Centro de Investigación en Alimentación y Desarrollo, A.C.

RELACIÓN ENTRE LA DIETA Y EL MICROBIOMA INTESTINAL DEL PEZ CEBRA (*Danio rerio*)

Por:

Erick Josué Navarro Barrón

TESIS APROBADA POR LA

COORDINACIÓN EN ACUICULTURA Y MANEJO AMBIENTAL

Como requisito parcial para obtener el grado de

DOCTOR EN CIENCIAS

Mazatlán, Sinaloa Septiembre 2021

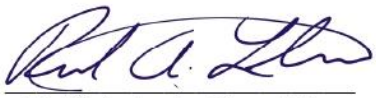
APROBACIÓN

Los miembros del comité designado para la revisión de la tesis de Erick Josué Navarro Barrón, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias.

Dr. Bruno Gómez Gil Rodríguez Sala
Director de tesis

Dra. Crisantema Hernández González
Integrante del comité de tesis

Dra. Alejandra García Gasca
Integrante del comité de tesis



Dr. Raúl Antonio Llera Herrera
Integrante del comité de tesis

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Dr. Pablo Wong González
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AGRADECIMIENTOS

Agradezco al Consejo Nacional de Ciencia y Tecnología (CONACYT) por la beca de doctorado otorgada.

Agradezco al Centro de Investigación en Alimentación y Desarrollo (CIAD), por permitirme desarrollar mis estudios e investigaciones para la elaboración de mi tesis doctoral.

Agradezco a mi director de tesis, Dr. Bruno Gómez Gil Rodríguez Sala, por permitirme formar parte de su equipo de trabajo, dirigir mi proyecto de tesis, y por ser parte fundamental de mi formación profesional.

Agradezco a la Dra. Crisantema Hernández Gonzáles por fungir con asesora de tesis, integrarme a su equipo de trabajo en el Laboratorio de Nutrición y por sus aportaciones al proyecto.

Agradezco a la Dra. Silvia Alejandra García Gasca por fungir como asesora de tesis, por el apoyo continuo durante el posgrado y sus aportaciones al proyecto.

Agradezco al Dr. Raúl Llera Herrera por fungir como asesor de tesis, por sus aportaciones al proyecto y el continuo apoyo durante el posgrado.

Agradezco al equipo técnico de Bacteriología y Genómica Microbiana, a la M.C. María del Carmen Bolán Mejía, a la M.C. Julissa Enciso Ibarra y a la M.C. Karen Enciso Ibarra por la capacitación y apoyo técnico.

Agradezco al personal técnico del CIAD, a la M.C. Selene Abad Rosales, a la M.C. Yazmín Sánchez Gutiérrez, al M.V.Z Rodolfo Lozano Olvera y a la M.C. Karla Aguilar por la capacitación y apoyo técnico.

Agradezco a mis amigos y compañeros de doctorado, quienes fueron un apoyo emocional y compartí grandes experiencias: Citlalic Pimentel, Ana Vargas, Mauricio Escalante, Andrés Galeana, Adrián Gonzáles, Rodolfo Martín y Marcelo Victorio.

Agradecimientos a Iris Navarro por el apoyo técnico en el laboratorio de nutrición y durante los muestreos.

Agradecimientos a Jenifer Rodríguez por el apoyo técnico durante los muestreos, las jornadas experimentales, y por acompañarme en todo momento.

Agradecimientos especiales a Jesús Navarro e Ismelda Barrón por el apoyo incondicional.

CONTENIDO

RESUMEN

En este estudio evaluamos el efecto de la dieta y la sobrealimentación en la microbiota y el microbioma intestinal de pez cebra (*Danio rerio*). La investigación se llevó a cabo en dos etapas, en la primera se evaluó el efecto de una dieta alta en grasa suministrada bajo un régimen basal (HF) y bajo sobrealimentación (HFO) contra una dieta control baja en grasa (LF); el experimento se llevó a cabo en machos y hembras por separado. Se utilizaron índices biométricos para determinar la obesidad. Además, se analizó la huella taxonómica de más de 2.6 millones de secuencias del gen 16S ARNr generadas por la plataforma Illumina Miniseq, las secuencias se agruparon con el 97% de similitud con vsearch y se clasificaron utilizando la base de datos de EzBioCloud. Los resultados revelaron que HFO induce la obesidad y perturba la microbiota del pez cebra ($p < 0.05$) afectando en mayor medida a los machos. Además, se reveló que HF no altera la microbiota intestinal, ni los parámetros biológicos con respecto a LF. En la segunda etapa se llevó a cabo un experimento con peces machos para determinar si la sobrealimentación con una dieta baja en grasa (LFO) replica los resultados del tratamiento HFO, además se incluyeron grupos de peces alimentados con una inclusión de agavina (5%) con el fin de evaluar su potencial prebiótico en sobrealimentación (LFAO) y en régimen basal (LFA). Los resultados mostraron que LFO altera la microbiota intestinal y aumenta la ganancia de peso e índices biométricos relacionados a la obesidad. La inclusión de agavina redujo significativamente la ingesta de alimento, la ganancia de peso y la grasa corporal del pez cebra en LFAO; además los análisis multivariados sugieren que la inclusión de agavina promueve la proliferación de *Fusobacteriaceae*. Finalmente, se llevó a cabo el análisis del microbioma intestinal de los grupos experimentales (LF, LFO, HF y HFO). Los resultados mostraron que la sobrealimentación también afecta las funciones del microbioma y dichas alteraciones son dependientes de la grasa en la dieta. Algunas funciones como la *fermentación de lisina* y *degradación de leucina* se encuentran relacionadas con la grasa en la dieta, mientras que la *síntesis de 3-hidroxi-3-metilglutaril-coenzima A* (*HMG CoA*) y las *conversiones de pirimidina* se relacionaron con el régimen alimentario. La sobrealimentación y la grasa en la dieta son factores que hacen una sinergia en la modulación de la microbiota y el microbioma intestinal.

Palabras clave: composición bacteriana intestinal, sobrealimentación, agavina, microbioma, grasa dietaria, análisis metagenómico, gen 16S rRNA.

ABSTRACT

In this study we evaluated the effect of the diet and overfeeding on the gut microbiota of zebrafish (*Danio rerio*). The research was carried out in two experimental stages, in the first stage the effect of a high-fat diet supplied under a basal regimen (HF) and overfeeding (HFO) was evaluated with a low-fat diet (LF) on males and females separately. Weight gain, condition factor and body mass index were used to determine obesity. 16S rRNA gene taxonomic fingerprint of more than 2.6 million sequences generated by the Illumina Miniseq platform were analyzed, the sequences were grouped with 97% similarity with vsearch and classified using the EzBioCloud database. Multivariate analyzes and diversity indices revealed that HFO induces obesity and disturbs the microbiota composition of zebrafish ($p < 0.05$), but these changes are dependent on sex affecting males to a greater extent. Moreover, the results indicated that HF does not alter the gut microbiota nor the biological parameters with respect to LF. In the second stage, an experiment was carried out with male fish to determine if overfeeding with a low-fat diet (LFO) replicates the results of HFO, in addition, some groups were fed with an inclusion of agavins (5%) in order to evaluate its prebiotic potential in overfeeding (LFAO) and basal regimen (LFA). LFO was shown to alter the gut microbiota, promote the weight gain and increase the biometric indices related to overweight and obesity. The inclusion of agavins significantly reduced feed intake, weight gain and body fat of zebrafish in LFAO; Furthermore, multivariate analyzes suggest that agavins inclusion promotes the proliferation of *Fusobacteriaceae*. Finally, the analysis of the gut microbiome in experimental groups (LF, LFO, HF and HFO) was carried out. The results showed that overeating also affects the microbiome functions and these alterations are dependent of dietary fat. Some functions such as *lysine fermentation* and *leucine degradation* were related to dietary fat, while *3-hydroxy-3-methylglutaryl coenzyme-A* (*HMG CoA*) *synthesis* and *pyrimidine conversions* were related to feeding regime. Overfeeding and dietary fat are synergistic factors in modulating the microbiota and the gut microbiome.

Keywords: gut bacterial composition, overfeeding, microbiome, dietary fat, agavins shotgun metagenomic analysis, 16S rRNA gene taxonomic fingerprinting.

1. SINÓPSIS

1.1. Justificación

La obesidad es uno de los principales problemas de salud pública a nivel mundial debido al gran número de individuos que la padecen y los trastornos metabólicos que esta conlleva. Según la Organización Mundial de la Salud, la obesidad se define como una acumulación anormal o excesiva de grasa que puede ser perjudicial para la salud. Entre los principales factores que conllevan a que un individuo incremente en exceso su peso corporal se encuentran los malos hábitos alimenticios. Uno de ellos es la sobrealimentación, la cual se define como la ingesta desmedida de nutrientes que genera un superávit calórico que promueve el sobrepeso y en casos severos la obesidad o algún otro padecimiento metabólico relacionada a esta como síndrome metabólico, aterosclerosis, diabetes mellitus tipo II, hígado graso, enfermedades coronarias, artrosis, etc. En los últimos años y gracias a los hallazgos recientes ha aumentado el interés por el estudio de la microbiota intestinal y el papel que juega en la salud metabólica. La microbiota intestinal es considerada un sistema complejo y dinámico que interviene en importantes procesos metabólicos y fisiológicos del organismo, que debido a su complejidad y dinamismo no hemos podido comprender completamente los alcances de sus funciones, pero gracias a las tecnologías de secuenciación masiva de nueva generación y técnicas bioinformáticas que surgieron hace algunos años es posible su estudio a través de enfoques integrales.

Mediante el estudio del pez cebra como organismo modelo y bajo un enfoque metagenómico, esta investigación pretende esclarecer aún más la relación que tiene la microbiota intestinal con factores como la grasa dietaria, la sobrealimentación y el efecto probiótico. Además, el análisis de índices biométricos nos permitirá aproximar y establecer una relación entre la condición de obesidad con la salud metabólica microbiana del organismo

1.2 Antecedentes

A los microorganismos que colonizan el tracto gastrointestinal se les denomina microbiota intestinal y ha evolucionado conjuntamente con el huésped durante miles de años para formar una relación intrincada y mutuamente beneficiosa (Cho y Blaser, 2012; Thursby y Juge, 2017). La microbiota intestinal se considera un órgano más del cuerpo, está compuesta por trillones de microorganismos y tiene una diversidad de genes al menos 100 veces mayor que el resto del organismo (Gill *et al*., 2006; Zhang y Yang, 2016). Además, es considerada un ecosistema microbiano complejo y dinámico en el que se encuentran una amplia gama de microbios derivados del ambiente donde reside el hospedero (Cho y Blaser, 2012). Entre los grupos microbianos que colonizan el tracto gastrointestinal, las bacterias (aerobias, anaerobias facultativas y anaerobias obligadas) son las más abundantes (Nayak, 2010). La composición y estructura de la microbiota intestinal puede variar dependiendo de diversos factores como la etapa de desarrollo del organismo (Mohajeri *et al*., 2018; Pangastuti 2010; Rungrassamee *et al*., 2013), el entorno en el que habita (Rungrassamee *et al*., 2014), el estado de salud (Clemente *et al*., 2012) y la dieta (Zhang *et al*., 2014).

Tanto en mamíferos como en organismos acuáticos la microbiota intestinal desempeña un papel en la absorción de nutrientes, la homeostasis metabólica y la defensa inmune (Gómez y Balcázar, 2008); aspectos directamente relacionados a la salud. Debido a la naturaleza simbionte de la microbiota intestinal y a sus capacidades metabólicas que benefician al organismo hospedero, en los últimos años se han llevado a cabo investigaciones para entender los alcances y la influencia que ejerce en el estado de salud. Algunos de estos hallazgos indican una relación de la microbiota intestinal con ciertos padecimientos como son los trastornos gastrointestinales (Marchesi *et al*., 2016), enfermedades renales (Al Khodor y Shatat, 2017), hepáticas (Qin *et al*., 2014), algunos tipos de cáncer (Zitvogel *et al*., 2015), diabetes autoinmune tipo 1 (Davis-Richardson y Triplett, 2015) y la obesidad (Duranti *et al*., 2017; Mekkes *et al*., 2014).

El establecimiento de una microbiota intestinal normal puede considerarse complementario al desarrollo del sistema digestivo, y en condiciones normales funciona como una barrera contra los patógenos invasores (Farzanfar, 2006). Una de las formas más sencillas y eficientes de inducir cambios en la microbiota intestinal es a través de la dieta (Zhang *et al*., 2014). Algunos de los

principales métodos de manipulación de la microbiota intestinal han comprendido la alteración o inclusión de ciertos nutrientes como proteínas y lípidos, así como también la adición de probióticos y prebióticos (Egerton *et al*., 2018). Por otro lado, la microbiota intestinal también puede ayudar en la regulación del balance energético en beneficio de la salud, ya que existe evidencia de la relación de ciertas bacterias benéficas con la activación de rutas relacionadas con el metabolismo de triglicéridos, glucosa y el apetito (Falcinelli *et al*., 2015, 2016a).

En cuanto a la obesidad, ésta se presenta como una condición causada principalmente por una alteración de la ingesta que promueve un balance energético positivo y que puede estar influenciado por factores genéticos y ambientales (Hou *et al*., 2017; López-Cepero y Palacios, 2015). A nivel mundial la obesidad prevalece y está aumentando a una tasa epidémica global; se estima que existen más de mil millones de adultos con sobrepeso y al menos 300 millones de ellos presentan esta condición, además se cree que la cantidad de personas obesas seguirá aumentando los próximos 20 o 30 años (Ghoorah *et al*., 2016). Esta condición generalmente conlleva al padecimiento de otros problemas de salud graves como la hipertensión, la diabetes mellitus tipo 2 y enfermedades cardiovasculares, entre otras (Yang *et al*., 2012).

Los hábitos dietarios como la sobrealimentación se han vuelto un problema porque originan obesidad (Zhang y Yang 2016). Además, al ser la dieta uno de los principales factores que modulan la microbiota intestinal, ésta va definir en gran parte su estructura o composición (Pray *et al*., 2013). En un estudio llevado a cabo por Zhong y colaboradores (2020) se plantea la evidencia de una relación entre la microbiota intestinal con enfermedades crónicas severas. Por ejemplo, se ha reportado que personas sanas presentan una mayor diversidad y riqueza de especies bacterianas en contraste con personas obesas con complicaciones metabólicas. Además, se reportan ciertas diferencias en la composición de los grupos más representativos lo que sugiere cambios importantes.

En otro estudio Porras y colaboradores (2017) reportan que el consumo de dietas altas en grasa que promueven el hígado graso se relaciona con las alteraciones de la composición bacteriana, asimismo existe una relación entre la sobreexpresión de genes pro-inflamatorios con la aparición de disbiosis.

Por otro lado, en modelos obesógenicos hipercalóricos (humanos) se han encontrado cambios específicos en las abundancias relativas de ciertos grupos bacterianos con respecto a los componentes de las dietas. Lo anterior se determinó por el aumento significativo de las

Proteobacterias en grupos alimentados con grasas saturadas, mientras que para grupos alimentados con grasas insaturadas el incremento se presentó en las bacterias fermentadoras de butirato (Jian *et al*., 2021), lo cual podría indicar un ambiente intestinal más saludable.

Okazaki y colaboradores (2019) mediante un modelo de pez cebra con diabetes tipo II revelaron que en organismos enfermos la diversidad microbiana intestinal es significativamente menor, lo cual es un signo de disbiosis. Al mismo tiempo, se reportó que varias rutas metabólicas de ciertos aminoácidos (arginina, prolina y fenilalanina) fueron sub-reguladas. En general, cada padecimiento metabólico relacionado a la ingesta calórica excesiva puede originar una disrupción en los parámetros normales de la microbiota intestinal afectando principalmente la diversidad, estructura o composición, medio intestinal y grupos bacterianos específicos que en casos más severos o prolongados puede convertirse en una disbiosis.

Una posible solución para restablecer las alteraciones en la microbiota intestinal por efecto de la sobrealimentación y la ingesta excesiva de grasa son los aditivos alimentarios llamados prebióticos, los cuales según Pressman *et al*. (2017) son definidos como ingredientes funcionales no digeribles (en su mayoría fibras) que benefician al huésped estimulando selectivamente el crecimiento de y/o actividad de grupos bacterianos específicos de la microbiota intestinal, mejorando así la salud (Gibson *et al*., 2004; Gibson y Roberfroid, 1995). A pesar de que todos los prebióticos son fibras, no toda fibra es prebiótica (Slavin, 2013). Así mismo según Gibson y Roberfroid (1995), para que un aditivo sea clasificado como prebiótico debe demostrarse científicamente que:

- El aditivo es resistente a la acidez gástrica, la hidrolisis enzimática, y la absorción del tracto gastrointestinal,
- es fermentado por la microbiota intestinal (bacterias probióticas), y
- estimula selectivamente la proliferación y/o actividad de otras bacterias intestinales asociadas a efectos benéficos hacia la salud y el bienestar.

Un mecanismo de acción importante en los prebióticos y fibras dietéticas en general es la fermentación de éstos en el colon y los cambios consecuentes en la microbiota intestinal. El ambiente intestinal es favorable para el crecimiento bacteriano debido al tránsito lento, la cantidad de nutrientes y el pH. En general, en presencia de prebióticos las bacterias que tienen un metabolismo sacarolítico casi exclusivo (es decir, sin actividad proteolítica) pueden considerarse beneficiadas (Slavin, 2013). Dicho perfil es típico de los lactobacilos, bifidobacterias, y fusobacterias las cuales ya han sido reportadas como bacterias probióticas en diversos organismos

(Cholan *et al*., 2020; Eid *et al.*, 2017; Gibson *et al.*, 2004; Rodes *et al*., 2011). De hecho, el principal mecanismo de acción de los prebióticos consiste en promover la proliferación de bacterias benéficas ya sea por su naturaleza como sustrato de las bacterias objetivo o generando las condiciones apropiadas en el tracto intestinal para la proliferación de bacterias simbiontes que no permitan la colonización de grupos patógenos (Slavin, 2013) y generen una mejor absorción de nutrientes (Krajmalnik-Brown *et al*., 2012).

Según Lupton (2004), las bacterias intestinales utilizan una variedad de enzimas hidrolizantes de carbohidratos para producir hidrógeno, metano, dióxido de carbono y ácidos grasos de cadena corta (SCFA, por sus siglas en inglés; principalmente acetato, propionato, butirato y lactato). A su vez, ciertas bacterias del colon generan energía a partir de la fermentación de estos productos. Los SCFA son solubles en agua y se absorben en el torrente sanguíneo, asimismo, el cerebro, los músculos y otros tejidos metabolizan el acetato sistémicamente mientras que el propionato se elimina por el hígado y puede disminuir la producción hepática de colesterol al interferir con su síntesis (Slavin, 2013).

Los prebióticos más comunes establecidos hasta la fecha son la inulina, fructooligosacáridos (FOS), fructooligosacáridos de cadena corta (scFOS), mananooligosacátidos (MOS), galactooligosacáridos (GOS), xilololigosacáridos (XOS) (Ringo *et al*., 2010) y recientemente las agavinas (Fuentes-Quesada *et al*., 2020; Huazano-García y López, 2015; Huazano-García *et al*., 2017). Los fructanos tipo inulina constituyen un grupo de oligosacáridos derivados de D-fructosa unidas en enlaces β 1-2, que son aislados de fuentes vegetales naturales y que pueden encontrarse en una gran variedad de granos comestibles, frutas, verduras y algas (Mussatto y Mancilha, 2007; Sarbini y Rastall, 2011). FOS y scFOS son oligosacáridos formados por monómeros de fructosa y además son algunos de los prebióticos más estudiados. Tanto en organismos acuáticos como en terrestres estas fibras han mostrado tener un efecto benéfico principalmente en las tasas de crecimiento, ingesta, conversión alimenticia y supervivencia; además de una relación con el incremento de la densidad y longitud de las microvellosidades intestinales lo que contribuye a una mejor adsorción de nutrientes (Huazano-García and López 2015; Miqdady *et al*., 2020; Ringo *et al*., 2010; Slavin 2013).

Según Cerezuela, *et al*. (2013 a,b) en sus estudios con inclusión de inulina en la Dorada *Sparaus aurata*, este prebiótico altera la diversidad bacteriana de forma específica, así como también la expresión de transferrina, una proteína de transporte involucrada en gran variedad de procesos

metabólicos y que en peces se encuentra relacionada con actividad inmune en tejidos de piel, sangre y bazo.

Aunque en general toda la gama de fibras utilizadas como prebióticos presentan beneficios similares para el hospedero independientemente de su hábitat (terrestre o acuático), las agavinas han tomado relevancia en los últimos años. Las agavinas son fibras prebióticas que se extraen de algunas especies de agave como *Agave tequilana*, *Agave angustifolia* y *Agave potatorum*. Se ha reportado que estas fibras promueven una mejora a los desórdenes metabólicos inducidos por la ingesta excesiva de dietas altas en grasa ayudando con la regulación del peso corporal y los niveles de glucosa, triglicéridos y colesterol (Huazano-García y López 2015). Asimismo, en estudios recientes se ha reportado que organismos tratados con inclusiones de agavina en la dieta incrementaron las concentraciones de SCFA en el colon, lo cual se relacionó con el decremento del colesterol y secreciones hormonales involucradas en la regulación del apetito (Alvarado-Jasso *et al*., 2020; Huazano-García *et al*., 2017).

Además de las propiedades que tiene la fermentación de los prebióticos en el tracto gastrointestinal, uno de los principales objetivos de su uso es que promueven la proliferación de las bacterias fermentadoras denominadas probióticos, las cuales son microorganismos que al ser administrados o encontrarse en cantidades adecuadas confieren algún beneficio en la salud intestinal del hospedero (Pandey *et al*., 2015). Sus mecanismos de acción son variados, los probióticos son los encargados de fermentar las fibras en el intestino produciendo los SCFA, generando así un pH ácido lo cual a su vez favorece el crecimiento de otros grupos bacterianos simbiontes o benéficos para el hospedero (Hai, 2015).

Otra característica es que poseen la capacidad de aumentar las mucinas ileocolónicas que coadyuvan en el recubrimiento del intestino con una capa de moco (Schiffrin y Blum 2002), el cual es un mecanismo inespecífico pero eficaz contra las infecciones por patógenos (Tormo, 2006). Probióticos de los géneros *Bifidobacterium* y *Lactobacillus* son directamente considerados antagónicos de patógenos, contribuyen a la modulación de la microbiota intestinal promueven la maduración del intestino desde etapas tempranas de desarrollo, pueden segregar antibióticos naturales (Costa-Ribeiro *et al*., 2003) y su administración por periodos continuos coadyuva en la proliferación de otros grupos aeróbicos benéficos (Spanhaak *et al*., 1998).

En cuanto a su relación con la salud metabólica, los probióticos se encuentran relacionados con la pérdida de peso, el metabolismo del colesterol y triglicéridos (Tormo, 2006). Además, se tiene

registro de un incremento de los ácidos biliares en las heces de organismos tratados con probióticos, esto indica una mayor degradación del colesterol lo que puede explicar su disminución en el organismo (Tormo, 2006). Recientemente una serie de estudios realizados en pez cebra han demostrado que la ingesta de probióticos (*Lactobacillus rhamnosus*) influye en la expresión de ciertos genes involucrados en el metabolismo de lípidos como: *dgat2*, *mgll*, *fit2*, entre otros, en favor a una reducción significativa de los niveles de triglicéridos (TAG) y colesterol (Falcinelli *et al*., 2015), además se ha reportado una disminución en los niveles de glucosa en la sangre y la ingesta alimenticia mediante la modulación de los genes *insulin* y *leptin*, respectivamente (Falcinelli *et al*., 2016a). En general cuando se estudia el uso de fibras como prebióticos es necesario considerar a los probióticos ya que son los encargados de metabolizar dichas fibras y generar beneficios al hospedero con su proliferación y producción de metabolitos.

Las investigaciones de la microbiota intestinal en relación a la obesidad humana no se limitan exclusivamente al análisis de muestras provenientes de humanos, de hecho, la gran mayoría de las investigaciones se llevan a cabo bajo un enfoque reduccionista donde se utilizan organismos modelo y/o se manipulan las condiciones experimentales.

