due to the carbon/nitrogen ratio. The carbon and nitrogen ratio was an important factor that improved the growth of *Lactobacillus plantarum* and that positively affected the lactic acid production in LAB [66, 67]. Several articles were published about the fermentation of vegetable protein origin [68, 69, 70, 71], or animal protein origin [72]. Lactic fermentation of vegetable origin and animal origin protein ingredients had several advantages in swine nutrition; in order to improved growth performance. Fermentation of feed protein was beneficial for pigs in two ways; first, anti-nutritional factors reduction for protein ingredients, as a result, the nutrient digestibility improved [64, 69, 70]. Second, the increase of intestinal microbiota [10, 71], with the purpose of reducing diarrhea and increasing the animals growth [73, 74, 75].

Fermented liquid feed has many advantages, but there are also some disadvantages. Fermentation caused loss of essential nutrients; specificallly essential amino acids. The amines formation; for example, cadaverine happens through decarboxylation of synthetic lysine [76]. The main recommendation to avoid the nutrients loss is to ferment only the cereal fraction; which contrasted with the results of this study, because it affected the carbon and nitrogen ratio, an important factor that influenced the lactic acid production in lactic acid bacteria [66, 67]. Therefore, more research is needed to achieve a high-quality fermented liquid feed with the minor or no loss of AA. The main factors are: protein concentration, origin, AA profile of the fraction to ferment, and the controlled fermentation conditions (temperature, inoculated lactic acid bacteria, and substrate disinfection). When obtaining a high-quality fermented liquid feed is important to speed up the process related to the transition from the initial phase (substrate with a high pH that allowed the coliform bacteria growth), to the stable phase (substrate with a lower pH that generates a bigger number of lactic acid bacteria, and it inhibits the multiplication of the pathogenic microorganisms) [61, 66]. The fermented liquid feed acceleration was an important factor for the fementation process, so it affected the lactic acid production and the lactic acid bacteria number by obtaining a quality fermentation [9, 76]. This study took the quality fermented liquid feed values reported by Missotten *et al.* [76]. Lactic acid has both bacteriostatic and bactericidal functions, which depend on the inclusion level or its concentration in fermented liquid feed [10]. Beal *et al.* [77] observed

that to prevent the growth of *Salmonella spp.*, fermented liquid feed required at least 75 mM of lactic acid. Brooks *et al.* [55] reported that by decreasing the number of enterobacteria, the lactic acid concentration must exceed 100 mM. Therefore, this high lactic acid concentration had a beneficial effect on the growth performance; by increasing not only daily feed intake, and daily weight gain but also in feed efficiency benefits, it was related to improving the lactic acid bacteria: Enterobacteria ratio for fermented liquid feed [76]. The results in phase III for this study proved that the molasses addition with 4% improved ($P < 0.05$) either the final pH or the lactic acid concentration. That´s why in this study was decided to work in the last phase with this level. Finally, adding by-products with a high content of available sugars (molasses) was beneficial not only in diets for weaned piglets [78, 79], but also it was an economical alternative to accelerate the growth of *Lactobacillus plantarum* in fermented corn.

Obtaining quality fermented liquid feed was important to reduce antibiotic as growth promoter in feed; the enterobacteria reduction in swine feed improved the animals' growth performance, especially for young animals [80, 81, 82]. A high lactic acid concentration in feed helps the intestinal microbiota development. Lactic acid reduces gastric pH and eliminates enterobacteria growth; which results in a better post-weaning growth performance [2, 3]. Weaning is a stressful event for piglets: with little or no feed intake, a new hierarchical order, diet transition, low production of pancreatic enzymes, and stomach hydrochloric acid. The stomach pH is a defense mechanism in piglets; it helps to reduce the enterobacteria amount in feed and improves the absorption of the nutrients [13, 14]. In pigs, protein digestion begins with the pepsin action, by secretion in the stomach mucosa of the precursor pepsinogen. The conversion rate from pepsinogen to pepsin depends on stomach pH; at a pH from 2 to 3.5, it is fast, but is very slow at a pH from 5 to 6 [18]. Therefore, fermented liquid feed helps to achieve an acidic stomach pH that reduces stress during weaning and improves the gut microbiota [46, 47, 48]. The increase in intestinal microbiota reduced diarrhea problems, improved [48, 83] the intestinal barrier, and intestinal villi [3, 47]. The quality fermented liquid feed development has the potential to reduce the antibiotics as growth promoters for the swine feed industry [3, 76]. Equally, the diet inclusion of protein ingredients depends on price, regional availability, protein content, AA profile, digestibility, and the pigs´ physiological stage; that is why it is necessary to develop quality fermented liquid feed with different ingredients from animal and vegetable protein origin. [52].

1.6. CONCLUSIONS

Through a four-stage statistical analysis, a high-quality fermented liquid diet corn-based was developed as a pig feed with the potential to replace antibiotics as growth promoters and reduce microbial resistance. This potential high-quality fermented liquid feed based on corn was obtained inoculating *Lactobacillus plantarum* to a mixture with a substrate: water ratio of 1: 2 and vegetable or animal protein origin with fermentation for 24 h at 30 ℃.

Abbreviations

FLF: Fermented liquid feed; AGP: Antibiotics´ ban as growth promoters; LAB: Lactic acid bacteria; PC: Crude protein; VO: Vegetable origin; OA: Animal origin; FT: Fermentation time; IA: Inoculum addition; HPLC: High performance liquid cromatography; mM: Milimolar; AA: Aminoacids.

Supplementary Information

It is not applicable.

Acknowledgements

It is not applicable.

Authors' contributions

Nicolás S. Espinosa-García designed the experiment, conducted the laboratory work, and wrote the manuscript. José Luis Figueroa-Velasco José Alfredo Martínez-Aispuro y María Teresa Sánchez-Torres-Esqueda analyzed the study data and helped to revise the manuscript. Ernesto Favela-Torres gave advice on the microbiological work and the HPLC analyzes. All authors have read and approved the final manuscript.

Funding

It is not applicable.

Availability of data and materials

The data analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

It is not applicable.

Consent for publication

It is not applicable.

Competing interests

The authors declare that they don't have any conflict of interest.

Author details

[1]Programa de Ganadería, Campus Montecillo, Colegio de Postgraduados. 56230. Montecillo, Estado de México. (jlfigueroa@colpos.mx). [2]Departamento de Biotecnología, Unidad Iztapalapa, Universidad Autónoma Metropolitana. 09340. Iztapalapa, Cuidad de México.

Availability of data and materials

The data analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

It is not applicable.

Consent for publication

It is not applicable.

Table 7. Effect of fermentation time, molasses and lactic acid addition in fermented corn with *Lactobacillus plantarum* on the final pH and the concentration of lactic acid, acetic acid and ethanol.

T1M1L1	T1M1L2	T1M2L1	T1M2L2	T2M1L1	T2M1L2	T2M2L1	T2M2L2	Probability						
								T	M	L	T*M	T*L	M*L	T*M*L
Final pH														
4.33±0.02	4.39±0.03	4.17±0.02	4.16±0.02	4.11±0.02	4.18±0.02	4.16±0.02	4.08±0.02	0.001	0.001	0.394	0.001	0.299	0.001	0.095
Lactic acid (mM)														
69.7±15	93.7±17	125.6±16	104.2±17	232.1±16	248.2±18	249.6±17	303.7±17	0.001	0.001	0.051	0.872	0.069	0.812	0.027
Acetic acid (mM)														
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ethanol (mM)														
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Data are means and standard error of each treatment; T = effect of fermentation time; T1 = fermentation time of 12h; T2 = fermentation time of 24h; M = effect of molasses addition; M1 = molasses addition of 2%; M2 = molasses addition of 4%; L = effect of lactic acid addition; L1 = lactic acid addition of 0 mM; L2 = lactic acid addition of 20 mM; T*M = effect of the interaction between fermentation time and molasses addition; T*L = effect of the interaction between fermentation time and lactic acid addition; M*L = effect of the interaction between molasses and lactic acid addition; T*M*L = effect of the interaction among fermentation time, molasses and lactic acid addition; ND = not detectable.

Table 8. Effect of fermentation time, vegetable or animal protein origin and inoculum size in fermented corn with *Lactobacillus plantarum* on the final pH and the concentration of lactic acid, acetic acid and ethanol.

T1O1I1	T1O1I2	T1O2I1	T1O2I2	T2O1I1	T2O1I2	T2O2I1	T2O2I2	Probability						
								T	O	I	T*O	T*I	O*I	T*O*I
Final pH														
4.33±0.02	4.39±0.03	4.17±0.02	4.16±0.02	4.11±0.02	4.18±0.02	4.16±0.02	4.08±0.02	0.001	0.001	0.661	0.003	0.028	0.001	0.001
Lactic acid (mM)														
69.7±15	93.7±17	125.6±16	104.2±17	232.1±16	248.2±18	249.6±17	303.7±17	0.001	0.001	0.019	0.001	0.073	0.001	0.001
Acetic acid (mM)														
1.46±0.71	1.76.0±0.6	ND	ND	6.1±0.98	ND	ND	5.8±1.04	0.014	0.293	1.000	0.383	0.854	0.001	0.001
Ethanol (mM)														
0.61±0.08	0.44±0.08	ND	ND	1.02±0.07	ND	ND	ND	0.932	0.003	0.071	0.932	0.224	0.071	0.224

Data are means and standard error of each treatment; T = effect of fermentation time; T1 = fermentation time of 12h; T2 = fermentation time of 24h; O = effect of vegetable or animal protein origin; O1 = vegetable protein origin; O2 = animal protein origin; I = effect of inoculum level; I1 = inoculum level of 240 µL; I2 = inoculum level of 480 µL; T*O = effect of the interaction between fermentation time and protein origin; T*I = effect of the interaction between fermentation time and inoculum level; O*I = effect of the interaction between protein origin and inoculum level; T*O*I = effect of the interaction among fermentation time, protein origin and inoculum level; ND = not detectable.

CAPÍTULO IV. HIGH-QUALITY FERMENTED LIQUID SWINE FEED WITH A LOW CONTENT OF CRUDE PROTEIN BASED ON CORN AND INOCULATED WITH LACTIC ACID BACTERIA COMBINATION

2.1. ABSTRACT

Background: Fermented liquid feed (FLF) for pigs has many advantages over traditional dry feeding, such as a lower pH and a higher lactic acid concentration. FLF produced with lactic acid bacteria (LAB) improves the intestinal microbiota and inhibits pathogenic microorganisms, hence reducing the need for the use of antibiotics as growth promoters (AGP). The use of AGP in animal feed industry contributes to increase and spread microbial resistance, resulting in an international animal and human health risk. The objective of this study was to develop a high quality FLF with a low content of crude protein based on corn inoculated with combinations of LAB to potentially replace AGP. **Methods:** A completely randomized experimental design with factorial arrangement was conducted in three phases with fermented corn as the main source of feed. In all laboratory phases: pH, lactic acid, acetic acid and ethanol concentrations were determined. In the first phase, the effect of the initial crude protein concentration (18 and 20%) and the addition of *Lactobacillus plantarum* (120 y 240 µL), and *Pediococcus pentosaceus* (120 and 240 µL) as inocula were evaluated. In the second phase, the effect of the initial crude protein concentration (18 and 20%) and the addition of *Lactobacillus plantarum* (120 y 240 µL), and *Pediococcus acidilacticiti* (120 and 240 µL) as inocula were evaluated. In the third phase, the effect of initial crude protein concentration (18 and 20%) and the addition of *Pediococcus pentosaceus* (120 y 240 µL), and *Pediococcus acidilacticiti* (120 and 240 µL) as inocula were evaluated. **Results:** The independent analysis of the variables for all phases showed that obtaining high quality FLF with low initial protein concentration (18%), and the addition of a mix inoculum was possible. Having a positive effect on the lactic acid production and the pH reduction. **Conclusions:** The fermentation conditions established in the evaluated variables developed 23 combinations of high quality FLF for pigs based on corn with a low crude protein content and combination of LAB.

Keywords: Lactic acid, pH, protein, fermented corn.

2.2. BACKGROUND

The use of antibiotics as growth promoters (AGP) in animal feed industry contributes to increase and spread microbial resistance, resulting in an international animal and human health risk [1–4]. It has been estimated to contribute to 10 million deaths per year in 2050, with an annual reduction from 2 to 3.5% in the world gross domestic product with an approximately cost of 100 billion US dollars [5]. Therefore, it is necessary to develop new feed alternatives that contribute by reducing the use of AGP in swine feed industry.

Feeding strategies avoiding the use of antibiotics include the dietary addition of: lactose [6–8], organic acids [9, 10], herbal extracts, essential oils, zinc, copper [11], antimicrobial peptides [12], intestinal microbiota transplantation [13–15], prebiotics [7, 16], and most commonly the use of probiotics [17–19]. Probiotics are defined as live microorganisms that, when consuming in adequate quantities, develop a benefit to the host in the intestinal microbiota [20, 21]. Currently the use of probiotics, prebiotics and food additives to obtain benefits represent an economic limitation for swine industry [22–24]. Fermented liquid feed (FLF) has advantages over traditional dry feeding, such as reduction of the pH and a high lactic acid concentration [25–27]. Lactic acid bacteria (LAB) improve the intestinal microbiota and inhibits pathogenic microorganisms; this inhibitory action is one of the most important characteristics for use in feeding pigs [28–30]. Moreover, FLF for pigs represents a good alternative to reduce the use of AGP [31–33].

FLF are an alternative for pig feeding to substitute AGP [34, 35]. When employing FLF, LAB with the greatest potential to improve gut microbiota and growth performance in pigs can be selected [36–38]. For that reason, FLF are not only a recent feeding strategy in the European swine industry, but their use is growing as an emergent technology in North America [39]. However, factors to obtain high quality FLF resulting in both viability and profitability in pig farms, by using cereals and by-products with combinations of LAB for intestinal microbiota and different levels of crude protein (CP) are required.

FLF with a low protein concentration would allow the use of this system either weaning pigs or grow-finisher pigs. The great advantages in reducing CP on diet, with amino acids (AA) addition to meet the requirements and to create a sustainable pig industry have been recently demonstrated [40, 41]. The decrease in CP for swine diets saves costly protein ingredients. It reduces not only nitrogen excretion, but also feeding costs, without affecting the growth performance compared to traditional diets [42, 43]. Reducing dietary protein content is a viable practice in corn-based pig

diets [41, 44]. The great advantages in reducing CP on diet, with AA addition to meet the requirements and to create a sustainable pig industry have been recently demonstrated [40, 41]. FLF with combination of LAB would permit the use of antimicrobial substances such as lactic acid, hydrogen peroxide, carbon dioxide, diacetyl, reuterin and bacteriocins, hence reducing the need for AGP in pig diets. [45, 46]. However, it is necessary to build evidence that a high quality FLF with LAB combinations is possible. Corn is the cereal with the highest production in the world; the American Continent produces up to 49.7% of this cereal. Corn is the most important cereal in the swine feed industry in the American continent [47]. According to Missotten *et al.* [26] the high quality FLF have the following features: a pH lower than 4.5; a lactic acid concentration of at least 150 mM; an acetic acid concentration lower than 40 mM; and an ethanol concentration of less than 0.8 mM. The objective of this study was to assess high-quality fermented liquid feed for pigs with a low crude protein concentration based on a mix of corn and inoculated with combinations of *Lactobacillus plantarum, Pediococcus pentosaceus and Pediococcus acidilacticiti* to potentially replace antibiotics as growth promoters and reduce microbial resistance.

2.3. MATERIALS AND METHODS

Microorganisms: *Lactobacillus plantarum, Pediococcus pentosaceus* and *Pediococcus acidilacticiti* strains were obtained from the lactic acid bacteria collection of the Solid-state fermentation pilot plant from the "Universidad Autónoma Metropolitana-Unidad Iztapalapa, México." The strains were preserved in tubes with cryoprotective beads (Technical Services Consultant LTD, England), in glycerol 20% (v v^{-1}) at -20 °C and lyophilized.

Inoculum preparation and experimental units

For inoculum production, a bead with the conserved strain (*Lactobacillus plantarum, Pediococcus pentosaceus* or *Pediococcus acidilacticiti*) was placed individually in a previously sterilized (120 °C, 15 min) 50 mL test tube with 30 mL of the Man, Rogosa and Sharpe (MRS) broth containing: (g L^{-1}) [Glucose 20, peptone 10, meat extract 8, sodium acetate 5, yeast extract 4, dipotassium phosphate 2, Tween-80, ammonium citrate 2, magnesium sulfate 2 and manganese sulfate 0.05] (Difco, Nueva Jersey, EUA). Cultures were incubated at 35 °C for 10 h.

Lactic fermentations

Falcon® 50 mL conical tubes were used as experimental units. In each tube, 10 g of substrate and 20 mL of distilled water at 90 ˚C were added (substrate:water ratio 1: 2). Each tube was mixed with a vortex for 1 min and cooled for one hour at environmental temperature before inoculation. Substrate composition, inoculum size, and lactic fermentation conditions are described further in this section. The incubation temperature was 30 ˚C in all phases.

Yellow corn flour (origin, USA; Productores Agropecuarios Tepexpan S.A. de C.V.), meat meal (origin, Mexico; Productores Agropecuarios Tepexpan S.A. de C.V.) and molasses (origin, Mexico; Productores Agropecuarios Tepexpan S.A. de C.V.) were used as substrates.

Experimental design. A completely randomized experimental design with factorial arrangement and distributed in three phases was used:

Phase I: Thirty two Falcon® plastic tubes of 50 mL with 10 g of a mix of yellow cornflour and meat meal, 4% of molasses, and a substrate:water ratio (1:2) were used. Tubes were divided into three factors: First, crude protein concentration with two levels: 18 and 20%. Second, inoculum addition of *Lactobacillus plantarum* (*Lp*) at two levels: 120 and 240 µL. Third, inoculum addition of *Pediococcus pentosaceus* (*Pp*) at two levels: 120 and 240 µL. Protein levels were calculated with the nutritional composition of yellow cornflour and meat meal, according to the NRC tables [48]. Tubes were distributed in eight treatments (Treatment 1: Crude protein concentration of 18%, *Lp* of 120 and *Pp* with 120 µL; Treatment 2: Crude protein concentration of 18%, *Lp* of 120 and *Pp* with 240 µL, Treatment 3: Crude protein concentration of 18%, *Lp* of 240 and *Pp* with 120 µL; Treatment 4: Crude protein concentration of 18%, *Lp* of 240 and *Pp* with 240 µL, Treatment 5: Crude protein concentration of 20%, *Lp* of 120 and *Pp* with 120 µL; Treatment 6: Crude protein concentration of 20%, *Lp* of 120 and *Pp* with 240 µL, Treatment 7: Crude protein concentration of 20%, *Lp* of 240 and *Pp* with 120 µL; Treatment 8: Crude protein concentration of 20%, *Lp* of 240 and *Pp* with 240 µL). All cultures were incubated at 30 ˚C. A completely randomized experimental design with a $2 \times 2 \times 2$ factorial arrangement (Table 9) and four replicates per treatment was used.

Phase II: Thirty two Falcon® plastic tubes of 50 mL with 10 g of a mix of yellow cornflour and meat meal, 4% of molasses, and a substrate:water ratio (1:2) were used. Tubes were divided into three factors: First, crude protein concentration with two levels: 18 and 20%. Second, inoculum addition of *Lactobacillus plantarum* (*Lp*) at two levels: 120 and 240 µL. Third, inoculum addition of *Pediococcus acidilacticiti* (*Pa*) at two levels: 120 and 240 µL. Protein levels were calculated

with the nutritional composition of cornflour and meat meal, according to the NRC tables [48]. Tubes were distributed in eight treatments (Treatment 1: Crude protein concentration of 18%, *Lp* of 120 and *Pa* with 120 µL; Treatment 2: Crude protein concentration of 18%, *Lp* of 120 and *Pa* with 240 µL, Treatment 3: Crude protein concentration of 18%, *Lp* of 240 and *Pa* with 120 µL; Treatment 4: Crude protein concentration of 18%, *Lp* of 240 and *Pa* with 240 µL, Treatment 5: Crude protein concentration of 20%, *Lp* of 120 and *Pa* with 120 µL; Treatment 6: Crude protein concentration of 20%, *Lp* of 120 and *Pa* with 240 µL, Treatment 7: Crude protein concentration of 20%, *Lp* of 240 and *Pa* with 120 µL; Treatment 8: Crude protein concentration of 20%, *Lp* of 240 and *Pa* with 240 µL). All cultures were incubated at 30 ˚C. A completely randomized experimental design with a 2 × 2 × 2 factorial arrangement (Table 9) and four replicates per treatment was used.

