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EFECTOS DE UN EXTRACTO DE ÁCIDOS HÚMICOS PROVENIENTE DE LOMBRICOMPOSTAS EN MODELOS *IN VITRO* E *IN VIVO* DE POLLOS DE ENGORDA

TESIS

QUE PARA OPTA POR EL GRADO DE MAESTRO EN CIENCIAS

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Dedicatorias

Quiero dedicar este trabajo a las personas más importantes en mi vida…

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RESUMEN

Este estudio se realizó para determinar el efecto de la suplementación dietética de ácidos húmicos (AH) en modelos *in vitro* e *in vivo*. En la primera parte de este estudio se evaluó la recuperación de *Salmonella Enteritidis* en un sistema digestivo *in vitro* e *in vivo*. En la prueba *in vitro* se probaron dos fuentes de AH (extracción comercial o natural) al 0.1% o al 0.2%. En la colonización intestinal, pollos de engorda machos de un día de edad fueron asignados aleatoriamente a uno de dos grupos (n = 25) con o sin 0.2% de AH aislados de lombricompostas, y se desafiaron a los 10 días de edad. La segunda parte de este estudio fue evaluar el efecto de los AH sobre la viscosidad intestinal, la permeabilidad intestinal y la excreción de amoníaco en un modelo de restricción de alimento (RA) de 24 horas para inducir permeabilidad intestinal. Pollos de engorda fueron asignados aleatoriamente a uno de dos grupos (n = 25 pollos) con o sin 0.2% de AH. La permeabilidad intestinal fue inducida a los 14 días de edad. Ambos grupos se les administró una dosis apropiada de dextrano de isotiocianato de fluoresceína (FITC-d) mediante sonda oral para determinar el grado de permeabilidad en el intestino. También se recogieron muestras de heces para evaluar la excreción de amoníaco, intestino e hígado para estimar la viscosidad y la translocación bacteriana (TB). Ninguna concentración de AH redujo los conteos de S. Enteritidis en ninguno de los compartimentos simulados (P >0.05). Se observó un aumento significativo (P <0.05) en la viscosidad intestinal del grupo experimental que consumió 0.2% de AH en comparación con el grupo de control no tratado. Además, el grupo tratado, mostró una reducción significativa en la filtración de FITC-d, TB al hígado y excreción de amoníaco en las heces en comparación con el grupo control no tratado. Estos resultados sugieren que los AH tiene un impacto positivo en la integridad intestinal en pollos de engorde.

Palabras clave: Pollos de engorda, Ácidos húmicos, Permeabilidad intestinal, *Salmonella* Enteritidis, Viscosidad intestinal.

ABSTRACT

This study was conducted to determine the effect of dietary supplementation of humic acids (HA) in an *in vitro* and *in vivo* model. In the first part of this study, the recovery of Salmonella Enteritidis in an *in vitro* and *in vivo* digestive system was evaluated. In the *in vitro* test, two sources of AH (commercial or natural extraction) were tested at 0.1% or 0.2%. In intestinal colonization, male one-day-old broiler chicks were randomly assigned to one of two groups (n = 25) with or without 0.2% of HA isolated from vermicomposts, and challenged at 10 days of age. The second part of this study was to evaluate the effect of HA on intestinal viscosity, intestinal permeability and ammonia excretion in a 24 hour food restriction (FR) model to induce intestinal permeability. Broilers were randomly assigned to one of two groups (n = 25 chickens) with or without 0.2% of HA. The intestinal permeability was induced at 14 days of age. Both groups were administered an appropriate dose of fluorescein isothiocyanate dextran (FITC-d) by oral gavage to determine the degree of permeability in the intestine. Also samples were also collected to evaluate the excretion of ammonia, intestine and liver to estimate viscosity and bacterial translocation (BT). No concentration of HA reduced the counts of S. Enteritidis in any of the simulated compartments ($p > 0.05$). A significant increase ($P < 0.05$) was observed in the intestinal viscosity of the experimental group that consumed 0.2% of HA compared to the control group. In addition, the treated group showed a significant reduction in the filtration of FITC-d, TB to the liver and excretion of ammonia in the feces compared to the untreated control group. These results suggest that HA has a positive impact on intestinal integrity in broiler chickens.

Keywords: Broilers, Humic acid, Intestinal permeability, *Salmonella* Enteritidis, Intestinal viscosity.

ÍNDICE

EFECTOS DE UN EXTRACTO DE ÁCIDOS HÚMICOS PROVENIENTE DE LOMBRICOMPOSTAS EN MODELOS *IN VITRO* E *IN VIVO* DE POLLOS DE ENGORDA

I. INTRODUCCIÓN.

La industria avícola ha sido el sector más dinámico dentro del mercado global de producción de carne destinada a la alimentación humana durante la última década, y ha mostrado el crecimiento más acelerado. Para el 2050, se proyecta que la población mundial va a incrementar en 50%; también se espera que la producción en el sector agropecuario se incremente el doble (FAO, 2016).

En México existe un déficit de proteína de origen animal por lo que no alcanza a cubrir la demanda de la población, aunque, de acuerdo con las Perspectivas Agrícolas OCDE - FAO 2016-2025, se proyecta que la producción de carne de ave crezca a una tasa promedio anual de 2.9 por ciento entre 2016 y 2025. Así, la producción y el consumo nacional continuarían con una tendencia ascendente (FIRA, 2016).

El incremento en la producción pecuaria se debe lograr con la mayor eficiencia en el uso de los recursos y sin comprometer la integridad ambiental ni la salud de los animales o personas. Siendo uno de los desafíos más importantes la prohibición del uso de antibióticos promotores del crecimiento (APC) como aditivos en los alimentos de los animales, debido a la preocupación por la resistencia de bacterias patógenas a los antibióticos, lo cual se convierte en un riesgo para la salud humana y animal. (Van, 2001). Por tal motivo, desde hace varias décadas se

empezaron a evaluar un sinnúmero de aditivos promotores del crecimiento no-farmacológicos, con el fin de sustituir o alternar el uso de los promotores tradicionales (Huyghebaert et al., 2011). Uno de los productos alternos que se ha evaluado desde hace algunos años como mejorador de la salud de los animales son los Ácidos Húmicos (AH).

Durante muchos años los AH han sido utilizados en la agricultura para suplementar el suelo, recientemente en ciencias ambientales y en la industria biomédica han retomado un gran interés debido a sus diferentes propiedades (Peña-Méndez et al., 2005; Aeschbacher et al., 2012). Los AH se han utilizado como un agente antidiarreico, analgésico, inmunoestimulador y antimicrobiano en las prácticas veterinarias en Europa (EMEA, 1999).

Varios estudios sugieren que los AH utilizados con prácticas adecuadas de nutrición, manejo y bioseguridad mejora la integridad intestinal y el rendimiento en aves de corral (Karaoglu et al., 2004; Ji et al., 2006; Ipek et al., 2008; Gomez-Rosales y Angeles, 2015,).

La mayoría de los AH probados en estos experimentos son productos comerciales derivados de elementos minerales (lignitos), donde la fuente húmica fue purificada. Una de las fuentes utilizadas por Gomez y Angeles (2015) procede de las lombricompostas, de donde se obtiene composta y lixiviados. Los resultados de éstas investigaciónes indican efectos benéficos sobre el rendimiento, digestibilidad ileal de la energía y una mayor retención de nutrientes de pollos de engorda,

ademas se observó beneficio en el rendimiento de la canal y la pechuga en los pollos suplementados (Maguey et al., 2016).

En el presente trabajo se propone evaluar un extracto de AH proveniente de una lombricomposta preparada con estiércol de animales, a través de un procedimiento alcalino (Stevenson, 1982; Baglieri et al., 2007).

Además, con la ayuda de modelos *in vitro* e *in vivo* desarrollados y estandarizados por el Poultry and Health Laboratory, AR, EUA, se procedió a desarrollar una serie de experimentos con la finalidad de evaluar los efectos de los AH extraídos/aislados de una lombricomposta sobre la colonización de *S.* Enteritidis, viscosidad y permeabilidad intestinal y la excreción de amonio en heces de pollos de engorda.

II.REVISIÓN DE LITERATURA.

II.I. Avicultura Global

La industria avícola ha sido el sector más dinámico dentro del mercado global de carnes durante la última década, y ha mostrado el crecimiento más acelerado en la demanda global de carnes. Para el 2050, se proyecta que la población mundial va a incrementar en 50%; también se espera que la producción en el sector agropecuario se incremente el doble (FAO, 2016).

El Departamento de Agricultura de los Estados Unidos (USDA, 2017) pronostica que la producción mundial crecerá un 1% en 2018, alcanzando 91.3 millones de tonelada de carne de pollo, principalmente por los Estados Unidos de América (EUA), Brasil, India y la Unión Europea (UE).

Para 2018 la expansión ascendente de la producción de pollo en EUA y Brasil se verá impulsada por el aumento de las exportaciones, mientras que el crecimiento de la UE e India se deberá al aumento lento pero constante debido a la demanda interna de cada país. Mientras tanto, se prevé que la producción de China disminuirá un 5%, estando limitado su crecimiento por la influenza aviar altamente patógena (H7N9), eventos que mermaron su producción en 2016 y 2017, con bajas del 8% y 6% respectivamente, también se verá afectada por la escasa disponibilidad de genética y por la poca demanda del mercado por sus productos cárnicos (USDA, 2017). EUA que actualmente no está castigada por la influenza aviar, cuenta con la mayor producción de carne de pollo a nivel mundial, misma

que es respaldada por el consumo local y el aumento de las exportaciones a México y otros mercados mundiales.

II.II. Avicultura Nacional

En México existe un déficit de proteína de origen animal por lo que no alcanza a cubrir la demanda de la población, aunque, de acuerdo con las Perspectivas Agrícolas OCDE-FAO, 2016-2025, se proyecta que la producción de carne de ave crecerá a una tasa promedio anual de 2.9 por ciento entre 2016 y 2025. Así, la producción y el consumo nacional continuarían con una tendencia ascendente (FIRA, 2016).

Por otra parte, se pronostica que el consumo per cápita de carne de pollo aumente a 36.7 kg en 2018, implicando un aumento en el consumo total de 3.27 millones de toneladas de carne de pollo al termino del año. En este entorno, se estima que las importaciones incrementen en 392 mil toneladas de carne de pollo, principalmente proveniente de EUA.

De acuerdo con el documento, Perspectivas de la agricultura y del desarrollo rural en las Américas: una mirada hacia América Latina y el Caribe 2017-2018, publicado por la CEPAL, FAO e IICA (2017), se posiciona a México como el segundo mayor productor de aves de corral (16%) de América Latina y el Caribe, con una expectativa de crecimiento del sector avícola en un 27% en los próximos 10 años. Sin embargo, el crecimiento continuo de la industria avícola deberá adecuarse a las preferencias de los consumidores y especialmente a las

emergencias sanitarias en las aves, además, se debe optar por tecnologías que le permitan mantener el intenso crecimiento del sector y proveer de proteína de buena calidad, minimizando el impacto ambiental (CEPAL, FAO e IICA, 2017).

II.III. Uso de antibióticos

En años recientes se ha detectado un aumento en la resistencia bacteriana a los antibióticos en la población humana y animal, aunado a la creciente preocupación de los consumidores por los residuos de medicamentos en la carne y productos de origen animal (Donaghue, 2003). Por lo tanto, en la unión europea el uso de antibióticos como agentes promotores de crecimiento en las aves de corral ha sido prohibido, y la presión de los consumidores probablemente conduzca a que sean retirados de otros países (Guban et al., 2006).

La inclusión de antibióticos en el alimento de aves de engorda se ha utilizado por varias décadas para modular la composición y la actividad de la microbiota intestinal, y para mejorar la salud y el rendimiento de los animales de producción (Argañaraz-Martínez et al., 2013). Esta práctica en la actualidad está prohibida en algunos países debido a la preocupación por la resistencia de bacterias patógenas, lo cual se convierte en un riesgo para la salud humana (Van, 2001). Por lo anterior, la Comisión Europea decidió prohibir el uso común de antibióticos en el alimento para consumo de animales, con la finalidad de disminuir la resistencia bacteriana en animales y en humanos (Huyghebaert et al., 2011).