Un organismo modelo que ha ganado relevancia para es este tipo de estudios es el pez cebra, que pese a ser un organismo acuático comparte una identidad genética del 70% con el humano y sus sistemas de órganos son funcionalmente similares (Seth *et al*., 2013ª; Davis *et al*., 2014). Entre sus principales ventajas se encuentra la fácil crianza, número de crías elevados, periodos cortos de desarrollo, y un cuerpo traslúcido que permite ver los órganos en etapas larvarias (Seth *et al*., 2013b). Además, a partir de 2013 su genoma (completamente secuenciado) se encuentra disponible (Howe *et al*., 2013), lo que permite el estudio de los efectos de la variación del ADN y facilita el entendimiento de su biología.

Al igual que otros modelos mamíferos, el pez cebra permite un estudio integral de la relación de la microbiota intestinal con factores como la obesidad y la sobrealimentación (Oka *et al*., 2010; Okazaki *et al*., 2019). Debido a que la dieta es considerada el factor principal que conduce a la obesidad humana (Hill *et al*., 2012; Hruby y Hu, 2015), sin importar el modelo, cualquier investigación dirigida a evaluar los cambios de la microbiota intestinal por efecto de dietas altas en grasa, carbohidratos o hipercalóricas, generará conocimiento útil a este campo de la investigación.

La homeostasis energética y la regulación metabólica del cuerpo implican interacciones complejas entre múltiples órganos y señalizaciones del sistema endócrino que permiten el equilibrio de la

ingesta, la utilización de nutrientes y el almacenamiento energético. Estas interacciones complejas que existen in vivo no pueden ser recreadas en estudios in vitro. Es por ello, que, para estudiar el metabolismo en un contexto multicelular, es conveniente adaptarse a un enfoque experimental de animales completos. El pez cebra posee todos los órganos clave necesarios para el control metabólico en humanos, desde los circuitos endócrinos del apetito presentes en el hipotálamo hasta el páncreas y otros tejidos sensibles a la insulina como el hígado, músculo y tejido adiposo blanco (Seth *et al*., 2013a).

Recientemente el pez cebra fue utilizado como modelo para evaluar el efecto probiótico de *Lactobacillus rhamnosus* en peces alimentados con dietas con distintos niveles de grasa (Falcinelli *et al*., 2017), en general, los resultados sugirieron que los grupos tratados con probióticos presentaron una regulación positiva en los genes anorexigénicos, así como una modulación en la expresión de genes involucrados en el metabolismo de triglicéridos y colesterol.

En otros estudios se han generado modelos de pez cebra obeso mediante la sobrealimentación y alteración de la dieta (Landgraf *et al*., 2017; Oka *et al*., 2010); en estos trabajos se ha demostrado que es posible la generación de fenotipos con trastornos metabólicos similares a los que se observan en el ser humano como obesidad, aumento del tejido adiposo, diabetes tipo II y enfermedades coronarias, entre otros (Zang *et al*., 2018).

En cuanto a la estructura y diversidad de su microbiota intestinal se sabe que comparte cierta similitud con la de la mayoría de organismos acuáticos, siendo su principal componente las *Proteobacteria*, seguido de otros grupos como, *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Actinobacteria*, etc. (Falcinelli *et al*., 2016b; Stephens *et al*., 2016; Udayangani *et al*., 2017). En general, todos los animales han co-evolucionado con los microorganismos de su entorno, es por ello que la microbiota intestinal del pez cebra es muy diferente a la de los mamíferos (desde nivel phylum), pero esta induce una respuesta muy conservada durante la colonización, el desarrollo y la susceptibilidad a enfermedades (Brugman, 2016). A pesar que los mamíferos y los peces viven en entornos diferentes y claramente tienen una fisiología distinta, la expresión génica intestinal y su regulación son similares (López Nadal *et al*., 2020).

Con lo anterior, el objetivo del presente proyecto fue evaluar en qué medida la sobrealimentación y el porcentaje de grasa en la dieta inducen la obesidad y modulan la estructura y diversidad de la microbiota y el microbioma intestinal del pez cebra (*Danio rerio*), así como también se evaluó el efecto prebiótico de la inclusión de agavina en la dieta. Este trabajo se llevó a cabo mediante el uso

de técnicas bioinformáticas y de secuenciación masiva del gen 16S ARNr y ADN metagenómico intestinal que fueron complementadas con el análisis de parámetros biológicos del pez para estudio integral de la microbiota intestinal en relación a con la salud metabólica.

1.3 Hipótesis

Acorde a la investigación antes expuesta, se formularon las siguientes hipótesis de investigación para el actual proyecto:

Hipótesis 1: La sobrealimentación promueve a la obesidad en pez cebra (*Danio rerio*) y modula la estructura y diversidad de la microbiota intestinal

Hipótesis 2: Los cambios en la microbiota intestinal por efecto de la sobrealimentación son dependientes del sexo del pez cebra y el porcentaje de grasa en la dieta.

Hipótesis 3: La inclusión de agavina al 5% en la dieta tiene un efecto benéfico en la microbiota intestinal del pez cebra y parámetros biológicos afectados por la sobrealimentación.

Hipótesis 4: Las funciones del microbioma intestinal de peces cebra son alteradas por efecto de la sobrealimentación y la cantidad de grasa en la dieta.

1.4 Objetivo General

Evaluar en qué medida la sobrealimentación y el porcentaje de grasa en la dieta inducen la obesidad y modulan la estructura y diversidad de la microbiota y el microbioma intestinal del pez cebra (*Danio rerio*); así como también determinar el efecto prebiótico de la inclusión de agavina en la dieta.

1.5 Objetivos Específicos

1. Determinar si una dieta alta grasa suministrada bajo un régimen basal altera la estructura y diversidad de la microbiota intestinal del pez cebra en comparación con una dieta estándar baja en grasa.
2. Determinar si la sobrealimentación con una dieta alta en grasa suministrada bajo 8 semanas induce al pez cebra a un estado de obesidad y evaluar la alteración de la microbiota intestinal.
3. Evaluar si los cambios en la microbiota intestinal por efecto del régimen en la dieta son dependientes del sexo.
4. Determinar si la sobrealimentación con dietas bajas en grasa induce al pez cebra a un estado de obesidad y por consecuencia altera la microbiota intestinal.
5. Evaluar el efecto prebiótico de la agavina en pez cebra sobrealimentado con una dieta baja en grasa.
6. Determinar los cambios en las funciones del microbioma intestinal por efecto de la sobrealimentación y la grasa dietaria en pez cebra macho

1.6 Sección Integradora del Trabajo

La presente tesis se integra en cinco capítulos. Dentro del esquema se incluyen: la sinopsis de la tesis (Capítulo 1; presente capítulo), un artículo de investigación original publicado (Capítulo 2) y un artículo sometido en formato de publicación (Capítulo 3), seguido por las conclusiones generales y las recomendaciones (Capítulos 4 y 5; respectivamente). Los artículos se anexan en el idioma en el cual fueron publicados y sometidos. A continuación, se describe una síntesis de las publicaciones que forman parte de los capítulos 2 y 3 de esta tesis.

En el segundo capítulo titulado "**Overfeeding a high-fat diet promotes sex-specific alterations on the gut microbiota of the zebrafish** (*Danio rerio*)", se realizó un ensayo experimental con pez cebra adulto de 3.5 mpf (meses post fertilización; por sus siglas en inglés). Los peces fueron distribuidos en tres tratamientos, el primer grupo fue alimentado con una dieta baja en grasa (LF),

el segundo con una dieta alta en grasa (HF) y un tercer grupo fue sobrealimentado con la dieta alta en grasa (HFO). Este ensayo se realizó por duplicado para peces machos y hembras durante ocho semanas. Al final del experimento se registraron algunos parámetros biológicos como la ingesta, ganancia en peso, factor de condición e índice de masa corporal, además se realizó una caracterización metagenómica de la diversidad y la composición de la microbiota intestinal. Los resultados metagenómicos fueron comparados mediante análisis estadísticos multivariados (PERMANOVA Y ANOSIM) tomando en como variables: la grasa dietaria, el régimen y el sexo. Los resultados de este capítulo indican cambios en la microbiota intestinal por efecto de la sobrealimentación y la cantidad de grasa en la dieta. Además, se observó que los cambios en la microbiota intestinal por efecto de las dietas son dependientes del sexo de pez cebra. Con el trabajo realizado en este capítulo se cumplieron los primeros tres objetivos de la tesis, así como las hipótesis de investigación 1 y 2. El resumen gráfico del capítulo 2 se representa en la figura 1.

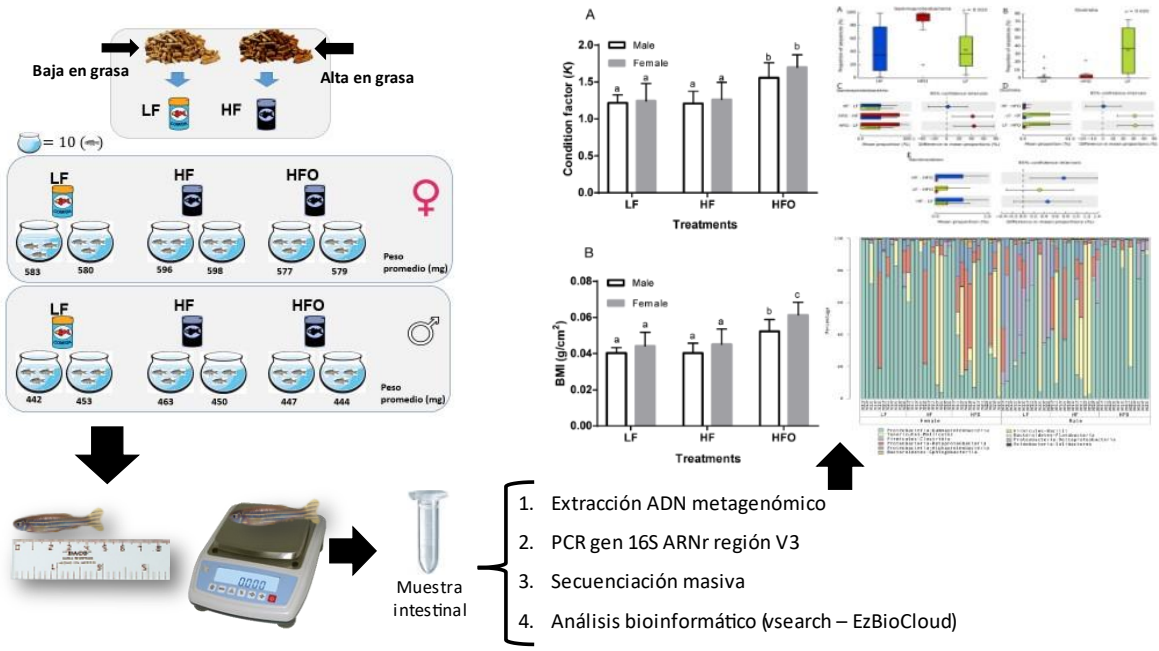

Figura 1. Resumen gráfico del capítulo 2.

En el tercer capítulo titulado "**Overfeeding, agavins and dietary fat; factors that modulate the microbiome of the zebrafish gut**", se realizó un ensayo experimental durante ocho semanas con peces machos de 2.5 mpf. Los peces fueron distribuidos en 4 tratamientos alimentados con dietas

bajas en grasa con y sin inclusión de agavina (5%) como prebiótico; dos grupos alimentados bajo régimen basal (LF y LFA) y dos grupos bajo sobrealimentación (LFO y LFAO). Posterior al experimento se registraron algunos parámetros biológicos como la ingesta, ganancia en peso, factor de condición, índices de masa corporal, viscerosomático y hepatosomático, además de la caracterización y comparación de los cambios en la microbiota intestinal y sus funciones. Los resultados de la microbiota fueron comparados mediante análisis estadísticos multivariados (PERMANOVA Y ANOSIM) tomando en cuenta el régimen y la inclusión del prebiótico. Además, se realizó un estudio comparativo para determinar los cambios de las funciones del microbioma tomando como variables: la grasa dietaria y el régimen alimenticio; esto comparando muestras de ambos experimentos.

Los resultados de este capítulos indican sinergia de la grasa dietaria y la sobrealimentación en la modulación del microbioma y una relación entre la familia *Fusobacteriaceae* con los grupos de peces alimentados con la inclusión agavina. Con el trabajo realizado en este capítulo se lograron cumplir los objetivos 4, 5 y 6, además se cumplieron las hipótesis 3 y 4. El resumen gráfico del capítulo 3 se presenta en la figura 2.

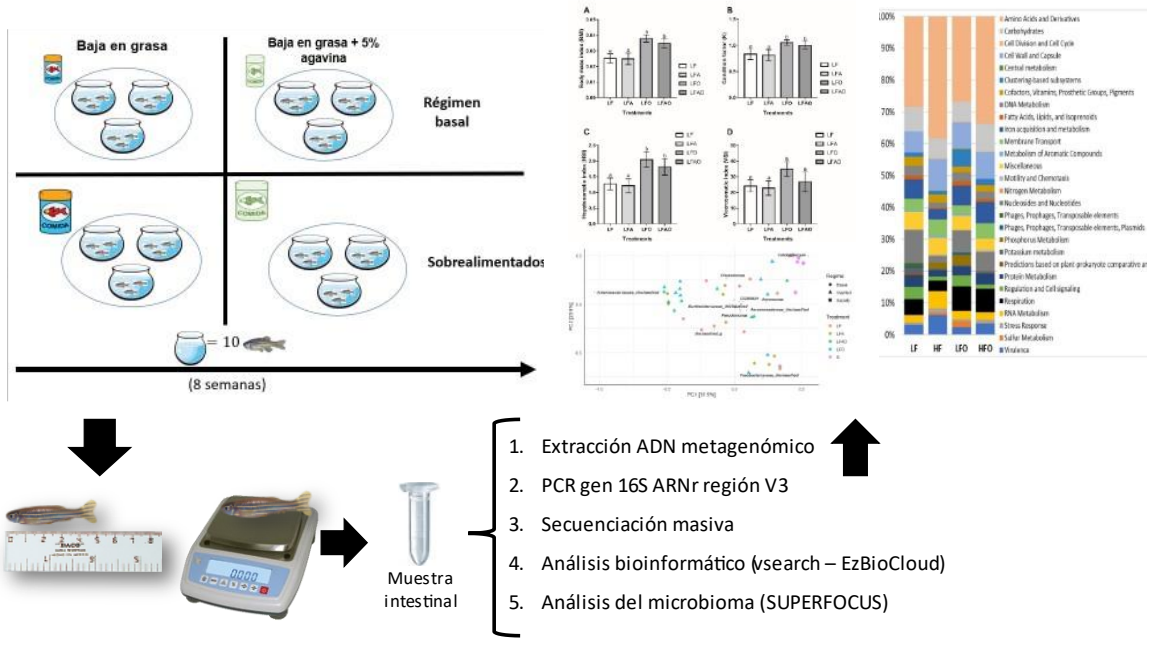

Figura 2. Resumen gráfico del capítulo 3

2. OVERFEEDING A HIGH-FAT DIET PROMOTES SEX-SPECIFIC ALTERATIONS ON THE GUT MICROBIOTA OF THE ZEBRAFISH (*Danio rerio*)

Zebrafish,

16(3), 268-279.

Navarro-Barrón, E., Hernández, C., Llera-Herrera, R., García-Gasca, A., & Gómez-Gil, B.

Artículo original publicado en la revista **Zebrafish**.
https://doi.org/10.1089/zeb.2018.1648

ZEBRAFISH
Volume 00, Number 00, 2019

DOI: 10.1089/zeb.2018.1648

Overfeeding a High-Fat Diet Promotes Sex-Specific Alterations on the Gut Microbiota of the Zebrafish (*Danio rerio*)

Erick Navarro-Barrón,[1] Crisantema Hernández,[1] Raúl Llera-Herrera,[2] Alejandra García-Gasca,[1] and Bruno Gómez-Gil[1]

Abstract

Diet modulates the gut microbiota and is one of the main factors promoting obesity and overweight. In the present study, we investigated the effect of a high-fat diet (HFD) on the gut microbiota of the zebrafish (*Danio rerio*). Fish were separated into three groups and fed in different regimes: low fat, high fat, and high fat overfed; the experiments were performed on males and females separately. We analyzed more than 2.6 million sequences of variable region V3 of the 16S rRNA gene generated by the Illumina Miniseq platform, clustered to 97% similarity with vsearch and classified with the EzBioCloud database. The weight gain, condition factor (K), and body mass index were calculated as indicators of obesity. Multivariate analysis (PERMANOVA and ANOSIM) and diversity indices (Shannon and Dominance) revealed that overfeeding a HFD disturbs the gut microbiota differently in males and females suggesting that sex is a significant factor ($p<0.05$) for the composition of the gut microbiota of zebrafish. The results also indicate that a HFD provided in a basal caloric regime does not promote obesity or alterations in the gut microbiota.

Keywords: gut bacterial community, high-fat diet, overfeeding, 16S ARNr metagenomic analysis

Introduction

The intestinal microbiota is now considered another component of the body, which has a diversity of genes at least 100 times greater than the rest of the body[1] due to the fact that it is composed of trillions of microorganisms.[2] In recent years, several studies have found that these microorganisms play an important role in the health of the host.[3] Diseases related to the immune system, such as autoimmune diabetes (type 1),[4] some types of cancer,[5] kidney disease,[6] liver disease,[7] gastrointestinal disorder,[2] and obesity[8] have been related to the intestinal microbiota. It is well known that the relationship between the immune system and the host is bilateral and this relationship allows alterations in the intestinal microbiota to be associated with the pathogenesis that compromises health.[9]

There are different factors that can cause alterations in the gut microbiota, as the environment of the host,[10] the lifestyle,[11] and the use of antibiotics[12]; however, the diet is the most important modulating factor due to the ability of the microbiota to adapt to the eating habits.[13] Even in organisms of the same species, environment, diet, and health are the main factors involved in the composition of the gut microbiota, however, other factors, such as sex,[14] should also be considered. Even if sex *per se* is not the main variation factor of the intestinal microbiota, it must be studied due to the effect generated by the interaction with the other factors.[15]

Microorganisms can influence the energy balance of the host; it has been shown that certain groups of bacteria induce the deposition and adsorption of fatty acids in the intestinal epithelium.[16] In some cases, bacterial species added to the diet as a probiotic have shown a beneficial effect in regulating the expression of genes related to the glucose and lipid metabolism to attenuate obesity.[17,18] The consumption of high-fat diet (HFD) is considered one of the main factors that contribute to the development of obesity[19] and changes in the composition of the gut microbiota,[20] such as the reduction of abundance and diversity.[21] The zebrafish (*Danio rerio*) is considered a good model organism for the study of metabolic diseases,[22] and because it shares obesity-related pathophysiological gene pathways with mammals, it is very useful for the analysis of the relationship between diet, obesity, and intestinal microbiota.[23,24] In zebrafish, a relationship

[1]CIAD, A.C. Mazatlán Unit for Aquaculture, Mazatlán, Mexico.
[2]Unidad Académica Mazatlán, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Mazatlán,

between fat levels in the diet and the composition of the intestinal microbiota in different stages of development has been demonstrated[23,25] and a recent study has reported that a HFD has an impact on metabolic and immunological functions.[26] Overfeeding has become a problem because it promotes weight gain[27] and is one of the main causes of obesity.[28] It has been reported in different species of vertebrates that in overfed organisms the intestinal bacterial composition is affected and the bacterial diversity is reduced.[29] Moreover, individuals with a low bacterial richness, gain weight more quickly over time,[30] demonstrating that a bilateral relationship exists between diet and the gut microbiota. Thus, the purpose of this study was to determine sex-specific alterations of the intestinal microbiota of the zebrafish induced by overfeeding of a HFD.

Materials and Methods

Animals, experimental design, and diets

Fully grown mature male and female adults (3.5 mpf) of wild-type zebrafish were obtained from a local pet store and maintained as previously described.[31] Fish were acclimatized to laboratory conditions (27.5°C ± 1°C under 12-h light/12-h dark period) during 2 weeks and fed with a commercial diet (Tetramin® Tropical Flakes) consisting of 46% crude protein, 11% crude fat, 3% crude fiber, and 6% moisture. The experimental diets were formulated to be isoproteic[32] and to contain two fat levels, 8% and 24% (Table 1). Diet formulation was performed according to Hernández *et al.*[33] Briefly, all the dry ingredients of the experimental diets were weighed, combined, and thoroughly mixed to homogeneity in a Hobart-type mixer. Oil was then added and thoroughly mixed for 5 min. The feed was manufactured by extrusion processing using a double screw (Brabender TSI 20/40). The pellets were reduced to a size of ~125–250 μm using sieves to remove fine particles. The pellets were stored at 4°C in labeled, sealed containers.

Table 1. Ingredient and Proximate Composition of the Experimental Diets for the Zebrafish (*Danio rerio*)

Ingredients	*Diets*	
(g kg^{-1} wet weight.)	*LF*	*HF*
Fish meal (sardine)[a]	613.9	613.9
Hydrolyzed tuna	135.0	135.0
Fish oil[b]	11.3	91.3
Corn oil	11.3	91.3
Dextrin[b]	225.8	65.6
Alginate[b]	30.0	30.0
Premix vitamin[c]	2.0	2.0
Premix minerals[c]	2.0	2.0
Antioxidants[d]	0.1	0.1
Soy bean lecithin (70%)[d]	15.0	15.0
Carotenoid (Carophyll pink)[d]	0.5	0.5
Monobasic phosphate[e]	6.0	6.0
Cellulose	30.0	30.0
Analyzed composition (% DM)[f]		
Crude protein	45.13 ± 0.86	46.57 ± 01.01
Crude lipid	8.92 ± 0.13	24.74 ± 0.12
Ash	11.48 ± 0.18	11.58 ± 0.12
NFE[g]	34.47 ± 1.04	17.11 ± 1.16
Gross energy (kJ g^{-1})[h]	19.9	23.5

[a]Premium-grade fish meal was obtained from Selecta de Guaymas, S.A. de C.V. Guaymas, Sonora, Mexico.
[b]Droguería Cosmopolita, S.A. de C.V. México, D.F., Mexico.
[c]Supplied by BASF Mexicana S.A. de C.V.
[d]DSM Nutritional Products Mexico S.A. de C.V., El Salto, Jalisco, Mexico.
[e]Sigma-Aldrich Chemical, S.A. de C.V. Toluca, Mexico State, Mexico.
[f]Mean ± SD, number of determinations = 3.
[g]Nitrogen-free extract (including fiber) = 100–(% protein+% lipid+% ash).
[h]Gross energy (kJ g−1) was calculated according to the physiological fuel values of protein, 20.93 kJ g^{-1}; lipids, 37.68 kJ g^{-1}; and nitrogen-free extract, 16.75 kJ g^{-1}.

The fish were randomly distributed in three treatments and fed with two different diets, low-fat (LF) and high-fat (HF), and another group was overfed (HFO) with the HFD. Males and females were kept in separate tanks and homogeneous groups were created according to fish weight. Each treatment consisted of four tanks (two tanks per each sex) with 10 fish per tank. The tanks were maintained in a water recirculation system containing a mechanical filter with a cellulose mesh, a chemical filter with activated carbon, and an ultraviolet light sterilizer. Before the experiment, the tanks were cleaned with soap and water, thoroughly rinsed, and dried with a cellulose paper towel. During the experiment, each tank was cleaned with soap and water at least once a week; meanwhile, the fish were carefully transferred to another clean tank. Twenty percent of the total water was exchanged daily with filtered and sterile water. Feces and other debris were removed daily by siphoning. The water temperature was maintained at 27.5°C, salinity = 0.51 g/L ± 0.04, and pH = 7.35 ± 0.18. LF and HF treatments were fed two times per day at a basal regime of 1.7% body weight (BW) (7.5 mg food/fish/day for males and 10 mg food/fish/day for females) without caloric restriction.[24] The HFO group were fed three times per day at an overfeeding regime of 8.5% BW (38.5 and 50 mg food/fish/day for males and females, respectively) to promote diet-induced obesity.[24] This feeding protocol was maintained for 8 weeks. The food intake was estimated by quantifying the food before and after feeding. After feeding, the remaining food was siphoned out, filtered, dried, and weighed.

