



**UNIVERSIDAD AUTÓNOMA DEL ESTADO DE MÉXICO**

**DOCTORADO EN CIENCIAS AGROPECUARIAS Y RECURSOS  
NATURALES**

**EFFECTO DEL EXTRACTO DE *Salix babylonica* Y ENZIMAS  
EXÓGENAS FIBROLITICAS SOBRE LA RESPUESTA  
PRODUCTIVA Y EL ESTADO INMUNOLÓGICO EN BORREGOS  
SUFFOLK.**

**TESIS**

**QUE PARA OBTENER EL GRADO DE DOCTORA EN CIENCIAS  
AGROPECUARIAS Y RECURSOS NATURALES**

**PRESENTA**

**MVZ. M. en C. NALLELY RIVERO PEREZ**

**El Cerrillo Piedras Blancas, Toluca, Estado de México**

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## RESUMEN

El costo de los insumos usados en la producción pecuaria, los conceptos de limpio, verde y ético y la compleja interacción existente entre la nutrición y la respuesta inmune, ha llevado a investigadores y productores a buscar alternativas alimenticias que les permitan cubrir los requerimientos de la sociedad moderna, sin demeritar la capacidad productiva en sus unidades de producción. Por tal motivo constantemente se están evaluando aditivos alimenticios tales como las enzimas exógenas y los extractos derivados de arbóreas y arbustivas forrajeras motivo de la presente investigación la cual tuvo como objetivo evaluar el efecto de las enzimas exógenas, el extracto de *Salix babylonica* y su combinación sobre parámetros hematológicos, inmunológicos y productivos. Para dar cumplimiento a este objetivo se realizaron dos experimentos. En el primer experimento se utilizaron 20 borregos Suffolk divididos en cuatro grupos experimentales de acuerdo a los siguientes tratamientos: 1) Control, los animales consumieron solo la dieta basal (DB), 2) EZ, los animales consumieron DB +10g de enzimas exógenas (ZADO®), 3) SB, los animales consumieron DB + 30ml de extracto de *Salix babylonica* y 4) EZSB, los animales consumieron DB + 10g de enzimas exógenas+30ml de extracto de *Salix babylonica*. Los tratamientos se ofrecieron del día 1 al día 60 del experimento, por las mañanas antes de proporcionarles el alimento. Las enzimas se mezclaron con 200g de concentrado y se ofrecieron 20 minutos antes de ofrecerles el total de alimento por día, el extracto se proporcionó directo en la boca con una jeringa. Se colectó sangre periférica por vía yugular los días 0, 15, 30, 45 y 60 y con ella se realizaron pruebas bioquímicas, hemogramas y determinación de inmunofenotipaje por citometría de flujo de linfocitos T colaboradores, T citotóxicos, granulocitos y monocitos y se determinaron parámetros productivos como ganancia diaria de peso y consumo voluntario.

En el segundo experimento se utilizaron 4 borregos Suffolk y 4 cabras Saanen los cuales fueron asignados de forma aleatoria a uno de los siguientes tratamientos 1) Control, los animales consumieron DB + 30ml de agua destilada, 2) EZ, los

animales consumieron DB+ 1µl/g/MS ingerida de de xilanasa (Dyadic International Inc. Jupiter, FL.) diluida en 30ml de agua destilada, 3)SB, los animales consumieron DB+ 30ml de extracto de *Salix babylonica*, 4)EZSB, los animales consumieron DB+ 1µl/g/MS ingerida de enzimas exógenas diluida en 30ml de agua destilada+ 30ml de extracto de *Salix babylonica*. El experimento se dividió en cuatro periodos de 15 días y los tratamientos se ofrecieron del día 1 al 15 de cada periodo, por vía oral con una jeringa. Se colectaron muestras de sangre vía yugular los días 15 de cada periodo experimental con las cuales se realizaron pruebas bioquímicas y hemogramas.

