
**UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO**

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**FACULTAD DE MEDICINA VETERINARIA Y ZOOTECNIA**

# CARACTERIZACIÓN DE ÁCIDOS HÚMICOS PROVENIENTES DE LOMBRICOMPOSTAS Y SU USO EN MODELOS *IN VITRO* QUE SIMULAN EL TRACTO DIGESTIVO DE LAS AVES DE ENGORDA

**TESIS**

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## RESUMEN

La presente investigación está constituida en dos etapas: en la primera etapa se realizó la caracterización de ácidos húmicos (AH) aislados de lombricompostas mediante las técnicas de potencial Z (ζ), punto de carga cero (pHpzc), espectroscopía de infrarrojo con transformada de Fourier con reflexión total atenuada (FTIR-ATR), microscopía electrónica de barrido (SEM) y espectroscopía de rayos X con dispersión de energía (EDS) para conocer las propiedades físicas y químicas de los AH, con la finalidad de elucidar el tipo de interacciones que podrían llevarse a cabo con la molécula de aflatoxina $B_1$ ($AFB_1$). Adicionalmente, se utilizó un modelo *in vitro* que simula el tracto digestivo de las aves de engorda para evaluar la capacidad de los AH de adsorber $AFB_1$. La inclusión de AH evaluada fue de 0.2% en una dieta contaminada con $AFB_1$ (100 µg/kg). En la segunda etapa experimental se evaluó el efecto de los AH sobre los conteos bacterianos de *Salmonella entérica* serotipo Enteritidis (SE)*, Escherichia coil* (EC), *Clostridium perfringens* (CP)*, Bacillus subtilis* (BS) y *Lactobacillus salivarius* (LS) en un modelo *in vitro*. Se realizaron pruebas independientes para cada bacteria con inclusiones ascendentes de AH: 1) Control negativo (sin bacteria), 2) Control positivo (bacteria), 3) 0.1% de AH + bacteria, 4) 0.2% de AH + bacteria, 5) 0.5% de AH + bacteria, y 6) 1% de AH + bacteria. Los resultados de la caracterización revelaron que los grupos funcionales primarios en los AH son los ácidos carboxílicos y fenólicos. Además, se determinó una superficie con una fuerte carga negativa en los pH simulados (3, 5 y 7). El porcentaje de adsorción de $AFB_1$ fue del 97.6% en comparación con el 81.5% de la zeolita utilizada como referencia ($P < 0.05$). Finalmente, en los conteos bacterianos se observó un aumento significativo de todas las bacterias utilizadas ($P \leq 0.0001$) en el último compartimiento simulado del modelo *in vitro.* En conclusión, los resultados de la presente investigación sugieren que los AH pueden ser utilizados como adsorbentes de aflatoxinas por su gran capacidad para unirse a la $AFB_1$, y como prebióticos por su capacidad estimulante del crecimiento bacteriano.



**Palabras clave:** Pollos de engorda, caracterización, ácidos húmicos, $AFB_1$, retos bacterianos.

## ABSTRACT

The present investigation is constituted in two phases: in the first phase , the characterization of humic acids (HA) isolated from vermicomposts was carried out using the techniques of potential Z (ζ), point of zero charge (pHpzc), Fourier transform infrared spectroscopy with attenuated total reflection (FTIR-ATR), scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) to determine the physical and chemical properties of HA, in order to elucidate the type of interactions that it could lead carried out with the aflatoxin $B_1$ ($AFB_1$) molecule. Additionally, an *in vitro* model that simulates the digestive tract of broiler chickens was used to evaluate the capacity of HA to adsorb $AFB_1$. The inclusion of HA evaluated was 0.2% in a diet contaminated with $AFB_1$ (100 µg/kg). In the second experimental stage, the effect of HA on the recovery of bacterial counts of *Salmonella enterica* serovar Enteritidis (SE), *Escherichia coil* (EC), *Clostridium perfringens* (CP), *Bacillus subtilis* (BS) and *Lactobacillus salivarius* (LS) was evaluated in an *in vitro* model. Independent tests were performed for each bacterium with ascending HA inclusions: 1) Negative control (no bacteria), 2) Positive control (bacteria), 3) 0.1% HA + bacteria, 4) 0.2% HA + bacteria, 5) 0.5% HA + bacteria, and 6) 1% HA + bacteria. The characterization results revealed that the main functional groups in HA are carboxylic and phenolic acids. Additionally, a surface with a strong negative charge was determined at the simulated pH (3, 5 and 7). The percentage of adsorption of $AFB_1$ was 97.6% compared to 81.5% of the zeolitic material used as reference ($P < 0.05$). Finally, in the bacterial counts, a significant increase of all the bacteria used ($P \leq 0.0001$) was observed in the last simulated compartment of the *in vitro* model. In conclusion, the results of the present investigation suggest that HA can be used as aflatoxin adsorbents due to their strong capacity to bind $AFB_1$, and as prebiotics due to their capacity to stimulate bacterial growth.



**Keywords**: Broilers, characterization, humic acids, $AFB_1$, bacterial challenges.

**ÍNDICE**

# CARACTERIZACIÓN DE ÁCIDOS HÚMICOS PROVENIENTES DE LOMBRICOMPOSTAS Y SU USO EN MODELOS *IN VITRO* QUE SIMULAN EL TRACTO DIGESTIVO DE LAS AVES DE ENGORDA

## I. INTRODUCCIÓN

Las sustancias húmicas (SH) son el componente principal de la materia orgánica del suelo, son macromoléculas orgánicas granulares de color marrón-negro resultantes principalmente de la descomposición de la materia vegetal (lignina, celulosa y hemicelulosa) y animal (lípidos y proteínas). Debido a su solubilidad, las SH se pueden dividir en ácidos húmicos (AH), ácidos fúlvicos (AF) y huminas. Los AH son solubles en medios alcalinos pero insolubles en medios ácidos. Los AF son solubles en ambos medios alcalinos y ácidos. Por otro lado, las huminas no son solubles en medios alcalino ni ácido. Sin embargo, la presencia de productos de descomposición asociados a la materia orgánica y minerales dificultan su aislamiento y caracterización (Peña-Méndez et al., 2005; Gomez-Rosales y Ángeles, 2015).

Los AH consisten principalmente de unidades alquilo/aromáticas, enlazadas por grupos carboxilos (COOH), fenólicos-OH, alcoholes-OH y carbonilo-OH (Saar y Weber 1979), además de la presencia de grupos amino y otros grupos funcionales de menor abundancia. La presencia de grupos carboxílicos y fenólicos son la principal razón por la cual los AH exhiben propiedades de intercambio catiónico (Gadad et al., 2007; Aeschbacher et al., 2011). Esta función en combinación con las propiedades coloidales, hacen a los AH agentes efectivos para enlazar y

transportar una gran variedad de agentes orgánicos e inorgánicos (Piccolo, 2002)

El potencial del uso de AH como agentes antivirales, antiinflamatorios, estrogénicos, profibrinolíticos y anticoagulantes, entre otras aplicaciones atraen el interés en la medicina y en la industria farmacéutica (Steinbüchel y Marchessault, 2005). En medicina veterinaria, han sido observadas propiedades antiinflamatorias, antioxidantes, antiparasitarias, antivirales y antibacterianas en diversas especies animales (Peña-Méndez et al., 2005; Aeschbacher et al., 2011).

Se han realizado numerosos estudios de los efectos de diversas concentraciones de AH sobre el peso vivo, el consumo de alimento, las características de la canal y las características gastrointestinales en pollos de engorda. La mayoría de los parámetros productivos, incluido el aumento de peso diario y el peso final de las canales de pollos de engorda, se mejoran al agregar AH al agua de bebida o al alimento (Kocabağli et al., 2002; Karaoglu et al., 2004; Rath et al., 2006; Ozturk et al., 2010; Arif et al., 2019).

El proceso por el cual los AH mejoran el rendimiento en las aves no es claro. Shermer et al (1998) propusieron la hipótesis de que los AH pueden afectar el desempeño de las aves al modificar la microbiota del tracto gastrointestinal, favoreciendo una mayor actividad enzimática y mejorando la digestibilidad de los nutrientes. En una investigación reciente, se descubrió que los AH aumentan la viscosidad intestinal, reducen la translocación bacteriana al hígado y la permeabilidad intestinal, influyendo de manera positiva a mantener la integridad

del epitelio intestinal (Maguey-Gonzalez et al., 2018 a). La evaluación de las propiedades fisicoquímicas de los AH es fundamental para el entendimiento de la interacción de estas sustancias con otros compuestos como las aflatoxinas. Por lo que en este trabajo se realizó la caracterización de los AH extraídos de lombricompostas, empleando diversas técnicas con la finalidad de elucidar las posibles interacciones con las bacterias y la aflatoxina $B_1$ en modelos *in vitro* que simula el tracto digestivo de las aves de engorda.

# II. ANTECEDENTES

## Sustancias húmicas

### Origen

Las SH son el componente principal de la materia orgánica, son macromoléculas orgánicas amorfas de color marrón-negro resultantes de la descomposición principalmente de la materia vegetal (lignina, celulosa y hemicelulosa) y animal (lípidos y proteínas). Las SH se forman por una serie de procesos físicos, químicos y biológicos complejos y no del todo esclarecidos, en los cuales, principalmente las eucariotas (detrivoros y hongos) y las procariotas (bacterias aeróbicas) descomponen la materia orgánica (Peña-Méndez et al., 2005; Gomez-Rosales y Ángeles, 2015).

Las SH están presentes en suelos, arroyos, lagos y océanos, constituye alrededor del 80% del carbón en suelos y el 60% del carbón disuelto en medios acuáticos (Lehmann y Kleber, 2015). Su presencia es generalizada a nivel mundial, se pueden encontrar desde las regiones tropicales hasta las árticas, como componente principal de grandes depósitos de turba, leonardita, carbón y esquisto bituminoso (Tan, 2003).

### Características generales de las sustancias húmicas

La composición elemental, la constitución de los principales grupos funcionales y las propiedades estructurales son técnicas comúnmente utilizadas para la

caracterización de las SH. Los principales elementos presentes en las SH son el C (55-59%), H (4.7-6.3%), O (33-41%), N (1.8-3.2%) y S (<1%). La composición elemental relativa en las SH se ve afectada por el origen, grado de maduración y técnica de aislamiento/extracción (Senesi et al., 1989; Ait Baddi et al., 2003; Giovanela et al., 2010; Xavier et al., 2012; Liu et al., 2020).

Senn y Kingman (1973) definieron que las SH son moléculas complejas con alto peso molecular de naturaleza hidrófila y ácida; además, precisaron la forma molecular de las SH por primera vez, determinando que las cargas negativas se atribuyen a la disociación de protones de los principales grupos funcionales en la molécula de las SH (Figura 1).

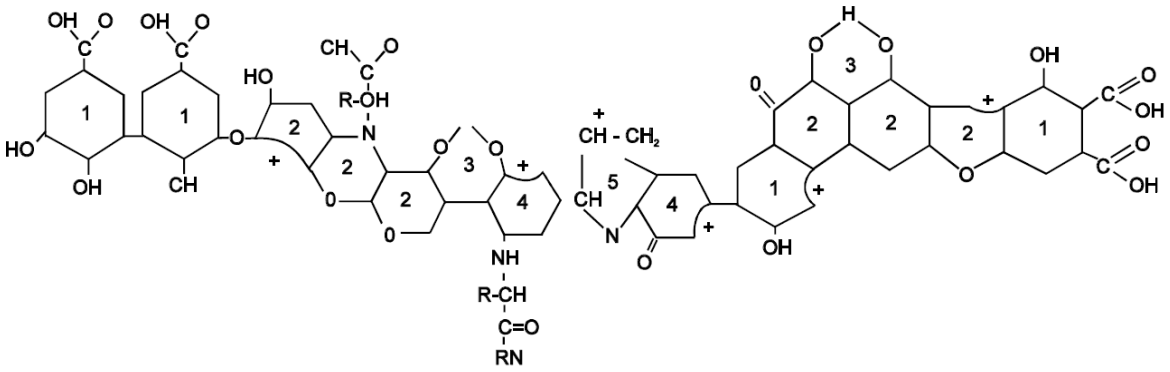


Figura 1. Estructura química de las sustancias húmicas (Senn y Kingman, 1973).

Las SH consisten de unidades alquilo/aromáticas, enlazadas principalmente por grupos carboxilos (COOH), fenólicos-OH, alcoholes-OH y carbonilo (Saar y Weber, 1979), además de la presencia de grupos amino y otros grupos funcionales de menor abundancia. La presencia de grupos carboxílicos y fenólicos

son la principal razón por la cual las SH exhiben propiedades de intercambio cationico (Gadad et al., 2007; Aeschbacher et al., 2011). Estos grupos carboxílicos disocian sus átomos de hidrógeno alrededor de un pH 3.0, mientras que los grupos fenólicos disocian sus protones alrededor de un pH 9.0. En general, estos dos principales grupos funcionales dan a las SH su comportamiento electroquímico (Stevenson y Schnitzer, 1982). Estas características químicas en las SH les confieren sus principales funciones de adsorción, intercambio catiónico, reacciones de quelación y reacciones complejas. En un rango de pH 4 a 8, las SH demuestran una carga negativa en su superficie. Cuando los grupos carboxilo y fenólico se encuentran disociados, da lugar a una función surfactante con la habilidad de unirse a diferentes compuestos que forman complejos químicos hidrofóbicos e hidrofílicos (Gaffney et al., 1996). Esta función en combinación con las propiedades coloidales, hacen a las SH agentes efectivos para transportar y ligar agentes orgánicos e inorgánicos (Piccolo, 2002).

Otras características interesantes de las SH son los altos rangos de peso molecular, que oscilan desde los cientos hasta varios miles de daltons, debido a procesos intermoleculares e intramoleculares de auto-organización. Factores como el pH, la fuerza iónica, la densidad de carga, el grado de ionización de los grupos ácidos, la concentración y las interacciones intermoleculares afectan la conformación estructural de las SH (Senesi et al., 1989; Ait Baddi et al., 2003). A un pH por debajo de 4, la estructura de las SH se encuentra protonada, dando como resultado una estructura agregada o compacta. A valores de pH entre 4 y 6,

la carga superficial es cercana a cero, minimizando la repulsión intramolecular y promoviendo una macro-estructura más extendida y dispersa (Sarlaki et al., 2020). A pH superior a 7, se puede observar una estructura abierta y completamente desplegada (Giovanela et al., 2010).

## Importancia de las sustancias húmicas en la medicina veterinaria

Las SH se han utilizado en humanos durante años como suplementos nutricionales y con fines terapéuticos. Actualmente, los diferentes atributos de las SH atraen el interés de su uso en la medicina y la industria farmacéutica por su potencial como agentes antivirales, antiinflamatorios, estrogénicos, profibrinolíticos, anticoagulantes, entre otros (Steinbüchel y Marchessault, 2005).

En medicina veterinaria, han sido observadas propiedades antiinflamatorias, antioxidantes, antiparasitarias, antivirales y antibacterianas (Peña-Méndez et al., 2005; Aeschbacher et al., 2011). Se ha documentado el uso profiláctico de las SH con dosis de 500 a 2000 mg/kg de peso en caballos, perros, cerdos y aves en terapias antidiarreicas o para aliviar dolencias gastrointestinales (EMEA, 1999). Cabe resaltar su uso como antídoto en la intoxicación por metales pesados como mercurio, cadmio y zinc en peces, ratones y aves (Tan, 2003). Pruebas clínicas sobre la seguridad y toxicología de las SH han demostrado que la dosis letal es de 10 g/Kg de peso vivo de los animales. Por lo tanto el uso de las SH es seguro (Islam et al., 2005).

En estudios *in vitro* sobre las propiedades antioxidantes de las SH, se encontró

que ayudan a mantener el equilibrio en las reacciones de óxido-reducción de las mitocondrias de ratas, además de auxiliar en la eliminación de los radicales libres y de los radicales superóxido (Vaskova et al., 2011). Yasar et al (2002), encontraron 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 las SH en el agua de bebida de ratas.

**Aves de engorda**

Se han realizado diversos estudios para determinar como las SH afectan el peso vivo, el consumo de alimento, los principales parámetros de la canal y las características gastrointestinales en pollos de engorda. La mayoría de los parámetros productivos, incluido el aumento de peso diario y el peso final de las canales de pollos de engorda, se mejoran al agregar SH al agua de bebida o al alimento (Arif et al., 2019; Kocabağli et al., 2002; Karaoglu et al., 2004; Rath et al., 2006; Ozturk et al., 2010).

Yörük et al (2004), reportaron que el índice de conversición alimenticia no se alteró a los 21 días, y mejoró después de los 42 días, lo que sugiere que el aumento en la ganancia de peso y la eficacia en la conversión alimenticia pueden ser causados por el efecto estimulante de las SH en el sistema digestivo, mejorando la utilización de nutrientes y los procesos metabólicos.

Debido a su capacidad adsorbente, las SH se han utilizado para reducir la micotoxicosis en las aves de corral (van Rensburg et al., 2006; Ghahri et al., 2010;

Arafat et al., 2017) además, diversos estudios han demostrado que las SH reducen las emisiones de amoníaco al medio ambiente (Henderson, 2005; Ji y Kim, 2006; Zralý et al., 2008).

El proceso por el cual las SH mejoran el rendimiento en las aves no es del todo claro. Shermer et al. (1998) propusieron la hipótesis de que las SH pueden afectar el desempeño de las aves al modificar la microbiota del tracto gastrointestinal, favoreciendo una mayor actividad enzimática y mejorando la digestibilidad de los nutrientes al aumentar la biodisponibilidad de minerales y otros compuestos mediante la quelación. De acuerdo con Hayirli et al. (2005), varios elementos traza presentes en las SH pueden servir como cofactores que aumentan la actividad de diferentes enzimas necesarias para la digestión y el transporte de nutrientes.

Finalmente, debido a sus características coloidales y la propiedad de formar agregados dentro de las soluciones, se ha propuesto que las SH tienen la capacidad de crear capas protectoras sobre la membrana mucosa epitelial del tracto digestivo, impidiendo la penetración de bacterias patógenas o sustancias tóxicas producidas por estas (EMEA, 1999; Islam et al., 2005; Maguey-Gonzalez et al., 2018 b). La capacidad de las SH para formar barreras protectoras, se atribuye a la creación de cadenas poliméricas en un medio con un pH ligeramente alcalino, como el intestino (Cozzolino y Piccolo, 2001; Piccolo, 2002). Las SH también interactúan con biomoléculas como el colágeno, promueven la resistencia y madurez de sus fibras, lo que resulta en un aumento de la integridad de las vellosidades intestinales (Yasar et al., 2002).

En una investigación reciente, se descubrió que las SH aumentaron la viscosidad intestinal, reduciendo la translocación bacteriana al hígado y la permeabilidad intestinal (Maguey-Gonzalez et al., 2018 a). Además, se ha demostrado que las SH influyen positivamente en la expresión del gen mucine-2 (MUC-2) en la mucosa del ciego (Mudroňová et al., 2020). MUC-2 es una mucina crucial que forma geles y funciona como el componente de barrera principal de las capas del glucocalix, así como un sitio de almacenamiento de inmunoglobulina A secretora (IgA).

**Uso de sustancias húmicas provenientes de lombricompostas en aves de engorda**

Gómez y Ángeles (2015) demostraron que la composta y los lixiviados provenientes de lombricompostas poseen niveles adecuados de SH. Los resultados de esta investigación muestran efectos positivos en la digestibilidad ileal de la energía y el rendimiento en los pollos de engorda. En un experimento posterior, se añadió lixiviado de humus de lombriz pasteurizado a los pollos para eliminar cualquier impacto potencial de los microorganismos en el lixiviado. Sin embargo, en este experimento solo se encontró un mayor rendimiento de la canal y pechuga de los pollos experimentales (Gómez-Rosales et al., 2021). Siguiendo la misma línea de investigación, fue fundamental crear una alternativa más sencilla de suplementar en la dieta de las aves, reduciendo los niveles de inclusión y amplificando los beneficios de los AH (Maguey-Gonzalez et al., 2018 a).

El aislamiento y extracción de los diferentes componentes de las SH (AH, AF y huminas) mediante el uso de una extracción alcalina con NaOH es una técnica estándar utilizada para muestras provenientes de suelos y compostas (de Souza, 2018). Mediante técnicas de caracterización para materiales se estimaron las principales propiedades químicas (Cuadro 1) y las estructuras planas de las SH con aromaticidad (Figura 2) utilizando el software de química ACD Lab v.12 (Advanced Chemistry Development, Toronto, Canadá) (Angeles et al., 2022).

Cuadro 1. Propiedades químicas estimadas de las moléculas de las sustancias húmicas provenientes de lombricompostas.

| | Ácido húmico | Ácido fúlvico |
|---|---|---|
| Fórmula | $C_{110}H_{105}N_7O_{50}$ | $C_{25}H_{17}N_0O_{18}$ |
| Peso molecular | 2325.02 | 619.39 |
| Composición elemental (%) | C (56.82), H (4.55), N (4.22), O (34.41) | C (48.48), H (2.77), N (2.26), O (46.49) |
| Densidad, $g/cm^3$ | 1.870 ± 0.10 | 1.935 ± 0.06 |

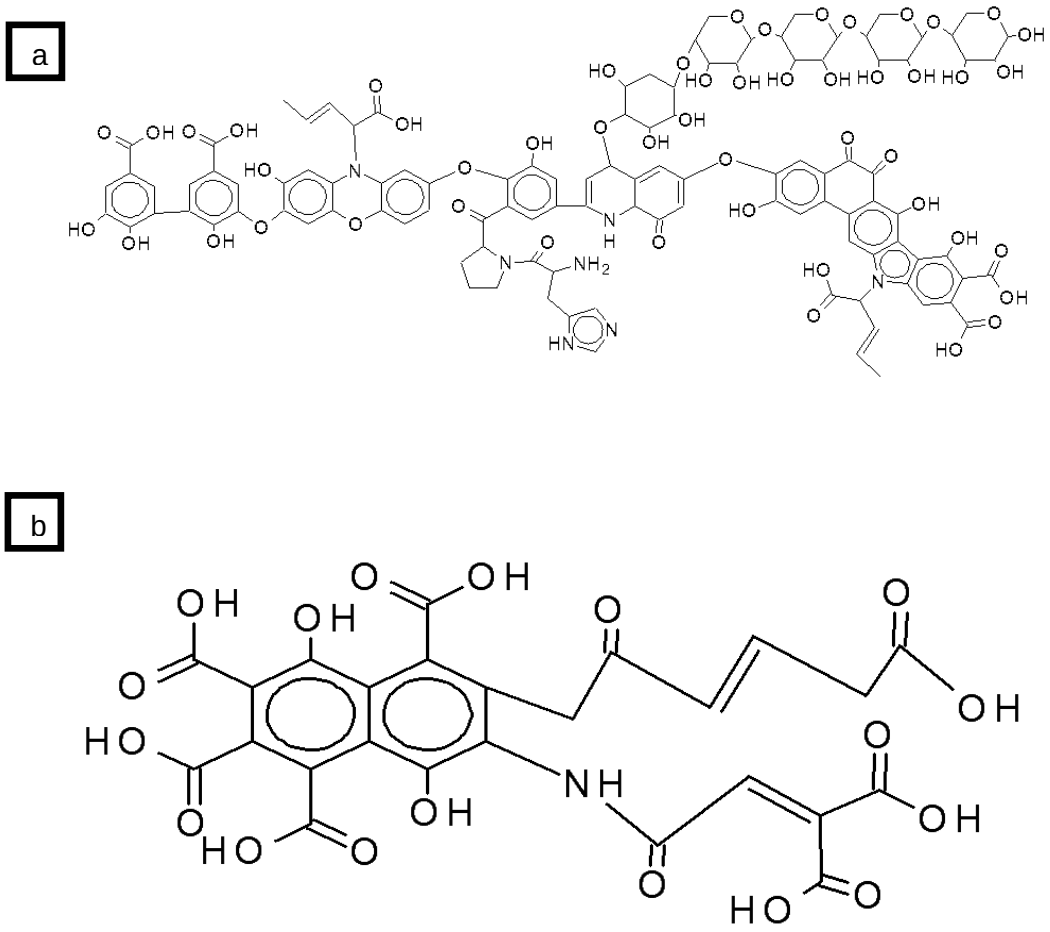


Figura 2. Representación de la estructura plana de una molécula de ácido húmico con aromaticidad (a) y la estructura plana de una molécula de ácido fúlvico con aromaticidad (b).

