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INSTITUTO DE INVESTIGACIONES EN CIENCIAS VETERINARIAS
INSTITUTO DE CIENCIAS AGRÍCOLAS

COMPUESTOS DE ALCALOIDES ISOQUINÓLICOS COMO ADITIVO POTENCIAL EN LA ALIMENTACIÓN DE RUMIANTES EN FINALIZACIÓN

TESIS
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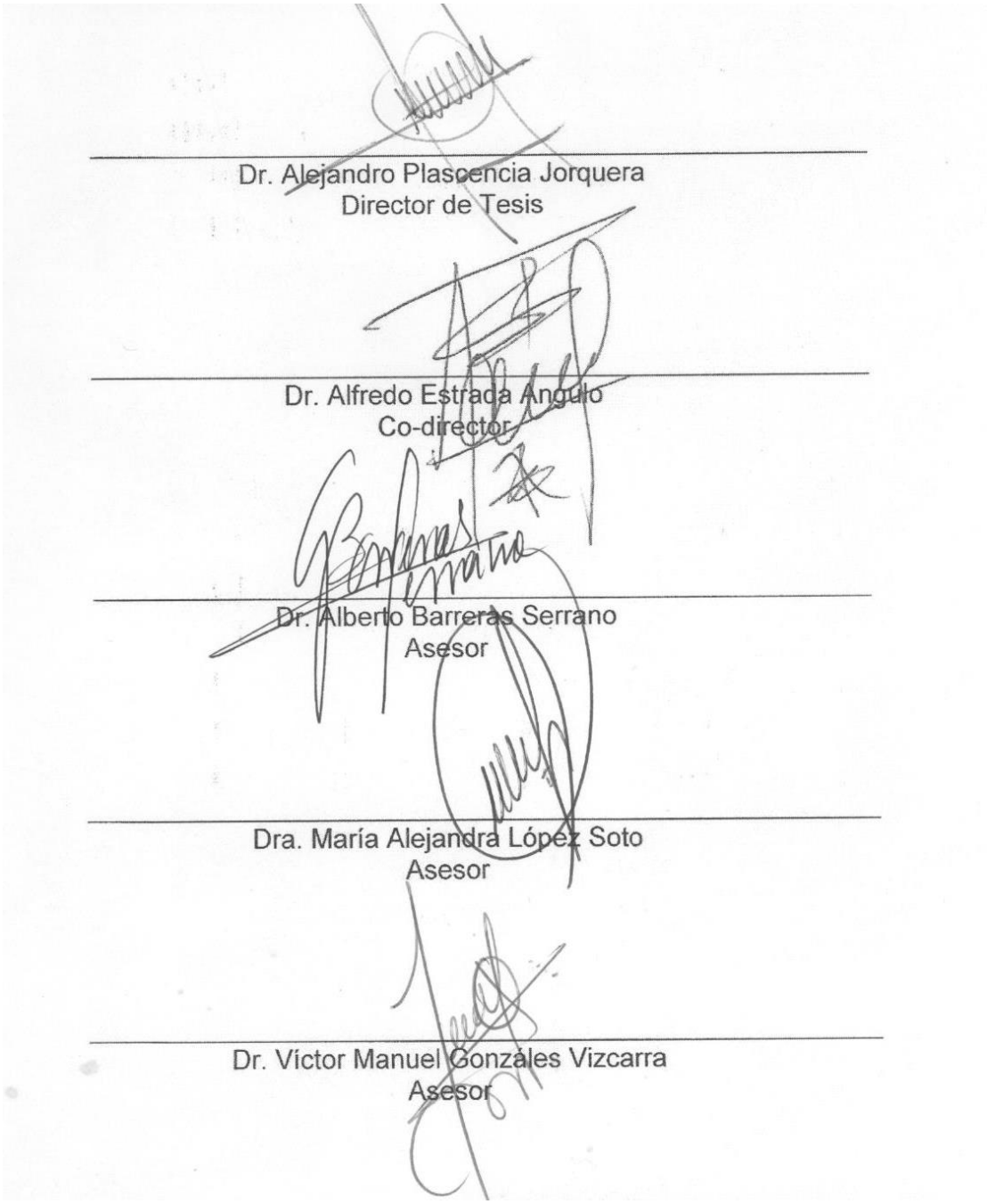
Compuestos de alcaloides isoquinólicos como aditivo potencial en la alimentación de rumiantes en finalización. Tesis presentada por **José Antonio Aguilar Hernández**. Ésta Tesis fue revisada bajo la dirección del consejo particular indicado, ha sido aprobada por el mismo y aceptada como requisito para obtener el grado de: Doctor en Ciencias Agropecuarias. Mexicali, Baja California, Septiembre de 2017.



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RESUMEN

Algunos alcaloides se utilizan en la alimentación animal como extracto de planta estandarizado que contiene alcaloides cuaternario benzofenantridina y protopina (QBA+PA), son compuestos fitogénicos con efecto antimicrobiano, antiinflamatorio y estimulante del apetito. La fuente de QBA+PA usado es SANGROVIT® RS (Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany) que contiene 3 g de QBA+PA por kg de producto. En la prueba 1, se utilizaron 4 novillos Holstein con cánulas ruminal y duodenal en un diseño cuadro latino 4 x 4 para conocer el efecto del consumo diario de 0, 2, 4 y 6 g/novillo/día de SANGROVIT® RS (SANG) en las características de fermentación ruminal y digestión. La dieta de finalización es a base de maíz rolado con 62% y 12% de heno de sudan, el resto está compuesto por granos secos de destilería, melaza, grasa y minerales. Con la inclusión de SANG se observó en el flujo duodenal un incremento lineal ($P=0.04$) de N no amoniacal y una disminución lineal ($P<0.01$) de N amoniacal, además, se mejoró ($P<0.05$) la eficiencia microbiana ruminal y eficiencia proteica, en la digestión de N en tracto total se observó incremento lineal ($P<0.01$) y la energía digestible de la dieta tendió a incrementar (efecto lineal, $P=0.09$) al suplementar SANG. La concentración de $N\text{-}NH_3$ disminuyó linealmente ($P=0.02$) y la proporción molar de acetato se incrementó ($P=0.04$) al aumentar los niveles de suplementación de SANG. Prueba 2, se utilizaron 20 ovejas Pelibuey x Katahdin (35 ± 2.3 kg) para ver los efectos sobre rendimiento, energética de la dieta, masa visceral y salud del epitelio ruminal en ovejas con estrés por calor, alimentados con una dieta a base de grano que contiene

49.7% de almidón y 15.3% de fibra detergente neutra, donde, los tratamientos fueron 0 y 0.5 g de SANG/oveja/día. Las ovejas se bloquearon por peso y se asignaron a 10 corrales (5 corrales/tratamiento) con 2 ovejas por corral en un período de 70 días de experimento, donde se observó un índice de temperatura y humedad de 81.7 ± 1.0 (peligro). No hubo efectos de tratamiento en consumo de agua, ni consumo de materia seca, pero si se observó una ganancia numérica mayor de 11.2% del grupo alimentado con SANG que el control, SANG fue eficiente al mejorar la ganancia un 8.3% (P=0.04), la energía neta de la dieta en 5.2% (P<0.01) y la energía neta observada/esperada en 5.9% (P<0.01). Sin embargo, disminuyó el peso de hígado un 10.3% (P=0.02) e incrementó la grasa visceral 16.9% (P=0.02). En el epitelio ruminal de las ovejas alimentadas con SANG tuvo puntuaciones más bajas en la degeneración celular (1.30 vs 2.34, P=0.02), paraqueratosis (1.30 vs 1.82, P=0.03) e infiltración de neutrófilos (2.08 vs 2.86, P=0.05) que el control. Se concluye que la suplementación de SANG mejoró la eficiencia de N en novillos alimentados con dietas de finalización, este efecto puede deberse a que se mejoró la eficiencia microbiana ruminal, disminuyó la degradación ruminal del N no amoniacal de la dieta y se incrementó la digestión de N posruminal, además, en condiciones de calor severo las ovejas alimentadas con dietas de finalización y suplementadas con SANG ayudo a mejorar los efectos negativos del calor severo en la engorda de ovejas, donde también se observó una mejora en la eficiencia energética mediada por los efectos antiinflamatorios de la suplementación de SANG y el aumento de la absorción de nutrientes.

Palabras clave: alcaloides isoquinolicos, fermentación ruminal, digestión, estrés por calor, epitelio ruminal, rumiantes

ABSTRACT

Some alkaloids are used in animal feed as a standardized plant extract containing quaternary benzophenanthridine and protopine alkaloids (QBA+PA) which are phytogenic compounds that have an antimicrobial, anti-inflammatory and appetite stimulant effect. The source of QBA+PA used is SANGROVIT® RS (Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany) which contain 3 g QBA+PA per kg of product.Trial 1, four Holstein steers with ruminal and duodenal cannulas were used in a 4 x 4 Latin square design to know the effect of daily intake of 0, 2, 4 and 6 g/stems/day of SANGROVIT®RS (SANG) on the characteristics of ruminal fermentation and digestion. The finishing diet is a steam-flaked corn–based with 62% and 12% sudangrass hay, the rest was composed of dried distillery grains, molasses, fat and minerals. With the inclusion of SANG was observed in the duodenal flow, a linear increase ($P=0.04$) of nonammonia N and a linear decrease ($P<0.01$) of ammoniacal N, in addition, the ruminal microbial efficiency was improved ($P<0.05$) and protein efficiency, in the digestion of N in total tract was observed linearly increase ($P<0.01$) and dietary digestible energy tended to increase (linear effect, $P= 0.09$) when to the supplementary SANG. Ruminal NH_3-N concentration linearly decreased ($P=0.02$) and the ruminal molar proportion of acetate increased ($P=0.04$) as the supplementation level of QBA+PA increased. Trial 2, twenty Pelibuey x Katahdin ewes (35 ± 2.3 kg) were used to see effects on performance, dietary energy, visceral mass and health of ruminal epithelial in ewe subjected to heat-stressed, fed on a diet a grain corn base containing

49.7% starch and 15.3% neutral detergent fiber, where, treatments were 0 and 0.5 g of SANG/ewe/day. The ewes were blocked by weight and assigned to 10 pens (5 pens/treatment) with two ewes per pen in a period of 70 days of experiment, where a temperature and humidity index of 81.7 ± 1.0 (danger) was observed. There were no treatment effects on water intake or dry matter intake, but if a numerical gain greater than 11.2% of the group fed with SANG than the control was observed, SANG was efficient to improve the gain by 8.3% (P=0.04), the dietary net energy in 5.2% (P<0.01) and the observed-to-expected net energy in 5.9% (P<0.01). However, decreased liver weight by 10.3% (P=0.02) and increased visceral fat by 16.9% (P=0.02).In the rumen epithelium of the ewes fed SANG had lower scores for cellular degeneration (1.30 vs 2.34, P=0.02), parakeratosis (1.30 vs 1.82, P=0.03) and neutrophil infiltration (2.08 vs 2.86, P=0.05) than the control. It is concluded that SANG supplementation improved efficiency of N in steers fed a finishing diet, this effect was due, to improved rumen microbial efficiency, decreased rumen degradation of nonammonia N in the diet and increased post-ruminal N digestion, in addition, in severe heat conditions the ewes fed with finishing diets and supplemented with SANG helped to improve the negative effects of severe heat in the feedlot ewes, were there was also an improvement in energy efficiency, mediated by the anti-inflammatory effects of supplemental SANG and the increase in nutrient absorption.

Key words: isoquinoline alkaloids, rumen fermentation, digestion, heat stress, ruminal epithelial, ruminants

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INTRODUCCIÓN

Las plantas producen diversos compuestos orgánicos como los metabolitos que son; a) primarios, son necesarios para el funcionamiento y supervivencia de las células y del organismo; b) secundarios, al parecer no tienen función directa en procesos fotosintéticos, respiratorios, asimilación de nutrientes, transporte de solutos o síntesis de proteínas, carbohidratos y lípidos, dentro de este grupo se encuentran los terpenos y esteroides, fenólicos y alcaloides, donde este último existen más de 12000 alcaloides, entre ellos los compuestos fitogénicos, como los QBA+PA extraídos de plantas como *Macleaya cordata*, que tienen efectos antimicrobiano (Kosina et al., 2010), antiinflamatorio (Tanaka et al., 1993), inmunomodulador (Chaturvedi et al., 1997) y estimulantes del apetito (Mellor, 2001). Los efectos antimicrobianos de los QBA+PA modulan el crecimiento de la microbiota gastrointestinal (Cushnie et al., 2014) y sus efectos parecen ser selectivos al mejorar la digestión de fibra en pollos de engorda (Sze y Chwen, 2011). En digestión *in vitro*, se observó que los QBA+PA tienen una capacidad para reducir la actividad de enzimas de degradación de AA como descarboxilasa (Drsata et al., 1996) y disminuir las concentraciones de N amoniacal (Smink y Van der Kolk, 2004), además, que el ganado pasa estrés por calor debido a un índice de temperatura y humedad alto y mayor concentración de granos en la dieta, por lo que se contrarresta haciendo uso de antibióticos como aditivos en la alimentación, ayudando a mantener un epitelio ruminal saludable a través de efectos selectivos sobre las poblaciones microbianas (Abdel-Samee, 1995).

Debido a la regulación de los diferentes fármacos que son utilizados para mejorar el proceso de producción en ganadería, junto con la creciente preocupación por la resistencia a los antibióticos y problemas de salud pública, y a un mayor riesgo de acidosis ruminal subclínica (Uyeno, 2015) del ganado sometido a estrés por calor ambiental y consumo de dietas altas en energía, se busca constantemente alternativas para el uso de aditivos orgánicos para la alimentación animal, además, la investigación del potencial de QBA+PA como aditivos para alimentación de rumiantes es limitada y no hay información disponible sobre los niveles de suplementación, parámetros de fermentación ruminal *in vivo*, síntesis de proteínas microbianas y digestión de los nutrientes en ganado de engorda alimentados con dietas altas en energía, ni efectos sobre el crecimiento y la energética de la dieta en rumiantes alimentados con dietas de finalización bajo condiciones de estrés por calor ambiental severa.