Diferentes alternativas a los antibióticos se han propuesto como una medida para eliminar patógenos o para mejorar el crecimiento y la conversión alimenticia en las aves de corral, incluyendo probióticos, enzimas, bacteriófagos y péptidos antimicrobianos (Joerger, 2003), así como una base de compuestos a base de hierbas y ácidos orgánicos. Estas sustancias ejercen sus efectos influyendo sobre la biota gastrointestinal y los procesos de digestión, de forma directa o indirecta.

Dentro de los ácidos orgánicos, se incluyen los ácidos húmicos que tienen una gran contribución en la rentabilidad de la avicultura, además de proveer productos sanos y nutritivos a los consumidores (Griggs y Jacob, 2005; Ozturk et al., 2010).

II.IV. Ácidos Húmicos

II.IV.I. Origen

Las sustancias húmicas (SH) son macromoléculas orgánicas que juegan un papel importante en la bioquímica, son una fracción de la materia orgánica del suelo y representa la mayor densidad del suelo y compostas, se producen por la biodegradación de materia orgánica, que involucra procesos físicos, químicos y microbiológicos (Peña-Méndez et al., 2005), en el cual, los eucariotas (gusanos y hongos) y los procariotas (bacterias aeróbicas) descomponen aún más la materia orgánica (Gomez-Rosales y Angeles, 2015). Las SH contienen la mayoría de los nutrientes del suelo, constituyendo alrededor del 80% del carbón en suelos y el 60% del carbón disuelto en el medio acuático, siendo componente natural de arroyos, lagos y océanos (Lehmann and Kleber, 2015).

Debido a su solubilidad, las sustancias húmicas se pueden dividir en ácidos húmicos (AH), ácidos fúlvicos, y huminas. Sin embargo, los productos de descomposición de la materia orgánica asociados con otros minerales dificultan el aislamiento y la caracterización de los constituyentes orgánicos del suelo (Peña-Méndez et al., 2005).

Senn y Kingman (1973) definieron la forma molecular de los AH por primera vez, determinando que los sitios oxidados dan a la molécula una carga negativa que le permite ligarse a iones de minerales (Figura 1). Otras características interesantes de los AH son los altos rangos de peso y talla de sus moléculas, que van en un rango desde los cientos hasta varios miles de unidades de masa atómica, que consisten de unidades alquilo/aromáticas, unidades enlazadas por oxígeno y nitrógeno, grupos funcionales mayormente unidos a ácidos carboxílicos, fenoles e hidroxilos de alcohol, cetona y grupos quinona (Saar and Weber, 1979). Estas características químicas de los AH les confieren una función surfactante con la habilidad de unirse a diferentes compuestos, formado complejos químicos hidrofóbicos e hidrofílicos (Gaffney et al., 1996). Esta función en combinación con las propiedades coloidales, hacen a los AH agentes efectivos para transportar y ligar agentes orgánicos e inorgánicos en el ambiente (Piccolo, 2002).

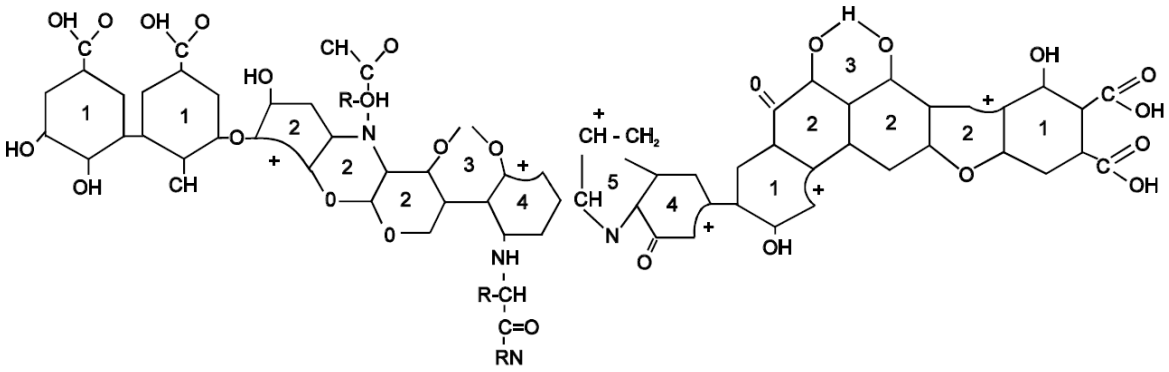

Figura 1. Estructura química de los Acidos húmicos (Senn and Kingman, 1973).

El principal interés de los AH es la capacidad de transferencia de electrones en reacciones de oxido-reducción (Aeschbacher, 2011), capacidad que les permite la transferencia de electrones cuando son reducidas y subsecuentemente re-oxidadas, teniendo una fuerte capacidad de intercambio de electrones (Nanny, 2007). Además la presencia de grupos carboxílicos y fenolatos confiere a los AH la habilidad para formar uniones con iones de Mg^{2+}, Ca^{2+}, Fe^{2+} y Fe^{3+}. Formando complejos de quelatos con uno o más de estos iones, siendo la formación de quelatos un importante aspecto en roles biológicos, regulando la biodisposición de iones metálicos (Stevenson, 1982).

II.IV.II. Uso animal

Durante siglos los AH han sido utilizados en la agricultura, recientemente en ciencias ambientales y en la industria biomédica han retomado un gran interés debido a sus diferentes propiedades antivirales, antioxidantes, inmunoestimulantes y antiinflamatorias (Peña-Méndez et al., 2005; Aeschbacher et al., 2012). Los AH se han utilizado como un agente antidiarreico, analgésico, inmunoestimulador y

antimicrobiano en las prácticas veterinarias en Europa (EMEA, 1999). Además, se ha demostrado que tienen una fuerte afinidad por ligarse a metales pesados, mutágenos, minerales, bacterias y aflatoxinas (Islam et al., 2005).

Estudios *in vitro* de propiedades antioxidantes de AH en dosis de 0.1% en mitocondrias de hígado de rata, órgano con la mayor función metabólica y responsable del metabolismo de compuestos farmacológicos, encontraron que los AH ayudan a mantener el equilibrio en las reacciones de óxido-reducción de las mitocondrias, además de auxiliar en la eliminación de los radicales libres y de los radicales superóxido (Vaskova et al., 2011). Demostrado que los AH a niveles profilácticos desempeñan un papel protector, además de limitar la formación de radicales libres potenciales de oxidación. Efecto también observado en ratas a las que se les extirpó 2/3 de hígado, resultando en una regeneración hepática con la aplicación de AH (Maslinski et al., 1993).

Yasar (2012), encontró una ganancia de peso significativa asociada con una mayor superficie epitelial, aumento en el largo de las vellosidades intestinales y profundidad en las criptas cuando se suministraron los AH en el agua de bebida de ratas.

II.IV.III. Uso en avicultura

Los efectos de los diferentes niveles de inclusión de los AH en la dieta sobre el peso vivo, consumo de alimento, características de la canal y características gastrointestinales en pollos de engorda han sido ampliamente estudiadas

(Kocabagli et al., 2002; Ceylan et al., 2003; Karaoglu et al., 2004; Rath et al., 2006; Oztruk et al., 2010), reportando que la adición de AH en el agua de bebida o en el alimento mejora la mayoría de los parámetros productivos, como la ganancia diaria de peso, además de conseguir un mayor rendimiento de la canal en pollos de engorda.

Jin et al. (1998) reportan que no se modifica la conversión alimenticia a los 21 días, pero mejora en el día 42 de edad, lo que sugiere que la mejora en la ganancia de peso y la eficacia de conversión alimenticia puede estar relacionada con el efecto promotor de los AH en los procesos metabólicos de la digestión y la utilización de nutrientes. Yoruk (2004) menciona un efecto lineal, mejorando los parámetros de producción, la reducción de mortalidad, la conversión alimenticia y el aumento en la producción de huevos, llegando a la conclusión de que la adición de AH pueden extender la rentabilidad de las aves ponedoras y de engorda.

El mecanismo por el cual los AH afectan al rendimiento de aves en corral es en gran parte desconocido. Shermer et al. (1996) tiene la hipótesis que los AH podrían influir en el rendimiento de las aves mediante la alteración de la microflora en el sistema gastrointestinal, especialmente en las poblaciones de *Escherichia coli* alterando el pH y la flora gastrointestinal para favorecer una mayor actividad de las enzimas intestinales y la digestibilidad de los nutrientes. Hayirli et al. (2005) mencionan que algunos elementos trazas de los AH pueden actuar como co-factores y aumentar la actividad de varias enzimas necesarias para la digestión y la utilización de nutrientes.

También se ha medido el efecto de los AH sobre el contenido de elementos traza en huevos de gallinas, existiendo una mayor absorción de Selenio (39.5%), Hierro (61.6%) y Cromo (15%), además de una mayor acumulación de Selenio en la yema y en menor medida en la albumina lo que sugiere una mejor biodisponibilidad de este mineral (Zbigniew et al., 2007). Shermer et al. (1996) refiere que los AH pueden aumentar la captación de nitrógeno, fósforo y otros nutrientes debido a sus propiedades quelantes, mejorando la biodisponibilidad de los nutrientes.

Debido a su capacidad adsorbente, los AH se han utilizado para reducir la micotoxicósis en las aves de corral (van Rensburg et al., 2006; Gahhri et al., 2010; Arafat et al., 2017); además, diversos estudios han demostrado que los AH reducen las emisiones de amoníaco al medio ambiente (Henderson, 2005; Ji et al., 2006; Zralý et al., 2008). Asimismo, los AH han mostrado tener un fuerte efecto anti-estrés en granjas de alta densidad, minimizando el efecto perjudicial del estrés crónico en gallinas de postura en producción (Cetin et al., 2011).

La mayoría de los AH probados en estos experimentos son productos comerciales derivados de elementos minerales (lignitos), donde la fuente húmica fue purificada. Una de las fuentes utilizadas por Gomez y Angeles (2015) procede de las lombricompostas, de donde se obtiene composta y lixiviados que escurren, resultantes de mantener el nivel óptimo de humedad; los resultados de ésta investigación indica efectos benéficos sobre el rendimiento, digestibilidad ileal de la energía y una mayor retención de nutrientes en pollos de engorda. En un

segundo trabajo, con la adición de lixiviado de humus de lombriz pasteurizado, para eliminar el posible efecto de los microorganismos presentes en el lixiviado, solamente se observó beneficio en el rendimiento de la canal y la pechuga en los pollos suplementados (Maguey, et al., 2016).

Siguiendo la misma línea de investigación, el desarrollo de una alternativa que sea más fácil de suplementar en el agua de bebida o en la dieta de las aves es necesario, reduciendo los nieles de inclusión y potencializando los efectos benéficos de los AH. Para esto, la extracción y aislamiento de AH de una lombricomposta es un proceso necesario.

II.IV.IV.Extracción/Aislamiento de ácidos húmicos.

Las sustancias húmicas en los suelos y sedimentos se pueden dividir en tres fracciones principales: ácidos húmicos, ácidos fúlvicos y huminas (Peña-Méndez et al., 2005; Lehmann y Kleber, 2015). Los ácidos húmicos y fúlvicos se extraen del suelo y otras fases sólidas por medio de una solución acuosa fuertemente básica de hidróxido de sodio o hidróxido de potasio. Los AH se precipitan en esta solución al ajustar el pH a 1 con acido clorhídrico o sulfúrico, dejando los ácidos fúlvicos en la solución (Stevenson, 1982; Baglieri et al., 2007). Ésta es la distinción principal, debida al pH, los ácidos fúlvicos son solubles en medios alcalinos, mientras que los ácidos húmicos son solubles en medios ácidos; en tanto que las huminas son insolubles a cualquier pH.

Se han propuesto varios procedimientos para la extracción de AH usando agentes quelantes, solventes orgánicos y soluciones salinas acuosas además de los solventes alcalinos (Stevenson, 1982). De estos, los solventes alcalinos siguen siendo los más eficientes y los más utilizados (Baglieri et al., 2007). La extracción con NaOH es utilizada como método estándar para el aislamiento de los AH, mostrando una eficacia de extracción superior al 80% de muestras provenientes de suelos (Tabla 1).