Recientemente se han establecido diversos modelos *in vitro* e *in vivo* para evaluar el comportamiento de diferentes sustancias químicas utilizadas como suplementos para aves de engorda (Tellez et al., 2014; Latorre et al., 2015; Vicuña et al., 2015; Kuttappan et al., 2015; Menconi et al., 2015; Baxter et al., 2017). Con la ayuda de estos modelos de investigación, se ha podido dilucidar algunos efectos de los AH. En primer lugar, se realizaron dos experimentos para evaluar los efectos de los AH

sobre los conteos bacterianos de *Salmonella* Enteritidis (SE) (Maguey-Gonzalez et al., 2018 b); no se encontraron efectos en la reducción de los conteos bacterianos en el modelo *in vitro*, o en la colonización intestinal, y tampoco se encontraron diferencias estadísticas en los conteos de coliformes y bacterias ácido lácticas (BAL) en el ciego de los pollos de engorda.

Adicionalmente, se realizó un segundo experimento con un modelo de restricción de alimentación de 24 horas, con el objetivo de investigar cómo los AH afectan la viscosidad y permeabilidad intestinal, así como la excreción de amoníaco en pollos de engorda. El grupo experimental que recibió 0.2 % de AH aumentó la viscosidad y disminuyó la permeabilidad intestinal, la translocación bacteriana al hígado, y la presencia amoníaco en las excretas. Por primera vez se confirmó que los AH tienen la capacidad de mejorar la viscosidad, y por ende, la integridad intestinal (Maguey-Gonzalez et al., 2018a).

En investigaciones posteriores en pollos de engorda suplementados con un extracto de SH, se observó un aumento del rendimiento de la canal y conteos de BAL, además se observó una reducción de la excreción de ooquistes de coccidia (Domínguez-Negrete et al., 2019). En un estudio recientemente realizado en pollos de engorda alimentados con niveles crecientes de SH, las aves mostraron un menor consumo de alimento, asociado a una mejor tasa de conversión alimenticia, además se observó una reducción de la mortalidad en comparación con las aves no suplementadas con SH y las suplementadas con antibióticos (Domínguez-Negrete et al., 2021).

La suplementación líquida de SH en el agua de bebida mostró la capacidad de quelar minerales, efecto asociado a la mejora de la mineralización ósea de tibias en pollos de engorda a los 21 y 42 días (Ángeles et al., 2022).

Adicionalmente, aves de engorda de 28 días de edad suplementadas con SH fueron sometidas a un cambio drástico en la dieta y a estímulo inmunológico mediante vacunación. Las SH aumentaron el número de células caliciformes y probablemente aumentaron la viscosidad del contenido intestinal, aunque no se observó reducción de la atrofia de la mucosa intestinal (López-García et al., 2023).

El efecto adsorbente de micotoxinas de los AH ha sido ampliamente estudiado (van Rensburg et al., 2006; Ghahri et al., 2010; Arafat et al., 2017), demostrando una reducción significativa en el efectos adversos causados por las micotoxinas en las aves de engorda. Cabe resaltar que el uso de SH en la mayoría de los estudios relacionados al efecto adsorbente de micotoxinas provenien de fuentes minerales. Hasta el momento no existe en la literatura disponible el efecto de adsobente de micotoxinas por AH provenientes de lombricompostas.

## III. JUSTIFICACIÓN

Debido a la prohibición del uso de antibióticos promotores del crecimiento como aditivos en los alimentos de los animales, desde hace varias décadas se empezaron a evaluar un sinnúmero de aditivos promotores del crecimiento no-farmacológicos. Uno de los productos que se ha estudiado desde hace algunos años como mejorador de la salud intestinal de los animales son los AH. La

mayoría de los AH comerciales son derivados de elementos minerales (lignitos), cuya fuente húmica fue purificada. Una de las fuentes alternas procede de las lombricompostas, de donde se obtiene composta y lixiviados con una buena concentración de AH. Algunos estudios sugieren que los AH utilizados con prácticas adecuadas de nutrición, manejo y bioseguridad mejoran la integridad intestinal y el rendimiento productivo de las aves de corral. No obstante, los efectos positivos observados, se desconoce la forma en cómo actúan dentro del organismo. Los posibles mecanismos de acción se remiten a la modulación de la microbiota intestinal y a la protección del epitelio intestinal, mecanismos que solo han sido teorizados. Recientemente, con el uso de un modelo que induce inflamación intestinal, se probó de forma experimental que los AH influyen de manera positiva para mantener la integridad del epitelio intestinal a través del aumento de la viscosidad intestinal. Actualmente, se requiere la ayuda de metodologías de caracterización para analizar los AH extraídos de lombricompostas y conocer más sobre sus interacciones con bacterias y aflatoxina $B_1$ en modelos *in vitro* que simulan el tracto digestivo de las aves de engorda.

## IV. HIPÓTESIS

La inclusión de un extracto de ácidos húmicos procedente de lombricompostas en dietas que no incluyan promotores del crecimiento y adsorbentes de micotoxinas, reducirá los conteos bacterianos, además de disminuir la $AFB_1$ libre en un modelo *in vitro* que simula el tracto digestivo de las aves de engorda.

## V. OBJETIVOS

### Objetivo general

Caracterizar ácidos húmicos provenientes de lombricompostas y evaluar su efecto en modelos *in vitro* que simulan el tracto digestivo de las aves de engorda

### Objetivos particulares

I. Caracterizar los ácidos húmicos mediante las técnicas de potencial Z (ζ), punto de carga cero (pHpzc), espectroscopía de infrarrojo con transformada de Fourier con reflexión total atenuada (FTIR-ATR), microscopía electrónica de barrido (SEM) y espectroscopía de rayos X con dispersión de energía (EDS).

II. Evaluar la capacidad adsorbente de los ácidos húmicos sobre Aflatoxina $B_1$, en un modelo *in vitro* que simula el tracto digestivo de las aves de engorda.

III. Evaluar los efectos de los ácidos húmicos en un modelo *in vitro* que simula el tracto digestivo de las aves de engorda bajo diferentes retos bacterianos (*Salmonella* Enteritidis*, Escherichia coli*, *Clostridium perfringens, Bacillus subtilis* y *Lactobacillus salivarius*).

## VI.ARTÍCULOS CIENTÍFICOS

## V.I Artículo 1

toxins

MDPI

*Article*

# Humic Acids Preparation, Characterization, and Their Potential Adsorption Capacity for Aflatoxin $B_1$ in an In Vitro Poultry Digestive Model

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**Abstract:** Vermicompost was used for humic acid (HA) preparation, and the adsorption of aflatoxin $B_1$ ($AFB_1$) was investigated. Two forms of HA were evaluated, natural HA and sodium-free HA (SFHA). As a reference, a non-commercial zeolitic material was employed. The adsorbents were characterized by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), energy-dispersive X-ray spectroscopy (EDS), zeta potential (ζ-potential), scanning electron microscopy (SEM), and point of zero charge (pHpzc). The adsorbent capacity of the materials when added to an $AFB_1$-contaminated diet (100 μg $AFB_1$/kg) was evaluated using an in vitro model that simulates the digestive tract of chickens. Characterization results revealed the primary functional groups in HA and SFHA were carboxyl and phenol. Furthermore, adsorbents have a highly negative ζ-potential at the three simulated pH values. Therefore, it appears the main influencing factors for $AFB_1$ adsorption are electrostatic interactions and hydrogen bonding. Moreover, the bioavailability of $AFB_1$ in the intestinal section was dramatically decreased when sorbents were added to the diet (0.2%, *w*/*w*). The highest $AFB_1$ adsorption percentages using HA and SFHA were 97.6% and 99.7%, respectively. The zeolitic material had a considerable adsorption (81.5%). From these results, it can be concluded that HA and SFHA from vermicompost could be used as potential adsorbents to remove $AFB_1$ from contaminated feeds.

**Keywords:** adsorption; aflatoxin $B_1$; humic acids; in vitro digestion model

**Key Contribution:** Humic acid and sodium-free humic acid are highly effective in the adsorption of $AFB_1$ and could be used as potential adsorbents to remove aflatoxins from contaminated feeds.

## 1. Introduction

Some toxigenic fungi can synthesize mycotoxins as secondary metabolites; mycotoxins have a wide range of chemical structures and a low molecular weight and threaten human and animal health [1]. The most common way to consume mycotoxins is through food contamination; however, exposure can also happen when spores are directly contacted or inhaled [2]. Of all the mycotoxins, aflatoxin $B_1$ ($AFB_1$) is the most harmful. Two closely related fungi, *Aspergillus flavus* and *Aspergillus parasiticus*, produce primarily aflatoxins. For instance, *A. togoensis* only synthesizes $AFB_1$; *A. flavus* and *A. pseudotamarii* synthesize $AFB_1$ and aflatoxin $B_2$ ($AFB_2$), while *A. aflatoxiformans*, *A. arachidicola*, *A. austwickii*, *A. cerealis*, *A. luteovirescens*, *A. minisclerotigenes*, *A. mottae*, *A. nomius*, *A. novoparasiticus*, *A. parasiticus*, *A. pipericola*, *A. pseudocaelatus*, *A. pseudonomius*, *A. sergii*, and *A. transmontanensis* produce $AFB_1$, $AFB_2$, aflatoxin $G_1$ ($AFG_1$), and aflatoxin $G_2$ ($AFG_2$) [3]. The toxic effects of $AFB_1$ on poultry are well known. Low production and a high vulnerability to illness are hazards of $AFB_1$ in poultry [2]. Hepatotoxic consequences include reduced liver-to-body weight ratios, changes in liver enzymes, abnormal blood-clotting pattern, and histological abnormalities, such hepatocellular necrosis and biliary hyperplasia [4].

The detoxification of mycotoxin-contaminated grains can be accomplished using a variety of procedures, including physical removal, chemical conversion to less toxic products, enzymatic detoxification, and microbial degradation, among others [5]. A better approach to reducing the harmful effects of mycotoxins on animal health would be to include more natural active ingredients in the feed. In the study of mycotoxin binders, substances derived from plants play a significant role [5,6]. Additionally, decontamination procedures must be integrated into animal diets in a simple, affordable, and safe manner Physical methods of removing or inactivating mycotoxins are less expensive and easier to use than chemical methods [7]. The effectiveness of adsorbents is related to their structure, charge distribution, and surface area. The shape and polarity of mycotoxins also affect their binding affinity [8].

Humic substances (HS), mainly composed of humic acids (HA), fulvic acids (FA), and humins, are heterogeneous macromolecules with numerous negatively charged functional groups, primarily carboxyl and phenol [9]. These groups are thought to be potential pollutant-binding sites, such as metallic species, herbicides, and pesticides [10], as well as complex formations with metal cations [11]. Recently, our research group studied an extract of HA from vermicompost under different experimental conditions to clarify its mechanism of action [12–14]. In previous in vitro and in vivo studies, several sources of HS containing mixtures of HA and FA have been used as mycotoxin binders [15]; however, purified HA from vermicompost has never been tested against $AFB_1$. HAs extracted from vermicompost are still in an early humification process and are considered immature; this factor may cause structural differences compared to HA extracted from lignites, leonardites, or other HA-aged sources

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), energy-dispersive X-ray spectroscopy (EDS), zeta potential (ζ-potential), scanning electron microscopy (SEM), and point of zero charge (pHpzc) are some important techniques for characterizing mycotoxins binders [16]. These techniques involve little material and are easily repeatable, non-destructive, and reasonably straightforward. These techniques may help to characterize the main chemical components of HA extracted from vermicompost, which may help to elucidate the possible mechanisms of action of HA as potential aflatoxin binder. As a result, the purpose of this study was to prepare and characterize

HA from vermicompost and evaluate its adsorption capacity for $AFB_1$ in an in vitro poultry digestive model.

## 2. Results and Discussion

### *2.1. Characterization*

#### 2.1.1. ATR-FTIR

Figure 1 shows the ATR-FTIR spectra of HA, SFHA, and the zeolite. The main FTIR bands and their corresponding assignments are shown in Table 1. In general, both HA adsorbents have a wide variety of functional groups, and their spectra showed high intensity for five principal bands: (A) 3238–3267 $cm^{-1}$ associated with OH-stretching vibrations, (B) 2926–2927 $cm^{-1}$ related to aliphatic groups, (C) 1571–1957 $cm^{-1}$ associated with carboxyl, amide, and aromatic vibrations, (E) 1412–1420 $cm^{-1}$ associated with carboxyl, aromatic, and phenol vibrations, and (G) 1120–1123 $cm^{-1}$ associated with C-O carbonyl. Furthermore, HA shows two distinctive bands at (I) 846 $cm^{-1}$ and (J) 620 $cm^{-1}$ assigned to the aromatic and aliphatic groups. The lack of certain bands in the FTIR spectra indicates a lower content of functional groups. Therefore, the absence of some bands in the SFHA spectra indicates HA has higher quantities of aromatic and aliphatic groups. On the other hand, SFHA exhibits three specific bands at (D) 1508 $cm^{-1}$ (carboxyl, amide, and aromatic groups), (F) 1215 $cm^{-1}$ (carboxyl vibrations), and (H) 1030 $cm^{-1}$ (aromatic groups). The band located between 1100 $cm^{-1}$ and 1035 $cm^{-1}$ corresponds to the Si-O stretching of silicate [17]. Its presence is attributed to impurities of aluminosilicates not eliminated during the HA extraction process from soils. However, the non-commercial zeolitic material also exhibited the distinctive band associated with $Al^{3+}$-OH at (A) 3621 $cm^{-1}$. Significant water absorption at the B (3387 $cm^{-1}$) and C bands (1630 $cm^{-1}$) proved the zeolite was also hydrated. These bands (B and C) are commonly associated with water molecules linked with $Na^{+}$ and $Ca^{2+}$ in the channels and cages of the zeolitic material. The band at (D) 1000 $cm^{-1}$ corresponds to the stretching vibration of T–O in $TO^4$ tetrahedra (T = Si and Al). Furthermore, the bands at (E) 793 $cm^{-1}$ and (H) 444 $cm^{-1}$ are related to the stretching vibration of O–T–O and the bending of T–O bonds, respectively [17]. Finally, the absorption band at (F) 600 $cm^{-1}$ is associated with the presence of heulandite [18]. These results are in accordance with those obtained by other researchers [19–23]. In general, HA contains a wide variety of acidic functional groups, such as carboxylic, carbonyl, hydroxyl, and phenolic (hydrophilic domains), as well as methyl, aliphatic, and aromatic moieties (hydrophobic domains). These functional groups are considered potential sites for binding pollutants [11,24], and for building complexes with certain metal cations [10]. Other studies indicate OH, C-O, C=O, and phenolic groups form hydrogen bonds with certain pollutants [17,25,26]. For instance, Vázquez-Durán et al [27] indicated OH could establish hydrogen bonds with the oxygen atoms in the methoxy, carbonyl, and ether groups of the $AFB_1$ molecule.

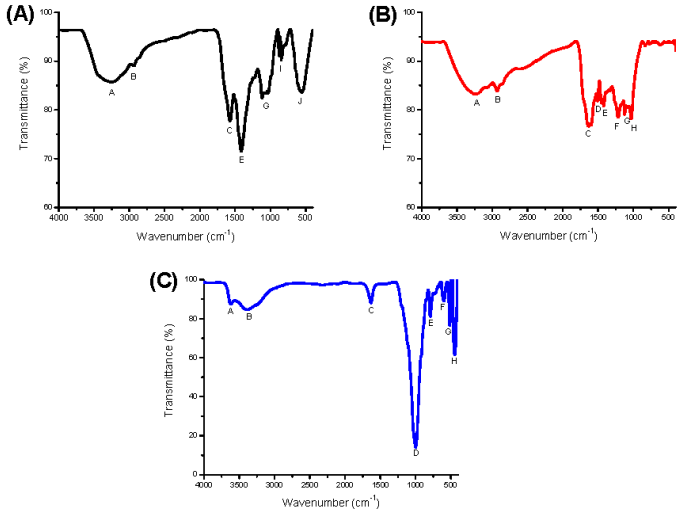


**Figure 1.** Comparative Fourier transform infrared spectra of: (**A**) humic acids, (**B**) sodium-free humic acids, and (**C**) the inorganic mycotoxin binder (zeolite).

**Table 1.** Band assignments of the vibrational frequencies in the humic acids and the inorganic mycotoxin binder (zeolite).

| Band | Wavenumber ($cm^{-1}$) | | Associated Functional Group |
|---|---|---|---|
| | HA | SFHA | |
| A | 3267 | 3238 | O-H stretching vibrations and partially N-H stretch |
| B | 2926 | 2927 | Aliphatic $CH_2$ and $CH_3$ |
| C | 1571 | 1597 | $COO^-$, amides ($NH_2$ of NH bonding) |
| D | | 1508 | C=O of carboxylic groups and amide; N–H stretch; aromatic C=C |
| E | 1412 | 1420 | $COO^-$ C=C stretch (aromatic ring), O–H and C–O of phenolic; C–N; C–H of $CH_3$, $CH_2$ and CH |
| F | | 1215 | C–O and O–H stretch of COOH and phenol |
| G | 1120 | 1123 | C–O stretch of alcohols, carbonyl, esters and ethers, O-H of phenol and carbohydrates |
| H | | 1030 | Aromatic ethers, C-O of carbohydrates; Si-O of silicate |
| I | 846 | | Aromatic groups |
| J | 620 | | Aliphatic –$CH_2$ |
| | | | Zeolite |
| A | 3621 | | O-H stretching ($Al^{3+}$-OH) |
| B | 3387 | | O-H stretching vibrations |
| C | 1630 | | H-O-H bending (water) |
| D | 1000 | | Si-O-Si antisymmetric stretch |
| E | 793 | | Si-O-Si stretching symmetric |
| F | 600 | | $SiO_4$ and $AlO_4$ tetrahedral |
| G | 515 | | Si-O bending vibration |
| H | 444 | | Si-O bending mode (-$SiO_4$-) |

2.1.2. SEM

The surface morphology and microstructure of the sorbents were evaluated using a series of acquired SEM images (Figure 2). The surface morphology of the zeolite is amorphous, and large clumps cover the pores. On the other hand, HA and SFHA had rough and uneven surfaces with aggregates of different shapes and sizes. Specifically for HA, most of the aggregated particles were approximately 163.42 ± 20.14 µm in size. Moreover, grooves on the particle surface with the trace of granules were also observed. These results are consistent with the granular structure of several HAs extracted from various sources [28,29]. HA's physical and chemical compositions differ considerably depending on its source, environmental conditions, and extraction procedure [20,24]. Several variables, including concentration, pH, ionic strength, charge density, acidic group ionization degree, and intermolecular interactions, influence the structural conformation of HA [30,31]. The HA structure is protonated at low pHs (below 4), resulting in a more condensed structure due to the establishment of H-bonds and van der Waals interactions. However, at pH levels between 4 and 7, HA adopts a more expanded and scattered macrostructure due to intra and intermolecular repulsion. Furthermore, at pH greater than 11, a condensed structure can also be observed [32]. According to this research, certain HA molecules engage in supramolecular interactions through dispersive forces, such as hydrogen bonds, van der Waals interactions, and π–π interactions [28,33].

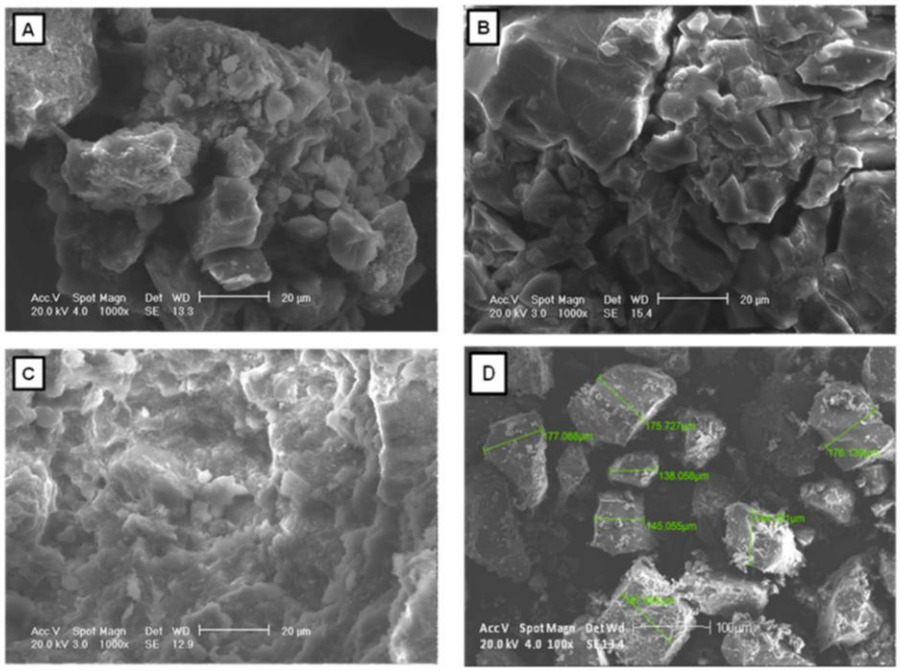


**Figure 2.** Structure of (**A**) humic acids, (**B**) sodium-free humic acids, and (**C**) the zeolite under SEM at ×1000. Measurements (**D**) of humic acids at ×100.

2.1.3. EDS

Figure 3 shows the energy-dispersive X-ray spectroscopy (EDS) spectra of the HA, SFHA, and the zeolite. Moreover, the elemental compositions of HA, SFHA, and the zeolite are shown in Table 2. Results indicated the principal elements in HA and SFHA were C with 35.42% and 43.13%, and O with 20.32% and 46.43%, respectively. The Na content of SFHA considerably dropped, decreasing from 27.68% to 1.78% due to its reduction by washing. Other minor elements were involved in HA materials, such as Al, Si, K, P, S, and Cl. Meanwhile, Mg and Fe were not detected. Parameters are found within published ranges in the literature [34,35]. Information on the elemental composition of HA is not particularly conclusive due to their complexity and a wide range of other factors [36]. In addition, the main elements in the zeolite were Si (40.93%), C (31.99%), O (14.13%), and Al (7.46%), and minor contents of Na (0.25%), K (1.80%), Mg (1.12%), Ca (1.45%), and Fe (0.87%). Furthermore, Cl, P, and S were not detected. Similar chemical compositions for this kind of material have been reported by other researchers [37–39].

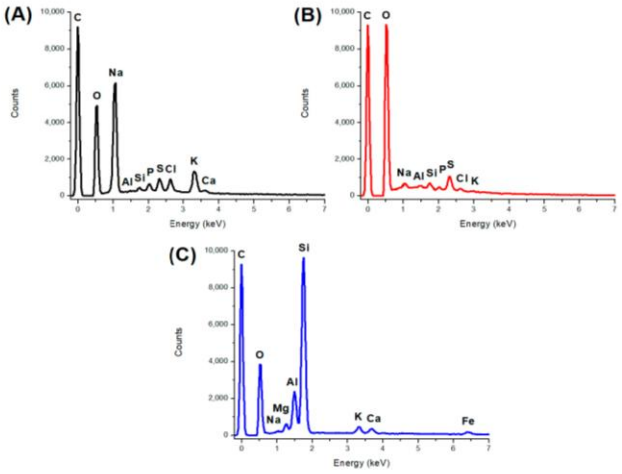


**Figure 3.** Representative energy-dispersive X-ray spectroscopy (EDS) spectra of (**A**) humic acids, (**B**) sodium-free humic acids, and (**C**) the zeolite.