El objetivo de los experimentos fue evaluar los efectos de la inclusión de diferentes niveles de QBA+PA sobre las características de la fermentación ruminal, digestión y síntesis de proteínas microbianas en novillos alimentados con una dieta de finalización, en ovejas fue evaluar la adición de QBA+PA en el rendimiento, energética de la dieta, características de canal y salud del epitelio ruminal, que fueron alimentados con dietas de alto valor energético bajo condiciones de índice de temperatura y humedad severo.

REVISIÓN DE LITERATURA

Productos naturales (metabolitos)

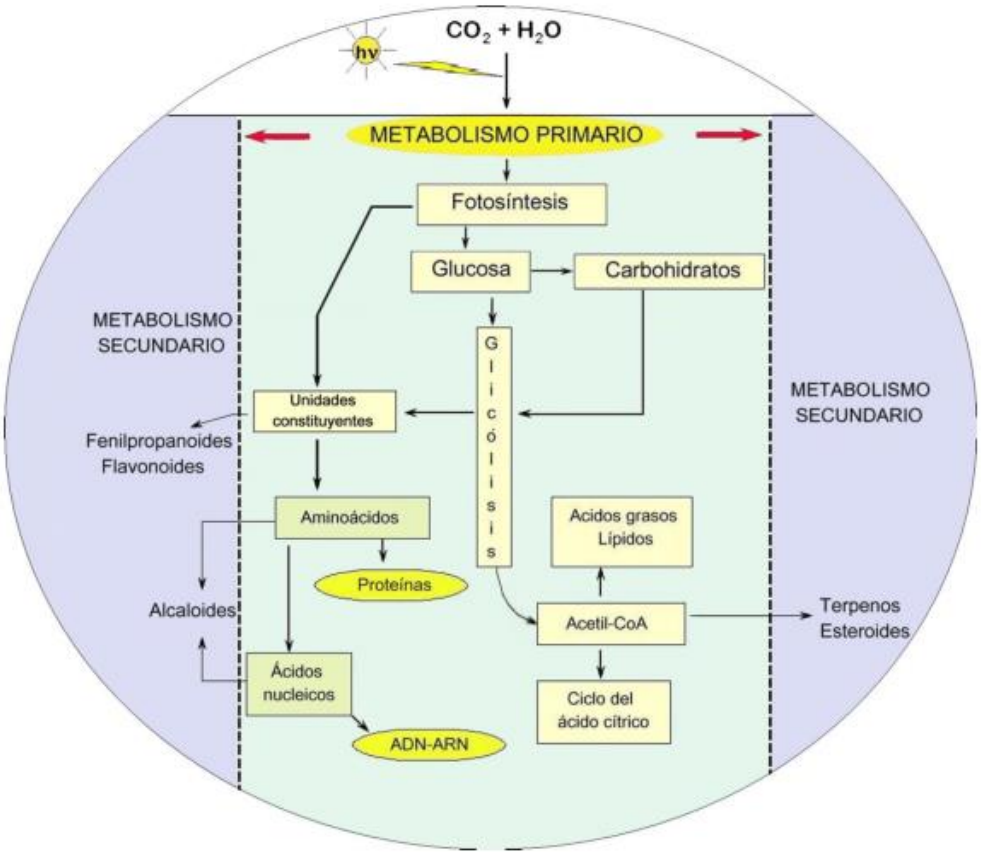
Las plantas producen una amplia y diversa variedad de compuestos orgánicos, la gran mayoría de los cuáles no parecen participar directamente en el crecimiento y desarrollo, tradicionalmente son conocidos como metabolitos secundarios que a menudo se distribuyen diferencialmente entre los grupos taxonómicos limitados dentro del reino vegetal, donde muchas de sus funciones siguen siendo desconocidos, en contraste los metabolitos primarios como fitoesteroles, lípidos acilo, nucleótidos, aminoácidos y ácidos orgánicos, se encuentran en todas las plantas realizando funciones metabólicas que son esenciales y por lo general evidente (Croteau et al., 2000).

Metabolitos primarios y secundarios

El conjunto de reacciones químicas que tienen lugar en un organismo constituye el metabolismo, donde la mayor parte del carbono, nitrógeno y energía termina en moléculas comunes, las cuáles son necesarias para el funcionamiento y supervivencia de las células y del organismo, tratándose de aminoácidos, nucleótidos, azúcares y lípidos, los cuales son denominados metabolitos primarios (Figura 1) y están presentes en todas las plantas, sin embargo, las plantas destinan una cantidad significativa del carbono asimilados y de energía a la síntesis de una amplia variedad de moléculas orgánicas que parecen no tener una función directa en procesos fotosintéticos, respiratorios,

asimilación de nutrientes, transporte de solutos o síntesis de proteínas, carbohidratos o lípidos, los cuáles se denominan metabolitos secundarios (Avalos y Pérez-Urria, 2009).

Los metabolitos secundarios se producen por lo general sólo en células especiales, diferenciadas y no son necesarios para las células mismas, además que, tienen importancia en la plantas para adaptarse a su entorno y han sido descritos como antibióticos, antifúngicos y antivirales, por lo tanto, son capaces de proteger a las plantas contra patógenos, también antigerminativas o tóxicos para otras plantas (Bourgaud et al., 2001; Stahl, 2004).



Avalos y Pérez-Urria, 2009

Figura 1. Elementos básicos del metabolismo primario y en relación con el metabolismo secundario de las plantas.

En los sistemas de alimentación de ganado actualmente se han estado regulando el uso de fármacos que potencian el crecimiento de los animales, por lo que existe la preocupación y a su vez el interés en la búsqueda de alternativas generalmente conocidos como seguros, entre estos se encuentra los compuestos fitogenéticos, como los alcaloides benzofenantridina y α-allocryptopine que han demostrado promesa como aditivo para la alimentación en pollos (Sze y Chwen, 2011) y cerdos (Jeroch et al., 2009).

Clasificación de los metabolitos secundarios

Los metabolitos secundarios de las plantas se clasifican según sus rutas biosintéticas en 3 familias de moléculas: terpenos y esteroides, fenólicos y alcaloides (Bourgaud et al., 2001).

***Terpenos y esteroides*:** a partir de los primeros miembros de la clase que se aislaron de la trementina (“terpentin” en alemán) se deriva el nombre de terpeno o terpenoide, los cuáles son derivados por fusión repetitiva de unidades de cinco carbonos (C_5) con ramificaciones basados en unidades del esqueleto isopentano, los cuáles son referidos como unidades de isopreno, los terpenos más pequeños que contienen un isopreno se denominan hemiterpenos, unidades con C10 se denominan monoterpenos, unidades con tres isoprenos (C15) se denominan sesquiterpenos, las unidades que contienen carbonos 20, 30, 40, >40 se llaman diterpenos, triterpenos, tetraterpenos y politerpenos respectivamente (Harborne, 1999; Croteau et al., 2000).

***Fenólicos*:** los aminoácidos aromáticos están presentes en el metabolismo primario como en el metabolismo secundario de las plantas, donde se sintetizan una gran variedad de productos secundarios que contienen un grupo fenol, que reciben el nombre de compuestos fenólicos, polifenoles o fenilpropanoides y derivan todas ellas del fenol, un anillo aromático con un grupo hidroxilo (Ávalos y Pérez-Urria, 2009).

***Alcaloides*:** la definición sencilla sugerida por Pelletier (1883) y mencionada por Ziegler y Facchini (2008) es que “son compuestos orgánicos cíclico que contienen nitrógeno (compuesto heterocíclico) en un estado de oxidación negativo con una distribución limitada entre los organismo vivos” donde el nitrógeno que contienen deriva principalmente de aminoácidos, los alcaloides desempeñan un sistema de defensa contra vertebrados, invertebrados y microorganismos patógenos y existe más de 12000 alcaloides que son explotados como productos farmacéuticos, estimulantes, narcóticos y venenos .

Clasificación de los alcaloides

Para la clasificación de los alcaloides existe diversa formas, estas puedes ser: a) en base a su estructura, diferenciando los distintos compuestos heterocíclicos; b) propiedades farmacológicas; c) distribución botánica; d) grupos de compuestos nitrogenados, aminas secundarias y terciarias, amino cuaternarios, amino neutral, N-óxidos (Roberts y Wink, 1998); e) origen biosintético, donde la mayoría de los alcaloides se forman a partir de L-aminoácidos como arginina/ornitina, lisina, triptófano, tirosina y fenilalanina; f)

según su ruta metabólica, donde encontramos los alcaloides de indole terpenoide, de tropano y nicotina, de purina y bencilisoquinolínicos (Facchini, 2001).

En esta ocasión tomaremos en cuenta la clasificación según su ruta metabólica para comprender un poco a cerca de los alcaloides de interés para su uso en la alimentación de los rumiantes.

Alcaloides de índole terpenoide: comprenden una familia de más de 3000 compuestos, que consisten en un resto de indol proporcionado por triptamine y un componente terpenoide derivado de iridoides glucósido secolaganina, en los que se incluyen el agente antineoplásico vinblastinay camptotecina, el antimalarico quinina y el veneno para ratas, estricnina (Facchini, 2001).

Alcaloides de tropano y nicotina: estos se originan de los aminoácidos ornitina y/o arginina, que tienen en común el esqueleto bicíclico de tropano que consta de siete miembros con puentes de nitrógeno (N) entre los carbono-1 (C-1) y C-5, el nitrógeno esta metilado. Muchos alcaloides tropano son esteres de alcoholes de tropina (tropano-3α-ol) o seudotropina (tropano-3β-ol) con ácidos alifáticos o aromáticos (Osbourn y Lanzotti, 2009).

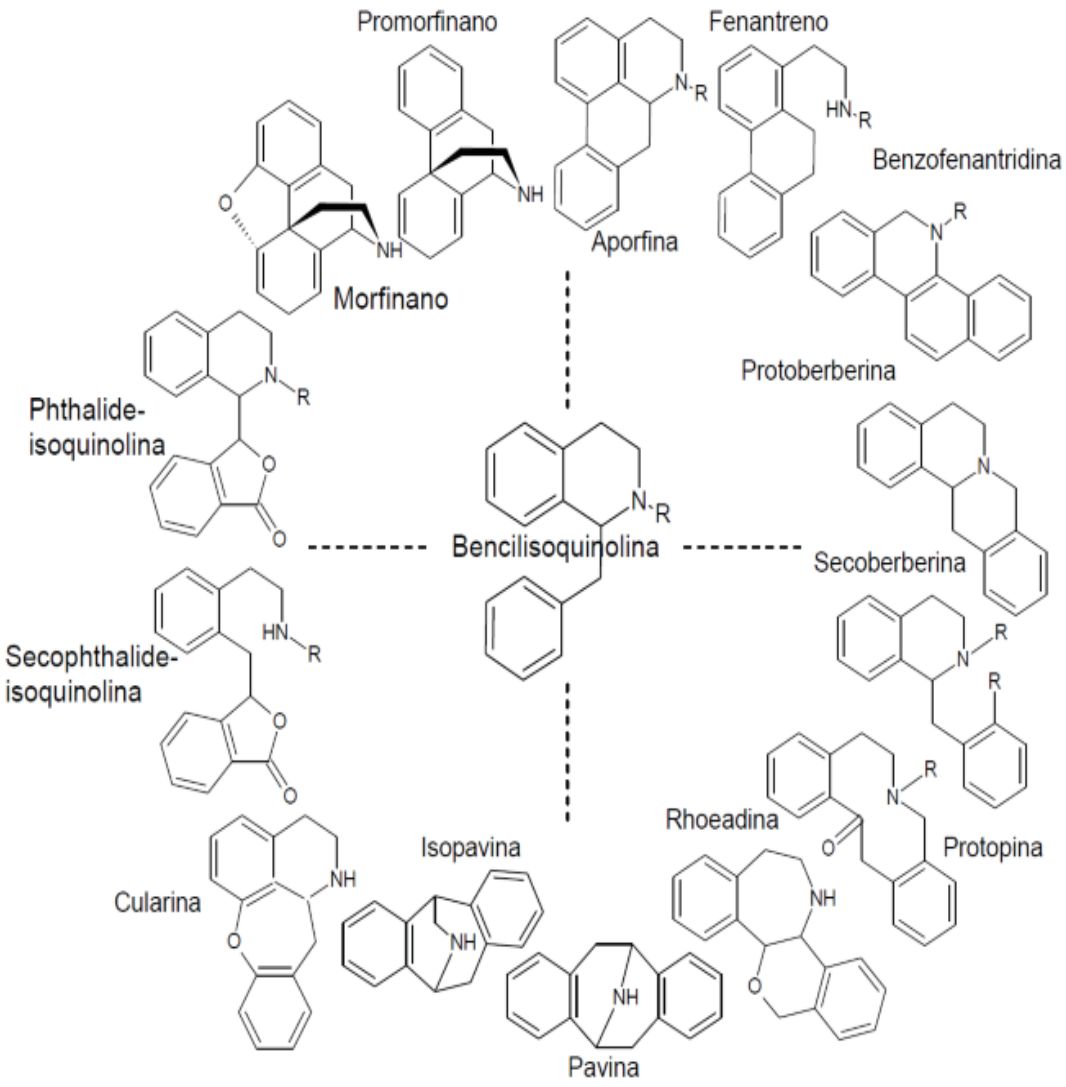
El tabaco (N*icotiana tabacum*) es una planta nativa de América que en un principio se olió, masticó, comió, bebió y se aplicó tópicamente para matar parásitos o en forma de gotas oculares y enemas, el principio activo en la mayoría de especies de *Nicotiana* es el alcaloide nicotina simple, que se compone de un anillo de piridina unido a un anillo de N-metilpirrolidina, donde el anillo de piridina se deriva del ácido quinolínico y el anillo de pirrolidina, como

con los alcaloides de tropano, proviene de putrescina. La ruta biosintética de nicotina consiste en al menos ocho pasos enzimáticos, en este proceso implica la formación de un catión N-metil-Δ-pyrrolinium, que es también un precursor de alcaloides tropano. La percepción del daño tisular en las hojas estimula la liberación de una molécula de señal (jasmonato) que induce biosíntesis de nicotina en las raíces y es transportada a los órganos aéreos a través del xilema (Zulak et al., 2006).