Sampling and DNA extraction

The condition factor (K) is commonly used to investigate growth patterns, whereas the body mass index (BMI) is mainly used to determine overweight and obesity. Both indices use a relationship between weight and length, and can indirectly and imperfectly determine the body fat and health.[34,35] After the feeding protocol, the weight and length of each fish were registered. Weight gain, condition factor (K), and BMI were calculated according to the following formulas:

$$Weight\ gain\ (\%) = \frac{final\ weight - initial\ weight}{initial\ weight} * 100$$

$$K = \frac{[weight(g) * 100]}{[length(cm)]^3}$$

$$BMI = \frac{weight\ (g)}{[length(cm)]^2}$$

Fish were aseptically dissected and the whole intestine, including its content, were collected in individual tubes

with ethanol 96% and preserved at −40°C. DNA was isolated using a modified CTAB method,[36] and DNA quality and concentration were measured in a DeNovix® DS-11 spectrophotometer.

DNA sequencing

Preparation of libraries for sequencing was done following the Illumina 16S Metagenomic Sequencing Library Preparation protocol (part # 15044223 Rev. B); briefly, PCR amplification of the V3 variable region of the 16S rRNA gene was amplified with universal primers[37] (lowercase) with Illumina adapters (uppercase): 16S-V3_338f (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG acy cct acg ggr ggc agc ag) and 16S-V3_533r (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G tta ccg cgg ctg ctg gca c). The amplification protocol was one cycle at 95°C/3 min, followed by 30 cycles of 95°C/30 s, 60°C/30 s, and 72°C/30 s, and a final cycle of 72°C/5 min. PCR amplicons were purified with AMPure XP magnetic beads and then Illumina indices were added for each sample in a second PCR at 95°C/3 min, followed by eight cycles of 95°C/30 s, 55°C/30 s, and 72°C/30 s, and a final cycle of 72°C/5 min, followed by a second cleanup with beads.

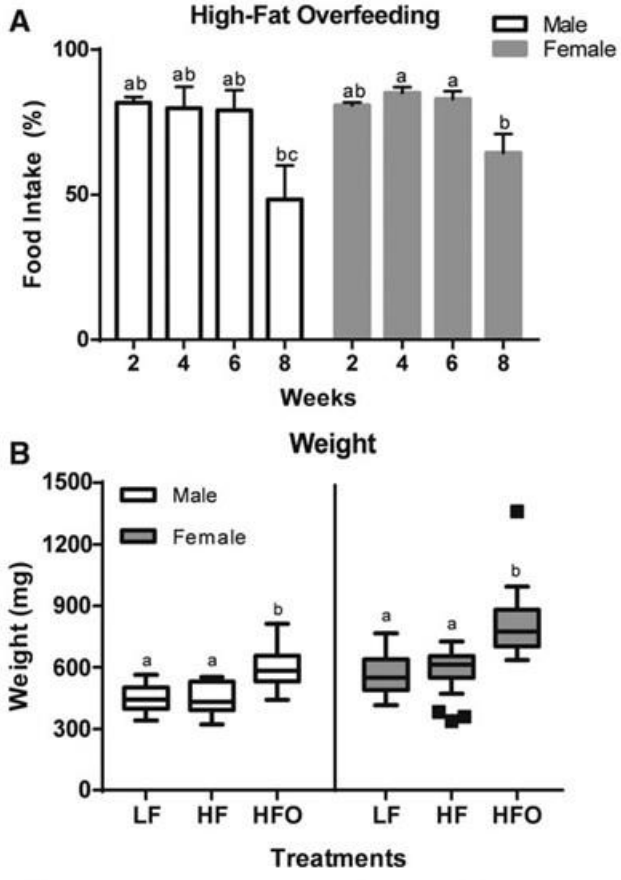

FIG. 1. Food intake evolution of overfed fish with a HF diet for 8 weeks in males and females **(A)**. Average weight of male and female zebrafish at HFO, LF basal regime, and HF basal regime **(B)**. Results are expressed in mean ± SD, *boxes* represent Q1–Q3 interquartile range and outliers are represented by *squares*. Mean with different *superscript letters* were significantly different ($p < 0.05$). HFO, high-fat overfed; LF, low-fat; HF, high-fat.

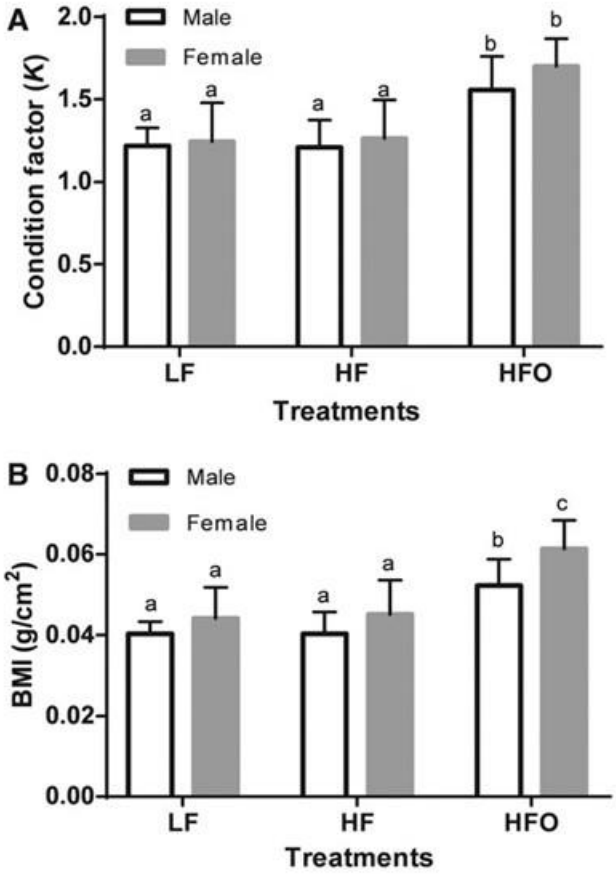

FIG. 2. Effect on the condition factor **(A)** and BMI **(B)** of male and female zebrafish under HFO, LF basal regime, and HF basal regime. Results are expressed in mean ± SD. Mean with different *superscript letters* were significantly different ($p < 0.05$). BMI, body mass index.

Final PCR amplicons were quantified with the Broad-Range dDNA Quantification Assay Kit (Thermo Scientific) for the Qubit fluorometer, and an equimolar pool was prepared. This pool was quantified with the HS assay for the Qubit, and adjusted to 4 nM for further denaturalization, dilution to 2 pM, and sequencing in a Mid-output flow cell (300 cycles, 2 × 150 pair-end library) in an Illumina MiniSeq.

Bioinformatic analysis

FASTQ sequences were processed with our metagenomic pipeline (https://github.com/GenomicaMicrob/metagenomic_pipeline); first, files were processed with the pair-end_cleaner v.0.9.9 pipeline (https://github.com/GenomicaMicrob/pair-end_cleaner): bases with a Phred quality lower than Q20 and ambiguous bases (n's) were deleted, short reads were removed (<140 bases), and they were also trimmed to no more than 150 bases with CUTADAPT.[38] Remaining pair-end sequences were assembled with PEAR (https://sco.h-its.org/exelixis/web/software/pear/doc.html) for a minimum assembly length of 170 bases, a maximum of 250 bases, and a minimum

TABLE 2. SUMMARY OF ILLUMINA SEQUENCING READ ANALYSIS: TOTAL SEQUENCES, OBSERVED SPECIES (OTUS), AVERAGE TAXA ASSIGNED (SAMPLE), SHANNON AND DOMINANCE INDICES; VALUES ARE REPRESENTED IN MEAN ± SD ($N=2$ IN EACH GROUP; ONLY IN FEMALE LF AND HF GROUPS $N=1$)

	Female			*Male*		
Groups	*LF*	*HF*	*HFO*	*LF*	*HF*	*HFO*
Total seq.	500,309	504,494	336,492	448,037	345,905	551,165
Total OTUs	1,055	1,249	1,424	1,144	1,211	839
OTU (avg. sample)	179 ± 89	206 ± 131	213 ± 140	187 ± 93	211 ± 109	143 ± 85
Shannon	1.91 ± 0.59	1.83 ± 0.64	2.20 ± 1.21	2.12 ± 0.75	2.28 ± 1.04	1.58 ± 0.29
Dominance	0.34 ± 0.12	0.35 ± 0.13	0.35 ± 0.26	0.29 ± 0.16	0.30 ± 0.21	0.38 ± 0.13

OTU, operational taxonomic unit.

overlap of 10 bases. Resulting sequences were converted to FASTA format. Chimera sequences were removed with the chimera_detector v.0.1.1 pipeline (https://github.com/GenomicaMicrob/chimera_detector); clean sequences were compared with the SILVA[39] v.1.28 database with vsearch[40] v.2.7.1 (*—uchime_ref*), those sequences that had a match (by 97% similarity) in the database, were not considered a chimera. Clean and chimera-free sequences were classified with the mg_classifier v.1.7.0 pipeline (https://github.com/GenomicaMicrob/mg_classifier); sequences were clustered to 97% similarity with vsearch v.2.7.1 *(—cluster_fast*), centroid sequences of each cluster were classified also with vsearch v.2.7.1 *(—usearch_global*) by matching to the EzBioCloud[41] database v.1.5 based on the prokaryotic taxonomic level thresholds proposed by Yarza.[42]

Statistical analyses

Results were expressed as the mean ± standard deviation (SD). Differences between the food intake, condition factor *K*, and BMI were determined by one-way ANOVA followed by the Tukey's test. Weight analysis was calculated separated for males and females due to differences in mean weight between sexes. Differences were considered significant when $p<0.05$. Statistical analysis and graph construction was performed using GraphPad Prism 7 (GraphPad Software). Univariate statistical analysis of bacterial community was performed using STAMP.[43] Diversity index and multivariate statistics were calculated using PAST3.[44] The Shannon index was calculated according to the following formula:

$$H = -\sum_{i=1}^{s} (p_i * \ln\ p_i)$$

Where:

H = Shannon diversity index; pi = proportion of the population made up of species i; s = number of species in a sample.

Results

Survival, food intake, and growth performance

Fish survival was 100% in all tanks except for one tank of the HFO treatment (male), where survival rate was 80%, even so, no significant differences were found. Food intake rate in basal regime (LF and HF) groups was 100% during the 8 weeks of the experiment. Overfed groups consumed 79%–82% (male) and 80%–5% (female) of the added food steadily during the first 6 weeks, but during the final 2 weeks the food intake rate decreased significantly ($p<0.05$) in both males and females to 48% and 64%, respectively (Fig. 1A). The absolute values of consumption are shown in the Supplementary Table S1.

Zebrafish fed with a basal regime (LF and HF) did not show significant differences in final weight, but those overfed (HFO) showed a significant increase ($p<0.05$) in weight regardless of sex. Average weight of males at the basal regime (LF and HF) was 442 and 448 mg, respectively, whereas the HFO group showed a weight of 592 mg; females showed the same growth pattern with 560, 586, and 808 mg, respectively (Fig. 1B).

Only HFO fish showed a significant growth, the weight gain rate was 38.0% and 40.1% for male and female zebrafish, respectively, whereas the groups at a basal regime showed minimal growth (3.0% to 3.5%), even females of the low-fat (LF) treatment presented a negative growth (−1.8%). Similarly, the condition factor (*K*) and BMI were significantly higher ($p<0.05$) in the overfed group (HFO, Fig. 2).

Sequencing and diversity

All the sequencing libraries (from all 70 samples) generated more than 5 million raw FASTQ sequences, once the Q-score was <20, chimeric and eukaryota sequences were excluded, 2.68 million of clean and high-quality bacterial sequences were classified with the EzBioCloud database. In general, male and female samples obtained the same number of sequences (1.3 million) distributed within treatments. The female groups obtained a high number of assigned Operational Taxonomic Units (OTUs) compared with the male groups, 1242 and 1064, respectively (Table 2).

Shannon's bacterial diversity index varied between HF and HFO females from 1.83 to 2.20, respectively; contrary to these results, males from the HFO group obtained the lowest value and HF the highest (1.58 and 2.28 for HFO and HF, respectively). Dominance index suggests that a HFD promotes the prevalence of some bacterial groups only in the HFO regime, as shown in the male groups, where LF and HF obtained 0.29 and 0.28, respectively; meanwhile the HFO obtained 0.38. However, this trend was not observed in females where the dominance indices were equivalent (0.34–0.35, Table 2). No significant differences in diversity were detected.

Gut bacterial composition of zebrafish at basal and overfeeding regimes

In general, more than 95% of the sequences were assigned to five bacterial classes, *Alphaprotebacteria*, *Betaproteobacteria*,

Gammaproteobacteria (phylum *Proteobacteria*), *Clostridia* (*Firmicutes*), and *Mollicutes* (*Tenericutes*); these taxa were detected in all treatments. *Gammaproteobacteria* was the most representative taxon accounting for more than 67% of the total sequences classified; *Gammaproteobacteria* was the only bacterial class present in every intestinal sample analyzed (Fig. 3).

At the genus level, 802 OTUs were found in all samples, the most representative genera were, in order of decreasing magnitude, *Aeromonas*, *Plesiomonas* (both *Gammaproteobacteria*), *Rombutsia* (*Clostridia*), *Mycoplasma* (*Mollicutes*), and *Paraburkholderia* (*Betaproteobacteria*). These five taxa represented 70% of total sequences (Table 3, Supplementary Fig. S1, Supplementary Table S2). Twenty percent of the total sequences could not be assigned to any known genus. At the family and class taxonomic levels, unclassified sequences were lower, 2.5% and less than 0.001%, respectively.

In general, a greater number of bacterial genera were detected in the intestines of female zebrafish: 333 for LF, 422 for HF, and 459 for HFO; whereas in males 328, 405, and 272 could be classified, respectively. These values coincide with the diversity indices, and thus, a higher gut bacterial richness in female fish was found. In both males and females, at least 30% of the total genera detected are shared between all groups (30.9% and 33.6%, respectively). Moreover, the most abundant genera (Table 3) were detected in both female and male groups. Unique genera, those detected in only one group, are mainly minority taxa with proportions lower than 0.01% (Fig. 4).

Effect of overfeeding a HFD in the intestinal bacterial microbiota

The diversity and abundance of microbiota were significantly different between sexes in the zebrafish analyzed. Therefore, it is possible that this variable or the interaction with other variables are generating a significant effect on the variation of the data. All samples (male and female) were tested in a two-way (sex and diet) permutational multivariate analysis of variance (PERMANOVA), which determined that sex (variable) and interaction promoted a significant effect on data variation ($p<0.05$). Based on this test, the diversity indices, and the abundance results, the tests to evaluate the diet effect, were performed separately by sex (Supplementary Table S3).

Males. To compare and detect differences in the bacterial community composition, groups were tested by One-Way Analysis of Similarity (ANOSIM), based on Bray–Curtis distances at the taxonomic class level. ANOSIM test detected a significant dissimilarity between the groups at the basal

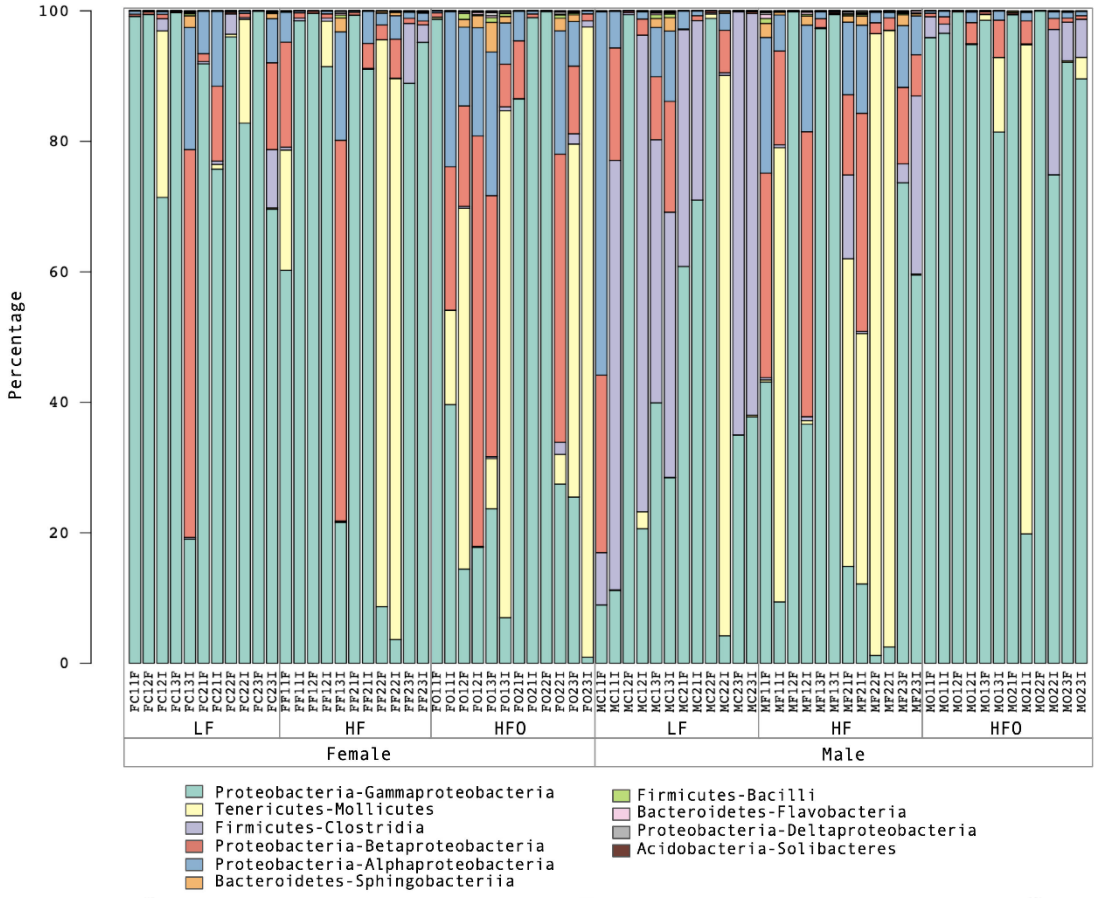

FIG. 3. Gut bacterial composition in male and female zebrafish under HFO, LF basal regime, and HF basal regime. The stacked bar chart represents in bacterial phyla and classes.

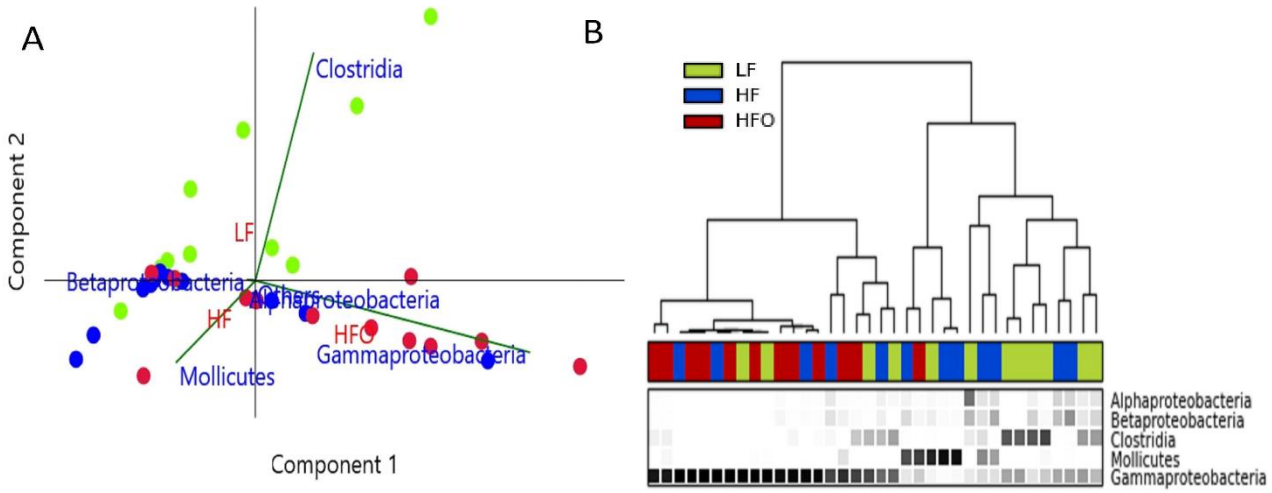

FIG. 5. Exploratory analysis of gut bacterial composition in zebrafish (male) in the HFO, LF, and HF regimes. Two-dimensional PCA biplots **(A)**; PC1 = 55.6%, PC2 = 24.9%. The heat map shows the most abundant classes, OTUs were organized according to their phylogenetic positions (UPGMA) and the taxa are shown on the *right* **(B)**. PCA, principal components analysis.

were tested by analysis of variance (Kruskal–Wallis); additionally, Storey's FDR correction was used to control the false discovery rate.[45] Only five taxa were detected as significantly different. Phylum *Bacteroidetes* was detected at higher proportion in HF against HFO ($p < 0.02$), as well as *Firmicutes*, which presented a higher proportion in LF against HF and HFO ($p < 0.001$, in both); on the contrary, *Proteobacteria* was detected at higher proportion in HFO against LF ($p < 0.05$), as shown in Supplementary Table S7. The classes *Gammaproteobacteria* and *Clostridia* presented significant differences ($p = 0.032$ and 0.025, respectively); Tukey–Kramer *post hoc*-identified pairs of groups may differ from each other ($p < 0.05$). Overfed zebrafish (HFO) presented a significantly higher mean proportion in *Gammaproteobacteria* compared against the basal groups (LF and HF). *Clostridia* obtained a higher mean proportion in zebrafish fed under LF diet, as shown in Figure 6. Statistical analyses were tested for all taxa, but only significant differences were detected at the phylum and class levels (Supplementary Table S7).

Females. Contrary to results obtained in male zebrafish, ANOSIM confirmed no significant dissimilarity between the treatments $R = 0.04$, $p = 0.12$; indicating that the variation within and between the groups was uniform and the bacterial community proportions were similar. Moreover, a close similarity was detected on basal groups (LF and HF) $R = -0.04$ (Supplementary Table S8). Nevertheless, SIMPER analysis was performed to detect the most variable taxa, these were in descending order, *Gammaproteobacteria*, *Mollicutes*, *Betaproteobacteria*, and *Alphaproteobacteria* (Supplementary Table S9). Due to the low diversity of Clostridia in the gut of female zebrafish, the contribution of this taxon to dissimilarity was minimal. Nevertheless, exploratory tests were performed (Fig. 7). PERNOVA did not detect significant differences between female zebrafish treatments. Even so, univariate analysis detected that one group, Enterobacteriales (order of *Gammaproteobacteria*) presented higher abundance in LF against HFO (Fig. 8).

Discussion

Obesity and overweight are two of the main problems affecting the human population worldwide. These conditions have been identified as factors influencing the increase of chronic diseases and disorders that can lead to death. Obesity is the result of an imbalance of energy that leads to the accumulation of fat in the body promoting an excessive increase in body mass. Among the main risk factors for obesity are genetic, socioeconomic, environmental, and those related to individual behavior, such as the diet.[46,47] Due to recent studies, where a relationship between intestinal microbiota and diet has been established,[23,29,48,49] it is important to understand the dynamics of bacterial communities and their role in metabolic health. So, we evaluated the effect of a HF diet provided under an overfed regime using the zebrafish as a model.

As expected, fish of the LF and HF treatments consumed 100% of the food supplied. According to our results of consumption and weight (Fig. 1 and Supplementary Table S1) the amount of food used in this study as a basal diet ($\approx 1.7\%$ of BW) can be used as a maintenance diet that satisfies the caloric requirements of zebrafish without increasing body weight. This value coincides with that reported in other studies, which mentions that amounts of <0.5% BW create a caloric restriction in the zebrafish,[24] whereas amounts of >2.4% BW can be considered excessive.[50] In this study, HFO groups consumed a quantity of food greater than >2.4% BW throughout the experiment; ensuring an overfeeding regime (Fig. 1A). In consequence, HFO groups presented a significant increase in body mass compared with LF and HF; for both males and females (Fig. 1B).