**Table 2.** The elemental composition (%) of humic acids (HA), sodium-free humic acids (SFHA) and the zeolite.

| Element | Adsorbent | | | SEM | *p*-Value |
|---|---|---|---|---|---|
| | HA | SFHA | Zeolite | | |
| C | 35.42 $^{b}$ | 43.13 $^{a}$ | 31.99 $^{c}$ | 0.20 | <0.0001 |
| O | 20.32 $^{b}$ | 46.43 $^{a}$ | 14.13 $^{c}$ | 0.61 | <0.0001 |
| Na | 27.68 $^{a}$ | 1.78 $^{b}$ | 0.25 $^{c}$ | 0.11 | <0.0001 |
| Al | 0.26 $^{c}$ | 0.78 $^{b}$ | 7.46 $^{a}$ | 0.17 | <0.0001 |
| Si | 0.82 $^{c}$ | 1.60 $^{b}$ | 40.93 $^{a}$ | 0.08 | <0.0001 |
| K | 6.66 $^{a}$ | 0.17 $^{c}$ | 1.80 $^{b}$ | 0.04 | 0.03 |
| P | 1.53 $^{a}$ | 0.59 $^{b}$ | ND | 0.24 | 0.02 |
| S | 3.00 $^{b}$ | 4.80 $^{a}$ | ND | 0.21 | 0.004 |
| Cl | 3.77 $^{a}$ | 0.71 $^{b}$ | ND | - | - |
| Mg | ND | ND | 1.12 a | - | - |
| Ca | 0.54 $^{b}$ | ND | 1.45 $^{a}$ | - | 0.02 |

| | | | | | |
|---|---|---|---|---|---|
| Fe | ND | ND | 0.87 | - | - |

[abc] Means with non-matching superscripts within rows indicates a significant difference at $p < 0.05$. ND = Not detected.

2.1.4. ζ-Potential

In colloidal systems, the ζ-potential is frequently employed to track the behavior of particles suspended in a liquid. Additionally, the magnitude of the ζ-potential indicates the strength of the electrostatic attraction or repulsion between particles; therefore, it may be used to describe the surface of charged particles [40]. Figure 4 shows the relationship between pH and ζ-potential of HA, SFHA, and the zeolite. The three adsorbents generally show the same behavior: as the pH increases (from 2 to 11), the ζ-potential value becomes more negative. The pH values used for the determination of ζ-potential were 2, 5, and 7, each one according to the simulated compartment (crop, proventriculus, and intestine) in the in vitro digestive model. At pH 2, values of −46.44 mV and −30.75 mV were observed; at pH 5 of −50.16 mV and −49.2 mV; and at pH 7 of −54.46 mV and −40.93 mV for HA and SFHA, respectively. As a result, at all three pH values tested, HA was more negative, followed by SFHA and the zeolite. Our findings are consistent with those reported by Hamza et al [41] who state as the pH rises, the acidic functional groups of HA deprotonate, resulting in a more negative surface. Moreover, Deng and Bai [42] also found when pH exceeds 1.9, HA has a negative ζ-potential. Omar et al. (2014) and Coles and Yong [43] also reported ζ-potential values of HA of −20 mV at pH 3 and −44 mV at pH 10. Finally, Loosli et al. [44] reported strong negative charge with ζ-potential values ranging from −30.2 mV at pH 3 to −69.0 mV at pH 11. Even at low pH, HAs are negatively charged because of the dissociation of their acidic functional groups (mainly carboxylic and phenolic hydroxyl) [26,45]. Instead of aromatic components, variations in acid group conformation can be used to explain how the interactions are pH-dependent [32]. The first decrease (pH 3) of ζ-potential corresponds to the dissociation of carboxylic acid groups, while the second (pH 6) corresponds to the start of ionization of phenolic acid groups [46]. Our findings show the three adsorbents have a highly negative ζ-potential at the three simulated pH levels of the in vitro model, particularly at pH 7. As a result, the $AFB_1$ molecule and the HA particle surface might be attracted by electrostatic forces since HAs are anionic polyelectrolytes that can interact with the positively charged $AFB_1$ molecules [47].

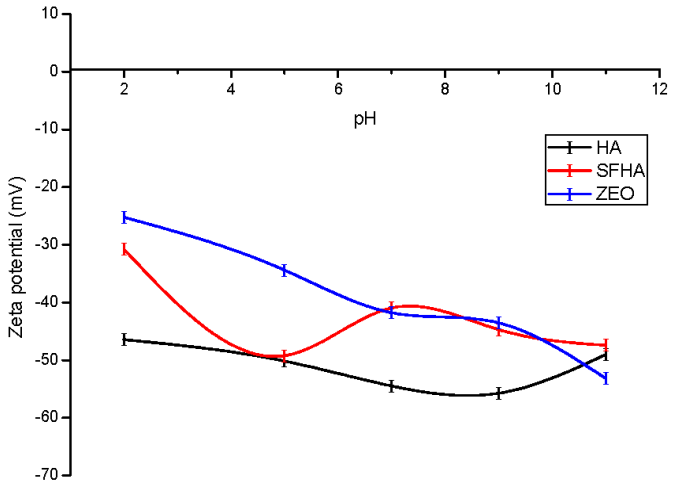


**Figure 4.** Relationship between zeta potential (ζ-potential) and pH of humic acids, sodium-free humic acids, and the zeolite. Mean of five replicates ± standard error.

2.1.5. pHpzc

Understanding the surface charge of the particles is greatly aided by the pHpzc, which stands for the point of zero charge where the sum of positive and negative charges is equal. Figure 5 shows the pHpzc of HA, SFHA, and the zeolite. It has been hypothesized that the surface of the adsorbent will be positively charged if pH < pHpzc and negatively charged if pH > pHpzc [47]. From the curves, the pHpzc for HA, SFHA, and the zeolite were 10.4, 2.2, and 8.8, respectively. According to Coles and Yong [43], the pHpzc of HA was less than 0.5. Moreover, Gjessing [48] reported pHpzc values for HA ranging from 1.2 to 1.8, which is very similar to the value of SFHA in our study. Furthermore, Giasuddin et al [49] reported the HA's surface charge was negative over a pH range of 5 to 9.3. However, the presence of organic matter in the HA determines whether the pHpzc decreases or increases [43].

Interestingly, compared to the pHpzc of HA, which was 10.4, the SFHA reduced their pHpzc value to 2.2 after washing. This significant change in the pHpzc value could be attributed to a change in organic matter composition or to the purification process used to reduce the sodium salts in the HA. In this context, $AFB_1$ adsorption is expected to be significant because SFHA has a negatively charged surface in all compartments simulated in the in vitro study. HA and the zeolite, on the other hand, have a positive surface charge on all three gastrointestinal sections, indicating electrostatic interactions do not govern $AFB_1$ adsorption. It is well known that other mechanisms, such as weak electrostatic interactions and moderate electron donor–acceptor attraction, contribute to the adsorption of aflatoxins into inorganic binders [37].

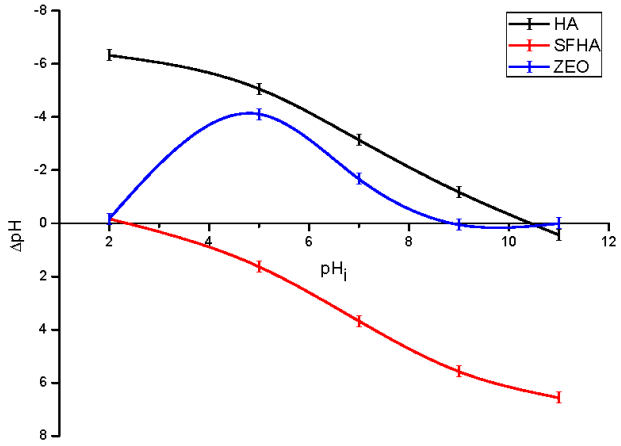


**Figure 5.** Point of zero charge (pHpzc) of humic acids, sodium-free humic acids, and the zeolite. Mean of five replicates ± standard error.

*2.2. In Vitro Digestive Model*

Figure 6 depicts the percentage of $AFB_1$ adsorption of the three tested sorbents. At the end of the in vitro model (the intestinal section), HA and SFHA adsorbents had 97.6% and 99.7% $AFB_1$ uptake, respectively. Using the zeolite, a moderate sorption uptake of 81.5% was reached. In contrast, controls (without the addition of sorbent materials) show a marked lack of $AFB_1$ adsorption (<3%). Following our findings, it was reported an in vitro model with 100 mg/mL of HA inclusion at 20 ng $AFB_1$/g achieved 90.50% of $AFB_1$ adsorption [50]. Moreover, Ye et al. [51] investigated the $AFB_1$ adsorption capacity in the

presence of sodium humate inclusions, various pH levels, interaction times, and $AFB_1$ concentrations. The highest $AFB_1$ adsorption percentages reported were 88.12% at pH 7 and 76.36% at pH 8. Furthermore, Vázquez-Durán et al. [27], using a dynamic in vitro model to assess the $AFB_1$ adsorption capacity of a non-commercial zeolite at 5% (*w*/*w*) inclusion, reported an adsorption percentage of 75.5%, which is consistent with this research.

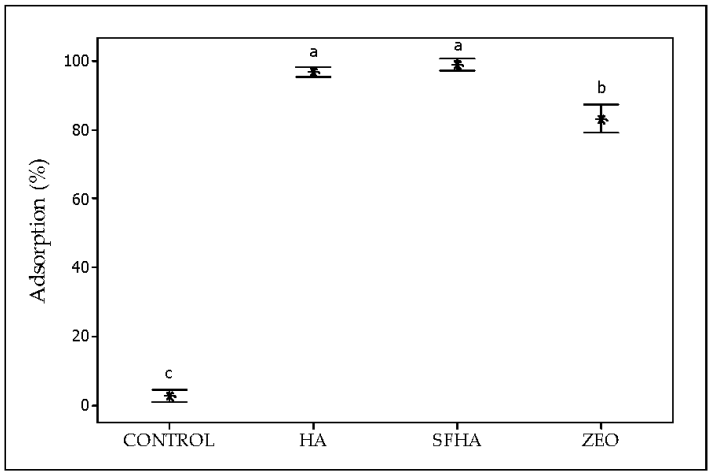


**Figure 6.** The adsorption capacity of humic acids (HA), sodium-free humic acids (SFHA), and the zeolite against $AFB_1$ using an in vitro digestive poultry model. Mean values ± standard error. [a-c] Means with different letter are statistically different (Tukey $p \leq 0.05$).

### *2.3. The Mechanism for $AFB_1$ Adsorption onto HA*

Because HA possesses highly hydrophobic surfaces and a wide variety of negatively charged functional groups [9], interactions between HA and $AFB_1$ may involve different mechanisms. Tan [52] lists seven potential ways HA might bind to gaseous, liquid, and solid components: (i) physical forces, (ii) chemical forces, (iii) hydrogen bonds, (iv) hydrophobic interactions, (v) electrostatic interactions, (vi) coordination reactions, and (vii) ligand exchange. The most important interactions between HA and $AFB_1$ are electrostatic interactions and hydrogen bonding. Nevertheless, other interactions could be considered, such as π–π stacking [53] and hydrophobic interactions (due to the many bond indices related to hydrophobic groups, including $CH_2$, $CH_3$, and C=C) [54] (Figure 7). Although this phenomenon is still not fully understood, aromatic structures and functional groups, such as OH and COOH, contributed to HA's high $AFB_1$ adsorption capacity [55]. Several techniques have been proposed to investigate the adsorption of different molecules onto HA. For example, physical modelling (Langmuir isotherm), kinetic modelling (Elovich kinetic model), surface complexation modelling, and Ligand and charge distribution model, among others [54]. However, to elucidate the nature of the molecular interactions between HA and $AFB_1$, future theoretical simulations using density functional theory (DFT) may be considered [56].

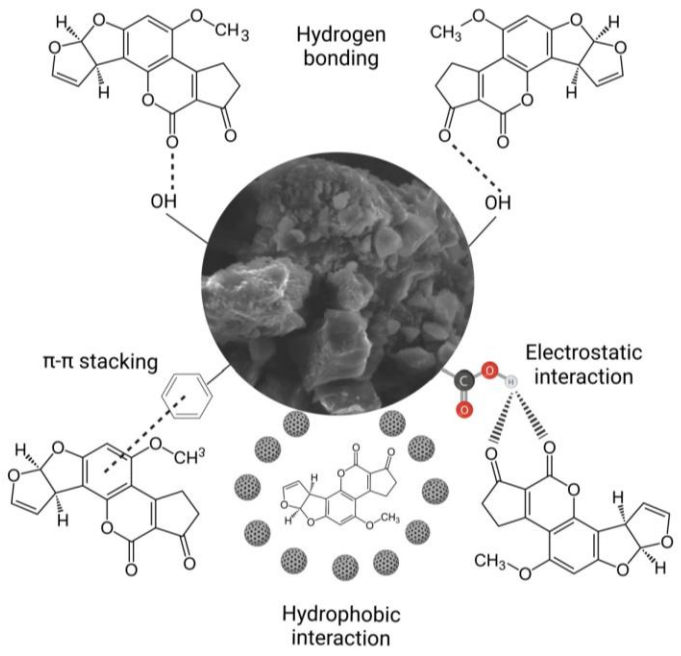


**Figure 7.** The hypothetical mechanism by which humic acids bind the $AFB_1$ molecule.

Nevertheless, because in vitro tests cannot fully simulate the conditions of a bird's digestive tract to determine the effectiveness of HA derived from vermicompost in reducing the harmful effects of $AFB_1$, in vivo experiments must be conducted. Data on the effectiveness of HA extracted from vermicompost to reduce the impact of $AFB_1$ in broilers are still meager. However, previous research has demonstrated the effectiveness of various humic substances as mycotoxins binders in in vivo trials. For instance, the addition of oxihumate (3.5 g/kg) was found to have a protective effect against liver, stomach, and heart damage in diets contaminated with $AFB_1$, and a significative decrease in several serum, and hematological biochemical indicators linked to aflatoxin toxicity was also seen in broilers [57]. Adding HA to chicken feed in amounts ranging from 0.2% to 0.4% (*w*/*w*) improved feed efficiency, reduced liver and bursa damage, and improved serum biochemical profiles associated with aflatoxin toxicity [58]. Additionally, adding HA (0.3% *w*/*w*) decreased $AFB_1$ residues in the liver and enhanced broiler antibody production against Newcastle disease [59].

## 3. Conclusions

In this study, two forms of HAs extracted from vermicompost were prepared, further characterized, and tested in $AFB_1$ adsorption experiments using an in vitro poultry digestive model. Despite differences in the microstructure, elemental composition, surface functional groups, pHpzc, and ζ-potential, both adsorbents demonstrated significant $AFB_1$ adsorption capacities when using an in vitro poultry digestive model. As a result, it can be concluded HA derived from vermicompost is highly effective in the adsorption of $AFB_1$. However, more in vivo studies will enhance our comprehension of HA efficacy to reduce the toxic effects of $AFB_1$ in poultry. There is now research being conducted in this area.

## 4. Materials and Methods

### *4.1. Humic Acids*

As previously described [12], HAs were extracted and isolated from a vermicompost. HAs were extracted with a sodium hydroxide solution (1M NaOH) in compost:alkali ratio of 1:4, then stirred for 2 h. A Whatman grade 40 filter paper was used to filter the suspension after it had been at room temperature for 24 h. The supernatant was then separated by decantation after the filtrate had been centrifuged for 15 min at 3500× *g*. The HA-containing supernatant was acidified with 10% HCl and agitated continuously until pH 2 was achieved, allowing the HA to precipitate. Centrifugation at 3500× *g* for 15 min separated HA from FA. Finally, the precipitate (HA) was normalized with 1M NaOH until pH 10 was achieved, then oven-dried at 60 °C. A black powder was produced as a result. To produce the second adsorbent material, hereinafter referred to as sodium-free humic acids (SFHA), HAs were redispersed in deionized water and neutralized with 10% HCl until pH 7 was reached, then washed (centrifugation and redispersion) ten times to remove excess sodium and subsequently oven-dried at 60 °C.

### *4.2. Characterization of HA*

#### 4.2.1. ATR-FTIR

Using a Fourier transform infrared spectrophotometer Frontier SP8000 (Perkin Elmer, Waltham, MA, USA) equipped with an attenuated total reflection (ATR) attachment (DuraSamplIR II, Smiths Detection, Warrington, UK), functional groups on the surface of the adsorbent materials were analyzed. Samples were deposited on the ATR diamond crystal, and spectra were collected in transmittance mode by combining 32 scans with a resolution of 4 $cm^{-1}$ in the 4000–400 $cm^{-1}$ region.

#### 4.2.2. Scanning Electron Microscopy (SEM)

Utilizing a scanning electron microscope (JSM-6010LA, Jeol Inc., MA, USA), the adsorbents' size and morphology was examined. A thin gold coating was applied to the samples to improve electron conductivity and image quality. Using a 20 kV accelerating voltage, microscopy was carried out. Secondary electron imaging mode was used to capture the images at a 1000× magnification.

#### 4.2.3. Energy-Dispersive X-ray Spectroscopy (EDS)

Using an energy-dispersive X-ray spectrometer with an environmental scanning electron microscope (Phillips XL30, EDS-ESEM, Eindhoven, The Netherlands), the multi-element analysis was carried out. A high-performance X Trace micro-spot X-ray source was used to analyze each sample three times, and an attached XFlash® 6/10 silicon drift detector was used to quantify the X-ray fluorescence spectrum it produced (Bruker Nano GmbH, Berlin, Germany).

#### 4.2.4. Zeta Potential (ζ-Potential)

The ZetaSizer Pro (Malvern Instruments, Worcestershire, UK) was used to determine zeta potential. The samples used for the measurements (20 mg dissolved in 10 mL distilled water) were adjusted to various pH levels using either HCl (0.1 M) or NaOH (0.1 M). To minimize the effects of viscosity and scattering, 100 µL of the aqueous phase were collected and diluted with 2 mL deionized water. Then, diluted samples were examined in a disposable capillary cell DTS1070 at room temperature with a 120-s equilibration time. Each measurement included eleven runs and three replicates of each sample to obtain a consistent result. The ZS Xplorer software was used to examine the results.

4.2.5. Point of Zero Charge (pHpzc)

In accordance with the instructions of Zavala-Franco et al [37], the pHpzc was measured. Briefly, equal quantities of sorbents were introduced to a series of flasks filled with distilled water at various pH levels (2, 5, 7, 9, and 11). The pH of the supernatant was measured after samples were agitated at 250 rpm for 195 min. The pH was measured using a combination glass electrode (Conductronic PC-45, Puebla, Mexico). The plot of ΔpH against pH was used to determine the pHpzc.

*4.3. In Vitro Adsorption Studies*

Preparation of the $AFB_1$-Contaminated Diet

Aflatoxin (100 μg $AFB_1$/mL) was made as a main stock in dimethyl sulfoxide. After that, distilled water was used to dilute the $AFB_1$ solution to 1 μg $AFB_1$/mL. An experimental maize-soybean meal diet was made to closely match the nutritional needs of broiler chickens, as suggested by the National Research Council [60]. There were no antibiotics or anticoccidial drugs in the diet (Table 3; adapted from Solís-Cruz et al. [61]). To achieve 100 μg $AFB_1$/kg, the diet was contaminated with 0.5 mL of the $AFB_1$ solution. Thereafter, five samples were chosen at random, and the content of $AFB_1$ was determined using the immunoaffinity column clean-up and liquid chromatography with fluorescence detection methodology. Levels of B-aflatoxins ($AFB_1$ and $AFB_2$), total fumonisins ($FB_1$, $FB_2$, and $FB_3$), and ochratoxin A (OTA) were also determined in the diet using monoclonal antibody-based affinity columns (VICAM Science Technology, Watertown, MA, USA) and fluorescence detection. In general, the experimental diet had no detectable levels of B-aflatoxins and total fumonisins; assayed contents of these mycotoxins were below the detection limits of the immunoaffinity column techniques employed (<1 ng/g and <0.016 mg/kg, respectively). OTA was present at a level of 7 ng/g.

**Table 3.** Ingredient composition of the experimental poultry diet.

| Ingredient | % |
|---|---|
| Maize | 55.07 |
| Soybean meal | 36.94 |
| Vegetable oil | 3.32 |
| Dicalcium phosphate | 1.58 |
| Calcium phosphate | 1.44 |
| Salt | 0.35 |
| DL-Methionine | 0.25 |
| Choline chloride 60% | 0.20 |
| L-Lysine HCL | 0.10 |
| Vitamin premix [1] | 0.30 |
| Mineral premix [2] | 0.30 |
| Antioxidant [3] | 0.15 |
| Protein | 19.5% |
| Metabolizable energy | 13 MJ/kg |

[1] Vitamin premix supplied the following per kg: vitamin A, 2000 IU; vitamin D3, 600, IU; vitamin E, 7.5 IU; vitamin K3, 9 mg; thiamine, 3 mg; riboflavin, 8 mg, pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamin, 16 mg; and ascorbic acid, 200 mg. [2] Mineral premix supplied the following per kg; manganese, 120 mg; zinc, 100 mg; iron, 120 mg; copper, 10–15 mg; iodine, 0.7 mg; selenium, 0.4 mg; and cobalt, 0.2 mg. [3] Ethoxyquin.

*4.4. In Vitro Digestive Model*

The $AFB_1$ adsorptive capacity of the tested materials was assessed using a previously described in vitro gastrointestinal poultry model [62] with minor modifications. The assay was carried out with one control (zeolite) and two different treatments (HA and SFHA). This model simulated the physiological conditions of broiler chicken crop, proventriculus, and intestine. Every tube was incubated at 40 °C while being shaken at 19 rpm at an angle of 30°. For each compartment, the pH, enzymes, and time windows were adjusted. In the beginning, 5 g of the $AFB_1$-contaminated feed and 10 mg of each adsorbent material were placed in polypropylene tubes (50 mL). Each tube received 10 mL of 0.03 M HCl to imitate the crop environment (pH reached values ~5.2). For 30 min, the tubes were incubated. A pH range of 1.4 to 2.0 was attained by adding 2.5 mL of 1.5 M HCl and 3000 U of pepsin (Merck KGaA, Darmstadt, Germany) per gram of feed in each tube following the incubation time. For a further 45 min, each tube was incubated. To simulate the third and final gastrointestinal compartment, 6.84 mg of 8×-pancreatin (Merck KGaA, Darmstadt, Germany) per gram of feed was added to 6.5 mL of 1.0 M $NaHCO_3$. The pH in this region was reached, between 6.4 and 6.8. Tubes were incubated for an additional 120 min. The entire in vitro digesting process took 195 min. Afterward, the supernatant from all tubes was collected and stored at −20 °C for further analysis after centrifuging them all at 7000× *g* for 30 min. To determine the real $AFB_1$ concentrations in each tube, controls (without the addition of adsorbent materials) were also prepared. The entire experiment was carried out in quintuplicate. The adsorption percentage of $AFB_1$ for each tested material was calculated as follows:

$$Adsorption\ (\%) = \frac{(Ci - Cs)}{Ci} \times 100 \quad (1)$$

where $Ci$ is the concentration of $AFB_1$ in the control (ng/mL); and $Cs$ is the concentration of $AFB_1$ in the supernatant of the treatments (ng/mL).

*4.5. Aflatoxin Assay*

Using monoclonal anti-body-based immunoaffinity columns (Afla-B, VICAM Science Technology, Watertown, MA, USA), $AFB_1$ was removed from the supernatants and then utilized for ultraperformance liquid chromatography (UPLC). A modified version of the procedure that Hernández-Ramirez et al [63] previously described was employed. A UPLC system (Waters ACQUITY H-class) was used, equipped with a quaternary solvent manager and a reverse phase column (2.1 mm × 100 mm, 1.7 μm particles). A mobile phase of water, methanol, and acetonitrile (64:18:18) was used to elute AFB1. Samples (1 μL) from the anti-body-based immunoaffinity columns were injected and eluted with a flow rate of 700 μL/min. A fluorescence detector with settings of 365 nm excitation and 429 nm emission was used to detect the toxin. The $AFB_1$ concentration was estimated using a calibration curve with a standard reference ($AFB_1$, Merck KGaA, Darmstadt, Germany).

*4.6. Method Validation*

The performance of the clean-up procedure was tested by measuring the percentage of $AFB_1$ recovery using the UPLC methodology, spiking four replicates of the experimental poultry diet with six different aflatoxin contents over the range of 8 to 250 ng $AFB_1$/g, attaining a toxin recovery of 92% with a standard deviation of 3.4, standard error of 1.7, and a coefficient variation value of 4.4%. Moreover, the validation of the UPLC method was performed based on the guidelines for single-laboratory validation of analytical methods for trace-level concentrations of organic chemicals elaborated by the AOAC/FAO/IAEA/IUPAC [64]. The following parameters were evaluated: limit of detection (LOD), limit of quantification (LOQ), and linearity. For linearity, a six-point calibration curve was plotted at concentrations over the range of 10 to 1000 ng $AFB_1$/L. In general, detection and quantification limits were found to be 2.0 and 6.7 ng $AFB_1$/L, respectively.