En ambos experimentos se observó que los tratamientos no produjeron efectos ( $P>0.05$ ) en los parámetros hematológicos, inmunológicos y productivos (ganancia diaria de peso y consumo voluntario). Se concluyó que el extracto de *Salix babylonica*, el complejo enzimático ZADO® y la xilanasa utilizados en los presentes experimentos no causan efectos en los parámetros medidos. Las variables hematológicas reportadas en ambos experimentos apoyan el hecho de que no son perjudiciales para la salud animal y pueden ser utilizados con seguridad en condiciones de campo.

**Palabras clave:** enzimas exógenas, extracto de *Salix babylonica*, parámetros hematológicos, inmunológicos y productivos, borregos, cabras.

## ABSTRACT

In recent years the high cost of inputs used in livestock production, the concepts of clean, green and ethical and the complex interaction between nutrition and the immune response, has led both researchers and farmers to seek alternatives that will meet the expectatives of the modern society, without discrediting the productive capacity of their production units. Therefore, there has been a constant evaluation of food additives such as exogenous enzymes and extracts derived from tree and shrub foliage. In this regard, the present investigation aimed to evaluate the effect of exogenous enzymes and *Salix babylonica* extract as well as their combination on haematological, immunological and productive parameters. In order to fulfill this objective, two experiments were performed. In the first experiment, 20 Suffolk sheep were randomly assigned to four experimental groups according to the following treatments: 1) Control animals consuming the basal diet (BD), 2) EZ, animals consuming BD +10g of exogenous enzymes (ZADO ®), 3 ) SB, animals consuming BD + 30ml *Salix babylonica* extract and 4) EZSB, animals consuming BD + 10g of exogenous enzymes + 30ml *Salix babylonica* extract. Treatments were offered from day 1 to day 60 of the experiment, in the morning before the daily feeding. Enzymes were mixed with 200g of concentrate offered and 20 minutes before offering total food per day, the extract was provided directly in the mouth with a syringe. Peripheral blood was collected by jugular vein on days 0, 15, 30, 45 and 60 and therewith biochemical test, blood count and determining immunophenotyping by flow cytometry of helper T lymphocytes, cytotoxic T lymphocytes, granulocytes and monocytes. Productive parameters such as daily weight gain and voluntary feed intake were also determined.

In the second experiment four Suffolk sheep and 4 Saanen goats were randomly assigned to one of the following treatments 1) Control, animals consuming BD plus 30ml of distilled water, 2) EZ , animals consuming BD + 1µl/g/DM intake of xylanase (Dyadic International Inc. Jupiter, FL) in 30ml of distilled water, 3 ) SB ,

animals consuming BD + 30ml *Salix babylonica* extract, 4 ) EZSB, animals consuming BD + 1µl/g/DM intake of xylanase (Dyadic International Inc. Jupiter, FL) in 30ml of distilled water + 30ml *Salix babylonica* extract. The experiment was divided in four periods of 15 days, the treatments were offered from day 1 to 15 every period, orally with a syringe. Blood samples were collected via jugular on day 15 of each experimental period for further biochemical tests and blood count analyses.

In both experiments there were no significant effects of treatments ( $P>0.05$ ) on haematological, immunological and productive parameters (daily weight gain and voluntary intake). It is concluded that *Salix babylonica* extract, the exogenous enzyme ZADO® and xylanase used in the present experiments did not affect the measured parameters. The hematological variables reported herein support the fact that they are not deleterious on animal health and might be safely used in field conditions.

**Keywords:** exogenous enzymes, *Salix babylonica* extract, hematological, immunological and productive parameters sheep, goats.

## I.- INTRODUCCIÓN

A nivel mundial los costos por alimentación en la producción pecuaria suponen más del 50% de los costos totales, por lo que constantemente se están buscando alternativas alimenticias que permitan obtener una óptima respuesta productiva a menor costo. En esta búsqueda se ha acudido al uso de pre y probióticos, ionóforos, enzimas, e incluso arbustivas y arbóreas forrajeras (Salem et al., 2006, 2010; Chung et al., 2012), en algunos estudios se han observado que estas plantas forrajeras tienen efectos positivos no solo en parámetros productivos sino también en parámetros reproductivos y sanitarios (Waterman et al., 1980; Owusu-Asiedu et al., 2010; Nayak, 2010).