Alcaloides de purina: son compuestos que contienen nitrógeno derivado del metabolismo de nucleósidos, donde la columna vertebral de purina se sintetiza a partir de varias moléculas pequeñas del metabolismo primario que incluye el ácido L-aspartico, L-glutamina, L-glicina y formiato (Osbourn y Lanzotti, 2009). Los alcaloides de purina se encuentran en una variedad de especies de plantas no relacionadas taxonómicamente, por ejemplo variedades de café (C*offea arabica*), te (C*amellia sinensis*, *Theaceae*), cacao (T*heobroma cacao*) guaraná (P*aullina cupana*, Spindaceae), donde la cafeína es el alcaloide de purina más abundante, seguida de teobromina y algunas purinas menores como teofilina y paraxantina (Ashihara y Suzuki, 2004). La cafeína es un estimulante central ampliamente consumido de forma cotidiana, aunque también sirve como medicina para el resfriado y como analgésico, donde el modo predominante de acción de la cafeína y los alcaloides de purina es el bloqueo de los receptores de adenosina que resulta de la liberación de neurotransmisores y a concentraciones altas se inhibe la fosfodiesterasa, enzima que hidroliza el segundo mensajero AMPc (Osbourn y Lanzotti, 2009).

Las especies de café tienen de 0.4 a 2.4% y C*amellia sinensis* de 2-3% de cafeína en materia seca, de las cuales existen dos hipótesis del papel de la cafeína en las plantas: a) La "teoría de la defensa química" propone que las altas concentraciones de cafeína en las hojas jóvenes, frutos y brotes de flor de las plantas actúa como defensa química para proteger los tejidos jóvenes blandos de los depredadores, como larvas de insectos; b) La "teoría de función alelopático propone que la cafeína en cubiertas de las semillas se libera en el suelo para inhibir la germinación de otras semillas (Ashihara et al., 2008).

Alcaloides bencilisoquinolínicos: la morfina fue el primer alcaloide bencilisoquinolínico (ABI) conocido que se aisló del opio por Friedrich Wilhelm Sertürner en 1804, constituyendo todo un suceso en la química de los productos naturales, hoy se conocen cerca de 2500 estructuras, que se caracterizan por presentar un esqueleto carbonado básico que proviene de un enlace entre un anillo isoquinolínico y otro bencil (sistema 1-benciltetrahidroisoquinolina, 1-btiq), donde, la diversidad estructural resulta de las modificaciones al esqueleto 1-benciltetrahidroisoquinolina por hidroxilaciones, reducciones, oxidaciones, formación de enlaces C-C y *O*- y *N*-metilaciones, de tal forma que se pueden distinguir básicamente 13 (Figura 2) subtipos: simples, aporfinas, benzofenantridinas, bisbencilisoquinolinas, cularinas, ftalideisoquinolinas, morfinanos, morfinandienonas, pavinas/isopavinas, protoberberinas, protopinas, rhoeadinas/paverrubinas y secoberberinas (De la cruz et al., 2012).



De la cruz et al., 2012

Figura 2.Diversidad estructural de alcaloides bencilisoquinolínicos.

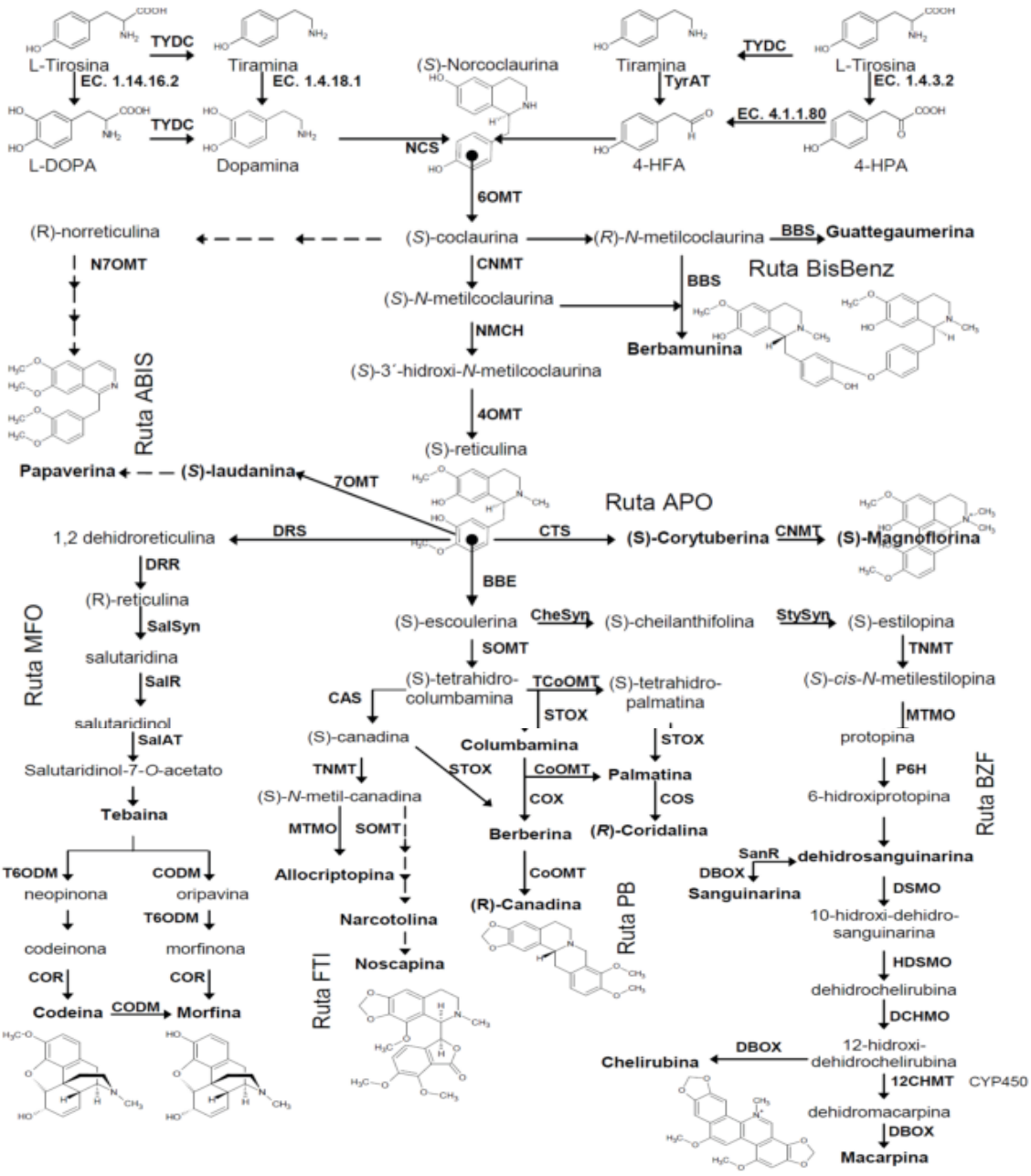
Aunque teóricamente los aminoácidos L-tirosina y L-fenilalanina son considerados como precursores básicos de toda la diversidad de ABI, únicamente con el primero se han reportado trabajos de incorporación a la ruta de biosíntesis (De la cruz et al., 2012).

Biosíntesis de los alcaloides Bencilisoquinolínicos

La biosíntesis de ABI puede agruparse artificialmente en tres intervalos a) la producción del precursor central de todos los ABI, *S*-norcoclaurina, desde dos moléculas de L-tirosina, b) la transformación de *S*-norcoclaurina a *S*-reticulina, intermediario principal de diversificación de la ruta y c) las rutas de diversificación que dan origen a los diferentes tipos de ABI. Las dos primeras partes que forman el núcleo de la biosíntesis están bastante bien caracterizadas a nivel proteómico y genómico, sin embargo, hay 4 enzimas que aún no han sido aisladas y/o caracterizadas de plantas productoras de ABI, aunque se ha reconocido su actividad catalítica sobre la diversificación de la ruta (Figura 3), donde, los avances más importantes se han centrado sobre seis ramas que inician con S-reticulina y permiten la biosíntesis de alcaloides a) bencilisoquinolínicos simples (ABIS, papaverina), b) protoberberina (PB, berberina), c) benzofenantridina (BZF, sanguinerina), d) morfinanos (MFO, morfina), e) aporfina (APO, magnoflorina) y f) fthalideisoquinolina (FTI, noscapina) (Ziegler y Facchini, 2008).

***Alcaloides benzofenantridina*:** el primer alcaloide de este tipo se aisló en 1908 y en la actualidad se conocen casi un centenar de estructuras ampliamente distribuidas en plantas de la familia de las Papaveráceas. Estos alcaloides se caracterizan por poseer un esqueleto básico de benzofenantridina, presentando algunos de ellos los anillos B y C hidrogenados (Díaz, 2003). Algunos alcaloides de isoquinolina extraídos de las plantas (como el benzofenantridina y α-allocryptopine) se sabe que tienen efectos

antimicrobianos (Colombo y Bosisio, 1996; Newton et al., 2002) y antiinflamatorios (Tanaka et al., 1993).



De la cruz et al., 2012

Figura 3. Rutas de biosíntesis de alcaloides bencilisoquinolínicos.

Los exudados de las raíces de muchas especies de *Papaveraceae* (*Sanguinaria canadensis* y *Eschsholzia californica*) como la sanguinaria son de color rojo debido a la acumulación de sanguinarina y otros alcaloides benzofenantridina (Zulak et al., 2006).

En un estudio que realizaron en plantas *Macleaya cordata* (cuadro 1) observaron que los alcaloides con mayor concentración en la parte aérea de la planta fue protopina (34.15%) y alocriptopina 29.16%), en la semilla se encontró mayor concentración de protopina (40%) y sanguinarina (35%), en caso de la cápsula los principales alcaloides fueron sanguinarina (73.9%) y queleritrina (16.9%) para proteger las semillas contra patógenos y depredadores herbívoros (Kosina et al., 2010).

Cuadro 1. Contenido de alcaloides en *Macleaya cordata*

	Sanguinarina*	Queleritrina*	Protopina*	Alocriptopina*	otros
Parte aérea	4.51 ± 0.23	2.88 ± 0.14	7.93 ± 0.34	6.77 ± 0.34	1.13±0.04
semilla	0.07 ± 0	0.02 ± 0	0.08 ± 0	0.03 ± 0	---
cápsula	32.08 ± 0.40	7.36 ± 0.01	0.29 ± 0.02	0.13 ± 0.08	3.50±0.21

* Medias ± desviación estándar, mg g^{-1} materia seca.

Kosina et al., 2010

Los alcaloides benzofenantridina 8-hidroxidihidrosanguinarine y 8-hidroxidihidroqueleritrina mostraron efectos potentes inhibitorios *in vitro* contra las cepas meticilina sensitiva a S*taphylococcus aureus* (MSSA) y meticilina

resistente a S*taphylococcus aureus* (MRSA) con una concentración mínima inhibitoria/concentración mínima bactericida de 0.49/1.95 y 0.98/7.81µg/ml respectivamente (Ying et al., 2008). La dihidrosanguinerina (DHSA) es el precursor biosintético de sanguinarina, sin embargo, sanguinarina puede ser convertida en DHSAy este puede ser una vía importante de desintoxicación en los animales y en el hombre tentativamente (Dvorák y Simánek, 2007),esto se corrobora con los resultados obtenidos tras la exposición al extracto de levadura como inductor del metabolismo secundario, donde se observó que a las 72 horas de exposición había un mayor contenido de DHSA y menor sanguinarina, posteriormente al día 20 se observa la mínima concentración de DHSA (6 nmol g DW^1) mientras sanguinarina aumentaba simultáneamente hasta alcanzar su máximo concentración (14 nmol g DW^1) siendo sanguinarina el resultado de la oxidación de DHSA (Guízar-González et al., 2012), este proceso de protección de autointoxicación en las plantas es regulado por la enzima sanguinarina reductasa (Vogel et al., 2010).

Queleritrina; es un alcaloide capaz de eludir las barrearas antiapoptóticas de las células cancerígenas, siendo así, un potencial terapéutico contra el cáncer, al tener actividad citotóxica contra células de carcinoma (Chan et al., 2003), además en otro estudios, queleritrina y sanguinarina en conjunto han demostrado inhibir el crecimiento de células cancerosas de melanoma uveal, por lo que se sugiere su aplicación como agente potencial anticanceroso (Kemeny-Beke et al., 2006).

Sanguinarina; alcaloide común en plantas de la familia *Papaveraceae* y *Fumariaceae*, una de las plantas de la familia *Papaveraceae* utilizadas por los nativos americanos es la *Sanguinaria canadensis* que acumula sanguinarina, ha sido utilizado para purificar la sangre, aliviar el dolor, curar heridas, reducir la fiebre, en productos de higiene oral para tratar la gingivitis y evitar la formación de placas debido a sus propiedades antimicrobianas y antiinflamatorias, demostrando así controlar la inflamación mediante la regulación de un factor de transcripción correspondiente, para modular la apoptosis como un potencial fármaco de quimioterapia y para inhibir la angiogénesis (Zulak et al., 2006).

Uso de alcaloides benzofenantridina (sanguinarina, queleritrina) en aves

Debido a las propiedades que tienen los alcaloides isoquinólicos como antiinflamatorios, antimicrobianos e inmunomoduladores, se han buscado obtener extractos de plantas para su uso en producción animal, tal es el caso de sangrovit® el cual su composición (g/kg) es: sanguinarina (13.51 ± 0.25), α-alocriptopine (20.26 ± 1.96), queleritrina (6.90 ± 0.09), protopina (4.30 ± 0.54), homochelidonine (1.63 ± 0.13), dihidrosanguinarina (0.25 ± 0.01) y trazas de oxisanguinarina, oxiqueleritrina y dihidroqueleritrina (Stiborova et al., 2008).

Una de las propiedades de sanguinarina es ser un potente inhibidor de la activación del factor nuclear (NF) kB que resulta de la inflamación y replicación viral, lo que le confiere ser un agente antiinflamatorio, ya que los compuestos antiinflamatorios como aspirina y glucocorticoides han demostrado inhibición de la activación de NFkB (Chaturvedi et al., 1997). Además, en otros estudios realizados sanguinarina y queleritrina en conjunto mostraron tener efecto como

agentes para el tratamiento de cáncer al inhibir el crecimiento de células e inducir la muerte celular por apoptosis en una variedad de células cancerosas (Malikova et al., 2006), como la apoptosis inducida en células del linfoma de efusión primaria (Hussain et al., 2007).