The linearity estimated with the coefficient of determination ($R^2$) was 0.9984. These results indicated the methodology used was acceptable.

### *4.7. Experimental Design and Statistical Analysis*

Data was subjected to one-way analysis of variance (one way-ANOVA) as a completely randomized design. Significant differences among the means were determined by the Tukey test. A value of $p = 0.05$ was used to detect significant differences between treatments.

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## References

1. Coulombe, R.A. Biological Action of Mycotoxins. *J. Dairy Sci.* **1993**, *76*, 880–891. https://doi.org/10.3168/jds.S0022-0302(93)77414-7.
2. Zain, M.E. Impact of Mycotoxins on Humans and Animals. *J. Saudi Chem. Soc.* **2011**, *15*, 129–144. https://doi.org/10.1016/j.jscs.2010.06.006.
3. Frisvad, J.C.; Hubka, V.; Ezekiel, C.N.; Hong, S.-B.; Nováková, A.; Chen, A.J.; Arzanlou, M.; Larsen, T.O.; Sklenář, F.; Mahakarnchanakul, W.; et al. Taxonomy of *Aspergillus* Section *Flavi* and Their Production of Aflatoxins, Ochratoxins and Other Mycotoxins. *Stud. Mycol.* **2019**, *93*, 1–63. https://doi.org/10.1016/j.simyco.2018.06.001.
4. Rawal, S.; Kim, J.E.; Coulombe, R. Aflatoxin B1 in Poultry: Toxicology, Metabolism and Prevention. *Res. Vet. Sci.* **2010**, *89*, 325–331. https://doi.org/10.1016/j.rvsc.2010.04.011.
5. Popescu, R.G.; Avramescu, S.; Marin, D.E.; Ţăranu, I.; Georgescu, S.E.; Dinischiotu, A. The Reduction of the Combined Effects of Aflatoxin and Ochratoxin A in Piglet Livers and Kidneys by Dietary Antioxidants. *Toxins* **2021**, *13*, 648. https://doi.org/10.3390/toxins13090648.
6. Wang, Y.; Liu, F.; Zhou, X.; Liu, M.; Zang, H.; Liu, X.; Shan, A.; Feng, X. Alleviation of Oral Exposure to B1-Induced Renal Dysfunction, Oxidative Stress, and Cell Apoptosis in Mice Kidney by Curcumin. *Antioxidants* **2022**, *11*, 1082. https://doi.org/10.3390/antiox11061082.
7. Fouad, A.; Ruan, D.; El-Senousey, H.; Chen, W.; Jiang, S.; Zheng, C. Harmful Effects and Control Strategies of Aflatoxin B1 Produced by Aspergillus Flavus and Aspergillus Parasiticus Strains on Poultry: Review. *Toxins* **2019**, *11*, 176. https://doi.org/10.3390/toxins11030176.
8. Kabak, B.; Dobson, A.D.W.; Var, I. Strategies to Prevent Mycotoxin Contamination of Food and Animal Feed: A Review. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 593–619. https://doi.org/10.1080/10408390500436185.
9. Piccolo, A. The Supramolecular Structure of Humic Substances: A Novel Understanding of Humus Chemistry and Implications in Soil Science. In *Advances in Agronomy*; Elsevier: Amsterdam, The Netherlands, 2002; Volume 75, pp. 57–134, ISBN 978-0-12-000793-6.
10. Guo, X.; Liu, H.; Wu, S. Humic Substances Developed during Organic Waste Composting: Formation Mechanisms, Structural Properties, and Agronomic Functions. *Sci. Total Environ.* **2019**, *662*, 501–510. https://doi.org/10.1016/j.scitotenv.2019.01.137.
11. Jiménez-González, M.A.; Álvarez, A.M.; Carral, P.; Almendros, G. Chemometric Assessment of Soil Organic Matter Storage and Quality from Humic Acid Infrared Spectra. *Sci. Total Environ.* **2019**, *685*, 1160–1168. https://doi.org/10.1016/j.scitotenv.2019.06.231.

12. Maguey-Gonzalez, J.A.; Michel, M.A.; Baxter, M.F.A.; Solis-Cruz, B.; Hernandez-Patlan, D.; Merino-Guzman, R.; Hernandez-Velasco, X.; Latorre, J.D.; Hargis, B.M.; Tellez, G.; et al. Effects of Humic Acids on Recovery of Salmonella Enterica Serovar Enteritidis. *Ann. Anim. Sci.* **2018**, *18*, 387–399. https://doi.org/10.1515/aoas-2017-0037.
13. Maguey-Gonzalez, J.A.; Michel, M.A.; Baxter, M.F.A.; Tellez, G.; Moore, P.A.; Solis-Cruz, B.; Hernández-Patlan, D.; Merino-Guzman, R.; Hernandez-Velasco, X.; Latorre, J.D.; et al. Effect of Humic Acids on Intestinal Viscosity, Leaky Gut and Ammonia Excretion in a 24 Hr Feed Restriction Model to Induce Intestinal Permeability in Broiler Chickens. *Anim. Sci. J.* **2018**, *89*, 1002–1010. https://doi.org/10.1111/asj.13011.
14. Maguey-González, J.A.; Gómez-Rosales, S.; de Lourdes Angeles, M.; López-Hernández, L.H.; Rodríguez-Hernández, E.; Solís-Cruz, B.; Hernández-Patlán, D.; Merino-Gúzman, R.; Téllez-Isaías, G. Effects of Humic Acids on the Recovery of Different Bacterial Strains in an in Vitro Chicken Digestive Model. *Res. Vet. Sci.* **2022**, *145*, 21–28. https://doi.org/10.1016/j.rvsc.2022.01.004.
15. de Lourdes Angeles, M.; Gómez-Rosales, S.; Téllez-Isaias, G. Mechanisms of Action of Humic Substances as Growth Promoters in Animals. In *Humus and Humic Substances—Recent Advances*; IntechOpen: London, UK, 2022; ISBN 978-1-80356-212-4.
16. Vázquez-Durán, A.; de Jesús Nava-Ramírez, M.; Téllez-Isaías, G.; Méndez-Albores, A. Removal of Aflatoxins Using Agro-Waste-Based Materials and Current Characterization Techniques Used for Biosorption Assessment. *Front. Vet. Sci.* **2022**, *9*, 897302. https://doi.org/10.3389/fvets.2022.897302.
17. Zhang, J.; Dai, J.; Wang, R.; Li, F.; Wang, W. Adsorption and Desorption of Divalent Mercury ($Hg^{2+}$) on Humic Acids and Fulvic Acids Extracted from Typical Soils in China. *Colloids Surf. A: Physicochem. Eng. Asp.* **2009**, *335*, 194–201. https://doi.org/10.1016/j.colsurfa.2008.11.006.
18. Mozgawa, W.; Sitarz, M.; Rokita, M. Spectroscopic Studies of Different Aluminosilicate Structures. *J. Mol. Struct.* **1999**, *511–512*, 251–257. https://doi.org/10.1016/S0022-2860(99)00165-9.
19. Alberts, J.J.; Filip, Z. Metal Binding in Estuarine Humic and Fulvic Acids: FTIR Analysis of Humic Acid-Metal Complexes. *Environ. Technol.* **1998**, *19*, 923–931. https://doi.org/10.1080/09593331908616750.
20. Manzak, A.; Kurşun, C.; Yıldız, Y. Characterization of Humic Acid Extracted from Aqueous Solutions with Polymer Inclusion Membranes. *J. Taiwan Inst. Chem. Eng.* **2017**, *81*, 14–20. https://doi.org/10.1016/j.jtice.2017.10.024.
21. Baglieri, A.; Vindrola, D.; Gennari, M.; Negre, M. Chemical and Spectroscopic Characterization of Insoluble and Soluble Humic Acid Fractions at Different PH Values. *Chem. Biol. Technol. Agric.* **2014**, *1*, 9. https://doi.org/10.1186/s40538-014-0009-x.
22. Ribeiro, J.S.; Ok, S.S.; Garrigues, S.; de la Guardia, M. FTIR Tentative Characterization of Humic Acids Extracted from Organic Materials. *Spectrosc. Lett.* **2001**, *34*, 179–190. https://doi.org/10.1081/SL-100002007.
23. Domínguez-Negrete, A.; Gómez-Rosales, S.; Angeles, M.d.L.; López-Hernández, L.H.; Reis-de Souza, T.C.; López-García, Y.; Zavala-Franco, A.; Téllez-Isaias, G. Effect of the Addition of Humic Substances as Growth Promoter in Broiler Chickens Under Two Feeding Regimens. *Animals* **2019**, *9*, 1101. https://doi.org/10.3390/ani9121101.
24. Rupiasih, N.N.; Vidyasagar, P.B. Analytical Study of Humic Acid from Various Sources Commonly Used as Fertilizer: Emphasis on Heavy Metal Content. *Int. J. Des. Nat. Ecodynamics* **2009**, *4*, 32–46. https://doi.org/10.2495/DNE-V4-N1-32-46.
25. Xu, D.; Xu, Z.; Zhu, S.; Cao, Y.; Wang, Y.; Du, X.; Gu, Q.; Li, F. Adsorption Behavior of Herbicide Butachlor on Typical Soils in China and Humic Acids from the Soil Samples. *J. Colloid Interface Sci.* **2005**, *285*, 27–32. https://doi.org/10.1016/j.jcis.2004.11.034.
26. Vermeer, A.W.P.; van Riemsdijk, W.H.; Koopal, L.K. Adsorption of Humic Acid to Mineral Particles. 1. Specific and Electrostatic Interactions. *Langmuir* **1998**, *14*, 2810–2819. https://doi.org/10.1021/la970624r.
27. Vázquez-Durán, A.; de Jesús Nava-Ramírez, M.; Hernández-Patlán, D.; Solís-Cruz, B.; Hernández-Gómez, V.; Téllez-Isaías, G.; Méndez-Albores, A. Potential of Kale and Lettuce Residues as Natural Adsorbents of the Carcinogen Aflatoxin B1 in a Dynamic Gastrointestinal Tract-Simulated Model. *Toxins* **2021**, *13*, 771. https://doi.org/10.3390/toxins13110771.
28. Prado, A.G.S.; Pertusatti, J.; Nunes, A.R. Aspects of Protonation and Deprotonation of Humic Acid Surface on Molecular Conformation. *J. Braz. Chem. Soc.* **2011**, *22*, 6.
29. Yang, F.; Zhang, S.; Cheng, K.; Antonietti, M. A Hydrothermal Process to Turn Waste Biomass into Artificial Fulvic and Humic Acids for Soil Remediation. *Sci. Total Environ.* **2019**, *686*, 1140–1151. https://doi.org/10.1016/j.scitotenv.2019.06.045.
30. Tarasevich, Y.I.; Dolenko, S.A.; Trifonova, M.Yu.; Alekseenko, E.Yu. Association and Colloid-Chemical Properties of Humic Acids in Aqueous Solutions. *Colloid J.* **2013**, *75*, 207–213. https://doi.org/10.1134/S1061933X13020166.
31. Sarlaki, E.; Paghaleh, A.S.; Kianmehr, M.H.; Vakilian, K.A. Chemical, Spectral and Morphological Characterization of Humic Acids Extracted and Membrane Purified from Lignite. *Chem. Chem. Technol.* **2020**, *14*, 353–361. https://doi.org/10.23939/chcht14.03.353.
32. Klučáková, M.; Věžníková, K. Micro-Organization of Humic Acids in Aqueous Solutions. *J. Mol. Struct.* **2017**, *1144*, 33–40. https://doi.org/10.1016/j.molstruc.2017.05.012.
33. Baigorri, R.; Fuentes, M.; González-Gaitano, G.; García-Mina, J.M. Analysis of Molecular Aggregation in Humic Substances in Solution. *Colloids Surf. A: Physicochem. Eng. Asp.* **2007**, *302*, 301–306. https://doi.org/10.1016/j.colsurfa.2007.02.048.
34. Giovanela, M.; Crespo, J.S.; Antunes, M.; Adamatti, D.S.; Fernandes, A.N.; Barison, A.; da Silva, C.W.P.; Guégan, R.; Motelica-Heino, M.; Sierra, M.M.D. Chemical and Spectroscopic Characterization of Humic Acids Extracted from the Bottom Sediments of a Brazilian Subtropical Microbasin. *J. Mol. Struct.* **2010**, *981*, 111–119. https://doi.org/10.1016/j.molstruc.2010.07.038.
35. Liu, X.; Hou, Y.; Li, Z.; Yu, Z.; Tang, J.; Wang, Y.; Zhou, S. Hyperthermophilic Composting of Sewage Sludge Accelerates Humic Acid Formation: Elemental and Spectroscopic Evidence. *Waste Manag.* **2020**, *103*, 342–351. https://doi.org/10.1016/j.wasman.2019.12.053.

36. Doskočil, L.; Burdíková-Szewieczková, J.; Enev, V.; Kalina, L.; Wasserbauer, J. Spectral Characterization and Comparison of Humic Acids Isolated from Some European Lignites. *Fuel* **2018**, *213*, 123–132. https://doi.org/10.1016/j.fuel.2017.10.114.
37. Zavala-Franco, A.; Hernández-Patlán, D.; Solís-Cruz, B.; López-Arellano, R.; Tellez-Isaias, G.; Vázquez-Durán, A.; Méndez-Albores, A. Assessing the Aflatoxin B1 Adsorption Capacity between Biosorbents Using an In Vitro Multicompartmental Model Simulating the Dynamic Conditions in the Gastrointestinal Tract of Poultry. *Toxins* **2018**, *10*, 484. https://doi.org/10.3390/toxins10110484.
38. Díaz-Nava, C.; Olguín, M.T.; Solache-Ríos, M. Water Defluoridation by Mexican Heulandite–clinoptilolite. *Sep. Sci. Technol.* **2002**, *37*, 3109–3128. https://doi.org/10.1081/SS-120005662.
39. Ostrooumov, M.; Cappelletti, P.; de'Gennaro, R. Mineralogical Study of Zeolite from New Mexican Deposits (Cuitzeo Area, Michoacan, Mexico). *Appl. Clay Sci.* **2012**, *55*, 27–35. https://doi.org/10.1016/j.clay.2011.09.011.
40. Lin, D.-Q.; Brixius, P.J.; Hubbuch, J.J.; Thömmes, J.; Kula, M.-R. Biomass/Adsorbent Electrostatic Interactions in Expanded Bed Adsorption: A Zeta Potential Study: Study of Zeta Potential in Expanded Bed Absorption. *Biotechnol. Bioeng.* **2003**, *83*, 149–157. https://doi.org/10.1002/bit.10654.
41. Hamza, Z.; El-Hashash, M.; Aly, S.; Hathout, A.; Soto, E.; Sabry, B.; Ostroff, G. Preparation and Characterization of Yeast Cell Wall Beta-Glucan Encapsulated Humic Acid Nanoparticles as an Enhanced Aflatoxin B1 Binder. *Carbohydr. Polym.* **2019**, *203*, 185–192. https://doi.org/10.1016/j.carbpol.2018.08.047.
42. Deng, S.; Bai, R.B. Aminated Polyacrylonitrile Fibers for Humic Acid Adsorption: Behaviors and Mechanisms. *Environ. Sci. Technol.* **2003**, *37*, 5799–5805. https://doi.org/10.1021/es034399d.
43. Coles, C.A.; Yong, R.N. Humic Acid Preparation, Properties and Interactions with Metals Lead and Cadmium. *Eng. Geol.* **2006**, *85*, 26–32. https://doi.org/10.1016/j.enggeo.2005.09.024.
44. Loosli, F.; Le Coustumer, P.; Stoll, S. TiO2 Nanoparticles Aggregation and Disaggregation in Presence of Alginate and Suwannee River Humic Acids. PH and Concentration Effects on Nanoparticle Stability. *Water Res.* **2013**, *47*, 6052–6063. https://doi.org/10.1016/j.watres.2013.07.021.
45. Illés, E.; Tombácz, E. The Role of Variable Surface Charge and Surface Complexation in the Adsorption of Humic Acid on Magnetite. *Colloids Surf. A: Physicochem. Eng. Asp.* **2003**, *230*, 99–109. https://doi.org/10.1016/j.colsurfa.2003.09.017.
46. Alvarez-Puebla, R.A.; Garrido, J.J. Effect of PH on the Aggregation of a Gray Humic Acid in Colloidal and Solid States. *Chemosphere* **2005**, *59*, 659–667. https://doi.org/10.1016/j.chemosphere.2004.10.021.
47. Ramales-Valderrama, R.; Vázquez-Durán, A.; Méndez-Albores, A. Biosorption of B-Aflatoxins Using Biomasses Obtained from Formosa Firethorn [Pyracantha Koidzumii (Hayata) Rehder]. *Toxins* **2016**, *8*, 218. https://doi.org/10.3390/toxins8070218.
48. Volk, B.G. *Physical and Chemical Characteristics of Aquatic Humus*; Gjessing, E.T., Ed.; Ann Arbor Science Publishers Inc.: Ann Arbor, MI, USA, 1976. https://doi.org/10.2134/jeq1976.00472425000500040039x.
49. Giasuddin, A.B.M.; Kanel, S.R.; Choi, H. Adsorption of Humic Acid onto Nanoscale Zerovalent Iron and Its Effect on Arsenic Removal. *Environ. Sci. Technol.* **2007**, *41*, 2022–2027. https://doi.org/10.1021/es0616534.
50. Abd El-Shafea, Y. In vivo and in vitro evaluation of efficacy of humic acid against aflatoxins. *Al-Azhar J. Pharm. Sci.* **2014**, *49*, 133–154. https://doi.org/10.21608/ajps.2014.6962.
51. Ye, S.-Q.; Lv, X.-Z.; Zhou, A.-G. In Vitro Evaluation of the Efficacy of Sodium Humate as an Aflatoxin B1 Adsorbent. *Aust. J. Basic Appl. Sci.* **2009**, *3*, 1296–1300.
52. Tan, K.H. *Humic Matter in Soil and the Environment: Principles and Controversies*; CRC Press: Boca Raton, FL, USA, 2003; ISBN 978-0-429-22322-8.
53. Tikhonov, V.; Drozdova, O.; Karpukhin, M.; Demin, V. Participation of Cadmium (Ll) and Copper (Ll) Ions in Intermolecular Forces of Humic Acids in Solutions. *IOP Conf. Ser. : Earth Environ. Sci.* **2019**, *368*, 012053. https://doi.org/10.1088/1755-1315/368/1/012053.
54. Islam, M.A.; Morton, D.W.; Johnson, B.B.; Angove, M.J. Adsorption of Humic and Fulvic Acids onto a Range of Adsorbents in Aqueous Systems, and Their Effect on the Adsorption of Other Species: A Review. *Sep. Purif. Technol.* **2020**, *247*, 116949. https://doi.org/10.1016/j.seppur.2020.116949.
55. Zhou, Q.; Ouyang, S.; Ao, Z.; Sun, J.; Liu, G.; Hu, X. Integrating Biolayer Interferometry, Atomic Force Microscopy, and Density Functional Theory Calculation Studies on the Affinity between Humic Acid Fractions and Graphene Oxide. *Environ. Sci. Technol.* **2019**, *53*, 3773–3781. https://doi.org/10.1021/acs.est.8b05232.
56. Vázquez-Durán, A.; Téllez-Isaías, G.; Hernández-Rodríguez, M.; Ruvalcaba, R.M.; Martínez, J.; Nicolás-Vázquez, M.I.; Aceves-Hernández, J.M.; Méndez-Albores, A. The Ability of Chlorophyll to Trap Carcinogen Aflatoxin B1: A Theoretical Approach. *Int. J. Mol. Sci.* **2022**, *23*, 6068. https://doi.org/10.3390/ijms23116068.
57. van Rensburg, C.J.; Van Rensburg, C.E.J.; Van Ryssen, J.B.J.; Casey, N.H.; Rottinghaus, G.E. In Vitro and In Vivo Assessment of Humic Acid as an Aflatoxin Binder in Broiler Chickens. *Poult. Sci.* **2006**, *85*, 1576–1583. https://doi.org/10.1093/ps/85.9.1576.
58. Ghahri, H.; Habibian, R.; Fam, M.A. Evaluation of the Efficacy of Esterified Glucomannan, Sodium Bentonite, and Humic Acid to Ameliorate the Toxic Effects of Aflatoxin in Broilers. *Turk. J. Vet. Anim. Sci.* **2010**, *34*, 385–391. https://doi.org/10.3906/vet-0903-19.
59. Arafat, R.Y.; Khan, S.H.; Saima Evaluation of Humic Acid as an Aflatoxin Binder in Broiler Chickens. *Ann. Anim. Sci.* **2017**, *17*, 241–255. https://doi.org/10.1515/aoas-2016-0050.
60. National Research Council; Board on Agriculture; Subcommittee on Poultry Nutrition. *Nutrient Requirements of Poultry: Ninth Revised Edition, 1994*; National Academies Press: Cambridge, MA, USA, 1994; ISBN 978-0-309-04892-7.

61. Solís-Cruz, B.; Hernández-Patlán, D.; Beyssac, E.; Latorre, J.D.; Hernandez-Velasco, X.; Merino-Guzman, R.; Tellez, G.; López-Arellano, R. Evaluation of Chitosan and Cellulosic Polymers as Binding Adsorbent Materials to Prevent Aflatoxin B1, Fumonisin B1, Ochratoxin, Trichothecene, *Deoxynivalenol*, and Zearalenone Mycotoxicoses Through an In Vitro Gastrointestinal Model for Poultry. *Polymers* **2017**, *9*, 529. https://doi.org/10.3390/polym9100529.
62. Latorre, J.D.; Hernandez-Velasco, X.; Kuttappan, V.A.; Wolfenden, R.E.; Vicente, J.L.; Wolfenden, A.D.; Bielke, L.R.; Prado-Rebolledo, O.F.; Morales, E.; Hargis, B.M.; et al. Selection of Bacillus Spp. for Cellulase and Xylanase Production as Direct-Fed Microbials to Reduce Digesta Viscosity and Clostridium Perfringens Proliferation Using an in Vitro Digestive Model in Different Poultry Diets. *Front. Vet. Sci.* **2015**, *2*, 25. https://doi.org/10.3389/fvets.2015.00025.
63. Hernández-Ramírez, J.O.; Merino-Guzmán, R.; Téllez-Isaías, G.; Vázquez-Durán, A.; Méndez-Albores, A. Mitigation of AFB1-Related Toxic Damage to the Intestinal Epithelium in Broiler Chickens Consumed a Yeast Cell Wall Fraction. *Front. Vet. Sci.* **2021**, *8*, 677965. https://doi.org/10.3389/fvets.2021.677965.
64. Alder, L.; Hill, A.; Holland, P.T.; Lantos, J.; Lee, S.M.; MacNeil, J.D.; O'Rangers, J.; van Zoonen, P.; Ambrus, A. Guidelines for single-laboratory validation of analytical methods for trace-level concentrations of organic chemicals. In *Principles and Practices of Method Validation*; Fajgelj, A., Ambrus, Á., Eds.; Royal Society of Chemistry: Cambridge, UK, 2000; pp 179–187, ISBN 978-0-85404-783-3.