Cuadro 1. Agentes utilizados para la extracción de ácidos húmicos

Agente	Extracción de ácidos húmicos (%)
NaOH	80
$Na_4P_2O_7$	30
Quelantes Orgánicos	30
Acetil-Acetona / Hidroxiquilona	30
Ácido Fórmico	55

(Stevenson, 1982)

II.V. Modelo *in vitro*

El desarrollo de modelos *in vitro* es una herramienta muy valiosa, siendo útiles como primera instancia para la evaluación del comportamiento de diferentes compuestos en la suplementación animal, incluyendo a las aves comerciales. El modelo de digestión *in vitro* (Bedford & Classen, 1993; Zyla *et al.* 1995), simula la temperatura corporal de los pollos de engorda, los movimientos peristálticos, las condiciones enzimáticas y de pH de cada compartimiento simulado (buche, proventrículo e intestino delgado). Este modelo está estandarizado con ligeras

modificaciones (Annett et al., 2002; Latorre et al., 2015) por “El Laboratorio de Salud Avícola” perteneciente al “Centro de excelencia para la ciencia avícola” de la “Universidad de Arkansas”, y entre sus aplicaciones, se utiliza para evaluar la persistencia de *Salmonella* Enteritidis.

II.VI. Modelo *in vivo*

Recientemente, en el mismo laboratorio se ha desarrollado diversos modelos para inducir la inflamación intestinal en aves. Estos modelos incluyen dietas altas en polisacáridos no amiláceos (Tellez et al., 2014; 2015); dexametasona (Vicuña et al., 2015a); sulfato de sodio dextrano (DSS) (Kuttappan et al., 2015a; Menconi et al., 2015); y 24 h de restricción alimenticia (RA) (Kuttappan et al., 2015b; Vicuña et al., 2015b). Estos modelos, generan la inflamación del epitelio gastronintestinal, generando una disrupción de las uniones estrechas, las cuales ayudan a mantener la integridad del intestino, incrementan la translocación de bacterias y la filtración del Dextrano de Isotiocinato de fluoresceína (FITC-d, por sus siglas en ingles) a la circulación sanguínea. Ya que, el FITC-d es una molécula grande (3-5 kDa), el cual, bajo condiciones normales no es capaz de cruzar la barrera epitelial (Yan et al., 2009). Sin embargo, durante la inflamación intestinal las uniones estrechas se interrumpen permitiendo que las moléculas de FITC-d entren a la circulación, lo que hace que el FITC-d sea un biomarcador viable para medir la función de la barrera intestinal (Baxter et al., 2017).

III. OBJETIVOS.

Objetivo general

Evaluar el efecto de la inclusión de un extracto de ácidos húmicos procedente de lombricompostas en modelos *in vitro* e *in vivo* con dietas que no incluyen antibióticos promotores del crecimiento para conocer más sobre sus mecanismos de acción.

Objetivos particulares

Evaluar el efecto sobre la persistencia de *Salmonella* Enteritidis en un modelo *in vitro* que simula el sistema digestivo del ave con dietas que no incluyen antibióticos.

Evaluar el efecto sobre el peso, la ganancia de peso y la persistencia de *Salmonella* Enteritidis, bacterias acido lácticas totales y bacterias gram negativas totales en ciegos de pollos de engorda de 10 días de edad.

Evaluar el efecto en las concentraciones de IgA y viscosidad en el contenido intestinal, la permeabilidad intestinal mediante las concentraciones séricas del isotiocianato de fluoresceína dextrano (FITC-d), la translocación bacteriana al hígado y el contenido amoniacal en heces de pollos de engorda de 14 días bajo un modelo de restricción alimenticia.

IV. HIPÓTESIS.

La inclusión de un extracto de acidos húmicos provenientes de una lombricomposta reducirá la persistencia de *Salmonella* Enteritidis en dietas ausentes de antibióticos.

La inclusión de un extracto de acidos húmicos provenientes de una lombricomposta disminuirá los efectos adversos de 24 h de restricción alimenticia.

V. ARTÍCULOS CIENTÍFICOS.

V.I Artículo 1.

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Effects of humic acids on recovery of *Salmonella enterica* serovar Enteritidis

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Abstract

Two experiments were conducted to evaluate the effects of humic acids (HA) on recovery of *Salmonella* Enteritidis, in an *in vitro* digestive system and on intestinal colonization in neonate broiler chickens. Experiment 1, two runs using an *in vitro* digestion model with two sources of HA (commercial or natural extraction) at 0.1 or 0.2 %, and inoculated with 10^7 CFU/tube of *S*. Enteritidis, were carried out. In experiment 2, one-day old male broiler chickens were randomly allocate to one of two groups (n=25) with or without 0.2% of isolated HA from worm-composte, and challenged with 10^6 CFU of *S*. Enteritidis per chicken at 10-d old. All chicks were euthanized 24-h hours post challenge, and were for serum fluorescein isothiocyanate dextran (FITC-d) determination. A section of ileum was removed to obtain total concentration of IgA. Ceca-cecal tonsils were removed to evaluate *Salmonella* recovery, total lactic acid bacteria (LAB) and total Gram negative bacteria. In experiment 1, neither concentration of commercial or natural HA were able to reduce the recovery of *S*. Enteritidis in any of the simulated compartments (P>0.05). Only the crop compartment showed significant differences in pH in both trials between control and treated groups. In experiment 2, no significant differences were observed in serum concentration of FITC-d, intestinal IgA, *S*. Enteritidis recovery, LAB or total Gram negative bacteria in the ceca between control and treated chickens. In conclusion, no effects of HA on recovery of *Salmonella* Enteritidis, in an *in vitro* digestive system and on intestinal colonization of *Salmonella*, bacterial counts in ceca, intestinal IgA and serum FITC-d in neonate broiler chickens were observed. Further studies to evaluate the effect of HA under feed restriction model as an inducer of intestinal inflammation are currently being conducted.

Keywords: Broilers, Intestinal IgA, Intestinal permeability, Humic acid, *Salmonella* Enteritidis.

1. Introduction

Humic acids (HA) are principal component of humic substances in organic constituents of soil, compost and coal; however HA are also important organic component in streams, lakes, and oceans (Lehmann and Kleber, 2015). Humic acids are produced by biodegradation of organic matter, hence, HA are a complex mixture of many different acids containing carboxyl and phenolate groups (Pandey et al., 2000). Therefore, HA behaves as a di- or tribasic acid and can interact with ions forming humic colloids (Chen and Elimelech, 2007). For centuries, HA have been used as a soil supplement in agriculture, and in humans as nutritional supplement (Peña-Méndez et al., 2005). Due to their heavy metal-binding abilities HA have been used to remove heavy metals from wastewater (Vaughan and MacDonald, 1976). In poultry, several studies have indicating that HA also have adsorbent mycotoxin capacity (van Rensburg et al., 2006; Arafat et al., 2017). Other studies suggest that HA used with proper nutritional, management and biosecurity practices, improve intestinal integrity and performance in poultry (Karaoglu et al., 2004; Ji et al., 2006; Ipek et al., 2008; Gomez-Rosales and Angeles, 2015). Nevertheless, these benefits in animal performance are poorly understood. Humic acid has been used as an antidiarrheal, analgesic, immunostimulatory, and antimicrobial agent in veterinary practices in Europe (EMEA, 1999). In an old study, it was found that HA extracted from different soils and two synthetic HA showed antimicrobial activity against many human pathogenic bacteria such as *St. epidermidis*, *St. aureus*, *Str. pyogenes*, *S. typhimurium*, *Prot. vulgaris*, *Ent. cloacae*, *Ps. aeruginosa* and *C. albicans* (Ansorg

and Rochus, 1978). In a recent report, it was observed that peat and coal HA showed a complete growth inhibition against St. *aureus* and *Candida*, and a decrease in the number of colonies from 78-80% in *E. coli* and from 58-70% in *S. Enteritidis* (Yarkova, 2011). It has been also speculated that HA stabilize the intestinal flora, and thus, ensure an improved utilization of nutrients in animal feed (Islam et al., 2005). Hence, the purpose of this study was to evaluate the effects of HA on recovery of *Salmonella enterica* serovar Enteritidis in and *in vitro* digestive system and intestinal colonization in neonate broiler chickens.

2. Material and methods

2.1 Humic acid

Humic acid from Sigma-Aldrich (catalog no. 53680) was used only in the *in vitro* digestive system to compare its effect on the recovery of *S*. Enteritidis with a natural source of HA. The isolation and extraction of HA from worm compost was performed as decribed by Stevenson (1982). For the alkaline extraction process of HA, sodium hydroxide (0.1M NaOH) was used in a ratio of 5 parts of NaOH to one part of compound (g/mL), allowed to stand for 24 h at room temperature, filtering in a 125 µm mesh and adding 10% sulfuric acid (H_2SO_4), rectifying a pH of 2. The solids and liquids were separated by decantation. The solid fraction (HA) was washed 2 times with distilled water to remove sulfuric acid residues, and between each wash, it was centrifuged for 20 min at 5000 rpm. In a roto-evaporator the sample was desiccated at 60 °C until it had a gel consistency. Finally, it was dry it in an oven at 60 °C. The result was a yellow-brown powder with a pH of 7 to 8.

2.2 Bacterial strain and culture conditions

The organism used in all experiments was a poultry isolate of *Salmonella enterica* serovar Enteritidis, bacteriophage type 13A, obtained from the USDA National Veterinary Services Laboratory, Ames, IA. This isolate was resistant to 25 µg/mL of novobiocin (NO, catalog no.N-1628, Sigma) and was selected for resistance to 20 µg/mL of nalidixic acid (NA, catalog no.N-4382, Sigma) in our laboratory. For the present studies, 100 µL of *S*. Enteritidis from a frozen aliquot was added to 10 mL of tryptic soy broth (Catalog no. 22092, Sigma) and incubated at 37°C for 8 h, and passed three times every 8 h to ensure that all bacteria were in log phase. Post-incubation, bacterial cells were washed 3 times with sterile 0.9% saline by centrifugation at 1,864 × *g* for 10 min, reconstituted in saline, quantified by densitometry with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific), and diluted to an approximate concentration of 10^8 CFU per milliliter. Concentrations of *S*. Enteritidis were further verified by serial dilution and plating on brilliant green agar (BGA**,** Catalog no. 70134, Sigma) with NO and NA for enumeration of actual CFU used to challenge the chickens.

2.3 Experiment 1. *In vitro* digestion model

Experiment 1 consisted of two independent *in vitro trials.* In each trial, all the steps of the *in vitro* digestion model were performed by quintuplicate at 40^{o}C to simulate poultry body temperature according to previous publications with minor modifications (Annett et al. 2002; Latorre et al. 2015). In this experiment, two diets with two sources of HA, from commercial (Sigma) or natural extraction were tested at 0.1 % and 0.2 % inoculated with 10^7 CFU/tube of SE (Table 1). Briefly, for all the

gastrointestinal compartments simulated during the *in vitro* digestion model, a BOD incubator (Biochemical oxygen demand incubator, model 2020, VWR, Houston, TX, USA) customized with an orbital shaker (Standard orbital shaker, model 3500, VWR, Houston, TX, USA) was used for mixing the feed content in the experimental tubes at 19 rpm. Additionally, all tube samples were hold in a 30 degrees inclination position to facilitate proper blending of feed particles and the enzyme solutions incorporated throughout the assay. The first gastrointestinal compartment simulated was the crop, where 5 g of feed and 10 ml of 0.03 M hydrochloric acid (HCL, catalog no. HX0607-2, EMD Millipore corporation, Billerica, MA, USA) were placed in 50 mL polypropylene centrifuge tubes and mixed vigorously reaching a pH value around 5.20, next the tubes were incubated for 30 min. The second gastrointestinal compartment simulated was the proventriculus, where 3,000 U of pepsin per g of feed where used (catalog no. P700, Sigma-Aldrich, St Louis, MO, USA) and 2.5 ml of 1.5 M HCl were added to each of the tubes, reaching a pH between 1.4 to 2.00, then all tubes were incubated for 45 min. The third and final gastrointestinal compartment simulated was the intestinal section. In this case, 6.84 mg of 8 x pancreatin (catalog no. P7545, Sigma-Aldrich, St Louis, MO, USA) were used per g of feed and included in 6.5 ml of 1.0 M sodium bicarbonate ($NaHCO_3$, catalog no. S6014, Sigma-Aldrich, St Louis, MO, USA), the pH ranged between 6.4 and 6.8, and all tube samples were incubated for 2 h. The complete *in vitro* digestion process took 3 h and 15 min. After the incubation time in each compartment, a sample was collected to enumerate *S*. Enteritidis.