Phase III: Thirty two Falcon® plastic tubes of 50 mL with 10 g of a mix of yellow cornflour and meat meal, 4% of molasses, and a substrate:water ratio (1:2) were used. Tubes were divided into three factors: First, crude protein concentration with two levels: 18 and 20%. Second, inoculum addition of *Pediococcus pentosaceus* (*Pp*) at two levels: 120 and 240 µL. Third, inoculum addition of *Pediococcus acidilacticiti* (*Pa*) at two levels: 120 and 240 µL. Protein levels were calculated with the nutritional composition of cornflour and meat meal, according to the NRC tables [48]. Tubes were distributed in eight treatments (Treatment 1: Crude protein concentration of 18%, *Pp* of 120 and *Pa* with 120 µL; Treatment 2: Crude protein concentration of 18%, *Pp* of 120 and *Pa* with 240 µL, Treatment 3: Crude protein concentration of 18%, *Pp* of 240 and *Pa* with 120 µL; Treatment 4: Crude protein concentration of 18%, *Pp* of 240 and *Pa* with 240 µL 480 µL, Treatment 5: Crude protein concentration of 20%, *Pp* of 120 and *Pa* with 120 µL; Treatment 6: Crude protein concentration of 20%, *Pp* of 120 and *Pa* with 240 µL, Treatment 7: Crude protein concentration of 20%, *Pp* of 240 and *Pa* with 120 µL; Treatment 8: Crude protein concentration of 20%, *Pp* of 240 and *Pa* with 240 µL). All cultures were incubated at 30 ˚C. A completely randomized experimental design with a 2 × 2 × 2 factorial arrangement (Table 9) and four replicates per treatment was used.

Measurement of response variables. At the end of fermentation time, fermented substrate was homogenized in vortex and two fractions were taken: 3 g of fermented substrate were used to measure pH (Conductronic pH120); 3 g of fermented substrate was centrifuged at 5,000 rpm for 15 min and the supernatant was used for lactic acid, acetic acid and ethanol analysis with an HPLC

Perkin Elmer LC250, SHIMADZU, by using the isocratic method described by Diano *et al.* [49]. An Aminex HPX-87H column was used at 60 ˚C, with a mobile phase of H_2SO_4 at 5 mM and a flow of 0.6 mL min^{-1}. A refractive index detector (LC-30, SHIMADZU) was used.

Table 9. Treatments in fermented corn with two crude protein levels and inoculum addition of *Pediococcus pentosaceus*, *Pediococcus acidilacticiti* and *Lactobacillus plantarum*.

Phase I combinations	Phase II combinations	Phase III combinations
N1 *Pp1 Lp1*	N1 *Pa1 Lp1*	N1 *Pp1 Pa1*
N1 *Pp1 Lp2*	N1 *Pa1 Lp2*	N1 *Pp1 Pa2*
N1 *Pp2 Lp1*	N1 *Pa2 Lp1*	N1 *Pp2 Pa1*
N1 *Pp2 Lp2*	N1 *Pa2 Lp2*	N1 *Pp2 Pa2*
N2 *Pp1 Lp1*	N2 *Pa1 Lp1*	N2 *Pp1 Pa1*
N2 *Pp1 Lp2*	N2 *Pa1 Lp2*	N2 *Pp1 Pa2*
N2 *Pp2 Lp1*	N2 *Pa2 Lp1*	N2 *Pp2 Pa1*
N2 *Pp2 Lp2*	N2 *Pa2 Lp2*	N2 *Pp2 Pa2*

Crude protein level (N1=18% and N2=20%), *Pediococcus pentosaceus* inclusion (*Pp1*=120 and *Pp2*=240 μL), *Pediococcus acidilacticiti* addition (*Pa1*=120 and *Pa2*=240 μL) and *Lactobacillus plantarum* addition (*Lp1*=120 and *Lp2*=240 μL).

Statistical analysis. For all phases, a completely randomized experimental design with a $2 \times 2 \times 2$ factorial arrangement (Table 9) with four replicates per treatment was used. The experimental design used in each of the three phases met the statistical assumptions: normality (Shapiro's test), variances homogeneity (Bartlett's test), and independence. The last was completed when assigning the experimental units for the different treatments by using randomization with R program version 3.5.1. [50]. The statistical analysis of variance for three stages was evaluated by employing GLM procedure with the statistical program, SAS (2012) version 9.4. Analysis of variance (ANOVA) was used and compared in Tukey's multiple range tests. Differences between treatments and interactions were considered significant when $P < 0.05$, and all values are expressed as the means ± standard error.

2.4. RESULTS

Phase I. Effect of crude protein concentration, *Lactobacillus plantarum* and *Pediococcus pentosaceus* addition in fermented corn

The crude protein concentration and *Lactobacillus plantarum* addition (Table 13) affected ($P < 0.001$) the final pH in fermented corn, where the best levels to decrease the final pH (Figure 12) were the crude protein concentration of 20% and *Lp* addition with 240 µL. *Pediococcus pentosaceus* addition did not affect ($P > 0.05$) the individual response to the final pH, or lactic acid, acetic acid and ethanol production in fermented corn.
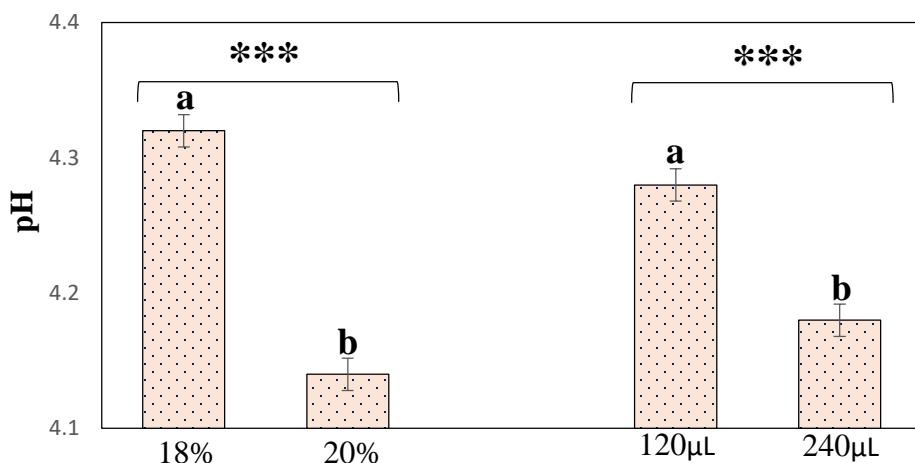
Figure 12. Final pH in response to crude protein level (18 and 20%) [left side], and inoculum addition of *Lactobacillus plantarum* (120 and 240 µL) [right side] in fermented corn. [ab]Values without similar letters are significantly different ($P < 0.05$), ***($P < 0.001$).

The crude protein concentration affected ($P < 0.001$) lactic acid production in fermented corn; the best level (Figure 2) was with the crude protein concentration of 18%. The analysis of variance (Table 2) showed no significant interaction ($P > 0.05$) among crude protein concentration, *Lactobacillus plantarum* and *Pediococcus pentosaceus* addition for the final pH. But there were several significant interactions for the lactic acid concentration. The first significant interaction ($P < 0.001$) was between the crude protein concentration and the inclusion of *Pediococcus pentosaceus;* the best combination (Figure 2) for the lactic acid concentration was the crude protein concentration of 18% and *Pp* addition with 120 µL. The second significant interaction ($P < 0.05$) was between the crude protein concentration and the inclusion of *Lactobacillus plantarum;* the biggest lactic acid concentration was (Figure 2) with the combination between crude protein concentration of 18% and *Lp* addition with 120 µL.
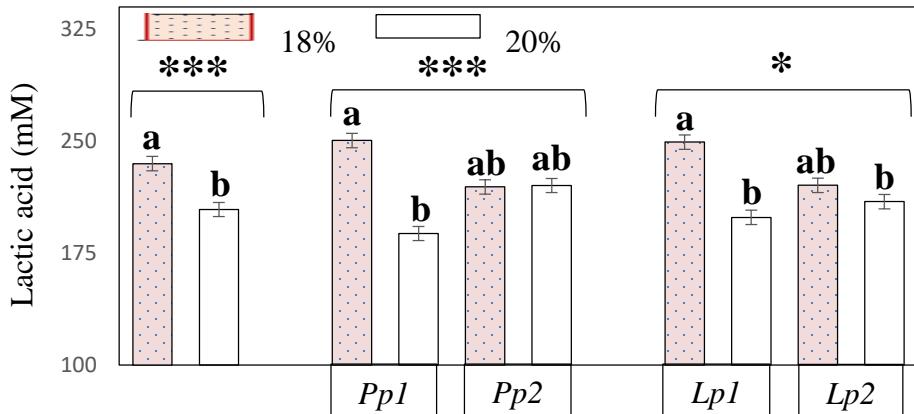
Figure 13. Lactic acid concentration in response to crude protein level (18% and 20%) [left side], interaction of crude protein level and *Pediococcus pentosaceus* addition (*Pp1*=120 and *Pp2*=240 µL) [central side] and interaction of crude protein level and *Lactobacillus plantarum* addition (*Lp1*=120 and *Lp2*=240 µL) [right side] in fermented corn. [ab]Values without similar letters are significantly different (P < 0.05), *(P < 0.05) ***(P < 0.001).

It´s very important to remark that for the first and second interactions both combinations were the same. In other hands, the best combination for lactic acid production was not only with crude protein concentration of 18% but also the inoculum inclusion with 120 µL.

The third significant interaction (P = 0.004) was between *Pediococcus pentosaceus* and *Lactobacillus plantarum* addition levels where the best combination (Figure 3) for the lactic acid concentration was the *Pp* of 240 µL and *Lp* addition with 120 µL.

Finally, the fourth significant interaction (P = 0.0003) was among all factors, the crude protein concentration, *Pediococcus pentosaceus* and *Lactobacillus plantarum* addition; the most important combination (Figure 15) for the lactic acid production was the crude protein concentration of 18%, the *Pp* of 120 µL and *Lp* addition with 240 µL.
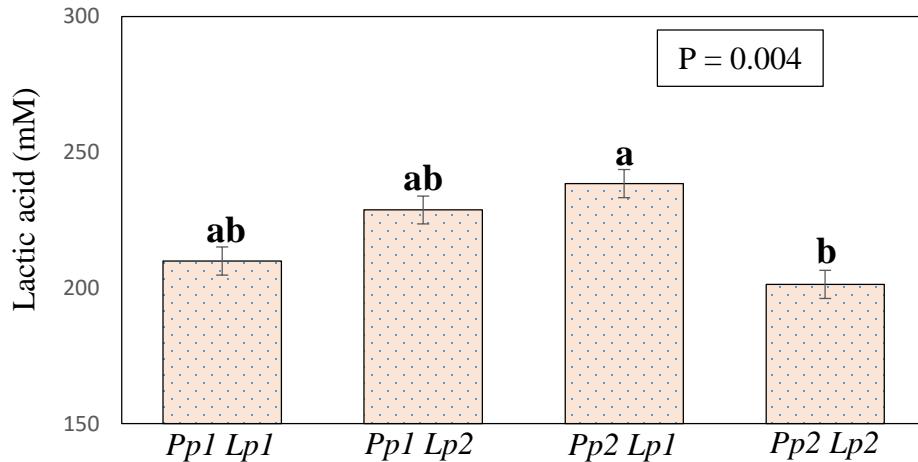
Figure 14. Lactic acid concentration in response to *Pediococcus pentosaceus* (*Pp1*=120 and *Pp2*=240 μL) and *Lactobacillus plantarum* addition (*Lp1*=120 and *Lp2*=240 μL) in fermented corn. [ab]Values without similar letters are significantly different (P < 0.05).

High quality FLF based on corn was possible in the phase I because of factors and levels. Eight combinations were developed of high quality FLF based on corn with 24 h of fermentation (Table 3), four combinations were for crude protein concentration of 18% and the other four for crude protein concentration of 20%.
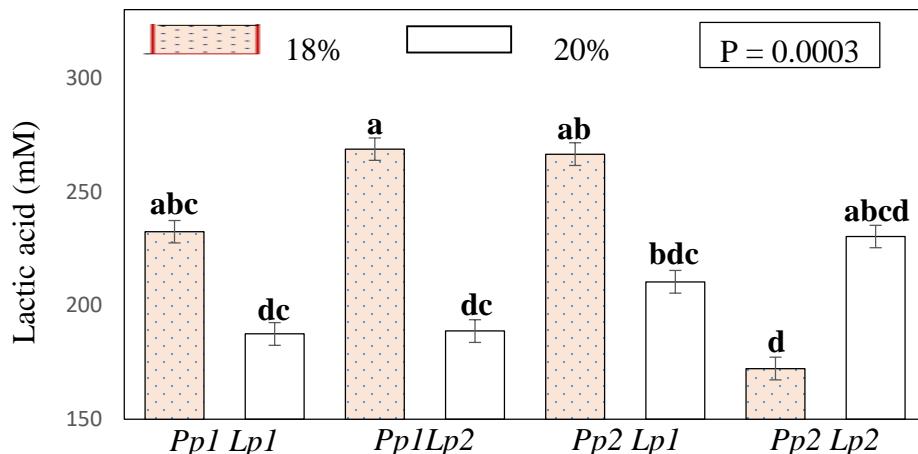
Figure 15. Lactic acid concentration in response to crude protein level (18% and 20%), *Pediococcus pentosaceus* (*Pp1*=120 and *Pp2*=240 μL), and *Lactobacillus plantarum* addition (*Lp1*=120 and *Lp2*=240 μL) in fermented corn. [ab]Values without similar letters are significantly different (P < 0.05).

Table 10. Quality treatments in fermented corn with two crude protein levels and inoculum addition of *Pediococcus pentosaceus and Lactobacillus plantarum.*

Variable	Final pH	Lactic acid (mM)	Acetic acid (mM)	Ethanol (mM)
Combinations		Quality values		
	4.5	150	40	0.8
N1 *Pp1 Lp1*	4.23	232.4	5.63	ND
N1 *Pp1 Lp2*	4.06	268.7	9.45	ND
N1 *Pp2 Lp1*	4.17	266.6	6.75	ND
N1 *Pp2 Lp2*	4.12	172.3	4.21	ND
N2 *Pp1 Lp1*	4.4	187.5	5.01	ND
N2 *Pp1 Lp2*	4.29	188.8	5.39	ND
N2 *Pp2 Lp1*	4.35	210.4	6.39	ND
N2 *Pp2 Lp2*	4.26	230.3	5.71	ND

Crude protein level (N1=18% and N2=20%), *Pediococcus pentosaceus* inclusion (*Pp1*=120 and *Pp2*=240 µL), and *Lactobacillus plantarum* addition (*Lp1*=120 and *Lp2*=240 µL). ND = not detectable.

Phase II. Effect of crude protein concentration, *Pediococcus acidilacticiti* and *Lactobacillus plantarum* addition in fermented corn

The crude protein concentration (Table 14) did not affect the final pH ($P > 0.05$). On the contrary, *Pediococcus acidilacticiti* as an individual addition modified ($P < 0.0001$) the final pH; where the best level to decrease the final pH was the *Pa* addition (Figure 5) with 240 µL. The first significant interaction ($P < 0.0001$) was between the inoculum size of *Pediococcus pentosaceus* and the crude protein level; the best combination (Figure 16) for the final pH was the *Pa* of 240 µL and crude protein concentration of 18%.

Similarly, the individual addition of *Lactobacillus plantarum* changed ($P < 0.0001$) the final pH; the best level to decrease the final pH was the *Lp* addition (Figure 17) with 240 µL. The second significant interaction ($P < 0.01$) was between the inoculum size of *Lactobacillus plantarum* and the crude protein level; the best combination (Figure 17) for the final pH was the *Lp* of 240 µL and crude protein concentration of 18%.
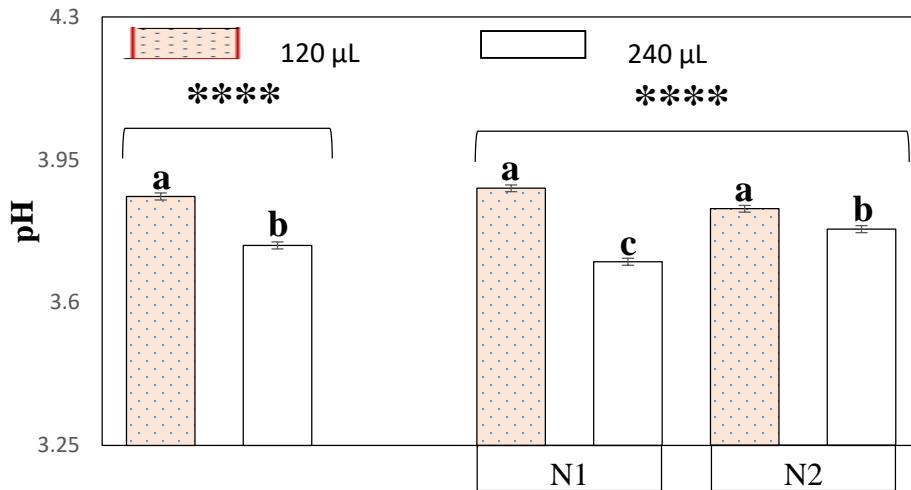
Figure 16. Final pH in response to *Pediococcus acidilacticiti* addition (120 and 240 μL) [left side], and interaction of inoculum size and crude protein level (N1=18 and N2=20%) [right side]. [ab]Values without similar letters are significantly different (P < 0.05). ****(P < 0.0001).
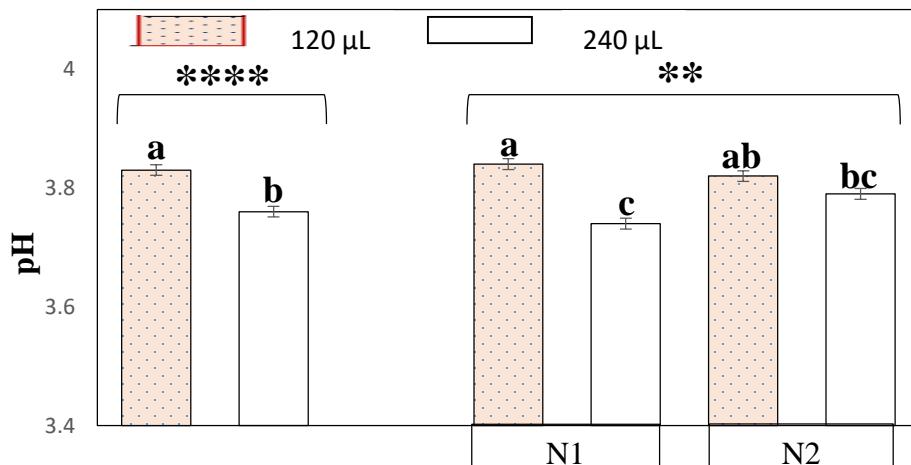
Figure 17. Final pH in response to *Lactobacillus plantarum*, addition (120 and 240 μL) [left side], and interaction of inoculum size and crude protein level (N1=18 and N2=20%) [right side]. [ab]Values without similar letters are significantly different (P < 0.05). **(P < 0.01), ****(P < 0.0001).