In general, the K value indicates that zebrafish fed a basal diet (LF and HF) can be considered healthy in terms of weight/length ratio. The female zebrafish presented a slightly higher K value than the male zebrafish, and it has been reported that this growth pattern is normal for this species.[51] K and BMI indices suggest that a HFD and overfeeding can promote obesity. The overfed groups (HFO) showed K and BMI values significantly higher than the basal-fed groups (LF

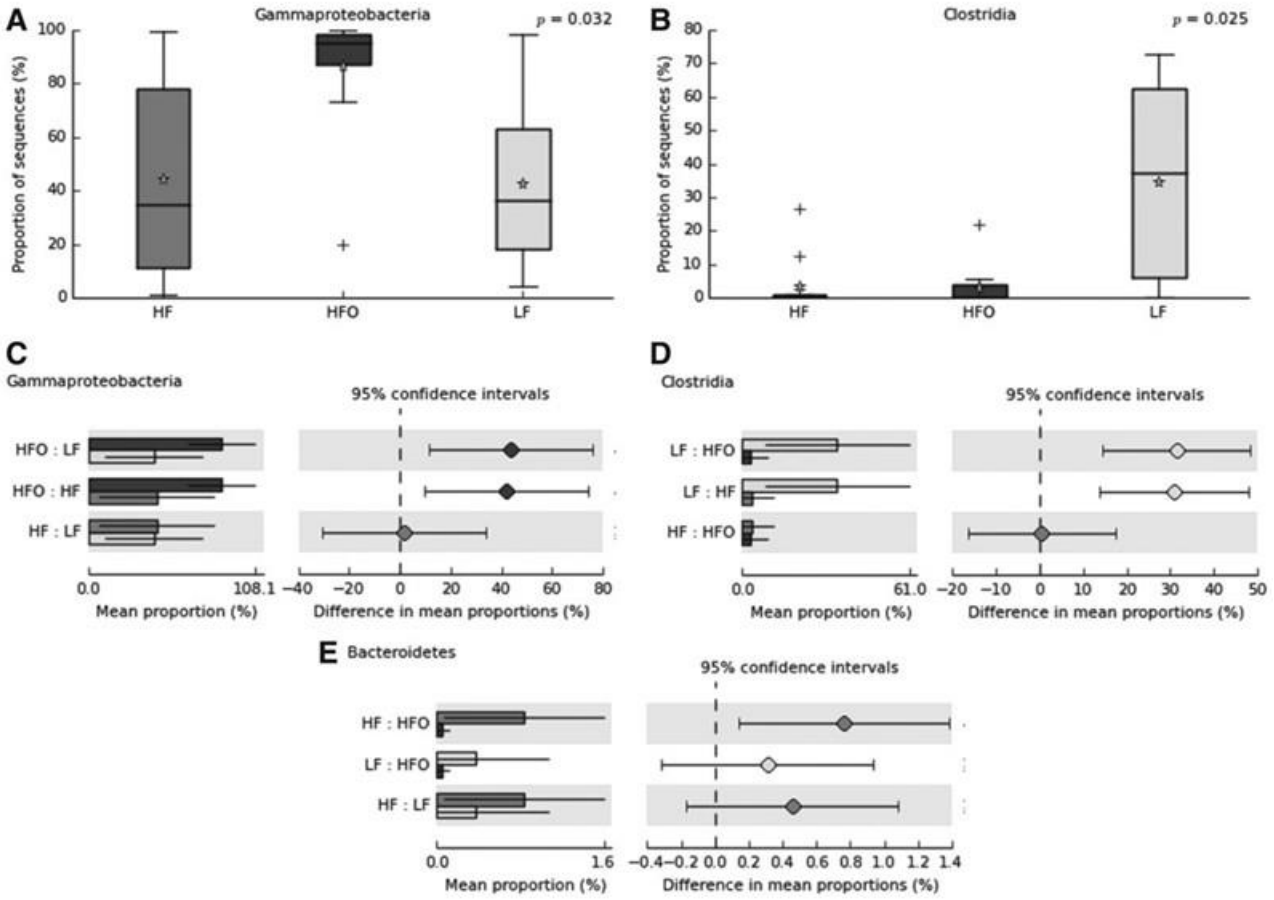

FIG. 6. Box plot showing the distribution in the proportion of Gammaproteobacteria **(A)** and Clostridia **(B)** assigned to LF, HF, and HFO groups. The median value is shown as a *line* within the *box* and the mean value as a *star*. *Whiskers* extend to the most extreme value within 1.5×IQR. Outliers are shown as crosses. *Post hoc* plots indicating the mean proportion of sequences within each group and the difference in mean proportions ($p<0.05$); Gammaproteobacteria **(C)**, Clostridia **(D)**, and Bacteroidetes **(E)**.

and HF). Similar results have been reported in an early stage of development[24] and in adult zebrafish[26,52]; in both cases, fish considered obese showed a BMI higher than 0.05 (g/cm^2). In this work, HFO male and female fish presented BMI values of 0.052 (g/cm^2) and 0.061 (g/cm^2), respectively; consequently, we can infer that HFO zebrafish became obese at the end of the experiment.

The effect of obesity induced by overfeeding on the specific diversity of the intestinal bacterial community was evaluated by calculating the Shannon index.[53] Previous

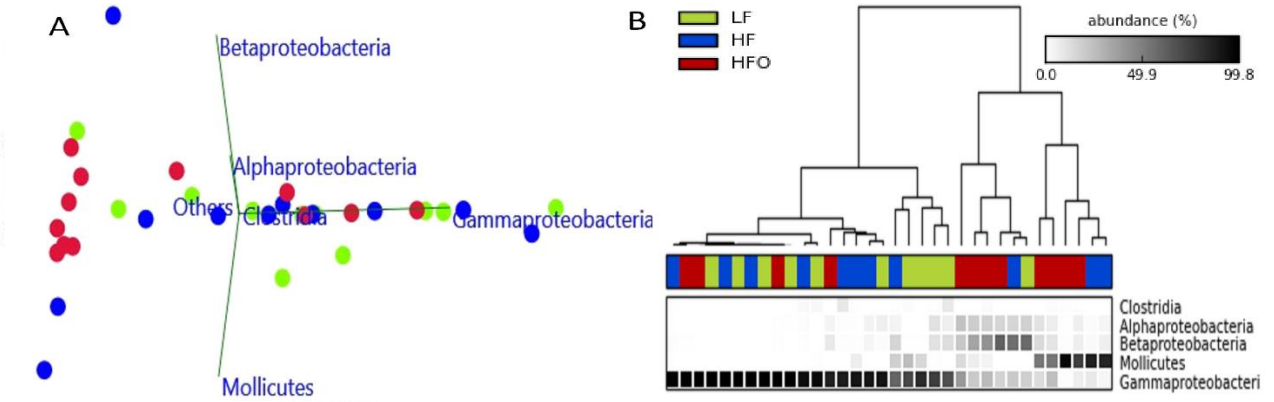

FIG. 7. Exploratory analysis of gut bacterial composition in zebrafish (female) under HFO, LF basal regime, and HF basal regime. Two-dimensional PCA biplots **(A)**; PC1 = 86.1%, PC2 = 8.1%. The heat map shows the most abundant classes, OTUs were organized according to their phylogenetic positions (UPGMA) and the taxa of OTUs are shown on the *right* **(B)**.

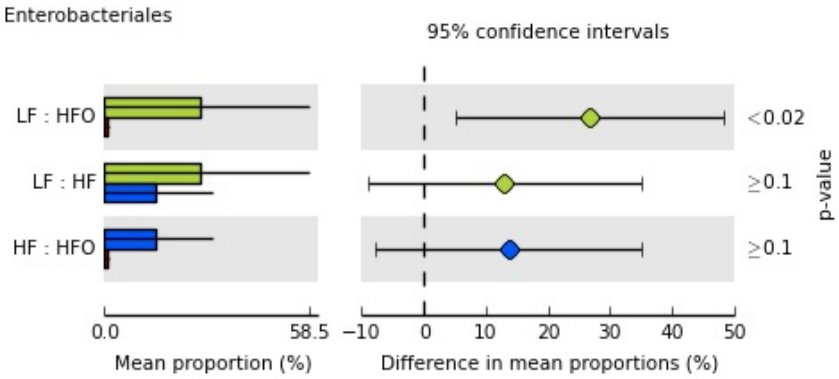

FIG. 8. *Post hoc* plots indicating the mean proportion of sequences within each group and the difference in mean proportions ($p<0.05$); Enterobacteriales (Class: Gammaproteobacteria).

studies have reported that intestinal bacterial diversity is significantly reduced with the increase of dietary fat, between 5% and 15%.[23] It has been also shown that bacterial diversity is reduced in organisms that suffer from obesity[54,55] as well as in organisms with a disorder or disease related to the digestive system.[56–58] In this experiment, HFO males showed a lower Shannon index (1.58 ± 0.29) compared with the LF and HF groups with similar values (2.12 ± 0.75 and 2.28 ± 1.04, respectively) but the dominance was higher in HFO males than in the other two groups (Table 2). These results suggest an alteration of intestinal bacterial diversity in males caused by induced obesity by overfed HFD. Contrary to the diversity observed in HFP males, HFO females showed a higher Shannon index (2.20 ± 1.21) than LF and HF females (1.91 ± 0.59 and 1.83 ± 0.64; respectively), but the dominance was similar among them (Table 2).

Sequencing data suggest that *Proteobacteria*, *Firmicutes,* and *Tenericutes* were the dominant phyla in the gut of adult zebrafish. Other studies have also reported Proteobacteria as the predominant phyla in the gut of zebrafish at embryo, juvenile, and adult stages[16,18,59]; even in other fish and shrimp species.[10,49,60–62] In all treatments, *Aeromonas* (*Gammaproteobacteria*) was the most abundant genus, except in male LF, where *Romboutsia* was the most abundant (Table 3). Similar results have been reported in other studies, where the *Aeromonadaceae* family was detected in the gut of zebrafish at the embryo, juvenile, and adult stages.[63] Moreover, some of the representative genera detected in this study, *Aeromonas, Pseudomonas, Plesiomonas,* and *Shewanella* have been detected as the most abundant genera and could be considered as part of core gut microbiota of zebrafish.[59] Possibly the colonization by genera of the *Gammaproteobacteria* class in the zebrafish gut can be explained because this is one of the most abundant groups in aquatic environments[64]; additionally, it has already been reported that these taxa are present in the surrounding water in experiments with zebrafish.[16]

In males, *Proteobacteria* and *Gammaproteobacteria* showed significantly higher proportions in HFO compared with LF and HF (Fig. 6A). It has been proposed that a higher prevalence of *Proteobacteria* is a possible signal of an imbalance of the intestinal microbiota (dysbiosis) that can lead to disease; increases in this phylum have been reported in mammals with metabolic disorders, such as genetically- and diet-induced obesity, T2DM, intestinal inflammation, and colorectal cancer.[65]

In zebrafish, decreases in the abundance of *Proteobacteria* in organisms that were treated with probiotics (*Lactobacillus rhamnosus*) have been reported, and showed a decrease in appetite, blood glucose, cholesterol, triacylglycerides (TAG), and the expression of genes involved in the synthesis of TAG and phospholipids (*dgat2* and *agpat4*, respectively), and increased expression of the *leptin* gene that promotes satiety.[17,18,66]

Bacteroidetes was detected in a fewer proportion in the HFO group, but only significant differences were detected ($p<0.05$) with the HF group (Fig. 6E); this coincides with studies that report significant decreases of *Bacteroidetes* in obese people compared with lean people and people after a diet therapy.[67] Similarly, it was reported that the abundance of *Bacteroidetes* in the cecal microbiota of mice with genetically induced obesity (*ob/ob*) was significantly reduced.[68] The proportion of *Bacteroidetes* in HF males was higher than in LF males ($p<0.1$, Supplementary Table S9); this coincides with other results reported in zebrafish, where a correlation between dietary fat levels and abundance of *Bacteroidetes* was observed.[25] It has also been reported that the significant increase of *Bacteroidetes* in zebrafish fed a fat-enriched diet is correlated with a certain degree of inflammation and an increase in goblet cells responsible for regulating the production of intestinal mucus.[20]

Mammalian studies have shown a relationship between obesity and consumption of HFD with an increased number of *Firmicutes*,[68,69] but recent studies in mice showed that animals fed with a HFD and with a greater weight gain showed a 17% decrease in this phylum.[66] Moreover, studies in zebrafish treated with probiotics (*L. rhamnosus*) showed a greater abundance of *Firmicutes* with a lower expression of the *npy* gene and therefore a lower intake of food and blood glucose compared with the control group.[18] This coincides with the results obtained in this study; the abundance of *Firmicutes* was the lowest in the HFO group, getting significant differences with the LF group (Supplementary Table S7). In several studies, especially with mammals, the proportion of Firmicutes/Bacteroidetes is used as an indicator of dysbiosis related to metabolic problems.[14,66,70] Similarly, zebrafish have shown lower proportions of *Firmicutes* when fed a fat-enriched diet with respect to a control.[20] It is possible that in aquatic animals, it could be more appropriate to consider the proportions of *Proteobacteria*, because this taxon is the most abundant in the intestine of numerous aquatic organisms, also, is one of the most responsive groups to the modifications of microbiota when health is affected.

In female zebrafish, the composition of the bacterial community was different from males. Two-way PERMANOVA

(Supplementary Table S3) suggests that most of the variations of the gut microbiota in this experiment is given by the interaction of the variables (diet and sex), and for this reason, a different response was obtained in the female gut microbiota composition (and diversity) by effect of the diet, with respect to males. It has been reported that the microbiota in mice is different in healthy males and females, and these differences caused different metabolic responses when the animals were treated with a streptozotocin-HFD (STZ-HFD) to induce nonalcoholic steatohepatitis–hepatocellular carcinoma. Consequently, a significant loss of diversity (Simpson index) was observed only in male mice with respect to the control. Moreover, the authors reported that tumor-suppressive miRNAs were significantly lower in STZ-HFD mice versus control, but males showed significantly lower levels than females.[14] Similarly, we observed a lower Shannon index and a higher dominance in HFO males versus HFO females (Table 2). Significant differences were detected in the composition of the bacterial community between HFO males versus HF and LF males, but not in the female groups (only *Enterobacteriales*). In zebrafish, the culturable gut microbiota between males and females was compared and the results did not reveal significant differences related to sex.[71] A more recent study reported that zebrafish exposure to silver nanoparticles promotes a sex-dependent effect on gut microbiota affecting the richness and diversity of intestinal bacterial community in males but not in females.[72] The authors state that sex-dependent response may be due to the fact that male and female zebrafish harbor different gut bacterial communities.

It has been previously shown that the effect of the diet on composition and bacterial diversity depends largely on sex-specific interactions, and sex hormones seem to be responsible (at least in part) for the differentiation of the bacterial communities between males and females. Nonetheless, the mechanisms, paths, and other factors remain unknown.[73] We speculate that the zebrafish reproductive functions (or reproductive cycles) affect the microbiota, because the caloric demand that requires the production of eggs is higher than in males, eggs contain a large amount of lipids, and their biomass can exceed 29% BW of female zebrafish unlike the testis that only represents less than 2% BW of male zebrafish.[26] Despite the reports revealing sex-dependent effects affecting the gut microbiota, a comparison of the gut microbiota between males and females before the experiment would have provided more arguments to determine whether a high fat overfeeding regime alters the gut microbiota in a sex-specific manner.

Conclusion

We have shown that a HFD fed at a basal regime did not alter the intestinal microbiota in male and female zebrafish; however, overfeeding did cause a dysbiosis altering the diversity and composition of the intestinal microbiota in a sex-specific manner. In this particular case, sex was a determining variable in the microbiota response to a HFD.

Acknowledgments

This study was supported by CIAD internal project. The authors thank Karen Enciso-Ibarra, Carmen Bolán-Mejía, and Patricia Domínguez-Jiménez for technical help.

Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Table S4
Supplementary Table S5
Supplementary Table S6
Supplementary Table S7
Supplementary Table S8
Supplementary Table S9

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Address correspondence to:
Bruno Gómez-Gil, PhD
CIAD
A.C. Mazatlán Unit for Aquaculture
AP. 711
Mazatlán 82000
Mexico

E-mail: bruno@ciad.mx

3. OVERFEEDING, AGAVINS AND DIETARY FAT; FACTORS THAT MODULATE THE MICROBIOME OF THE ZEBRAFISH GUT

Navarro-Barrón, E., Hernández, C., Llera-Herrera, R., García-Gasca, A., Mercedes López-Pérez & Gómez-Gil, B.

Artículo enviado a la revista **Zebrafish**.

OVERFEEDING, AGAVINS AND DIETARY FAT; FACTORS THAT MODULATE THE MICROBIOME OF THE ZEBRAFISH GUT.

Erick Navarro-Barrón[a], Crisantema Hernández-González[a], Raúl Llera-Herrera[b], Alejandra García-Gasca[a], Mercedes López-Pérez[c], and Bruno Gómez-Gil[a]*

[a]CIAD, A.C. Mazatlán Unit for Aquaculture, AP 711, Mazatlán, Sinaloa, México 82000.

[b]Unidad Académica Mazatlán, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México. Av. José Montes Camarena S.N., PO Box 811; Mazatlán, Sinaloa, CP82040. México.

[c]Departamento de Biotecnología y Bioquímica, Centro de Investigación y de Estudios Avanzados del IPN, México

*Corresponding author. bruno@ciad.mx, +52 (669) 989 8700.

Abstract

In this study we evaluated the effect of overfeeding with a low-fat diet (LFO) in some biological parameters and in the gut microbiota of adult male zebrafish (*Danio rerio*). Low-fat overfeeding of zebrafish promoted weight gain and an increase in biometric indices related to overweight and obesity; moreover, overfeeding modulated the gut microbiome. The inclusion of 5% agavins in the diet promoted a statistically significant reduction ($p < 0.05$) in the zebrafish food intake under overfeeding regime, as well as a decrease in body weight and body fat. Multivariate analyzes (PERMANOVA and ANOSIM) suggested that overfeeding with a low-fat diet supplemented with agavins (LFAO) induces an effect that can modulate the intestinal microbiota as estimated by taxonomic fingerprinting of the 16S rRNA gene (V3 region). Additionally, metagenomic sequences showed that overfeeding also affects the functional gut microbiome and is dependent on the fat in the diet. Functional metagenomic analysis suggested that *lysine fermentation* and *leucine degradation* genes abundances were related to dietary fat, while *HMG CoA synthesis* and *pyrimidine conversions* were related to the food regime.

Keywords: gut bacterial composition, overfeeding, microbiome, dietary fat, shotgun metagenomic analysis, 16S rRNA gene taxonomic fingerprinting.

Introduction

Overfeeding is the excessive nutrients intake that promotes a caloric surplus in the body[1], it is one of the main causes that promote weight gain[2], and is determined by various factors such as environmental, behavior, and genetics.[3–5]. Overfeeding promotes overweight and in severe cases obesity[6], these conditions increase the risk of contracting other diseases such as type 2 diabetes, cancer, cardiovascular diseases, and other hormonal and metabolic disruptions[7,8]. A therapeutic alternative with potential to treat the effects caused by obesity and metabolic disorders is the inclusion of prebiotics in the diet[9]. Prebiotics are mostly indigestible fibers that benefit health through the modification of certain bacterial groups of the host intestinal microbiota[10]. Agavins are prebiotics obtained from *Agave spp*. (*A. tequilana*, *A. angustifolia*, and *A. potatorum*) that were shown to reverse metabolic disorders induced by consumption of high-fat diets[11]. The inclusion of agavins in the diet has been reported to regulate body weight, and levels of glucose, triglycerides, and cholesterol[11]; which can be indicators of obesity. More recently, agavins has been reported to increase the concentration of short-chain fatty acids (SCFA) that are implicated in hormonal secretion involved in appetite control[12,13], also improves the histological condition of the digestive tract[14] and plays an important role in the regulation of gut microbiota promoting health[12,14]. Due to its structure, diversity, functionality, and the important role it plays in the health of host, the gut microbiota is considered another organ of the body[15,16] and owing to its nature and complexity it behaves as a dynamic system that can be altered by different factors to which the host is exposed, such as environment, lifestyle, diet, drugs, stage of development, or state of health, among others.[17–21] Various metabolic and gastrointestinal diseases have been reported as related to the gut microbiota[15,22–24]. Although in most cases the changes in the gut microbiota are the effect caused by some health conditions or diseases suffered by the host, the gut microbiota can also become the cause that leads to loss of health; for example, when a dysbiosis exists. This complex two-way relationship and the dynamic nature of the gut microbiota are challenging to understand the clinical potential for human health benefits. A recently proposed model organism for the study of metabolic diseases is the zebrafish[25] (*Danio rerio*) due to its high homology with human genes and organs, common metabolic pathways, high growth and reproduction rates, and relatively easy breeding[26]. In addition, in recent years it has served as a study model for changes in its gut microbiota due to different factors and from different research approaches[27–30]. In a previous study, our group showed that overfeeding with a high-fat diet (HFO) promotes obesity and dysbiosis in the intestinal

microbiota of zebrafish[31]. To continue our research, the purpose of this study was to determine if overfeeding with a low-fat diet (LFO) replicates these alterations and if the inclusion of agavins (5%) in the diet is capable of dampening the LFO effect and also to evaluate if effects of overfeeding on the gut microbiome (microbiota and its functions) are dependent on dietary fat.

Materials and Methods

Animals, experimental design, and diets

Fully grown mature male adults (2.5 months post fertilization, mpf) of wild-type zebrafish were obtained from a local pet store and maintained as described earlier[32]. Fish were acclimatized to laboratory conditions (27.5°C ± 1°C under 12-h light/12-h dark period) during 2 weeks and fed with a commercial diet (Tetramin® Tropical Flakes) consisting of 46% crude protein, 11% crude fat, 3% crude fiber, and 6% moisture. Before starting the experiment, some gut samples of acclimatized fish were preserved and processed as explained later (S group). Two experimental diets were formulated to be isoenergetic and contain the same macronutrients proportions (Table 1). Diet formulation was performed according to Hernández *et al*[33]. Briefly, all the dry ingredients of the experimental diets were weighted, combined and thoroughly mixed to homogeneity in a Hobart-type mixer. Oil was then added and thoroughly mixed for 5 min. The feed was manufactured using a meat grinder (TorRey M-12FS). The pellets were reduced to size of between 125-250 µm using sieves, then were stored at 4 °C in labeled sealed containers. Fish were randomly distributed in four treatments, two groups were fed with low-fat (LF) and low-fat with agavins included (LFA) diets and others two groups were overfed (LFO and LFAO) with both diets respectively; agavins used were extracted and purified from *Agave tequilana* Weber Blue variety plants as described by Huazano-García and López[11]. Fish were kept in separate tanks and homogeneous groups were selected based on fish weight. Each treatment consisted on three tanks with 10 fish per tank. The tanks were maintained in water recirculation system with a mechanical filter with a cellulose mesh, a chemical filter with activated carbon, and an ultraviolet light sterilizer. During the experiment, fish were carefully transferred to another clean tank at least once a week. The temperature water was maintained at 27.5 °C, salinity = 0. 48 g/L ± 0.06 and pH = 7.25 ± 0.24. Twenty percent of the water in the recirculation system was exchanged daily with filtered and sterile water. Stools and other debris were removed daily by siphoning. Low-fat (LF) and low-fat with agavins included (LFA) treatments were fed two times per day at basal regime of

1.7% BW[31] (≈3.6 mg food/fish/day). Overfed groups (LFO and LFAO) were fed three times per day at an overfeeding regime of 8.5% BW (18 mg food/fish/day) in order to promote diet-induced obesity. Experimental and feeding protocols were maintained for eight weeks. The food intake was estimated by quantifying the food before and after feeding. After feeding, the remaining food was siphoned out, filtered, dried, and weighed.