## V.II. Artículo 2

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# Effects of humic acids on the recovery of different bacterial strains in an *in vitro* chicken digestive model

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ABSTRACT

Humic acids (HA) have been evaluated as growth promoters in poultry, but their effects on the gut microbiota remains controversial using *in vitro* and *in vivo* models. The objective of this study was to determine the effect of HA extracted from a wormcompost on the recovery of bacteria: *Salmonella Enteritidis* (*S. Enteritidis*), *Escherichia coli* (*E. coli*), *Clostridium perfringens* (*C. perfringens*), *Bacillus subtilis* (*B. subtilis*) and *Lactobacillus salivarius* (*L. salivarius*) using an *in vitro* chicken digestive system. Independent *in vitro* trials were run for each bacteria using six treatments: 1) Negative control with no bacteria added (Control-), 2) Positive control added with bacteria (Control+), 3) 0.1% HA + bacteria, 4) 0.2% HA + bacteria, 5) 0.5% HA + bacteria and 6) 1% HA + bacteria. Data was subjected to analysis of variance and linear regression. In the crop, *S. Enteritidis* was lower, C. perfringes and *B. subtilis* were not affected by HA, while *E. coli* and L. *salivarius* were higher at 0.5 and 1% HA inclusion ($P \leq 0.0001$). In the proventriculus, *S. Enteritidis*, *E. coli* and *B. subtilis* were higher at 0.5 and 1% HA inclusion ($P \leq 0.0001$); *C. perfringens* and L. *salivarius* were not affected by HA. In intestine, significant increases of all bacteria strains were observed ($P \leq 0.0001$). In conclusion, the results suggests that HA can be used as prebiotic, but their mechanisms of action to stimulate the growth of gut bacteria remains to be elucidated.

## 1. Introduction

Antibiotics have been used in poultry at sub-therapeutic dosages for growth promotion purposes to improve the performance and health of poultry by the control of pathogenic bacteria, modifying the immune status (Lee et al., 2012) and their anti-inflammatory effects (Niewold, 2007). However, scientific evidence suggests that the overuse of antimicrobials at sub-therapeutic doses in animal production promotes bacterial resistance. An increase in numbers of antibiotic resistant bacteria has been observed in fecal samples, slaughter facilities and environments. Therefore, antibiotic-resistant bacteria may adversely affect human health (Apata, 2009; Van den Bogaard et al., 2001; Zhang et al., 2017; Ashbolt et al., 2013). Consequently, in 2006 the European Union completely banned the use of growth-promoting antibiotics in animal production and, more recently, in 2017, the United States of America adopted the same restriction (Salim et al., 2018). Likewise, the World Health Organization guidelines on use of medically important antimicrobials in food-producing animals recommended complete restriction of all antibiotics in food-producing animals for growth promotion (Aidara-Kane et al., 2018).

This restriction has forced the poultry industry to investigate alternatives to replace or reduce de use of antibiotics growth promoters while maintaining high production efficiency and producing safe products. Among some of the alternatives evaluated in poultry diets are probiotics,

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prebiotics, enzymes, herbal products, organic acids (Ferket, 2004) and other feed additives as Humic Substances (HS) (Arif et al., 2019). However, the use of HS in poultry nutrition as an alternative to growth promoting has gained increasing importance (Galip et al., 2010). HS are the main component of soil organic matter and are divided into three principal fractions: humic acids (HA) (acid-insoluble fraction), fulvic acids (alkali- and acid-soluble fraction) and humin (alkali- and acid-insoluble fraction) (Peña-Méndez et al., 2005; Abd El-Hack, 2016). HA represent the principal fraction of HS (Schnitzer and Khan, 1972).

Despite the fact that humic acids have been reported to have promising advantages in poultry production as growth promoters given their antioxidant, antifungal and immunostimulant properties, mainly, there are contrasting results regarding their antimicrobial activity (Riede et al., 1991; Yarkova, 2011; Mudrovnová et al., 2020); Shermer et al., 1998; Maguey-Gonzalez et al., 2018a,b) Therefore, the objective of this study was to determine the effect of HA extracted from a wormcompost on the recovery of *Salmonella Enteritidis, Escherichia coli, Clostridium perfringens, Bacillus subtilis* and *Lactobacillus salivarius* using an *in vitro* chicken digestive system.

## 2. Material and methods

### 2.1. Humic acids

The extraction and isolation of HA from a wormcompost was performed as described by Parsons (1983). The HA were extracted in a proportion 1:4 (compost:alkali) using a sodium hydroxide solution (1 M NaOH), followed by stirring for 2 h. The suspension was allowed to stand for 24 h at room temperature and was filtered using a Whatman grade 40 filter paper. Then, the filtrate was centrifuged for 15 min at 3500g, and the supernatant was separated by decantation. The supernatant, which contained the HA, was acidified adding hydrochloric acid (10% HCl) with constant stirring until a pH 2 was reached to precipitate the HA. Which, were then separated from fulvic acids by centrifugation at 3500g for 15 min. Finally, the precipitated (HA) was alkalinized until a pH 10 was reached with 1 M NaOH, and dried at 60 °C. The result was a black powder with a pH of 10.

### 2.2. Bacterial strains and culture conditions

The organisms used in the experiments were: *Salmonella enterica* subsp. enterica serovar Enteritidis (*S. Enteritidis*) derived from ATCC® 13076™ (Catalog no. 0345P); *Escherichia coli* (*E. coli*) derived from ATCC® 35218™ (Catalog no. 0495P) and *Clostridium perfringens* (*C. perfringens*) derived from NCTC® 8678™ (Catalog no. 0547P), obtained from Microbiologics. Furthermore, *Bacillus subtilis* (*B. subtilis*) and *Lactobacillus salivarius* (*L. salivarius*) isolated in our laboratory (identification by 16S rRNA sequence analyses, Midi labs, Newark, DE 19713, USA) from broiler chickens, and potentially used as probiotics were tested. One hundred μL of each bacterium from a frozen aliquot was added to 10 mL of nutrient broth (BD Bioxin, Edo. de México, México) for SE, EC, BS and LS; or 10 mL of reinforced clostridial medium (Acumedia, Neogen, MI, USA) for CP. Each organism was incubated at 37 °C for 24 h, and passed three times every 8 h to ensure that all bacteria were in log phase. After incubation, bacteria were centrifuged at 1864g for 10 min, washed 3 times with sterile 0.9% saline, and reconstituted in saline, quantified by densitometry, using a spectrophotometer (Genesysm 10s UV–Vis, ThermoFisher Scientific) and diluted to obtain an approximate concentration of $10^8$ CFU/mL. Bacterial concentration was verified by serial dilution and plating on specific agar for each organism.

### 2.3. Experimental design

The experiment consisted of five independent *in vitro* trials by quintuplicate, considering each bacteria strain (*S. Enterica, E. coli, C. perfringens, B. subtilis and* L. *salivarius*). Treatments for each *in vitro* trail were: 1) Negative control with no bacteria added (Control-), 2) Positive control added with bacteria (Control+), 3) 0.1% of HA + bacteria, 4) 0.2% of HA + bacteria, 5) 0.5% of HA + bacteria and 6) 1% of HA + bacteria.

### 2.4. In vitro chicken digestive model

Antimicrobial activity of HA was measured using a previously described chicken digestive model with minor modifications (Latorre et al., 2015), the model simulated the three main compartments of the gastrointestinal tract of broilers. To simulate the first compartment (crop), 5 g of sterile starter chicken feed (Table 1) were placed in 50 mL polypropylene centrifuge tubes, each of the treatments at their proper concentration, 9 mL of 0.03 M HCl and 1 mL of a suspension of bacteria with a concentration of $10^8$ CFU/mL, reaching a pH value around 5.20. The tubes were incubated for 30 min at 40 °C in an incubator-shaker (MaxQ 4450 Benchtop orbital shakers, ThermoFisher Scientific) with constant movement at 20 rpm in a 30° degrees inclination position to facilitate proper blending of the content. The next compartment simulated was the proventriculus, 2.5 mL of 1.5 M HCl and 3000 U of pepsin/g of feed (Sigma-Aldrich, St Luois, Mo, USA) were added to each tube, to achieve a pH between 1.4 and 2.00, then all tubes were incubated for 45 min. Finally, the last simulated compartment was an intestinal section; 6.5 mL of 1.0 M sodium bicarbonate ($NaHCO_3$) and 6.84 mg of 8-x USP pancreatin (Sigma-Aldrich, St Luois, Mo, USA) per g of feed were added to the tubes, the pH ranged between 6.4 and 6.8; all the tubes were incubated for 2 h. After the incubation time in each simulated compartment, 1 mL sample was collected, diluted, and plated on specific agar to enumerate the different microorganisms. The complete *in vitro* digestion model took 3 h and 15 min.

### 2.5. Enumeration of bacteria

Ten-fold dilutions from each sample were made in a sterile 96 well Bacti flat bottom plate, plated and incubated according to the microorganism to evaluate the total number of bacteria. *S. Enteritidis* was

**Table 1**
Composition of the starter diet.

| Ingredients | % |
|---|---|
| Corn | 63.72 |
| Soybean meal | 29.40 |
| Vegetable oil | 2.10 |
| Calcium orthophosphate | 1.70 |
| Calcium carbonate | 1.60 |
| Salt | 0.32 |
| DL-Methionine | 0.24 |
| L-Lysine HCL | 0.26 |
| Threonine | 0.08 |
| Sodium bicarbonate | 0.20 |
| Vitamin premix[a] | 0.10 |
| Mineral premix[b] | 0.10 |
| Choline chloride | 0.09 |
| Total | 100.00 |
| Calculated composition | |
| ME, kcal/kg | 3000 |
| Dig. Lys, % | 1.10 |
| Dig. Met, % | 0.52 |
| Dig. Thr, % | 0.71 |
| Total Ca, % | 1.00 |
| Av. P, % | 0.50 |

[a] Vitamin premix per Kg: vitamin A, 6500 IU; vitamin D, 32000 IU; vitamin E, 15 IU; vitamin K,1.5 mg; thiamine, 1.5 mg; riboflavin, 5 mg; niacin, 35 mg; pyrixodine, 3.5 mg; pantothenic acid, 10 mg; choline, 1500 mg; Folic acid, 0.6 mg; biotin, 15 mg; .vitamin b12, 15 mg;
[b] Mineral premix per Kg: manganese, 100 mg; zinc, 100 mg; iron, 50 mg; copper, 10 mg; iodine, 1 mg.

plated on Xylose Lysine Deoxycholate agar (XLD, BD Bioxin); *E. coli* on Eosin Methylene Blue Agar Levine (EMB, BD Bioxin); *C. perfringens* on Reinforced Clostridial Agar (Acumedia); *B. subtilis* on Tryptic Soy Broth (TSB, BD Bioxin) + Bacteriological agar (BD Bioxin); and *L. salivarius* on de Man, Rogosa and Sharpe agar (MRS, BD Difco).

### 2.6. Data and statistical analysis

$Log_{10}$ CFU/g of each bacterium, as well as the pH was subjected to analysis of variance as a completely randomized design, using the General Linear Models procedure of SAS (SAS. SAS User's Guide, 2002). Statistical differences among the means were determined by the least statistical difference test at $p < 0.05$. Simple lineal regression analysis were used to determine linear (L), quadratic (Q), and cubic (C) effects of HA addition on recovery of bacteria. Significant differences were considered with a value of $P < 0.05$.

## 3. Results

Table 2 summarizes the results of the effect of HA on *S. Enteritidis* counts and pH under an *in vitro* poultry digestive model. In the Control- treatment (standard diet only) no *S. Enteritidis* was detected in any of the simulated compartments. In the crop, the *S. Enteritidis* counts were similar between the Control+ and the 0.1 and 0.2% HA, while a significant reduction on the *S. Enteritidis* counts was observed at 0.5 and 1% HA inclusion (Cubic effect, $P \leq 0.0001$). In the proventriculus, the *S. Enteritidis* counts were also similar between the Control+ and the 0.1 and 0.2% HA inclusion, but a significant increase at 0.5 and 1% HA was observed (Lineal effect, $P \leq 0.0001$). In the intestine, a linear increase of *S. Enteritidis* counts were observed at each levels of HA inclusion up to 0.5%, and then a plateau was observed between 0.5 and 1% HA inclusion (Quadratic effect, $P \leq 0.0001$).

In the crop, the Control-, Control+ and 0.1% HA had similar pH, but it linearly increased from 0.1 to 1% HA inclusion (Quadratic effect, $P \leq 0.0001$). In the proventriculus, the pH was similar between the Control- and Control+ groups, it increased at 0.1 to 0.5% HA, but it dropped at 1% HA inclusion compared to the rest of the treatments (Cubic effect, $P \leq 0.0001$). In the intestine, the pH was higher from 0.1–0.5% HA inclusion compared to the Control- and Control+ groups, then the pH significantly dropped at 1% HA inclusion (Quadratic effect, $P \leq 0.0001$).

Table 3 summarizes the results of the effect of HA on *E. coli* counts and pH under an *in vitro* poultry digestive model. In the Control- no *E. coli* was detected in any of the simulated compartments. In the crop, proventriculus and intestine, the *E. coli* counts were lower in the Control+ compared to the HA groups, with the highest *E. coli* counts at 0.5 and 1% HA inclusions (Quadratic effect, $P \leq 0.0001$).

The pH in the crop was similar between the Control+ and 0.1% HA and then linearly increased as the addition of HA increased (Quadratic effect, $P \leq 0.0001$), while in the proventriculus the pH increased linearly up to the 0.5% HA addition, and then dropped at 1% HA (Cubic effect, $P \leq 0.0001$). In the intestine, the pH was similar between the Control- and Control+ compared to the treatments with 0.1, 0.2 and 0.5% HA, but at 1% HA the pH significantly dropped (Quadratic effect, $P \leq 0.0001$).

The results of the effect of HA on the counts of *C. perfringens* and pH under an *in vitro* poultry digestive model are summarized in Table 4. In the Control- no *C. perfringens* was detected in any of the simulated compartments. No differences were observed on the *C. perfringens* counts between the Control+ and the treatments added with HA in the crop and proventriculus. In the intestine, the *C. perfringens* counts were lower in the Control+ and the 0.1 HA and were higher at 0.5 and 1% HA, while at 0.2% HA the counts were intermediate (Quadratic effect, $P \leq 0.0001$).

In the crop, the pH was similar between the Control- and Control+ compared to 0.1% HA, then a linear increase was observed up to 1% HA (Quadratic effect, $P \leq 0.0001$). In the proventriculus, the pH was similar between the Control- and Control+ and 0.1% HA, then it increased at 0.2 and 0.5% HA, and suddenly dropped at 1% HA (Quadratic effect, $P \leq 0.0001$). In the intestine, the pH was similar between the Control- and Control+ and the 0.1, and 0.2 HA inclusion, it increased at 0.5% HA and then dropped at 1% HA (Cubic effect, $P \leq 0.0001$).

Table 5 shows the results of the effect of humic acids on counts of *B. subtilis* under an *in vitro* poultry digestive model. In the Control- no *B. subtilis* was detected in any of the simulated compartments. In the crop, the *B. subtilis* counts were lower at 0.5% HA compared to the Control+ and the HA levels of 0.1, 0.2 and 1%; between the Control+ and 0.1, 0.2 and 1% HA no differences were detected (Quadratic effect, $P \leq 0.0001$). In the proventriculus, similar *B. subtilis* counts were shown in the Control+ and 0.1, 0.2 and 0.5% HA levels, but the counts were higher at 1% HA (Cubic effect, $P \leq 0.0001$). In the intestine, similar *B. subtilis* counts for the Control+ and 0.1 and 0.2% HA levels were observed, but the counts were higher at 0.5 and 1% HA (Cubic effect, $P \leq 0.0001$).

In the crop, the Control- and Control+ had similar pH, and it increased from 0.1 to 1% HA inclusion (Lineal effect, $P \leq 0.0001$). In the proventriculus, the pH was similar between the Control- and Control+, and it increased from 0.1 to 0.5% HA, but it dropped at 1% HA inclusion compared to the rest of the treatments (Cubic effect, $P \leq 0.0001$). In the intestine, the Control-, Control+ and from 0.1–0.5% HA had similar pH, then it significantly fell at 1% HA (Quadratic effect, $P \leq 0.0001$).

The effect of HA on the counts of L. *salivarius* under an *in vitro* poultry digestive model is summarized in Table 6. In the Control- no L. *salivarius* was detected in any of the simulated compartments. In the crop, the L. *salivarius* counts were lower in the Control+ and 0.1% HA, then significant increases were observed at 0.2, 0.5 and, then a further increase was observed at 1% HA addition (Cubic effect, $P \leq 0.0001$). In the proventriculus, all the HA supplemented groups and the Control+ had similar counts. In the intestine, the *L. salivarius* counts were higher at 1% HA

**Table 2**
Effect of humic acids on the counts of *Salmonella Enteritidis*[*] and pH under an *in vitro* poultry digestive model.[**]

| Treatment | Crop | | Proventriculus | | Intestine | |
|---|---|---|---|---|---|---|
| | Counts | pH | Counts | pH | Counts | pH |
| Control- | 0.00[a] | 5.23[a] | 0.00[a] | 2.15[a] | 0.00[a] | 6.84[a] |
| Control+ | 7.18[b] | 5.23[a] | 2.30[b] | 2.21[a] | 6.41[b] | 6.73[b] |
| Humic acid, 0.1% | 7.07[b] | 5.24[a] | 2.30[b] | 2.45[b] | 6.82[c] | 6.94[c] |
| Humic acid, 0.2% | 7.18[b] | 5.36[b] | 2.59[b] | 2.49[b] | 7.49[d] | 6.90[c] |
| Humic acid, 0.5% | 6.79[c] | 5.47[c] | 3.73[c] | 2.46[b] | 8.15[e] | 6.94[c] |
| Humic acid, 1.0% | 6.89[c] | 5.69[d] | 4.72[d] | 1.98[c] | 8.15[e] | 6.16[d] |
| SEM[***] | 0.069 | 0.014 | 0.303 | 0.077 | 0.136 | 0.079 |
| Fit of the regression model | Quadratic | Quadratic | Lineal | Cubic | Quadratic | Quadratic |
| $R^2$ | 0.89 | 0.98 | 0.98 | 0.99 | 0.99 | 0.98 |

a-e Values in columns with different letters differ significantly ($P \leq 0.0001$).
[*] The initial inoculum of *S. Enteritidis* in the feed was $10^8$ CFU/g.
[**] Data are expressed as $log_{10}$ CFU.
[***] Standard error of the mean.

**Table 3**
Effect of humic acids on the counts of *Escherichia coli*[*] and pH under an *in vitro* poultry digestive model.[**]

| Treatment | Crop | | Proventriculus | | Intestine | |
|---|---|---|---|---|---|---|
| | Counts | pH | Counts | pH | Counts | pH |
| Control- | 0.00[a] | 5.09[a] | 0.00[a] | 2.09[a] | 0.00[a] | 6.93[a] |
| Control+ | 7.18[b] | 5.21[b] | 2.32[b] | 2.15[a] | 6.43[b] | 6.94[a] |
| Humic acid, 0.1% | 7.54[c] | 5.29[b] | 2.48[bc] | 2.35[ab] | 6.82[c] | 6.95[a] |
| Humic acid, 0.2% | 7.52[c] | 5.46[c] | 2.84[c] | 2.36[bc] | 7.24[d] | 6.94 [a] |
| Humic acid, 0.5% | 7.72[d] | 5.54[c] | 3.48[d] | 2.43[c] | 7.68[e] | 6.94 [a] |
| Humic acid, 1.0% | 7.88[d] | 6.14[d] | 4.06[d] | 2.02[a] | 8.40[f] | 6.27[b] |
| SEM[***] | 0.104 | 0.040 | 0.189 | 0.079 | 0.112 | 0.035 |
| Fit of the regression model | Cubic | Quadratic | Quadratic | Cubic | Quadratic | Quadratic |
| $R^2$ | 0.94 | 0.97 | 0.99 | 0.97 | 0.98 | 0.99 |

a-f Values in columns with different letters differ significantly ($P \leq 0.0001$).
[*] The initial inoculum of *Escherichia coli* in the feed was $10^6$ CFU/g.
[**] Data are expressed as $\log_{10}$ CFU.
[***] Standard error of the mean.

**Table 4**
Effect of humic acids on the counts of *Clostridium perfringens*[*] and pH under an *in vitro* poultry digestive model[**].

| Treatment | Crop | | Proventriculus | | Intestine | |
|---|---|---|---|---|---|---|
| | Counts | pH | Counts | pH | Counts | pH |
| Control- | 0.00[a] | 5.42[a] | 0.00[a] | 2.14[a] | 0.00[a] | 6.78[a] |
| Control+ | 2.19[b] | 5.45[a] | 1.12[b] | 2.13[a] | 1.61[b] | 6.77[a] |
| Humic acid, 0.1% | 2.16[b] | 5.49[a] | 1.18[b] | 2.21[a] | 1.81[bc] | 6.81 [a] |
| Humic acid, 0.2% | 2.34[b] | 5.66[b] | 1.18[b] | 2.30[b] | 1.94[c] | 6.84[a] |
| Humic acid, 0.5% | 2.28[b] | 5.73[b] | 1.12[b] | 2.41[c] | 2.21[d] | 7.03[b] |
| Humic acid, 1.0% | 2.36[b] | 6.11[c] | 1.16[b] | 2.03[d] | 2.27[d] | 6.22[c] |
| SEM[***] | 0.126 | 0.046 | 0.072 | 0.035 | 0.076 | 0.037 |
| Fit of the regression model | – | Quadratic | – | Quadratic | Quadratic | Cubic |
| $R^2$ | | 0.97 | | 0.99 | 0.99 | 0.99 |

a-d Values in columns with different letters differ significantly ($P \leq 0.0001$).
[*] The initial inoculum of *Clostridium perfringens* in the feed was $10^6$ CFU/g.
[**] Data are expressed as $\log_{10}$ CFU mean ± SE.
[***] Standard error of the mean.

**Table 5**
Effect of humic acids on the counts of *Bacillus subtilis*[*] and pH under an *in vitro* poultry digestive model[**].

| Treatment | Crop | | Proventriculus | | Intestine | |
|---|---|---|---|---|---|---|
| | Counts | pH | Counts | pH | Counts | pH |
| Control- | 0.00[a] | 5.47[a] | 0.00[a] | 2.13[a] | 0.00[a] | 6.90[a] |
| Control+ | 6.72[b] | 5.51[ab] | 1.46[b] | 2.12[a] | 4.33[b] | 6.89[a] |
| Humic acid, 0.1% | 6.69[bc] | 5.55[b] | 1.67[b] | 2.19[b] | 4.30[b] | 6.89[a] |
| Humic acid, 0.2% | 6.57[cd] | 5.62[c] | 1.68[b] | 2.20[b] | 4.40[b] | 6.90[a] |
| Humic acid, 0.5% | 6.52[d] | 5.68[c] | 1.70[bc] | 2.30[c] | 5.00[c] | 6.92[a] |
| Humic acid, 1.0% | 6.71[b] | 5.86[d] | 1.89[c] | 2.04[d] | 5.01[c] | 6.29[b] |
| SEM[***] | 0.054 | 0.019 | 0.079 | 0.021 | 0.065 | 0.011 |
| Fit of the regression model | Quadratic | Lineal | Cubic | Cubic | Cubic | Quadratic |
| $R^2$ | 0.94 | 0.97 | 0.97 | 0.98 | 0.99 | 0.98 |

a-d Values in columns with different letters differ significantly ($P \leq 0.0001$).
[*] The initial inoculum of *Bacillus subtilis* in the feed was $10^6$ CFU/g.
[**] Data are expressed as $\log_{10}$ CFU mean ± SE.
[***] Standard error of the mean.

**Table 6**
Effect of humic acids on the counts of *Lactobacillus salivarius*[*] and pH under an *in vitro* poultry digestive model.[**]

| Treatment | Crop | | Proventriculus | | Intestine | |
|---|---|---|---|---|---|---|
| | Counts | pH | Counts | pH | Counts | pH |
| Control- | 0.00[a] | 5.56[ab] | 0.00[a] | 2.17[a] | 0.00[a] | 6.90[a] |
| Control+ | 4.64[b] | 5.53[a] | 2.51[b] | 2.18[a] | 7.49[b] | 6.89[a] |
| Humic acid, 0.1% | 4.50[b] | 5.58[bc] | 2.45[b] | 2.20[a] | 7.50[b] | 6.90[a] |
| Humic acid, 0.2% | 5.13[c] | 5.61[c] | 2.52[b] | 2.20[a] | 7.54[b] | 6.89[a] |
| Humic acid, 0.5% | 5.12[c] | 5.72[d] | 2.52[b] | 2.33[b] | 7.58[b] | 6.91[a] |
| Humic acid, 1.0% | 5.49[d] | 6.04[e] | 2.49[b] | 2.07[c] | 8.16[c] | 6.73[b] |
| SEM[***] | 0.140 | 0.014 | 0.079 | 0.016 | 0.096 | 0.013 |
| Fit of the regression model | Cubic | Lineal | – | Cubic | Quadratic | Quadratic |
| $R^2$ | 0.81 | 0.98 | | 0.99 | 0.99 | 0.97 |

a-e Values in columns with different letters differ significantly ($P \leq 0.0001$).
[*] The initial inoculum of *Lactobacillus salivarius* in the feed was $10^8$ CFU/g.
[**] Data are expressed as $\log_{10}$ CFU mean ± SE.
[***] Standard error of the mean.

inclusion compared to the rest HA levels and the Control+ (Quadratic effect, $P \leq 0.0001$).