Estudios realizados en pollos de engorda donde se midió la producción de ácidos grasos (AG) en el contenido cecal y que al comparar los tratamientos control (sin sangrovit®) vs SANG30 (30mg sangrovit®/kg de alimento), se observó que la producción de ácidos grasos de cadena corta (AGCC) total fueron menores (P=0.001) para SANG30 (109 ± 2.83 µmol/g) con respecto al control (120 ± 3.58 µmol/g), dado principalmente por el ácido graso acético (69.8 ± 2.34 vs 79.7 ± 2.8 µmol/g) y propiónico (11 ± 0.86 vs 13.4 ± 0.85 µmol/g), sin embargo, al observar los datos en porcentaje solo se refleja diferencia estadística en butirato, con un mayor (P<0.05) porcentaje para el tratamiento SANG30 (22.2 ± 0.84) con respecto al control (17.9 ± 0.95) (Juskiewicz et al., 2013), estos resultados son similares a los reportados por Zdunczyk et al. (2010), donde se observó mayor (P=0.016) concentración de ácido butírico (32.8 vs 26.8 µmol/g de digesta cecal) y mayor (P=0.008) porcentaje del mismo con respecto al total de AGCC (23 vs 19%) para el tratamiento SANG30 vs el control, además, tiene una tendencia (P=0.060) en la disminución del pH cecal del tratamiento SANG30 (5.97) respecto al control (6.11).

Por otra parte, la adición de 15 mg de Sangrovit®/kg de alimento en pollos, disminuye (P<0.05 y P=0.075 respectivamente) las actividades potencialmente dañinas de los β-glucuronidasa y β-glucosidasa, sin embargo se

observó un aumento (P=0.001) significativo en la actividad de las enzimas glucolíticas bacterianas α-glucosidasa, α-galactosidasa y β-galactosidasa en comparación con el grupo control (Juskiewicz et al., 2011).

En otro estudio realizado en pollos de un día de edad expuestos a *Salmonella enteritidis* y 24 horas pos-exposición se aplicaron los tratamientos control (sin Sangrovit®) y SAN100 (100g de Sangrovit®/1000 L de agua por 6 días), al día 7 pos-inoculación se tomaron muestras cecales y cultivos para determinar cuántos fueron positivos/negativos a *Salmonella enteritidis*, para las muestras cecales fue menor (P<0.05) los positivos en el tratamiento SAN100 (0/12) vs el control (11/1), en las muestras de cultivo fue menor (P<0.05) para SAN100 (5/7) que para el control (10/2), por lo que, Sangrovit® redujo significativamente el número de positivos a *Salmonella,* observando mejores resultados en las heces que en los cultivos (Pickler et al., 2013).

En los aspectos de producción Viera et al. (2008a), reportaron una mejor (P=0.004) conversión alimenticia (1.51) en pollos de engorda del día 1 a 35 en el tratamiento SAN37 (37.5 ppm sangrovit®) vs los tratamientos suplementados con 0 (1.55), 12.5 (1.56) y 50 ppm (1.55) de sangrovit®, pero sin diferencia significativa (P>0.05) con el tratamiento de 25 ppm (1.53), sin embargo, no se observó diferencia estadística por semana. En otro estudio donde los tratamientos fueron 50 ppm de sangrovit® del día 1 a 21 y 25 ppm del día 22 a 42 (SAN50) y el control (sin sangrovit®), se observó que el tratamiento SAN50 tuvo mejor (P<0.05) respuesta en la conversión alimenticia que el control en el período 8 a 14 días (1.259 vs 1.299), con respecto al período completo de engorda (día 1 a 42) fue mejor (P<0.05) la conversión alimenticia del

tratamiento SAN50 vs control (1.597 vs 1.635), los cuáles estos resultados se mejoraron en 2.32% con el tratamiento SAN50 (Viera et al. 2008b).

Sin embargo, en el trabajo reportado por Yakhkeshi et al. (2011) en pollos, no se observó diferencia estadística en consumo, ganancia de peso ni conversión alimenticia, pero si se observó una respuesta del sistema inmune posterior a la segunda inmunización (35 días) de 1 ml al 5% de glóbulos rojos-antígeno de ovejas, suspendido al 0.9% en solución salina donde fue mayor ($P<0.05$) la relación heterófilo/linfocitos en el tratamiento SAN (29.69) que el control (23.41).

Por otra parte, la suplementación con sanguinarina en pollos aumentó la digestión de la fibra (Sze et al., 2010), además, disminuyó la degradación de proteína alimenticia (Drsata et al., 1996), por lo tanto disminuye la concentración de nitrógeno amoniacal *in vitro* (Smink y Van der Kolk, 2004) e in vivo (Plascencia and Zinn, 2014). Basándose en resultados de estudios *in vitro*, donde se observa un efecto inhibitorio de la sanguinarina sobre la descarboxilasa de aminoácidos aromáticos (Drsata et al., 1996), se supone que Sangrovit® mejora la retención de proteína mediante la reducción de la descarboxilación de aminoácidos aromáticos (Mellor, 2001).

Uso de alcaloides benzofenantridina (sanguinarina, queleritrina) en cerdos

Estudios realizados en cerdos indican que los alcaloides sanguinarina y queleritrina tienen mínima absorción, esto es corroborado en estudios de cerdos suplementados con 2 ppm (SAN2) y 100 ppm (SAN100) de extracto de *Macleaya cordata,* donde se observó que en ambos tratamientos la mayor parte

de los alcaloides sanguinarina (92.34%) y queleritrina (96.71%) se observaron en heces, solo una pequeña cantidad se absorbe (cuadro 2), además, las pequeñas cantidades de alcaloides detectados en hígado no provocaron en los animales ningún signo de intoxicación (Kosina et al., 2004).

Al comparar los tratamientos control (sin sangrovit®) vs SAN3 (30 g Sangrovit®/ton de alimento) en engorda de cerdos en el período de inicio (7 a 20 kg) se observó que el índice fagocítico fue menor (P>0.05) para el tratamiento control en comparación con SAN3, esto se debió a la mejora de la potencia activa de los leucocitos en lugar de un aumento en el número de leucocitos, por lo tanto, Sangrovit® estimula el índice de fagocitosis en los cerdos durante el período de inicio, lo que podría aliviar perturbaciones y desequilibrios de la flora intestinal y restablecer el equilibrio de la flora normal (Gudev et al., 2004).

Cuadro 2. Niveles de Sanguinarina y Queleritrina en plasma, tejidos y heces

	2 ppm		100 ppm	
	Sanguinarina	Queleritrina	Sanguinarina	Queleritrina
Plasma *	4	-	108	24
Hígado**	13	5	113	40
Musculo**	-	-	-	-
Encía**	79	36	514	50
Lengua**	10	-	32	-
Estomago**	7	-	52	-
Intestino**	15	-	124	49
Heces**	1180	834	16110	8412

* ng/ml, ** ng/g.

Kosina et al. 2004

Jeroch et al. (2009), reportan que adicionar 15 mg de sangrovit®/kg de alimento (SANG15) en lechones mejora ($P<0.05$) la ganancia de peso 8.5%, el peso final del lechón en 6.17% (21.11 ± 1.4 kg vs 22.50 ± 1.33 kg), en la etapa de engorda se observó mejor ($P<0.05$) ganancia de peso en 2.8% para el tratamiento SANG15 que el control (889 ± 29 vs 864 ± 24 gramos), sin embargo no se observó diferencia estadística ($P>0.05$) en el peso final del cerdo (98.27 vs 99.90 kg).

Índice de temperatura y humedad

El ITH sirve para determinar el grado de estrés por calor al cual está sometido un animal bajo condiciones ambientales, que se calcula mediante la siguiente ecuación $ITH = 0.81\times T + [RH \times (T-14.40)] + 46.40$, esto a partir del registro diario de las mínimas y máximas de temperatura y humedad, que se clasifican de la siguiente manera: Normal <74, Alerta 75-78, Peligro 79-83 y Emergencia> 84, donde, un ITH> 84 el ganado necesita de tres a cuatro días para que pueda equilibrar adecuadamente una producción metabólica reducida (menor consumo de alimento) y las capacidades de disipar el calor para limitar el aumento de la temperatura corporal (Hahn, 1999). En México, la mayoría de ganado de engorda y corderos finalizados en corral están donde predomina el clima semiárido, tropicales y subtropicales (Partida et al., 2013), estas regiones tienen climas extremos (ITH>77) la mayor parte del año, que son estresantes para los animales.

Estrés por calor

Los factores medioambientales en el ganado influencia directamente sobre la fisiología, comportamiento y la salud del ganado, no obstante aunque se adaptan a las condiciones medioambientales en viven, tienen mecanismos fisiológicos más restringidos para hacer frente al exceso de calor proveniente de la combinación de dietas de alta densidad energética (granos), altas temperaturas y alta humedad relativa (Brown-Brandl et al., 2006). Sin embargo, los animales hacen frente a estos períodos de estrés con modificaciones de comportamiento, cambios en los nutrientes, siendo el agua y la energía los más afectados cuando el ganado se encuentra fuera de la zona termo-neutral (Arias et al., 2008), como se observó en ganado de finalización con ITH promedio de 32.4 ± 0.2 y 69.0 ± 0.1 puntos donde se incrementó hasta un 87% el consumo de agua (17.3 ± 0.1 L/día vs 32.4 ± 0.1 L/día), sin embargo el consumo de alimento (11.20 ± 0.02 vs 9.57 ± 0.02 materia seca/animal/día) disminuyó 15% (Arias y Mader, 2011), el aumento del consumo de agua diaria se asocia a las variaciones en la cantidad de sangre circulando en el organismo, así como la tasa a la cual ésta se evapora de la piel y del tracto respiratorio (Arias et al., 2008).

La disminución del consumo de alimento diario afecta directamente el aumento de peso diario y la eficiencia alimenticia, como un bajo consumo de energía y bajo rendimiento en los corderos (Bernabucci et al., 2009), además, una alteración de la función inmunológica, particularmente del tracto digestivo (Uyeno, 2015), entonces, para evitar el bajo consumo de energía, las dietas se incrementan en su contenido energético al aumentar los carbohidratos solubles

(principalmente granos de cereales), por lo tanto, el ganado es más susceptible a sufrir acidosis ruminal subaguda.

Acidosis ruminal subaguda

La acidosis ruminal subaguda (ARSA) se refiere a una serie de condiciones que refleja la disminución del pH (5.0 a 5.5) en el rumen del ganado, la cual no es suficiente para desencadenar los síntomas de la acidosis clínica (Lean et al., 2007).

Cuando se suplementa raciones altas en carbohidratos que son de rápida fermentación se incrementa el nivel de producción de ácidos grasos volátiles (AGV) y ácido láctico en rumen (Nagaraja and Titgemeyer, 2007), al inicio de la producción de AGV (principalmente ácido propionico) se estimula el desarrollo de la mucosa ruminal hasta un 50% que favorece la absorción de los ácidos, pero puede llegar a exceder su capacidad de absorción (Gómez et al., 2014), entonces, la acidosis ruminal sólo se produce cuando la tasa de producción de AGV's excede la velocidad a la que el ácido se puede quitar del rumen a través de la neutralización y eliminación (Penner y Aschenbach, 2011). La forma de neutralizarla por la capacidad de la saliva en rumen muestran estimaciones cuantitativas que representar el 30% (Allen, 1997), entonces, la eliminación es el mecanismo más importante para reducir el riesgo de ARSA, que en condiciones normales la absorción de AGV a través de la pared del rumen representa hasta un 53% de la eliminación total de ácido, entonces la absorción tiene un papel central en la eliminación del ácido del rumen (Gäbel et al., 1991).

Además, durante la acumulación de ácido y un pH bajo permite el desarrollo de poblaciones de C*lostridium* y coliformes los que provocan una inflamación de la mucosa y el desarrollo de paraqueratosis, el cual actúa como barrera física para la absorción de AGV (Niño, 2009), además que aumenta la presión osmótica de la digesta y puede conducir al daño e inflamación del epitelio del rumen y permitir la entrada sistémica de endotoxinas, bacterias o aminas que pueden contribuir aún más a la inflamación o infección (Owens et al, 1998; Plaizier et al., 2008), por lo tanto, disminuye la digestibilidad de la ración provocando oscilaciones en el consumo de materia seca (Niño, 2009), debido a que el epitelio ruminal es responsable de varias funciones fisiológicamente importantes como la absorción y transporte de nutrientes, el metabolismo de ácidos grasos de cadena corta y la protección (Odongo et al., 2006).

Para disminuir el riesgo de presentación de ARSA debido a que su origen es multifactorial (población microbiana, producción de saliva, motilidad ruminal) y difícil de lograr el éxito, Penner y Aschenbach (2011) señalan que un enfoque más práctico sería diseñar estrategias nutricionales que mejoren la función del epitelio ruminal, en donde además se puede considerar que el ganado es alimentado con dietas de finalización altas en energía y con un ITH > 77 en la mayor parte del año.

JUSTIFICACIÓN

En la actualidad, la investigación del potencial de QBA+PA como aditivo para la alimentación de rumiantes es limitada y no hay información disponible sobre los niveles de suplementación, parámetros de fermentación ruminal in vivo, síntesis de proteínas microbianas y digestión de los nutrientes en ganado de engorda alimentados con dietas altas en energía, ni efectos sobre el crecimiento y la energética de la dieta en rumiantes alimentados con dietas de finalización bajo condiciones de estrés por calor ambiental severa. Además, existe una creciente preocupación por la resistencia a los antibióticos y a un mayor riesgo de acidosis ruminal subaguda del ganado con ITH > 77 y consumo de dietas altas en energía, por lo que se busca constantemente alternativas para el uso de aditivos orgánicos para la alimentación animal.

HIPÓTESIS

Los compuestos fitogénicos como los alcaloides cuaternarios benzofenantridina y protopina (QBA+PA), disminuyen la degradación de aminoácidos y concentración ruminal de nitrógeno amoniacal, así también mejoran la salud del epitelio ruminal en rumiantes alimentados con dietas de finalización y riesgo de acidosis ruminal subaguda.