Sampling and DNA extraction

Once the experiment was completed, the length and weight of each fish were registered to calculate the weight gain, condition factor (K), and body mass index (BMI). Both indexes use a relationship between length and weight to indirectly although imperfectly determinate the body fat and health. Body mass index (BMI) is mainly used to determinate overweight and obesity while the condition factor (K) is commonly used to investigate growth patterns. Both indices use a relationship between weight and length, and can indirectly and imperfectly determine the body fat and health[34,35]. Additionally, some fishes were anesthetized[36], euthanized and dissected[37] to calculate the Hepatosomatic and Viscerosomatic indices (HSI and VSI, respectively). HSI and VSI are considered important for assessing the food value and as indicators of the fish status. These indices were calculated according to the following formulas:

$$Weight\ gain\ (\%) = \frac{final\ weight - initial\ weight}{initial\ weight} * 100$$

$$K = \frac{[weight(g) * 100]}{[length(cm)]^3}$$

$$BMI = \frac{weight\ (g)}{[length(cm)]^2}$$

$$HSI = \frac{liver\ weight}{body\ weight} * 100$$

$$VSI = \frac{Visceral\ weight}{body\ weight} * 100$$

After biometric parameters were recorded, fish were aseptically dissected[37]. The whole intestine including its content were collected in individual 1.5 mL Eppendorf tubes with ethanol 96 % and preserved at -40 °C. DNA was isolated using a modified CTAB method[38], DNA quality and concentration was measured in a DeNovix® DS-11 spectrophotometer.

16S taxonomic fingerprinting sequencing and bioinformatic analysis

Preparation of libraries for sequencing was done following the Illumina 16S Metagenomic Sequencing Library Preparation protocol (part # 15044223 Rev. B); briefly, PCR amplification of the V3 variable region of the 16S rRNA gene was amplified with universal primers[39] (lowercase) with Illumina adapters (uppercase): 16S-V3_338f (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG acy cct acg ggr ggc agc ag) and 16S-V3_533r (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G tta ccg cgg ctg ctg gca c). The amplification protocol was one cycle at 95°C/3 min, followed by 30 cycles of 95°C/30 s, 60C/30 s, and 72°C/30 s, and a final cycle of 72°C/5 min. PCR amplicons were purified with AMPure XP magnetic beads and then Illumina indices were added for each sample in a second PCR at 95°C/3 min, followed by eight cycles of 95°C/30 s, 55°C/30 s, and 72°C/30 s, and a final cycle of 72°C/5 min, followed by a second cleanup with beads. Final PCR amplicons were quantified with the Broad-Range dDNA Quantification Assay Kit (Thermo Scientific) for the Qubit fluorometer, and an equimolar pool was prepared. This pool was quantified with the HS assay for the Qubit, and adjusted to 4 nM for further denaturalization, dilution to 2 pM, and sequencing in a Mid-output flow cell (300 cycles, 2 × 150 pair-end library) in an Illumina MiniSeq.

FASTQ sequences were processed with our metagenomic pipeline (https://github.com/GenomicaMicrob/metagenomic_pipeline); first, files were processed with the pair-end_cleaner v.0.9.9 pipeline (https://github.com/GenomicaMicrob/pairend_cleaner): bases with a Phred quality lower than Q20 and ambiguous bases (n's) were deleted, short reads were removed (<140 bases), and they were also trimmed to no more than 150 bases with CUTADAPT[40]. Remaining pair-end sequences were assembled with PEAR (https://sco.h-its.org/exelixis/web/software/pear/doc.html) for a minimum assembly length of 170 bases, a maximum of 250 bases, and a minimum overlap of 10 bases. Resulting sequences were converted to FASTA format. Chimera sequences were removed with the chimera_detector v.0.1.1 pipeline (https://github.com/GenomicaMicrob/chimera_detector); clean sequences were compared with the

SILVA[41] v.1.28 database with vsearch[42] v.2.7.1 (—uchime_ref), those sequences that had a match (by 97% similarity) in the database, were not considered a chimera. Clean and chimera-free sequences were classified with the mg_classifier v.1.7.0 pipeline (https://github.com/GenomicaMicrob/mg_classifier); sequences were clustered to 97% similarity with vsearch v.2.7.1 (—cluster_fast), centroid sequences of each cluster were classified also with vsearch v.2.7.1 (—usearch_global) by matching to the EzBioCloud[43] database v.1.5 based on the prokaryotic taxonomic level thresholds proposed by Yarza[44].

Metagenome sequencing (shotgun) and bioinformatic analysis

Gut zebrafish metagenomic DNA concentration was determined by Qubit fluorometer using dsDNA HS Assay Kit (Invitrogen™). Metagenomic libraries were created following the Illumina Nextera XT DNA Library Prep Kit and sequencing in an Illumina Miniseq. Pair-end sequences were processed with our shotgun metagenomic pipeline (https://sites.google.com/a/ciad.mx/bioinformatica/home/metagenomica-funcional); first, sequences were trimmed with CUTADAPT[40] to remove sequencing adaptors (first 15 and beyond 190 bases) and delete those lower Q30 sequences. Trimmed sequences were assembled with PEAR, those reads that were out of sync due to trimming were pairing using PAIRFQ (https://github.com/sestaton/Pairfq) and results files were convert to FASTA format. Clean sequences were classified with SUPERFOCUS[45] with a 98% identity database (DB_98) to obtain a SEED[46] subsystem (sets of protein families with a similar function) levels (1-3;) of functional annotations reads.

Statistical analysis

Mean and standard deviation (SD) were calculated from biometrics and indexes. Significant differences between food intake, growth, body fat, BMI, K, HSI and VSI were determined by one-way ANOVA followed by the Tukey's test. Differences were considered significant when $p < 0.05$. Statistical analysis and graph construction were performed using GraphPad Prism 7 (GraphPad Software). Heatmaps and univariate statistical analysis of bacterial community and functional metagenomic were performed using STAMP[47]. Diversity index, principal components analysis (PCA) and multivariate statistics were calculated using PAST3[48]. The Dominance and Shannon index were calculated according to the following formula:

$$H = -\sum_{i=1}^{s}(pi * \ln pi)$$

$$D = \sum_{i}\left(\frac{n_i}{n}\right)^2$$

Where:

H = Shannon diversity index; *pi* = proportion of the population made up of species *i*; *s* = number of species in a sample.

D = Dominance; n_i = number of individuals of taxon *i*.

Results

Food intake, growth and biometric indices

Fish survival was 100% in all treatments. Food intake rate in basal regime (LF and LFA) was 100% during the eight weeks of the experiment. Overfed groups (LFO and LFAO) did not consume all the food offered; in average LFO food intake was 142 mg–168.9 mg fish/day (78%–93.8% of total supplied) while LFAO consumed 133.5 mg–155.4 mg (74.2%–86.3%; Suppl. Table 1). During the initial 6 weeks' food intake was similar in overfeed groups, except the final 2 weeks; where LFO food intake was significantly higher than LFAO (Fig.1A). Zebrafish under basal treatments did not show a significantly growth compared to the initial weight; overfed groups showed a significant growth. Average weight of basal groups was 229 mg and 233 mg (LF and LFA, respectively) while overfed groups reached 492 mg and 430 mg (LFO and LFAO, respectively); final weight of LFO group was significantly higher than LFAO (Fig. 1B). The weight gain rate was 130% and 101% for LFO and LFAO, respectively; whereas the basal groups showed minimal growth 7.5% and 9.3% (LF and LFA, respectively). Similar results were obtained for the zebrafish body fat. The overfed groups showed a significantly higher percentage of body fat than basal groups (Fig. 1C). In overfed groups, LFO presented a higher amount of body fat than LFAO (avg. 16% and 13%, respectively). In general, the condition factor (K), BMI, and HSI were significantly higher in the overfed groups; but in VSI only LFO group showed a significantly higher index meanwhile LFA showed similar values than basal groups (Fig. 2).

16S taxonomic fingerprinting sequencing and diversity

Sequencing of 42 gut zebrafish samples generated more than 2.9 million raw FASTQ sequences, before Q-score <20, chimeric, singleton, and Eukaryota sequences were excluded. 1.53 million of clean and high-Q sequences were classified with the EzBioCloud database. In average, each group produced 300 thousand reads. The LF produced the higher number of assigned Operational Taxonomic Units (OTUs) whereas with LFA the lowest OTUs were obtained; 972 and 576, respectively (Table 2). Shannon and Dominance indexes did not show significant differences between treatments, but groups with agavins inclusion produced the lower values. LF and LFO showed Shannon index values of 2.3 and 2.1, respectively; whereas LFA and LFAO produced 1.8 and 1.9 respectively. Dominance index values were higher in agavins inclusion groups (Table 2); LFA and LFAO produced 0.29 and 0.28 while LF and LFAO, 0.23 and 0.23, respectively.

Gut bacterial composition

More than 99% of the sequences were assigned to six bacterial classes, *Actinobacteria* (phylum *Actinobacteria*), *Alphaproteobacteria*, *Betaproteobacteria* y *Gammaproteobacteria* (*Proteobacteria*), *Fusobacteria* (*Fusobacteria*) and *Mollicutes* (*Firmicutes*), besides these taxa were detected in all treatments (Fig. 3). The Proteobacteria classes mentioned above were the only ones detected in all samples, with *Gammaproteobacteria* as the most abundant (> 55.8% of total). All OTUs and taxa detected are showed in Suppl. Table 2. The 2,125 OTUs detected in all samples were classified into 712 genera. In decreasing order, the most representative were: an unclassified genus of the *Enterobacteriaceae*, *Cetobacterium (Fusobacteriaceae)*, unclassified genus of *Fusobacteriaceae*, *Plesiomonas* (*Enterobacteriaceae*), and *Aeromonas* (*Aeromonadaceae*); these five family-genus taxa count more than 76% of total sequences (Suppl. Table 3). The most abundant genera of each treatment are shown in Suppl. Table 4. About 10% of total sequences were not classified to any know taxa at family level, moreover only 41% of total sequences could be classified to genus level (Suppl. Fig. 1 shows relative abundance at family-genus level). Different bacterial genera could be classified depending on the treatments: 456, 245, 350 y 390 for LF, LFA, LFO and LFAO respectively. At least, 20% (148) of the total genera detected are shared between all groups (Fig. 4). Basal treatments share 26.8% of bacterial genera, whereas the overfed treatments share 32.9%; the agavins treatments share 26.7%, and treatments without prebiotic inclusion share 30.4%. In the LF treatment, the highest number of unique genera (163) were

detected and in the LFA treatment, the lowest (26).

Effect of a low-fat diet overfeeding and agavins inclusion on gut zebrafish microbiota composition.

The abundance of some taxa in the zebrafish gut bacterial composition had significant differences depending on the diet supplied. The permutational multivariate analysis of variance (PERMANOVA) detected significant differences ($p < 0.05$) in the bacterial composition between the initial group (S) and the rest of the treatments at the class taxonomic level; except for the LFA (Suppl. Table 5). These differences are magnified at lower taxonomic hierarchical levels. An exploratory analysis (PCA plot, Fig. 5 and Sup. Fig. 1) at the genus level shows that the bacterial composition of the initial group (S) is shaped mainly by genera of *Fusobacteriaceae* (*Cetobacterium* and an unclassified genus) and lacks genera of the *Enterobacteriaceae* family. Additionally, most of the S samples clustered together (Fig. 5) indicating certain homogeneity and a bacterial composition different from all treatments; this significantly different taxa ($p < 0.05$) are presented in the Suppl. Fig. 2.

Subsequently, the results of all samples were tested in a two-way PERMANOVA (regime and agavins inclusion), which determined that regime and factors interaction promoted the most data variation ($p = 0.11$ and 0.16; respectively), while agavins generated a minimal data variation ($p > 0.6$; Suppl. Table 6). Differences in gut bacterial composition between experimental groups were determined by one-way analysis of similarity[49] (ANOSIM), based on Bray-Curtis distances at genus level. ANOSIM showed a significant dissimilarity between LFA and LFAO ($p = 0.038$; Suppl. Table 7). No significant differences were detected between other groups. Similarity percentage analysis (SIMPER) between LFA and LFAO groups detected that an unclassified genus of *Enterobacteriaceae*, another unclassified genus of *Fusobacteriaceae*, *Plesiomonas* (*Enterobacteriaceae*), and *Cetobacterium* (*Fusobacteriaceae*) contributed mostly to the dissimilarity (Suppl. Table 8). Comparing LFA vs LFAO, two taxa were detected significantly higher: an unclassified *Fusobacteriaceae* ($p = 0.004$) and *Aeromonas* ($p = 0.01$) in LFA (Fig. 6B). Similarly, the proportion of the unclassified *Fusobacteriaceae* was lower in LF vs LFA ($p = 0.47$; Fig. 6A). As mentioned above the diet factor promoted a considerable data variation; statistics detected that the genus *Paraburkholderia* proportion was higher ($p = 0.02$) in overfeed samples than basal, agavins inclusion-independent (Fig. 6C).

Effect of low-fat and high-fat overfeeding on the gut zebrafish functional microbiome.

Because overfeeding was the factor that most significantly modulated the gut bacterial composition, some representative samples from LF and LFO were compared to determinate significant differences in the zebrafish gut functional metagenome. To have a better understanding, some samples from our previous work were also included[31]; these previous groups were fed with a high-fat diet at a basal (HF) and overfed regimes (HFO). About 40 million metagenomic sequences were classified into functional subsystem levels (1-3; Suppl. Table 9). The functional profile of the zebrafish gut microbiome was shaped from 28 subsystems (level 1) of which the 15 most abundant were present in all groups. In general, the most abundant SEED systems (http://pubseed.theseed.org/) were related to the functional subsystems *amino acids and derivatives*, *cell wall and capsule*, *carbohydrates*, *nucleosides and nucleotides*, *respiration*, *iron acquisition and metabolism* (Fig. 7). All metagenomic functional results were tested in a two-way (fat and regime) PERMANOVA which suggests that fat promoted a significant effect on data variation ($p = 0.01$); furthermore, the factors interaction generated a higher effect on the variability of intestinal metagenome ($p = 0.001$; Suppl. Table 10) suggesting a synergy of experimental effects (fat and regime). One-way ANOSIM detected a significant dissimilarity between HFO against HF and LFO ($p = 0.03$ and 0.001; respectively), also between low-fat feeding groups LF vs LFO ($p = 0.02$; Suppl. Table 11). Non-parametric univariate analysis between experimental groups exhibited the significantly different metagenomic functions at subsystem level 3; the low-fat diet groups comparison showed a difference *in 3-Hydroxy-3-methylglutaryl coenzyme A* (*HMG CoA*) *synthesis*, while LFO presented a higher mean proportion (Fig. 8A). Between overfed groups, HFO obtained a higher mean proportion of *lysine fermentation* than LFO while *leucine degradation* is lower (Fig. 8B-C). *HMG CoA synthesis* mean proportion was higher in HF against HFO while *pyrimidine conversions* and *hemin transport system* functions presented higher proportions (Fig. 8D-F). Only those functions that presented a mean proportion difference above 1% and $p < 0.05$ were considered.

Discussion

Overfeeding is the excessive intake of nutrients that generates a caloric surplus in the organism. It is considered a disorganized regime that leads to obesity and overweight, conditions that affect more than one third of the world's population[50]. Obesity is an energy imbalance that leads to the

excessive generation of body fat, becoming a risk factor for the appearance of chronic diseases that can lead to death. To better understand the factors and mechanisms that influence the overfeeding-obesity relationship, different studies have been carried out from different approaches[51–55]; including metagenomic methods[31,56] that suggest a relationship between the intestinal microbiota and the physiological state of overfed organisms. Due the importance of better understanding the role of the microbiome in metabolic health, we used zebrafish as a model to assess the effects of overfeeding and dietary fat on the gut microbiota. The relative food intake in overfed groups remained stable during the experimental period (3.6% - 5.1% of BW) promoting significant differences in the final weights against basal groups; reaffirming that intakes above 2.4% BW generate a caloric surplus[57] in the zebrafish. Results match our previous work where it was proven that an intake of 1.7% BW covers the basal needs of zebrafish without increasing the body weight[31]. Overfeeding and excessive weight gain lead to increased biological indices that allow determining obesity through BMI, K, HSI and VSI; in others studies, it has been possible to induce levels of obesity in zebrafish using overfeeding with normal (*Artemia* nauplii) and high-fat diets[31,51,54,58]. Our results showed the possibility that overfeeding with even low-fat diets lead to zebrafish obesity. In general, the overfed groups (LFO and LFAO) showed significantly higher indices values than the control groups (LF and LFA; Fig.2). Considering BMI of male zebrafish our results match with a reported diet-induce obesity model fed with *Artemia*[59], moreover, the values in hepatosomatic index and body fat percentage coincide with results reported with other obesogenic model of zebrafish by metabolic disruption[60]. Inclusion of agavins did not cause any effect on biological indices among basal groups (LF and LFA). Conversely, in overfed groups LFA consumption was significant lower ($p < 0.05$; Fig. 1A) than LFAO at final stages of experiment suggesting that a 5% agavin inclusion reduces appetite. Agavin effects have been evaluated in mammals models (mice) demonstrating that it plays a role in the regulation of appetite, body weight, and blood levels of glucose, triglycerides and cholesterol[11], as well as in combination with other additives, it offers a synergistic protection against metabolic sequelae of obesity[13]. The body fat percentage and VSI were significantly higher ($p < 0.05$) in LFO than LFAO, suggesting that the 5% agavins inclusion in diet could be used to regulate obesity in other species.

Overfeeding and the prebiotic inclusion had no effect on the diversity of the zebrafish gut microbiota. In general, less diversity was observed in the prebiotic groups but these changes were not significant (Table 2) due to the high variability within groups. The most abundant phyla found

were *Proteobacteria*, *Fusobacteria*, and *Tenericutes* (> 98%; Fig. 3); these phyla have been also found in the gut zebrafish at different proportions, but in most cases, *Proteobacteria* is the most abundant phylum[14,27,31,61,62]. *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* were detected in all individual samples, similarly *Gammaproteobacteria* was detected in all samples of gut zebrafish fed with a high-fat diet[31]; this taxon is one of the most abundant in aquatic environments[63] as well as in the gut of different fish species[14,64–66]. At the genus level, the Venn's diagram[67] exposed that the identified most abundant taxa (*Acinetobacter*, *Aeromonas*, *Cetobacterium*, *Paraburkholderia*, *Plesiomonas*, *Pseudomonas,* and *Roseiarcus*; Table 3) were detected in all experimental groups, which belong to 20.8% of the shared taxa (Fig. 4). That outcome could suggest that these genera belong to the autochthonous zebrafish gut microbiota. One-way ANOSIM in experimental groups revealed dissimilarity in the gut zebrafish microbiota composition in LFA vs LFAO by overfeeding effect (Suppl. Table 7), but this upshot was not replicated between LF vs LFO.

The inclusion of agavins showed no dissimilarities in the microbial composition of feces compared to the controls, these results are supported by the two-way PERMANOVA analysis, which shows that the greatest variability of microbial groups was determined by the overfeeding factor (Suppl. Table 6). Interestingly, the interaction between the regime and prebiotic factors (overfeeding and agavins) promote a higher variability than only the prebiotic factor. As it has been shown, overfeeding and prebiotics are factors that modulate the gut microbiota[31,56,68,69], but the physiological effects caused by overfeeding in animals is greater than the effects generated by probiotics and that could be reflected in the gut microbiota; therefore, it could be one more argument to consider the gut microbiota as an indicator of metabolic health condition[70–72]. According to the SIMPER analysis (Suppl. Table 8), the genera that contributed the greatest variability to the difference in gut bacterial composition between agavins included groups (LFA vs LFAO) were those detected in the higher proportions (Table 3). Particularly, unidentified genera of *Fusobacteriaceae* were detected in a significantly higher proportion in LFA vs LF and LFAO ($p < 0.47$ and $p < 0.004$ respectively; Fig. 6). The *Fusobacteriaceae* family has been related positively to intestinal health[73], it belongs to the *Fusobacteria* phylum which is a group that prevails in zebrafish throughout its development stage[74], as well as in other warm water fishes[64]. These anaerobes produce butyrate in the gut zebrafish[75], and are also related to an increase of goblet cells and villi length[61] allowing better absorption of nutrients and intestinal health of the immune system.

A recent study reported an increase of these taxa in gut zebrafish fed with a supplementation of chitosan as a prebiotic; an aminopolysaccharide biopolymer[61]. More studies are needed to link the intake of prebiotics with fermentative bacteria that improve intestinal health. Interestingly, the unidentified genera of *Fusobacteriaceae* were detected in lower proportion in LFAO compared to LFA despite the agavins inclusion; this could be due to the overfeeding effect, which strongly modulates the gut microbiota. Even so, it is important to consider that prebiotics inclusions could increase the abundance of these taxa in zebrafish when there is no overfeeding.

Paraburkholderia is a genus that was previously detected in the gut of zebrafish as one of the most abundant regardless of the diet. In this study, *Paraburkholderia* was significantly more abundant in the overfeeding groups (LFO and LFAO) regardless of the prebiotic factor. Although there is no record of any important relationship of *Paraburkholderia* with physiological characteristics in zebrafish to our knowledge, the importance of this genus should be considered for future zebrafish studies.

Although it was not the main objective of this research, it is important to mention that significant differences in diversity (Table 2) and composition (Suppl. Table 5 and Suppl. Figure 2) of gut microbiota in initial samples vs experimental groups could be due to diverse factors such as changes in the environment, feeding regime, stress by manipulation, water quality, and prophylactic treatment to which the zebrafish was subjected during acclimatization and adaptation to the water recirculation system before start the experiment. All these factors can affect the gut microbiota[76–79] and this might be the reason why microbiota in acclimatized stock (S) vs the experimental groups, is totally different.

Finally, the microbiomes were compared to determine how overfeeding and dietary fat modulate the gut bacteria functions. As mentioned in the results section (Suppl. Table 10), the dietary fat and the interaction fat-overfeeding had a significant effect on the microbiome variability of the experimental groups. The results indicate that excessive fat (24%) in the diet significantly affects the gut microbiome (even more than low-fat overfeeding), but when the fish were overfed with the high-fat diet, the effect was magnified. Each basal regimen treatment produced a gut microbiome significantly different from its overfed counterpart. The genes related to the mevalonic acid synthesis (an intermediate in the biosynthesis of ketone bodies and cholesterol[80]) by *HMG CoA synthesis* were more abundant in HF vs HFO, in the same way in LFO vs LF (Fig. 8A and 9D; respectively). The genes related to *lysine fermentation* were more abundant in HFO than in LFO

(Fig. 8B), which suggests a relationship between these functions with the dietary fat in the overfeeding regime. Lysine is a growth-related essential amino acid[81] and plays a fundamental role in the production of carnitine, a nutrient that transports long-chain fatty acids to the mitochondria for energy production and helps lower cholesterol levels[82]. Functions related to *leucine degradation* were significantly higher in LFO vs HFO (Fig. 8 C); it has been reported that the supplementation with amino acids such as leucine promote reduction of fat deposits, reduction of obesity and glucose homeostasis[83]. Although this effect may be related to the fact that zebrafish in HFO treatment showed a BMI and K higher than LFO, this comparison is not adequate as they were at different stages of development (about 1 month apart). Based on the results obtained in both our studies (this and previous one[31]), we showed that overfeeding alters both physiological parameters and the intestinal microbiome independent of diet, but the effects are more adverse as dietary fat increases.

Conclusion

Overfeeding with a low-fat diet promotes weight gain in zebrafish, moreover, the inclusion of agavins in the diet (5%) decrease the effects of overfeeding on the food intake, which is reflected on the weight gain, body fat percentage, and vicerosomatic index. Even though, diets with agavins included (5%) did not promote a significant effect to mitigate the overfeeding repercussions on the microbial composition of feces. The agavins inclusion in the diet showed a positive relation with the increment of desirable *Fusobacteriaceae* genera. Additionally, the effect of overfeeding on the modulation of the gut bacterial composition and its functions (microbiota and microbiome) is dependent on the fat included in the diet and is magnified with overfeeding.