2.4 Experiment 2. Animal source, diets, and experimental design

One-day old male Cobb-Vantress broiler chickens (Fayetteville, AR, USA) were neck-tagged, weighted and randomly allocate to one of two groups (*n* = 25 chickens), with or without 0.2% of isolated HA from worm-composte, and placed in heated brooder batteries with a controlled age-appropriate environment. Chicks had *ad libitum* access to water and feed for 10 days. The experimental diet was formulated to approximate the nutritional requirements of broiler chickens as recommended by the National Research Council (1994), and adjusted to breeder's recommendations (Cobb-Vantress Inc. 2015). No antibiotics were added to the diet (Table 1). All animal handling procedures complied with Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville. Specifically, the IACUC approved this study under the protocol #15006. Chickens were orally gavaged with 10^6 CFU of *S*. Enteritidis alive per chicken at 10-d old. Twenty-four h post challenge, all chickens were euthanized and bled (femoral vain) to obtain serum for FITC-d determination. A section of the middle ileum was removed to obtain total concentration of IgA. Ceca-cecal tonsils were removed to evaluate *Salmonella* recovery as describe below. A small number of chicks (*n* = 10) were humanely killed upon arrival with CO_2 asphyxiation. Ceca-cecal tonsils, liver and spleen were aseptically cultured in tetrathionate enrichment broth (Catalog no. 210420, Becton Dickinson, Sparks, MD). Enriched samples were confirmed negative for *Salmonella* by streak plating the samples on Xylose Lysine Tergitol-4 (XLT-4, Catalog no. 223410, BD Difco™) selective media.

2.5 *Salmonella* recovery

Ceca-cecal tonsils (CCT) were homogenized and diluted with saline (1:4 by wt/vol) and ten-fold dilutions were plated on BGA with NO and NA, incubated at 37°C for 24 h to enumerate total *S*. Enteritidis colony forming units. However, in both trials of experiment 1, following plating to enumerate total SE, the CCT samples were enriched in double strength tetrathionate enrichment broth and further incubated at 37°C for 24 h to enrich. Following this, enrichment samples were plated on BGA with NO and NA and incubated at 37°C for 24 h to confirm presence/absence of typical lactose-negative colonies of *Salmonella*.

2.6 Enumeration of bacteria

Whole duodenum, ileum, and both ceca were aseptically removed and separated into sterile bags and homogenized. Samples were weighed and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Ten-fold dilutions of each sample, from each group were made in a sterile 96 well Bacti flat bottom plate and the diluted samples were plated on two different plates of medium to evaluate total number of lactic acid bacteria (LAB) in Man Rogosa Sharpe (MRS Agar VWR Cat. No. 90004-084 Suwanee, GA 30024) or total Gram-negative bacteria in MacConkey Agar (VWR Cat. No. 89429–342 Suwanee, GA 30024).

2.7 Serum determination of FITC-d leakage

Intestinal leakage of fluorescein isothiocyanate dextran (FITC-d) (MW 3-5 KDa; Sigma-Aldrich Co., St. Louis, MO) and the measurement of its serum concentration as a marker of paracellular transport and mucosal barrier dysfunction (Yan et al. 2009; Baxter et al. 2017). At 24 h, post *S*. Enteritidis challenge, chickens in all

groups were given an oral gavage dose of FITC-d (8.32 mg/kg). Following 1 h, they were euthanized and blood samples were collected from the femoral vein kept at room temperature for 3 h and centrifuged (500 x *g* for 15 min) to separate the serum from the red blood cells. FITC-d levels of diluted serum samples (1:5 PBS) were measured at excitation wavelength of 485 nm, gain 40 and emission wavelength of 528 nm with a Synergy HT, Multi-mode microplate fluorescence reader (BioTek Instruments, Inc., Vermont, USA). Fluorescence measured was then compared to a standard curve with known FITC-d concentrations. Gut leakage for each bird was reported as ng of FITC-d/mL of serum (Baxter et al. 2017).

2.8 Enzyme-linked immunosorbent assay for total IgA levels

An indirect ELISA was performed to quantify IgA as described previously (Merino-Guzmán et al. 2017). The commercial chicken IgA ELISA quantitation set (Cat. E30-103, Bethyl Laboratories Inc. Montgomery, TX 77356) was used according with the manufacturer's instructions. In brief, 96-well plates (Cat. 439454, Nunc MaxiSorp, Thermo Fisher Scientific, Rochester, NY) were coated with 1 µg/100 µL of goat polyclonal anti-chicken IgA diluted in 0.05 M Carbonate-Bicarbonate, pH 9.6. The plates were covered with a lid and allowed to incubate overnight at 4°C. Then the contents of the plates were emptied, tapped on a dry paper towel, and rinsed 5 times with washing solution (50 mM/L Tris, 0.14 M/L NaCl, 0.05% Tween 20, pH 8.0), 350 µL/well. Individual wells were then blocked (125 µL/well) with 20% Superblock (Pierce Inc., Rockford, IL) in PBS for 60 min at room temperature. The plates were again emptied, tap dried and stored desiccated without further washing

step. Samples were thawed to room temperature and diluted in sample/conjugate diluent (50 mM/L Tris, 0.14 M/L NaCl, 1% Bovine serum albumin, 0.05% Tween 20) and 100 µL were added to the respective wells. A standard curve was used in order to quantify the total IgA in the samples, chicken reference IgA serum from the quantitation kit was serially diluted in sample/conjugate diluent to get concentrations of 1,000, 500, 250, 125, 62.5, 31.25 and 15.625 ng/mL, sample/conjugate; diluent alone was used as the zero standard (blank). Standard dilutions were added to the respective wells, 100 µL. Plates were then incubated for 1 h at room temperature and rinsed 5 times with washing solution. Goat anti chicken IgA-HRP conjugated detection antibody from the IgA quantitation set was diluted 1:40,000 in sample/conjugate diluent and 100 µl were transferred to each well. The plate was incubated 60 min at room temperature. After incubation, horseradish peroxidase (HRP) detection antibody was removed and the plate washed again five times as previously described. After washing, 100 µL of tetramethylbenzidine substrate (Cat. TMBS-1000-01, TMB Super Sensitive one component HRP microwell substrate, SurModics IVD, Eden Prairie, MN 55344 USA) was added to each well and incubated for 15 min at room temperature, protected from light. The reaction was stopped with 100 µL of 3-7% maleic acid solution (Cat. LSTP-1000-01, BioFx® 450 nm liquid stop solution for TMB microwell substrates, SurModics IVD, Eden Prairie, MN 55344 USA), and absorbance was measured at 450nm using an ELISA plate reader (Synergy HT, multi-mode microplate reader, BioTek Instruments, Inc., Winooski, VT, USA). The 450 nm absorbance value minus the blank value for each standard concentration

was plotted on the vertical (Y) axis versus the corresponding chicken IgA concentration on the horizontal (X) axis using the Gen5^{TM} software (BioTek Instruments, Inc., Winooski, VT, USA). Chicken IgA concentration obtained were multiplied by the dilution factor to determine the amount of chicken IgA in the undiluted samples. Optimum dilution for total IgA quantitation in different samples was: lachrymal fluid 1:2 000, tracheal swab 1:10, saliva 1:20, cloacal swab 1:10, gut rinse 1:100.

2.9 Data and statistical analysis

Log_{10} CFU/g of *S*. Enteritidis, pH, body weight (BW), body weight gain (BWG), total intestinal IgA and serum FITC-d concentration were subjected to analysis of variance as a completely randomized design, using the General Linear Models procedure of SAS (SAS Institute 2002). Significant differences among the means were determined by Duncan's multiple-range test at $p < 0.05$. The enrichment data were expressed as positive/total chickens (%), and the percent recovery of SE was compared using the Chi-Squared test of independence, testing all possible combinations to determine the significance ($p < 0.05$).

3. Results

Table 2 summarizes the results of the effect of HA on recovery of SE during *in vitro* digestion, under variable biochemical conditions simulating different sections of the gastrointestinal tract of poultry in two independent trials of experiment 1. In both trials, neither concentration of 0.1 % or 0.2 % of natural HA or HA from Sigma were able to reduce the recovery of *S*. Enteritidis in any of the simulated compartments of the *in vitro* digestive model ($p > 0.05$). Similarly, no significant differences in pH

were observed in proventriculus or intestine in both trials between control and treated groups. However, a significant reduction on pH in the crop compartment was observed for both concentrations of commercial or natural HA when compared with the control non-treated group. Interestingly, with the supplementation of both HA and both concentrations evaluated, a tendency (p = 0.07) to increase the numbers of *S*. Enteritidis was observed in the intestine compartment, and this increase was associated with a similar tendency (p = 0.07) of higher pH when compared with control non-treated group (Table 2).

Table 3 shows the results of the evaluation of BW and BWG in chickens consuming a corn-based diet with or without inclusion of 0. 2% of Natural HA of experiment 2. No significant differences were observed in BW or BWG between control and treated chickens at day 10 of age (Table 3).

The results of the evaluation of intestinal IgA, serum FITC-d and bacterial counts in ceca of 10-day old broiler chickens treated with or without 0.2 % natural humic acid and challenged with *S.* Enteritidis of experiment 2 are summarized in Table 4. No significant differences in any of the variables were observed between control or treated chickens (Table 4).

4. Discussion

The biodegradation product of organic matter, the compost, has been used in agriculture for centuries, as fertilizer and soil amendment (Lehmann and Kleber, 2015). Modern composting includes several meticulous steps that include the addition of water, air, and carbon- and nitrogen-rich materials to the organic matter to be composted (Peña-Méndez et al., 2005). In the process, eukaryotes (worms

and fungi) as well as prokaryotes (aerobic bacteria) further break up the organic matter (Gomez-Rosales and Angeles, 2015). Some of the end chemical products are carbon dioxide and ammonium, which is an important source of nitrogen for plants. Other important nutrients such as humus or HA are also produced during composting of organic matter. In soil science, humus (from the Latin *humus*: earth) is a fraction of soil organic matter and represents the majority density of soil and contributes to moisture and nutrient retention. Hence, humus contains most of the nutrients of the soil. However, decomposition products of organic matter associated with other minerals makes difficult to isolate and characterize soil organic constituents (Peña-Méndez et al., 2005). Back in the 18th century, chemists used alkaline extractions to isolate some organic constituents present in soil and the substances isolated were identified as 'humic acid', 'fulvic acid', and 'humin' (Peña-Méndez et al., 2005; Lehmann and Kleber, 2015). Interestingly, in spite of the scientific and accurate evidence of 'humification', the concept persists in the current literature and textbooks. Nonetheless, humic substances are still in the main are of interest of soil scientists, as well as researchers in other areas of basic and applied science (Islam et al., 2005; Klocking and Helbig, 2005; Peña-Méndez et al., 2005). Several procedures have been proposed for the extraction of HA using alkaline solvents, chelating agents, organic solvents and aqueous saline solutions (Stevenson, 1982; Baglieri et al., 2007). From these, alkaline solvents remain the most efficient and most widely used (Baglieri et al., 2007).

Humic acid has been used as an antidiarrheal, analgesic, immunostimulatory, and antimicrobial agent in veterinary practices in Europe (EMEA, 1999). Furthermore,

due to their adsorbent capacity, HA have been used to reduce mycotoxicosis (van Rensburg et al., 2006; Ghahri et al., 2010; Arafat et al., 2017) and to reduce ammonia emissions from pig manure (Ji et al., 2006; Písaříková et al., 2010). In poultry, several investigators have reported that HA improves performance, gut morphology, carcass traits and meat quality and reduces social stress (Karaoglu et al., 2004; Cetin et al., 2011; Gomez-Rosales and Angeles, 2015). As far as we are aware, this is the first study trying to evaluate the effect of HA extracted from a worm-compost on *S*. Enteritidis recovery *in vitro* or *in vivo*. In the present study, the supplementation of 0.1 % or 0.2 % of a commercial HA product from Sigma-Aldrich or a natural HA product extracted from worm-compost in an *in vitro* digestive model, did not affect the numbers of *S*. Enteritidis recovered in the three compartments evaluated. Interestingly, a numerical tendency to increase the recovery of *S*. Enteritidis was observed in those groups supplemented with commercial or natural HA at both concentrations when compared with control-non treated group, and this increase was associated with a numerical increase in pH in the intestine compartment. These findings were confirmed when chickens received 0.2 % of HA in the diet for 10 days prior to *S*. Enteritidis administration, HA had no effect on *S*. Enteritidis recovery from CCT 24 h after *S*. Enteritidis challenge. These results do not agree with previous reports based on *in vitro* and *in vivo* assays. In the study of Ansorg and Rochus (1978) it was found that HA extracted from different soils and two synthetic HA showed antimicrobial activity against many human pathogenic bacteria such as St. epidermidis, St. aureus, Str. pyogenes, S. typhimurium, Prot. vulgaris, Ent. cloacae, Ps. aeruginosa and C. albicans. Yarkova

(2011) observed that peat and coal HA showed complete growth inhibition of St. aureus and Candida, and a decrease in the number of colonies from 78-80% in *E. coli* and from 58-70% in S. Enteritidis. Aksu and Bozkurt (2009) fed broiler chickens diets supplemented with a commercial source of HA (Farmagulator Dry-Humic Acid^{TM}) and found that the CFU of *E. coli* in the digesta of birds fed either a diet with antibiotic and diets with HA were significantly lower than in those given the control. Opposite to this, in the report of Jansen van Rensburg and Naude (2009), the coliforms and *E. coli* counts in the caecum were not affected by the addition of potassium humate in the drinking water of broilers; whereas Shermar et al. (1998) reported an increase between 10 to 100 times in the *E. coli* populations from birds receiving a commercial mined humate (Menefee Humate^{TM}) compared to the control birds.