In the crop, the Control- and Control+ had similar pH, and it increased from 0.1 to 1% HA inclusion (Lineal effect, $P \leq 0.0001$). In the proventriculus, the pH was similar between the Control- and Control+ and 0.1 and 0.2% HA, it increased at 0.5% HA, and then fell at 1% HA inclusion compared to the rest of the treatments (Cubic effect, $P \leq 0.0001$). In the intestine, the Control-, Control+ and from 0.1–0.5% HA had similar pH, then it significantly dropped at 1% HA (Quadratic effect, $P \leq 0.0001$).

## 4. Discussion

Humic acids are the principal components of humic substances, which are produced during the humification of decomposing organic matter, particularly from amino acids, lignins, pectins or carbohydrates of plants, leading to the formation of complex macromolecular structures, and are natural components of drinking water, soils and lignite (Islam et al., 2005; Peña-Méndez et al., 2005). HS are fundamentally composed by an aromatic backbone made of nitrogenous heterocycles surrounded by different functional groups such as quinones, ketones, alcohols, phenols, carboxylic and carbonic acids (Stevenson, 1994; Calace et al., 2001). Due to this polyfunctionality, HS interact with anions through positively charged groups and with cations through negatively charged groups (Peña-Méndez et al., 2005). It has been described that a low pH values HA are positively charged, but at pH greater than 4.7 they are negatively charged due to dissociation of protons from carboxylic acids or alcohols, favoring ionic interactions (Kinniburgh et al., 1998; Shao et al., 2013).

In this regard, and assuming that the *in vitro* digestion model used considered enzymatic conditions and three pH, 1.2–2, 5.2 and 6.4–6.8 (Latorre et al., 2015), the effect of HA on the counts of bacteria of importance in poultry as *S. Enteritidis, E. coli, C. perfringens, L. salivarius* and *B. subtilis* was evaluated. The results of the present study showed significant increases in the counts of all five species of bacteria evaluated in the compartment that simulated the intestine in an *in vitro* chicken model (Figs. 1–5).

In the simulated crop the counts of each of the bacteria were different due to their differences on the nutritional and environmental conditions, as well as, their specific growing time to each bacterium. Although the general trend in bacterial counts was upward, there was a significant decrease in *S. Enteritidis* counts as the concentration of HA increased. In a previous experiment, using 0.1 or 0.2% HA the recovery of *S. Enteritidis* in the same compartment under the same *in vitro* digestive model was not affected by the addition of HA (Maguey-Gonzalez et al., 2018b) which agree with the present results.

In the proventriculus, bacterial counts were significantly reduced compared to those observed in the intestine and crop. This reduction can be explained considering the low pH since it is an important factor for bacterial growth (Jin and Kirk, 2018). However, the counts of Gram-negative bacteria were less affected (*S. Enteritidis* and *E. coli*) compared to Gram-positive bacteria (*C. perfringens*, *B. subtilis* and L. *salivarius*). This could be due to the presence of the outer wall in Gram negative bacteria, which can protect the bacteria from its acidic environment. Another possible reason could be associated with the amphiphilic characteristics of HS since they can act as natural surfactants absorbing on different surfaces including biological membranes of bacteria, being stronger at low pH (Fein et al., 1999; Visser, 1985; Pflug, 1982). Protective properties of HS against the cell-wall disruption of Micrococcus luteous by the enzyme lysozyme (Pflug, 1982) and to the exposure of bacterial and cultures of human cells to UV-B radiation (Klöcking et al., 2013) have been reported. Whether HA is able to protect certain type of bacteria from acidifying in the proventriculus by interacting with the bacterial wall remains to be clarified.

In the simulated intestine *S. Enteritidis, E. coli, C. perfringens* and *B. subtilis* counts linearly increased due to the additions of HA. The higher bacterial counts found in the simulated intestine may be due to better environmental conditions such as a close to neutral pH and the very high availability of nutrients released from the feed components. In line with our results, it has been reported that in the small intestine reside bacterial communities, dominated by the phyla Firmicutes, predominating the genus *Lactobacillus* with 70% of the total, and Clostridiales (Yadav and Jha, 2019). Other phyla commonly found in the small intestine are the Bacteroidetes (Bacteroides), Actinobacteria (Bifidobacterium) and Proteobacterias that includes the enterobacteriacea family (such as *E. coli* and *Salmonella spp*). (Clavijo and Flórez, 2018; Shang et al., 2018; Díaz Carrasco et al., 2019).

Although the effect of HA on the growth of microorganisms present in the digestive tract are unclear, in an *in vitro* study, in which natural and synthetic HA were tested, the spectrum and degree of antimicrobial activity against several human pathogenic bacteria varied according to the origin and mode of extraction of natural HS (Ansorg and Rochus, 1978), as well as the modifications made at the structural level since it has been reported that they can decrease from 78 to 80% and from 58 to 70% the number of *E. coli* and *S. Enteritidis*, respectively, compared to natural HA (Yarkova, 2011).

In the present study HA increased bacterial counts. It has been reported that HA can be used as substrates providing organic carbon or nutrient such as nitrogen, phosphorus, trace elements and vitamins in plants, fungi and bacteria, as well as, enhancing nutrient supply by increasing the uptake of nitrogen, minerals and micronutrients (Filip and Demnerova, 2009; Kulikova et al., 2005; Tan, 2014). In addition, some reports indicate that HS stimulates the growth and diversity of bacterial communities in the soil and the environment (Ueno et al., 2016; Yang and Antonietti, 2020; Visser, 1985). Based on this, it could

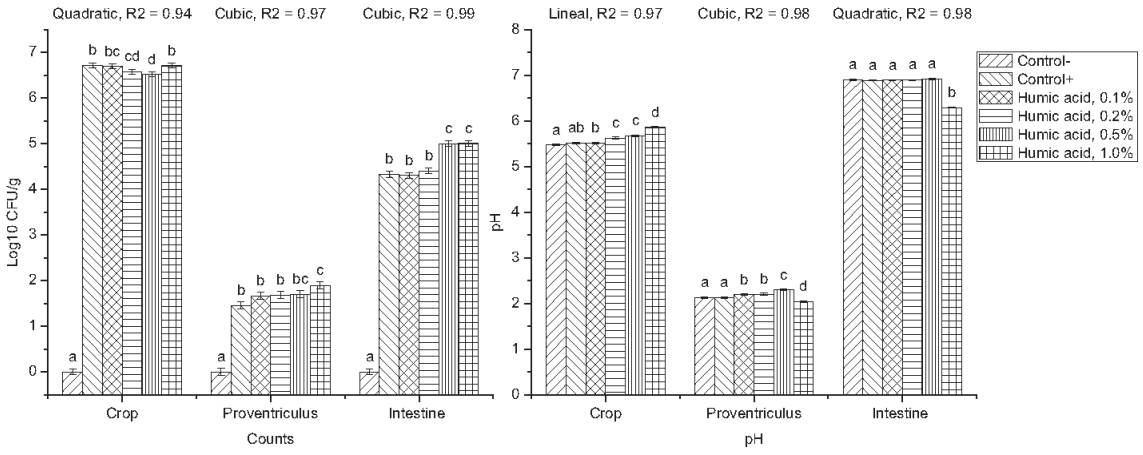


**Fig. 1.** Effect of humic acids on the counts of *Salmonella Enteritidis** and pH under an *in vitro* poultry digestive model.
*The initial inoculum of *S. Enteritidis* in the feed was $10^8$ CFU/g. [a-e] Values in columns with different letters differ significantly ($P \leq 0.0001$).

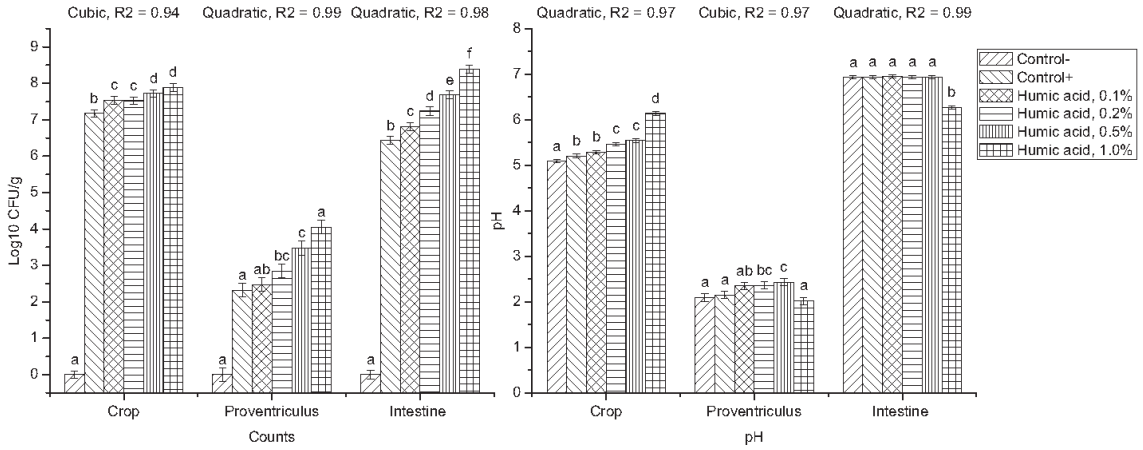


**Fig. 2.** Effect of humic acids on the counts of *Escherichia coli** and pH under an *in vitro* poultry digestive model.
*The initial inoculum of *Escherichia coli* in the feed was $10^6$ CFU/g.
[a-f] Values in columns with different letters differ significantly ($P \leq 0.0001$).

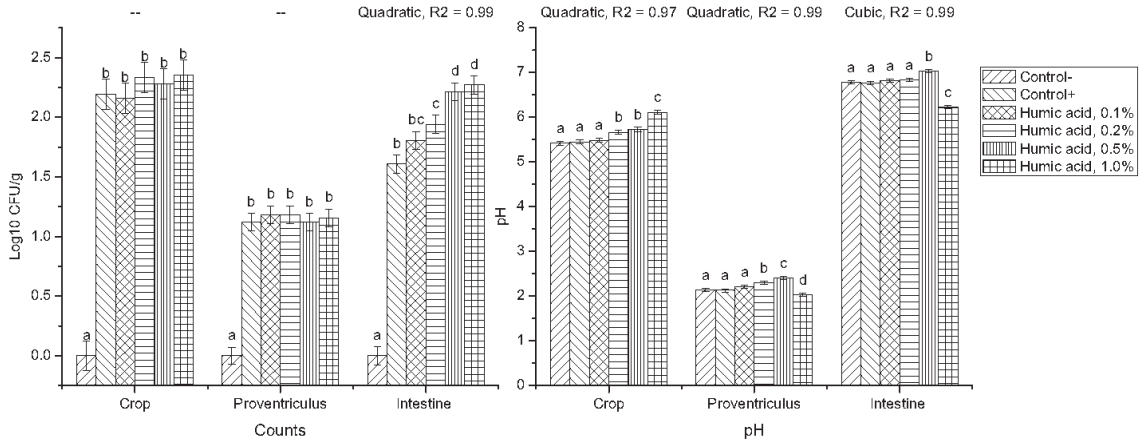


**Fig. 3.** Effect of humic acids on the counts of *Clostridium perfringens** and pH under an *in vitro* poultry digestive model.
*The initial inoculum of *Clostridium perfringens* in the feed was $10^6$ CFU/g.
[a-d] Values in columns with different letters differ significantly ($P \leq 0.0001$).

be mentioned that HA are improving the assimilation of nutrients found in the feed that was used in the *in vitro* digestion model, which is causing an increase in bacterial counts in the intestinal compartment.

One main difference when comparing our results with other *in vitro* studies is that real digestive environmental conditions were used in which pH and transit time through the digestive tract was closely simulated. Pepsins and pancreatic enzymes added to the proventriculus and intestine probably released in the medium different types of dietary components ready to be used by bacteria which are also commonly available in real situations. *In vitro* experiments, a cytotoxic effect of HS against many mammalian and bacterial cells have been demonstrated due to the accumulation of free-radicals during long-term culture times, inducing lower oxygen uptake, lower electron transfer to acceptors and lipid peroxidation in cell membranes (Hassett et al., 1987; Ho et al., 2003).

It is probably that in some long-term *in vitro* experiments the antimicrobial activity of HS was due to the accumulation of toxic metabolites in the culture. It is probably that in our simulated digestive system the rapid transit time through the digestive compartments, the addition of the buffering solutions and the presence of several dietary components in the intestine overcome the possible accumulation of toxic substances or neutralize the effects of free-scavenging radicals. This is a topic that remains to be clarified.

When comparing these *in vitro* results with *in vivo* studies conducted in our laboratory, it is a fact that the inclusion concentration of HA in the diets is important to increase the counts of beneficial bacteria and have marked effects on the productive parameters since the differences observed are only numerical (Maguey-Gonzalez et al., 2018a,b; Dominguez-Negrete et al., 2019).

It should be considered that other mechanisms of action have been proposed to explain the benefits observed in broiler chickens supplemented with HS, including the ability to create protective layers over the epithelial mucosal membrane of the digestive tract against the penetration of toxic and other bacterial contaminated substances (Kühnert

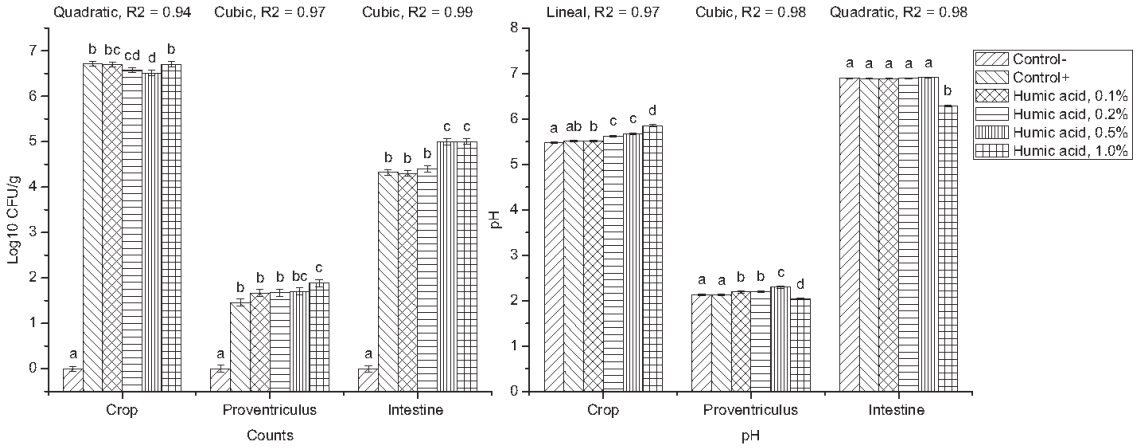


**Fig. 4.** Effect of humic acids on the counts of *Bacillus subtilis** and pH under an *in vitro* poultry digestive model.
*The initial inoculum of *Bacillus subtilis* in the feed was $10^8$ CFU/g.
[a-d] Values in columns with different letters differ significantly ($P \leq 0.0001$).

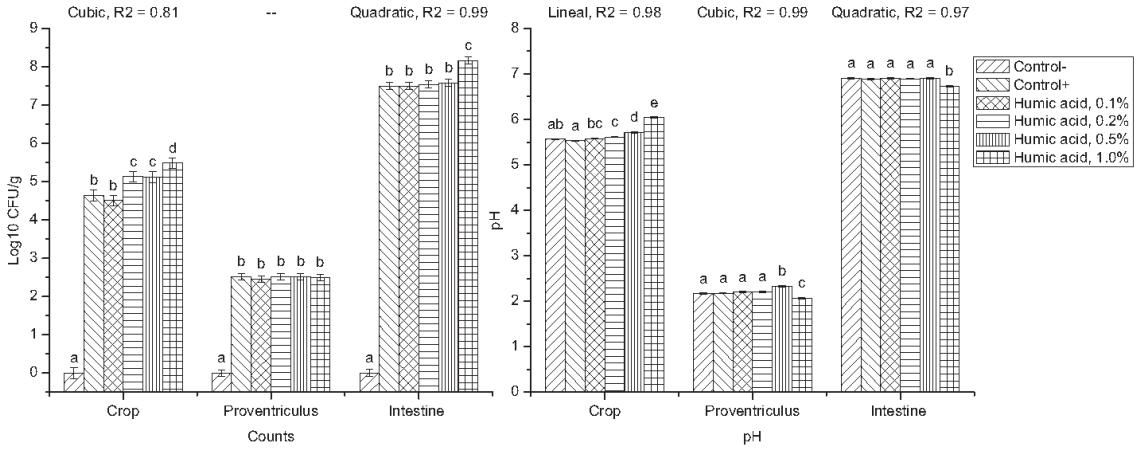


**Fig. 5.** Effect of humic acids on the counts of *Lactobacillus salivarius** and pH under an *in vitro* poultry digestive model.
*The initial inoculum of *Lactobacillus salivarius* in the feed was $10^8$ CFU/g.
[a-e] Values in columns with different letters differ significantly ($P \leq 0.0001$).

et al., 1991; Maguey-Gonzalez et al., 2018b). These effects have been associated to the macro colloidal structure of HA ensuring a good shielding on mucous membranes of the stomach and gut, but also to the induction of higher mucins production in the GIT (Mudrovnová et al., 2020).

As growth promoters HA improve nutrient absorption and could alter the intestinal microbiota by stimulating their growth, providing a protective barrier against the penetration of pathogens and other toxic substances in the intestine, which translates into better intestinal health (Pukalchik et al., 2019).These effects have been associated to the macro colloidal structure of HA ensuring a good shielding on mucous membranes of the stomach and gut, but also to the induction of higher mucins production in the GIT (Mudrovnová et al., 2020).

## 5. Conclusions

In conclusion, the addition of HA extracted from worm compost in an *in vitro* chicken digestive system caused a trend of increasing bacterial counts in the crop as the concentration of HA increased, with the exception of *S. Enteritidis*. Bacterial counts in the proventriculus were significantly lower than in the intestine and crop, but Gram-negative bacteria (*S. Enteritidis and E. coli*) were less affected than Gram-positive bacteria (*C. perfringens, B. subtilis and* L. *salivarius*). The addition of HA increased the counts of *S. Enteritidis*, *E. coli*, *Clostridium perfringens*, and *Bacillus subtilis* in the simulated intestine. It is clear that HA can be used by bacteria as substrates since they are organic sources of carbon, nitrogen, phosphorus and other nutrients. However, they can also improve nutrient assimilation, as probably occurred during our experiments as bacterial counts were increased. The mechanisms by which HA enhanced the growth of the different bacterial strains and the pH changes in every simulated compartment need to be clarified. Further studies to evaluate the changes in structure, composition and colloidal properties of HA under different pH conditions are currently being conducted.

## Declaration of Competing Interest

None.