The lactic acid production was similar (P > 0.05) because of the crude protein concentration; in which both levels of protein, 18 and 20%, produced (Figure 7) the same response. The individual response to inoculum levels of *Pediococcus acidilacticiti* affected (P = 0.004) the lactic acid production; the best level (Figure 7) for the lactic acid concentration was the *Pa* addition with 120 μL. Likewise, the individual response to inoculum levels of *Lactobacillus plantarum* changed (P=0.004) the lactic acid production: the best level (Figure 7) for the lactic acid concentration was the *Lp* addition with 120 μL. It´s very important to mention that the individual response in both

Pediococcus acidilacticiti (P < 0.01) and *Lactobacillus plantarum* (P < 0.05) addition the level (120 µL) to achieve the biggest lactic acid production was the same (Figure 7). The analysis of variance (Table 4) showed no significant interaction (P > 0.05) among crude protein concentration, *Pediococcus acidilacticiti* and *Lactobacillus plantarum* addition for the lactic acid concentration.
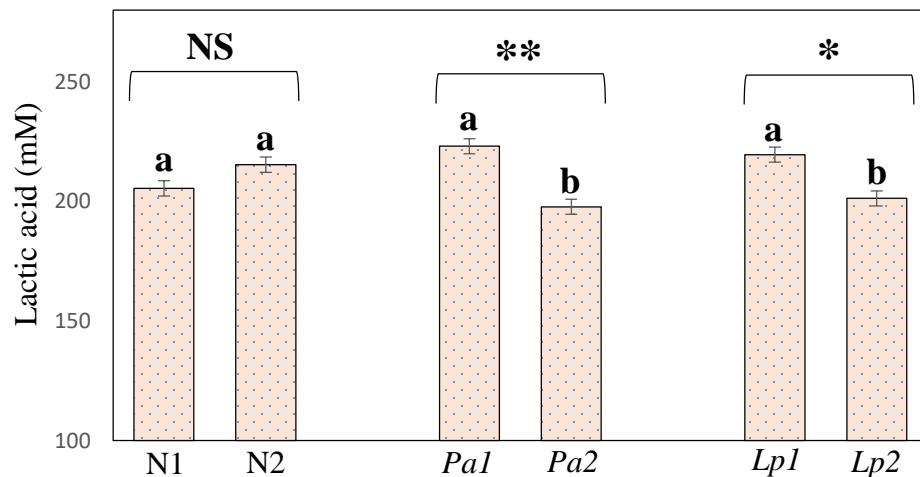
Figure 18. Lactic acid concentration in response to crude protein level (18 and 20%) [left side], *Pediococcus acdilacticiti* (*Pa1*=120 and *Pa2*=240 µL) [central side], and *Lactobacillus plantarum* addition (*Lp1*=120 and *Lp2*=240 µL) [right side]. [ab]Values without similar letters are significantly different (P < 0.05). *(P < 0.05), **(P < 0.01), NS (No significant).

High quality fermented liquid feed based on corn was possible in the phase II because of factors and levels. Eight combinations were developed of high quality fermented liquid feed based on corn with 24 h of fermentation (Table 11), four combinations were for crude protein concentration of 18% and the other four for crude protein concentration of 20%.

Table 11. Quality treatments in fermented corn with two crude protein levels and inoculum addition of *Pediococcus acidilacticiti* and *Lactobacillus plantarum*.

Variable	Final pH	Lactic acid (mM)	Acetic acid (mM)	Ethanol (mM)
Combinations		Quality values		
	4.5	150	40	0.8
N1 *Pa1 Lp1*	3.93	242.7	4.02	ND
N1 *Pa1 Lp2*	3.83	200.4	2.65	ND
N1 *Pa2 Lp1*	3.75	197.7	2.16	ND
N1 *Pa2 Lp2*	3.65	179.2	2.18	ND
N2 *Pa1 Lp1*	3.84	224.6	4.31	ND
N2 *Pa1 Lp2*	3.83	224.5	3.56	ND
N2 *Pa2 Lp1*	3.83	211.3	3.51	ND
N2 *Pa2 Lp2*	3.75	200.1	2.45	ND

Crude protein level (N1=18% and N2=20%), *Pediococcus acidilacticiti* addition (*Pa1*=120 and *Pa2*=240 µL), and *Lactobacillus plantarum* addition (*Lp1*=120 and *Lp2*=240 µL). ND = not detectable.

Phase III. Effect of crude protein concentration, *Pediococcus pentosaceus* and *Pediococcus acidilacticiti* addition in fermented corn

The crude protein concentration (Table 15) affected ($P < 0.001$) the final pH; the best level (Figure 8) to reduce the final pH was the crude protein concentration of 20%. Additionally, both *Pediococcus pentosaceus* ($P < 0.01$) and *Pediococcus acidilacticiti* as an individual addition modified ($P < 0.001$) the final pH, where the best levels to decrease the final pH were the *Pp* addition (Figure 19) with 240 µL and the *Pp* addition (Figure 19) with 240 µL.
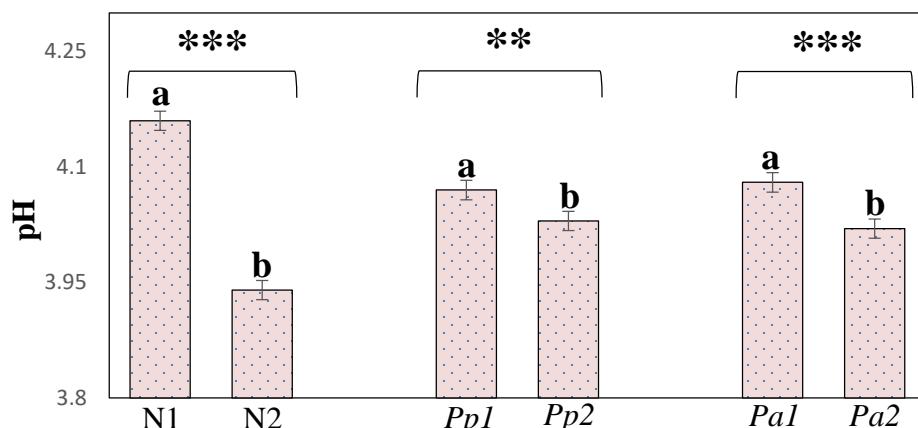
Figure 19. Final pH in response to crude protein level (N1=18 and N2=20%) [left side], *Pediococcus pentosaceus* (*Pp1*=120 and *Pp2*=240 µL) [central side], and *Pediococcus acidilacticiti* addition (*Pa1*=120 and *Pa2*=240 µL) [right side]. [a,b]**Values without similar letters are significantly different ($P < 0.05$). **($P < 0.01$), ***($P < 0.001$).**

The lactic acid production was increased ($P < 0.05$) due to the crude protein concentration, in which the protein level of 20% produced (Figure 9) the highest response. The individual response to inoculum levels of *Pediococcus pentosaceus* affected ($P < 0.001$) the lactic acid production; the best level (Figure 20) for the lactic acid concentration was the *Pp* addition with 120 µL. However, the individual response to inoculum levels of *Pediococcus acidilacticiti* did not change ($P > 0.05$) the lactic acid production (Figure 20).
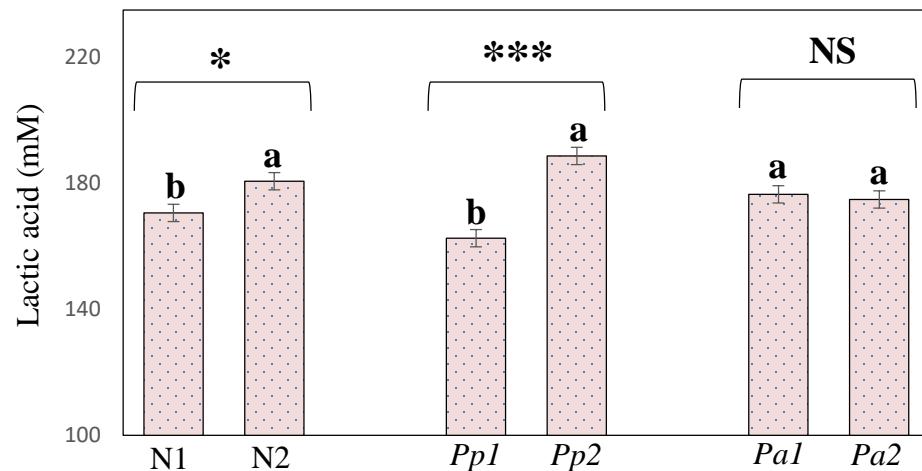
Figure 20. Lactic acid concentration in response to crude protein level (N1=18 and N2=20%) [left side], *Pediococcus pentosaceus* (*Pp1*=120 and *Pp2*=240 µL) [central side], and *Pediococcus acidilacticiti* addition (*Pa1*=120 y *Pa2*=240 µL) [right side]. [ab]Values without similar letters are significantly different ($P < 0.05$). *($P < 0.05$), *($P < 0.001$). NS (No significant).**

The analysis of variance (Table 15) indicated a couple of significant interactions for the final pH. The first significant interaction ($P < 0.001$) was between the crude protein concentration and the inclusion of *Pediococcus pentosaceus;* the best combination (Figure 21) to reduce the final pH was the crude protein concentration of 20% and *Pp* addition with 120 µL. The second significant interaction ($P = 0.036$) was between the crude protein concentration and *Pediococcus acidilacticiti* addition; the most important combination (Figure 21) to reduce the final pH was the crude protein concentration of 20%, and *Pa* addition with 240 µL. The analysis of variance (Table 15) showed no significant interaction ($P > 0.05$) among crude protein concentration, *Pediococcus pentosaceus* and *Pediococcus acidilacticiti* addition for the final pH.
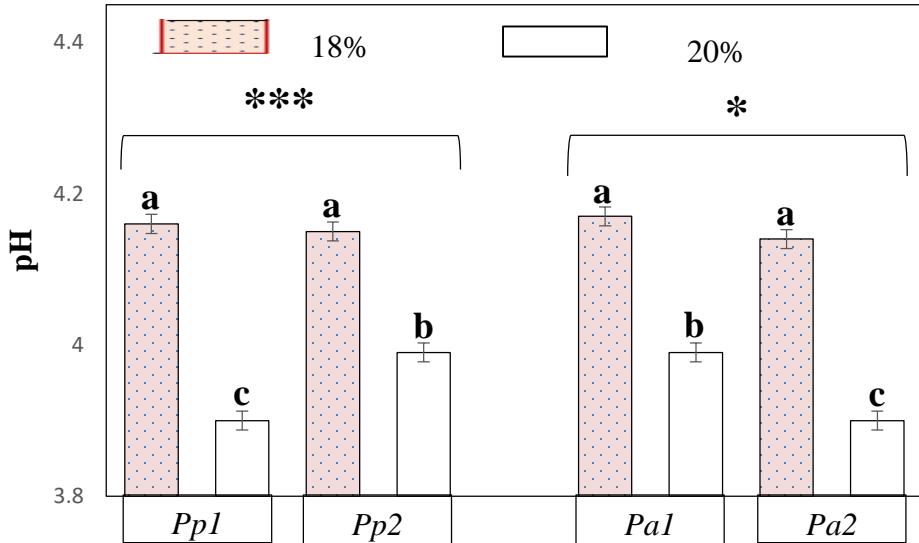
Figure 21. Final pH in response to the interaction between crude protein level (18 and 20%) and *Pediococcus pentosaceus* addition (*Pp1*=120 and *Pp2*=240 µL) [left side], and the interaction between crude protein level and *Pediococcus acidilacticiti* addition (*Pa1*=120 and *Pa2*=240 µL) [right side]. [ab]Values without similar letters are significantly different (P < 0.05). *(P < 0.05), ***(P < 0.001).

There were two significant interactions in this phase for the lactic acid concentration. The first significant interaction (P = 0.021) was between the crude protein concentration and the inclusion of *Pediococcus pentosaceus;* the best combination (Figure 22) for the lactic acid concentration was with both levels 18 and 20% of crude protein concentration and *Pp* addition of 240 µL. Finally, the second significant interaction (P < 0.001) was between the crude protein concentration and the inclusion of *Pediococcus acidilacticiti;* the biggest lactic acid concentration was (Figure 22) with the combination between crude protein concentration of 20% and *Pa* addition with 240 µL. The analysis of variance (Table 15) showed no significant interaction (P > 0.05) among crude protein concentration, *Pediococcus pentosaceus* and *Pediococcus acidilacticiti* addition for the lactic acid production.
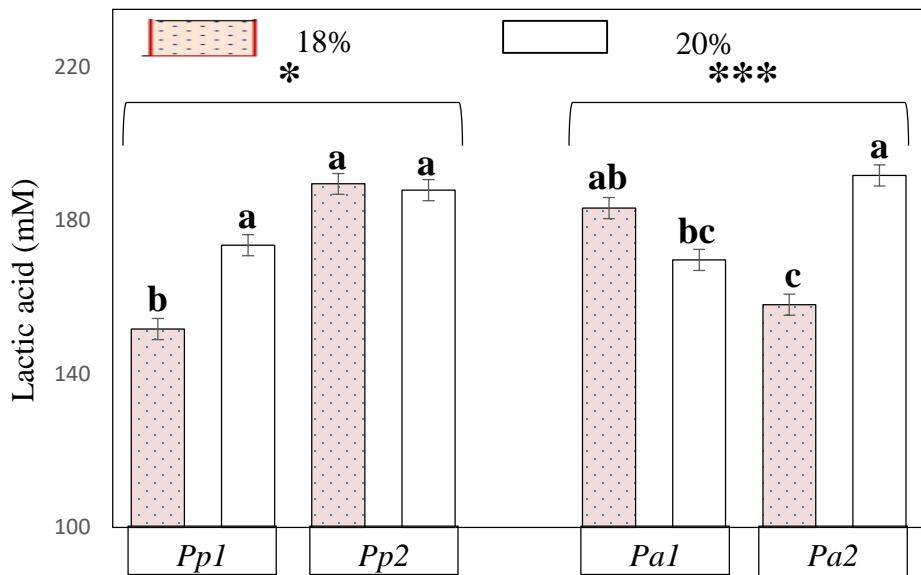
Figure 22. Lactic acid concentration in response to the interaction between crude protein level (18 and 20%) and *Pediococcus pentosaceus* addition (*Pp1*=120 and *Pp2*=240 µL) [left side], and the interaction between crude protein level and *Pediococcus acidilacticiti* addition (*Pa1*=120 and *Pa2*=240 µL) [right side]. [ab]Values without similar letters are significantly different (P < 0.05). *(P < 0.05), *(P < 0.001).**

High quality fermented liquid feed based on corn was possible in the phase III because of factors and levels. Seven combinations were developed for high quality fermented liquid feed based on corn with 24 h of fermentation (Table 7), three of them were for crude protein concentration of 18% and the other four for crude protein concentration of 20%.

Table 12. Quality treatments in fermented corn with two crude protein levels and inoculum addition of *Pediococcus pentosaceus and Pediococcus acidilacticiti*.

Variable	Final pH	Lactic acid (mM)	Acetic acid (mM)	Ethanol (mM)
Combinations		Quality values		
	4.5	150	40	0.8
N1 *Pp1 Pa1*	4.19	165.5	ND	ND
N1 *Pp1 Pa2*	4.13	137.9	ND	ND
N1 *Pp2 Pa1*	4.16	200.0	ND	ND
N1 *Pp2 Pa2*	4.15	178.1	ND	ND
N2 *Pp1 Pa1*	3.94	169.9	ND	ND
N2 *Pp1 Pa2*	3.85	177.3	ND	ND
N2 *Pp2 Pa1*	4.03	169.7	ND	ND
N2 *Pp2 Pa2*	3.94	206.1	ND	ND

Crude protein level (N1=18% and N2=20%), *Pediococcus pentosaceus* inclusion (*Pp1*=120 and *Pp2*=240 µL), and *Pediococcus acidilacticiti* addition (*Pa1*=120 and *Pa2*=240 µL). ND = not detectable.

2.5. DISCUSSION

The use of fermented liquid feed in pigs is particularly beneficial for weaned piglets and it has two main advantages: First, animals obtain food and water simultaneously, which not only reduces the time for animals when finding both nutrients [24, 51], but also this diet represents a less drastic change than the transition from milk to a traditional dry diet during weaning [52–54]. Second, fermented liquid feed reduces the pH in diet, therefore the animal's stomach improves its role as the first defense against pathogenic microorganisms [37, 52, 55]. However, the use of fermented liquid feed has shown different results, because of the animal weight, ingredient composition, and the quality of fermented liquid feed [56, 57].

Fermented liquid feed in weaned piglets with European ingredients. Bunte *et al.* [58] reported that feeding fermented liquid feed (cereal composition of rye, barley and wheat) in piglets had an influence on the bacterial composition in the small intestine, large intestine and up to faeces. But Bunte *et al.* [58] informed no differences on growth performance neither between control and full fermented diet nor between control and partially fermented diet were found. Bunte *et al.* [58] used an inoculum of *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Lactococcus lactis*, a temperature of 34–38 °C with 24 h of fermentation. These conditions, except temperature, were similar to our three stages conditions.

Fermented liquid feed in in grow-finisher pigs with European ingredients. O'Meara *et al.* [59] described that fermented whole diet liquid feeding results not only into a lower carcass growth but also in a poorer feed efficiency in grow-finisher pigs compared to cereal fraction fermented liquid feed. Based on O'Meara *et al.* [59] when offering fermented liquid feed (cereal composition of barley and wheat) in grow-finisher pigs in whole fermentation diet decreased growth and feed utilization in this treatment; as a result, whole fermented liquid feed is not recommended for grow-finisher pigs. O'Meara *et al.* [59] used an inoculum of *Lactobacillus plantarum*, and *Pediococcus acidilacticiti*, with 48 h of fermentation. Their study did not achieve fermented liquid feed quality values; especially, about ethanol concentration (quality fermented liquid feed contain less than 0.8 mM). Ethanol concentration of fermented cereal fraction had 50, 41 and 37 mM for fermented tank, mixing tank and through, respectively. Additionally, ethanol concentration of whole fermented liquid feed had 63, 63 and 51 mM for fermented tank, mixing tank and through, respectively. The elevated ethanol and acetate concentrations have a negative impact of feed intake in pigs [60, 61]. That is why it is very important to produce a quality fermented liquid feed for pigs; in the phase I of the present study a high quality fermented liquid feed based on corn was possible with 24 h of fermentation, four combinations were with crude protein of 18% and the other four with crude protein of 20%.

Lactic acid has both bacteriostatic and bactericidal functions, which depends on the inclusion level or its concentration in fermented liquid feed [62, 63]. Beal *et al.* [64] observed that to prevent the growth of *Salmonella spp*., fermented liquid feed required at least 75 mM of lactic acid. Brooks *et al.* [51] reported that by decreasing the number of enterobacteria, the lactic acid concentration must exceed 100 mM. However, the use of fermented liquid feed has shown different results especially on ingredients different from the swine European feeding [65, 66].

Fermented liquid feed in both piglets and grow-finisher pigs with corn by product ingredient. Rho *et al.* [39] described that treating corn DDGS with xylanases with or without liquid fermentation improved feed efficiency in piglets for phase 1. However, these benefits were not observed in phase 2. Rho *et al.* [39] used spontaneous fermentation without inoculum addition, a temperature of 40 °C after 10 d of fermentation. These conditions, explicitly about both inoculum inclusion and temperature, were very different from our three stages conditions. Rho *et al.* [39] were looking for lactic acid concentrations above 100 mM to prevent unwanted pathogens and reduce gastric pH, it is a lower concentration than our quality value (150 mM) for the present study. Rho *et al.*

[39] reported that the pH for the steeped corn DDGS decreased to an acceptable range (less than 4.5 mM) from day 3 to day 10 of fermentation. But lactic acid and acetic acid concentrations were within the quality ranges only on day 3. In other words, fermentation of corn DDGS did not reach stable conditions as indicated by the reduction of pH and lactic acid and acetic acid concentrations from day 3 to day 10 of fermentation.

Fermented liquid feed in both piglets and grow-finisher pigs with corn as a main ingredient. Xin *et al.* [67] indicated that fermented liquid feed (composition of corn, rice bran and wheat bran) improved the growth performance and digestibility of nutrients not only for piglets but also for grow-finisher pigs. According to Xin *et al.* [67] FLF regulated the secretion of gastrointestinal hormones, improved intestinal microbiota and increased volatile fatty acids in colon digesta and improved intestinal microbiota. Xin *et al.* [67] used controlled fermentation; the process was developed according to patent (no.201910736307.0). Xin *et al.* [67] reported good results for fermented liquid feed in pigs with lactic acid concentration above 100 mM, but again it is a lower concentration than our quality value (150 mM) for our study. In sum, fermented liquid feed has shown better results including in both piglets and grow-finisher pigs only with quality fermented liquid feed, and it is possible under controlled fermentation conditions [67–69].

The quality fermented liquid feed development has the potential to reduce the antibiotic as growth promoter for swine feed industry [70–72]. The main recommendation to avoid the nutrients loss is by fermenting only the cereal fraction; which contrasted with the results of our present study, because it affects the carbon and nitrogen ratio, an important factor for lactic acid bacteria [59, 73]. Spontaneous fermentation caused loss of essential nutrients; specifically essential AA. [73]. Spontaneous fermentation and its associated issues are commonplace when fresh liquid feeding is practiced on commercial pig production units [73]. The best way to reduce the nutrients loss is with implementation of suitable control strategies, by developing good guidelines for the sanitation practices management of liquid feeding systems [59, 73]. However, a high quality fermented liquid feed based on corn was possible in all phases of the present study with 24 h of fermentation, with two crude protein levels (18 and 20%).