It has been also speculated that HA stabilize the intestinal flora, and thus, ensure an improved utilization of nutrients in animal feed (Islam et al., 2005). The results of the present study do not agree with the above suggestion because the total counts of LAB or total Gram negative bacteria isolated from the ceca (Experiment 2) were not different whether the diet was supplemented or not supplemented with HA, indicating that HA does not impact the numbers of gut microbiota. Our results are in agreement with the findings of Jansen van Rensburg and Naude (2009), in which, the aerobic mesophiles, lactococci and lactobacilli counts in the caecum of broilers were not affected by the addition of potassium humate in the drinking water; but are opposite to the increased CFU of Lactobacilli reported in broilers supplemented with HA (Aksu and Bozkurt, 2009).

The supplementation of 0.2 % of natural HA did not affect the concentration of total intestinal IgA (Table 4), which do not support the suggestion that HA has immunostimulant effects (EMEA, 1999; Klocking and Helbig, 2005). A positive effect on the antibody titres against Newcastle Disease Virus have been reported in broilers supplemented with HA (Aksu and Bozkurt, 2009; Kamel et al., 2015). In another study with broilers fed a commercial source of HA (Farmagulator) an increase in the antibody production against SRBC was reported (El-Husain et al., 2008). In addition, Jansen van Rensburg and Naude (2009), reported that the addition of potassium humate in the drinking water of broilers significantly inhibited the release of TNF-α, IL-1β, IL-6 and IL-10 by phytohaemaglutinin A stimulated mononuclear lymphocyte.

One of the proposed mechanisms of action of HA its related to the ability to create protective layers over the epithelial mucosal membrane of the digestive tract against the penetrations of toxic and other bacterial contaminated substances (Rath et al., 2002; Taklimi et al., 2012); however, the results of our study do not support this suggestion because the supplementation of HA did not affect the concentration of serum FITC-d in chicks used in Experiment 2 (Table 4). FITC-d is a large molecule (3-5 kDa), which does not usually leak through the intact gastrointestinal tract barrier. However, when there are conditions which disrupt the tight junctions between epithelial cells, the molecule can enter circulation, demonstrated by an increase in trans-mucosal permeability associated with chemically induced disruption of tight junctions by elevated serum levels of FITC-d after oral administration (Yan et al., 2009).

The lack of effects of HA on *Salmonella* recovery in the *in vitro* assay and on intestinal colonization, bacterial counts in ceca, intestinal IgA and serum FITC-d in broilers may have been due to differences in the dosage and chemical composition of HA (Ansorg and Rochus, 1978; Yarkova, 2011). In the present study, the HA dosages were chosen from the reports of Kocabagli et al. (2002) and Yoruk et al. (2004). Regarding the differences in chemical composition, it has been pointed out that HA with different characteristics such as chain length, side chain composition and origin (plant, soil, peat, and coal derived) can be found in commercial or purified sources of HA (Gomez-Rosales and Angeles, 2015).

In some of the studies reviewed above, different sources of HA were tested, such as HA extracted from different soils, peat and coal, a source of potassium humate, as well as commercial and synthetic HA; furthermore, different dosages in the *in vitro* and *in vivo* tests were used, which could have been caused the differences in the response variables compared to our study, in that an extracted HA source from a worm-compost was tested. It has also been suggested that responses to alternatives to antibiotic growth promoters may be greater in a more challenging environment (Ozturk et al., 2010, 2012) and that feed additives such as HA are not effective if there are no stress factors. Elucidation of the effects of HA under stress models as inducers of intestinal inflammation deserves further clarification.

It can be concluded that, not effects of HA on recovery of *Salmonella* Enteritidis, in an *in vitro* digestive system and on intestinal colonization of *Salmonella*, bacterial counts in ceca, intestinal IgA and serum FITC-d in neonate broiler chickens were observed. Further studies to evaluate the effect of HA under feed restriction model

as an inducer of intestinal inflammation are currently being conducted.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville. Specifically, the IACUC approved this study under the protocol #15006.

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Table 1. Ingredients (%). Diet based on corn

Item	Diet based on corn	Diet based on corn
Ingredients		
Corn	54.64	54.64
Soybean meal	36.94	36.94
HA Sigma-Aldrich	0.1% / 0.2%	
HA Worm composte		0.1%/0.2%
Vegetable oil	3.32	3.32
Dicalcium phosphate	1.58	1.58
Calcium carbonate	1.44	1.44
Salt	0.35	0.35
DL-Methionine	0.25	0.25
Vitamin premix[a]	0.30	0.30
L-Lysine HCl	0.10	0.10
Choline chloride 60%	0.10	0.10
Mineral premix[b]	0.30	0.30
Antioxidant[c]	0.15	0.15
Total	100.00	100.00

[a] Vitamin premix supplied the following per kg: vitamin A, 20,000,000 IU; vitamin D3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g (Nutra Blend LLC, Neosho, MO 64850).

[b] Mineral premix supplied the following per kg: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10–15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g (Nutra Blend LLC, Neosho, MO 64850).

[c] Ethoxyquin.

Table 2. Effect of humic acid on recovery of *Salmonella* Enteritidis[*] during *in vitro* digestion, under variable biochemical conditions simulating different sections of the gastrointestinal tract of poultry. Experiment 1**

Treatment	Crop		Proventriculus		Intestine	
	S. Enteritidis	pH	*S.* Enteritidis	pH	*S.* Enteritidis	pH
Trial 1						
Control	7.05 ± 0.05	5.13 ± 0.01 [d]	3.50 ± 0.73	2.71 ± 0.10	7.37 ± 0.19	7.37 ± 0.19
Humic acid Natural (0.1%)	6.84 ± 0.18	5.25 ± 0.008 [b]	2.90 ± 0.60	2.21 ± 0.07	7.51 ± 0.11	7.51 ± 0.11
Humic acid Sigma (0.1%)	6.94 ± 0.05	5.20 ± 0.004 [c]	3.10 ± 0.80	2.33 ± 0.03	7.61 ± 0.07	7.61 ± 0.07
Humic acid Natural (0.2%)	7.05 ± 0.07	5.39 ± 0.008 [a]	4.76 ± 0.66	2.34 ± 0.04	6.96 ± 0.44	6.96 ± 0.44
Humic acid Sigma (0.2%)	6.93 ± 0.09	5.21 ± 0.001 [c]	3.10 ± 0.80	2.27 ± 0.07	7.60 ± 0.20	7.60 ± 0.20
Trial 2						
Control	7.47 ± 0.09	5.39 ± 0.01 [c]	2.30 ± 0.70	2.29 ± 0.05	7.28 ± 0.48	6.77 ± 0.01
Humic acid Natural (0.1%)	7.62 ± 0.01	5.51 ± 0.01 [b]	3.02 ± 0.72	2.42 ± 0.07	7.36 ± 0.35	6.73 ± 0.007
Humic acid Sigma (0.1%)	7.63 ± 0.01	5.40 ± 0.01 [c]	3.55 ± 0.77	2.43 ± 0.04	7.94 ± 0.11	6.72 ± 0.01
Humic acid Natural (0.2%)	7.53 ± 0.09	5.56 ± 0.01 [a]	2.90 ± 0.60	2.49 ± 0.07	7.60 ± 0.19	6.69 ± 0.01
Humic acid Sigma (0.2%)	7.54 ± 0.06	5.43 ± 0.01 [c]	2.99 ± 0.69	2.49 ± 0.06	7.88 ± 0.01	6.48 ± 0.17

[*] The initial inoculum of *S*. Enteritidis in the feed was 10^7 CFU/g.

[**] Data are expressed as $\log_{10}$ CFU mean ± SE.

[a- d] Values within columns with different superscripts in lower case letters differ significantly (p <0.05).

Table 3. Evaluation of body weight (BW) and body weight gain (BWG) in chickens consuming a corn-based diet with or without inclusion of 0.2% of natural humic acid. Experiment 2[a]

Item	BW 0-d (g/broiler)	BW 10-d (g/broiler)	BWG 0 to 10-d (g/broiler)
Control	44.28 ± 0.67	228.4 ± 7.85	183.76 ± 7.5
Natural humic acid (0.2 %)	45.54 ± 0.69	230.38 ± 6.25	184.83 ± 6.14

[a] Data are expressed as mean ± SE. n = 25/group, $p > 0.05$

Table 4. Evaluation of intestinal IgA, serum FITC-d and bacterial counts in ceca of 10-day old broiler chickens treated with or without 0.2 % natural humic acid and challenged with *Salmonella* Enteritidis[a]. Experiment 2[b]

Item	Intestinal IgA ng/mL	Serum FITC-d ng/mL	Ceca Log_{10} /g			
			S. Enteritidis		Total Gram (-)	Total LAB
			Log_{10} SE/g of ceca content	Enrichment culture[c]		
Control *SE*	1666.09 ± 58.18	718.6 ± 61.43	2.02 ± 0.35	9/12 (75%)	7.81 ± 0.09	7.72 ± 0.09
Humic acid *SE*	1547.09 ± 73.39	718.6 ± 47.84	2.47 ± 0.22	11/12 (92%)	7.40 ± 0.21	7.98 ± 0.20

[a]Chickens were orally gavaged with 10^6 CFU of *S.* Enteritidis per chicken at 10-d old, samples were collected 24 h later.

[b]Data expressed in Log_{10} CFU/g of tissue. Mean ± SE from 12 chickens, $p > 0.05$.

[c]Data expressed as positive/total chickens (%).

S. Enteritidis: Total *Salmonella* Enteritidis recovered; Total Gram: Total Gram negative bacteria recovered; Total LAB: Total lactic acid bacteria recovered.

V.II. Artículo 2.

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Effect of humic acids on intestinal viscosity, leaky gut and ammonia excretion in a 24 hours feed restriction model to induce intestinal permeability in broiler chickens

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Abstract

The purpose of this study was to evaluate the effect of HA on intestinal viscosity, leaky gut, and ammonia excretion in a 24 h feed restriction (FR) model to induce intestinal permeability in chickens. One-day old male Cobb-Vantress broilers were randomly allocated to one of two groups (*n* = 25 chickens), with or without 0.2% of isolated HA from worm-compost, and placed in brooder batteries. Chicks had *ad libitum* access to water and feed for 14 days. Intestinal permeability was induced by 24 h FR starting at 14 d. At 15 d of age, chickens in both groups were given an appropriate dose of fluorescein isothiocyanate dextran (FITC-d) by oral gavage. Intestine and liver samples were also collected to evaluate viscosity and bacterial translocation (BT) respectively. An increase ($P<0.05$) in intestinal viscosity was observed in the experimental group consuming 0.2% of HA and confirmed in with a published *in vitro* digestion model that simulates the chemical and physical conditions of the crop, proventriculus and intestine of chickens. Furthermore, the treated group also showed a significant reduction in FITC-d, liver BT and ammonia in the manure. These results suggest that HA have a positive impact in intestinal integrity in chickens.

Keywords: Humic acids, chicken, Intestinal viscosity, intestinal permeability, ammonia.