Las arbóreas y arbustivas forrajeras pueden producir efectos positivos y negativos en la producción pecuaria, ya que además de ser fuentes de proteína, energía y fibra también son fuentes de metabolitos secundarios producidos por la propia planta como defensa frente a los depredadores y a algunos patógenos (Barahona et al., 1997; Provenza et al., 2000).

Los metabolitos secundarios pueden mejorar algunos parámetros productivos en rumiantes, además de ejercer un efecto antiparasitario (taninos y saponinas), anticancerígeno antioxidante e inmunoestimulante (compuestos fenólicos, saponinas alcaloides y terpenos). La presencia o ausencia de estos efectos dependerá del tipo de compuesto secundario y la cantidad y la frecuencia con la que se consuman ya que al sobrepasar las dosis óptimas pueden causar alteraciones a nivel sanguíneo por su acción hemolítica (saponinas), daño renal, hepático y neurológico e incluso causar la muerte (Waterman et al., 1980; Reed, 1995; Athanasiadou and Kyriazakis, 2004).

Las enzimas son catalizadores orgánicos que permiten optimizar el uso de los nutrientes presentes en la dieta ya que desdoblan los sustratos y los hacen más

disponibles para el proceso digestivo, estas se usan principalmente en aves y cerdos; sin embargo, su uso en rumiantes es cada vez es más frecuente sobre todo de las enzimas exógenas fibrolíticas por su tipo de alimentación (Bedford and Partridge, 2010).

Las ventajas principales del uso de enzimas exógenas fibrolíticas en la nutrición de rumiantes son favorecer la digestión de la fibra (polisacáridos no amiláceos), lo que permite reducir el uso de granos debido al mayor aporte de energía que realizan los sustratos fibrosos, mejoran el valor energético de los cereales lo que supone un ahorro económico, reduce la excreción y las pérdidas de ciertos compuestos como el fósforo y el nitrógeno, y su liberación al medio ambiente, previenen alteraciones digestivas y diarreas. Lo anterior resulta en una mejora en parámetros productivos, reproductivos, sanitarios e inmunológicos dado el mejor aprovechamiento de nutrientes en cantidad y calidad, ya que el animal tiene a su disposición todos los sustratos que requiere para cumplir con estas funciones, bajo el entendido de que un animal bien alimentado es un animal inmunocompetente y por lo tanto productivo (Keith and Jeejeebhoy, 1997; Bedford and Partridge, 2010).

Los efectos del uso de enzimas exógenas fibrolíticas (ZADO® y xilanasa Dyadic International Inc Jupiter, FL) (Salem et al., 2011a; Arriola et al., 2011; Salem et al., 2013) así como de los compuestos secundarios presentes en el extracto de *Salix babylonica* (Salem et al., 2011b) sobre respuesta productiva en rumiantes ya han sido documentados; sin embargo los efectos que estos aditivos alimenticios puedan ejercer sobre ciertos parámetros hematológicos indicadores del estado de salud de los animales no han sido evaluados siendo que existen evidencias de que ambos pueden producir efectos positivos como negativos sobre dichos parámetros (Chandra, 1997; Turner et al., 2005; Mahgoub et al., 2008a; Ramirez-Restrepo et al., 2010; Velizadeh et al., 2010; Vasta and Luciano, 2011). Además de ejercer una acción inmunomoduladora que puede ser directa o indirecta (Keith and Jeejeebhoy, 1997; Provenza and Villalba, 2010).

## II. REVISIÓN DE LITERATURA

El uso del follaje de arbustos y árboles en la alimentación de los rumiantes es una alternativa para el desarrollo de una producción animal sostenible; sin embargo, en ocasiones el follaje contiene metabolitos secundarios con actividad anti-nutricional, que actúan como mecanismos de defensa contra microorganismos, insectos y depredadores principalmente taninos (Camacho et al., 2010a). No obstante, algunos de ellos pueden ser benéficos para los animales.