Potential advantages of fermented liquid feed with reduction of crude protein. A high quality fermented liquid feed with reduction of crude protein is possible. As a result, it has several advantages for fermented liquid feed: prevents loss of AA during fermentation, saves protein ingredients, decreases not only nitrogen excretion, but also feeding cost without affecting growth

performance compared to traditional diets for pigs [74–77]. Finally, fermented liquid feed affects the gut ecology in a different way than antibiotics and contributes to the enhanced bacterial diversity in the gastrointestinal tract [45]. The increase for intestinal microbiota reduces diarrhea, improves the intestinal barrier and intestinal villi in pigs. Animal intestines are colonized by many microorganisms, and these organisms differ in both type and number. Among these microorganisms, lactic acid bacteria, which are dominant intestinal microbiota, have received worldwide attention and examination [79, 80].

Potential advantages of fermented liquid feed with lactic acid bacteria combination. Studies have shown that lactic acid bacteria can regulate the common ratio of intestinal microbiota and maintain the intestinal balance of this ecosystem [46, 80]. Lactic acid bacteria are capable of producing inhibitory substances, other than organic acids, that have inhibitory activities against different microorganisms. Lactic acid bacteria produce antimicrobial substances such as hydrogen peroxide, carbon dioxide, diacetyl, reuterin and bacteriocins; therefore, fermented liquid feed provides a good opportunity to use these antimicrobial substances by reducing antibiotic as growth promoter in pigs. However, not all lactic acid bacteria will reduce food-borne pathogens in farm animals; therefore, the careful selection of strains to be supplied under appropriate conditions is important [45, 81]. That is why it is essential to produce a quality fermented liquid feed for pigs; in all phases of the present study a high quality fermented liquid feed based on corn was possible with 24 h of fermentation, with different levels and combinations of *Lactobacillus plantarum, Pediococcus pentosaceus and Pediococcus acidilacticiti* and potential to replace antibiotics as growth promoters and reduce microbial resistance.

2.6. CONCLUSIONS

Through a three-phase statistical analysis, the fermentation conditions established in the evaluated variables developed 23 combinations of a high quality fermented liquid corn-based for pigs with a low crude protein content and inoculating *Lactobacillus plantarum, Pediococcus pentosaceus* and *Pediococcus acidilacticiti*. This high quality diet was obtained as a pig feed with the potential to replace antibiotics as growth promoters and reduce microbial resistance. It is possible to reduce the amount of crude protein by 2% in fermented corn avoiding quality reduction, and even in phase I the quality improves by reducing the protein content. It is also possible to obtain high-quality fermented liquid swine feed with lactic acid bacteria combination, hence LAB are capable of

producing inhibitory substances against microorganisms, and these antimicrobial substances provide a good opportunity for the future to reduce AGP in pigs.

Abbreviations

FLF: Fermented liquid feed; LAB: Lactic acid bacteria; AGP: Antibiotics as growth promoters; CP: Crude protein; AA: Amino acids; FT: Fermentation time; USA: United States of America; CPC; Crude protein concentration; Lp: Inoculum addition of *Lactobacillus plantarum*; Pp: Inoculum addition of *Pediococcus pentosaceus;* Pa: Inoculum addition of *Pediococcus acidilacticiti;* NRC: National Research Council; HPLC: High performance liquid chromatography; GLM: General lineal models; mM: Milimolar.

Supplementary Information

It is not applicable.

Authors' contributions

Nicolás S. Espinosa-García designed the experiment, conducted the laboratory work, and wrote the manuscript. José Luis Figueroa-Velasco, José Alfredo Martínez-Aispuro and María Teresa Sánchez-Torres-Esqueda analyzed the study data and helped to revise the manuscript. Ernesto Favela-Torres gave advice on the microbiological work and the HPLC analyzes. All authors have read and approved the final manuscript.

Availability of data and materials

The data analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

It is not applicable.

Consent for publication

It is not applicable.

Competing interests

The authors declare that they don't have any conflict of interest.

Author details

[1]Programa de Ganadería, Campus Montecillo, Colegio de Postgraduados. 56230. Montecillo, Texcoco, Estado de México. (jlfigueroa@colpos.mx). [2]Departamento de Biotecnología, Unidad Iztapalapa, Universidad Autónoma Metropolitana. 09340. Iztapalapa, Cuidad de México.

Table 13. Effect of crude protein level, *Pediococcus pentosaceus*, *Lactobacillus plantarum* addition in fermented corn on the final pH and the concentration of lactic acid, acetic acid and ethanol.

N1$Pp1Lp1$	N1$Pp1Lp2$	N1$Pp2Lp1$	N1$Pp2Lp2$	N2$Pp1Lp1$	N2$Pp1Lp2$	N2$Pp2Lp1$	N2$Pp2Lp2$	Probability						
								N	Pp	Lp	N*Pp	N*Lp	Pp*Lp	N*Pp*Lp
Final pH														
4.23±0.03	4.06±0.03	4.17±0.03	4.12±0.02	4.40±0.03	4.29±0.03	4.35±0.02	4.26±0.02	0.001	0.343	0.001	0.482	0.801	0.182	0.318
Lactic acid (mM)														
232.4±11	268.7±11	266.6±11	172.3±11	187.5±9	188.8±9	210.4±9	230.3±9	0.001	0.952	0.307	0.001	0.033	0.004	0.001
Acetic acid (mM)														
5.63±0.4	9.45±0.5	6.75±0.4	4.21±0.5	5.01±0.3	5.39±0.4	6.39±0.3	5.71±0.4	0.012	0.078	0.464	0.001	0.243	0.001	0.001
Ethanol (mM)														
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Data are means and standard error of each treatment; N = effect of crude protein level; N1 = crude protein level of 18%; N2 = crude protein level of 20%; Pp = effect of *Pediococcus pentosaceus* addition; $Pp1$ = *Pediococcus pentosaceus* addition of 120 µl; $Pp2$ = *Pediococcus pentosaceus* addition of 120 µl; Lp = effect of *Lactobacillus plantarum* addition; $Lp1$ = *Lactobacillus plantarum* addition of 120 µl; $Lp2$ = *Lactobacillus plantarum* addition of 240 µl; N*Pp = effect of the interaction between crude protein level and *Pediococcus pentosaceus* addition; N*Lp = effect of the interaction between crude protein level and *Lactobacillus plantarum* addition; Pp*Lp = effect of the interaction between *Pediococcus pentosaceus* and *Lactobacillus plantarum* addition; N*Pp*Lp = effect of the interaction among crude protein level, *Pediococcus pentosaceus* and *Lactobacillus plantarum* addition. ND = not detectable.

Table 14. Effect of crude protein level, *Pediococcus acidilacticiti*, *Lactobacillus plantarum* addition in fermented corn on the final pH and the concentration of lactic acid, acetic acid and ethanol.

N1*Pa1Lp1*	N1*Pa1Lp2*	N1*Pa2Lp1*	N1*Pa2Lp2*	N2*Pa1Lp1*	N2*Pa1Lp2*	N2*Pa2Lp1*	N2*Pa2Lp2*	Probability						
								N	*Pa*	*Lp*	N*Pa	N*Lp	*Pa**Lp	N*Pa*Lp
Final pH														
3.93±0.02	3.83±0.02	3.75±0.02	3.65±0.02	3.84±0.02	3.83±0.02	3.83±0.02	3.75±0.02	0.292	0.001	0.001	0.001	0.013	0.489	0.611
Lactic acid (mM)														
242.7±8	200.4±7	197.7±7	179.2±6	224.6±6	224.5±6	211.3±5	200.1±5	0.235	0.004	0.032	0.405	0.119	0.725	0.331
Acetic acid (mM)														
4.02±0.3	2.65±0.2	2.16±0.2	2.18±0.5	4.31±0.3	3.56±0.2	3.51±0.3	2.45±0.2	0.006	0.001	0.002	0.653	0.631	0.258	0.084
Ethanol (mM)														
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Data are means and standard error of each treatment; N = effect of crude protein level; N1 = crude protein level of 18%; N2 = crude protein level of 20%; *Pa* = effect of *Pediococcus acidilacticiti* addition; *Pa1* = *Pediococcus acidilacticiti* addition of 120 µl; *Pa2* = *Pediococcus acidilacticiti* addition of 240 µl; *Lp* = effect of *Lactobacillus plantarum* addition; *Lp1* = *Lactobacillus plantarum* addition of 120 µl; *Lp2* = *Lactobacillus plantarum* addition of 240 µl; N*Pa = effect of the interaction between crude protein level and *Pediococcus acidilacticiti* addition; N*Lp = effect of the interaction between crude protein level and *Lactobacillus plantarum* addition; *Pa**Lp = effect of the interaction between *Pediococcus acidilacticiti* and *Lactobacillus plantarum* addition; N*Pa*Lp = effect of the interaction among crude protein level, *Pediococcus acidilacticiti* and *Lactobacillus plantarum* addition. ND = not detectable.

Table 15. Effect of crude protein level, *Pediococcus pentosaceus*, *Pediococcus acidilacticiti* addition in fermented corn on the final pH and the concentration of lactic acid, acetic acid and ethanol.

N1*Pp1Pa1*	N1*Pp1Pa2*	N1*Pp2Pa1*	N1*Pp2Pa2*	N2*Pp1Pa1*	N2*Pp1Pa2*	N2*Pp2Pa1*	N2*Pp2Pa2*	Probability						
								N	*Pp*	*Pa*	N*Pp	N*Pa	*Pp**Pa	N*Pp*Pa
Final pH														
4.19±0.02	4.13±0.03	4.16±0.02	4.15±0.02	3.94±0.03	3.85±0.03	4.05±0.02	3.94±0.03	0.001	0.003	0.001	0.001	0.036	0.831	0.253
Lactic acid (mM)														
165.5±6	137.9±6	201.0±6	178.1±5	169.9±5	177.3±5	169.7±5	206.1±5	0.042	0.001	0.729	0.021	0.001	0.085	0.205
Acetic acid (mM)														
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ethanol (mM)														
ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Data are means and standard error of each treatment; N = effect of crude protein level; N1 = crude protein level of 18%; N2 = crude protein level of 20%; *Pp* = effect of *Pediococcus pentosaceus* addition; *Pp1* = *Pediococcus pentosaceus* addition of 120 µl; *Pp2* = *Pediococcus pentosaceus* addition of 240 µl; *Pa* = effect of *Pediococcus acidilacticiti* addition; *Pa1* = *Pediococcus acidilacticiti* addition of 120 µl; *Pa2* = *Pediococcus acidilacticiti* addition of 240 µl; N*Pp = effect of the interaction between crude protein level and *Pediococcus pentosaceus* addition; N*Pa = effect of the interaction between crude protein level and *Pediococcus acidilacticiti* addition; *Pp**Pa = effect of the interaction between *Pediococcus pentosaceus* and *Pediococcus acidilacticiti* addition; N*Pp*Pa = effect of the interaction among crude protein level, *Pediococcus pentosaceus* and *Pediococcus acidilacticiti* addition. ND = not detectable.

CONCLUSIONES GENERALES

En el primer estudio, a través de una investigación en cuatro etapas, se desarrolló una dieta líquida fermentada de calidad a base de maíz como alimento para cerdos con el potencial de reemplazar a los antibióticos como promotores del crecimiento y reducir la resistencia microbiana. La dieta líquida fermentada de calidad se obtuvo con la adición de *Lactobacillus plantarum* a una mezcla de maíz y proteína de origen vegetal o animal con fermentación durante 24 h a 30 ℃ y una relación sustrato: agua de 1: 2. La mejor respuesta en maíz fermentado con *Lactobacillus plantarum* se mostró sin la inclusión de goma xantana, con 1: 2 en la relación sustrato:agua y con 20% de proteína cruda de origen animal. La inclusión de melaza y el tiempo de cultivo de 24 h, produce un maíz fermentado con *Lactobacillus plantarum* de calidad.

Para el segundo estudio, se desarrolló una dieta líquida fermentada de alta calidad a base de maíz como alimento para cerdos con el potencial de reemplazar a los antibióticos como promotores del crecimiento y reducir la resistencia microbiana. La dieta líquida fermentada de alta calidad a base de maíz se obtuvo al inocular *Lactobacillus plantarum*, *Pediococcus pentosaceus* y *Pediococcus acidilacticiti* en una mezcla con una relación sustrato: agua de 1: 2, los niveles de proteína cruda de 18 y 20% y con fermentación durante 24 h a 30 ℃.

Fue posible obtener una dieta líquida fermentada de alta calidad basada en maíz para cerdos con la reducción del contenido de proteína cruda en el sustrato y con la combinación de bacterias ácido lácticas, lo que permite el uso de dietas líquidas fermentadas de alta calidad con base en maíz en lechones destetados o cerdos en crecimiento.

LITERATURA CITADA

Introducción general

Canibe, N., H. Miettinen, and B. B. Jensen. 2008. Effect of adding *Lactobacillus plantarum* or a formic acid containing-product to fermented liquid feed on gastrointestinal ecology and growth performance of piglets. Livest. Sci. 114:251–262.

Canibe, N., and B. B. Jensen. 2012. Fermented liquid feed – microbial and nutritional aspects and impact on enteric diseases in pigs. Anim. Feed Sci. Technol. 173:17–40.

Canibe, N., M. O'Dea, and S. Abraham. 2019. Potential relevance of pig gut content transplantation for production and research. J. Anim. Sci. Biotechnol. 10: 55.

Cho, J. H., Z. F. Zhang, and I. H. Kim. 2013. Effects of fermented grains as raw cereal substitutes on growth performance, nutrient digestibility, blood profiles, and fecal noxious gas emission in growing pigs. Livest. Sci. 154:131–136.

Dong, X., N. Zhang, M. Zhou, Y. Tu, K. Deng, and Q. Diao. 2014. Effects of dietary probiotics on growth performance, faecal microbiota and serum profiles in weaned piglets. Anim. Prod. Sci. 54:616–621.

Dou, S., P. Gadonna-Widehem, V. Rome, D. Hamoudi, L. Rhazi, L. Lakhal, T. Larcher, N. Bahi-Jaber, A. Pinon-Quintana, A. Guyonvarch, I. L. E. Huerou-Luron, and L. Abdennebi-Najar. 2017. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhea. PLoS ONE. 12: e0169851.

FAO: FAOSTAT Organización de las naciones unidas para la alimentación y la agricultura. 2019.

FDA, 2012. Guidance for Industry. The judicious use of medically important antimicrobial drugs in food-producing animals, department of health and human services (Food and Drug Administration), U.S.A.

FDA, 2016. New animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209. FDA Guidance for industry #213. available at: https://www.fda.gov/media/83488/download.

Guo, P., K. Zhang, X. Ma, and P. He. 2020. *Clostridium* species as probiotics: potentials and challenges. J. Anim. Sci. Biotechnol. 11:24.

He, Y., C. Jinno, K. Kim, Z. Wu, B. Tan, X. Li, R. Whelan, and Y. Liu. 2020. Dietary *Bacillus spp.* enhanced growth and disease resistance of weaned pigs by modulating intestinal microbiota and systemic immunity. J. Anim. Sci. Biotechnol. 11: 101.

Heo, J. M., F. O. Opapeju, J. R. Pluske, J. C. Kim, D. J. Hampson, and C. M. Nyachoti. 2013. gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J. Anim. Physiol. Anim. Nutr. 97:207–37.

Holmes, A. H., L. S. P. Moore, A. Sundsfjord, M. Steinbakk, S. Regmi, A. Karkey, P. J. Guerin, and L. J. Piddock. 2016. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 387:176–87.

Hu, L., S. Geng, Y. Li, S. Cheng, X. Fu, X. Yue, and X. Han. 2017a. Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. Front. Microbiol. 8:2663.

Hu, S., L. Wang, Z. Jiang. 2017b. Dietary additive probiotics modulation of the intestinal microbiota. Protein. Peptide Lett. 24:382–387.

Jensen, H. H., and D. J. Hayes. 2014. Impact of Denmark's ban on antimicrobials for growth promotion. Curr. Opin. Microbiol. 19:30–36.

Jeong, Y. D., H. S. Ko, A. Hosseindoust, Y. H. Choi, B. J. Chae, D. J. Yu, E. S. Cho, Y. H. Kim, S. M. Shim, C. S. Ra, Y. I. Kim, and J. S. Kim. 2018. Effects of dietary lactose levels and supplementation of prebiotics on growth performance in weanling pigs. J. Anim. Sci. 96:42–3.

Kim, K., Y. He, X. Xiong, A. Ehrlich, X. Li, H. Raybould, E. R. Atwill, E. A. Maga, J. Jørgensen, and Y. Liu. 2019. Dietary supplementation of *Bacillus subtilis* influenced intestinal health of weaned pigs experimentally infected with a pathogenic *E. coli.* J. Anim. Sci. Biotechnol. 10: 52.

López-Gálvez, G., M. López-Alonso, A. Pechova, B. Mayo, N. Dierick, and J. Gropp. 2020. Alternatives to antibiotics and trace elements (copper and zinc) to improve gut health and zootechnical parameters in piglets: A review. Anim. Feed Sci. and Technol. 114727.

Mao, X., C. Gu, H. Hu, J. Tang, D. Chen, B. Yu, J. He, J. Yu, J. Luo, and G. Tian. 2016. Dietary *Lactobacillus rhamnosus* gg supplementation improves the mucosal barrier function in the intestine of weaned piglets challenged by porcine rotavirus. PLoS One. 11: e0146312.

Markowiak, P., and K. Śliżewska. 2018. The role of probiotics, prebiotics and synbiotics in animal nutrition. Gut Pathog. 10: 21.

Missotten, J. A. M., J. Michiels, A. Ovyn, S. De Smet and N. A. Dierick. 2010. Fermented liquid feed for pigs. Arch. Anim. Nutr. 64:437–466.

Mocherla, V. A., N. Suiryanrayna, and J. V. Ramana. 2015. A review of the effects of dietary organic acids fed to swine. J. Anim. Sci. Biotechnol. 6: 45.

Nawab, A., W. Liu, G. Li, F. Ibtisham, D. P. Fox, Y. Zhao, J. Wo, M. Xiao, Y. Nawab, and L. An. 2019. The potential role of probiotics (nutraceuticals) in gut health of domestic animals; an alternative to antibiotic growth promoters. J. Hell. Vet. Med. Soc. 69: 1169.

Nhung, N., N. Cuong, G. Thwaites, and J. Carrique-Mas. 2016. Antimicrobial usage and antimicrobial resistance in animal production in Southeast Asia: A review. Antibiotics. 5: 37.

Plumed-Ferrer, C., and von Wright, A., 2011. Antimicrobial activity of weak acids in liquid feed fermentations, and its effects on yeasts and lactic acid bacteria. J. Sci. Food Agric. 91:1032–1040.

Rho, Y., D. Wey, C. Zhu, E. Kiarie, K. Moran, E. Van Heugten, and C. F. M de Lange. 2018. Growth performance, gastrointestinal and digestibility responses in growing pigs when fed corn-soybean meal-based diets with corn DDGS treated with fiber degrading enzymes with or without liquid fermentation. J. Anim. Sci. 96:5188–5197.

Robinson, K., Z. Deng, Y. Hou, and G. Zhang. 2015. Regulation of the intestinal barrier function by host defense peptides. Front. Vet. Sci. 2:57.

Salim, H. M. D., K. S. Huque, K. M. Kamaruddin, and M. D. A. H. Beg. 2018. Global restriction of using antibiotic growth promoters and alternative strategies in poultry production. Sci. Progr. 101:52–75.

Shi, C., Y. Zhang, Z. Lu, and Y. Wang. 2017. Solid-state fermentation of corn-soybean meal mixed feed with *Bacillus subtilis* and *Enterococcus faecium* for degrading antinutritional factors and enhancing nutritional value. J. Anim. Sci. Biotechnol. 8:50.

Tang, K. L., N. P. Caffrey. D. B. Nobrega, S. C. Cork, P. E. Ronksley, H. W. Barkema, A. J. Polachek, H. Ganshorn, N. Sharma, J. D. Kellner, S. L. Checkley, and W. A. Ghali. 2019. Comparison of different approaches to antibiotic restriction in food-producing animals: stratified results from a systematic review and meta-analysis. B. M. J. Glob. Health. 4: e001710.

Vidovic, N., and S. Vidovic. 2020. Antimicrobial resistance and food animals: influence of livestock environment on the emergence and dissemination of antimicrobial resistance. Antibiotics. 9:52.

Vieco-Saiz, N., Y. Belguesmia, R. Raspoet, E. Auclair, F. Gancel, I. Kempf, and D. Drider. 2019. Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. Front. Microbiol. 10:57.