1. Introduction

Humic acids (HA) are principal components of humic substances in organic constituents of soil, compost, coal and are also a primary organic component of streams, lakes, and oceans (Lehmann & Kleber, 2015). Humic acids are produced by biodegradation of organic matter that involves physical, chemical and microbiological processes, hence, HA are a complex mixture of many different acids containing carboxyl and phenolate groups (Pandey et al., 2000). The relevance of HA is that they constitute about 80% of the carbon on the ground, and 60% of the carbon dissolved in aquatic media. Due to their solubility, they are divided in HA, fulvic acids and humin and they have been used for centuries as a soil supplement in agriculture. More recently the environmental and biomedical industries have had a growing interest in HA due to its antiviral, anti-oxidant, immune stimulant and anti-inflammatory properties (Peña-Méndez et al., 2005; Aeschbacher et al., 2012). Furthermore, HA extracted from compost as a washing agent for removal Cu, Cd, Zn, Pb and Ni from soil, indicate that HA are suitable for remediating soil contaminated with multiple heavy metals in extremely high concentrations. (Kulikowska et al., 2015).

In poultry, several studies indicate that HA have the ability to adsorb mycotoxins and improve performance (Ji et al., 2006; van Rensburg et al., 2006; Gomez-Rosales & Angeles, 2015; Arafat et al., 2017). In addition, several studies have shown that HA reduces emissions of ammonia from the environment (Henderson, 2005; Ji et al., 2006; Zralý et al., 2008). Ammonia has been recognized as one of

the most stressful gases found in commercial chicken houses (Moore et al., 2011). On the other hand, HA has been reported to stabilize the intestinal microbiota and improve nutrient digestibility in animals (Islam et al., 2005; Maysa & El-Sheikh, 2008; Aksu and Bozkurt, 2009). Furthermore, dietary administration of HA has been shown to has strong anti-stress effect in high-density barns, minimizing the harmful effect of chronic stress on performance in laying hens (Cetin et al., 2011). Since chronic stress has profound effects in the biology of metazoans, mainly by inducing chronic inflammation (Stenvinkel, et al., 1999), the anti/stress effects of HA may not only be associated with reduction of ammonia in the chicken houses, but also, by the colloidal and antioxidant properties of HA described to increase intestinal viscosity and cellular integrity (Salminen and Isolauri, 2006; Salzman, 2011; Elson and Cong, 2012; Vašková et al., 2011).

Recently, our laboratory has developed several models to induce intestinal inflammation in poultry. Those models include high non-starch polysaccharides diets (Tellez et al., 2014, 2015); dexamethasone (Vicuña et al., 2015a); dextran sodium sulfate (DSS) (Kuttappan et al., 2015a; Menconi et al., 2015); and 24 h FR (Kuttappan et al., 2015b; Vicuña et al., 2015b). In the above models, inflammation causes disruption of the epithelial tight junctions increasing liver bacterial translocation and leakage of serum fluorescein isothiocyanate dextran (FITC-d) to systemic blood circulation. FITC-d is a large molecule (3-5 kDa), which under normal conditions is not able to cross the epithelial barrier (Yan et al., 2009). However, during intestinal inflammation the tight junctions are disrupted allowing

the FITC-d molecule to enter circulation, making FITC-d a viable biomarker to measure intestinal barrier function (Baxter et al., 2017). Due to the remarkable physical and chemical properties of HA, these organic compounds may have important direct or indirect effects on intestinal integrity. Hence, the purpose of this study was to evaluate the effects of HA on intestinal viscosity, leaky gut and ammonia excretion in a 24 h FR model to induce intestinal permeability in broiler chickens.

2. Material and methods

2.1 Isolation/Extraction of Humic Acids

The isolation and extraction of HA from worm compost was performed as described by Stevenson (1982). For the alkaline extraction process of HA, sodium hydroxide (0.1N NaOH) was used in a ratio of 5 parts of NaOH to one part of compound (g/mL), allowed to stand for 24 h at room temperature, filtered through a 125 µm mesh and acidified using 10% sulfuric acid (H_2SO_4), rectifying a pH of 2. The solids and liquids were separated by decantation. The solid fraction (HA) was washed 2 times with distilled water to remove sulfuric acid residues, and between each wash, it was centrifuged for 20 min at 3500 G. The sample was desiccated in a roto-evaporator at 60 °C until it had a gel consistency. Finally, it was dried it in an oven at 60 °C. The result was a yellow-brown powder with a pH of 7 to 8.

2.2 Animal source, diets, and experimental design

One-day old male Cobb-Vantress broiler chickens (Fayetteville, AR, USA) were neck-tagged, weighted and randomly allocated to one of two groups (*n* = 25

chickens), with or without 0.2% of isolated HA from worm-compost, and placed in heated brooder batteries with a controlled age-appropriate environment. Chicks had *ad libitum* access to water and feed for 14 days. The experimental diet was formulated to approximate the nutritional requirements of broiler chickens as recommended by the National Research Council (1994), and adjusted to breeder's recommendations (Cobb-Vantress Inc. 2015). No antibiotics were added to the diet (Table 1). Intestinal permeability was induced using FR as previously published (Kuttappan et al., 2015b; Vicuña et al., 2015b). Chickens were randomly assigned to each experimental group, and had unrestricted access to feed and water from 1 d to 14 d of age. Beginning at 14 d, chickens were subjected to 24 h of FR. Concentration of FITC-d was calculated based on group body weight (Kg/BW), therefore groups were weighed the day before FR began. At 15 d of age, chickens in both groups were given an appropriate dose of FITC-d by oral gavage. One hour post gavage chickens were euthanized and blood samples were collected from the femoral vein and centrifuged (500 x *g* for 15 min) to separate the serum from the red blood cells for FITC-d determination. Intestinal content and liver samples were also collected to evaluate viscosity and bacterial translocation as described below. All animal handling procedures complied with Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville. Specifically, the IACUC approved this study under the protocol #15006.

2.3 Viscosity

Total intestinal digesta contents were collected from Meckel's diverticulum to the ileocecocolonic junction. For viscosity analysis, approximately 1.5 g (wet weight) of

the fresh digesta was immediately placed in a microcentrifuge tube and centrifuged at 12,000 X *g* at 4^{o}C for 5 min. The supernatant fluid was collected and stored on ice until viscosity measurement was determined using a LVDV-I Brookfield digital cone-plate viscometer fitted with a CP-40 spindle (Brookfield Engineering, Middleboro, MA, USA). The analyzed samples and the viscometer cup were maintained at 40 °C during viscosity measurement. Viscosity was measured in centipoise (cP = 1/100 dyne s/cm^2) and the results were reported as $\log_{10}$ cP.

2.4 Bacterial translocation

Briefly, the right half of the liver was removed from each chicken, collected in sterile bags, homogenized, weighed and 1:4 w/v dilutions were made with sterile 0.9% saline. Ten-fold dilutions of each sample, from each group were made in a sterile 96 well Bacti flat bottom plate and the diluted samples were plated on tryptic soy agar (TSA, catalog no. 211822, Becton Dickinson, Sparks, MD, USA). Samples were then enriched on tryptic soy broth and further incubated at 37 °C for 24 h. Following this, enrichment samples were plated on TSA and incubated at 37 °C for 24 h to confirm presence/absence of colonies.

2.5 Serum determination of FITC-d leakage

Intestinal leakage of fluorescein isothiocyanate dextran (FITC-d) (MW 3-5 KDa; Sigma-Aldrich Co., St. Louis, MO, USA) and the measurement of its serum concentration as a marker of paracellular transport and mucosal barrier dysfunction was performed as previously described by Baxter et al. (2017). FITC-d levels of diluted serum samples (1:5 PBS) were measured at excitation wavelength of 485 nm, gain 40 and emission wavelength of 528 nm with a Synergy HT, Multi-mode

microplate fluorescence reader (BioTek Instruments, Inc., Winooski,VT, USA). Fluorescence measured was then compared to a standard curve with known FITC-d concentrations. Gut leakage for each bird was reported as ng of FITC-d/mL of serum (Baxter et al., 2017).

2.6 Physiochemical analysis of the poultry manure

Eight samples of manure from each group were collected from the trays of the batteries at day 15 to obtain the physicochemical analysis of the manure. Moisture was determined by drying the litter at 65 °C overnight and comparing the weight before and after drying. The water potential of incubated litter was measured at 23 °C using a dew point potentiometer (Model WP4; Decagon Inc., Pullman, WA). The instrument measures water potentials from 0 to −80 MPa with a precision of ± 0.1 MPa. Water potential was obtained by placing 2 g of litter in the potentiometer chamber and allowing it to equilibrate for 5 to 10 min. Litter pH was determined using a combination electrode (Fisher Scientific, Hampton, NH, USA) at a 5:1 deionized water to litter ratio (Wolf, 2003). Total N and total C were determined by combustion (Watson et al., 2003) of the litter using a Vario Max CN analyzer (Elementar Americas, Inc., Mt. Laurel, NJ, USA) The NH_4–N content of litter was determined using a 1:10 litter to 1 N KCl extraction (Choi & Moore, 2008) followed by colorimetric analysis on a Skalar using chemical method no.155-324 (Skalar Inc., Buford, GA, USA). A SevenMulti Mettler Toledo probe (Mettler-Teledo, LLC, Columbus, OH, USA) was used to measure pH on the 1:10 1N KCl extractions.

The NO3–N content was also assessed after this KCl extraction using Quickchem FIA+, method #12-107-04-1-B (Lachat Instruments, Loveland, CO, USA). The

remaining total elemental composition of poultry litter was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis after HNO3 and HCl microwave digestion (Walter et al., 1997). Microwave digestion was performed using in a Mars 5 Microwave (CEM Corp., Matthews, NC, USA). The procedure consisted of mixing 0.5 g litter with 9 mL HNO3 and 3 mL HCl in a Teflon microwave digestion vessel. This mixture was allowed to predigest for 45 min at room temperature and was then placed in the microwave. A 6.5-min ramp time was used to achieve a digestion temperature of 175 °C, which was held for 12 min. Samples were allowed to cool to room temperature and then filtered through a Whatman 42 filter before ICP-OES analysis. Total N and NH4–N analyses were performed on wet litter. Microwave digestion for analysis by ICP-OES was performed on litter dried at 65 °C. Organic-N was estimated by subtracting the NH4–N and NO3–N values from the total N value. Organic N mineralization and total N loss were determined by mass balance. All N values were adjusted for moisture content and are reported on a dry weight basis.

2.7 *In vitro* digestion model

The *in vitro* digestion model was performed by quintuplicate at 40^{o}C to simulate poultry body temperature according to previous publications with minor modifications (Annett et al., 2002; Latorre et al., 2015). In this experiment, two diets were tested. A control non-treated diet (Table 1) and a control diet supplemented with 6 % HA. Briefly, for all the gastrointestinal compartments simulated during the *in vitro* digestion model, a BOD incubator (Biochemical oxygen demand incubator, model 2020, VWR, Houston, TX, USA) customized with an orbital shaker

(Standard orbital shaker, model 3500, VWR, Houston, TX, USA) was used for mixing the feed content in the experimental tubes at 19 rpm. Additionally, all tube samples were hold in a 30 degrees inclination position to facilitate proper blending of feed particles and the enzyme solutions incorporated throughout the assay. The first gastrointestinal compartment simulated was the crop, where 5 g of feed and 10 mL of 0.03 M hydrochloric acid (HCL, catalog no. HX0607-2, EMD Millipore corporation, Billerica, MA, USA) were placed in 50 mL polypropylene centrifuge tubes and mixed vigorously reaching a pH value around 5.20, next the tubes were incubated for 30 min. The second gastrointestinal compartment simulated was the proventriculus, where 3,000 U of pepsin per g of feed where used (catalog no. P700, Sigma-Aldrich, St Louis, MO, USA) and 2.5 mL of 1.5 M HCl were added to each of the tubes, reaching a pH between 1.4 to 2.0, then all tubes were incubated for 45 min. The third and final gastrointestinal compartment simulated was the intestinal section. In this case, 6.84 mg of 8 x pancreatin (catalog no. P7545, Sigma-Aldrich, St Louis, MO, USA) were used per g of feed and included in 6.5 mL of 1.0 M sodium bicarbonate ($NaHCO_3$, catalog no. S6014, Sigma-Aldrich, St Louis, MO, USA), the pH ranged between 6.4 and 6.8, and all tube samples were incubated for 2 h. The complete *in vitro* digestion process took 3 h and 15 min. After the incubation time in each compartment, a sample was collected to evaluate viscosity and pH.