### 2.1. Arbóreas forrajeras

Un gran número de árboles son forrajeros y ramoneables, por lo que constituyen un recurso alimenticio importante para los rumiantes debido a su alto contenido de proteína cruda y minerales (calcio y fósforo), además de que son un complemento en la temporada de secas y mejoran la cantidad y la calidad de los pastos (Camacho et al., 2010b).

Hasta hace poco, los árboles como recurso alimenticio habían sido ignorados por científicos debido al conocimiento inadecuado de su uso potencial y a la carencia de iniciativa para desarrollar sistemas alimenticios más innovadores actualmente los sistemas de producción agropecuarios han retomado como objetivo alcanzar una comunidad estable, con varios estratos de plantas productoras de follaje y/o frutos con valor nutritivo complementario a los monocultivos (Olivares Pérez et al., 2005).

Los requisitos para que un árbol sea clasificado como forrajero son: 1) Que su consumo por los animales sea adecuado como para esperar cambios en los parámetros de respuesta; 2) Que el contenido de nutrientes, sea atractivo para la producción animal; 3) Que sea tolerante a la poda 4) Que el rebrote sea lo suficientemente vigoroso, para obtener niveles significativos de producción de biomasa comestible, por unidad de área; y 5) Que los niveles de metabolitos

secundarios no afecten el consumo, parámetros productivos y la salud del animal (Sosa et al., 2004).

Existen muchas especies con potencial forrajero, entre las que se destacan las integrantes de la familia Leguminosae por su excelente producción de biomasa en el periodo seco, los altos contenidos de proteína y su naturaleza multipropósito (Camacho et al., 2010b). Asimismo, en América Continental y el Caribe, algunas de las leguminosas forrajeras de mayor importancia lo constituyen las especies pertenecientes a los géneros Albizia, Cassia, Pithecellobium, Leucaena, Lysiloma y Enterolobium, las cuales son excelentes fuentes de fibra, energía proteína y algunos minerales (Aregheore and Perera, 2004; Aregheore et al., 2006).

El valor nutritivo de arbóreas y arbustivas varía en los diferentes componentes de la biomasa arbórea. Las hojas presentan mayores concentraciones de nutrientes que las ramas y los tallos, la variación también se ha relacionado con la edad y con la posición en el árbol, siendo las hojas jóvenes más ricas en proteínas que las viejas y estas presentan porcentajes de digestibilidad bajos, debido a las concentraciones mayores de lignina y posiblemente de factores anti-nutricionales como los taninos (Carmona Agudelo, 2007).

Debido a las perspectivas y bondades de estas plantas para la ganadería, se precisa conocer las particularidades esenciales de su composición fitoquímica, el valor nutritivo y su repercusión en la aceptabilidad, por parte de los rumiantes, para poder establecer las principales ventajas y limitaciones en el uso de cada planta (García and Medina, 2006).

## **2.2. Metabolitos secundarios**

A raíz del déficit alimentario y la crisis económica mundial, los países latinoamericanos han tenido que incursionar en otras estrategias de alimentación para incrementar la producción animal en las condiciones tropicales;

específicamente ofertándole al ganado una mayor cantidad de proteína y minerales que, en sentido general, se encuentran de forma deficitaria en los pastos. En este sentido, el follaje de los árboles ha tenido un papel protagónico por sus considerables niveles de proteína (bancos de proteína) y aceptable valor nutritivo (Aregheore and Perera, 2004; García and Medina, 2006; García et al., 2008).