## Acknowledgments

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## References

Abd El-Hack, M.E., 2016. Impacts of dietary humic acid supplementation on growth performance, some blood metabolites and carcass traits of broiler chicks. Indi. J. Animal. Sci. 86 (9), 1073–1078.
Aidara-Kane, A., Angulo, F.J., Conly, J.M., Minato, Y., Silbergeld, E.K., McEwen, S.A., Collignon, P.J., 2018. World Health Organization (WHO) guidelines on use of medically important antimicrobials in food-producing animals. Anti. Resist. Infec. Con. 7 (1), 1–8.
Ansorg, R., Rochus, W., 1978. Studies on the antimicrobial effect of natural and synthetic humic acids. Arzneimittelforsch. 28, 2195–2198.
Apata, D., 2009. Antibiotic resistance in poultry. Inter. J. Poult. Sci 8 (4), 404–408.
Arif, M., Alagawany, M., Abd El-Hack, M., Saeed, M., Arain, M., Elnesr, S., 2019. Humic acid as a feed additive in poultry diets: a review. Irani. J. Vet. Res. 20 (3), 167.
Ashbolt, N.J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., Coors, A., 2013. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. Enviro. Health. Perspec. 121 (9), 993–1001.
Calace, N., Liberatori, A., Petronio, B., Pietroletti, M., 2001. Characteristics of different molecular weight fractions of organic matter in landfill leachate and their role in soil sorption of heavy metals. Enviro. Poll. 113 (3), 331–339.
Clavijo, V., Flórez, M.J.V., 2018. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: a review. Poul. Sci. 97 (3), 1006–1021.
Diaz Carrasco, J.M., Casanova, N.A., Fernández Miyakawa, M.E., 2019. Microbiota, gut health and chicken productivity: what is the connection? Micro. 7 (10), 374.
Dominguez-Negrete, A., Gómez-Rosales, S., Angeles, M., López-Hernández, L.H., Reis-de Souza, T.C., López-Garcia, Y., Zavala-Franco, A., 2019. Effect of the addition of humic substances as growth promoter in broiler chickens under two feeding regimens. Animals 9 (12), 1101.
Fein, J.B., Boily, J.-F., Güçlü, K., Kaulbach, E., 1999. Experimental study of humic acid adsorption onto bacteria and Al-oxide mineral surfaces. Chem. Geo. 162 (1), 33–45.
Ferket, P.R., 2004. Alternatives to antibiotics in poultry production: responses, practical experience and recommendations. Nut. Biotech. Feed. Food. Indus. 57–67.
Filip, Z., Demnerova, K., 2009. Humic substances as natural interacting agents: their characteristics and possible fate in environment. Exposure and Risk Assessment of Chemical Pollution. Contem. Metho, 247–257.
Galip, N., Polat, U., Biricik, H., 2010. Effects of supplemental humic acid on ruminal fermentation and blood variables in rams. Ital. J. Animal. Sci. 9 (4), e74.
Hassett, D.J., Bisesi, M.S., Hartenstein, R., 1987. Bactericidal action of humic acids. Soil. Bio. Biochem. 19 (1), 111–113.
Ho, K.J., Liu, T.K., Huang, T.S., Lu, F.J., 2003. Humic acid mediates iron release from ferritin and promotes lipid peroxidation in vitro: a possible mechanism for humic acid-induced cytotoxicity. Archiv. Toxi. 77 (2), 100–109.
Islam, K.M.S., Schuhmacher, A., Gropp, J., 2005. Humic acid substances in animal agriculture. Pakis. J. Nut. 4 (3), 126–134.
Jin, Q., Kirk, M.F., 2018. pH as a primary control in environmental microbiology: 2. Kinetic perspective. Front. Enviro. Sci. 6, 101.
Kinniburgh, D.G., Van Riemsdijk, W.H., Koopal, L.K., Benedetti, M.F., 1998. Ion binding to humic substances: measurements, models, and mechanisms. Adsorp. Metals. Geo. 483–520.
Klöcking, R., Felber, Y., Guhr, M., Meyer, G., Schubert, R., Schoenherr, J.I., 2013. Development of an innovative peat lipstick based on the uv-b protective effect of humic substances. Mire. Peat. 11.
Kühnert, V.M., Bartels, K.P., Kröll, S., Lange, N., 1991. Huminsäurehaltige Tierarzneimittel in Therapie und Prophylaxe bei gastrointestinalen Erkrankungen von Hund und Katze. Monatshefte Vet. Med. 46, 4–8.
Kulikova, N., Stepanova, E., Koroleva, O., 2005. Mitigating activity of humic substances: direct influence on biota. Use of humic substances to remediate polluted environments: from theory to practice, pp. 285–309.
Latorre, J.D., Hernandez-Velasco, X., Kuttappan, V.A., Wolfenden, R.E., Vicente, J.L., Wolfenden, A.D., Bielke, L.R., 2015. Selection of Bacillus spp. for cellulase and xylanase production as direct-fed microbials to reduce digesta viscosity and Clostridium perfringens proliferation using an in vitro digestive model in different poultry diets. Front. Vet. Sci 2.
Lee, K.-W., Hong, Y.H., Lee, S.-H., Jang, S.I., Park, M.-S., Bautista, D.A., Ritter, G.D., 2012. Effects of anticoccidial and antibiotic growth promoter programs on broiler performance and immune status. Reser. Vet. Sci. 93 (2), 721–728.
Maguey-Gonzalez, J.A., Michel, M.A., Baxter, M.F., Solis-Cruz, B., Hernandez-Patlan, D., Merino-Guzman, R., Hernandez-Velasco, X., et al., 2018b. Effects of humic acids on recovery of Salmonella enterica serovar Enteritidis. Annals. Animal Sci. 18 (2), 387–399.
Maguey-Gonzalez, J.A., Michel, M.A., Baxter, M.F., Tellez Jr., G., Moore Jr., P.A., Solis-Cruz, B., Hernández-Patlan, D., 2018a. Effect of humic acids on intestinal viscosity, leaky gut and ammonia excretion in a 24 hr feed restriction model to induce intestinal permeability in broiler chickens. Animal. Sci. J. 89 (7), 1002–1010.
Mudrovnová, D., Karaffová, V., Pevsulová, T., Kovsvcová, J., Maruvsvcáková, I.C., Bartkovsk'y, M., Marcinvcáková, D., 2020. The effect of humic substances on gut microbiota and immune response of broilers. Food. Agri. Immuno. 31 (1), 137–149.
Niewold, T.A., 2007. The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. Poul. Sci. 86 (4), 605–609.
Parsons, J., 1983. Humus chemistry—genesis, composition. Reactions. Soil Science 135 (2), 129–130.
Peña-Méndez, E.M., Havel, J., Patovcka, J., 2005. Humic substances-compounds of still unknown structure: applications in agriculture, industry, environment, and biomedicine. J. Appl. Biomed. 3 (1), 13–24.
Pflug, W., 1982. Humic Acids and the Disruption of Bacterial Cell Walls by Lysozyme.
Pukalchik, M., Kydralieva, K., Yakimenko, O., Fedoseeva, E., Terekhova, V., 2019. Outlining the potential role of humic products in modifying biological properties of the soil—a review. Front. Enviro. Sci. 7, 80.
Riede, U.N., Zeck-Kapp, G., Freudenberg, N., Keller, H., Seubert, B., 1991. Humate-induced activation of human granulocytes. Virchows Archiv B 60 (1), 27–34.
Salim, H.M., Huque, K.S., Kamaruddin, K.M., Haque Beg, A., 2018. Global restriction of using antibiotic growth promoters and alternative strategies in poultry production. Sci. Prog. 101 (1), 52–75.
SAS. SAS User's Guide, Versión 9.0, 2002. SAS Institute Inc, Cary, NC, USA.
Schnitzer, M., Khan, S., 1972. Humic Substances in the Environment. Marcel Dekker, New York.
Shang, Y., Kumar, S., Oakley, B., Kim, W.K., 2018. Chicken gut microbiota: importance and detection technology. Front. Vet. Sci. 5, 254.
Shao, J., Zhao, L., Chen, X., He, Y., 2013. Humic acid rejection and flux decline with negatively charged membranes of different spacer arm lengths and charge groups. J. Mem. Sci. 435, 38–45.
Shermer, C., Maciorowski, K., Bailey, C., Byers, F., Ricke, S., 1998. Caecal metabolites and microbial populations in chickens consuming diets containing a mined humate compound. J. Sci. Food. Agri. 77 (4), 479–486.
Stevenson, F.J., 1994. Humus Chemistry: Genesis, Composition, Reactions. John Wiley and Sons.
Tan, K.H., 2014. Humic Matter in Soil and the Environment: Principles and Controversies.
Ueno, A., Shimizu, S., Tamamura, S., Okuyama, H., Naganuma, T., Kaneko, K., 2016. Anaerobic decomposition of humic substances by Clostridium from the deep subsurface. Sci. Rep. 6 (1), 1–9.
Van den Bogaard, A., London, N., Driessen, C., Stobberingh, E., 2001. Antibiotic resistance of faecal Escherichia coli in poultry, poultry farmers and poultry slaughterers. J. Anti. Chemo. 47 (6), 763–771.
Visser, S., 1985. Physiological action of humic substances on microbial cells. Soil. Bio. Biochem. 17 (4), 457–462.
Yadav, S., Jha, R., 2019. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. J. Animal. Sci. Biotech. 10 (1), 1–11.
Yang, F., Antonietti, M., 2020. The sleeping giant: a polymer view on humic matter in synthesis and applications. Prog. Poly. Sci. 100, 101182.
Yarkova, T., 2011. Chemical modification of humic acids by the introduction of indole-containing fragments. Sol. Fuel. Chem. 45 (4), 261.
Zhang, Y.-J., Hu, H.-W., Gou, M., Wang, J.-T., Chen, D., He, J.-Z., 2017. Temporal succession of soil antibiotic resistance genes following application of swine, cattle and poultry manures spiked with or without antibiotics. Enviro. Poll. 231, 1621–1632.

# VII. DISCUSIÓN

## Caracterización de los ácidos húmicos

### Composición elemental

Respecto a la composición elemental, el espectro de la EDS indica que el carbón representa el 35.42% de los AH. Seguido por el oxígeno (20.32%) nitrógeno (27.18 %), potasio (6.66 %) y otros elementos menores como el cloro (3.77 %), azufre (3.00 %), fósforo (1.53 %), silicio (0.82 %), aluminio (0.26 %) y calcio (0.54 %). Los parámetros coinciden con los rangos publicados por Giovanela et al (2010) y Liu et al (2020). Los principales elementos utilizados en el análisis de los AH son el carbono, hidrógeno, oxígeno y nitrógeno. Las proporciones de estos elementos puede variar de acuerdo a la fuente y la forma de extracción, pero manteniendo una proporción de estos principales elementos. Aunque la presencia de elementos traza dificulta la caracterización, la identificación de los principales grupos funcionales sigue siendo la forma más adecuada para el estudio de los AH (Doskočil et al., 2018).

### Principales grupos funcionales

En general, los espectros FTIR-ATR mostraron que los AH contienen una amplia variedad de grupos funcionales ácidos como carboxílicos, carbonilos, hidroxilos y fenólicos (dominios hidrofílicos), así como grupos alifáticos, aromáticos y metilo (dominios hidrofóbicos). Estos grupos funcionales se consideran sitios potenciales para la unión de contaminantes (Rupiasih y Vidyasagar, 2009; Jiménez-González

et al., 2019), y para la formación de complejos con ciertos cationes metálicos (Guo et al., 2019). Otros estudios indican que los grupos fenólicos asociados a OH, C-O, C=O forman enlaces de hidrógeno con ciertos compuestos orgánicos e inorgánicos (Vermeer et al., 1998; Xu et al., 2005; Zhang et al., 2009). Por ejemplo, Vázquez-Durán et al (2021) indicaron que el OH podría establecer enlaces de hidrógeno con los átomos de oxígeno en los grupos metoxi, carbonilo y éter de la molécula de $AFB_1$.

**Microestructura**

La morfología superficial y la microestructura de los AH se observó utilizando microscopía electrónica de barrido (SEM). La superficie de los AH es rugosa e irregular con la presencia de agregados en diversas formas y tamaños. La mayoría de las partículas tienen un tamaño promedio de 163 ± 20,1 µm. Además, se observaron surcos en la superficie de las partículas con trazas de gránulos. Estos resultados son consistentes con la estructura granular de varios AH extraídos de diversas fuentes (Prado et al., 2011; Yang et al., 2019). La composición física y química de los AH difiere según su fuente, las condiciones ambientales y el procedimiento de extracción (Chen et al., 2007; Rupiasih et al., 2009; Manzak et al., 2017). Asimismo, diversas variables, como la concentración, el pH, la fuerza iónica, el grado de ionización de los grupos ácido y las interacciones intermoleculares, influyen en la conformación estructural de los AH (Tarasevich et al., 2013; Sarlaki et al., 2020). La estructura de los AH se protona a un pH inferior a 4, lo que da como resultado una estructura agregada. Mientras que la

carga superficial es casi nula a niveles de pH entre 4 y 6, lo que reduce la repulsión intramolecular y promueve una macroestructura más expandida y dispersa (Prado et al., 2011). A pH superior a 7, se puede observar una estructura abierta y completamente desplegada (Klučáková et al., 2017). Ciertas moléculas de AH participan en interacciones supramoleculares a través de fuerzas dispersivas como enlaces de hidrógeno, interacciones de Van der Waals e interacciones π-π, lo que da lugar a diferentes configuraciones estructurales que pueden presentar estos compuestos (Baigorri et al., 2007; Prado et al., 2011).

**Carga eléctrica superficial**

El potencial ζ se emplea con frecuencia para conocer el comportamiento de las partículas suspendidas en un líquido. Además, la magnitud del potencial ζ indica la fuerza de la atracción o repulsión electrostática entre partículas; por lo tanto, puede usarse para describir la superficie de partículas cargadas (Lin et al., 2003; Butt et al., 2013). Los valores de pH que se utilizaron para la determinación del potencial ζ fueron 2, 5 y 7, cada uno de acuerdo con el compartimento simulado del tracto intestinal (buche, proventrículo e intestino) en el modelo *in vitro*. En ambos materiales utilizados el valor del potencial ζ se volvió más negativo a medida que el pH se aumento. Respecto a los AH el valor del potencial ζ fue de -46.44 mV, -50.16 mV, -54.46 mV, respectivamente a cada compartimiento. Nuestros hallazgos son consistentes con los informados por Hamza et al (2019), quienes afirman que a medida que aumenta el pH, los grupos funcionales ácidos de los AH se desprotonan, lo que da como resultado una superficie con una mayor

carga negativa. Además, Deng y Bai (2003) también encontraron que cuando el pH excede 1.9, los AH tienen un potencial ζ negativo. Coles y Yong (2006), informaron valores de potencial ζ de -20 mV a pH 3 y -44 mV a pH 10. Finalmente, Loosli et al (2013) informaron una fuerte carga negativa con valores de potencial ζ que oscilan entre -30.2 mV a pH 3 y -69.0 mV a pH 11. Incluso a pH bajo, los AH tienen carga negativa debido a la disociación de sus grupos funcionales ácidos (principalmente carboxílicos y hidroxilo fenólico) (Vermeer et al., 1998; Illés y Tombácz, 2003). Las variaciones en la conformación de los grupos ácidos pueden usarse para explicar cómo las interacciones dependen del pH (Klučáková et al., 2017). Se reconoce que la primera disminución del potencial ζ (pH 3) corresponde a la disociación de los grupos ácido carboxílico, mientras que la segunda disminución del potencial ζ (pH 6) corresponde al inicio de la ionización de los grupos ácido fenólicos (Alvarez-Puebla et al., 2005). En este trabajo de investigación se encontró que los AH tienen un potencial ζ altamente negativo en los tres compartimientos del modelo *in vitro*, particularmente a pH 7. Por lo tanto, la molécula de la $AFB_1$ y la superficie de los AH podrían interactuar por fuerzas electrostáticas, ya que los AH son polielectrolitos aniónicos que pueden interactuar con las moléculas $AFB_1$ cargadas positivamente (van Rensburg et al. 2006; Vázquez-Durán et al., 2016; Hamza et al., 2019).

El pHpzc representa el punto de carga cero, donde la suma de las cargas positivas y negativas es igual, esta técnica ayuda a comprender la carga superficial de las partículas. Se ha reportado que la superficie del adsorbente estará cargada

positivamente si el pH < pHpzc y negativamente si el pH > pHpzc (Ramales-Valderrama et al., 2016). El pHpzc para los AH fue a pH 10.4. De acuerdo con Coles y Yong (2006), el pHpzc de los AH que analizaron fue inferior a 0.5. De la misma forma, Volk (1976) informó valores de pHpzc que oscilan entre 1.2 y 1.8. Por otro lado, Giasuddin et al (2007) informaron que el pHpzc de los AH se encuentra en un rango de pH de 5 a 9.3. Sin embargo, se conoce que la presencia de materia orgánica o impurezas en los AH determina si el pHpzc disminuye o aumenta (Coles y Yong, 2006). Teniendo en cuenta lo anterior, los AH provenientes de lombricompostas tienen una carga superficial positiva en las tres secciones gastrointestinales simuladas, lo que indica que las interacciones electrostáticas no gobiernan la adsorción de la $AFB_1$. Otros mecanismos, como la atracción moderada entre donador y aceptor de electrones, contribuyen a la adsorción de micotoxinas en adsorbentes inorgánicos, mecanismos que posiblemente pueden llevarse acabo por los AH (Zavala-Franco et al., 2018).

### Capacidad adsorbente de $AFB_1$

Los AH tuvieron una capacidad adsortiva de $AFB_1$ en el modelo *in vitro* del 97.6%, en comparación al 81.5% de adsorción de la zeolita utilizada como control. En un estudio *in vitro* con 100 mg/ml de inclusión de AH y un reto de 20 ng de $AFB_1$/g de alimento, se observó una adsorción de $AFB_1$ del 90.50 % (El-Shafea, 2014). Además, Ye et al (2009) investigaron la capacidad adsortiva de $AFB_1$ en presencia de diferentes niveles de AH, bajo diferentes pH, tiempos de interacción y concentraciones de $AFB_1$. Los mayores porcentajes de adsorción fueron 88.12 %

a pH 7 y 76.36 % a pH 8. Mientras tanto, Vázquez-Durán et al (2021) informaron un porcentaje de adsorción del 75.5 %, cuando utilizaron un modelo dinámico *in vitro* para evaluar la capacidad de adsorción de $AFB_1$ de una zeolita no comercial con una inclusión del 5 % (p/p), lo cual es consistente con los resultados de esta investigación.

### Potencial mecanismo de absorción

Debido a que los AH poseen superficies altamente hidrofóbicas y una amplia variedad de grupos funcionales cargados negativamente (Kabak et al., 2006), las interacciones entre los AH y la $AFB_1$ pueden involucrar diferentes mecanismos. Tan (2003) enumera siete posibles formas en que los AH podrían interactuar con componentes gaseosos, líquidos y sólidos: (1) fuerzas físicas, (2) fuerzas químicas, (3) enlaces de hidrógeno, (4) interacciones hidrofóbicas, (5) interacciones electrostáticas, (6) reacciones de coordinación, y (7) intercambio de ligandos. Las interacciones más importantes entre los AH y la $AFB_1$ son las electrostáticas y los enlaces de hidrógeno. Sin embargo, podrían considerarse otras interacciones, como el apilamiento π-π (Tikhonov et al., 2019) y las interacciones hidrofóbicas debido a los numerosos enlaces relacionados con grupos hidrofóbicos, incluidos $CH_2$, $CH_3$ y C=C (Islam et al., 2020). Aunque el mecanismo por el cual la unión AH-$AFB_1$ aún no se comprende completamente, se reconoce que las estructuras aromáticas y los grupos funcionales como -OH y -COOH contribuyen a la alta capacidad de adsorción de los AH (Zhou et al., 2019). Se han propuesto diversas técnicas para investigar la absorción de los AH en

diferentes moléculas. Por ejemplo, los modelos físico (isotermia de Langmuir), cinético (modelo cinético de Elovich), de complejación de superficies y de distribución de ligandos y cargas, entre otros (Islam et al., 2020). Para dilucidar la naturaleza de las interacciones moleculares entre HA y AFB1, se pueden considerar futuras simulaciones teóricas utilizando la teoría funcional de la densidad (DFT) (Vázquez-Durán et al., 2022).

### Conteos bacterianos

Aunque se ha demostrado el efecto promotor del crecimiento de las SH provenientes de lombricompostas en la producción de pollos de engorda, sus efectos antimicrobianos son inconsistentes. Se evaluó el efecto de los AH sobre los conteos bacterianos de *Salmonella* Enteritidis (SE), *Escherichia coli* (EC), *C. perfringens* (CP), *Bacillus subtilis* (BS), y *Lactobacillus salivarius* (LS) con el uso de un modelo *in vitro* que simula el tracto digestivo de las aves de engorda.

En general, los conteos bacterianos de los cinco microorganismos utilizados aumentaron en el último comportamiento simulado (intestino) conforme se aumento la inclusión de AH. Estos hallazgos pueden deberse a que en el intestino simulado, se dan condiciones ambientales favorables para cada uno de los microorganismos utilizados, además de una alta disponibilidad de nutrientes liberados de los componentes del alimento. Se ha reportado que en el intestino delgado residen comunidades bacterianas dominadas por los filos Firmicutes, en los que predomina el género *Lactobacillus* con el 70%, y Clostridiales con el 10%

del total de las poblaciones bacterianas (Yadav y Jha, 2019). Otros filos que se encuentran comúnmente en el intestino delgado son Bacteroidetes (Bacteroides), Actinobacteria (Bifidobacterium) y Proteobacterias que incluye la familia de las enterobacterias (como *E. coli* y *Salmonella* spp) (Clavijo y Flórez, 2018; Shang et al., 2018; Diaz-Carrasco et al., 2019). Adicionalmente, los AH pueden ser utilizados como sustrato por plantas, hongos y bacterias, ya que proporcionan nutrientes como carbono, nitrógeno, fósforo, oligoelementos y vitaminas. Además, se ha observado que los AH mejoran la absorción de minerales y micronutrientes en las plantas (Tan, 2003; Kulikova et al., 2005; Filip y Demnerova, 2009). Algunos estudios indican que las SH estimulan el crecimiento y la diversidad de comunidades bacterianas en el suelo y en el ambiente (Visser, 1985; Ueno et al., 2016; Yang y Antonietti, 2020).

Actualmente, se ha estudiado el efecto prebiótico de los AH en conjunto con diferentes microorganismos benéficos para la salud intestinal de las aves. Ceylan et al (2003) mencionan que el uso de AH y probióticos mejoró la conversión alimenticia y la microbiota intestinal de las aves de engorda. Arpášová et al (2016) indican que la suplementación de 0.5% de AH en la dieta de aves de postura mejoró significativamente la producción y la calidad de los huevos. Es claro que los AH pueden ser utilizados por los microorganismos como substrato, ya que son una fuente rica en carbón, nitrógeno, fosforo y otros nutrientes. El mecanismo por el cual los AH promueven el crecimiento bacteriano en las aves de engorda necesita ser aclarado.

## VIII. CONCLUSIONES

En la primera parte de este estudio, se puede concluir que los AH derivados de lombricomposta son altamente efectivos en la adsorción de $AFB_1$. Sin embargo, las pruebas *in vitro* no pueden simular completamente las condiciones del tracto digestivo de las aves de engorda. Por lo tanto, es necesario realizar experimentos *in vivo* para mejorar nuestra comprensión sobre la eficacia de los AH para reducir los efectos tóxicos de la $AFB_1$ en aves y posiblemente en otras especies. De la misma forma, se pueden considerar futuras simulaciones teóricas para conocer al detalle las interacciones físicas y químicas entre los AH y la $AFB_1$.

Respecto al efecto de los AH sobre el comportamiento de los microorganismos utilizados en el modelo *in vitro*, se podría mencionar que los AH mejoran la asimilación de los nutrientes que se encuentran en el alimento que se utilizó provocando un aumento en los conteos bacterianos en el compartimento intestinal. Por lo tanto, se puede concluir que los AH podrían ser utilizados como sustrato por la microbiota intestinal y mejorar la disposición de nutrientes, promoviendo el desarrollo de una microbiota beneficiosa para las aves de engorda. Es importante esclarecer como influyen los AH en grupos bacterianos de interés para la industria avícola. Actualmente se están realizando investigaciones para conocer a detalle como influyen los AH en la microbiota intestinal en aves de engorda.

## IX. LITERATURA CITADA


Abd El-Shafea, Yasser. 2014. «In vivo and in vitro evaluation of efficacy of humic acid against aflatoxins». *Al-Azhar Journal of Pharmaceutical Sciences* 49(1):133-54. doi: 10.21608/ajps.2014.6962.

Aeschbacher, Michael, Daniele Vergari, René P. Schwarzenbach, y Michael Sander. 2011. «Electrochemical Analysis of Proton and Electron Transfer Equilibria of the Reducible Moieties in Humic Acids». *Environmental Science & Technology* 45(19):8385-94. doi: 10.1021/es201981g.

Ait Baddi, Ghita, Mohamed Hafidi, Véronique Gilard, y Jean-Claude Revel. 2003. «Characterization of Humic Acids Produced during Composting of Olive Mill Wastes: Elemental and Spectroscopic Analyses (FTIR and $^{13}$ C-NMR)». *Agronomie* 23(7):661-66. doi: 10.1051/agro:2003042.

Alvarez-Puebla, R. A., y J. J. Garrido. 2005. «Effect of PH on the Aggregation of a Gray Humic Acid in Colloidal and Solid States». *Chemosphere* 59(5):659-67. doi: 10.1016/j.chemosphere.2004.10.021.

Angeles, Ml, S. Gómez-Rosales, Yr López-Garcia, y A. Montoya-Franco. 2022. «Growth Performance and Tibia Mineralization of Broiler Chickens Supplemented with a Liquid Extract of Humic Substances». *Brazilian Journal of Poultry Science* 24(3):eRBCA-2021-1450. doi: 10.1590/1806-9061-2021-1450.

Arafat, Rana Yaser, Sohail Hassan Khan, y Saima. 2017. «Evaluation of Humic Acid as an Aflatoxin Binder in Broiler Chickens». *Annals of Animal Science* 17(1):241-55. doi: 10.1515/aoas-2016-0050.

Arif, M., M. Alagawany, M. E. Abd El-Hack, M. Saeed, M. A. Arain, y S. S. Elnesr. 2019. «Humic Acid as a Feed Additive in Poultry Diets: A Review». *Iranian Journal of Veterinary Research* 20(3):167-72.

Arpášová, Henrieta, Miroslava Kačániová, Veronika Pistová, Branislav Gálik, Martin Fik, y Lukáš Hleba. 2016. «Effect of Probiotics and Humic Acid on Egg Production and Quality Parameters of Laying Hens Eggs». *Scientific Papers*.

Baigorri, Roberto, Marta Fuentes, Gustavo González-Gaitano, y Jose Ma García-Mina. 2007. «Analysis of Molecular Aggregation in Humic Substances in Solution». *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 302(1-3):301-6. doi: 10.1016/j.colsurfa.2007.02.048.

Butt, Hans-Jürgen, Karlheinz Graf, y Michael Kappl. 2013. *Physics and Chemistry of Interfaces*. John Wiley & Sons.

Ceylan, NecmettiN, İBrahiM ÇiFtçi, y ZiYa İLhan. 2003. «The Effects of Some Alternative Feed Additives for Antibiotic Growth Promoters on the Performance and Gut Microflora of Broiler Chicks».

Chen, Changlun, Xiangke Wang, Hui Jiang, y Wenping Hu. 2007. «Direct Observation of Macromolecular Structures of Humic Acid by AFM and SEM». *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 302(1-3):121-25. doi: 10.1016/j.colsurfa.2007.02.014.

Clavijo, Viviana, y Martha Josefina Vives Flórez. 2018. «The Gastrointestinal Microbiome and Its Association with the Control of Pathogens in Broiler Chicken Production: A Review». *Poultry Science* 97(3):1006-21. doi: 10.3382/ps/pex359.

Coles, Cynthia A., y Raymond N. Yong. 2006. «Humic Acid Preparation, Properties and Interactions with Metals Lead and Cadmium». *Engineering Geology* 85(1-2):26-32. doi: 10.1016/j.enggeo.2005.09.024.

Cozzolino, A., P. Conte, y A. Piccolo. 2001. «Conformational Changes of Humic Substances Induced by Some Hydroxy-, Keto-, and Sulfonic Acids». *Soil Biology and Biochemistry* 33(4-5):563-71. doi: 10.1016/S0038-0717(00)00196-6.

Deng, Shubo, y Renbi B. Bai. 2003. «Aminated Polyacrylonitrile Fibers for Humic Acid Adsorption: Behaviors and Mechanisms». *Environmental Science & Technology* 37(24):5799-5805. doi: 10.1021/es034399d.