Wang, J., Y. Han, J. Z. Zhao, Z. J. Zhou, and H. Fan. 2017. Consuming fermented distillers' dried grains with solubles (DDGS) feed reveals a shift in the faecal microbiota of growing and fattening pigs using 454 pyrosequencing. J. Integr. Agric. 16:900–910.

Xu, B., L. Zhu, J. Fu, Z. Li, Y. Wang, and M. Jin. 2019. Overall assessment of fermented feed for pigs: a series of meta-analyses. J. Anim. Sci. 97:4810–4821.

Yu, M., Z. Li, W. Chen, T. Rong, G. Wang, and X. Ma. 2019. *Hermetia illucens* larvae as a potential dietary protein source altered the microbiota and modulated mucosal immune status in the colon of finishing pigs. J. Anim. Sci. Biotechnol. 10: 50.

Zhao, J., Z. Zhang, S. Zhang, G. Page, and N. W. Jaworski. 2021. The role of lactose in weanling pig nutrition: a literature and meta-analysis review. J. Anim. Sci. Biotechnol. 12:10.

Zhu, J., M. Gao, R. Zhang, Z. Sun, C. Wang, F. Yang, T. Huang, S. Qu, L. Zhao, and Y. Li. 2017. Effects of soybean meal fermented by *L. plantarum*, *B. subtilis* and *S. cerevisieae* on

growth, immune function and intestinal morphology in weaned piglets. Microb. Cell Fact. 16: 191.

Zijlstra, R. T., and E. Beltranena. 2013. Swine convert co-products from food and biofuel industries into animal protein for food. Anim. Frontiers. 3:48–53.

Revisión de literatura

Abedi, E., S. and M. B. Hashemi. 2020. Lactic acid production - producing microorganisms and substrates sources-state of art. Heliyon. 6(10):e04974.

Aidara-Kane, A., J. F. Angulo, J. M. Conly, Y. Minato, E. K. Silbergeld, S. A. McEwen, and P. J. Collignon. 2018. World Health Organization (WHO) guidelines on use of medically important antimicrobials in food-producing animals. Antimicrob. Resist. Infect. Control. 7:7.

Balan, P., M. Staincliffe, and P. J. Moughan. 2021. Effects of spray-dried animal plasma on the growth performance of weaned piglets-A review. J. Anim. Physiol. Anim. Nutr. 00:1–16.

Blavi, L., D. Solà-Oriol, P. Llonch, S. López-Vergé, S. M. Martín-Orúe, and J. F. Pérez. 2021. Management and feeding strategies in early life to increase piglet performance and welfare around weaning: A Review. Animals (Basel). 11(2):302.

Beal, J. D., S. J. Niven, A. Campbell, and P. H. Brooks. 2002. The effect of temperature on the growth and persistence of Salmonella in fermented liquid pig feed. Int. J. Food Microbiol. 79: 99–104.

Beal, J. D., S. J. Niven, P. H. Brooks, and B. P. Gill. 2005. Variation in short chain fatty acid and ethanol concentration resulting from the natural fermentation of wheat and barley for inclusion in liquid diets for pigs. J. Sci. Food Agr. 85:433–440.

Blajman, J. E., R. B. Páez, C. G. Vinderola, M. S. Lingua, and M. L. Signorini. 2018. A meta-analysis on the effectiveness of homofermentative and heterofermentative lactic acid bacteria for corn silage. J. Appl. Microbiol. 14084.

Bonetti, A., B. Tugnoli, A. Piva, and E. Grilli. 2021. Towards zero zinc oxide: feeding strategies to manage post-weaning diarrhea in piglets. Animals. 11:642.

Bonis, V., C. Rossell, and H. Gehart. 2021. The intestinal epithelium - fluid fate and figid fsructure from crypt bottom to villus tip. Front. Cell Dev. Biol. 9:661931.

Brooks, P. H. 2008. Fermented liquid feed for pigs. CAB Reviews: Perspectives in agriculture, veterinary science. Nutr. Nat. Resour. 73: 1–18.

Brooks, P. H., and C. A. Tsourgiannis. 2003. Weaning the pig, concepts and consequences. Pluske J. R., Le Dividich J., Verstegen M. W. A. (Eds.). Wageningen academic publishers, Wageningen, The Netherlands. Factors affecting the voluntary feed intake of the weaned pig; p. 81–116.

Brooks, P. H., J. D. Beal, S. Niven, and V. Demečková. 2003. Liquid feeding of pigs II. Potential for improving pig health and food safety. In: Animal science papers and reports. Presented at the Conference: Effect of genetic and non-genetic factors on carcass and meat quality of pigs; 24–25 April 2003; Siedlce, Poland. Vol 21, Supplement. 1, 23–39.

Bunte, S., R. Grone, B. Keller, C. Keller, E. Galvez, T. Strowig, J. Kamphues, and J. Hankel. 2020. Intestinal microbiota of fattening pigs offered non-fermented and fermented liquid feed with and without the supplementation of non-fermented coarse cereals. Microorganisms. 8(5):638.

Burki, T. K. 2018. Tackling antimicrobial resistance in food-producing animals. Lancet. Respir. Med. 6:93–94.

Canibe, N., and B. B. Jensen. 2003. Fermented and nonfermented liquid feed to growing pigs: effect on aspects of gastrointestinal ecology and growth performance. J. Anim. Sci. 81:2019–2031.

Canibe, N., and B. B. Jensen. 2012. Fermented liquid feed – microbial and nutritional aspects and impact on enteric diseases in pigs. Anim. Feed Sci. Technol. 173:17–40.

Chen, C., Y. Yin, Q. Tu, and H. Yang. 2018. Glucose and amino acid in enterocyte: absorption, metabolism and maturation. Front. Biosci. 23:1721–1739.

Chikindas, M. L., R. Weeks, D. Drider, V. A. Chistyakov, L. M. Dicks. 2018. Functions and emerging applications of bacteriocins. Curr. Opin. Biotechnol. 49:23–28.

Dou, S., Gadonna-Widehem, P., Rome, V., Hamoudi, D., Rhazi, L., Lakhal, L., Larcher, T., Bahi-Jaber, N., Pinon-Quintana, A., Guyonvarch, A., Huerou-Luron, I.L.E., and Abdennebi-Najar, L., 2017. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. PLoS ONE. 12: e0169851.

Dowarah, R., A. K. Verma, N. Agarwal, P. Singh, and B. R. Singh. 2018. Selection and characterization of probiotic lactic acid bacteria and its impact on growth, nutrient digestibility, health and antioxidant status in weaned piglets. PLoS One. 13(3):e0192978.

EFSA, 2017. Safety of lactic acid and calcium lactate when used as technological additives for all animal species. EFSA J. 15(7):4938.

Enan, G., Mamdouh, M., Negm, S., Ismaiel, A. and Abdel-Shaf, S. 2018. Classifcation, antimicrobial potential, industrial applications and probiotic capability of lactic acid bacteria: A review article. Res. J. Appl. Sci. 13:742–757.

Ensari, A., and M. N. Marsh. 2018. Exploring the villus. Gastroenterol. Hepatol. Bed. Bench. Summer. 11(3):181–190.

Everts, H., N. Salden, A. G. Lemmens, J. Wijers, and A. C. Beynen. 2000. High intakes of L+ and D- lactic acid are efficiently metabolized by pigs and rats. J. Anim. Physiol. Anim. Nutr. 83:224–230.

FDA, 2012. Guidance for Industry. The judicious use of medically important antimicrobial drugs in food-producing animals. Department of health and human services (Food and Drug Administration), U.S.A.

Gao, J., J. Yin, K. Xu, T. Li, and Y. Yin. 2019. What is the impact of diet on nutritional diarrhea associated with gut microbiota in weaning piglets: A system review. Biomed. Res. Int. 2019:6916189.

Guo, P., K. Zhang, X. Ma, and P. He. 2020. Clostridium species as probiotics: potentials and challenges. J. Anim. Sci. Biotechnol. 11:24.

Ha, T. Q., and Hoa T. M. T. 2016. Selection of lactic acid bacteria producing bacteriocin. J. Viet. Env. 271–276.

He, Y., C. Jinno, K. Kim, Z. Wu, B. Tan, X. Li, R. Whelan, and Y. Liu. 2020. Dietary *Bacillus spp.* enhanced growth and disease resistance of weaned pigs by modulating intestinal microbiota and systemic immunity. J. Anim. Sci. Biotechnol. 11: 101.

Hosain, M. Z., S. M. L. Kabir, and M. M. Kamal. 2021. Antimicrobial uses for livestock production in developing countries. Vet. World. 14:210–221.

Hosoe, N., K. Takabayashi, H. Ogata, and T. Kanai. 2019. Capsule endoscopy for small-intestinal disorders: Current status. Dig. Endosc. 31:498–507.

Huting, A. M. S., A. Middelkoop, X. Guan, and F. Molist. 2021. Using nutritional strategies to shape the gastro-intestinal tracts of suckling and weaned piglets. Animals. 11:402.

Khalid, K. 2011. An overview of lactic acid bacteria. Int. J. Biosci. 1:1–13.

Kim, K., Y. He, X. Xiong, A. Ehrlich, X. Li, H. Raybould, E. R. Atwill, E. A. Maga, J. Jørgensen, and Y. Liu. 2019. Dietary supplementation of bacillus subtilis influenced intestinal health of weaned pigs experimentally infected with a pathogenic *E. coli*. J. Anim. Sci. Biotechnol. 10: 52.

Liu, Y., C. D. Espinosa, J. J. Abelilla, G. A. Casas, L. V. Lagos, S. A. Lee, W. B. Kwon, J. K. Mathai, D. M. D. L. Navarro, N. W. Jaworski, and H. H. Stein. 2018. Non-antibiotic feed additives in diets for pigs: A review. Anim Nutr. 4:113–125.

López-Gálvez, G., M. López-Alonso, A. Pechova, B. Mayo, N. Dierick, and J. Gropp. 2020. Alternatives to antibiotics and trace elements (copper and zinc) to improve gut health and zootechnical parameters in piglets: A review. Anim. Feed Sci. Technol. 114727.

Luissint, A. C., C. A. Parkos, and A. Nusrat. 2016. Inflammation and the intestinal barrier: leukocyte-epithelial cell interactions, cell junction remodeling, and mucosal repair. Gastroenterol. 151(4):616-32.

Martini, E., S. M. Krug, B. Siegmund, M. F. Neurath, and C. Becker. 2017. Mend your fences: The epithelial barrier and its relationship with mucosal immunity in inflammatory bowel disease. Cell. Mol. Gastroenterol. Hepatol. 4:33–46.

Missotten, J. A. M., J. Michiels, J. Goris, L. Herman, M. Heyndrick, S. De Smet, and N. A. Dierick. 2007. Screening of two probiotic products for use in fermented liquid feed. Livest. Sci. 108:232–235.

Missotten, J. A. M., J. Mitchiels, J. Degroote, and S. De Smet. 2015. Fermented liquid feed for pigs: an ancient technique for the future. J. Anim. Sci. Biotechnol. 6: 4.

Mocherla, V. A., N. Suiryanrayna, and J. V. Ramana. 2015. A review of the effects of dietary organic acids fed to swine. J. Anim. Sci. Biotechnol. 6: 45.

Moeser, A. J., C. S. Pohl, and M. Rajput. 2017. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. Anim. Nutr. 3:313–321.

Monteagudo-Mera, A., R. A. Rastall, G. R. Gibson, D. Charalampopoulos, and A. Chatzifragkou. 2019. Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health. Appl. Microbiol. Biotechnol. 103(16):6463–6472.

Montoro-Huguet, M. A., B. Belloc, and M. Domínguez-Cajal. 2021. Small and large intestine (I): malabsorption of nutrients. Nutrients. 13:1254.

Moran, C. A. 2001. Development and benefits of liquid diets for newly weaned pigs [PhD thesis]. [Plymouth, UK]: University of Plymouth.

Mou, Q., H. S. Yang, Y. L.Yin, and P. F. Huang. 2019. Amino acids influencing intestinal development and health of the piglets. Animals. 9:302.

Nawab, A., W. Liu, G. Li, F. Ibtisham, D. P. Fox, Y. Zhao, J. Wo, M. Xiao, Y. Nawab, and L. An. 2019. The potential role of probiotics (nutraceuticals) in gut health of domestic animals; an alternative to antibiotic growth promoters. J. Hell. Vet. Med. Soc. 69: 1169.

Nayak, J. V., A. Rathor, J. W. Grayson, D. T. Bravo, N. Velasquez, J. Noel, D. M. Beswick, K. O. Riley, Z. M. Patel, D. Y. Cho, R. L. Dodd, A. Thamboo, G. W. Choby, E. Walgama, G. R. Harsh, P. H. Hwang, L. Clemons, D. Lowman, J. S. Richman, and B. A. Woodworth. 2018. Porcine small intestine submucosal grafts improve remucosalization and progenitor cell recruitment to sites of upper airway tissue remodeling. Int. Forum Allergy Rhinol. 00:1–7.

Nhung, N., N. Cuong, G. Thwaites, and J. Carrique-Mas. 2016. Antimicrobial usage and antimicrobial resistance in animal production in southeast Asia: A review. Antibiotics 5: 37.

Niba, A. T., J. D. Beal, A. C. Kudi, P. H. Brooks. 2009. Potential of bacterial fermentation as a biosafe method of improving feeds for pigs and poultry. Afri. J. Biotechn. 8:1758–1767.

Nishiyama, K., M. Sugiyama, T. Mukai. 2016. Adhesion properties of lactic acid bacteria on intestinal mucin. Microorganisms. 4:34.

Niven, S. J., Beal, J. D., and Brooks, P. H. 2006. The effect of controlled fermentation on the fate of synthetic lysine in liquid diets for pigs. Anim. Feed Sci. Technol. 129:304–315.

NRC. 2012. Nutrient requirements of swine. 11th rev. ed. Natl. Acad. Press, Washington, DC.

Ortega. A. D. S. V., and C. Szabó. 2021. Adverse effects of heat stress on the intestinal integrity and function of pigs and the mitigation capacity of dietary antioxidants: A review. Animals. 11:1135.

Plumed-Ferrer, C., and A. Von Wright. 2009. Fermented pig liquid feed: nutritional, safety and regulatory aspects. J. Appl. Microbiol. 106:351–368.

Reddy, G., M. Altaf, B. J. Naveena, M. Venkateshwar, E. V. Kumar. 2008. Amylolytic bacterial lactic acid fermentation - a review. Biotechnol. Adv. 26(1):22–34.

Rist, V. T., E. Weiss, M. Eklund, R. Mosenthin. 2013. Impact of dietary protein on microbiota composition and activity in the gastrointestinal tract of piglets in relation to gut health: a review. Animal. 7:1067–1078.

Salim, H. M. D., K. S. Huque, K. M. Kamaruddin, and M. D. A. H. Beg. 2018. Global restriction of using antibiotic growth promoters and alternative strategies in poultry production. Sci. Progr. 101:52–75.

Scholten, R. H. J., C. M. C. Van der Peet-Schwering, L. A. den Hartog, M. Balk, J. W. Schrama, and W. M. A. Verstegen. 2002. Fermented wheat in liquid diets: Effects on gastrointestinal characteristics in weanling piglets. J. Anim. Sci. 80:1179–1186.

Suresh, G., R. K. Das, S. Kaur Brar, T. Rouissi, A. Avalos Ramirez, Y. Chorfi, S. Godbout. 2017. Alternatives to antibiotics in poultry feed: molecular perspectives. Crit. Rev. Microbiol. 44:318–335.

Tang, K. L., N. P. Caffrey. D. B. Nobrega, S. C. Cork, P. E. Ronksley, H. W. Barkema, A. J. Polachek, H. Ganshorn, N. Sharma, J. D. Kellner, S. L. Checkley, and W. A. Ghali. 2019. Comparison of different approaches to antibiotic restriction in food-producing animals: stratified results from a systematic review and meta-analysis. B. M. J. Glob. Health. 4: e001710.

Van Boeckel, T. P., C. Brower, M. Gilbert, B. T. Grenfell, S. A. Levin, T. P. Robinson, A. Teillant, and R. Laxminarayan. 2015. Global trends in antimicrobial use in food animals. Proc. Natl. Acad. Sci. U. S. A. 112:5649–5654.

Van Winsen, R. L., L. J. A. Lipman, S. Biesterveld, B. A. P. Urlings, J. M. A. Snijders, and F. Van Knapen. 2000. Mechanism of *Salmonella* reduction in fermented pig feed. J. Sci. Food Agr. 81:342–346.

Vanrolleghem, W., S. Tanghe, S. Verstringe, G. Bruggeman, D. Papadopoulos, P. Trevisi, J. Zentek, S. Sarrazin, and J. Dewulf. 2019. Potential dietary feed additives with antibacterial effects and their impact on performance of weaned piglets: a meta-analysis. Vet. J. 249:24–32.

Vidovic, N., and S. Vidovic. 2020. Antimicrobial resistance and food animals: influence of livestock environment on the emergence and dissemination of antimicrobial resistance. Antibiotics. 9:52.

Vieco-Saiz, N., Y. Belguesmia, R. Raspoet, E. Auclair, F. Gancel, I. Kempf, and D. Drider. 2019. Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. Front. Microbiol. 10:57.

Walia, K., M. Sharma, S. Vijay, B. R. Shome. 2019. Understanding policy dilemmas around antibiotic use in food animals & offering potential solutions. Indian J. Med. Res. 149:107–118.

Walton, K. D., D. Mishkind, M. R. Riddle, C. J. Tabin, and D. L. Gumucio. 2018. Blueprint for an intestinal villus: species-specific assembly required. Wiley Interdiscip. Rev. Dev. Biol. 7(4):e317.

Wang, Y., J. Zhou, G. Wang, S. Cai, X. Zeng, and S. Qiao. 2018. Advances in low-protein diets for swine. J. Anim. Sci. Biotechnol. 9: 1-14.

Wang, Y. M., H. T. Yu, J. Y. Zhou, X. F. Zeng, G. Wang, S. Cai, and S. Y. Qiao. 2019. Effects of feeding growing-finishing pigs with low crude protein diets on growth performance, carcass characteristics, meat quality and nutrient digestibility in different areas of China. Animal Feed Science and Technology, 114256. Anim. Feed Sci. and Technol. 256: 114256.

Wensley, M. R., M. D. Tokach, J. C. Woodworth, R. D. Goodband, J. T. Gebhardt, J. M. DeRouchey, and D. McKilligan. 2021. Maintaining continuity of nutrient intake after weaning. A review of pre-weaning strategies. Transl. Anim. Sci. 5:1–12.

Weström, B., E. Arévalo Sureda, K. Pierzynowska, S. G. Pierzynowski, and F. J. Pérez-Cano. 2020. The immature gut barrier and its importance in establishing immunity in newborn mammals. Front. Immunol. 11:1153.

Wu, Y., J. Zhao, C. Xu, N. Ma, T. He, J. Zhao, X. Ma, P. A. Thacker. 2018. Progress towards pig nutrition in the last 27 years. J. Sci. Food Agric. 14:5102–5110.

Xiong, X., B. Tan, M. Song, P. Ji, K. Kim, Y. Yin, and Y. Liu. 2019. Nutritional intervention for the intestinal development and health of weaned pigs. Front. Vet. Sci. 6:46.

Xu, B., L. Zhu, J. Fu, Z. Li, Y. Wang, and M. Jin. 2019. Overall assessment of fermented feed for pigs: a series of meta-analyses. J. Anim. Sci. 97:4810–4821.

Yadav, A. K., A. Tyagi, A. Kumar, S. Panwar, S. Grover, A. C. Saklani, R. Hemalatha, V. K. Batish. 2017. Adhesion of Lactobacilli and their anti-infectivity potential. Crit. Rev. Food Sci. Nutr. 57:2042–2056.

Yang, F., C. Hou, X. Zeng, S. Qiao. 2015. The use of lactic acid bacteria as a probiotic in swine diets. Pathogens. 4:34–45.

Yang, Z., S. and F. Liao, 2019. Physiological effects of dietary amino acids on gut health and functions of swine. Front. Vet. Sci. 6:169.

Yuan, L., J. Chang, Q. Q. Yin, M. Lu, Y. R. Di, P. Wang, Z. X. Wang, E. Z. Wang, and F. S. Lu. 2017. Fermented soybean meal improves the growth performance, nutrient digestibility, and microbial flora in piglets. Anim. Nutr. 3:19–24.