OBJETIVO GENERAL

El objetivo de este trabajo de investigación es valorar a influencia de QBA+PA como aditivo potencial en la alimentación de rumiantes alimentados con dietas de finalización en fermentación ruminal, digestión de nutrientes, rendimiento, características de canal y salud del epitelio ruminal.

OBJETIVOS ESPECÍFICOS

1. Evaluar diferentes niveles de suplementación de QBA+PA sobre la síntesis de proteína microbiana, fermentación ruminal y digestibilidad de nutrientes en novillos alimentados con una dieta de finalización de alta energía.

2. Evaluar la suplementación de QBA+PA en corderos de finalización en condiciones de estrés por calor, alimentados con dietas de finalización y riesgo de padecer acidosis subaguda, en el rendimiento, características de la canal, energética de la dieta y cambios histológicos en el epitelio ruminal.

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ARTÍCULOS PUBLICADOS

Running head: Isoquinoline alkaloids for feedlot steers

Evaluation of isoquinoline alkaloids supplementation levels on ruminal fermentation, characteristics of digestion and microbial protein synthesis in steers fed a high-energy diet

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ABSTRACT: Four Holstein steers with ruminal and duodenal cannulas were used in a 4×4 Latin square design to examine the effect of daily intake of 0, 2, 4 or 6 g/steer of standardized plant extract containing a mixture of quaternary benzophenanthridine alkaloids and protopine alkaloids (QBA+PA) on the characteristics of ruminal fermentation and characteristics of digestion. The basal diet consisted of a steam-flaked corn-based finishing diet that contained 62% corn and 12% sudangrass hay and the rest of diet was composed mainly by dried distillers grains, molasses, fat, and minerals. The source of QBA+PA used was Sangrovit-RS (Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany) and supplementation levels of 2, 4 and 6 g Sangrovit-RS/steer/day represented a net daily ingestion of approximately 6, 12 and 18 mg of QBA+PA compounds. Inclusion of QBA+PA linearly increased (P = 0.04) flow to duodenum of nonammonia-N and linearly decreased (P < 0.01) duodenal flows of ammonia-N. Ruminal microbial efficiency (duodenal MN, $g \cdot kg^{-1}$ OM fermented in the rumen) and protein efficiency (duodenal non-ammonia-N, $g \cdot g^{-1}$ N intake) were increased (P < 0.05) as level of QBA+PA increased. There were no effects of QBA+PA supplementation on ruminal, postruminal and total tract digestion of OM, starch, and NDF, but postruminal and total tract digestion of N increased (P < 0.01) as level of QBA+PA increased. Digestible energy of the diet tended to increase (linear affect, P = 0.09) with QBA+PA supplementation. Ruminal pH and total VFA molar concentration was not different between treatments. Ruminal NH_3-N concentration decreased linearly (P = 0.02) with QBA+PA supplementation. Ruminal molar proportion of acetate increased (P = 0.04) as supplementation level of QBA+PA increased. It is concluded that

QBA+PA supplementation enhances efficiency of N utilization in feedlot steers fed a steam-flaked corn-based finishing diet. This effect was due in part to enhanced ruminal microbial efficiency, decreased ruminal degradation of dietary non-ammonia N, and enhanced postruminal N digestion.

Keywords: digestion, feedlot cattle, isoquinoline alkaloids, rumen fermentation

INTRODUCTION

Concern over the use of regulated growth-enhancing drugs in feed formulations for livestock has furthered interest in the search for generally-recognized-as-safe alternatives. Among these, phytogenic compounds such as some isoquinoline alkaloids extracted from plants have shown promise as feed additives in broilers and pigs (Greathead, 2003). Quaternary benzophenanthridine and protopine alkaloids (QBA+PA) have been shown to have antimicrobial (Newton et al., 2002), anti-inflammatory (Tanaka et al., 1993) and immunomodulatory effects (Chaturvedy et al., 1997). With respect to digestion, these compounds suggested an ability to reduce in vitro the activity level of amino acids degradation enzymes such as decarboxylase (Drsata et al., 1996) and decreases in vitro ammonia-N concentrations (Smink and Van der Kolk, 2004). The antimicrobial effects of QBA+PA modulate growth of gastrointestinal microbiota (Juśkiewicz et al., 2011; Cushnie et al., 2014) and their effects appear to be selective, since QBA+PA supplementation potentiated the effects of fibrolytic enzymes improving fiber digestion in broilers (Sze and Chwen, 2011). Theoretically, all these effects are advantageous for ruminants

fed high-energy diets; however, research of the potential of QBA+PA as feed additives for cattle is limited and there is no information available on the effects of QBA+PA supplementation levels on in vivo ruminal fermentation parameters, microbial protein synthesis and site and extent of digestion of nutrients in feedlot cattle fed high-energy diets.

Considering that isoquinoline alkaloids have shown promising results in N retention, fiber digestion and on improving nutrient absorption in non-ruminant species, it was hypothesized that QBA+PA supplementation have the potential benefits as feed additive to cattle fed a finishing diets. The objective of this trial was to evaluate the effect of different inclusion levels of standardized plant extract containing QBA+PA on the characteristics of ruminal fermentation, characteristics of digestion and microbial protein synthesis in steers fed a finishing diet.

MATERIALS AND METHODS

The trial was conducted at the Ruminant Metabolism Experimental Unit of the Instituto de Investigaciones en Ciencias Veterinarias of the Universidad Autónoma de Baja California located 10 km south of Mexicali City in northwestern México (32° 40' 7”N and 115° 28' 6”W). The area isabout 10 m above sea level, and has Sonoran desert conditions (BWh classification according Köppen). All animal management procedures were conducted within the guidelines of locally-approved techniques for animal use and care.

Animals, treatments, and sampling

Four Holstein steers (253± 9 kg live weight)with ruminal (“T” tygon cannula with 3.8 cm i.d.) and duodenal (“T” tygon cannula 1.9 cm i.d.) cannulas

were used to examine the effect of different inclusion levels of standardized plant extract contained QBA+PA on the characteristics of ruminal fermentation, characteristics of digestion and microbial protein synthesis in steers fed a finishing diet. The ruminal and duodenal cannulas were placed as described by Zinn and Plascencia (1993). The source of QBA+PA used was Sangrovit-RS (Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany) which is a standardized plant extract preparation consisting of the QBA+PA extract from *Macleaya cordata*. The extract is standardized to contain 3 g of quaternary benzophenathridine and protopine alkaloids per kg of product.

Steers were housed in individual pens (3.9 m^2) in an indoor facility, with a concrete floor covered with a neoprene mat, automatic waterers and individual feed bunks. All steers received *ad libitum* access to the basal diet (Table 1) for 21 days before the initiation of the trial.Although there is no comparable data regarding the effects of QBA+PA in ruminant studies; however, previous reports (Jankowski, et al., 2009; Kantas et al., 2015) indicated that doses from 0.75 to 3 mg of QBA+PA/kg of diet can positively impact non-ruminant species and the dosage level can affect the levels of responses (Rawling et al., 2009). Therefore, the treatments consisted of a basal diet (Table 1) supplemented to provide 0, 2, 4, or 6 g/steer/day of Sangrovit-RS which represents a net daily ingestion of approximately 0, 6 , 12 and 18 mg of QBA+PA, which approximately corresponds to 1, 2 and 3 mg of QBA+PA/kg of feed) .The dose of QBA+PA of each treatment was weighed using a precision balance (Ohaus, mod AS612, Pine Brook, NJ) and was added in equal proportions to the basal diet (top-dressed) at time of feeding. The basal diet was fed in two equal proportions at 0800 and 2000

h daily. Chromic oxide (3.0 g/kg of diet air-dry basis) was used as an indigestible marker to estimate nutrient flow and digestibility (as dry matter basis, the daily ingestion of chromic oxide averaged 14.87±0.71 g). Chromic oxide was premixed with minor ingredients (urea and mineral supplement composed by limestone and trace mineral salt) in a 2.5 m^3 capacity concrete mixer (mod 30910-7, Coyoacán, Mexico) for 5 min and then, the final product was incorporated after that steam-flaked corn was added to the mixer. Steam-flaked corn was prepared to provide flake density of 0.36 kg/L, while sudangrass hay was ground in a hammer mill (Bear Cat #1A-S, Westerns Land and Roller Co., Hastings, NE) with a 3.81cm screen, before incorporation into complete mixed diets. To avoid feed refusals, DM intake was restricted during experiment to 5.31 kg/d (90% of observed DM intake during a 14-d preliminary period before start of the trial). Experimental periods consisted of 21 days, with 17 days for dietary treatment adjustment and 4 days for sample collection. During the collection period, duodenal and fecal samples were taken from all steers, twice daily as follows: d 1, 0750 and 1350 h; d 2, 0900 and 1500 h; d 3, 1050 and 1650 h; and d 4, 1200 and 1800 h. Individual samples consisted of approximately 500 mL of duodenal chyme and 200 g (wet basis) of fecal material. Feed, duodenal and fecal samples from each steer and within each collection period were prepared for analysis as follows: Samples were first oven-dried at 70°C and then ground in a laboratory mill (Micro-Mill, Bell-Arts Products, Pequannock, N J). Samples were then oven-dried at 105°C until constant weight and stored in sealed glass jars. During the final day of each collection period, ruminal samples were obtained from each steer at 4 and 8 h after feeding via the ruminal cannula.

Ruminal fluid pH was determined on fresh samples. Samples were then strained through four layers of cheese cloth. For VFA analysis, 2 mL of freshly prepared 25% (w/vol) meta-phosphoric acid was added to 8 mL of strained ruminal fluid, centrifuged (17,000 × *g* for 10 min) and supernatant fluid stored at -20°C. For ammonia-N analysis, 10 mL of strained ruminal fluid was acidified with 0.5 mL of 6 N HCL and stored at -20°C. Upon completion of the trial, at 1200 h (4 h after the morning feeding), ruminal fluid (approximately 500 mL) was obtained from all steers and composited for isolation of ruminal bacteria via differential centrifugation (Bergen et al., 1968) as follows: 1) ruminal fluid was diluted 50:50 with 0.16N saline (37^{o}C) agitate gently for about 30 seconds and strained through 4 layers of cheesecloth; 2) strained fluid was promptly transferred into centrifuge bottles and spun at 2000 × *g* for 10 min at 10^{o}C; 3) supernate was decanted and centrifuged at 43,000 × *g* for 20 minutes at 10^{o}C, and 4) supernate was decanted and the pellet isolated, oven-dried (70°C) and then ground with a mortar and pestle. The microbial isolate served as the purine:N reference for the estimation of microbial N contribution to chyme entering the small intestine (Zinn and Owens, 1986). Time required to consume approximately 90% of the morning feed allowance (meal duration) was recorded for each steer during the last 14 days of each experimental period.

Sample analysis and calculations

Feed, duodenal and fecal samples were subject to the following analysis: dry matter (oven drying at 105°C until no further weight loss; method 930.15; AOAC, 2000); ash (method 942.05; AOAC, 2000), Kjeldahl N (method 984.13; AOAC, 2000); aNDFom [Van Soest et al., 1991, corrected for NDF-

ash,incorporating heat stable α-amylase (Ankom Technology, Macedon, NY) at 1mL per 100 mL of NDF solution (Midland Scientific, Omaha, NE)]; chromic oxide (Hill and Anderson, 1958), and starch (Zinn, 1990). In addition, gross energy (GE, using the adiabatic bomb model 1271; Parr Instrument Co., Moline, IL. USA) were determined for feed and fecal samples. Ammonia-N (method 941.04; AOAC, 2000) and purines (Zinn and Owens, 1986) were determined in duodenal samples. Concentrations of VFA in ruminal fluid were determined by gas chromatography using a 10% SP-1200/1% H_3PO_4 on 80/100 Chromsorb W AW packing in a 183 cm × 22 cm i.d. glass column with column, inlet and detector temperatures maintained at 120, 195 and 200 °C, respectively, and with N_2 carrier gas flow rate at 20 mL/min (Zinn, 1988). Ammonia-N in ruminal fluid was determined by procedures adapted from Fawcett and Scott (1960).

Organic matter of feed and digesta samples were estimated as the difference of DM minus ash content. Microbial organic matter (MOM) and microbial nitrogen (MN) entering to duodenum (measured from duodenal cannula placed 6 cm from phyloric sphincter) were calculated using purines as a microbial marker (Zinn and Owens, 1986). Organic matter fermented in the rumen was considered equal to the OM intake minus the difference between the amount of total OM reaching the duodenum and the MOM reaching the duodenum. Feed N escape to the small intestine was considered equal to the total N leaving the abomasum minus ammonia-N and MN and, thus, includes any endogenous contributions. Ruminal microbial efficiency was estimated as duodenal MN, $g \cdot kg^{-1}$ OM fermented in the rumen and protein efficiency represent the duodenal non-ammonia-N (NAN), $g \cdot g^{-1}$ N intake. Methane

production was calculated based on the theoretical fermentation balance for observed molar distribution of VFA and OM fermented in the rumen (Wolin, 1960).

Statistical analysis

The effects of the QBA+PA level on characteristics of digestion was analyzed as a 4 × 4 Latin square design using the MIXED procedure (SAS Inst. Inc., Cary, NC). The fixed effects consisted of treatment and period, and steer as a random effect. The statistical model for the trial was as follows:

$Y_{ijk} = \mu + S_i + P_j + T_k + E_{ijk}$, where: Y_{ijk} is the response variable, μ is the common experimental effect, S_i is the steer effect (n=4), P_j is the period effect, T_k is the treatment effect (doses level of 0, 2, 4,and 6 g/steer/day which were randomised assigned to the steers in the first period), and E_{ijk} is the residual error. Treatments effects on digestion and fermentation variables were tested by means of polynomial contrasts (SAS Inst., Inc., Cary, NC; Version 9.3). Duration of meal (offered in the morning) which was recorded in each animal in the last 14-d of each period (56 observations per treatment), and ruminal fermentation data which are presented as average of the samples obtained at 4 and 8-h post-feeding (8 observations per treatment) were analyzed with a linear mixed model for repeated measures in a 4 × 4 Latin square design according to SAS (SAS Inst., Inc., Cary, NC; Version 9.3). The model included the effects of treatment, repeated measure (duration of meal or average of the samples obtained at 4 and 8-h post-feeding), period, and the interaction of repeated measure × treatment as fixed effects, with animal as a random effect. As the repeated measurements are taken over time on the same animal, they show correlation or

exhibit variability that changes, then, three covariance structures were evaluated by fit: UN (unstructured), CS (compound symmetry), and AR1 (first-order autoregresive), utilizing Akaike's information criterion (AIC) and Schwarz's Bayesian information criterion (BIC), being that covariance structure with smaller values, the better. The analysis was carried out with the MIXED procedure of SAS software. In all cases, least squares means and standard error are reported and contrasts were considered significantly when the *P* value was ≤ 0.05, and tendencies are identified when the *P*-value was > 0.05 and ≤ 0.10.