Diaz Carrasco, Juan M., Natalia A. Casanova, y Mariano E. Fernández Miyakawa. 2019. «Microbiota, Gut Health and Chicken Productivity: What Is the Connection?» *Microorganisms* 7(10):374. doi: 10.3390/microorganisms7100374.

Domínguez-Negrete, Alejandra, Sergio Gómez-Rosales, María de Lourdes Angeles, Luis Humberto López-Hernández, Tercia Cesaria Reis de Souza, Juan David Latorre-Cárdenas, y Guillermo Téllez-Isaias. 2021. «Addition of Different Levels of Humic Substances Extracted from Worm Compost in Broiler Feeds». *Animals* 11(11):3199. doi: 10.3390/ani11113199.

Domínguez-Negrete, Alejandra, Sergio Gómez-Rosales, María de Lourdes Angeles, Luis Humberto López-Hernández, Tercia Cesaria Reis-de Souza, Yair López-García, Anai Zavala-Franco, y Guillermo Téllez-Isaias. 2019. «Effect of the Addition of Humic Substances as Growth Promoter in Broiler Chickens Under Two Feeding Regimens». *Animals* 9(12):1101. doi: 10.3390/ani9121101.

Doskočil, Leoš, Jana Burdíková-Szewieczková, Vojtěch Enev, Lukáš Kalina, y Jaromír Wasserbauer. 2018. «Spectral Characterization and Comparison of Humic Acids Isolated from Some European Lignites». *Fuel* 213:123-32. doi: 10.1016/j.fuel.2017.10.114.

Filip, Zdenek, y Katerina Demnerova. 2009. «Humic Substances as Natural Interacting Agents: Their Characteristics and Possible Fate in Environment». Pp. 247-57 en *Exposure and Risk Assessment of Chemical Pollution — Contemporary Methodology*, *NATO Science for Peace and Security Series C: Environmental Security*, editado por L. I. Simeonov y M. A. Hassanien. Dordrecht: Springer Netherlands.

Gadad, Praveen, Hongxia Lei, y Mark A. Nanny. 2007. «Characterization of Noncovalent Interactions between 6-Propionyl-2-Dimethylaminonaphthalene (PRODAN) and Dissolved Fulvic and Humic Acids». *Water Research* 41(19):4488-96. doi: 10.1016/j.watres.2007.06.011.

Gaffney, Jeffrey S., Nancy A. Marley, y Sue B. Clark, eds. 1996. *Humic and Fulvic Acids: Isolation, Structure, and Environmental Role*. Vol. 651. Washington, DC: American Chemical Society.

Ghahri, Hasan, Reza Habibian, y Mehdi Abdollah Fam. 2010. «Evaluation of the Efficacy of Esterified Glucomannan, Sodium Bentonite, and Humic Acid to Ameliorate the Toxic Effects of Aflatoxin in Broilers». *Turkish Journal of Veterinary & Animal Sciences*. doi: 10.3906/vet-0903-19.

Giasuddin, Abul B. M., Sushil R. Kanel, y Heechul Choi. 2007. «Adsorption of Humic Acid onto Nanoscale Zerovalent Iron and Its Effect on Arsenic Removal». *Environmental Science & Technology* 41(6):2022-27. doi: 10.1021/es0616534.

Giovanela, M., J. S. Crespo, M. Antunes, D. S. Adamatti, A. N. Fernandes, A. Barison, C. W. P. da Silva, R. Guégan, M. Motelica-Heino, y M. M. D. Sierra. 2010. «Chemical and Spectroscopic Characterization of Humic Acids Extracted from the Bottom Sediments of a Brazilian Subtropical Microbasin». *Journal of Molecular Structure* 981(1-3):111-19. doi: 10.1016/j.molstruc.2010.07.038.

Gomez-Rosales, S., y M. de L. Angeles. 2015. «Addition of a Worm Leachate as Source of Humic Substances in the Drinking Water of Broiler Chickens». *Asian-Australasian Journal of Animal Sciences* 28(2):215-22. doi: 10.5713/ajas.14.0321.

Guo, Xiao-xia, Hong-tao Liu, y Shu-biao Wu. 2019. «Humic Substances Developed during Organic Waste Composting: Formation Mechanisms, Structural Properties, and Agronomic Functions». *Science of The Total Environment* 662:501-10. doi: 10.1016/j.scitotenv.2019.01.137.

Hamza, Zeinab, Maher El-Hashash, Soher Aly, Amal Hathout, Ernesto Soto, Bassem Sabry, y Gary Ostroff. 2019. «Preparation and Characterization of Yeast Cell Wall Beta-Glucan Encapsulated Humic Acid Nanoparticles as an Enhanced Aflatoxin B1 Binder». *Carbohydrate Polymers* 203:185-92. doi: 10.1016/j.carbpol.2018.08.047.

Hayirli, A., N. Esenbuğa, M. Macit, M. A. Yörük, A. Yıldız, y H. Karaca. 2005. «Nutrition Practice to Alleviate the Adverse Effects of Stress on Laying Performance, Metabolic Profile and Egg Quality in Peak Producing Hens: II. The Probiotic Supplementation». *Asian-Australasian Journal of Animal Sciences* 18(12):1752-60. doi: 10.5713/ajas.2005.1752.

Henderson, Peter A. 2005. «The Growth of Tropical Fishes». Pp. 85-100 en *Fish Physiology*. Vol. 21. Elsevier.

Illés, Erzsébet, y Etelka Tombácz. 2003. «The Role of Variable Surface Charge and Surface Complexation in the Adsorption of Humic Acid on Magnetite». *Colloids and*

*Surfaces A: Physicochemical and Engineering Aspects* 230(1-3):99-109. doi: 10.1016/j.colsurfa.2003.09.017.

Islam, K. M. S. A. Schumacher, y . J. M. Gropp. 2005. «Humic Acid Substances in Animal Agriculture». *Pakistan Journal of Nutrition* 4(3):126-34. doi: 10.3923/pjn.2005.126.134.

Islam, Md. Aminul, David W. Morton, Bruce B. Johnson, y Michael J. Angove. 2020. «Adsorption of Humic and Fulvic Acids onto a Range of Adsorbents in Aqueous Systems, and Their Effect on the Adsorption of Other Species: A Review». *Separation and Purification Technology* 247:116949. doi: 10.1016/j.seppur.2020.116949.

Ji, F., J. J. McGlone, y S. W. Kim. 2006. «Effects of Dietary Humic Substances on Pig Growth Performance, Carcass Characteristics, and Ammonia Emission1». *Journal of Animal Science* 84(9):2482-90. doi: 10.2527/jas.2005-206.

Jiménez-González, Marco A., Ana M. Álvarez, Pilar Carral, y Gonzalo Almendros. 2019. «Chemometric Assessment of Soil Organic Matter Storage and Quality from Humic Acid Infrared Spectra». *Science of The Total Environment* 685:1160-68. doi: 10.1016/j.scitotenv.2019.06.231.

Kabak, Bulent, Alan D. W. Dobson, y Iş[idot]l Var. 2006. «Strategies to Prevent Mycotoxin Contamination of Food and Animal Feed: A Review». *Critical Reviews in Food Science and Nutrition* 46(8):593-619. doi: 10.1080/10408390500436185.

Karaoglu, Mevlüt, Muhlis Macit, Nurinisa Esenbuga, Hülya Durdag, Leyla Turgut, y O. Cevdet Bilgin. 2004. «Effect of supplemental humate at different levels on the growth performance, slaughter and carcass traits of broilers». *International Journal of Poultry Science* 3(6):406-10.

Klučáková, Martina, y Kateřina Věžníková. 2017. «Micro-Organization of Humic Acids in Aqueous Solutions». *Journal of Molecular Structure* 1144:33-40. doi: 10.1016/j.molstruc.2017.05.012.

Kocabağli, N., M. Alp, N. Acar, y R. Kahraman. 2002. «The Effects of Dietary Humate Supplementation on Broiler Growth and Carcass Yield». *Poultry Science* 81(2):227-30. doi: 10.1093/ps/81.2.227.

Kulikova, N. A., E. V. Stepanova, y O. V. Koroleva. 2005. «Mitigating Activity of Humic Substances: Direct Influence on Biota». Pp. 285-309 en *Use of Humic Substances to Remediate Polluted Environments: From Theory to Practice*, *NATO Science Series*, editado por I. V. Perminova, K. Hatfield, y N. Hertkorn. Dordrecht: Springer Netherlands.

Lehmann, Johannes, y Markus Kleber. 2015. «The Contentious Nature of Soil Organic Matter». *Nature* 528(7580):60-68. doi: 10.1038/nature16069.

Lin, Dong-Qiang, Peter J. Brixius, Jürgen J. Hubbuch, Jörg Thömmes, y Maria-Regina

Kula. 2003. «Biomass/Adsorbent Electrostatic Interactions in Expanded Bed Adsorption: A Zeta Potential Study: Study of Zeta Potential in Expanded Bed Absorption». *Biotechnology and Bioengineering* 83(2):149-57. doi: 10.1002/bit.10654.

Liu, Xiaoming, Yi Hou, Zhen Li, Zen Yu, Jia Tang, Yueqiang Wang, y Shungui Zhou. 2020. «Hyperthermophilic Composting of Sewage Sludge Accelerates Humic Acid Formation: Elemental and Spectroscopic Evidence». *Waste Management* 103:342-51. doi: 10.1016/j.wasman.2019.12.053.

Loosli, Frédéric, Philippe Le Coustumer, y Serge Stoll. 2013. «TiO2 Nanoparticles Aggregation and Disaggregation in Presence of Alginate and Suwannee River Humic Acids. PH and Concentration Effects on Nanoparticle Stability». *Water Research* 47(16):6052-63. doi: 10.1016/j.watres.2013.07.021.

López-García, Y. R., S. Gómez Rosales, M. L. Angeles, H. Jiménez-Severiano, R. Merino-Guzmán y G. Téllez- Isaías. 2023. « Effect of the Addition of Humic Substances on Morphometric Analysis and Number of Goblet Cells in the Intestinal Mucosa of Broiler Chickens ». *Animals, 13(2), 212. https://doi.org/10.3390/ani13020212*.

Maguey-Gonzalez, Jesus A. a, Matias A. Michel, Mikayla F. A. Baxter, Bruno Solis-Cruz, Daniel Hernandez-Patlan, Ruben Merino-Guzman, Xochitl Hernandez-Velasco, Juan D. Latorre, Billy M. Hargis, Guillermo Tellez, y Sergio Gomez-Rosales. 2018. «Effects of Humic Acids on Recovery of Salmonella Enterica Serovar Enteritidis». *Annals of Animal Science* 18(2):387-99. doi: 10.1515/aoas-2017-0037.

Maguey-Gonzalez, Jesús A. b, Matias A. Michel, Mikayla F. A. Baxter, Guillermo Tellez, Philip A. Moore, Bruno Solis-Cruz, Daniel Hernández-Patlan, Rubén Merino-Guzman, Xochitl Hernandez-Velasco, Juan D. Latorre, Billy M. Hargis, Sergio Gomez-Rosales, y Guillermo Tellez-Isaias. 2018. «Effect of Humic Acids on Intestinal Viscosity, Leaky Gut and Ammonia Excretion in a 24 Hr Feed Restriction Model to Induce Intestinal Permeability in Broiler Chickens». *Animal Science Journal* 89(7):1002-10. doi: 10.1111/asj.13011.

Manzak, Aynur, Cem Kurşun, y Yasemin Yıldız. 2017. «Characterization of Humic Acid Extracted from Aqueous Solutions with Polymer Inclusion Membranes». *Journal of the Taiwan Institute of Chemical Engineers* 81:14-20. doi: 10.1016/j.jtice.2017.10.024.

Mudroňová, D., V. Karaffová, T. Pešulová, J. Koščová, I. Cingeľová Maruščáková, M. Bartkovský, D. Marcinčáková, Z. Ševčíková, y S. Marcinčák. 2020. «The Effect of Humic Substances on Gut Microbiota and Immune Response of Broilers». *Food and Agricultural Immunology* 31(1):137-49. doi: 10.1080/09540105.2019.1707780.

Ozturk, E., N. Ocak, I. Coskun, S. Turhan, y G. Erener. 2010. «Effects of Humic Substances Supplementation Provided through Drinking Water on Performance, Carcass Traits and Meat Quality of Broilers». *Journal of Animal Physiology and Animal Nutrition* 94(1):78-85. doi: 10.1111/j.1439-0396.2008.00886.x.

Peña-Méndez, Eladia M., Josef Havel, y Jiří Patočka. 2005. «Humic Substances - Compounds of Still Unknown Structure: Applications in Agriculture, Industry, Environment, and Biomedicine». *Journal of Applied Biomedicine* 3(1):13-24. doi: 10.32725/jab.2005.002.

Piccolo, Alessandro. 2002. «The Supramolecular Structure of Humic Substances: A Novel Understanding of Humus Chemistry and Implications in Soil Science». Pp. 57-134 en *Advances in Agronomy*. Vol. 75. Elsevier.

Prado, Alexandre G. S., Jonas Pertusatti, y Alécio R. Nunes. 2011. «Aspects of Protonation and Deprotonation of Humic Acid Surface on Molecular Conformation». *J. Braz. Chem. Soc.* 22(8):6.

Ramales-Valderrama, Rosa, Alma Vázquez-Durán, y Abraham Méndez-Albores. 2016. «Biosorption of B-Aflatoxins Using Biomasses Obtained from Formosa Firethorn [Pyracantha Koidzumii (Hayata) Rehder]». *Toxins* 8(7):218. doi: 10.3390/toxins8070218.

Rath, N. C., W. E. Huff, y G. R. Huff. 2006. «Effects of Humic Acid on Broiler Chickens1». *Poultry Science* 85(3):410-14. doi: 10.1093/ps/85.3.410.

van Rensburg, C. Jansen, C. E. J. Van Rensburg, J. B. J. Van Ryssen, N. H. Casey, y G. E. Rottinghaus. 2006. «In Vitro and In Vivo Assessment of Humic Acid as an Aflatoxin Binder in Broiler Chickens». *Poultry Science* 85(9):1576-83. doi: 10.1093/ps/85.9.1576.

Rupiasih, N. N., y P. B. Vidyasagar. 2009. «Analytical Study of Humic Acid from Various Sources Commonly Used as Fertilizer: Emphasis on Heavy Metal Content». *International Journal of Design & Nature and Ecodynamics* 4(1):32-46. doi: 10.2495/DNE-V4-N1-32-46.

Saar, Robert A., y James H. Weber. 1979. «Complexation of cadmium(II) with water- and soil-derived fulvic acids: effect of pH and fulvic acid concentration». *Canadian Journal of Chemistry* 57(11):1263-68. doi: 10.1139/v79-206.

Sarlaki, Ehsan, Ali Sharif Paghaleh, Department of Agrotechnology, College of Abouraihan, University of Tehran, Tehran, Iran, Mohammad Hossein Kianmehr, Department of Agrotechnology, College of Abouraihan, University of Tehran, Tehran, Iran, Keyvan Asefpour Vakilian, y Department of Agrotechnology, College of Abouraihan, University of Tehran, Tehran, Iran. 2020. «Chemical, Spectral and Morphological Characterization of Humic Acids Extracted and Membrane Purified from Lignite». *Chemistry & Chemical Technology* 14(3):353-61. doi: 10.23939/chcht14.03.353.

Senesi, N., T. M. Miano, M. R. Provenzano, y G. Brunetti. 1989. «Spectroscopic and Compositional Comparative Characterization of I.H.S.S. Reference and Standard Fulvic and Humic Acids of Various Origin». *Science of The Total Environment* 81-82:143-56. doi: 10.1016/0048-9697(89)90120-4.

Senn, T. L., y A. R. Kingman. s. f. «A review of humus and humic acids». 5.

Sergio Gómez-Rosales, María Angeles, y Jesús Maguey-González. 2021. «Respuesta productiva y balance de nitrógeno en pollos adicionados con sustancias húmicas en el agua de bebida». *Abanico Veterinario* 11. doi: 10.21929/abavet2021.30.

Shang, Yue, Sanjay Kumar, Brian Oakley, y Woo Kyun Kim. 2018. «Chicken Gut Microbiota: Importance and Detection Technology». *Frontiers in Veterinary Science* 5.

Shermer, C. L., K. G. Maciorowski, C. A. Bailey, F. M. Byers, y S. C. Ricke. 1998. «Caecal Metabolites and Microbial Populations in Chickens Consuming Diets Containing a Mined Humate Compound». *Journal of the Science of Food and Agriculture* 77(4):479-86. doi: 10.1002/(SICI)1097-0010(199808)77:4<479::AID-JSFA607>3.0.CO;2-L.

de Souza, Fabiana, y Saulo Roca Bragança. 2018. «Extraction and Characterization of Humic Acid from Coal for the Application as Dispersant of Ceramic Powders». *Journal of Materials Research and Technology* 7(3):254-60. doi: 10.1016/j.jmrt.2017.08.008.

Steinbüchel, Alexander, y R. H. Marchessault. 2005. *Biopolymers for Medical and Pharmaceutical Applications*. Weinheim: Wiley VCH.

Stevenson, I. L., y M. Schnitzer. 1982. «Transmission electron microscopy of extracted fulvic and humic acids»: *Soil Science* 133(3):179-85. doi: 10.1097/00010694-198203000-00007.

Tan, Kim H. 2003. *Humic Matter in Soil and the Environment: Principles and Controversies*. Boca Raton: CRC Press.

Tarasevich, Yu. I., S. A. Dolenko, M. Yu. Trifonova, y E. Yu. Alekseenko. 2013. «Association and Colloid-Chemical Properties of Humic Acids in Aqueous Solutions». *Colloid Journal* 75(2):207-13. doi: 10.1134/S1061933X13020166.

The European Agency for the Evaluation of Medicinal Products. 1999. «humic-acids-their-sodium-salts-summary-report-committee-veterinary-medicinal-products_en.pdf».

Tikhonov, V., O. Drozdova, M. Karpukhin, y V. Demin. 2019. «Participation of Cadmium (Ll) and Copper (Ll) Ions in Intermolecular Forces of Humic Acids in Solutions». *IOP Conference Series: Earth and Environmental Science* 368(1):012053. doi: 10.1088/1755-1315/368/1/012053.

Ueno, Akio, Satoru Shimizu, Shuji Tamamura, Hidetoshi Okuyama, Takeshi Naganuma, y Katsuhiko Kaneko. 2016. «Anaerobic Decomposition of Humic Substances by Clostridium from the Deep Subsurface». *Scientific Reports* 6(1):18990. doi: 10.1038/srep18990.

Vázquez-Durán, Alma, María de Jesús Nava-Ramírez, Daniel Hernández-Patlán, Bruno

Solís-Cruz, Víctor Hernández-Gómez, Guillermo Téllez-Isaías, y Abraham Méndez-Albores. 2021. «Potential of Kale and Lettuce Residues as Natural Adsorbents of the Carcinogen Aflatoxin B1 in a Dynamic Gastrointestinal Tract-Simulated Model». *Toxins* 13(11):771. doi: 10.3390/toxins13110771.

Vázquez-Durán, Alma, Guillermo Téllez-Isaías, Maricarmen Hernández-Rodríguez, René Miranda Ruvalcaba, Joel Martínez, María Inés Nicolás-Vázquez, Juan Manuel Aceves-Hernández, y Abraham Méndez-Albores. 2022. «The Ability of Chlorophyll to Trap Carcinogen Aflatoxin B1: A Theoretical Approach». *International Journal of Molecular Sciences* 23(11):6068. doi: 10.3390/ijms23116068.

Vermeer, A. W. P., W. H. van Riemsdijk, y L. K. Koopal. 1998. «Adsorption of Humic Acid to Mineral Particles. 1. Specific and Electrostatic Interactions». *Langmuir* 14(10):2810-19. doi: 10.1021/la970624r.

Visser, S. A. 1985. «Physiological Action of Humic Substances on Microbial Cells». *Soil Biology and Biochemistry* 17(4):457-62. doi: 10.1016/0038-0717(85)90009-4.

Volk, B.G. Physical and chemical characteristics of Aquatic Humus; Gjessing, E.T., Ed.; Ann Arbor Science Publishers Inc.: Ann Arbor, MI, USA, 1976 de 2022 (https://scholar.google.com/scholar_lookup?title=Physical+and+chemical+characteristics+of+aquatic+humus.&author=Gjessing+E.T.&publication_year=1976).

Xavier, Dm, Silva Vs, Santos Rp, Mesko Mf, Costa Sn, Freire Vn, y Cavada Bs. 2012. «Characterization of the coal humic acids from the candiota coalfield, brazil». *International Journal of Agriculture Sciences* 4(5):238-42. doi: 10.9735/0975-3710.4.5.238-242.

Xu, Duanping, Zhonghou Xu, Shuquan Zhu, Yunzhe Cao, Yu Wang, Xiaoming Du, Qingbao Gu, y Fasheng Li. 2005. «Adsorption Behavior of Herbicide Butachlor on Typical Soils in China and Humic Acids from the Soil Samples». *Journal of Colloid and Interface Science* 285(1):27-32. doi: 10.1016/j.jcis.2004.11.034.

Yadav, Sudhir, y Rajesh Jha. 2019. «Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry». *Journal of Animal Science and Biotechnology* 10(1):2. doi: 10.1186/s40104-018-0310-9.

Yang, Fan, y Markus Antonietti. 2020. «The Sleeping Giant: A Polymer View on Humic Matter in Synthesis and Applications». *Progress in Polymer Science* 100:101182. doi: 10.1016/j.progpolymsci.2019.101182.

Yang, Fan, Shuaishuai Zhang, Kui Cheng, y Markus Antonietti. 2019. «A Hydrothermal Process to Turn Waste Biomass into Artificial Fulvic and Humic Acids for Soil Remediation». *Science of The Total Environment* 686:1140-51. doi: 10.1016/j.scitotenv.2019.06.045.

Yasar, S., A. Gokcimen, I. Altuntas, Z. Yonden, y E. Petekkaya. 2002. «Performance and Ileal Histomorphology of Rats Treated with Humic Acid Preparations*». *Journal of Animal Physiology and Animal Nutrition* 86(7-8):257-64. doi: 10.1046/j.1439-

0396.2002.00383.x.

Ye, Sheng-Qun, Xing-Zuo Lv, y An-Guo Zhou. 2009. «In Vitro Evaluation of the Efficacy of Sodium Humate as an Aflatoxin B1 Adsorbent». 5.

Yörük, M. A., M. Gül, A. Hayirli, y M. Macit. 2004. «The Effects of Supplementation of Humate and Probiotic on Egg Production and Quality Parameters During the Late Laying Period in Hens». *Poultry Science* 83(1):84-88. doi: 10.1093/ps/83.1.84.

Zavala-Franco, Anai, Daniel Hernández-Patlán, Bruno Solís-Cruz, Raquel López-Arellano, Guillermo Tellez-Isaias, Alma Vázquez-Durán, y Abraham Méndez-Albores. 2018. «Assessing the Aflatoxin B1 Adsorption Capacity between Biosorbents Using an In Vitro Multicompartmental Model Simulating the Dynamic Conditions in the Gastrointestinal Tract of Poultry». *Toxins* 10(11):484. doi: 10.3390/toxins10110484.

Zhang, Jie, Jiulan Dai, Renqing Wang, Fasheng Li, y Wenxing Wang. 2009. «Adsorption and Desorption of Divalent Mercury (Hg2+) on Humic Acids and Fulvic Acids Extracted from Typical Soils in China». *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 335(1-3):194-201. doi: 10.1016/j.colsurfa.2008.11.006.

Zhou, Qixing, Shaohu Ouyang, Zhimin Ao, Jing Sun, Guanlan Liu, y Xiangang Hu. 2019. «Integrating Biolayer Interferometry, Atomic Force Microscopy, and Density Functional Theory Calculation Studies on the Affinity between Humic Acid Fractions and Graphene Oxide». *Environmental Science & Technology* 53(7):3773-81. doi: 10.1021/acs.est.8b05232.

Zralý, Z., B. Písaříková, M. Trčková, y M. Navrátilová. 2008. «Effect of Humic Acids on Lead Accumulation in Chicken Organs and Muscles». *Acta Veterinaria Brno* 77(3):439-45. doi: 10.2754/avb200877030439.