Acknowledgements

This study was supported by CIAD internal project. Thanks to Karen Enciso-Ibarra, Carmen Bolán-Mejía, and Erika Sánchez-Gutierrez for technical help. Agavins was donated by Natural Products Chemical Laboratory from CINVESTAV Irapuato unit.

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Table 1. Ingredient and proximate composition of the experimental diets for the zebrafish (*Danio rerio*).

Ingredients	**Diets**	
(g kg^{-1} wet weight.)	**C**	**A**
Fish meal (sardine)[a]	800.0	800.0
Fish oil[b]	15.0	15.0
Corn oil	15.0	15.0
Dextrin[b]	98.0	48.0
Alginate[b]	30	30
Premix vitamin[c]	2	2
Premix minerals[c]	2	2
Antioxidants[d]	0.1	0.1
Soy bean lecithin (70%)[d]	1.4	1.4
Carotenoid (Carophyll pink)[d]	0.5	0.5
Monobasic phosphate[e]	6	6
Cellulose	30	30
Agavin[f]	0	50
Analysed Composition (% DM)[g]		
Crude protein	54.8	55.0
Crude lipid	8.2	8.8
Ash	12.6	12.7
NFE[h]	24.4	23.6
Gross energy (kJ g^{-1})[i]	18.6	18.7

[a] "Premium" grade fish meal was obtained from Selecta de Guaymas, S.A. de C.V. Guaymas, Sonora, Mexico.[b] Droguería Cosmopolita, S.A. de C.V. México, D.F., Mexico.[c] Supplied by BASF Mexicana S.A. de C.V.[d] DSM Nutritional Products Mexico S.A. de C.V., El Salto, Jalisco, Mexico.[e] Sigma-Aldrich Chemical, S.A. de C.V. Toluca, Mexico State, Mexico.[f] Supplied by CINVESTAV Irapuato, Guanajuato, Mexico.[g] mean ± SD, number of determinations=3.[h] Nitrogen-free extract (including fiber)=100−(% protein+% lipid+% ash).[i] Gross energy (kJ g−1) was calculated according to the physiological fuel values of protein, 20.93 kJ g^{-1}; lipids, 37.68 kJ g^{-1}; and nitrogen-free extract, 16.75 kJ g^{-1}.

Table 2. Summary of Illumina sequencing read analysis: total sequences, observed species (OTUs), average taxa assigned (sample), Shannon and dominance indexes; values are represented in means ± SD (n = 9 in each group; only start group (S) n = 6).

Groups	Initial (S)	Low-fat (LF)	Low-fat with agavins (LFA)	Low-fat overfed (LFO)	Low-fat with agavins and overfed (LFAO)
Total sequences	311,355	232,601	348,244	306,342	334,887
Total OTUs	142	972	576	849	914
OTUs (avg. sample)	46 ± 13.6^{a}	174 ± 170^{b}	118 ± 54^{b}	159 ± 95^{b}	170 ± 112^{b}
Shannon index	1.26 ± 0.14^{a}	2.30 ± 0.94^{b}	1.83 ± 0.56^{ab}	2.10 ± 0.35^{b}	1.95 ± 0.67^{ab}
Dominance	0.41 ± 0.05^{a}	0.23 ± 0.13^{b}	0.29 ± 0.12^{ab}	0.26 ± 0.07^{ab}	0.28 ± 0.16^{ab}

OTU, operational taxonomic unit.

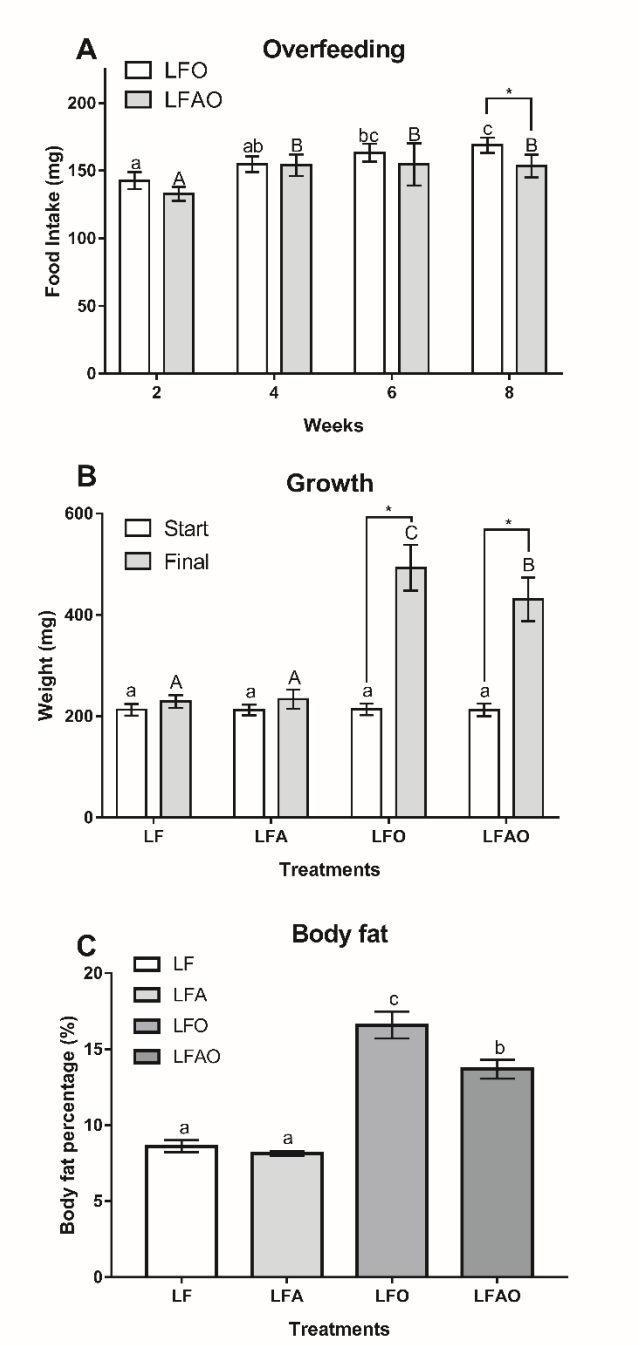

Figure 1. Food intake evolution of overfed fish with a low-fat (LFO) and low-fat agavin (LFAO) included diets for eight weeks (A). Initial and final weight of low-fat (LF), low-fat agavin included (LFA) and respective overfed groups (LFO and LFAO; B). The body fat percentage at the end of the experiment of all groups (C). Results are expressed in mean ± SD. Bars with different superscript letters were significantly different ($p < 0.05$); capital and lowercase indicate differences with respect to the graph legend. * means a statistical difference between stages.

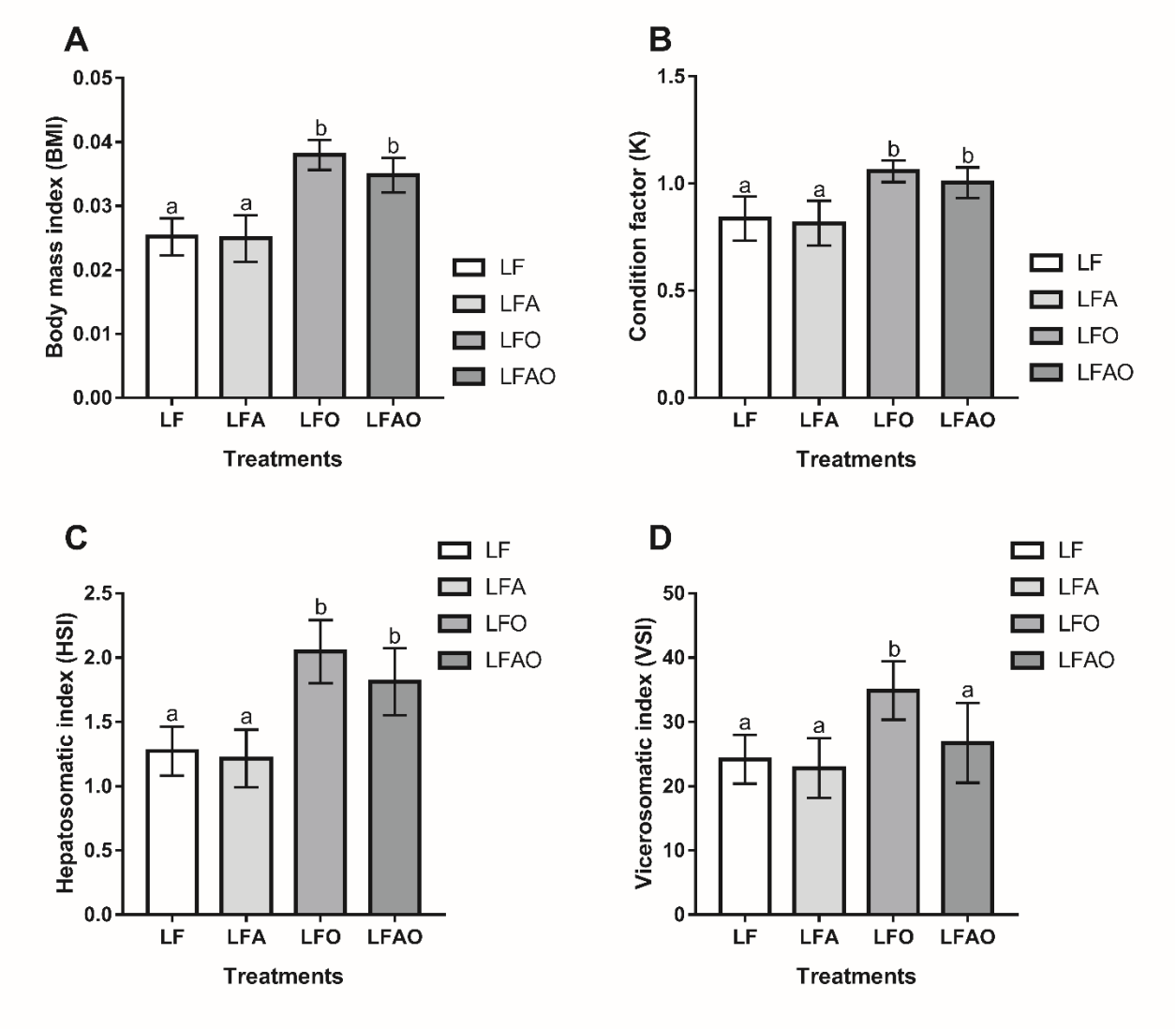

Figure 2. Effect on the body mass index (A), condition factor (B), hepatosomatic index (C) and viscerosomatic index (D) of zebrafish. Results are expressed in mean ± SD. Means with different superscript letters were significantly different ($p < 0.05$).

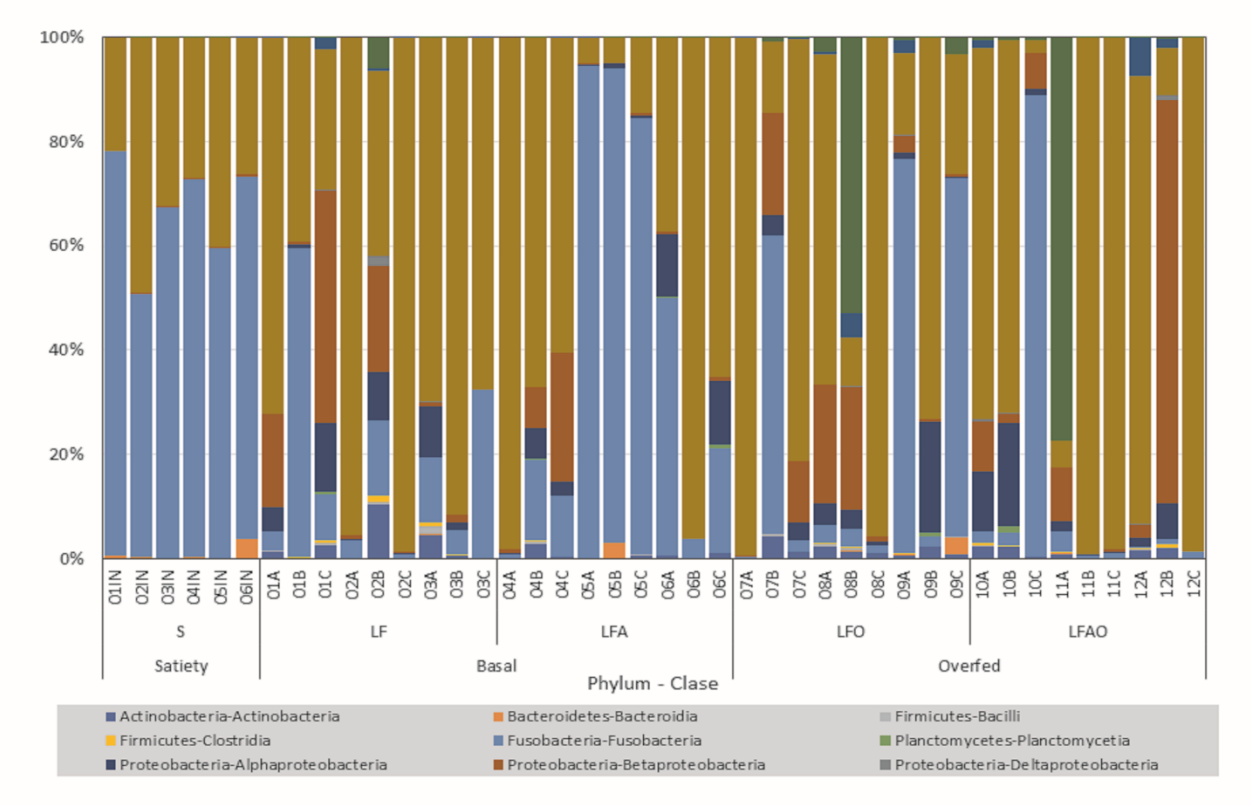

Figure 3. Read abundance of zebrafish gut bacterial composition under low-fat (LF), low-fat agavin included (LFA) and respective overfed groups (LFO and LFAO). Initial group (S) is included.

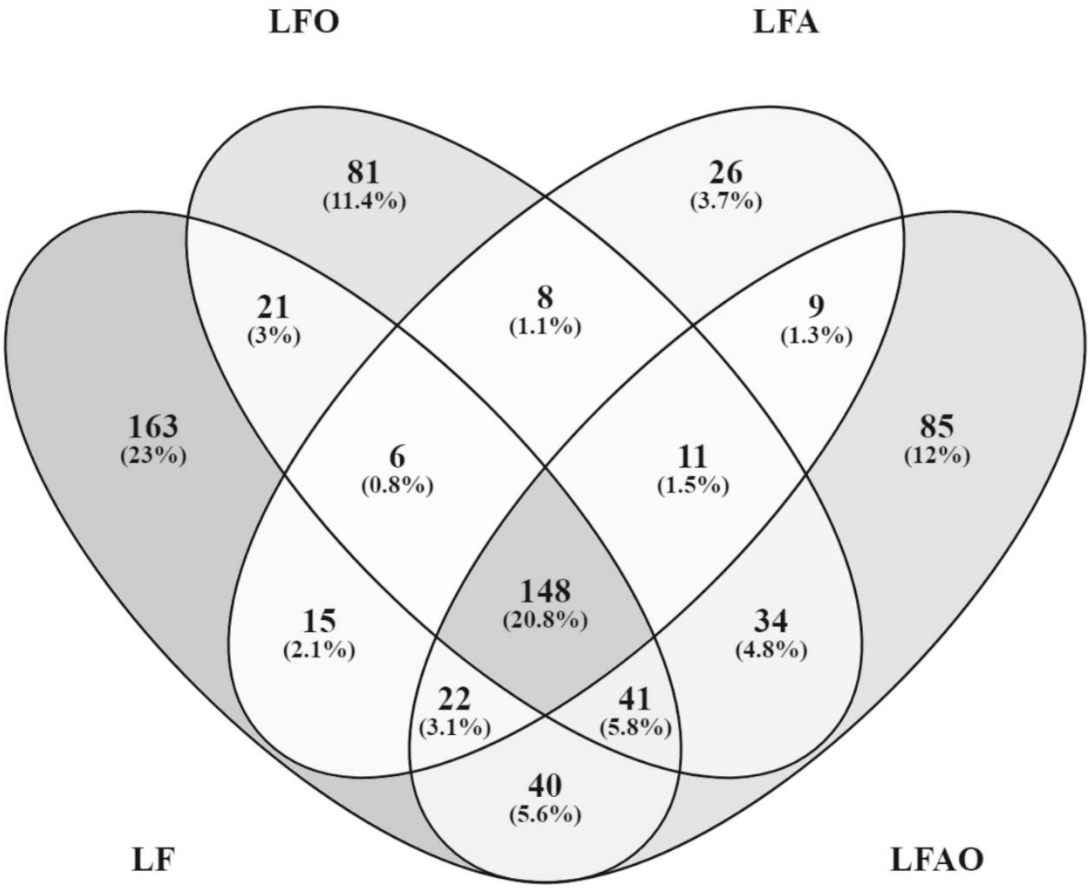

Figure 4. Intestinal bacterial genera shared between zebrafish groups. The Venn diagram represents unique and shared genera between groups at low-fat (LF), low-fat agavin included (LFA) and the respective overfed groups (LFO and LFAO).

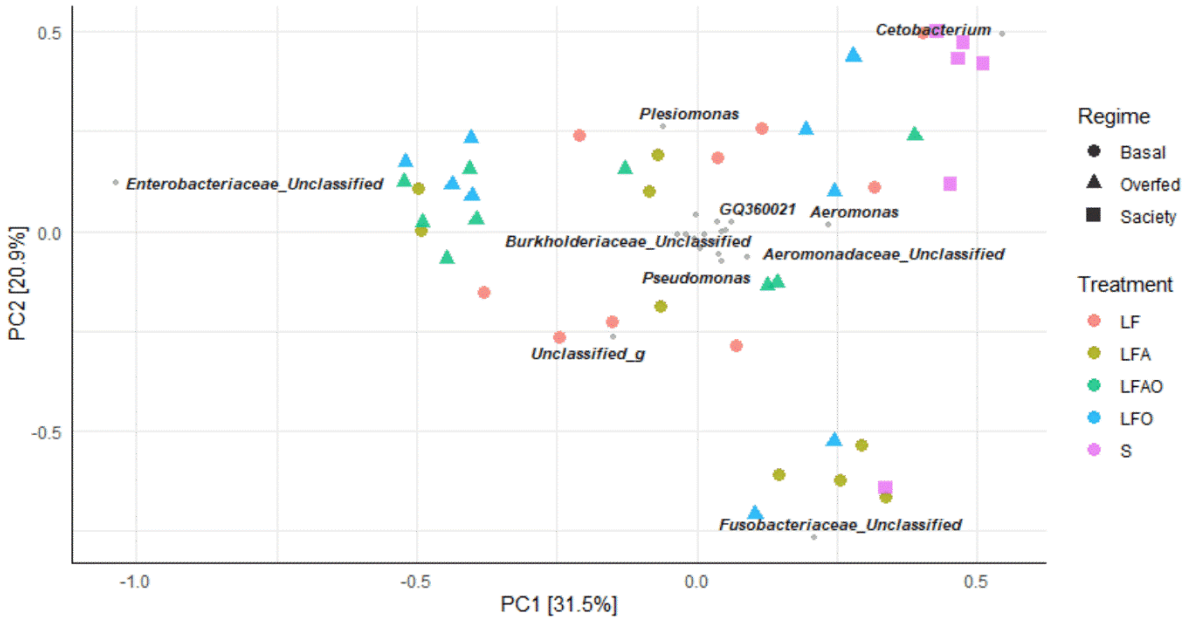

Figure 5. 2D PCA bi-plots of gut bacterial composition in zebrafish at low-fat (LF), low-fat agavin included (LFA) and the respective overfed groups (LFO and LFAO). Red circles, LF; yellow circles, LFA; blue triangles, LFO and; green triangles, LFAO. The group of initial samples (S) was including as pink squares. PC1 =31.5%, PC2 = 20.9%.

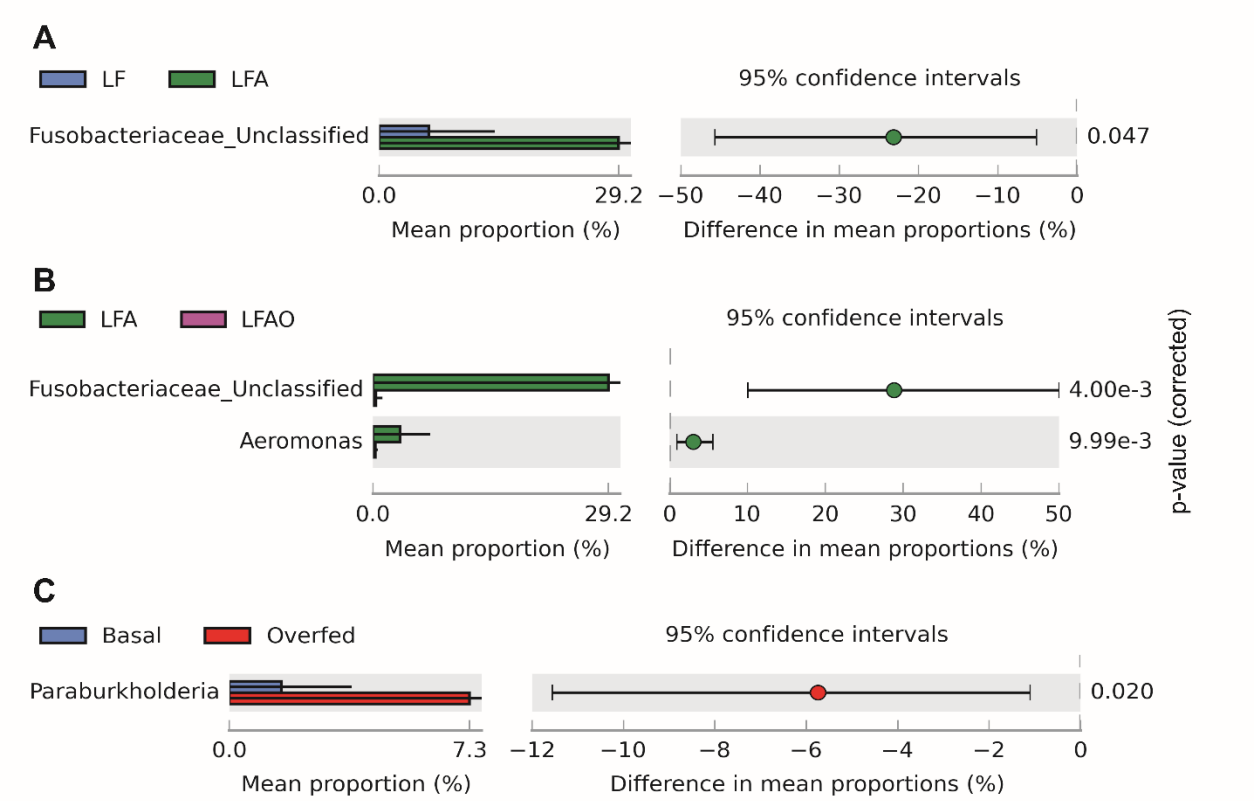

Figure 6. Post-hoc plots of taxa (genera level) detected as significantly different. Graphs indicate the mean proportion of sequences within each group and the difference in mean proportions between groups ($p < 0.05$). A, shows a difference in U. of *Fusobacteriaceae* in LF vs LFA. B, shows differences in U. of *Fusobacteriaceae* and *Aeromonas* in LFA vs LFAO. C, shows difference in all samples considering the food regime as factor.