RESULTS AND DISCUSSION

There were no feed refusals. Rate of feed consumption was similar (*P* = 0.56) across treatments. Time required to consume 90% of the morning feeding averaged 31.3 min (5.9 ± 0.6 min/kg DM). This rate of DM intake is consistent with previously reported ranges of 4 to 10 min/kg DM (Grant and Albright, 2000; Plascencia et al., 2011).

Treatment effects on characteristics of ruminal and total tract digestion are summarized in Table 2. Flows of OM, NDF and starch to duodenum did not differ (P ≥ 0.27) among treatments, but QBA+PA supplementation increased (linear effect, *P* = 0.04) flow of NAN and decreased (linear effect, *P* < 0.01) duodenal flows of NH_3-N. Consequently, ruminal N efficiency (NAN flow to the duodenum/N intake) was linearly (*P*< 0.05) increased with QBA+PA supplementation. The increased duodenal flow of NAN was due in part to the trends for linear increases on ruminal escape of feed N (P = 0.10) and on duodenal flow of microbial N (P = 0.06). Decreases of ruminal degradation of feed N in steers supplemented with certain antimicrobials (such ionophores)

have been reported previously (Zinn, 1988; Zinn et al., 1994). The reduction on peptide degradation and amino acid deamination is mainly attributed to inhibition of high ammonia- producing bacteria (Yang and Russell, 1993). However, in vitro studies by Drsata et al. (1996) revealed that QBA+PA supplementation decreased breakdown of amino acids, mainly by inhibition of aromatic amino acid decarboxylase.

The numeric trend (P = 0.06) for increased net microbial N flow to the small intestine with increasing QBA+PA supplementation was surprising in that supplementation with antimicrobial compounds often result in decreased net microbial protein synthesis (Zinn, 1987; Zinn et al., 1994), largely through inhibition of *Gram*-positive bacteria (Chen and Russell, 1991). Sanguinarine and chelerthryne, principal compounds of QBA in Sangrovit-RS, have significant dose dependent antibacterial activity against *Gram*-positive and *Gram*-negative bacteria (Opletal et al., 2014). It has been stated that recycling of microbial protein is a factor that depresses microbial growth efficiency and most recycling thought to be mediated by protozoa predation. Decreasing or removing protozoa from the rumen have shown to increase microbial efficiency (VanSoest, 1994). Because the selective antimicrobial effects of QBA+PA in rumen environment have been not evaluated more research is needed to further asses the role of QBA+PA in modulating ruminal microbial (protozoa and bacteria) growth in vivo and its effect on the ruminal dynamic of N.

There were no effects (P > 0.50) of QBA+PA supplementation on ruminal digestion of OM, NDF and starch. Due to antimicrobial properties of QBA+PA some decrease in ruminal NDF degradation was expected. Indeed, a

characteristic response to antibiotic supplementation has been decreased ruminal fiber digestion (Zinn, 1993). However, effects may differ according to source (selective effects) and level of supplemental antibiotic. For example, in an experiment conducted by Salinas-Chavira et al. (2009), ruminal NDF digestion was depressed in steers supplemented with monensin, but unaffected in steers supplemented with virginamycin.

The absence of effect of QBA+PA on OM ruminal digestion with increases on net microbial N synthesis resulted in greater (P< 0.05) ruminal microbial efficiency (MN, g·kg^{-1} OM fermented in the rumen) as level of QBA+PA increased.

There were no treatment effects on postruminal and total tract digestion of OM, NDF, and starch. However, postruminal and total tract digestion of N were increased (P < 0.01) as level of QBA+PA was increased. Considering that steers were fed with the same level of protein (22 g of N daily), the reasons for this difference in digestion of protein is not apparent (Holter and Reid, 1959). A possible explanation is the higher ratio of dietary-to-microbial protein entering the intestine for QBA+PA treatments (ratio of dietary-to-microbial protein entering to intestine for QBA+PA treatments averaging 95.6, while for controls was 93.0). Feed protein is usually more digestible than microbial protein (Van Soest, 1994) and this phenomenon can explain the increased nitrogen digestibility observed in QBA+PA treatments. Improvement in protein retention by reducing the intestinal decarboxylation of aromatic amino acids have been argued as a possible cause of greater N retention in non-ruminants species when supplemented with QBA+PA (Vieira et al., 2008). The above assumption

is based on the findings of Drsata et al. (1996). In an in vitro test, those researchers observed a strong and irreversible inhibitory effect of QBA+PA on the enzyme aromatic amino acid decarboxylase extracted from the liver of the rat. Inhibitory effects were effective at low concentrations of QBA+PA ($1.2 \times 10^{-4}M$), although it should be noted that the effects of QBA+PA has not been evaluated in the rumen environment.

Digestible energy value of the diet tended to increase (linear effect, $P = 0.09$) as QBA+PA supplementation increased. There is no other information reported in the literature with which to compare effects of QBA+PA on dietary digestible energy in ruminants. But the improvement in total tract DM digestion in pigs and broilers that were fed with supplemental QBA+PA was attributed to an enhanced absorption and/or improvement in fermentative processes in the lower GIT (Vieira et al., 2008; Jeroch et al., 2009).

Treatment effects on ruminal pH, ammonia-N, VFA molar proportions and estimated methane production are summarized in Table 3. The ruminal pH (sampling 4 and 8 h post-feeding) averaged 6.31 ± 0.27, and was not affected ($P \geq 0.16$) by treatments. The effect of QBA+PA supplementation on ruminal pH in cattle has not been previously reported. In broilers QBA+PA supplementation did not affect pH of cecal digesta (Zduńczyk et al., 2010;Juśkiewicz et al., 2011).

Consistent with decreased duodenal ammonia-N (Table 2), QBA+PA supplementation decreased (linear effect, $P< 0.01$) ruminal ammonia-N concentration. According to Chen and Russell (1991), this effect is attributable to decreased proteolysis and deamination of amino acids.

Consistent with lack of treatment effects on ruminal OM digestion, total ruminal VFA concentration was also unaffected by QBA+PA supplementation. However, QBA+PA supplementation increased (linear effect, P = 0.03) ruminal molar proportion of acetate. Differences in molar proportions of propionate and butyrate, acetate:propionate molar ratio, and estimated methane production did not differ (P > 0.20). Likewise, Smink and Van der Kolk (2004) observed an effect on increases on molar proportion of acetate without effect on total VFA production and molar proportions of propionate and butyrate when they studied in vitro effects of QBA+PA on fermentation of concentrate based diets. Generally, the effects of supplemental antimicrobials on ruminal VFA molar proportions have not been consistent. Some reports showed that ionophores can be affective in altering ruminal fermentation, specifically increasing molar proportions of propionate and decreasing molar proportions of acetate and estimated methane production (Quinn et al., 2009; Wingard, 2014). However, numerous other studies (Zinn, 1987; Galyean et al., 1992; Salinas-Chavira et al., 2009) revealed little or no change in ruminal VFAmolar proportions or estimated methane production with ionophore supplementation.

Although no differences were detected in butyrate molar proportion (P > 0.16), QBA+PA supplementation resulted in a numeric decrease (24.5%) in butyrate molar proportion. Jankowski et al. (2009) observed a 28% decrease (P< 0.01) in cecal butyrate production in broilers supplemented with QBA+PA extracted from *Macleaya cordata.* Declines in butyrate production in some studies may reflect reductions in Clostridium populations (Van den Abbeele et al., 2013). Rahman et al. (2009) demonstrated potent antibacterial activity of

QBA+PA extract *in vitro* against to several *Gram*-positive bacterial including Clostridium. Further work is warranted to quantify this potential modulating effect of QBA on ruminal and postruminal clostridial growth in feedlot cattle.

As level of QBA+PA increased, valerate production decreased ($P< 0.01$). Since changes in valerate production reflects microbial fermentation of polypeptides and amino acids (Russell et al., 1991), therefore, this reduction of ruminal valerate is consistent with decreases in ruminal feed protein degradation and in the lower concentration of ruminal NH_3-N observed in the present experiment (Table 2).

In conclusion, daily consumption of Sangrovit-RS, a standardized source of QBA+PA used in this experiment, at levels of up to 6 g/day (approximately 18 mg of QBA+PA compounds/steer/day) does not affect feeding behavior of cattle fed high concentrate diets. Supplementation with QBA+PA enhanced efficiency of N utilization in feedlot steers fed a steam-flaked corn-based finishing diet. This effect was due in part to enhanced ruminal microbial efficiency, decreased ruminal degradation of dietary feed N, and enhanced postruminal N digestion.

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Table 1. Ingredients and composition of basal diet fed to steers

Item	Diet composition (% DMB)
Ingredient	
Steam-flaked corn	61.80
Distillers dried grains with solubles	15.00
Sudan grass hay	12.00
Yellow grease	2.00
Molasses	6.00
Limestone	1.50
Urea	1.00
Trace mineral salt[1]	0.40
Chromic oxide	0.30
Chemical composition, % DM basis[2]	
Crude protein	14.35
Starch	47.76
NDF	16.43
Ash	5.78
Calculated net energy, Mcal/kg of DM basis[3]	
Maintenance	2.16
Gain	1.50

[1]Trace mineral salt contained: $CoSO_4$, 0.068%; $CuSO_4$, 1.04%; $FeSO_4$, 3.57%; ZnO, 1.24%; $MnSO_4$, 1.07%, KI 0.052%; and NaCl, 92.96%.

[2]Dietary chemical composition was determined by analyzing subsamples collected and composited throughout the experiment. Accuracy was ensured by adequate replication with acceptance of mean values that were within 5% of each other.

[3]Net energy was calculated based on tabular net energy (NE) values for individual feed ingredients (NRC, 2000) with the exception of supplemental fat, which was assigned NE_m and NE_g values of 6.03 and 4.79, respectively (Zinn, 1988).

Table 2. Influence of supplementation level of QBA+PA on characteristics of apparent ruminal and total tract digestion in cannulated Holstein steers (253 kg BW)

Item	QBA+PA[1], mg/steer/day				SEM	Contrast *P*-value		
	0	6	12	18		Linear	Quadratic	Cubic
Meal duration, min/feeding[2]	30.5	31.0	32.1	31.7	1.7	0.56	0.75	0.94
Intake, g/d								
DM	5,314	5,316	5,318	5,320				
OM	5,007	5,007	5,007	5,007				
NDF	874	874	874	874				
Starch	2,538	2,538	2,538	2,538				
N	122	122	122	122				
GE, Mcal/d	23.62	23.62	23.62	23.62				
Flow to duodenum, g/d								
Apparent OM	2,438	2,486	2,615	2,485	49	0.27	0.12	0.17
Microbial OM	615	628	659	644	12	0.06	0.27	0.27
NDF	473	471	485	472	28	0.95	0.83	0.74
Starch	419	393	397	370	52	0.58	0.89	0.91
N	123	125	131	131	2.31	0.05	0.68	0.52

NH_3-N	3.91	3.29	2.49	2.54	0.15	<0.01	0.07	0.17
NAN	118.7	121.7	128.1	127.8	2.88	0.04	0.59	0.46
MN	61.48	62.81	65.83	64.43	1.13	0.06	0.27	0.27
Feed N	57.19	58.83	62.27	63.39	2.62	0.10	0.93	0.76
Ruminal digestion, % of intake								
OM[3]	59.90	59.26	57.40	59.58	0.92	0.52	0.17	0.25
NDF	45.85	46.03	44.45	46.05	3.20	0.94	0.83	0.74
Starch	83.51	84.52	84.35	85.38	2.11	0.58	0.99	0.81
Feed N	53.13	51.78	48.96	48.04	1.92	0.08	0.73	0.65
Microbial efficiency[4]	19.37	19.95	21.58	20.36	0.40	0.04	0.11	0.11
N efficiency[5]	0.97	1.00	1.05	1.05	0.02	0.04	0.59	0.45
Postruminal digestion, % entering								
OM	59.53	60.62	64.90	62.02	1.83	0.20	0.32	0.26
NDF	13.79	8.41	14.89	12.65	7.38	0.92	0.84	0.55
Starch	93.04	91.76	91.49	94.18	1.69	0.68	0.29	0.80
N	71.13	73.21	76.28	75.79	0.68	<0.01	0.11	0.19
Fecal excretion, g/d								
DM	1,113	1,118	1,050	1,083	41.2	0.28	0.56	0.43
OM	985	978	918	943	38.9	0.32	0.70	0.46

NDF	406	424	413	407	13.7	0.84	0.42	0.59
Starch	27.7	32.6	32.3	20.5	4.19	0.29	0.09	0.75
N	35.3	33.4	30.9	31.5	0.75	<0.01	0.16	0.35
GE, Mcal/d	5.14	5.10	4.65	4.82	0.16	0.09	0.55	0.20
Total-tract digestion, % of intake								
DM	78.67	78.86	80.24	79.61	0.77	0.28	0.58	0.43
OM	80.34	80.46	81.67	81.17	0.78	0.32	0.70	0.46
NDF	53.48	51.45	52.74	53.40	1.57	0.88	0.43	0.60
Starch	98.91	98.72	98.73	99.19	0.17	0.29	0.09	0.75
N	71.18	72.64	74.60	74.17	0.62	<0.01	0.16	0.35
DE, %	78.25	78.40	80.31	79.59	0.68	0.09	0.54	0.20
DE diet, Mcal/kg	3.48	3.48	3.57	3.54	0.03	0.09	0.54	0.20

[1]The source of QBA+PA used was Sangrovit-RS (Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany).