2.8 Data and statistical analysis

Log_{10} cfu/g of liver bacterial traslocation, BW, body weight gain (BWG), viscosity, pH, and serum FITC-d concentration were subjected to one way analysis of

variance as a completely randomized design, using the General Linear Models procedure of SAS (SAS Institute, 2002). Significant differences among the means were determined by Duncan's multiple-range test at $P<0.05$. The enrichment data were expressed as positive/total chickens (%), and the percent recovery of bacteria was compared using the Chi-Squared test of independence, testing all possible combinations to determine the significance ($P<0.05$).

3. Results

The results of the evaluation of BW and BWG in broiler chickens consuming a corn-based diet with or without the inclusion of 0.2% of HA for 14 days are summarized in Table 2. No significant differences were observed in BW or BWG between both groups (Table 2).

Table 3 shows the results of the evaluation of intestinal viscosity, serum FITC-d, and liver BT in chickens consuming a corn-based diet with or without inclusion of 0.2% of HA following 24 h of FR in broiler chickens. A significant increase in intestinal viscosity was observed in the experimental group consuming 0.2% of HA when compared with control non-treated group. The treated group also showed a significant reduction in FITC-d when compared to the control non-treated group. Similarly, a significant reduction in $\log_{10}$ cfu/g and the number of positive, liver enrichment samples, were observed in the treated group (58%) when compared to the non-treated control chickens (100%) (Table 3).

The results of the physiochemical analysis of the poultry manure from broiler chickens consuming a corn-based diet with or without inclusion of 0.2% of HA

following 24 h of FR are summarized in Table 4. A significant reduction in litter dry weight, pH, Ammonia-N, Ammonia-N wet and Ammonia-N dry was observed in treated chickens when compared with the control non-treated group. Interestingly, water content (%) was significant higher in treated chickens when compared with control non-treated chickens (Table 4).

Table 5 shows the results of the effect of humic acids on viscosity and pH during *in vitro* digestion, under variable biochemical conditions simulating different sections of the gastrointestinal tract of poultry. A significant increase in viscosity was observed in the crop and intestine compartments in the treated group with 6 % HA when compared with control non-treated group. As expected, this increase in viscosity was associated with increases in pH in both compartments. In contrast, in the proventriculus compartment, a significant reduction in viscosity was observed in the treated group when compared with control non-treated group. In this compartment, 6 % HA also increase the pH from 1.90 in the control non-treated group to 3.38, hence affecting the polymerization of HA and therefore the viscosity (Table 5).

4. Discussion

Humans have been using HA for over two centuries; however, there is still little knowledge regarding their structure and properties, since HA cannot be classified as any other chemical class of compounds such as polysaccharides or proteins (Islam et al., 2005; Peña-Méndez et al., 2005). Due to their solubility, fulvic acids are those organic materials that are soluble in water at all pH values, while HA are insoluble at acidic pH values (pH < 2) but are soluble at higher pH values. On the

other hand, humin is the fraction of natural organic materials that is insoluble in water at all pH values. These definitions reflect the traditional methods for separating the different fractions from the original humic materials (Gaffney et al., 1996). Another interesting characteristic of HA is that they have a wide range of molecular weights and sizes, ranging from a few hundred to several hundred thousand atomic mass units consisting of alkyl/aromatic units cross-linked mainly by oxygen and nitrogen groups with the major functional groups being carboxylic acid, phenolic and alcoholic hydroxyls, ketone, and quinone groups (Saar & Weber, 1979). These chemical characteristics allow HA functioning as surfactants, with the ability to bind both hydrophobic and hydrophilic materials (Gaffney et al., 1996). This function in combination with their colloidal properties, makes HA effective agents in transporting and binding both organic and inorganic contaminants in the environment (Piccolo, 2002).

In the present study, a remarkable increase in intestinal viscosity was observed in chickens consuming 0.2% HA for 14 days. As far as we know, this is the first report showing this effect in poultry. This increase in intestinal viscosity was also confirmed using an *in vitro* digestive model that simulates the physical and chemical environment of the crop, proventriculus and intestine of chickens. Interestingly, the increase viscosity observed during the crop and proventriculus compartments, was associated with an increase in pH in the group that contained HA as compared with control-non treated group. This increase in intestinal viscosity may be due to the rheological behavior of HA, since they have a colloidal character which can enhance chemical and physical interactions due to the large

surface areas of its colloidal particles (Gaffney et al., 1996). The colloidal character of HA material are thought to consist of coiled, long-chain, or three-dimensional cross-linked macromolecules with electrical charges variously distributed on the particle. The presence of charged sites, arising from ionized acidic groups, results in mutual repulsion and causes maximum expansion of the molecule. All these physicochemical properties are closely related to the solution chemistry, like ionic strong or pH (Klučáková & Věžníková, 2017). Furthermore, it has been reported that polymerization of HA occurs to a further extent at pH 7 than at pH 4, due to the larger mobility of reacting molecules as was confirmed by the increasing *in vivo* viscosity (Cozzolino & Piccolo, 2002). Additionally, it has been confirmed that HA interact with biomolecules such as collagen promoting resistance and maturity of collagen fibers (Riede et al., 1992) and increasing integrity of the ileal epithelium (Yasar et al., 2002). Hence, it is possible that the intestinal polymerization of HA were responsible of increasing the viscosity, and intestinal integrity as was evidenced by a significant reduction in intestinal permeability. Intestinal epithelial cells are not only responsible for digestion, secretion and absorption but act as a physical barrier separating external environmental agents from the internal host environment, hence preventing the entry of intraluminal microbiota, antigens, and toxins, yet, providing tolerance to nutrients, water and electrolytes (Salminen and Isolauri, 2006; Salzman, 2011; Elson & Cong, 2012). Any microscopic damage that alters gut permeability is associated with BT to the portal vein and/or systemic circulation leading to systemic bacterial infections (Ilan, 2012). Stress is known to affect gastrointestinal tract (GIT) homeostasis by altering gut motility, permeability,

as well as alterations in ion, fluid, and mucus secretion and absorption (Alverdy & Aoys, 1991; Collins & Bercik, 2009; Verbrugghe et al., 2011; Karavolos et al., 2013).

Stress is a biological mechanism of defense and survival of all living organisms. However, chronic stress leads to chronic inflammation which has been recognized to have dramatic effects on the health of individuals (Stenvinkel et al., 1999). For instance, several investigators have reported that acute or chronic stress modify gut permeability associated with a temporary redistribution of tight junction proteins (Maejima et al., 1984; Koh et al., 1996; Matter and Balda, 2007; Assimakopoulos et al., 2011). Some of these alterations are linked to Mast cells in the brain-gut axis which secrete several neurotransmitters and pro inflammatory cytokines, with profound effects on GIT physiology (Groschwitz & Hogan, 2009; Bailey et al., 2011; Lamprecht & Frauwallner, 2012). Another hormone that increases during acute or chronic stress is corticotropin releasing factor, which increases intestinal paracellular permeability via mast cell dependent release of TNF-α and proteases (Taché & Perdue, 2004; Teitelbaum et al., 2008; Overman et al., 2012). Moreover, excessive cortisol may lead to GIT disturbances, opportunistic infections, and impaired wound healing (Moeser et al., 2007; Smith et al., 2010; Galley & Bailey, 2014). Oxidative stress also increases disruption of the tight junctions by H_2O_2 or by nitric oxide, leading to changes in tyrosine kinase and/or protein tyrosine-phosphatase activities, altering the phosphorylated state of junctional proteins (Sander et al., 2005). In the present study, it was remarkable to observe that dietary administration of 0.2% of HA, in chickens that received 24 h FR, showed a

significant reduction in intestinal permeability, as confirmed by reduction in leakage of FITC-d and liver BT when compared with control non-treated chickens. Dissolved HA are taken up by organisms and interact on various molecular and biochemical levels, and it has been shown that may transcriptionally control biotransformation, ant-oxidant and anti-stress defense systems and modulate the respective enzyme activities. In addition, HA are potent chelating agents of heavy metals such as iron, copper (Vaughan and MacDonald, 1976). Both minerals are strong generators of reactive oxygen species (ROS), and several reports indicate that free radical destabilize the paracellular pathway, increasing ion leakage rates (Ferruzza et al., 2002; Henderson, 2005). By recapturing the radicals, HA can increase the host antioxidant defensive mechanism (Vašková et al., 2011; Aeschbacher et al., 2012). Furthermore, the aromatic groups of HA, have been shown to stimulate active Na+ uptake, K+-ATPase activity, and reduces paracellular permeability, then these direct beneficial effects would oppose the toxic effects of metals (Wood et al., 2003,2011).

Poultry litter is a valuable nutrient source for soil as it contains high levels of protein (up to 30% crude protein), nitrogen (N), and other minerals, including phosphorous, potassium, and calcium (Kelleher et al., 2002). However, a major issue with poultry litter is the loss of N as ammonia due to microbial mineralization of urea and uric acid, which represent up to 80% of the total N in poultry litter (Nahm, 2003). In addition, ammonia volatilization in the chicken houses results in malodorous emissions and loss of poultry litter value as a fertilizer due to N loss, but most importantly, it causes severe stress and health issues in the birds with negative

impacts on performance (Moore et al., 2011). The results of the present study showed a significant reduction in the concentration of ammonia in the manure of chickens fed 0.2% of HA during the first 14 days. These findings are in agreement with several investigators that have shown similar results in poultry and pigs (Kelleher et al., 2002; Ji et al., 2006). Humic acids play an important role in the nitrogen cycle by influencing the distribution, bioavailability, and ultimate fate of organic nitrogen (Nahm, 2003). Ammonium is oxidized by autotrophic ammonia-oxidizing bacteria in the manure; however HA have been shown to cause inhibition of the urease activity modifying the microbial biomass in the litter (Vaughan and MacDonald, 1976; Clinton et al., 1995). These microbiological changes caused by HA reduce the negative effects of the direct application of urea and other chemical fertilizers on soil bacteria or fungi (Dong et al., 2009). In summary, the results of the present study suggest that supplementation of 0.2% of HA in the diet of chickens for 14 days, increase intestinal viscosity and intestinal integrity, and confirm its benefits reducing ammonium of poultry manure.

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Conflict of interest. The authors declare that they have no conflict of interest.

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Table 1 Ingredient composition of starter broiler diet based on corn and soybean meal with or without inclusion of 0.2% of humic acids

Item	Control diet	Experimental diet
Ingredients (%)		
Corn	57.34	57.14
Soybean meal	34.66	34.66
HA worm composte	0.00	0.20
Vegetable oil	3.45	3.45
Dicalcium phosphate	1.86	1.86
Calcium carbonate	0.99	0.99
Salt	0.38	0.38
DL-Methionine	0.33	0.33
L-Lysine HCl	0.31	0.31
Threonine	0.16	0.16
Vitamin premix[a]	0.20	0.20
Choline chloride 60%	0.20	0.20
Mineral premix[b]	0.10	0.10
Antioxidant[c]	0.02	0.02
Calculated analysis		
Metabolizable energy (kcal/kg)	3,03	3,03
Crude protein (%)	22.15	22.15
Lysine (%)	1.36	1.36
Methionine (%)	0.65	0.65
Threonine (%)	0.91	0.91
Total Calcium (%)	0.90	0.90
Available Phosphorus (%)	0.45	0.45

[a] Vitamin premix supplied the following per kg: vitamin A, 20,000,000 IU; vitamin D3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g (Nutra Blend LLC, Neosho, MO 64850).

[b] Mineral premix supplied the following per kg: manganese, 120 g; zinc, 100 g; iron, 120 g; copper, 10–15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g (Nutra Blend LLC, Neosho, MO 64850). [c] Ethoxyquin.

Table 2 Evaluation of body weight (BW) and body weight gain (BWG) in broiler chickens consuming a corn-based diet with or without inclusion of 0.2% of humic acids for 14 days.

Variable	Control	0.2% Humic acids
BW 1-d	44.84 ± 0.57	45.00 ± 0.60
BW 14-d	327.16 ± 9.37	342.76 ± 9.01
BWG 14-d	282.32 ± 9.28	297.76 ± 9.00

[1]Data expressed as Mean ± SE in g, *n* = 25/group. P>0.05

Table 3 Evaluation of intestinal viscosity, serum FITC-d, and liver bacterial translocation in chickens consuming a corn-based diet with or without inclusion of 0.2% of humic acids following 24 hours of feed restriction (FR) in broiler chickens.