Zhao, Y., G. Tian, D. Chen, P. Zheng, J. Yu, J. He, and B. Yu. 2019. Effect of different dietary protein levels and amino acids supplementation patterns on growth performance, carcass characteristics and nitrogen excretion in growing-finishing pigs. J. Anim. Sci. Biotechnol. 1: 75-84.

Capítulo I

1. Missotten JAM, Michiels J, Ovyn A, De Smet S, Dierick NA. Fermented liquid feed for pigs. Arch Anim Nutr. 2010;64(6):437–466.

2. Plumed-Ferrer C, von Wright A. Antimicrobial activity of weak acids in liquid feed fermentations, and its effects on yeasts and lactic acid bacteria. J Sci Food Agric. 2011;91(6):1032–1040.

3. Xu B, Zhu L, Fu J, Li Z, Wang Y, Jin M. Overall assessment of fermented feed for pigs: a series of meta-analyses. J Anim Sci. 2019;97(12):4810–4821.

4. Canibe N, Miettinen H, Jensen BB. Effect of adding *Lactobacillus plantarum* or a formic acid containing-product to fermented liquid feed on gastrointestinal ecology and growth performance of piglets. Livest Sci. 2008;114(2):251–262.

5. Vieco-Saiz N, Belguesmia Y, Raspoet R, Auclair E, Gancel F, Kempf I, Drider D. Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. Front Microbiol. 2019;10:57.

6. Niba AT, Beal JD, Kudi AC, Brooks PH. Potential of bacterial fermentation as a biosafe method of improving feeds for pigs and poultry. Afri J Biotechn. 2009;8(9):1758–1767.

7. Canibe N, Jensen BB. Fermented liquid feed – microbial and nutritional aspects and impact on enteric diseases in pigs. Anim Feed Sci Technol. 2012;173(1–2):17–40.

8. Shi C, Zhang Y, Lu Z, Wang Y. Solid-state fermentation of corn-soybean meal mixed feed with *Bacillus subtilis* and *Enterococcus faecium* for degrading antinutritional factors and enhancing nutritional value. J Anim Sci Biotechnol. 2017;8:50.

9. Wang J, Han Y, Zhao JZ, Zhou ZJ, Fan H. Consuming fermented distillers' dried grains with solubles (DDGS) feed reveals a shift in the faecal microbiota of growing and fattening pigs using 454 pyrosequencing. J Integr Agric. 2017;16(4):900–910.

10. Zhu J, Gao M, Zhang R, Sun Z, Wang C, Yang F, Huang T, Qu S, Zhao L, Li. Y. Effects of soybean meal fermented by *L. plantarum*, *B. subtilis* and *S. cerevisieae* on growth, immune function and intestinal morphology in weaned piglets. Microb Cell Fact. 2017;16:191.

11. Rho Y, Wey D, Zhu C, Kiarie E, Moran K, Van Heugten E, de Lange C. F. M. Growth performance, gastrointestinal and digestibility responses in growing pigs when fed corn-soybean meal-based diets with corn DDGS treated with fiber degrading enzymes with or without liquid fermentation. J Anim Sci. 2018;96(12):5188–5197.

12. Lalles JP, Bosi P, Smidt H, Stokes CR. Nutritional management of gut health in pigs around weaning. Proc Nutr Soc. 2007;66(2):260–8.

13. Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J Anim Physiol Anim Nutr. 2013;97(2):207–37.

14. Dong X, Zhang N, Zhou M, Tu Y, Deng K, Diao Q. Effects of dietary probiotics on growth performance, faecal microbiota and serum profiles in weaned piglets. Anim Prod Sci. 2014;54(5):616–621.

15. Yang X, Kim JS, Baidoo SK. Dietary effect of lactose on growth performance of nursery pigs on a liquid feeding system. J Anim Sci. 2016:94:131–2.

16. Jeong YD, Ko HS, Hosseindoust A, Choi YH, Chae BJ, Yu DJ, Cho ES, Kim YH, Shim SM, Ra CS, Kim YI, Kim JS. Effects of dietary lactose levels and supplementation of prebiotics on growth performance in weanling pigs. J Anim Sci. 2018;96:42–3.

17. Zhao J, Zhang Z, Zhang S, Page G, Jaworski NW. The role of lactose in weanling pig nutrition: a literature and meta-analysis review. J Anim Sci Biotechnol. 2021;12(1):10.

18. Mocherla VA, Suiryanrayna N, Ramana JV. A review of the effects of dietary organic acids fed to swine. J Anim Sci Biotechnol. 2015;6:45.

19. López-Gálvez G, López-Alonso M, Pechova A, Mayo B, Dierick N, Gropp J. Alternatives to antibiotics and trace elements (copper and zinc) to improve gut health and zootechnical parameters in piglets: A review. Anim Feed Sci and Technol. 2020;114727.

20. Robinson K, Deng Z, Hou Y, Zhang G. Regulation of the intestinal barrier function by host defense peptides. Front Vet Sci. 2015;2:57.

21. Aroniadis OC, Brandt LJ. Fecal microbiota transplantation: past, present and future. Curr Opin Gastroenterol. 2013;29(1):79–84.

22. Hu L, Geng S, Li Y, Cheng S, Fu X, Yue X, Han X. Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. Front Microbiol. 2017;8:2663.

23. Canibe N, O'Dea M, Abraham S. Potential relevance of pig gut content transplantation for production and research J Anim Sci Biotechnol. 2019;10(1):55.

24. Yu M. Li Z, Chen W, Rong T, Wang G, Ma X. *Hermetia illucens* larvae as a potential dietary protein source altered the microbiota and modulated mucosal immune status in the colon of finishing pigs. J Anim Sci Biotechnol. 2019;10:50.

25. Kenny M, Smidt H, Mengheri E, Miller B. Probiotics - do they have a role in the pig industry? Animal. 2011;5(3):462–70.

26. Kim K, He Y, Xiong X, Ehrlich A, Li X, Raybould H, Atwill ER, Maga EA, Jørgensen J, Liu. Y. Dietary supplementation of *Bacillus subtilis* influenced intestinal health of weaned pigs experimentally infected with a pathogenic *E. coli*. J Anim Sci Biotechnol. 2019;10:52.

27. Guo P, Zhang K, Ma X, He P. *Clostridium* species as probiotics: potentials and challenges. J. Anim. Sci. Biotechnol. 2020;11:24.

28. Krishna Rao R, Samak G. Protection and restitution of gut barrier by probiotics: Nutritional and clinical implications. Curr Nutr Food Sci. 2013;9(2): 99–107.

29. Liao SF, Nyachoti M. Using probiotics to improve swine gut health and nutrient utilization. Anim Nutr. 2017;3(4):331–343.

30. Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, Yuan Z. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Front. Microbiol. 2014;5:217.

31. Hu S, Wang L, Jiang Z. Dietary additive probiotics modulation of the intestinal microbiota. Protein Peptide Lett. 2017;24(5):382–387.

32. Nawab A, Liu W, Li G, Ibtisham F, Fox DP, Zhao Y, Wo J, Xiao M, Nawab Y, An L. The potential role of probiotics (nutraceuticals) in gut health of domestic animals; an alternative to antibiotic growth promoters. J Hell Vet Med Soc. 2019;69:1169.

33. Spreeuwenberg MAM, Verdonk JMAJ, Gaskins HR, Verstegen MWA. Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. J Nutr. 2001;131(5):1520–7.

34. Han X, Yan F, Nie X, Xia W, Chen S, Zhang X, Qian L. Effect of replacing antibiotics using multi-enzyme preparations on production performance and antioxidant activity in piglets. J Integr Agric. 2017;16:640–647.

35. Dou S, Gadonna-Widehem P, Rome V, Hamoudi D, Rhazi L, Lakhal L, Larcher T, Bahi-Jaber N, Pinon-Quintana A, Guyonvarch A, Huerou-Luron ILE, Abdennebi-Najar L. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. PLoS ONE. 2017;12(1): e0169851.

36. He Y, Jinno C, Kim K, Wu Z, Tan B, Li X, Whelan R, Liu Y. Dietary *Bacillus spp.* enhanced growth and disease resistance of weaned pigs by modulating intestinal microbiota and systemic immunity. J Anim Sci Biotechnol. 2020:11;101.

37. Holmes AH, Moore LSP, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, Guerin PJ, Piddock LJ. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 2016;387(10014):176–87.

38. Vidovic N, Vidovic S. Antimicrobial resistance and food animals: Influence of livestock environment on the emergence and dissemination of antimicrobial resistance. Antibiotics. 2020;9(2):52.

39. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, Laxminarayan R. Global trends in antimicrobial use in food animals. Proc Natl Acad Sci. U. S. A. 2015;112(18):5649–5654.

40. Nhung N, Cuong N, Thwaites G, Carrique-Mas J. Antimicrobial usage and antimicrobial resistance in animal production in Southeast Asia: A review. Antibiotics. 2016;5(4):37.

41. Tang KL, Caffrey NP, Nobrega DB, Cork SC, Ronksley PE, Barkema HW, Polachek AJ, Ganshorn H, Sharma N, Kellner JD, Checkley SL, Ghali W. A. Comparison of different approaches to antibiotic restriction in food-producing animals: stratified results from a systematic review and meta-analysis. B M J Glob Health. 2019;4(4):e001710.

42. Jensen HH, Hayes DJ. Impact of Denmark's ban on antimicrobials for growth promotion. Curr. Opin. Microbiol. 2014;19:30–36.

43. Salim HMD, Huque KS, Kamaruddin KM, Beg MDAH. Global restriction of using antibiotic growth promoters and alternative strategies in poultry production. Sci Progr. 2018;101(1):52–75.

44. FDA, 2012. Guidance for Industry. The judicious use of medically important antimicrobial drugs in food-producing animals, Department of health and human services (Food and Drug Administration), U.S.A.

45. FDA, 2016. New animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209. FDA Guidance for Industry #213. Available at: https://www.fda.gov/media/83488/download.

46. Cani PD, Delzenne NM. The gut microbiome as therapeutic target. Pharmacol Ther. 2011;130(2):202–212.

47. Mao X, Gu C, Hu H, Tang J, Chen D, Yu B, He J, Yu J, Luo J, Tian G. Dietary *Lactobacillus rhamnosus* GG supplementation improves the mucosal barrier function in the intestine of weaned piglets challenged by porcine rotavirus. PLoS One. 2016;11(1):e0146312.

48. Markowiak P, Śliżewska K. The role of probiotics, prebiotics and synbiotics in animal nutrition. Gut Pathog. 2018;10:21.

49. Cho JH, Zhang ZF, Kim IH. Effects of fermented grains as raw cereal substitutes on growth performance, nutrient digestibility, blood profiles, and fecal noxious gas emission in growing pigs. Livest Sci. 2013;154:131–136.

50. Zijlstra RT, Beltranena. E. Swine convert co-products from food and biofuel industries into animal protein for food. Anim. Frontiers. 2013;3(2):48–53.

51. FAO: FAOSTAT Organización de las Naciones Unidas para la Alimentación y la Agricultura. 2019.

52. NRC. 2012. Nutrient requirements of swine. 11th rev. ed. Natl. Acad. Press, Washington, DC.

53. Diano A, Bekker-Jensen S, Dynesen J, Nielsen J. Polyol synthesis in *Aspergillus niger*: Influence of oxygen availability, carbon and nitrogen sources on the metabolism. Biotechnol. Bioeng. 2006;94(5): 899–908.

54. R Core Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/. (Consulted: July 2020).

55. Brooks PH. Fermented liquid feed for pigs. CAB Reviews: Perspectives in Agriculture, Veterinary Science. Nutrition and Natural Resources. 2008;73(3):1–18.

56. Kraler M, Schedle K, Schwarz C, Domig KJ, Pichler M, Oppeneder A, Wetscherek W, Pruckler M, Pignitter M, Pirker KF, Somoza V, Heine D, Kneifel W. Fermented and extruded wheat bran in piglet diets: impact on performance, intestinal morphology, microbial metabolites in chyme and blood lipid radicals. Arch Anim Nutr. 2015;69(5):378–398.

57. Acosta JA, Gabler NK, Frank JW, Bass BE, Patience JE. Effect of lactose, inulin, *lactobacillus acidophilus* fermentation product, or dietary antibiotics on intestinal function of nursery pigs. J Anim Sci. 2017;97(4):1310–1315.

58. Roura E, Fu M. Taste, nutrient sensing and feed intake in pigs (130 years of research: then, now and future). Anim Feed Sci Technol. 2017;233:3–12.

59. Choi JY, Shinde PL, Ingale SL, Kim JS, Kim YW, Kim KH, Kwon IK, Chae BJ. Evaluation of multi-microbe probiotics prepared by submerged liquid or solid substrate fermentation and antibiotics in weaning pigs. Livest Sci. 2011;138(1–3):144–151.

60. Plumed-Ferrer C, Von Wright A. Fermented pig liquid feed: nutritional, safety and regulatory aspects. J. Appl. Microbiol. 2009;106(2):351–368.

61. Park JW, Kim IH. Effect of dietary fermented corn in different energy dense diets on growth performance, nutrient digestibility, ileal microorganisms, and fecal noxious gas emission of growing pigs. Can J Anim Sci. 2018;98(2):325–332.

62. Missotten JAM, Michiels J, Ovyn A, De Smet S, Dierick N. Fermented liquid feed for weaned piglets: impact of sedimentation in the feed slurry on performance and gut parameters. Czech J Anim Sci. 2015;60(5):195–207.

63. Hurst D, Clarke L, Lean IJ. Effect of feeding at different water-to-feed ratios on the growth performance of growing-finishing pigs. Animal. 2008;2(9):1297–1302.

64. Jones CK, DeRouchey JM, Nelssen JL, Tokach MD, Dritz SS, Goodband RD. Effects of fermented soybean meal and specialty animal protein sources on nursery pig performance. J Anim Sci. 2010;88(5):1725–1732.

65. Hou Y, Wu Z, Dai Z, Wang G, Wu G. Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. J Anim Sci Biotechnol. 2017;8(1):24.

66. Abdel-Rahman MA, Tashiro Y, Sonomoto K. Recent advances in lactic acid production by microbial fermentation processes. Biotechnol Adv. 2013;31(6):877–902.

67. Wang, Y, Tashiro Y, Sonomoto K. Fermentative production of lactic acid from renewable materials: recent achievements, prospects, and limits. J Biosci Bioeng. 2015;119(1):10–18.

68. Amadou I, Kamara MT, Tidjani A, Foh MBK, Guo-Wei L. Physicochemical and nutritional analysis of fermented soybean protein meal by *Lactobacillus plantarum* Lp6. World J Dairy Food Sci. 2010;5(2):114–118.

69. Wang Y, Lu WQ, Li DF, Liu XT, Wang HL, Niu S, Piao XS. Energy and ileal digestible amino acid concentrations for growing pigs and performance of weanling pigs fed fermented or conventional soybean meal. Asian-Australas. J Anim Sci. 2014;27(5):706–716.

70. Yuan L, Chang J, Yin QQ, Lu M, Di YR, Wang P, Wang ZX, Wang EZ, Lu. FS. Fermented soybean meal improves the growth performance, nutrient digestibility, and microbial flora in piglets. Anim Nutr. 2017;3(1):19–24.

71. Zhang Y, Shi C, Wang C, Lu Z, Wang F, Feng J, Wang Y. Effect of soybean meal fermented with *Bacillus subtilis* BS12 on growth performance and small intestinal immune status of piglets. Food Agric Immunol. 2018;29(1):133–146.

72. Zhang B, Yang W, Zhang H, Meng Q, Bi C, Shan A. Effect of fermented blood cells on growth performance and intestinal characteristics of weaned piglets. J Anim Physiol Anim Nutr. 2019;103(6):1875–1884.

73. Wang M, Donovan SM. Human microbiota-associated swine: current progress and future opportunities. ILAR J. 2015;56(1):63–73.

74. Yang, Q, Huang X, Zhao S, Sun W, Yan Z, Wang P, Li S, Huang W, Zhang S, Liu L, Gun S. Structure and function of the fecal microbiota in diarrheic neonatal piglets. Front Microbio. 2017;8:502.

75. Tang W, Qian Y, Yu B, Zhang T, Gao J, He J, Huang Z, Zheng P, Mao X, Luo J, Yu J, Chen D. Effects of *Bacillus subtilis* DSM32315 supplementation and dietary crude protein level on performance, gut barrier function and microbiota profile in weaned piglets. J Anim Sci. 2019;97(5):2125–38.

76. Missotten JAM, Mitchiels J, Degroote J, De Smet S. Fermented liquid feed for pigs: an ancient technique for the future. J Anim Sci Biotechnol. 2015;6:4.

77. Beal JD, Niven SJ, Campbell A, Brooks PH. The effect of temperature on the growth and persistence of *Salmonella* in fermented liquid pig feed. Int J Food Microbiol. 2002;79(1–2): 99–104.

78. Mavromichalis I, Hancock JD, Hines RH, Senen BW, Cao H. Lactose, sucrose, and molasses in simple and complex diets for nursery pigs. Anim Feed Sci Technol. 2001;93:127–35.

79. Guo JY, Phillips CE, Coffey MT, Kim SW. Efficacy of a supplemental Candy coproduct as an alternative carbohydrate source to lactose on growth performance of newly weaned pigs in a commercial farm condition. J Anim Sci. 2015;93(11):5304–12.

80. Beal JD, Niven SJ, Brooks PH, Gill BP. Variation in short chain fatty acid and ethanol concentration resulting from the natural fermentation of wheat and barley for inclusion in liquid diets for pigs. J Sci Food Agri. 2005;85(3):433–444.

81. Kobashi Y, Ohmori H, Tajima K, Kawashima T, Uchiyama H. Reduction of chlortetracycline-resistant *Escherichia coli* in weaned piglets fed fermented liquid feed. Anaerobe. 2008;14(4):201–204.

82. Pearnil BV, Muthuvel S, Govidasamy P, Villavan M, Alagawany M, Farag MR, Dhama K, Gopi M. Role of acidifiers in livestock nutrition and health: A review. J Anim Physiol Anim Nutr. 2020;104(2):1–12.

83. Sugiharto S, Lauridsen C, Jensen BB. Gastrointestinal ecosystem and immunological responses in *E. coli* challenged pigs after weaning fed liquid diets containing whey permeate fermented with different lactic acid bacteria. Anim Feed Sci Tech. 2015;207:278–282.

Capítulo II.

1. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, Laxminarayan R. Global trends in antimicrobial use in food animals. Proc Natl Acad Sci. U. S. A. 2015;112(18):5649–5654.

2. Holmes AH, Moore LSP, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, Guerin PJ, Piddock LJ. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 2016;387(10014):176–87.

3. Nhung N, Cuong N, Thwaites G, Carrique-Mas J. Antimicrobial usage and antimicrobial resistance in animal production in Southeast Asia: A review. Antibiotics. 2016;5(4):37.

4. Vidovic N, Vidovic S. Antimicrobial resistance and food animals: Influence of livestock environment on the emergence and dissemination of antimicrobial resistance. Antibiotics. 2020;9(2):52.

5. Tang KL, Caffrey NP, Nobrega DB, Cork SC, Ronksley PE, Barkema HW, Polachek AJ, Ganshorn H, Sharma N, Kellner JD, Checkley SL, Ghali W. A. Comparison of different approaches to antibiotic restriction in food-producing animals: stratified results from a systematic review and meta-analysis. B M J Glob Health. 2019;4(4):e001710.

6. Yang X, Kim JS, Baidoo SK. Dietary effect of lactose on growth performance of nursery pigs on a liquid feeding system. J Anim Sci. 2016:94:131–2.

7. Jeong YD, Ko HS, Hosseindoust A, Choi YH, Chae BJ, Yu DJ, Cho ES, Kim YH, Shim SM, Ra CS, Kim YI, Kim JS. Effects of dietary lactose levels and supplementation of prebiotics on growth performance in weanling pigs. J Anim Sci. 2018;96:42–3.

8. Zhao J, Zhang Z, Zhang S, Page G, Jaworski. N. W. The role of lactose in weanling pig nutrition: a literature and meta-analysis review. J Anim Sci Biotechnol. 2021;12(1):10.

9. Plumed-Ferrer C, von Wright A. Antimicrobial activity of weak acids in liquid feed fermentations, and its effects on yeasts and lactic acid bacteria. J Sci Food Agric. 2011;91(6):1032–1040.

10. Mocherla VA, Suiryanrayna N, Ramana JV. A review of the effects of dietary organic acids fed to swine. J Anim Sci Biotechnol. 2015;6:45.