[2]Duration of meal (offered in the morning) was registered in each animal in the last 14-d of all periods (56 observations per treatment).

[3] Organic matter fermented in the rumen was considered equal to the OM intake minus the difference between the amount of total OM reaching the duodenum and the MOM reaching the duodenum.

[4]Microbial efficiency estimated as duodenal MN, g kg^{-1} OM truly fermented in the rumen.

[5]N efficiency estimated as duodenal nonammonia-N, g g^{-1} N intake.

Table 3. Influence of supplementation level of QBA+PA on ruminal pH, NH_3-N, VFA concentration, and estimated methane production[1]

Item	QBA+PA[2], mg/steer/day				SEM	Contrast *P*-value		
	0	6	12	18		Linear	Quadratic	Cubic
pH	6.27	6.29	6.23	6.44	0.09	0.16	0.55	0.42
Ruminal ammonia-N, mg/dL	17.1[a]	13.1[ab]	10.9[b]	11.7[b]	0.57	<0.01	0.05	0.84
Total VFA, moles	72.6	73.8	72.1	70.2	4.3	0.66	0.73	0.90
Ruminal VFA, mol/100 mol								
Acetate	48.8	53.7	54.4	54.4	1.35	0.03	0.12	0.56
Propionate	30.5	32.3	31.4	30.5	2.65	0.93	0.64	0.82
Isobutyrate	0.25	0.30	0.43	0.50	0.18	0.11	0.53	0.93
Butyrate	15.2	10.9	11.6	11.9	1.75	0.16	0.20	0.56
Isovalerate	2.5	1.7	1.6	1.7	0.22	0.06	0.09	0.51
Valerate	2.7[a]	1.4[b]	1.3[b]	1.4[b]	0.11	<0.01	0.01	0.06
Acetate:propionate ratio	1.60	1.66	1.76	1.79	0.24	0.80	0.84	0.96
Methane production[3]	0.44	0.46	0.48	0.47	0.03	0.39	0.78	0.62

[1]Average of the samples taken at 4 and 8-h post-feeding.

[2]The source of QBA+PA used was Sangrovit-RS (Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany).

[3]Methane, mol/mol of glucose equivalent fermented (Wolin, 1960).

Running title: Sangrovit supplementation in heat stressed finishing ewes

Influence of quaternary benzophenantridine and protopine alkaloids on growth performance, dietary energy, carcass traits, visceral mass and rumen health in finishing ewes under conditions of severe temperature humidity index

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ABSTRACT: Twenty Pelibuey × Katahdin ewes (35 ± 2.3 kg) were used in order to determine the effects of the consumption of standardized plant extract containing a mixture of quaternary benzophenanthridine alkaloids and protopine alkaloids (QBA+PA)on growth performance, dietary energetics, visceral mass, and ruminal epithelial health in heat-stressed ewes fed with a high-energy corn-based diet. The basal diet [13.9% crude protein and 2.09 Mcal of net energy (NE) of maintenance/kg of dry matter (DM)] contained 49.7% starch and 15.3% neutral detergent fiber. Source of QBA+PA was SANGROVIT® RS (SANG) which contain 3 g of quaternary benzophenathridine and protopine alkaloids per kg of product. Treatments consisted of a daily consumption of 0 or 0.5 g SANG/ewe. Ewes were grouped by weight and assigned to 10 pens (5 pens/treatment), with two ewes per pen. The experimental period lasted 70 days. The mean Temperature Humidity Index (THI) during the course of this experiment was 81.7± 1.0 (severe heat stress). There were no treatment effects on water intake. Dry matter intake was not affected ($p=0.70$) by treatments, but as a consequence of group fed SANG had a numerically (11.2%) higher gain in comparison to the control group, SANG improved gain efficiency (8.3%, $p=0.04$), dietary net energy (5.2%, $p<0.01$) and the observed-to-expected net energy (5.9%, $p<0.01$). Supplemental SANG did not affect ($p\geq0.12$) carcass characteristics, chemical composition of shoulder, and organ weights (g/kg EBW) of stomach complex, intestines, and heart/lung. Supplemental SANG decreased liver weight (10.3%, $p=0.02$) and increased visceral fat (16.9%, $p=0.02$). Rumen epithelium of ewes fed SANG had lower

scores for cellular dropsical degeneration (1.30 vs 2.34, p=0.02), parakeratosis (1.30 vs 1.82, p=0.03) and neutrophil infiltration (2.08 vs 2.86, p=0.05) than controls. It is concluded that SANG supplementation helped ameliorate the negative effects of severe heat on growth performance of feedlot ewes fed high-energy corn-based diets. Improvement in energetic efficiency may have been mediated, in part, by anti-inflammatory effects of supplemental SANG and corresponding enhancement of nutrient uptake.

Key words: isoquinoline alkaloids, heat stress, high-energy diets, fed efficiency, small ruminants, ruminal epithelial

INTRODUCTION

Heat stress negatively affects daily weight gain and/or feed efficiency of feedlot cattle. Additionally, cattle under heat stress show impaired immune function, particularly as related to the digestive tract, resulting in increased risk of subclinical rumen acidosis (Uyeno, 2015). Antibiotic feed additives have benefited cattle under heat stress, helping to maintain a healthy ruminal epithelium through selective effects on microbial populations (Abdel-Samee, 1995). Niewold (2007) indicate that the effects of dietary supplementation with subtherapeutic levels of antimicrobials may be mediated through anti-inflammatory mechanisms. However, there is mounting concern regarding this practice and the development of antibiotic resistance and public health. Thus, considerable effort has been directed toward advancing the use of more “organic” alternatives. Among these, phytogenic compounds, such as isoquinoline alkaloids extracted from plants as *Macleaya cordata*, have shown

promise as feed additives in broilers and pigs (Kantas et al., 2014). The quaternary benzophenanthridine and protopine alkaloids (QBA+PA) have both anti-inflammatory and immunomodulatory effects (Kosina et al., 2010). These alkaloids have selective effects on microbial growth along the digestive tract (Cushnie et al., 2014). In feedlot cattle, QBA+PA supplementation decreased ruminal ammonia N concentration (Plascencia and Zinn, 2014). Theoretically, all these effects are advantageous for ruminants under heat stress; however, research of the potential of QBA+PA as feed additives for cattle is limited and there is no information available on the effects of QBA+PA supplementation in finishing ruminants under conditions of severe ambient heat load. The aim of this experiment was to evaluate the effects of inclusion of standardized plant extract containing isoquinoline alkaloids (QBA+PA) on growth performance, dietary energy, carcass traits and health of the ruminal epithelium in feedlot ewes fed finishing high-energy diets under conditions of severe temperature-humidity index.

MATERIALS AND METHODS

All animal management procedures were conducted within the guidelines of locally-approved techniques for animal use and care (NOM-051-ZOO-1995: humanitarian care of animals during mobilization of animals; NOM-062-ZOO-1995: technical specifications for the care and use of laboratory animals. Livestock farms, farms, centres of production, reproduction and breeding, zoos and exhibition hall, must meet the basic principles of animal welfare; NOM-024-ZOO-1995: animal health stipulations and characteristics during transportation of animals.

Weather measurement and THI estimation

Climatic variables (ambient temperature and relative humidity) were obtained every hour from on-site weather equipment (Thermohygrometer Avaly, Mod. DTH880, Mofeg S.A., Zapopan, Jalisco) throughout the experimental period. The temperature humidity index was calculated using the following formula: THI = 0.81× *T* + RH (*T* – 14.40) + 46.40 (Hahn, 1999).

Animals, diet and experimental design

Twenty Pelibuey × Katahdin (35 ± 2.3 kg body weight, BW) ewes were grouped by weight and assigned to 10 pens, with two ewes per pen. Pens had a size of 6 m^2 with overhead shade, automatic waterers and 1 m fence-line feed bunks. Two weeks before initiation of the experiment the ewes were treated for parasites (Tasasel 5%, Fort Dodge, Animal Health, México) and injected with 1×10^6 IU vitamin A (Synt-ADE, Fort Dodge Animal Health, México).The source of QBA+PA used was Sangrovit-RS (SANG; Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany) which is a standardized plant extract preparation consisting of the QBA+PA extract from *Macleaya cordata*. The extract is a standardized to contain 3 g of quaternary benzophenathridine and protopine alkaloids per kg of product with a carrier based on rye flour plus salt. Treatments consisted of a basal diet (Table 1) supplemented to provide 0 or 0.5 g/ewe/day of SANG.Ewes were fed 0.3 kg (as fed basis) of the basal diet in the morning feeding (0800 h), and the remainder in the afternoon feeding. To ensure daily consumption, the total daily dosage of SANG was provided in the morning feeding as part of the complete mixed diet. This was accomplished by combining 50 g of SANG (hand-weighed using a precision balance, Ohaus, mod. SCOUT PRO

SP401, Pine Brook, NJ) with 30 kg of the basal diet in a 90-kg capacity paddle mixer (Leon Weill mixer, model 30910-7, Coyoacán, Mexico) and mixing for 10 min before feeding to ewes. Ewes were weighed before the morning meal on day 1 and day 70 (harvest). The initial BW was converted to shrunk body weight (SBW) by multiplying the weight by 0.96 to adjust for the gastrointestinal fill, and all lambs were fasted (feed, but not drinking water was withdrawn) for 18 h before recording the final BW. Ewes were allowed *ad libitum* access to dietary treatments. Daily feed allotments to each pen were adjusted to allow minimal (<5%) feed refusals in the feed bunk. The amounts of feed offered and of feed refused were weighed daily. Lambs were provided fresh feed twice daily at 0800 and 1400 hours. Feed bunks were visually assessed between 0740 and 0750 hours each morning, refusals were collected and weighed and feed intake was determined. Water consumption was measured daily at 0700 h by dipping a graduated rod into the tank drinker (one watering tank for each pen). Once the measure was taken, the remaining water was drained, and the tanks were refilled with fresh water.

Laboratory analyses and calculations

Feed samples were subject to the following analysis: Dry matter (oven drying at 105°C until no further weight loss; method 930.15; AOAC 2000); ash (method 942.05; AOAC, 2000), Kjeldahl N (method 984.13; AOAC, 2000); aNDFom [Van Soest et al., 1991, corrected for NDF-ash,incorporating heat stable α-amylase (Ankom Technology, Macedon, NY) at 1mL per 100 mL of NDF solution (Midland Scientific, Omaha, NE)]; and starch (method 996.11; AOAC, 2000).

The estimations of dietary energetic and expected dry matter intake were performed based on the estimated initial and final SBW. Average daily gains (ADG) was determined by subtracting the initial BW from the final BW and dividing that result by the number of days on feed. The efficiency of BW gain was computed by dividing ADG by the daily dry matter intake (DMI). The estimation of expected DMI was determined based on observed ADG and average SBW according to the following equation: expected DMI, kg/d = (EM/NE_m) + (EG/NE_g), where EM (energy required for maintenance, Mcal/d = $0.056 \times SBW^{0.75}$ (NRC, 1985), EG (energy gain, Mcal/d) = $0.276 \times ADG \times SBW^{0.75}$ (NRC, 1985), and NE_m and NE_g are 2.05 and 1.38 Mcal/kg, respectively (derived from tabular values based on the ingredient composition of the experimental diet; NRC, 1985). The coefficient (0.276) was estimated assuming a mature weight of 113 kg for Pelibuey × Kathdin (Estrada-Angulo et al., 2013). Dietary NE was estimated by means of the quadratic formula: $x = (-b - \sqrt{b^2} - 4ac)/ 2c$, where $x = NE_m$, a =-0.41EM, b = 0.877 EM + 0.41 DMI + EG, and c = -0.877 DMI (Estrada-Angulo et al., 2013).

Carcass and visceral mass data

After harvest, ewes were skinned, and the gastrointestinal (GIT) organs were separated and weighed. After carcasses (with kidneys and internal fat included) were chilled in a cooler at -2 to 1°C for 48 h, the following measurements were obtained: (1) body wall thickness (distance between the 12^{th} and 13^{th} ribs beyond the ribeye, five inches from the midline of the carcass); (2) fat thickness perpendicular to the *M. longissimus thoracis* (LM),measured over the center of the*longissimus muscle* between the 12th and 13th rib; (3) LM

surface area, measure using a grid reading of the cross sectional area of the *longissimus muscle* between 12th and 13th rib, and 4) kidney, pelvic and heart fat (KPH). The KPH was removed manually from the carcass, and afterwards weighed and reported as a percentage of the cold carcass weight (USDA, 1982).Shoulders were obtained from the forequarter. The weights of shoulder were subsequently recorded. The shoulder composition was assessed using physical dissection by the procedure described by Luaces et al. (2008).

All tissue weights are reported on a fresh tissue basis. Organ mass was expressed as gramsof fresh tissue per kilogram of final empty body weight (EBW). Final EBW represents the final full BW minus the totaldigesta weight. The stomach complex was calculated as the digesta-free sum of the weights of the rumen, reticulum, omasum and abomasum. The ruminal epithelial histology was evaluated on samples obtained from the rumen (cranial, dorsal and ventral sac) immediately following evisceration. The fixation protocol of ruminal papillae was based upon methodology reported by Graham and Simmons (2005).

Statistical analyses

Performance (DMI, gain, gain efficiency, observed dietary NE, observed-to expected dietary NE ratio, and observed-to-expected DMI ratio) and carcass data were analyzed as a randomized complete block design using pen as the experimental unit. The MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) was used to analyze variables, with hot carcass weight (HCW) as a covariate when it represented a significant ($p \leq 0.05$) source of variation in analysis of carcass measures. Shoulder composition was analyzed as a general complete block design, including the effect of block × treatment interaction, together with the

effect of cold carcass weight (CCW) as covariate. When the covariate represented a non-significant ($p>0.05$) source of variation it was not included into the model. The analysis was realized using the MIXED procedure (SAS Inst. Inc., Cary, NC). Visceral organ mass data were analyzed as a complete block design, including the effect of block × treatment interaction. The MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) was used to analyze the variables. Histological changes to ruminal epithelium (edema or inflammation process) were evaluated based on degree changes (considering dropsical degeneration, neutrophil infiltration and parakeratosis), coded as 1=normal, 2=moderate, and 3= intense. Histological data were analyzed using ANOVA, Kruskal-Wallis and Fisher's exact tests ($\alpha = 0.05$). Multiple test comparisons were performed among treatments (LSD). Differences were considered significant when the p-value was ≤ 0.05, and tendencies were identified when the p-value was >0.05 and ≤ 0.10.