Sin embargo, la gran mayoría de especies vegetales presentan metabolitos secundarios, como taninos, saponinas, compuestos fenólicos y alcaloides, que pueden tener un efecto negativo sobre el valor nutricional de estos forrajes (Salem et al., 2010). Estas sustancias son elaboradas por la planta y cumplen funciones como la defensa contra herbívoros, hongos, bacterias y virus, la protección contra la radiación ultravioleta, sirven además como mecanismo para evitar la deshidratación de sus tejidos y protegen a la planta de factores bióticos y abióticos que le puedan producir stress (Zhao et al., 2005; Salem et al., 2006; Augustin et al., 2011). La presencia de estos componentes confiere a las plantas un sabor desagradable, que en muchos casos genera baja aceptabilidad (disminuye consumo voluntario) para su consumo por parte de los animales herbívoros y hace que se disminuya la presión de pastoreo (Salem et al., 2006, Varón and Granados, 2010).

El término de metabolitos secundarios engloba sustancias químicamente muy diversas y se establece como contraposición a los productos del metabolismo primario, que aparecen en el citoplasma de todas las células vegetales y cuyas diferencias entre plantas son únicamente de índole cuantitativo (Augustin et al., 2011). En este sentido, se ha propuesto denominar a los metabolitos secundarios sustancias ecológicamente eficaces, frente a los compuestos primarios que serían sustancias fisiológicamente eficaces.

Hasta 2007 se habían reportado cerca de 8000 polifenoles, 270 aminoácidos no proteicos, 32 cianógenos, 10,000 alcaloides y varias saponinas y esteroides (Carmona Agudelo, 2007).

Los metabolitos secundarios han sido durante mucho tiempo ignorados en los trabajos de nutrición. No obstante, su carácter claramente ventajoso para la planta y, en muchos casos, en obvio detrimento de los herbívoros, ha conducido a que actualmente se valore su incidencia en la producción agroganadera, especialmente en aquellos sistemas basados en el aprovechamiento de pastos (Ramos et al., 1998).

En las plantas existe una enorme diversidad bioquímica de metabolitos secundarios, fruto, tal vez, de su imposibilidad de huida frente a las agresiones (Cheeke, 1994). Ahora bien, debido al elevado costo energético que implica su síntesis, las plantas encauzan su metabolismo hacia un tipo u otro de compuesto secundario dependiendo de los recursos disponibles (Bryant et al., 1992). Frente a condiciones severas, como las que se dan en climas áridos, las plantas tienden a aumentar sus defensas, sobre todo de tipo cualitativo (alcaloides, glicósidos cianogenéticos, etc.), ya que, en estos casos, les resulta mucho más difícil regenerar los tejidos dañados por los herbívoros (Johnson et al., 1985; Lundberg and Palo, 1993). Esto resulta en una intoxicación más fácil del animal en estas condiciones, siendo de especial incidencia en el ganado doméstico cuando su manejo en pastoreo no es el más adecuado, o cuando se enfrenta a la escasez de otros recursos vegetales (James et al., 1992).

### **2.3. Adaptación a los metabolitos secundarios en rumiantes**

Los herbívoros han desarrollado algunas adaptaciones fisiológicas y etológicas que les ha permitido reducir el efecto nocivo de las metabolitos secundarios (Provenza et al., 1990).

A modo de ejemplo, numerosas especies basan su alimentación en plantas con un elevado contenido en taninos: arbustos, árboles, etc., y prácticamente en todas ellas, se ha observado en la saliva la presencia de proteínas ricas en el aminoácido prolina que tiene gran afinidad con los taninos con los que forma un complejo soluble (Robbins et al., 1987).

Hasta hace algunos años se pensaba que los animales que consumían plantas con altos contenidos de taninos sufrían un proceso de adaptación a nivel de glándulas parótidas que consistía en secretar saliva con altos contenidos de prolina que protegía a los animales de los efectos negativos de los taninos en el proceso digestivo; sin embargo, Robbins et al.(1987) y Ammar et al.(2011) realizaron estudios para determinar si la ingestión de plantas con mayores contenidos de taninos (quebracho) en ovejas, puede causar cambios en la saliva secretada, que mejore la digestión *in vitro* de arbustos ricos en taninos, en el cual se demostró que la saliva de animales alimentados con plantas taniníferas no producen más proteínas de las que se producen normalmente por lo que no protegen a los animales de los efectos negativos de los taninos durante el proceso de digestión (Ammar et al., 2011).