11. López-Gálvez G, López-Alonso M, Pechova A, Mayo B, Dierick N, Gropp J. Alternatives to antibiotics and trace elements (copper and zinc) to improve gut health and zootechnical parameters in piglets: A review. Anim Feed Sci and Technol. 2020;114727.

12. Robinson K, Deng Z, Hou Y, Zhang G. Regulation of the intestinal barrier function by host defense peptides. Front Vet Sci. 2015;2:57.

13. Aroniadis OC, Brandt LJ. Fecal microbiota transplantation: past, present and future. Curr Opin Gastroenterol. 2013;29(1):79–84.

14. Hu L, Geng S, Li Y, Cheng S, Fu X, Yue X, Han X. Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. Front Microbiol. 2017;8:2663.

15. Canibe N, O'Dea M, Abraham S. Potential relevance of pig gut content transplantation for production and research J Anim Sci Biotechnol. 2019;10(1):55.

16. Yu M. Li Z, Chen W, Rong T, Wang G, Ma X. *Hermetia illucens* larvae as a potential dietary protein source altered the microbiota and modulated mucosal immune status in the colon of finishing pigs. J Anim Sci Biotechnol. 2019;10:50.

17. Kenny M, Smidt H, Mengheri E, Miller B. Probiotics - do they have a role in the pig industry? Animal. 2011;5(3):462–70.

18. Kim K, He Y, Xiong X, Ehrlich A, Li X, Raybould H, Atwill ER, Maga EA, Jørgensen J, Liu. Y. Dietary supplementation of *Bacillus subtilis* influenced intestinal health of weaned pigs experimentally infected with a pathogenic *E. coli.* J Anim Sci Biotechnol. 2019;10:52.

19. Guo P, Zhang K, Ma X, He P. *Clostridium* species as probiotics: potentials and challenges. J. Anim. Sci. Biotechnol. 2020;11:24.

20. Krishna Rao R, Samak G. Protection and restitution of gut barrier by probiotics: nutritional and clinical implications. Curr Nutr Food Sci. 2013;9(2): 99–107.

21. Liao SF, Nyachoti M. Using probiotics to improve swine gut health and nutrient utilization. Anim Nutr. 2017;3(4):331–343.

22. Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, Yuan Z. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Front. Microbiol. 2014;5:217.

23. Hu S, Wang L, Jiang Z. Dietary additive probiotics modulation of the intestinal microbiota. Protein Peptide Lett. 2017;24(5):382–387.

24. Nawab A, Liu W, Li G, Ibtisham F, Fox DP, Zhao Y, Wo J, Xiao M, Nawab Y, An L. The potential role of probiotics (nutraceuticals) in gut health of domestic animals; an alternative to antibiotic growth promoters. J Hell Vet Med Soc. 2019;69:1169.

25. Missotten JAM, Michiels J, Ovyn A, De Smet S, Dierick NA. Fermented liquid feed for pigs. Arch Anim Nutr. 2010;64(6):437–466.

26. Missotten JAM, Mitchiels J, Degroote J, De Smet S. Fermented liquid feed for pigs: an ancient technique for the future. J Anim Sci Biotechnol. 2015;6:4.

27. Xu B, Zhu L, Fu J, Li Z, Wang Y, Jin M. Overall assessment of fermented feed for pigs: a series of meta-analyses. J Anim Sci. 2019;97(12):4810–4821.

28. Canibe N, Miettinen H, Jensen BB. Effect of adding *Lactobacillus plantarum* or a formic acid containing-product to fermented liquid feed on gastrointestinal ecology and growth performance of piglets. Livest Sci. 2008;114(2):251–262.

29. Niba AT, Beal JD, Kudi AC, Brooks PH. Potential of bacterial fermentation as a biosafe method of improving feeds for pigs and poultry. Afri J Biotechn. 2009;8(9):1758–1767.

30. Vieco-Saiz N, Belguesmia Y, Raspoet R, Auclair E, Gancel F, Kempf I, Drider D. Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. Front Microbiol. 2019;10:57.

31. Canibe N, Jensen BB. Fermented liquid feed – microbial and nutritional aspects and impact on enteric diseases in pigs. Anim Feed Sci Technol. 2012;173(1–2):17–40.

32. Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhea without using in-feed antimicrobial compounds. J Anim Physiol Anim Nutr. 2013;97(2):207–37.

33. Dong X, Zhang N, Zhou M, Tu Y, Deng K, Diao Q. Effects of dietary probiotics on growth performance, faecal microbiota and serum profiles in weaned piglets. Anim Prod Sci. 2014;54(5):616–621.

34. Wang J, Han Y, Zhao JZ, Zhou ZJ, Fan H. Consuming fermented distillers' dried grains with solubles (DDGS) feed reveals a shift in the faecal microbiota of growing and fattening pigs using 454 pyrosequencing. J Integr Agric. 2017;16(4):900–910.

35. Zhu J, Gao M, Zhang R, Sun Z, Wang C, Yang F, Huang T, Qu S, Zhao L, Li. Y. Effects of soybean meal fermented by *L. plantarum*, *B. subtilis* and *S. cerevisieae* on growth, immune function and intestinal morphology in weaned piglets. Microb Cell Fact. 2017;16:191.

36. Cani PD, Delzenne NM. The gut microbiome as therapeutic target. Pharmacol Ther. 2011;130(2):202–212.

37. Mao X, Gu C, Hu H, Tang J, Chen D, Yu B, He J, Yu J, Luo J, Tian G. Dietary *Lactobacillus rhamnosus* GG supplementation improves the mucosal barrier function in the intestine of weaned piglets challenged by porcine rotavirus. PLoS One. 2016;11(1):e0146312.

38. Markowiak P, Śliżewska K. The role of probiotics, prebiotics and synbiotics in animal nutrition. Gut Pathog. 2018;10:21.

39. Rho Y, Wey D, Zhu C, Kiarie E, Moran K, Van Heugten E, de Lange C. F. M. Growth performance, gastrointestinal and digestibility responses in growing pigs when fed corn-

soybean meal-based diets with corn DDGS treated with fiber degrading enzymes with or without liquid fermentation. J Anim Sci. 2018;96(12):5188–5197.

40. Chen J, Kang B, Jiang Q, Han M, Zhao Y, Long L, Yao K. α-ketoglutarate in low-protein diets for growing pigs: Effects on cecal microbial communities and parameters of microbial metabolism. Front Microbiol. 2018;31(9):1057.

41. Wang Y, Zhou J, Wang G, Cai S, Zeng X, Qiao S. Advances in low-protein diets for swine. J Anim Sci Biotechnol. 2018;19(9)60.

42. Zhao Y, Tian G, Chen D, Zheng P, Yu J, He J, Yu B. Effect of different dietary protein levels and amino acids supplementation patterns on growth performance, carcass characteristics and nitrogen excretion in growing-finishing pigs. J Anim Sci Biotechnol. 2019;16:(10):75–84.

43. Wang YM, Yu HT, Zhou JY, Zeng XF, Wang G, Cai S, Qiao SY. 2019. Effects of feeding growing-finishing pigs with low crude protein diets on growth performance, carcass characteristics, meat quality and nutrient digestibility in different areas of China. Anim Feed Sci Technol. 256: 114256.

44. Chen J, Su W, Kang B, Jiang Q, Zhao Y, Fu C, Yao K. Supplementation with α-ketoglutarate to a low-protein diet enhances amino acid synthesis in tissues and improves protein metabolism in the skeletal muscle of growing pigs. Amino Acids. 92018;50(11): 1525–1537.

45. Tajima K, Ohmori H, Aminov RI, Kobashi Y, Kawashima T. Fermented liquid feed enhances bacterial diversity in piglet intestine. Anaerobe. 2010;(1):6-11.

46. Enan G, Mamdouh M, Negm S, Ismaiel A, Abdel-Shaf S. Classifcation, antimicrobial potential, industrial applications and probiotic capability of lactic acid bacteria: A review article. Res J Appl Sci. 2018;(13) 742–757.

47. FAO: FAOSTAT Organización de las Naciones Unidas para la Alimentación y la Agricultura. 2019.

48. NRC. 2012. Nutrient requirements of swine. 11th rev. ed. Natl. Acad. Press, Washington, DC.

49. Diano A, Bekker-Jensen S, Dynesen J, Nielsen J. Polyol synthesis in *Aspergillus niger*: Influence of oxygen availability, carbon and nitrogen sources on the metabolism. Biotechnol. Bioeng. 2006;94(5): 899–908.

50. R Core Team. 2018. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. https://www.R-project.org/. (Consulted: July 2020).

51. Brooks PH. Fermented liquid feed for pigs. CAB Reviews: Perspectives in agriculture, veterinary science. Nutr Nat Resour. 2008;73(3):1–18.

52. Kraler M, Schedle K, Schwarz C, Domig KJ, Pichler M, Oppeneder A, Wetscherek W, Pruckler M, Pignitter M, Pirker KF, Somoza V, Heine D, Kneifel W. Fermented and

extruded wheat bran in piglet diets: impact on performance, intestinal morphology, microbial metabolites in chyme and blood lipid radicals. Arch Anim Nutr. 2015;69(5):378–398.

53. Acosta JA, Gabler NK, Frank JW, Bass BE, Patience JE. Effect of lactose, inulin, *lactobacillus acidophilus* fermentation product, or dietary antibiotics on intestinal function of nursery pigs. J Anim Sci. 2017;97(4):1310–1315.

54. Roura E, Fu M. Taste, nutrient sensing and feed intake in pigs (130 years of research: then, now and future). Anim Feed Sci Technol. 2017;233:3–12.

55. Choi JY, Shinde PL, Ingale SL, Kim JS, Kim YW, Kim KH, Kwon IK, Chae BJ. Evaluation of multi-microbe probiotics prepared by submerged liquid or solid substrate fermentation and antibiotics in weaning pigs. Livest Sci. 2011;138(1–3):144–151.

56. He Y, Mao C, Wen H, Chen Z, Lai T, Li L, Lu W, Wu H. Influence of ad libitum feeding of piglets with *Bacillus Subtilis* fermented liquid feed on gut flora, luminal contents and health. Sci Rep. 2017;(7):44553.

57. Torres-Pitarch A, Gardiner GE, Cormican P, Rea M, Crispie F, O'Doherty JV, Cozannet P, Ryan T, Cullen J, Lawlor PG. Effect of cereal fermentation and carbohydrase supplementation on growth, nutrient digestibility and intestinal microbiota in liquid-fed grow-finishing pigs. Sci Rep. 2020;10(1):13716.

58. Bunte S, Grone R, Keller B, Keller C, Galvez E, Strowig T, Kamphues J, Hankel J. Intestinal microbiota of fattening pigs offered non-fermented and fermented liquid feed with and without the supplementation of non-fermented coarse cereals. Microorganisms. 2020;8(5):638.

59. O' Meara FM, Gardiner GE, O' Doherty JV, Clarke D, Cummins W, Lawlor PG. Effect of wet/dry, fresh liquid, fermented whole diet liquid, and fermented cereal liquid feeding on feed microbial quality and growth in grow-finisher pigs. J Anim Sci. 2020;98(6):skaa166.

60. Beal JD, Niven SJ, Brooks PH, Gill BP. Variation in short chain fatty acid and ethanol concentration resulting from the natural fermentation of wheat and barley for inclusion in liquid diets for pigs. J Sci Food Agri. 2005;85(3):433–444.

61. Pearnil BV, Muthuvel S, Govidasamy P, Villavan M, Alagawany M, Farag MR, Dhama K, Gopi M. Role of acidifiers in livestock nutrition and health: A review. J Anim Physiol Anim Nutr. 2020;104(2):1–12.

62. Plumed-Ferrer C, Von Wright A. Fermented pig liquid feed: nutritional, safety and regulatory aspects. J. Appl. Microbiol. 2009;106(2):351–368.

63. Abdel-Rahman MA, Tashiro Y, Sonomoto K. Recent advances in lactic acid production by microbial fermentation processes. Biotechnol Adv. 2013;31(6):877–902.

64. Beal JD, Niven SJ, Campbell A, Brooks PH. The effect of temperature on the growth and persistence of *Salmonella* in fermented liquid pig feed. Int J Food Microbiol. 2002;79(1–2): 99–104.

65. Wiseman M, McBride B, Li J, Wey D, Zhu J, de Lange CFM. Effects of steeped or fermented distillers dried grains with solubles on growth performance in weanling pigs. J Anim Sci. 2017;95(8):3563-3578.

66. Park JW, Kim IH. Effect of dietary fermented corn in different energy dense diets on growth performance, nutrient digestibility, ileal microorganisms, and fecal noxious gas emission of growing pigs. Can J Anim Sci. 2018;98(2):325–332.

67. Xin H, Wang M, Xia Z, Yu B, He J, Yu J, Mao X, Huang Z, Luo Y, Luo J, Yan H, Wang H, Wang Q, Zheng P, Chen D. Fermented diet liquid feeding improves growth performance and intestinal function of pigs. Animals. 2021;11(5):1452.

68. Sugiharto S, Lauridsen C, Jensen BB. Gastrointestinal ecosystem and immunological responses in *E. coli* challenged pigs after weaning fed liquid diets containing whey permeate fermented with different lactic acid bacteria. Anim Feed Sci Tech. 2015;207:278–282.

69. Yuan L, Chang J, Yin QQ, Lu M, Di YR, Wang P, Wang ZX, Wang EZ, Lu. FS. Fermented soybean meal improves the growth performance, nutrient digestibility, and microbial flora in piglets. Anim Nutr. 2017;3(1):19–24.

70. Choi JY, Shinde PL, Ingale SL, Kim JS, Kim YW, Kim KH, Kwon IK, Chae BJ. Evaluation of multi-microbe probiotics prepared by submerged liquid or solid substrate fermentation and antibiotics in weaning pigs. Livest Sci. 2011;138(1–3):144–151.

71. Wang Y, Lu WQ, Li DF, Liu XT, Wang HL, Niu S, Piao XS. Energy and ileal digestible amino acid concentrations for growing pigs and performance of weanling pigs fed fermented or conventional soybean meal. Asian-Australas. J Anim Sci. 2014;27(5):706–716.

72. Roura E, Fu M. Taste, nutrient sensing and feed intake in pigs (130 years of research: then, now and future). Anim Feed Sci Technol. 2017;233:3–12.

73. O'Meara FM, Gardiner GE, Clarke D, Cummins W, O'Doherty JV, Lawlor PG. Microbiological assessment of liquid feed for finisher pigs on commercial pig units. J Appl Microbiol. 2021;130(2):356-369.

74. Li YH, Li FN, Duan YH, Guo QP, Wen CY, Wang WL, Huang XG, Yin YL. Low-protein diet improves meat quality of growing and finishing pigs through changing lipid metabolism, fiber characteristics, and free amino acid profile of the muscle. J Anim Sci. 2018;96(8):3221-3232.

75. Nguyen DH, Lee SI, Cheong JY, Kim IH. Influence of low-protein diets and protease and bromelain supplementation on growth performance, nutrient digestibility, blood urine nitrogen, creatinine, and faecal noxious gas in growing–finishing pigs. Can J Anim Sci. 2018;(3):488–497.

76. Niyazov NSA, Kalnitsky BD. Effects of low-protein diets with different levels of essential amino acids and metabolizable energy on productivity and metabolic processes in pigs. Russ Agri Sci. 2018;(1):75–79.

77. Presto-Åkerfeldt M, Lindberg JE, Göransson L, Andersson K. Effects of reducing dietary content of crude protein and indispensable amino acids on performance and carcass traits of single-phase- and 2-phase-fed growing-finishing pigs. Livest Sci. 2019;(224)96–101.

78. Wang M, Donovan SM. Human microbiota-associated swine: current progress and future opportunities. ILAR J. 2015;56(1):63–73.

79. Yang Q, Huang X, Zhao S, Sun W, Yan Z, Wang P, Li S, Huang W, Zhang S, Liu L, Gun S. Structure and function of the fecal microbiota in diarrheic neonatal piglets. Front Microbio. 2017;8:502.

80. Shin D, Chang SY, Bogere P, Won K, Choi JY, Choi YJ, Lee HK, Hur J, Park BY, Kim Y, Heo J. Beneficial roles of probiotics on the modulation of gut microbiota and immune response in pigs. PLoS One. 2019;14(8):e0220843.

81. Satessa GD, Tamez-Hidalgo P, Hui Y, Cieplak T, Krych L, Kjærulff S, Brunsgaard G, Nielsen DS, Nielsen MO. Impact of dietary supplementation of lactic acid bacteria fermented rapeseed with or without macroalgae on performance and health of piglets following omission of medicinal zinc from weaner diets. Animals (Basel). 2020;10(1):137.

ANEXOS

Corrida en SAS para el pH de la fase 1 en el primer estudio

```
Data ejemplo1;
DO A=1 TO 2;
DO X=1 TO 2;
DO REP=1 TO 5;
INPUT Y@@;
COMVAX=A*10+X;
OUTPUT;
END;
END;
END;
DATALINES;

3.66    3.62    3.64    3.70    3.65 3.6     3.63    3.65    3.67    3.65 3.5      3.51    3.55
        3.52    3.51 3.63        3.9     3.62    3.52    3.54
;
PROC GLM;
CLASS A X;
MODEL Y = A|X;
LSMEANS A X A*X/ADJUST=TUKEY ALPHA=0.05 LINES;
LSMEANS A*X/ SLICE= A SLICE=X ADJUST=TUKEY LINES;
OUTPUT OUT=NEW P=YHAT R=RESID;
PROC SORT DATA= ejemplo1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= ejemplo1; BY X;
PROC MEANS MEAN STDERR; BY X;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*X/STDERR;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
RUN;
```

Corrida en SAS para la concentración de ácido lactico de la fase 1 en el primer estudio

```
Data ejemplo1;
DO A=1 TO 2;
DO X=1 TO 2;
DO REP=1 TO 5;
INPUT Y@@;
COMVAX=A*10+X;
OUTPUT;
END;
END;
END;
DATALINES;
61.73   131.35 116.45 67.48   126.90 53.34   87.70   111.64 62.88   66.76   80.25   85.71   78.02
        101.53 93.70   37.12   70.97   47.23   75.03   69.38
;
PROC GLM;
CLASS A X;
MODEL Y = A|X;
LSMEANS A X A*X/ADJUST=TUKEY ALPHA=0.05 LINES;
LSMEANS A*X/ SLICE= A SLICE=X ADJUST=TUKEY LINES;
OUTPUT OUT=NEW P=YHAT R=RESID;
PROC SORT DATA= ejemplo1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= ejemplo1; BY X;
PROC MEANS MEAN STDERR; BY X;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*X/STDERR;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
RUN;
```

Corrida en SAS para la concentración de ácido acetico de la fase 1 en el primer estudio

```
Data ejemplo1;
DO A=1 TO 2;
DO X=1 TO 2;
DO REP=1 TO 5;
INPUT Y@@;
COMVAX=A*10+X;
OUTPUT;
END;
END;
END;
DATALINES;
2.27    3.29    2.52    2.39    5.13    2.09    2.77    3.40    2.84    2.74    1.31    2.46    2.35
        3.17    4.35    4.00    3.64    2.46    3.05    2.08
;
PROC GLM;
CLASS A X;
MODEL Y = A|X;
LSMEANS A X A*X/ADJUST=TUKEY ALPHA=0.05 LINES;
LSMEANS A*X/ SLICE= A SLICE=X ADJUST=TUKEY LINES;
OUTPUT OUT=NEW P=YHAT R=RESID;
PROC SORT DATA= ejemplo1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= ejemplo1; BY X;
PROC MEANS MEAN STDERR; BY X;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*X/STDERR;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
RUN;
```

Corrida en SAS para el pH de la fase 2 en el primer estudio

```
Data ejemplo1;
DO P=1 TO 3;
DO O=1 TO 2;
DO REP=1 TO 4;
INPUT Y@@;
COMVPO=P*10+O;
OUTPUT;
END;
END;
END;
DATALINES;
3.71    3.82    3.62    3.72    3.95    3.9    3.81    3.82    3.95    3.72    3.8    4    3.82
        4.06    4.08    4.04    4.14    4.02    3.99    3.98    3.96    4.2    4.23    4.17
;
PROC GLM;
CLASS P O;
MODEL Y = P|O;
LSMEANS P O P*O/ADJUST=TUKEY ALPHA=0.05 LINES;
LSMEANS P*O/ SLICE= P SLICE=O ADJUST=TUKEY LINES;
OUTPUT OUT=NEW P=YHAT R=RESID;
PROC SORT DATA= ejemplo1; BY P;
PROC MEANS MEAN STDERR; BY P;
PROC SORT DATA= ejemplo1; BY O;
PROC MEANS MEAN STDERR; BY O;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS P*O/STDERR;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
RUN;
```