Treatment	Intestinal viscosity (cP Log_{10})[3]	Serum FITC-d (ng/mL)[2]	Liver bacterial translocation (Log_{10} cfu/g)[4]	
			cfu Log_{10} of liver	Liver enrichment culture[5]
Control FR	0.13 ± 0.01 [b]	828.58 ± 32.85 [a]	2.83 ± 0.09 [a]	12/12(100%)
0.2% Humic acids FR	0.24 ± 0.01 [a]	544.62 ± 41.84 [b]	1.60 ± 0.04 [b]	7/12 (58%)*

[a-b] Superscripts within columns indicate significant difference at P<0.05.

[1] Data expressed as Mean ± SE.

[2] Serum (FITC-d) was evaluated in 20 chickens/group.

[3] Intestinal viscosity evaluated in Log_{10} (in centipoise, cP = 1/100 dyne sec/cm^2), *n* = 5 chickens/group.

[4] Liver bacterial translocation was evaluated in 12 chickens/group.

[5] Data expressed as positive/total chickens (%).

*P<0.001

Table 4 Physiochemical analysis of the poultry manure from broiler chickens consuming a corn-based diet with or without inclusion of 0.2% of Humic acid following 24 hours of feed restriction of 10 days chicken feces.[1]

Variable	Control	0.2% Humic Acids
Litter Wet Weight (g)	5.05 ± 0.003 [a]	5.02 ± 0.001 [a]
Litter Dry Weight (g)	2.21 ± 0.06 [a]	1.98 ± 0.05 [b]
Water content (%)	56.01 ± 1.26 [b]	60.43 ± 1.03 [a]
pH	6.73 ± 0.03 [a]	6.62 ± 0.04 [b]
Ammonia-N(mg/L)	90.85 ± 3.01 [a]	58.07 ± 7.60 [b]
Ammonia-N Wet (mg/kg)	905.25 ± 30.03 [a]	579.25 ± 75.35 [b]
Ammonia-N Dry (mg/kg)	2064.80 ± 38.16 [a]	1459.80 ± 177.95 [b]

[1]Data is expressed as mean ± standard error. Values within rows with different lowercase superscripts differ significantly ($P<0.05$)

Table 5

Effect of humic acid on viscosity and pH during *in vitro* digestion, under variable biochemical conditions simulating different sections of the gastrointestinal tract of poultry.

Treatment	Crop		Proventriculus		Intestine	
	Viscosity	pH	Viscosity	pH	Viscosity	pH
Control	0.782 ± 0.004 [b]	5.486 ± 0.010 [b]	0.918 ± 0.007 [a]	1.900 ± 0.004 [b]	0.942 ± 0.004 [b]	6.826 ± 0.005
Humic acid (6%)	1.082± 0.004 [a]	8.464 ± 0.004 [a]	0.872 ± 0.004 [b]	3.388 ± 0.004 [a]	1.104 ± 0.002 [a]	7.054 ± 0.002

[a-d] Values in columns with different letters differ significantly (P<0.05).

VI. DISCUSIÓN.

En el presente estudio con la suplementación de 0.1% o 0.2% de AH de origen comercial de Sigma-Aldrich (lignito purificado) o un producto de AH natural, extraído de una composta de lombriz, en un modelo de digestión *in vitro* que simula diferentes secciones del tracto gastrointestinal (buche, proventrículo e intestino) de las aves de engorda, no se vieron afectados los conteos de SE recuperada en los tres compartimentos evaluados. Curiosamente, se observó una tendencia ($p=0.07$) en los conteos de SE en ambos grupos suplementados con AH de origen comercial o natural en ambas concentraciones en comparación con el grupo control no tratado, este aumento se asoció con un incremento numérico del pH en el compartimiento simulado del intestino. Estos hallazgos se confirmaron en el experimento *in vivo*, donde, los pollos que fueron suplementadas con 0.2% de AH en la dieta durante 10 días, previos al desafio con SE, no se observó ningún efecto sobre la persistencia de SE en los ciegos, después de 24 horas del desafío.

Con la suplementación de AH no se observaron diferencias significativas en las evaluaciones de peso corporal y ganancia de peso corporal, en los conteos de IgA intestinal, el paso de FITC-d en suero sanguíneo, ni en los conteos totales de bacterias gram negativas y ácido lácticas, en comparación con el grupo control no tratado.

Estos resultados no concuerdan con informes previos basados en ensayos *in vitro* e *in vivo*. Yarkova (2011) observó que los AH provenientes de lixiviados y ligados

a carbón mostraron inhibición completa del crecimiento de *St. aureus* y *Candida*, y una disminución en el número de colonias de 78-80% en *E. coli* y de 58-70% en *S. enteritidis*. Aksu y Bozkurt (2009) mencionan que, en pollos de engorda suplementados en el alimento con una fuente comercial de AH (Farmagulator Dry-Humic AcidTM), encontraron que las UFC de *E. coli* en la digesta de aves fueron significativamente menores en la dietas suplementadas con antibióticos y dietas con AH que en el grupo control. Frente a esto, el informe de Jansen van Rensburg y Naude (2009), refieren que, los coliformes totales y los recuentos de *E. coli* en el ciego no se vieron afectados por la adición de humato de potasio en el agua de bebida de los pollos de engorda; mientras que Shermar et al. (1998) informaron un aumento entre 10 y 100 veces en las poblaciones de *E. coli* de pollos que recibieron un humato comercial mineral (Menefee HumateTM) en comparación con el control.

Ademas, se observó un marcado incremento en la viscosidad intestinal en pollos de engorda que consumieron 0.2% de AH durante 14 días. El incremento en la viscosidad intestinal en este grupo puede deberse a las características coloidales de los AH, las cuales pueden mejorar las interacciones químicas y físicas a lo largo de las superficies de estas partículas (Gaffney et al., 1996). Otras características interesantes de los AH son los altos rangos de peso y talla de sus moléculas, que van en un rango desde los cientos hasta los varios miles de unidades de masa atómica, que consisten de unidades alquilo/aromáticas, unidades enlazadas por oxígeno y nitrógeno, grupos funcionales mayormente

unidos a ácidos carboxílicos, fenoles e hidroxilos de alcohol, cetona y grupos quinona (Saar and Weber, 1979). Estas características químicas de los AH tienen la función surfactante con la habilidad de unirse tanto al material hidrofóbico como al hidrofílico (Gaffney et al., 1996). La presencia de sitios cargados, prevalentes en grupos ionizados acidificados, resulta en una repulsión mutua y causa la máxima expansión de la molécula. Todas estas propiedades fisicoquímicas están estrechamente relacionadas con la solución química, así como la fuerza iónica y el pH (Klučáková and Věžníková, 2017). Además se ha reportado que la polimerización de los AH ocurre tanto a pH 7 como a pH 4, como lo confirma el incremento *in vivo* e *in vitro* de la viscosidad (Cozzolino and Piccolo, 2002). Confirmando que las interacciones de los AH con biomoléculas como el colágeno, ayuda a promover la resistencia y maduración de las fibras de colágeno (Riede et al., 1992); lo cual, incrementa la integridad del epitelio ileal (Yasar et al., 2002).

Consecuentemente, es posible que la polimerización del intestino debido a los AH fuera la responsable del incremento en la integridad intestinal. Las células del epitelio intestinal no solo son responsables de la digestión, secreción y absorción, también actúan como una barrera física, que separa los agentes externos del ambiente de los hospederos internos, por lo tanto, previenen la entrada de la microbiota intraluminal, antígenos y toxinas, e incrementar la tolerancia hacia algunos nutrientes (Salminen and Isolauri, 2006; Salzman, 2011; Elson and Cong, 2012). Sin embargo, durante la inflamación intestinal las uniones estrechas se interrumpen permitiendo que el FITC-d y la microbiota entren en circulación

sanguínea. En el presente estudio, se observó que la administración dietética de 0.2% de HA, en pollos sometidos a RA durante 24 h, mostró una notable reducción significativa en la permeabilidad intestinal, confirmada por la reducción del paso de FITC-d y TB hepática, comparado con el grupo control de pollos no tratados.

El organismo de los animales absorbe los AH disueltos e interactúan a diversos niveles moleculares y bioquímicos; se ha señalado que pueden controlar transcripcionalmente sistemas de biotransformación, antioxidación, asi como activar mecanismos para contrarrestar el estrés y modular las actividades enzimáticas respectivas. Además, los AH son potentes agentes quelantes de metales pesados como hierro y cobre (Vaughan y MacDonald, 1976). Ambos minerales son fuertes generadores de especies reactivas de oxígeno (ROS), y varios informes indican que los radicales libres desestabilizan la vía paracelular, aumentando las tasas de fuga de iones (Ferruzza et al., 2002; Henderson, 2005). Al recapturar los radicales, los AH puede aumentar el mecanismo defensivo antioxidante del huésped (Vašková et al., 2011; Aeschbacher et al., 2012). Igualmente, se ha demostrado que los grupos aromáticos de los AH estimulan la captación activa de Na^+, la actividad de K^+-ATPasa y reducen la permeabilidad paracelular, dichos beneficios neutralizan los efectos tóxicos de los metales pesados (Wood et al., 2003; 2011).

Respecto al análisis fisicoquímico de las heces, se encontró una reducción significativa en la concentración de amoníaco en la excreta de pollos alimentados

con 0.2% de AH durante los primeros 14 días. Estos hallazgos coinciden con los de varios investigadores que han demostrado resultados similares en aves de corral y cerdos (Kelleher et al., 2002; Ji et al., 2006). Los AH desempeñan un papel importante en el ciclo del nitrógeno al influir en la distribución, la biodisponibilidad y el destino final del nitrógeno orgánico (Nahm, 2003). El amonio se oxida por bacterias autótrofas que oxidan amoníaco en el estiércol; sin embargo, se ha demostrado que los AH causan inhibición de la actividad de la ureasa modificando la biomasa microbiana en la excreta (Vaughan y MacDonald, 1976; Clinton et al., 1995). Estos cambios microbiológicos causados por los AH reducen los efectos negativos de la aplicación directa de urea y otros fertilizantes químicos en las bacterias u hongos del suelo (Dong et al., 2009).

La excreta de aves es una valiosa fuente de nutrientes para el suelo ya que contiene altos niveles de proteína (hasta 30% de proteína cruda), nitrógeno (N) y otros minerales, incluyendo fósforo, potasio y calcio (Kelleher et al., 2002). Sin embargo, un problema importante con la excreta de aves es la pérdida de N como amoníaco debido a la mineralización microbiana de la urea y ácido úrico, que representan hasta el 80% del N total en la excreta (Nahm, 2003). Además, la volatilización de amoníaco en las casetas de pollo produce emisiones malolientes y pérdida de valor de la excreta como fertilizante. Ocasionando estrés severo y problemas de salud en las aves, lo que impacta de forma negativa el rendimiento productivo (Moore et al., 2011).

VII. CONCLUSIONES

En el presente estudio, la suplementación de 0.1% o 0.2% de AH de origen comercial de Sigma-Aldrich (lignito purificado) o un producto de AH natural, extraído de la composta de lombriz en un modelo de digestión *in vitro*, no afectó los conteos de S. Enteritidis recuperada en los tres compartimentos evaluados.

En resumen, los resultados del presente estudio sugieren que la suplementación de 0.2% de AH en la dieta de pollos durante 14 días, aumenta la viscosidad intestinal y la integridad intestinal, y confirma sus beneficios reduciendo el amonio de la excreta de aves de corral.

Las diferencias en resultados entre el artículo 1 y el artículo 2 se deben a las condiciones bajo las cuales se llevaron a cabo los experimentos, con el uso del modelo de restricción alimenticia como inductor de la inflamación intestinal se simuló también las condiciones de estrés a las que normalmente están sometidas las aves bajo la producción intensiva, denotando que el uso de AH bajo éste tipo de condiciones es recomendado

Cabe resaltar que éste es uno de los primeros trabajos en el cual se estudia los efectos de los AH en retos bacterianos como con S. Enteritidis de manera *in vitro* e *in vivo*, además, de ser un estudio en el cual se demuestra por primera vez el mecanismo de acción de los AH a nivel del tracto digestivo sobre el mantenimiento de la integridad intestinal en las aves de corral.

El seguir estudiando las diferentes aplicaciones de los AH extraidos y/o provenientes de lombricomposta en las aves de corral, es un planteamiento a futuro para poder dilucidar de manera mas precisa sus m ecanismos de acción y esclarecer sus diferentes interacciones en los distintos niveles, molecular, celular y sistémico.

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