Es probable que el mecanismo primario por el que los rumiantes pueden tolerar altos contenidos de taninos en la ración sea mediante la adaptación de los microorganismos ruminales y su capacidad de detoxificar estos compuestos siendo, por ello, los rumiantes menos susceptibles que el resto de herbívoros a los efectos perjudiciales de los taninos (más las cabras que los borregos y las vacas) aunque la detoxificación puede tener lugar en el intestino delgado, riñón e hígado (Ramos et al., 1998).

Los herbívoros seleccionan su dieta entre una amplia variedad de especies vegetales, tratando, por una parte, de cubrir sus necesidades nutritivas y, por otra, de evitar la ingestión de compuestos tóxicos. Dicha selección está basada en un proceso de aprendizaje tipo prueba/error. Parece claro que los animales son

capaces de asociar el sabor de un alimento con las consecuencias negativas postprandiales, pudiendo esta asociación conducir a una aversión condicionada hacia el alimento (Provenza, 1995).

Los herbívoros posiblemente adquieren aversiones a corto plazo repetidas veces a lo largo de su vida, como consecuencia de su comportamiento ingestivo, basado en el muestreo. Evidentemente, las aversiones a largo plazo podrían jugar un papel fundamental cuando el alimento es muy tóxico. Sin embargo, cuando la toxicidad no es muy alta, las aversiones se mantienen durante periodos cortos. Esto permite a los rumiantes un re-aprendizaje sobre qué plantas, o partes de éstas, pueden seleccionar en un momento determinado, teniendo en cuenta que sus consecuencias negativas pueden ser paliadas con el tiempo gracias a la adaptación fisiológica de los animales a los metabolitos secundarios de sus dietas (Ramos et al., 1998).

Finalmente, hay que precisar que los animales salvajes disponen de mejores adaptaciones a las toxinas vegetales que los animales domésticos, ya que, para éstos, el hombre ha transformado grandes extensiones de territorio en praderas aptas para su alimentación, e impedido, mediante riego, siega, pastoreo y abonado, la proliferación de plantas tóxicas. No es casual, por tanto, que los mayores problemas de rendimiento e intoxicación del ganado ocurran en pastos no modificados por el hombre y en regiones áridas debido a las particulares adaptaciones de las plantas al estrés ambiental (Ramos et al., 1998).

#### **2.4. La relación entre la respuesta productiva y el estado inmunológico**

El sistema inmunitario actúa para proteger al huésped frente a los agentes extraños o antígenos (bacterias, virus, hongos, parásitos, células malignas) que puedan invadir el organismo. El funcionamiento adecuado de este sistema de defensa está condicionado por la nutrición así como por el estado nutricional del individuo. La malnutrición debida a la ingesta deficitaria de energía y

macronutrientes y/o deficiencias de micronutrientes específicos puede dañar el sistema inmunitario, suprimiendo sus funciones que son fundamentales para la protección del huésped; estas deficiencias afectan tanto la respuesta inmune innata como la adquirida. Y están asociadas a un mayor riesgo de infecciones, que a su vez, producen cambios que deterioran el estado nutricional y por consiguiente la respuesta productiva (Chandra, 2002; Calder and Kew, 2002; Maggini et al., 2007; Wintergerst et al., 2007).

Hay interacciones, sinergismos y antagonismos extremadamente importantes entre nutrición e inmunidad que afectan marcadamente la productividad. Existen dos clases de interacciones. En primer lugar, la nutrición puede influir en la inmunocompetencia y de ahí en su resistencia a las enfermedades. En segundo lugar, las respuestas del sistema inmune a los procesos infecciosos afectan al crecimiento, metabolismo y necesidades de nutrientes. Además, los organismos patógenos pueden reducir la absorción de nutrientes. Estas dos importantes interacciones pueden estar relacionadas entre sí, resultando un ciclo malnutrición-infección.