RESULTS AND DISCUSSION

The experiment was carried out from June to August of 2014, the minimum and maximum estimated THI were 64.50 and 104.20, respectively (Table 2). Daily maximal THI exceeded the threshold THI value of 74 (Mader et al., 2006) for every day of the 70-d study. Average daily THI was 81.7± 0.97. Pelibuey breeds and their crosses adapt well to elevated ambient temperatures (Romero et al., 2013). Nevertheless, based on THI code (Normal THI < 74; Alert 75 -79; Danger 79-84, and Emergency > 84), ewes were exposed, on a daily basis, to conditions of severe ambient heat load.

Treatment effects on growth performance and dietary energetics are shown in Table 3. Water intake averaged 7.3 L/d and was not affected (P=0.19) by SANG supplementation. Based on NRC (2007) expected water intake was 6.1 L/d, where water intake, L/d = (1.25 + 0.18 × average temperature) × DMI. This estimate is in good (95%) agreement with measures for non-supplemented ewes, but is considerable less (75%) than observed water intake of SANG supplemented ewes. There is no information related to the effects of supplementation of SANG on water intake. The effect of QBA+PA on water intake of ruminants has not been previously reported. However, the numeric increase is consistent with diuretic effects of QBA+PA observed in rats (Zdarilova et al., 2008).

Dry matter intake averaged 0.890 kg/d and was not affected (p=0.70) by treatments. This value is 24% lower than the average DMI of previous reports utilizing lambs with similar breeding and dietary NE concentration (Robles-Estrada et al., 2009; Estrada-Angulo et., 2013). However, the observed marked depression in DMI was in close agreement with the expected reduction in intake (25%) by feedlot lambs subjected to severe heat load (NRC, 1987).

Even though daily gain was not statistically different (averaging 113 g/day), ewe that fed SANG had numerically higher gain (11%) than controls. Under “normal” THI conditions, expected ADG for lambs (NRC, 1985) is about 0.270 kg/d. Previous reports (Robles-Estrada et al. 2009; Estrada-Angulo et., 2013) using lambs of similar breeding observed ADG from 0.200 to 0.300 kg/d.Decreased in ADG is linked in part with reduced feed intake, as well as metabolic changes, during adaptation to “severe” ambient heat load.

SANG improved gain efficiency by 8.3% (p=0.04). Increased maintenance requirement in lambs due to metabolic demands to dissipate heat load, reduces energy available for weight gain (NRC, 2007). The estimation of dietary energy and the ratio of observed-to-expected DMI revealed differences in efficiency independent of ADG, providing important insight into potential treatment effects on the efficiency of energy utilisation of the diet. Compared to controls, dietary NE and observed-to-expected dietary NE ratio were 5.2 and 5.8% greater ($p<0.01$), and observed-to-expected DMI ratio was 3.6% lower ($p<0.02$) in lambs supplemented with SANG. Barajas et al. (2014) observed a 3.2% increase in dietary NE of feedlot bulls supplemented with 4 g SANG/day. An alternative approach for expressing SANG effects on animal energetics in the present experiment is to keep the net energy value of the diet constant and present treatment effects solely as a function of changes in the maintenance coefficient, as follows: $MQ = (NE_m \times(DMI-(EG/NE_g)))/SBW^{0.75}$, where NE_m correspond to the NE_m of diet (Table 1), $EG= 276\times ADG\times SBW^{0.75}$ and SBW is the average SBW. Accordingly, ambient heat load increased the maintenance coefficient of control and SANG supplemented ewes by 14.7 and 9.4%, respectively. It was previously reported (Plascencia and Zinn, 2014) that supplemental SANG increased supply of non-ammonia N to the small intestine, as a consequence of a decreased ruminal feed protein degradation and increased microbial protein synthesis in cattle that were fed a high energy finishing diet. Even when the effects of SANG have been not evaluated on ruminal microorganisms in vivo, the reduction on peptide degradation and amino acid deamination could be attributed to the selective antimicrobial effects of

SANG that could affect the high ammonia- producing bacteria, and/or by inhibition of aromatic amino acid decarboxylase (Drsta et al., 1996). While the increase on microbial protein synthesis could be a reflex of a decrease or the removing protozoa from the rumen by SANG supplementation. Therefore, decreases of the negative impact of high temperatures on energy efficiency may be also partially explained by improves on protein efficiency.

There were no treatment effects ($p \geq 0.12$) on carcass characteristics and chemical composition of shoulder (Table 4). Barajas et al. (2014) also did not observe an effect of supplemental SANG on carcass characteristics of finishing bulls. Likewise, supplemental SANG did not affect carcass characteristics of pigs (Boddington and McOrist, 2011) or breast muscle weight of broilers (Zduńczyk et al., 2010).

There were no supplemental SANG effects on weights (g/kg EBW) of stomach complex, intestines, and heart/lung (Table 5). However, SANG decreased liver weight (10.3%, $p=0.02$), and increased visceral fat (16.9%, $p=0.02$). To our knowledge, no information is available related to the effects of supplementation of SANG on visceral mass of ruminants. In a previous report (Jankowski et al., 2009), supplemental SANG decreased (11.1%) the liver mass in broilers. The basis for increased visceral fat with SANG supplementation is not clear. Previously (Plascencia and Zinn, 2014), supplemental SANG increased ruminal acetate molar concentration, and increased ruminal acetate may contribute to greater visceral fat deposition (Smith and Crouse, 1984).

Rumen epithelium of ewes fed SANG showed less inflammation than that of controls (Figure 1). The score for dropsical degeneration (2.08 vs 2.34,

p=0.02), parakeratosis (1.30 vs 1.82, p=0.03) and neutrophil infiltration (2.08 vs 2.86, p=0.05) were lower for SANG than for controls. Similarly, supplementation of QBA and PA mixtures improved gut health by increasing villi height, villi width, villi height:crypt-depth ratio and surface area of jejunum in broilers under conditions of high ambient heat load (Reansoi, 2002). Likewise, the anti-inflammatory effects of SANG have been reported previously (Kosina et al., 2010), and appears to be more pronounced in animals under conditions that contribute to greater risk of inflammation. Considering that the proportion of soluble sugars and fiber in basal diet (49.7 and 15.3%, respectively, Table 1) and the duration of the finishing period (70 days) the risk factor of presentation of subacute acidosis was high (NRC, 2007), and in ruminants, sub-acute acidosis is a primary cause of inflammatory processes in ruminal rumen epithelial tissue (Plaizier et al., 2012). Niewold (2007) suggested that the effects of growth promoters maybe mediated through anti-inflammatory mechanisms.According to Vieira et al. (2008), this opens a new window of opportunity in the search for additives to promote animal growth. Therefore, it is possible that the search for alternatives to growth promoters does not necessarily mean a search for substances with the same mode of action as antimicrobials.

It is concluded that SANG supplementation helped ameliorate negative effects of severe ambient heat load on dietary energetics of ewes fed a high-energy finishing diet, resulting in improved gain efficiency. This effect may have been mediated, in part, by anti-inflammatory effects of supplemental SANG and corresponding enhancement of nutrient uptake.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Table 1. Ingredients and composition of basal diet fed to ewe (% of dry matter)

Item	Diet composition (% DMB)
Ingredient	
Dry-rolled white corn	70.00
Soybean meal	8.00
Sudan grass hay	10.00
Molasses cane	7.50
Yellow grease	2.00
Trace mineral salt[1]	2.50
Chemical composition [b] (% DM basis)	
Crude protein	13.86
Ether extract	5.13
NDF	15.35
Starch	49.65
Calculated net energy (Mcal/kg of DM basis)	
Maintenance	2.05
Gain	1.38

[1] Mineral premix contained: CP, 50%; Calcium, 28%; Phosphorous, 0.55%; Magnesium, 0.58%; Potassium, 0.65%; NaCl, 15%; vitamin A, 1,100 IU/kg; vitamin E, 11 UI/kg; [b]Based on tabular net energy (NE) values for individual feed ingredients (NRC, 2007).

Table 2. Ambient temperature (Ta), relative humidity (RH) and calculated temperature-humidity index (THI) [1] registered every hour and expressed as a weekly average)

Week	Mean T_a, °C	Min T_a,°C	Max T_a, °C	Mean RH, %	Min RH, %	Max RH, %	Mean THI	Min THI	Max THI
1	33.10± 1.6	21.40±2.0	41.10± 1.0	48.2±8.8	16.0± 12.0	92.0± 10.9	82.2±8.3	64.50±2.6	104.2±4.3
2	31.80±1.4	20.89±1.0	39.51±1.3	49.60±3.4	24.29±7.9	92.43±3.0	80.80±2.8	64.89±0.7	101.62±2.7
3	31.28±1.2	21.50±0.9	39.26±0.7	51.48±7.4	27.89±3.6	79.29±17.3	80.42±6.9	65.79±0.7	97,91±4.9
4	29.90±1.5	23.10±0.9	37.14±0.8	69.14±7.7	41.34±10.1	93.40±5.0	81.33±6.6	68.71±1.4	97.73±1.3
5	31.99±2.0	23.93±0.5	38.61±0.6	58.74±5.9	38.10±3.6	91.57±5.1	82.65±4.6	69.41±0.4	99.85±1.0
6	32.03±1.9	24.81±1.6	38.51±1.5	59.15±7.2	35.81±4.9	84.43±6.5	82.77±6.1	70.23±2.2	97.96±3.3
7	33.14±0.9	23.69±1.4	39.06±1.6	51.49±5.1	32.60±3.9	87.31±3.8	82.89±4.9	68.61±1.8	99.57±3.2
8	30.16±1.6	23.84±0.9	37.44±1.8	60.76±9.9	36.73±3.7	87.36±2.8	81.52±0.8	69.18±0.8	96.86±3.3
9	30.24±1.3	24.57±1.1	36.69±1.3	65.19±6.5	40.87±5.8	88.74±1.8	81.22±5.5	70.46±1.2	95.89±2.2
10	29.76±1.9	20.93±1.1	30.83±2.2	65.18±7.8	35.87±5.5	77.64±1.6	80.52±6.4	65.69±1.6	84.13±3.6
Mean.	31.34±1.3	22.86±1.6	37.82±2.8	57.89±7.4	32.95±8.0	87.41±5.6	81.67±1.0	67.74±2.3	97.57±5.4

[1] THI = 0.81 × ambient temperature + [(relative humidity × (ambient temperature- 14.4)] + 46.4. THI code (Normal THI < 74;Alert 75-79; Danger 79-84, and Emergency > 84).

Table 3. Effects of SANGROVIT-RS supplementation (0.5 g/head/day) on growth-performance and dietary energy in ewes under hot environment fed with high-energy corn-based diet

Item	SANG intake, g/d		SEM[1]	P-value[2]
	0	0.5		
Days on feed	70	70		
Water intake (L/pen)	6.44	8.10	1.06	0.19
Body weight (kg)[3]				
Initial	38.19	38.14	0.02	0.31
Final	45.64	46.45	0.38	0.21
DM intake (g/d)	880	895	26	0.70
DM intake (% LW)	2.11	2.12	0.05	0.85
ADG (g/d)	107	119	6	0.20
Gain for feed	0.121	0.133	0.002	0.04
NE diet (Mcal/kg)				
Maintenance	1.87	1.96	0.01	<0.01
Gain	1.23	1.31	0.009	<0.01
Observed- to-expected NE				
Maintenance	0.91	0.96	0.005	<0.01
Gain	0.89	0.95	0.006	<0.01
Observed-to-expected DMI[4]	1.10	1.06	0.007	0.012

[1] SEM= standard error of mean.

[2] *P* = observed treatment effect.

[3] The initial BW was reduced by 4% to adjust for the gastrointestinal fill, and all ewes were fasted (food but not drinking water was withdrawing) for 18 h before recording the final BW.

[4]Observed-to-expected DMI is according to ADG and NE diet concentration.

Table 4. Effects of SANGROVIT-RS supplementation (0.5 g/head/day) on carcass characteristics and shoulder composition in ewes under hot environment fed with high-energy corn-based diet

	SANG intake, g/d			
Item	0	0.5	SEM[a]	P-value[b]
HCW (kg)	26.45	27.05	0.29	0.21
Dressing percentage	57.95	58.25	0.43	0.64
CCW (kg)	25.88	26.56	0.28	0.17
LM area (cm^2)	15.41	15.55	0.35	0.76
Fat thickness (mm)	5.71	5.32	0.30	0.41
KP fat (%)	2.40	2.76	0.34	0.50
Body wall thickness (mm)	20.3	23.1	0.79	0.06
Shoulder composition (g/kg)				
Muscle	615	612	0.96	0.83
Fat	192	205	14.2	0.56
Bone	182	178	5.2	0.29

[1] SEM= standard error of mean.

[2] *P* = observed treatment effect.

Table 5. Effects of SANGROVIT-RS supplementation (0.5 g/head/day) visceral organ mass in ewes under hot environment fed with high-energy corn-based diet

Item	SANG intake, g/d		SEM[1]	P-value[2]
	0	0.5		
Organ weight (g/kg EBW)				
Stomach complex	30.14	33.39	1.35	0.12
Intestines	48.03	49.60	2.20	0.62
Liver	17.05	15.29	0.44	0.02
Heart/ lungs	24.61	24.98	0.66	0.69
Visceral fat	40.20	48.42	2.17	0.02
Histological changes of ruminal epithelium[3]				
Dropsical degeneration	2.34	1.30	0.30	0.02
Neutrophil infiltration	2.86	2.08	0.25	0.05
Parakeratosis	1.82	1.30	0.15	0.03

[1] SEM= standard error of mean.

[2] *P* = observed treatment effect.

[3] Histological changes were coded as 1=normal, 2=moderated, and 3= intense.