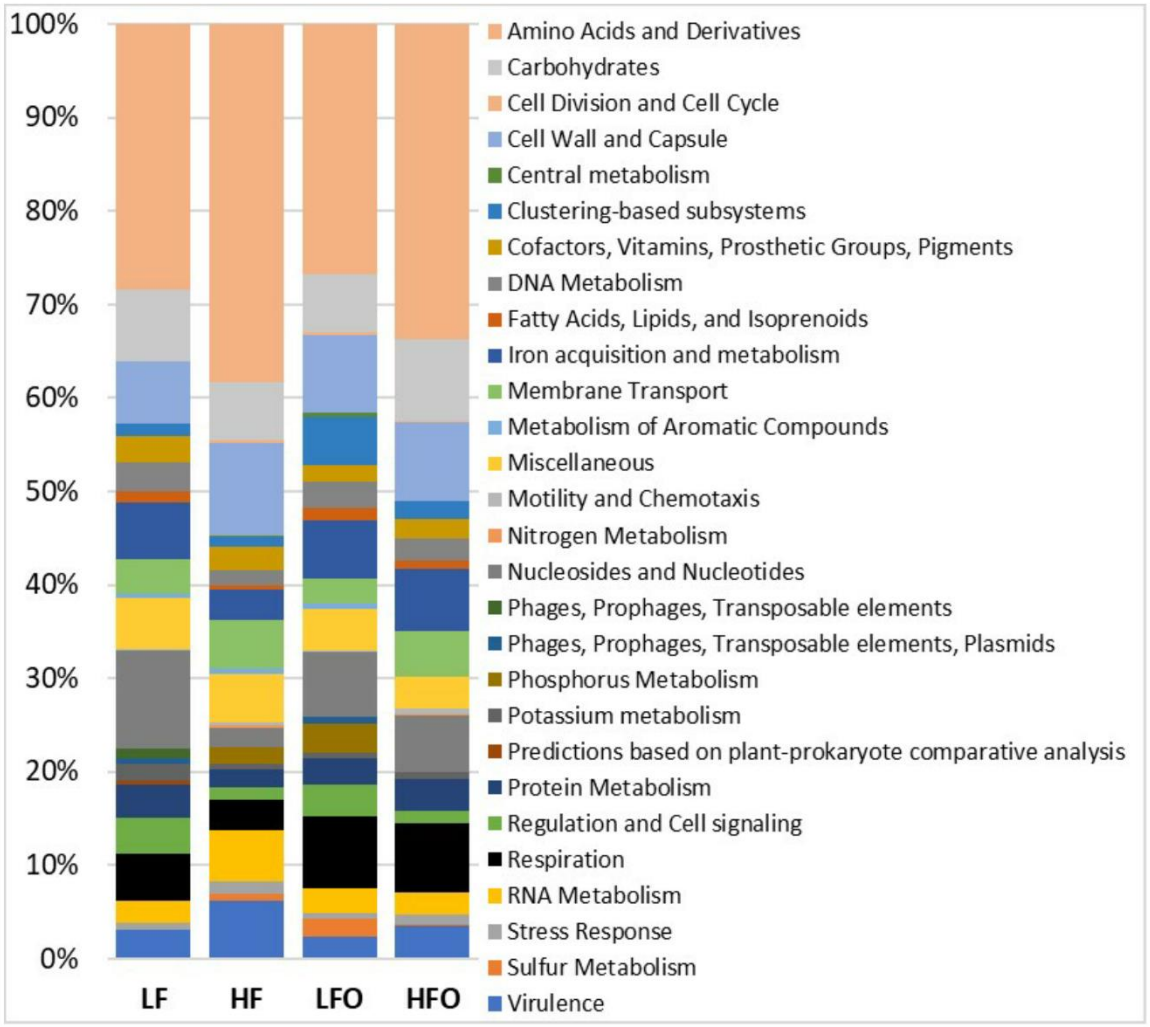

Figure 7. Relative abundance of zebrafish gut microbiome functions at subsystem level 1. LF, low-fat; HF, high-fat; LFO, low-fat overfed; and HFO, high-fat overfed.

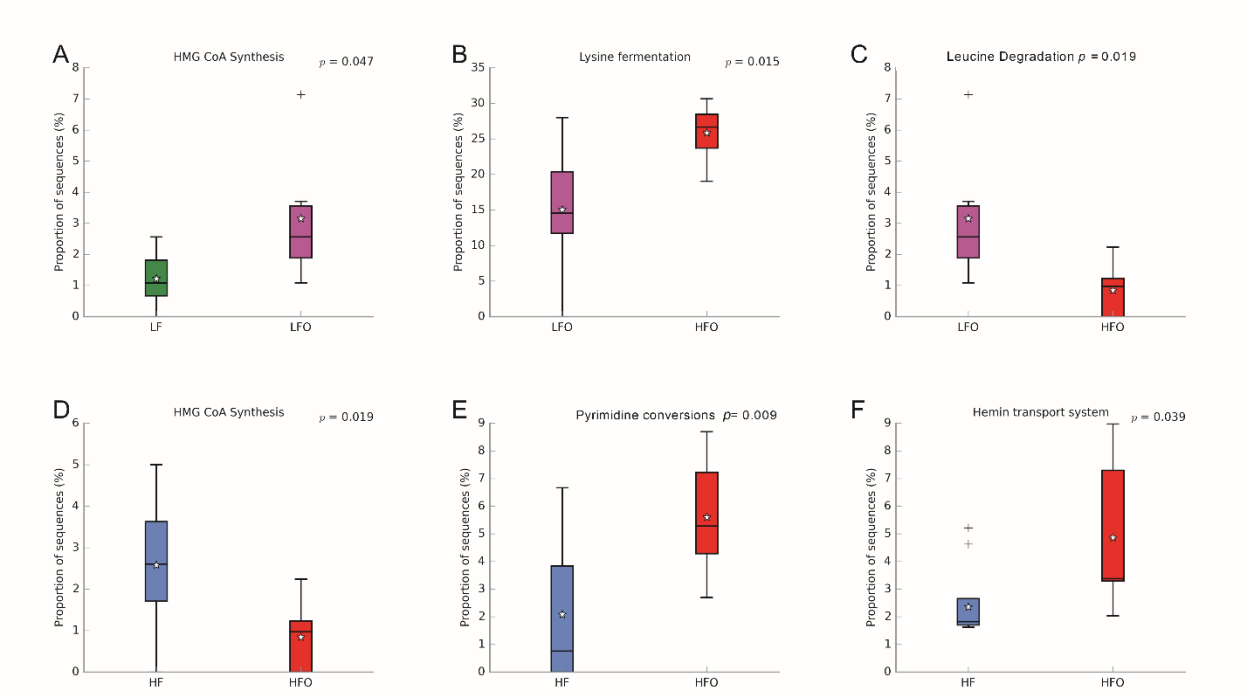

Figure 8. Box plot showing the proportions of intestinal metagenomic functions at subsystem level 3 detected as significantly different between experimental groups. A, shows differences between LF vs LFO; B and C, show differences between overfed groups (LFO vs HFO); and D-F, show differences between high-fat diet groups (HF and HFO) The median value is shown as a line within the box and the mean value as a star. Whiskers extend to the most extreme value within 1.5*IQR. Outliers are shown as crosses.

4. DISCUSIÓN GENERAL

Existen muchos estudios sobre la relación entre la dieta y la microbiota intestinal usando distintos modelos, entre ellos el pez cebra (Gardiner *et al.*, 2020; González-Olmo *et al*., 2021; Leeming *et al*., 2019; Parata *et al*., 2020), además otras investigaciones ya han reportado las alteraciones o cambios que sufre la microbiota cuando el hospedero padece de algún trastorno metabólico incluidos aquellos relacionados con la obesidad (Faillaci *et al*., 2018). En este trabajo, se utilizó al pez cebra como modelo para entender aún mejor la relación de la dieta y la obesidad con la microbiota intestinal, y como ésta se altera por efecto de la sobrealimentación y la grasa dietaria. Además, se evaluó el potencial prebiótico de la agavina en la microbiota intestinal de peces sobrealimentados y alimentados bajo un régimen basal.

Los protocolos de alimentación utilizados en los experimentos de los capítulos 2 y 3 mostraron ser eficaces. Por un lado, la cantidad propuesta como dieta basal (1.7% del peso corporal) funcionó en ambos experimentos para mantener los parámetros biométricos del pez en estado saludable, mientras que el suministro excesivo de alimento en los grupos sobrealimentados (8.5% del peso corporal) demostró inducir a la ganancia excesiva de peso con una ingesta general de ≈4%. Estos resultados concuerdan con la información de trabajos previos que proponen cantidades mínimas y máximas (0.5% y 2.4%) de alimento para la especie con las que se podría promover la inanición y la sobrealimentación respectivamente (Oka *et al*., 2010; Pannevis y Earle, 1994). Los resultados del presente estudio podrían ser útiles para futuros modelos de obesidad por sobrealimentación alta y baja en grasa con pez cebra. Cabe mencionar que las dietas experimentales fueron adicionadas con dos principales fuentes lipídicas, el aceite de maíz y el aceite de pescado. Por un lado, el aceite de pescado aporta los omega-3 de mayor importancia nutricional para el desarrollo y la salud, los ácidos docosahexaenoico (DHA) y eicosapentaenoico (EPA), así como también un porcentaje considerable de ácidos grasos poliinsaturados (Swanson *et al.*, 2012). Mientras que el aceite de maíz tiene como principal componente un omega-6, el ácido linoleico (Dupont *et al*., 1990). Con esto se buscó tener una composición lipídica variada en las dietas.

Los índices biométricos basados en longitud y peso son una herramienta útil que permite determinar el estado físico de los organismos (Hu, 2007; Jin *et al*., 2015). En ambos experimentos los resultados de los índices biométricos fueron congruentes con el consumo alimenticio y la

ganancia en peso independientemente del tratamiento. De esta forma, la condición de obesidad en el pez cebra fue definida mediante los resultados de distintos estudios con la especie, por ejemplo, Oka y colaboradores (2020) con un protocolo similar al presentado en este estudio crearon un modelo de obesidad en pez cebra mediante la sobrealimentación con nauplios de *Artemia*, la cual se usó como referencia para el diseño de las dietas altas en grasa de este estudio, ya que son consideras por los autores como un alimento alto en grasa (Oka *et al*., 2010). Asimismo, otros autores (Landgraf *et al*., 2017; Zang *et al*., 2017) lograron fenotipos obesos en pez cebra alimentados con dietas altas en grasa utilizando protocolos de alimentación similares. En general, las ganancias en peso de los peces sobrealimentados, así como las diferencias en los índices biométricos coinciden con las reportadas en los estudios antes mencionados, donde además se registraron disrupciones en niveles de colesterol, glucosa y triglicéridos.

La pérdida de diversidad de la microbiota intestinal por efecto de la obesidad ya ha sido reportada (Francino, 2016) y si bien, no se presentaron cambios estadísticamente significativos, esto puede ser debido a la variabilidad dentro de los grupos a causa de las diferencias intrínsecas entre individuos (Davidson *et al*., 2018; Gatesoupe *et al.*, 2016), cabe resaltar que en los peces machos sobrealimentados se observó una diversidad menor y dominancia mayor con respecto a los grupos basales. Algo similar se observó por efecto de la agavina, los grupos basales y sobrealimentados presentaron valores menores de diversidad en comparación con los grupos no tratados. Resultados similares por efecto de este prebiótico fueron reportados por Huazano-García y colaboradores (2017); como se menciona en el capítulo 1 esto puede deberse a que los prebióticos estimulan selectivamente la proliferación de ciertos grupos bacterianos potencialmente benéficos.

En cuanto a la composición (proporción) de los taxones bacterianos, independientemente del tratamiento (capítulo 1 y 2) todos los phyla representativos que se identificaron ya han sido reportados y se sabe que forman parte de la microbiota normal de la especie (Falcinelli *et al*., 2018; Roeselers *et al*., 2011; Semova *et al*., 2012). Como se menciona en ambos capítulos, *Proteobacteria* es el orden más representativo en el pez cebra, y en diversas especies de ambiente acuático, es por ello que los cambios en las proporciones de este taxón fue lo que aportó mayor variabilidad a las diferencias en la composición. Otros estudios han reportado la sensibilidad de este taxón incrementando su proporción por efecto de la sobrealimentación y decreciendo por el efecto prebiótico de la agavina (Huazano-García *et al*., 2017). Por el contrario, *Bacteroidetes* fue un grupo que se encontró relacionado de forma negativa con la sobrealimentación alta en grasa

(capítulo 2), esto concuerda con los estudios de Ley y colaboradores (2006) donde observaron menores proporciones de este taxón en personas obesas contra personas sanas. Del mismo modo, *Firmicutes* se encontró relacionado negativamente con la sobrealimentación alta en grasa haciendo congruencia con lo reportado por Clarke y colaboradores (2012).

En cuanto al efecto prebiótico de la agavina (capítulo 3) se encontró una relación positiva con la Familia *Fusobacteriaceae*, pero solo en los grupos basales. Es posible que el aumento de esta familia no se haya presentado en el grupo sobrealimentado con agavina incluida debido a que el efecto de la sobrealimentación es mayor como factor alterante. Esto puede indicar que el efecto prebiótico en la microbiota intestinal se atenúa cuando el organismo está bajo régimen de sobrealimentación, aunque por otro lado se observó un efecto de pérdida de apetito ya que los grupos suplementados con el prebiótico presentaron un consumo menor (Cerdó *et al*., 2019) lo que podría deberse a la producción de ácidos grasos de cadena corta como el acetato que están relacionados con la estimulación de la leptina secretada por los adipocitos (Alvarado-Jasso *et al.*, 2020).

En cuanto al sexo como factor, los resultados obtenidos en el capítulo 2 indican que la modulación de la microbiota intestinal por efecto de la sobrealimentación con una dieta alta en grasa es dependiente del sexo del pez cebra, encontrándose una mayor variabilidad en la microbiota de los machos contra sus respectivos controles. El hallazgo sobre las alteraciones de la microbiota intestinal dependiente del sexo por factores dietarios ya ha sido reportado en otras especies de vertebrados (Bolnick *et al*., 2014), pero particularmente en pez cebra esta dependencia solo se había reportado a causa de la exposición de nanopartículas de plata (Ma *et al*., 2018). Es probable que las diferencias en la microbiota por efecto de la sobrealimentación se deban a aspectos metabólicos en la especie diferenciados por el sexo, se sabe que el pez cebra presenta un dimorfismo sexual y a pesar que los requerimientos nutricionales son similares, los requerimientos calóricos y algunos nutrientes pueden cambiar dependiendo del estado reproductivo (Watts *et al*., 2012), ya que la hembras distribuyen una mayor cantidad de recursos para la reproducción (Uusi-Heikkilä *et al*., 2012). Además, un estudió reporto que las hembras fértiles sobrealimentadas desarrollaron menos esteatosis en comparación a los machos bajo el mismo tratamiento (Turola *et al*., 2015). Considerando lo anterior, es probable que esas diferencias fisiológicas vuelvan a los machos más sensibles a la obesidad por sobrealimentación y por la tanto a las alteraciones en la microbiota intestinal.

Por último, y haciendo congruencia con los resultados de la microbiota poblacional, el análisis funcional determinó que la interacción de los factores (régimen-grasa dietaria) promueve un efecto significativo mayor que los mismos por separado.

Las variaciones estadísticas de las abundancias de los genes funcionales solo se encontraron en el nivel más bajo de los subsistemas en los cuales se clasifican las funciones del microbioma intestinal. Por ejemplo, los genes del subsistema *HMG CoA* que se encuentra relacionado con la síntesis de cuerpo cetónicos y colesterol (Bucher *et al*., 1960) fue más abundante en el grupo basal con dieta alta grasa (HF) en comparación con su homólogo sobrealimentado (HFO), mientras que en los alimentados con dieta baja en grasa *HMG CoA* fue más abundante en el sobrealimentado (LFO con respecto a LF).

Este resultado aparentemente disonante podría ser un indicio de que los genes funcionales *HMG CoA* y su regulación se encuentran relacionadas a niveles lipídicos específicos, ya que, a pesar de la sobrealimentación los grupos LFO y HFO proveen al pez aportes lipídicos muy distintos. Por su parte los genes funcionales relacionados a la *síntesis de lisina* fueron más abundantes en el grupo sobrealimentado alto en grasa (HFO) con respecto al bajo en grasa (LFO), la lisina es un aminoácido relacionado con la producción de carnitina la cual transporta a los aminoácidos de cadena larga a la mitocondria para la producción de energía y además ayuda a reducir el colesterol (Lin *et al*., 2014). Es posible que los grupos con mayor consumo de grasa (HFO) activen estas rutas metabólicas para obtener energía principalmente de los lípidos excedentes como forma de regulación.

En general, los datos de esta investigación sugieren que la microbiota es alterada por la sobrealimentación y a medida que la grasa dietaria aumenta, si ambos factores por separado ya tienen un efecto modulador en la microbiota intestinal, resulta interesante que la sinergia entre los factores incremente el grado de alteración.

5. CONCLUSIONES GENERALES

La sobrealimentación con una dieta baja en grasa genera una ganancia excesiva de peso en pez cebra, promueve la obesidad y modula algunos grupos bacterianos de la microbiota intestinal sin llegar a provocar una disbiosis como en el caso de la sobrealimentación con exceso de grasas.

Las dietas altas en grasa (24%) suministradas bajo un régimen basal no promueven un aumento significativo en el peso corporal con respecto a peces alimentados con dietas bajas en grasa (8%), no alteran los parámetros biológicos que determinan la obesidad (IMC y factor de condición) y no promueven un cambio significativo de la microbiota intestinal.

Las dietas altas en grasa suministradas bajo un régimen de sobrealimentación generan una ganancia excesiva de peso, promueven a la obesidad y causan disbiosis alterando la diversidad y composición de la microbiota intestinal del pez cebra.

Los cambios en la microbiota intestinal del pez cebra por efecto de la sobrealimentación con una dieta alta en grasa son dependientes del sexo; los peces machos presentan un mayor grado disbiosis que las hembras.

En general el efecto de la sobrealimentación promueve en mayor medida los cambios en las funciones del microbioma intestinal de pez cebra con respecto a la grasa dietaria, pero la sinergia de ambos factores potencia dicho efecto.

El phylum *Proteobacteria* es el grupo más abundante y se encuentra presenta en todos los organismos por lo que debe ser considerado como parte de la microbiota núcleo.

La inclusión de agavina en la dieta disminuye la ingesta del pez cebra mitigando la ganancia de peso por sobrealimentación, pero no promueve un efecto significativo en la mitigación de las alteraciones de la microbiota intestinal por sobrealimentación. No obstante, los grupos alimentados con inclusión del prebiótico mostraron un aumento de géneros deseables de *Fusobacterieaceae*.

Las funciones metabólicas del microbioma intestinal del pez cebra pueden ser alteradas significativamente por efecto de la grasa dietaria y la sobrealimentación.

Las funciones metabólicas más abundantes en el microbioma intestinal del pez cebra son aquellas relacionadas al metabolismo de aminoácidos, así mismo son las que presentan mayor variabilidad a los tratamientos.

Se recomienda realizar análisis proximales y de metabolitos a los organismos que sea sean

seleccionados para análisis metagenómico funcional, esto permitiría entender mejor los perfiles funcionales relacionados a la actividad metabólica.

Suministrar una cantidad similar al 1.7% del peso corporal del pez cebra en alimento balanceado bajo en grasa (8%) funciona como dieta de mantenimiento (basal) para protocolos experimentales.

6. RECOMENDACIONES

La microbiota intestinal de los organismos debe considerarse un sistema dinámico y abierto donde cualquier efecto externo puede alterar la estructura y diversidad de la misma; por lo tanto, es recomendable tratar de reducir en lo posible dichos factores o bien considerarlos como variables en los análisis estadísticos aplicando un criterio multivariado.

Debido a que los cambios en la microbiota por sobrealimentación son dependientes del sexo del pez cebra, debe considerarse al sexo como una variable en futuros estudios.

Se recomienda un suministro de alimento balanceado de ≈1.7% del peso corporal para estudios donde se busque un mantenimiento basal en pez cebra.

Aumentar aún más los niveles de grasa (>24%) permitiría llegar a mayores grados de disbiosis y obesidad, esto considerando que los efectos de la sobrealimentación en la microbiota y los parámetros biológicos se vieron potenciados con el aumento de grasa en la dieta.

Se recomienda continuar las investigaciones con inclusión de agavina como prebiótico en las dieta de peces; esto debido al efecto benéfico que mostró en contra de la ganancia excesiva de peso, el consumo y en la proliferación de grupos bacterianos benéficos.

Se recomienda usar técnicas bioinformáticas que permitan asociar los genes del microbioma funcional con grupos bacterianos específicos para establecer relaciones de causalidad.

Se recomienda estudiar los efectos de la agavina en el microbioma desde un perfil funcional para establecer posibles relaciones con genes reguladores del metabolismo energéticos.

Se recomienda realizar una limpieza con herramientas bioinformáticas como *deconseq* para eliminar secuencias eucariotas y virales previo a la clasificación funcional con SUPERFOCUS.

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7. ANEXOS

7.1. Información Suplementaría del Capítulo 1

Sup. Table 1. Absolute consumption values of the HFO groups.

	FEMALE			MALE		
	Food (mg)	**Consumed food**		**Food (mg)**	**Consumed food**	
WEEKS		**mg**	**%**		**mg**	**%**
2		404 ± 5.8	80.9		286 ± 6.2	81.7
4	500	424 ± 10.5	84.9	350	279 ± 26.8	79.9
6		413 ± 13.4	82.7		276 ± 24.5	79.0
8		321 ± 31.5	64.3		145 ± 36.4	41.6

Sup. Table 2. Representative bacterial genera in the intestine of the zebrafish (*Danio rerio*).

Genus	Sequences	Percentage of total	Cumulative percentage
Aeromonas	944,955	35.2	35.2
Unclassifieds	536,897	20.0	55.2
Plesiomonas	388,441	14.5	69.6
Romboutsia	207,153	7.7	77.3
Mycoplasma	135,820	5.1	82.4
Paraburkholderia	123,506	4.6	87.0
Alkanindiges	61,925	2.3	89.3
Pseudomonas	46,910	1.7	91.0
Shewanella	23,662	0.9	91.9
Sphingomonas	23,521	0.9	92.8
Acinetobacter	23,282	0.9	93.7
Ralstonia	16,291	0.6	94.3
Sphingobium	9,428	0.4	94.6
Bradyrhizobium	9,224	0.3	95.0
Asinibacterium	5,211	0.2	95.2
Prosthecomicrobium	4,946	0.2	95.3
Croceicoccus	4,887	0.2	95.5
Clostridium	4,479	0.2	95.7
Sellimonas	4,333	0.2	95.8

Sup. Table 3. Two-way PERMANOVA (permutation 9999) analysis multivariate test for all samples (classes).

Source	Sum of sqrs	df	Mean square	F	p
Sex	0.55238	1	0.55238	2.3046	0.0433
Diet	0.59568	2	0.29784	1.2427	0.2083
Interaction	0.82353	2	0.41177	1.718	0.0005
Residual	15.34	64	0.23968		
Total	17.311	69			

Sup. Table 4. Analysis of similarity in male groups

ANOSIM	**R values**		
	LF	HF	HFO
LF		0.11	0.22
HF	0.11		0.18
HFO	0.22	0.18	
	Bonferroni-corrected *p* values		
	LF	HF	HFO
LF		0.17	0.01
HF	0.17		0.03
HFO	0.01	0.03	

Low fat (LF) vs High fat overfed (HFO)			
Taxon	Av. dissim	Contrib. %	Cumulative %
Gammaproteobacteria	43.76	60.73	60.73
Mollicutes	17.18	23.84	84.58
Betaproteobacteria	4.219	5.856	90.43
Clostridia	2.833	3.932	94.37
Alphaproteobacteria	2.317	3.215	97.58
Actinobacteria	1.061	1.473	99.05

HF vs HFO			
Gammaproteobacteria	39.31	54.31	54.31
Clostridia	18.86	26.05	80.36
Mollicutes	7.816	10.8	91.16
Alphaproteobacteria	2.915	4.027	95.19
Betaproteobacteria	2.77	3.827	99.01
Actinobacteria	0.3848	0.5316	99.54

Sup. Table 6. PERMANOVA in male groups

PERMANOVA	**Bonferroni-corrected *p* values**		
	LF	HF	HFO
LF		0.1935	0.0045
HF	0.1935		0.0321
HFO	0.0045	0.0321	

Sup. Table 7. Analysis of similarity in female groups

ANOSIM	**R values**		
	LF	HF	HFO
LF		-0.04	0.12
HF	-0.04		0.05
HFO	0.12	0.05	
	Bonferroni-corrected *p* values		
	LF	HF	HFO
LF		1	0.15
HF	1		0.45
HFO	0.15	0.45	

Sup. Table 8. SIMPER analysis in female groups

	Comparison between all groups (LF vs HF vs HFO)		
Taxon	Av. dissim	Contrib. %	Cumulative %
Gammaproteobacteria	38.25	64.99	64.99
Mollicutes	8.926	15.17	80.16
Betaproteobacteria	7.354	12.5	92.66
Alphaproteobacteria	3.207	5.45	98.11
Clostridia	0.6673	1.134	99.24
Others	0.446	0.7579	100

Sup. Table 9. Summary of the taxa detected as significantly different by Kruskal-Wallis test with Storey FDR-correction in male groups.