La malnutrición implica un incremento de la incidencia, duración y mortalidad ligada a las enfermedades infecciosas, y las infecciones causan anorexia y malnutrición. Independientemente de que el ciclo comience por una subalimentación o por un control inadecuado de las enfermedades, tanto el sistema inmune como el status nutritivo pueden deteriorarse simultáneamente dando lugar a una mayor probabilidad de infecciones y a una baja productividad (Calder and Kew, 2002; Maggini et al., 2007).

Numerosos estudios han demostrado que deficiencias severas o crónicas de muchos nutrientes reducen la respuesta inmune. Esto no es sorprendente si se tiene en cuenta la elevada velocidad de división de las células y el gran número de cofactores enzimáticos que son necesarios para que se produzca la respuesta inmune (Calder and Kew, 2002). Afortunadamente, las deficiencias severas de

nutrientes son raras en la producción animal moderna como consecuencia de la formulación científica de las dietas, ya que a partir de que se estableció la relación entre nutrición e inmunidad se comenzaron a formular dietas que basadas en ingredientes selectivos en la cantidad requerida (Chandra, 1997).

Desde un punto de vista práctico, algunos nutrientes pueden modular la respuesta inmune tal es el caso del zinc, cobre, selenio, hierro, las vitaminas A, C, E y B6, además del ácido fólico, los cuales son fundamentales para el mantenimiento de la inmunocompetencia (Chandra, 1997; Calder and Kew, 2002; Chandra, 2002; Maggini et al., 2007; Wintergerst et al., 2007).

Además hay estudios que evidencian la importancia de los lípidos, energía y proteína sobre la modulación de la respuesta inmune, en el caso de los lípidos estos son de importancia crucial ya que los ácidos grasos forman parte de la estructura de los linfocitos y otras células del sistema inmune, mientras que la deficiencias de energía y proteína incrementan la frecuencia y severidad de las infecciones, causan atrofia del timo, depleción linfocitaria disminuyen la proliferación de células T (CD4+ Y CD8+) y debilitan a los nódulos linfáticos periféricos (Savino, 2002, Marcos et al., 2003, Lesourd, 2006).

## **2.5. Efecto de los metabolitos secundarios sobre la respuesta productiva**

Los metabolitos secundarios pueden disminuir las concentraciones de amonio, alterar la proporción acetato: propionato en el fluido ruminal, disminuir la producción de metano, estimular el metabolismo ruminal con lo cual incrementan la degradabilidad de la proteína cruda y de la pared celular, etc. (Jiménez-Peralta et al., 2011), Además disminuyen la producción de nitrógeno amoniacal reducen el olor de las heces, como ocurre con las saponinas presentes en dos plantas tropicales *Yucca schidigera* en México y *Quillaja saponaria* de origen chileno (Cheeke, 2000, Singer et al., 2008).

Se ha reportado que el valor nutritivo de los forrajes está determinado por su composición química. Pero el valor nutritivo *per se* está influenciado por un gran número de factores que pueden afectar la eficiencia con la cual los rumiantes utilizan estos forrajes. Consecuentemente, se ha reportado que el valor nutritivo depende no solo de la digestibilidad del forraje sino también del consumo voluntario. Este se ve influenciado por la palatabilidad, variación estacional y disponibilidad. Un aspecto que afecta el consumo y la digestibilidad de los componentes de los forrajes, y que es muy marcado en las especies leguminosas tropicales es la presencia de metabolitos secundarios, la presencia de algunos de estos factores pueden alterar la utilización de los nutrientes, la conversión alimenticia y por ende la productividad de los animales (Carmona Agudelo, 2007).