Corrida en SAS para la concentración de ácido lactico de la fase 2 en el primer estudio

```
Data ejemplo1;
DO P=1 TO 3;
DO O=1 TO 2;
DO REP=1 TO 4;
INPUT Y@@;
COMVPO=P*10+O;
OUTPUT;
END;
END;
END;
DATALINES;
59.79  47.82  33.29  41.57  74.20  84.93  70.40  97.76  97.41  88.42  102.57 102.57 115.33
       125.24 142.15 133.66 198.44 133.08 131.84 120.32 132.51 137.14 205.42 257.79
;
PROC GLM;
CLASS P O;
MODEL Y = P|O;
LSMEANS P O P*O/ADJUST=TUKEY ALPHA=0.05 LINES;
LSMEANS P*O/ SLICE= P SLICE=O ADJUST=TUKEY LINES;
OUTPUT OUT=NEW P=YHAT R=RESID;
PROC SORT DATA= ejemplo1; BY P;
PROC MEANS MEAN STDERR; BY P;
PROC SORT DATA= ejemplo1; BY O;
PROC MEANS MEAN STDERR; BY O;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS P*O/STDERR;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
RUN;
```

Corrida en SAS para el pH de la fase 3 en el primer estudio

```
Data tarea5_1;
DO A=1 TO 2;
DO B=1 TO 2;
DO C=1 TO 2;
DO R=1 TO 4;
INPUT Y@@;
COMAB=A*10+B;
COMAC=A*10+C;
COMBC=B*10+C;
COMABC=A*100+B*10+C;
OUTPUT;
END;
END;
END;
END;
CARDS;
4.31   4.35   4.4    4.27
4.42   4.41   4.37   4.37
4.22   4.18   4.15   4.15
4.19   4.21   4.14   4.12
4.16   4.14   4.03   4.08
4.18   4.19   4.17   4.18
4.15   4.17   4.15   4.18
4.07   4.1    4.06   4.09
;
PROC GLM;
CLASS A B C;
MODEL Y=A|B|C;
LSMEANS A B C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B A*C B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B/SLICE=B SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*C/SLICE=C SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=A SLICE=B*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=B SLICE=A*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=C SLICE=B*A ADJUST=TUKEY LINES ALPHA=0.05;
OUTPUT OUT=NUEVO P=YHAT R=RESID;
```

```
PROC SORT DATA= tarea5_1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= tarea5_1; BY B;
PROC MEANS MEAN STDERR; BY B;
PROC SORT DATA= tarea5_1; BY C;
PROC MEANS MEAN STDERR; BY C;
PROC SORT DATA= tarea5_1; BY A*B;
PROC MEANS MEAN STDERR; BY A*B;
PROC SORT DATA= tarea5_1; BY B*C;
PROC MEANS MEAN STDERR; BY B*C;
PROC SORT DATA= tarea5_1; BY A*C;
PROC MEANS MEAN STDERR; BY A*C;
PROC SORT DATA= tarea5_1; BY A*B*C;
PROC MEANS MEAN STDERR; BY A*B*C;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*B*C/STDERR;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
RUN;
```

Corrida en SAS para la concentración de ácido lactico de la fase 3 en el primer estudio

```
Data tarea5_1;
DO A=1 TO 2;
DO B=1 TO 2;
DO C=1 TO 2;
DO R=1 TO 4;
INPUT Y@@;
COMAB=A*10+B;
COMAC=A*10+C;
COMBC=B*10+C;
COMABC=A*100+B*10+C;
OUTPUT;
END;
END;
END;
END;
CARDS;
76.44   72.26   67.72   62.7
84.53   95.43   102.08  92.98
96.99   105.36  148.96  151.15
87.69   107.45  106.44  115.38
242.73  221.44  226.54  237.62
256.83  293.07  197     245.9
260.09  251.46  268.76  218.09
327.09  345.1   285.52  253.02
;
PROC GLM;
CLASS A B C;
MODEL Y=A|B|C;
LSMEANS A B C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B A*C B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B/SLICE=B SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*C/SLICE=C SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=A SLICE=B*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=B SLICE=A*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=C SLICE=B*A ADJUST=TUKEY LINES ALPHA=0.05;
OUTPUT OUT=NUEVO P=YHAT R=RESID;
PROC SORT DATA= tarea5_1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= tarea5_1; BY B;
PROC MEANS MEAN STDERR; BY B;
PROC SORT DATA= tarea5_1; BY C;
PROC MEANS MEAN STDERR; BY C;
PROC SORT DATA= tarea5_1; BY A*B;
PROC MEANS MEAN STDERR; BY A*B;
PROC SORT DATA= tarea5_1; BY B*C;
```

```
PROC MEANS MEAN STDERR; BY B*C;
PROC SORT DATA= tarea5_1; BY A*C;
PROC MEANS MEAN STDERR; BY A*C;
PROC SORT DATA= tarea5_1; BY A*B*C;
PROC MEANS MEAN STDERR; BY A*B*C;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*B*C/STDERR;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
RUN;
```

Corrida en SAS para el pH de la fase 4 en el primer estudio

```
Data tarea5_1;
DO A=1 TO 2;
DO B=1 TO 2;
DO C=1 TO 2;
DO R=1 TO 4;
INPUT Y@@;
COMAB=A*10+B;
COMAC=A*10+C;
COMBC=B*10+C;
COMABC=A*100+B*10+C;
OUTPUT;
END;
END;
END;
END;
CARDS;
        4.84            4.72            4.75            4.91
        4.59            4.55            4.62            4.8
        4.96            4.83            4.95            5.02
        5.21            5.25            5.21            5.25
        4.02            3.99            3.99            4.02
        3.92            3.99            3.98            3.95
        4.3             4.19            4.18            4.21
        4.23            4.19            4.13            4.17
;
PROC GLM;
CLASS A B C;
MODEL Y=A|B|C;
LSMEANS A B C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B A*C B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B/SLICE=B SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*C/SLICE=C SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=A SLICE=B*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=B SLICE=A*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=C SLICE=B*A ADJUST=TUKEY LINES ALPHA=0.05;
OUTPUT OUT=NUEVO P=YHAT R=RESID;
PROC SORT DATA= tarea5_1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= tarea5_1; BY B;
PROC MEANS MEAN STDERR; BY B;
PROC SORT DATA= tarea5_1; BY C;
PROC MEANS MEAN STDERR; BY C;
PROC SORT DATA= tarea5_1; BY A*B;
PROC MEANS MEAN STDERR; BY A*B;
PROC SORT DATA= tarea5_1; BY B*C;
PROC MEANS MEAN STDERR; BY B*C;
PROC SORT DATA= tarea5_1; BY A*C;
PROC MEANS MEAN STDERR; BY A*C;
PROC SORT DATA= tarea5_1; BY A*B*C;
PROC MEANS MEAN STDERR; BY A*B*C;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
```

```
PROC MEANS A*B*C/STDERR;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
RUN;
```

Corrida en SAS para la concentración de ácido lactico de la fase 4 en el primer estudio

```
Data tarea5 1;
DO A=1 TO 2;
DO B=1 TO 2;
DO C=1 TO 2;
DO R=1 TO 4;
INPUT Y@@;
COMAB=A*10+B;
COMAC=A*10+C;
COMBC=B*10+C;
COMABC=A*100+B*10+C;
OUTPUT;
END;
END;
END;
END;
CARDS;
105.26  111.21  106.27  106.53
120.08  107.78  134.74  93.12
87.21   133.83  107.9   107.05
84.51   87.52   93.35   107.42
194.67  250.92  173.47  140.61
232.55  245.05  229.73  230.71
331     352     351     342
219.11  255.68  238.77  231.55
;
PROC GLM;
CLASS A B C;
MODEL Y=A|B|C;
LSMEANS A B C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B A*C B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B/SLICE=B SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*C/SLICE=C SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=A SLICE=B*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=B SLICE=A*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=C SLICE=B*A ADJUST=TUKEY LINES ALPHA=0.05;
OUTPUT OUT=NUEVO P=YHAT R=RESID;
PROC SORT DATA= tarea5_1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= tarea5_1; BY B;
PROC MEANS MEAN STDERR; BY B;
PROC SORT DATA= tarea5 1; BY C;
PROC MEANS MEAN STDERR; BY C;
PROC SORT DATA= tarea5_1; BY A*B;
PROC MEANS MEAN STDERR; BY A*B;
PROC SORT DATA= tarea5_1; BY B*C;
PROC MEANS MEAN STDERR; BY B*C;
PROC SORT DATA= tarea5_1; BY A*C;
PROC MEANS MEAN STDERR; BY A*C;
PROC SORT DATA= tarea5_1; BY A*B*C;
PROC MEANS MEAN STDERR; BY A*B*C;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*B*C/STDERR;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
RUN
```

Corrida en SAS para el pH de la fase 1 en el segundo estudio

```
Data tarea5_1;
DO A=1 TO 2;
DO B=1 TO 2;
DO C=1 TO 2;
DO R=1 TO 4;
INPUT Y@@;
```

```
COMAB=A*10+B;
COMAC=A*10+C;
COMBC=B*10+C;
COMABC=A*100+B*10+C;
OUTPUT;
END;
END;
END;
END;
CARDS;
```

4.5	4.16	4.15	4.13
4.07	4.07	4.06	4.07
4.13	4.18	4.15	4.22
4.11	4.11	4.14	4.12
4.46	4.44	4.39	4.31
4.32	4.29	4.29	4.28
4.38	4.34	4.36	4.32
4.26	4.27	4.26	4.26

```
;
PROC GLM;
CLASS A B C;
MODEL Y=A|B|C;
LSMEANS A B C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B A*C B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B/SLICE=B SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*C/SLICE=C SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=A SLICE=B*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=B SLICE=A*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=C SLICE=B*A ADJUST=TUKEY LINES ALPHA=0.05;
OUTPUT OUT=NUEVO P=YHAT R=RESID;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
PROC SORT DATA= tarea5_1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= tarea5_1; BY B;
PROC MEANS MEAN STDERR; BY B;
PROC SORT DATA= tarea5_1; BY C;
PROC MEANS MEAN STDERR; BY C;
PROC SORT DATA= tarea5_1; BY A*B;
PROC MEANS MEAN STDERR; BY A*B;
PROC SORT DATA= tarea5_1; BY B*C;
PROC MEANS MEAN STDERR; BY B*C;
PROC SORT DATA= tarea5_1; BY A*C;
PROC MEANS MEAN STDERR; BY A*C;
PROC SORT DATA= tarea5_1; BY A*B*C;
PROC MEANS MEAN STDERR; BY A*B*C;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*B*C/STDERR;
RUN;
```

Corrida en SAS para la concentración de ácido lactico de la fase 1 en el segundo estudio

```
Data tarea5_1;
DO A=1 TO 2;
DO B=1 TO 2;
DO C=1 TO 2;
DO R=1 TO 4;
INPUT Y@@;
COMAB=A*10+B;
COMAC=A*10+C;
COMBC=B*10+C;
COMABC=A*100+B*10+C;
OUTPUT;
END;
```

```
END;
END;
END;
CARDS;
```

209.61	252.23	249.58	218.25
231.39	274.02	309.99	259.44
224.46	312.43	264.34	264.96
199.8	166.62	152.95	170.1
236.16	173.62	165.27	175.1
188.69	183.78	202.7	180.27
218.62	189.42	214.2	219.43
208.89	251.59	222.28	238.55

```
;
PROC GLM;
CLASS A B C;
MODEL Y=A|B|C;
LSMEANS A B C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B A*C B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B/SLICE=B SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*C/SLICE=C SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=A SLICE=B*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=B SLICE=A*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=C SLICE=B*A ADJUST=TUKEY LINES ALPHA=0.05;
OUTPUT OUT=NUEVO P=YHAT R=RESID;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
PROC SORT DATA= tarea5_1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= tarea5_1; BY B;
PROC MEANS MEAN STDERR; BY B;
PROC SORT DATA= tarea5_1; BY C;
PROC MEANS MEAN STDERR; BY C;
PROC SORT DATA= tarea5_1; BY A*B;
PROC MEANS MEAN STDERR; BY A*B;
PROC SORT DATA= tarea5_1; BY B*C;
PROC MEANS MEAN STDERR; BY B*C;
PROC SORT DATA= tarea5_1; BY A*C;
PROC MEANS MEAN STDERR; BY A*C;
PROC SORT DATA= tarea5_1; BY A*B*C;
PROC MEANS MEAN STDERR; BY A*B*C;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*B*C/STDERR;
RUN;
```

Corrida en SAS para el pH de la fase 2 en el segundo estudio

```
Data tarea5_1;
DO A=1 TO 2;
DO B=1 TO 2;
DO C=1 TO 2;
DO R=1 TO 4;
INPUT Y@@;
COMAB=A*10+B;
COMAC=A*10+C;
COMBC=B*10+C;
COMABC=A*100+B*10+C;
OUTPUT;
END;
END;
END;
END;
CARDS;
```

3.99	3.94	3.89	3.92
3.85	3.86	3.82	3.8

3.75	3.78	3.74	3.76
3.71	3.67	3.62	3.6
3.85	3.79	3.84	3.9
3.85	3.83	3.87	3.76
3.78	3.83	3.77	3.84
3.76	3.77	3.72	3.77

```sas
;
PROC GLM;
CLASS A B C;
MODEL Y=A|B|C;
LSMEANS A B C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B A*C B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B/SLICE=B SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*C/SLICE=C SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=A SLICE=B*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=B SLICE=A*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=C SLICE=B*A ADJUST=TUKEY LINES ALPHA=0.05;
OUTPUT OUT=NUEVO P=YHAT R=RESID;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
PROC SORT DATA= tarea5_1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= tarea5 1; BY B;
PROC MEANS MEAN STDERR; BY B;
PROC SORT DATA= tarea5_1; BY C;
PROC MEANS MEAN STDERR; BY C;
PROC SORT DATA= tarea5_1; BY A*B;
PROC MEANS MEAN STDERR; BY A*B;
PROC SORT DATA= tarea5_1; BY B*C;
PROC MEANS MEAN STDERR; BY B*C;
PROC SORT DATA= tarea5_1; BY A*C;
PROC MEANS MEAN STDERR; BY A*C;
PROC SORT DATA= tarea5_1; BY A*B*C;
PROC MEANS MEAN STDERR; BY A*B*C;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*B*C/STDERR;
RUN;
```

Corrida en SAS para la concentración de ácido lactico de la fase 2 en el segundo estudio

```sas
Data tarea5 1;
DO A=1 TO 2;
DO B=1 TO 2;
DO C=1 TO 2;
DO R=1 TO 4;
INPUT Y@@;
COMAB=A*10+B;
COMAC=A*10+C;
COMBC=B*10+C;
COMABC=A*100+B*10+C;
OUTPUT;
END;
END;
END;
END;
CARDS;
266.13 262.92 250.31 191.67
182.91 225.23 219.41 174.27
205.15 235.35 169.8  188.46
183.85 186.37 165.44 181.14
206.62 222.65 244.71 224.65
238.4  174.67 249.58 235.55
217.4  209.73 213.33 204.99
201.84 209.53 190.99 201.46
;
PROC GLM;
```

```
CLASS A B C;
MODEL Y=A|B|C;
LSMEANS A B C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B A*C B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B/SLICE=B SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*C/SLICE=C SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=A SLICE=B*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=B SLICE=A*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=C SLICE=B*A ADJUST=TUKEY LINES ALPHA=0.05;
OUTPUT OUT=NUEVO P=YHAT R=RESID;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
PROC SORT DATA= tarea5_1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= tarea5_1; BY B;
PROC MEANS MEAN STDERR; BY B;
PROC SORT DATA= tarea5 1; BY C;
PROC MEANS MEAN STDERR; BY C;
PROC SORT DATA= tarea5_1; BY A*B;
PROC MEANS MEAN STDERR; BY A*B;
PROC SORT DATA= tarea5_1; BY B*C;
PROC MEANS MEAN STDERR; BY B*C;
PROC SORT DATA= tarea5_1; BY A*C;
PROC MEANS MEAN STDERR; BY A*C;
PROC SORT DATA= tarea5_1; BY A*B*C;
PROC MEANS MEAN STDERR; BY A*B*C;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*B*C/STDERR;
RUN;
```

Corrida en SAS para el pH de la fase 3 en el segundo estudio

```
Data tarea5_1;
DO A=1 TO 2;
DO B=1 TO 2;
DO C=1 TO 2;
DO R=1 TO 4;
INPUT Y@@;
COMAB=A*10+B;
COMAC=A*10+C;
COMBC=B*10+C;
COMABC=A*100+B*10+C;
OUTPUT;
END;
END;
END;
END;
CARDS;
```

4.2	4.23	4.15	4.18
4.15	4.1	4.15	4.14
4.14	4.17	4.18	4.17
4.17	4.15	4.14	4.14
4.02	3.95	3.9	3.9
3.88	3.84	3.88	3.83
4.14	4.1	4	3.98
3.93	3.96	3.98	3.9

```
;
PROC GLM;
CLASS A B C;
MODEL Y=A|B|C;
LSMEANS A B C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B A*C B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B/SLICE=B SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*C/SLICE=C SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/ADJUST=TUKEY LINES ALPHA=0.05;
```

```
LSMEANS A*B*C/SLICE=A SLICE=B*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=B SLICE=A*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=C SLICE=B*A ADJUST=TUKEY LINES ALPHA=0.05;
OUTPUT OUT=NUEVO P=YHAT R=RESID;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
PROC SORT DATA= tarea5_1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= tarea5_1; BY B;
PROC MEANS MEAN STDERR; BY B;
PROC SORT DATA= tarea5_1; BY C;
PROC MEANS MEAN STDERR; BY C;
PROC SORT DATA= tarea5_1; BY A*B;
PROC MEANS MEAN STDERR; BY A*B;
PROC SORT DATA= tarea5_1; BY B*C;
PROC MEANS MEAN STDERR; BY B*C;
PROC SORT DATA= tarea5_1; BY A*C;
PROC MEANS MEAN STDERR; BY A*C;
PROC SORT DATA= tarea5_1; BY A*B*C;
PROC MEANS MEAN STDERR; BY A*B*C;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*B*C/STDERR;
RUN;
```

Corrida en SAS para la concentración de ácido lactico de la fase 3 en el segundo estudio

```
Data tarea5_1;
DO A=1 TO 2;
DO B=1 TO 2;
DO C=1 TO 2;
DO R=1 TO 4;
INPUT Y@@;
COMAB=A*10+B;
COMAC=A*10+C;
COMBC=B*10+C;
COMABC=A*100+B*10+C;
OUTPUT;
END;
END;
END;
END;
CARDS;
```

196.22	152.32	166.61	146.98
144.79	142.73	141.32	122.91
227.35	192.83	183.87	200.01
172.36	185.61	181	173.32
178.77	172.08	165.93	162.8
161.05	191.09	177.73	179.13
174.46	163.75	162.26	177.98
209.72	210.62	186.71	217.69

```
;
PROC GLM;
CLASS A B C;
MODEL Y=A|B|C;
LSMEANS A B C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B A*C B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B/SLICE=B SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*C/SLICE=C SLICE=A ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=A SLICE=B*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=B SLICE=A*C ADJUST=TUKEY LINES ALPHA=0.05;
LSMEANS A*B*C/SLICE=C SLICE=B*A ADJUST=TUKEY LINES ALPHA=0.05;
OUTPUT OUT=NUEVO P=YHAT R=RESID;
PROC UNIVARIATE NORMAL DATA=NEW;
VAR RESID;
```

```
PROC SORT DATA= tarea5_1; BY A;
PROC MEANS MEAN STDERR; BY A;
PROC SORT DATA= tarea5_1; BY B;
PROC MEANS MEAN STDERR; BY B;
PROC SORT DATA= tarea5_1; BY C;
PROC MEANS MEAN STDERR; BY C;
PROC SORT DATA= tarea5_1; BY A*B;
PROC MEANS MEAN STDERR; BY A*B;
PROC SORT DATA= tarea5_1; BY B*C;
PROC MEANS MEAN STDERR; BY B*C;
PROC SORT DATA= tarea5_1; BY A*C;
PROC MEANS MEAN STDERR; BY A*C;
PROC SORT DATA= tarea5_1; BY A*B*C;
PROC MEANS MEAN STDERR; BY A*B*C;
PROC PLOT DATA=NEW;
PLOT YHAT*RESID;
PROC MEANS A*B*C/STDERR;
RUN;
```