	Multiple groups	**Between groups Tukey-Kramer: *p*- values**		
	p-values	LF vs HF	LF vs HFO	HF vs HFO
Phylum				
Bacteroidetes	= 0.022	≥ 0.1	≥ 0.1	**< 0.02**
Firmicutes	= 0.015	**< 0.001**	**< 0.001**	≥ 0.1
Proteobacteria	= 0.044	≥ 0.1	**< 0.05**	≥ 0.1
Class				
Clostridia	= 0.025	**< 0.001**	**< 0.001**	≥ 0.1
Gammaproteobacteria	= 0.032	≥ 0.1	**< 0.01**	**< 0.01**

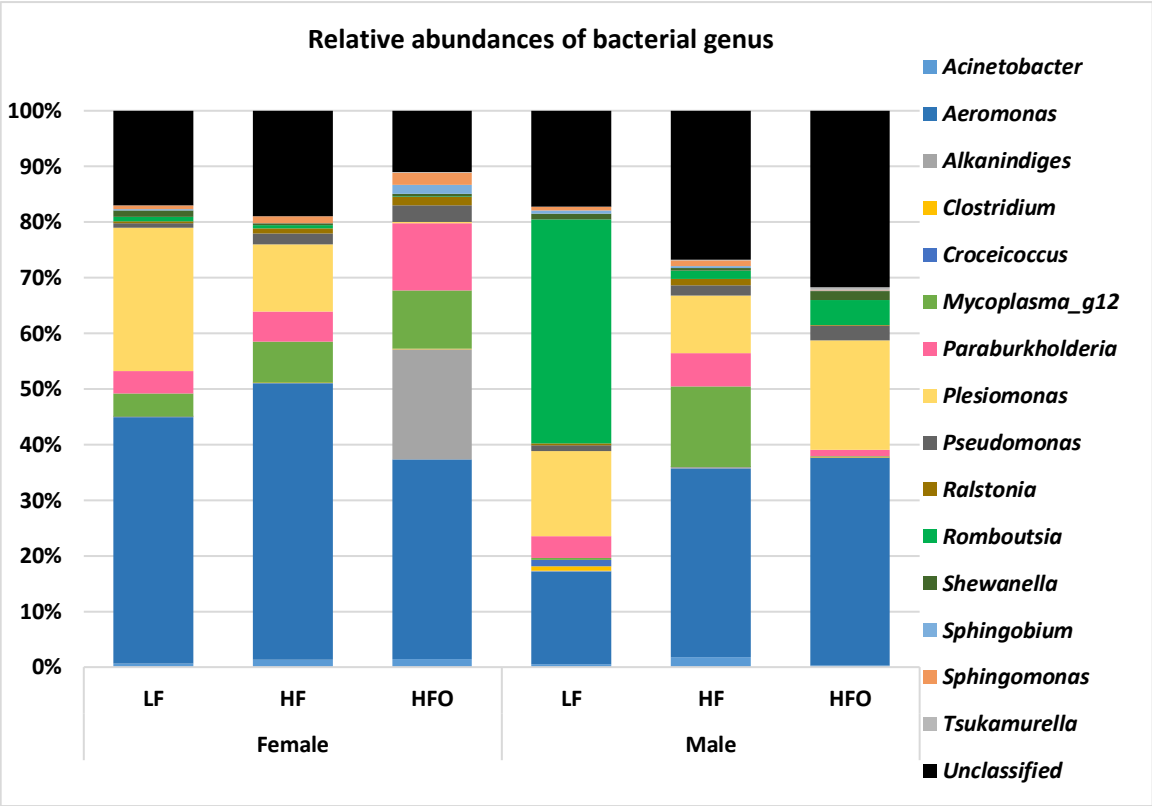


Figure Sup. 1. Gut bacterial composition (genera) in male and female zebrafish at a high-fat overfeeding (HFO) regime, low-fat (LF) basal regime, and high-fat (HF) basal regime.

7.2. Información Suplementaria del Capítulo 2

Suppl. Table 1. Absolute consumption values of the overfed groups.

	LFO			LFAO		
	Food (mg)	**Consumed food**		**Food (mg)**	**Consumed food**	
WEEKS		**mg**	**%**		**mg**	**%**
2		143 ± 8.6	79.4		133.5 ± 6.8	74.2
4	180	154.7 ± 8.3	85.9	180	151.7 ± 8.9	84.3
6		163.3 ± 8.6	90.7		154.7 ± 20.2	85.9
8		168.9 ± 7.7	93.8		155.4 ± 11.5	86.3

Suppl. Table 2. OTUs and taxa classified.

Hierarchical level	***Taxa***
Phylum	27
Class	67
Order	143
Family	316
Genus	712
Specie	1,197
OTUs	*2,125*

Suppl. Table 3. Representative bacterial genera in the intestine of the zebrafish (*Danio rerio*).

Family	***Genera***	***Sequences***	***Percentage of total***	***Cumulative percentage***
Enterobacteriaceae	***Unclassified_genera***	447,623	29.19	29.19
Fusobacteriaceae	***Cetobacterium***	249,383	16.26	45.45
Fusobacteriaceae	***Unclassified_genera***	210,420	13.72	59.18
Enterobacteriaceae	***Plesiomonas***	197,935	12.91	72.08
Unclassified_family	***Unclassified_genera***	159,239	10.38	82.47
Aeromonadaceae	***Aeromonas***	61,724	4.03	86.49
Aeromonadaceae	***Unclassified_genera***	34,558	2.25	88.75
Burkholderiaceae	***Paraburkholderia***	22,693	1.48	90.23
Pseudomonadaceae	***Unclassified_genera***	15,453	1.01	91.24
Pseudomonadaceae	***Pseudomonas***	15,402	1.00	92.24
Burkholderiaceae	***Unclassified_genera***	13,857	0.90	93.14
Porphyromonadaceae	***GQ360021_genera***	6,294	0.41	93.55
Beijerinckiaceae	***Roseiarcus***	5,652	0.37	93.92
Propionibacteriaceae	***Propionibacterium***	3,903	0.25	94.18
Bradyrhizobiaceae	***Bradyrhizobium***	3,619	0.24	94.41
Shewanellaceae	***Shewanella***	3,547	0.23	94.64
Fusobacteriaceae	***Fusobacterium***	3,348	0.22	94.86
Moraxellaceae	***Acinetobacter***	3,121	0.20	95.07

Suppl. Table 4. Top ten abundant genera in gut zebrafish for all treatments (LF, LFO, LFA and LFAO) and samples before starting the experiment (S). Unclassified groups were included as a taxon.

Family	Genera	Of total %	Cumulative %	Family	Genera	Of total%	Cumulative %
Initial (S)				**Low-fat overfed (LFO)**			
Fusobacteriaceae	*Cetobacterium*	47.5	47.5	*Enterobacteriaceae*	*Unclassified*	42.1	42.1
Fusobacteriaceae	*Unclassified*	18.0	65.5	*Fusobacteriaceae*	*Cetobacterium*	19.8	61.9
Aeromonadaceae	*Aeromonas*	12.5	78.0	*Enterobacteriaceae*	*Plesiomonas*	9.3	71.2
Aeromonadaceae	*Unclassified*	10.2	88.2	*Fusobacteriaceae*	*Unclassified*	7.7	78.9
Enterobacteriaceae	*Plesiomonas*	9.5	97.7	*Unclassified*	*Unclassified*	3.7	82.6
Shewanellaceae	*Shewanella*	0.6	98.3	*Aeromonadaceae*	*Aeromonas*	3.1	85.7
Porphyromonadaceae	*GQ360021*	0.3	98.6	*Burkholderiaceae*	*Paraburkholderia*	1.4	87.1
Fusobacteriaceae	*Ilyobacter*	0.2	98.8	*Beijerinckiaceae*	*Roseiarcus*	0.9	88.0
Fusobacteriaceae	*Fusobacterium*	0.1	98.9	*Porphyromonadaceae*	*GQ360021*	0.9	88.9
Burkholderiaceae	*Paraburkholderia*	0.1	99.0	*Burkholderiaceae*	*Unclassified*	0.7	89.7
Low-fat (LF)				**Low-fat with agavins and overfed (LFAO)**			
Enterobacteriaceae	*Plesiomonas*	26.2	26.2	*Enterobacteriaceae*	*Unclassified*	43.9	43.9
Enterobacteriaceae	*Enterobacteriaceae*	24.1	50.3	*Unclassified*	*Unclassified*	20.1	64.0
Unclassified	*Unclassified*	16.4	66.7	*Enterobacteriaceae*	*Plesiomonas*	15.4	79.5
Fusobacteriaceae	*Cetobacterium*	7.9	74.7	*Fusobacteriaceae*	*Cetobacterium*	5.0	84.4
Fusobacteriaceae	*Unclassified*	7.4	82.0	*Burkholderiaceae*	*Paraburkholderia*	4.3	88.7
Pseudomonadaceae	*Unclassified*	6.5	88.6	*Burkholderiaceae*	*Unclassified*	1.3	90.0
Burkholderiaceae	*Unclassified*	2.3	90.8	*Beijerinckiaceae*	*Roseiarcus*	0.6	90.6
Aeromonadaceae	*Aeromonas*	1.4	92.2	*Fusobacteriaceae*	*Unclassified*	0.5	91.1
Burkholderiaceae	*Paraburkholderia*	0.6	92.8	*Pseudomonadaceae*	*Pseudomonas*	0.3	91.4
Pseudomonadaceae	*Pseudomonas*	0.5	93.3	*Moraxellaceae*	*Acinetobacter*	0.3	91.7

Low-fat with agavins (LFA)			
Enterobacteriaceae	*Unclassified*	33.2	33.2
Fusobacteriaceae	*Unclassified*	32.2	65.4
Unclassified	*Unclassified*	12.1	77.6
Enterobacteriaceae	*Plesiomonas*	7.8	85.4
Pseudomonadaceae	*Pseudomonas*	3.4	88.8
Aeromonadaceae	*Aeromonas*	2.6	91.4
Fusobacteriaceae	*Cetobacterium*	1.6	93.0
Burkholderiaceae	*Paraburkholderia*	0.6	93.7
Burkholderiaceae	*Unclassified*	0.5	94.2
Porphyromonadaceae	*GQ360021*	0.5	94.7

Suppl. Table 5. PERMANOVA in all groups at class level.

PERMANOVA	***p* values matrix**				
	LF	LFA	LFO	LFAO	S
LF		0.3172	0.8287	0.6676	0.0006
LFA	0.3172		0.7776	0.2607	0.2546
LFO	0.8287	0.7776		0.615	0.0432
LFAO	0.6676	0.2607	0.615		0.0003
S	0.0006	0.2546	0.0432	0.0003	

Suppl. Table 6. Two-way PERMANOVA (permutation 9999), test in all experimental samples at genus level.

Source	**Sum of sqrs.**	**df**	**Mean square**	**F**	***p***
Regime	0.49894	1	0.49894	1.5648	0.1159
Prebiotic	0.23595	1	0.23595	0.74	0.6636
Interaction	0.38351	1	0.38351	1.2028	0.165
Residual	10.203	32	0.31885		
Total	11.322	35			

Suppl. Table 7. One-way ANOSIM in experimental groups at genus level.

ANOSIM	***p* values matrix**			
	LF	LFA	LFO	LFAO
LF		0.298	0.8508	0.1273
LFA	0.298		0.3481	0.038
LFO	0.8508	0.3481		0.3686
LFAO	0.1273	0.038	0.3686	

Suppl. Table 8. SIMPER analysis between LFA and LFAO groups (genus level)

Taxon	**Av. dissim**	**Contrib. %**	**Cumulative %**
Enterobacteriaceae_Unclassified	27.49	34.96	34.96
Fusobacteriaceae_Unclassified	16.38	20.84	55.8
Unclassified_g	11.99	15.25	71.05
Plesiomonas	9.271	11.79	82.84
Cetobacterium	3.873	4.925	87.77
Paraburkholderia	2.633	3.348	91.11
Pseudomonas	2.149	2.733	93.85
Aeromonas	1.457	1.853	95.7
Burkholderiaceae_Unclassified	1.243	1.581	97.28

Suppl. Table 9. Percentage of sequence classified at different functional subsystem levels.

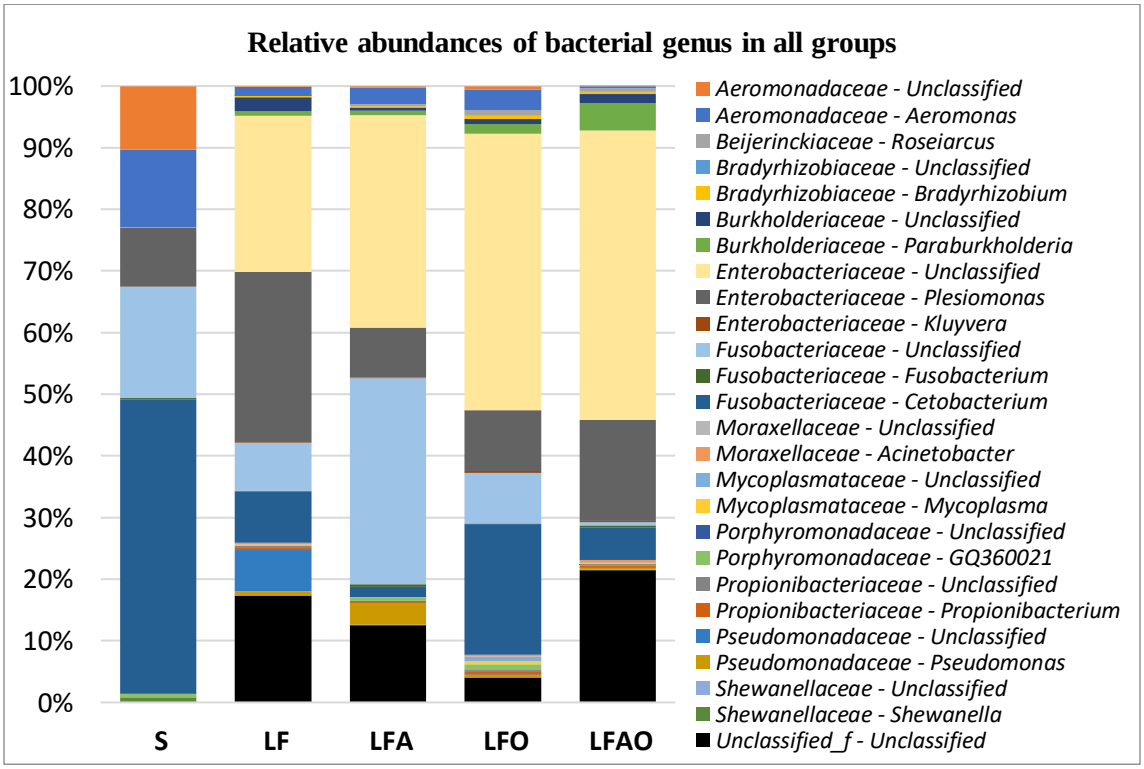
Subsystem level 1	%	% Acc.	Subsystem level 2	%	% Acc.	Subsystem level 3	%	% Acc.
Amino Acids and Derivatives	32.4	32.4	Lysine, threonine, methionine, and cysteine	24.3	24.3	Lysine fermentation	22.0	22.0
Cell Wall and Capsule	8.5	40.9	Gram-Negative cell wall components	7.6	31.8	Major Outer Membrane Proteins	6.0	28.0
Carbohydrates	7.2	48.1	Branched-chain amino acids	6.6	38.4	pyrimidine conversions	5.7	33.8
Nucleosides and Nucleotides	6.1	54.2	Pyrimidines	5.8	44.2	Hemin transport system	4.2	38.0
Respiration	5.8	60.0	Iron acquisition and metabolism	5.4	49.6	Respiratory Complex I	3.9	41.8
Iron acquisition and metabolism	5.5	65.5	Electron donating reactions	4.2	53.8	Cobalt-zinc-cadmium resistance	3.3	45.1
Miscellaneous	4.6	70.0	Central carbohydrate metabolism	3.9	57.7	Ton and Tol transport systems	3.3	48.4
Membrane Transport	4.2	74.2	Resistance to antibiotics and toxic compounds	3.8	61.5	TCA Cycle	3.2	51.6
Virulence	4.0	78.1	Membrane Transport	3.6	65.1	Metabolite repair	3.0	54.6
RNA Metabolism	3.3	81.5	Miscellaneous	3.1	68.2	RNA polymerase III initiation factors	2.2	56.8
Protein Metabolism	2.9	84.4	Transcription	2.5	70.7	HMG CoA Synthesis	1.9	58.7
DNA Metabolism	2.4	86.7	Protein biosynthesis	1.7	72.5	Leucine Degradation and HMG-CoA Metabolism	1.9	60.6
Regulation and Cell signaling	2.3	89.0	DNA repair	1.6	74.1	Core oligosaccharide biosynthesis	1.5	62.2
Cofactors, Vitamins, Prosthetic Groups, Pigments	2.3	91.3	Clustering-based subsystems	1.6	75.8	Isoleucine degradation	1.4	63.5
Clustering-based subsystems	2.3	93.6	Fermentation	1.5	77.3	Valine degradation	1.3	64.9
Phosphorus Metabolism	1.2	94.8	Plant-Prokaryote comparative genomics	1.4	78.7	Acetyl-CoA fermentation to Butyrate	1.3	66.2
Stress Response	0.9	95.7	Proteolytic pathway	1.3	80.0	Lysine fermentation MCB 432	1.3	67.5
Fatty Acids, Lipids, and Isoprenoids	0.9	96.7	Phosphorus Metabolism	1.2	81.2	Campylobacter Iron Metabolism	1.2	68.8
Potassium metabolism	0.9	97.6	Potassium metabolism	0.9	82.1	CBSS-316057.3.peg.563	1.1	69.9
Sulfur Metabolism	0.7	98.2	RNA processing and modification	0.8	82.9	Ribosome SSU eukaryotic and archaeal	1.1	71.0
Metabolism of Aromatic Compounds	0.4	98.7	Regulation and Cell signaling	0.8	83.7	Potassium homeostasis	0.9	71.9
Motility and Chemotaxis	0.3	99.0	Sugar alcohols	0.8	84.5	Phosphate metabolism	0.8	72.7
Others	1	100	Others	15.5	100	Others	27.3	100

Suppl. Table 10. Two-way PERMANOVA (permutation 9999, test applied at subsystem level 3 from functional matrix.

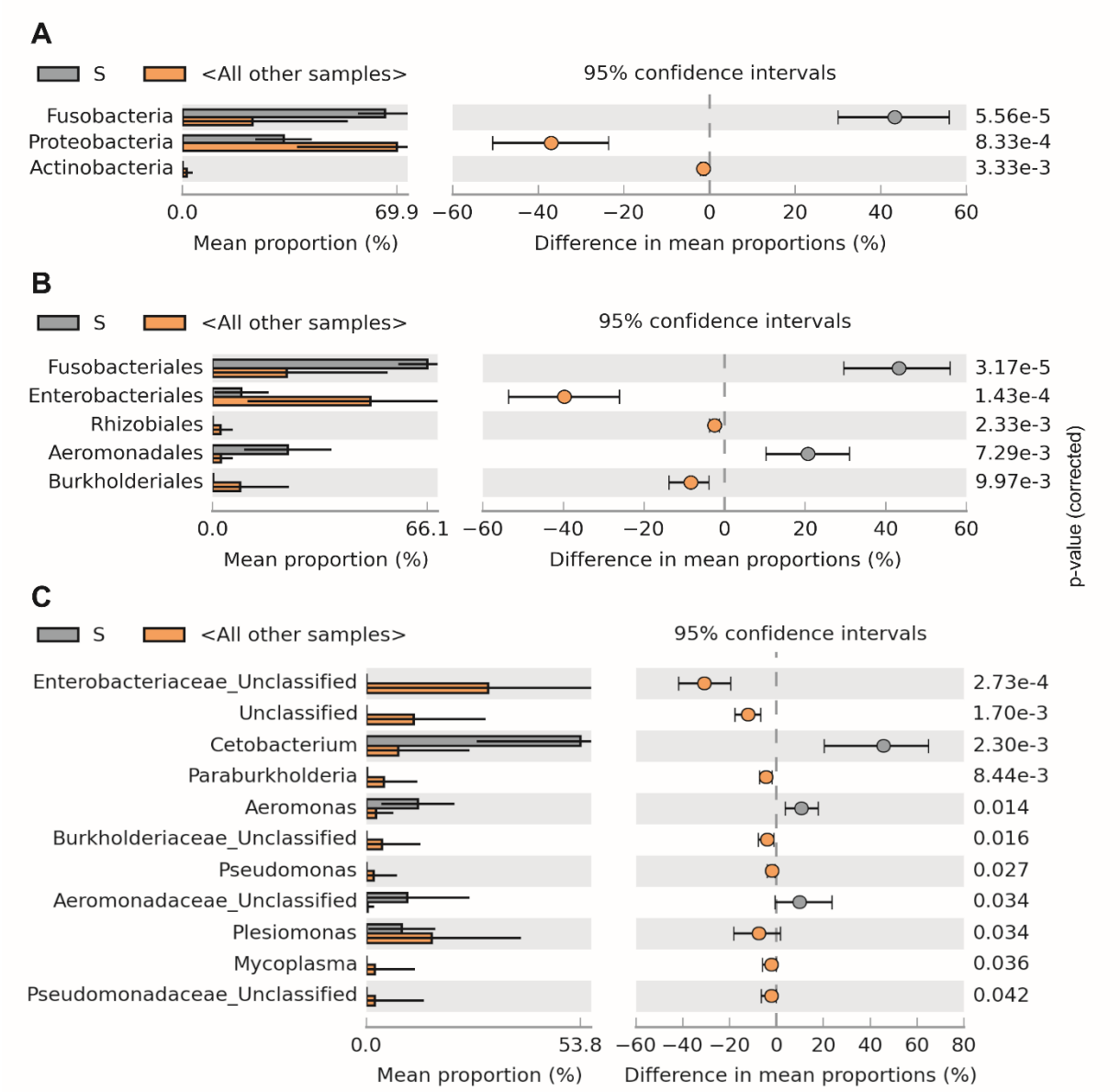
Source	Sum of sqrs.	df	Mean square	F	*p*
Fat	0.40257	1	0.40257	1.4887	0.0145
Regime	0.33966	1	0.33966	1.256	0.0927
Interaction	0.35924	1	0.35924	1.3284	0.0017
Residual	6.4901	24	0.27042		
Total	7.5916	27			

Suppl. Table 11. One-way ANOSIM, test applied in at subsystem level 3.

ANOSIM	***p* values matrix**			
	LFO	LFB	HFB	HFO
LFO		0.022	0.0608	0.0007
LFB	0.022		0.2321	0.1124
HFB	0.0608	0.2321		0.0388
HFO	0.0007	0.1124	0.0388	



Suppl. Figure 1. Zebrafish gut bacterial composition (Family-genera) at start (S), low-fat without and with agavin under basal regime (LF and LFA) and low-fat without and whit agavina under overfed regime (LFO and LFAO).



Suppl. Figure 2. Post hoc plots indicating the difference in mean proportions ($p < 0.05$) of taxa detected in S vs all treatments in gut zebrafish at class (A), order (B) and family-genus level (C).