Son tal vez los taninos: compuestos fenólicos de alto peso molecular, los compuestos que revisten una mayor importancia, no solo por su diversidad y concentración, sino también por sus múltiples efectos en la dinámica digestiva de los animales se sabe que el efecto en el animal va a depender de su concentración en la planta y del nivel de suministro del forraje que los contiene, en la dieta del animal (Carmona Agudelo, 2007). Se ha observado que altas concentraciones de taninos en la dieta disminuyen el consumo voluntario así como la digestión de la fibra y la proteína, mientras que a bajos niveles favorece la absorción de nitrógeno a nivel de intestino delgado reduciendo su degradación a nivel ruminal (Barahona et al., 1997; Provenza et al., 2000). Incluso se ha sugerido que la utilización de plantas forrajeras con niveles altos de taninos, mezcladas con especies altas en nitrógeno soluble, con la finalidad de mejorar el uso del nitrógeno por los rumiantes, reduciendo la degradación de proteína soluble en rumen y diluyendo el efecto de los compuestos tóxicos.

La afinidad de los taninos por las proteínas depende de sus características químicas y de las condiciones fisicoquímicas del sistema. En base a su origen químico los taninos se han dividido en dos grupo: hidrolizables y condensados (Reed, 1995).

Los complejos formados por los taninos son estables e insolubles a un pH entre 3 y 7, pero son liberados a un pH menor a 3 o mayor a 8. Esta propiedad para ligar las proteínas a un pH neutro y liberarlas a valores de pH bajo, ha llevado a muchos investigadores a pensar que podría ser una herramienta útil para reducir la degradación de la proteína en el rumen, siendo posible así incrementar, el flujo y la absorción de N amoniacal y aminoácidos esenciales en el intestino delgado (Reed, 1995, Jackson et al., 1996; Barahona et al., 1997).

Desde hace algunos años se ha intentado reducir el nivel de taninos principalmente condensados con el uso de polietilenglicol ya que se estos presentan mayor afinidad por este compuesto que por las proteínas por lo que al unirse a este compuesto reducen sus efectos tóxicos además de no afectar notablemente el valor nutritivo de los forrajes (Provenza et al., 2000, Salem et al., 2007). Barahona et al. (1997) cita que cuando el nivel de taninos condensados, presentes en las plantas (leguminosas), se reduce por diferentes métodos, se observa un incremento en la degradación de la proteína en el rumen y consecuentemente una disminución en el N que alcanza el duodeno. Además un incremento en el consumo y la digestibilidad de la fibra (Barahona et al., 1997).

También se han encontrado efectos negativos sobre la digestibilidad de los carbohidratos estructurales y solubles (Carmona Agudelo, 2007); además, reducen la producción de subproductos energéticos de la fermentación microbiana, como los ácidos grasos volátiles (Provenza et al., 2000).

Así mismo se sabe que la reducción del consumo voluntario de forrajes ricos en taninos se debe a que estos se unen a las proteínas de la saliva, ocasionando el efecto de astringencia. La interacción con los componentes proteicos parecen tener efecto inhibitorio sobre activadores de algunas enzimas tanto aminolíticas como lipídicas y peptídicas.

Otro compuesto secundario que produce grandes efectos en la dinámica digestiva de los rumiantes, es la mimosina, alcaloide encontrado en *Leucaena leucocephala* en una proporción que oscila entre un 3 – 5% el cual puede ser incluido en dietas tanto de monogástricos (5 – 10%) como rumiantes (más del 30%) sin presentar efectos tóxicos ya que al ser un aminoácido no proteico resulta sumamente toxico a niveles mayores a los anteriormente misionados tanto en no rumiantes como en rumiantes no adaptados a su consumo (Hammond, 1995).

En rumiantes con previo periodo de adaptación al consumo de mimosina, este compuesto es metabolizado por los microorganismos del rumen a 3-hydroxy-4(1H)-pyridon (3-4 DPH) a pesar de los altos niveles de mimosina en estos forrajes, su presencia en las heces es muy baja, debido a que tanto la mimosina como el DHP (metabolito producido a nivel ruminal, derivado de la mimosina) son rápidamente absorbidos. Pero su excreción urinaria es rápida, lo cual permite explicar porque los efectos tóxicos en los rumiantes no son tan frecuentes, además se sabe que existen bacterias ruminales capaces de degradar y utilizar esta sustancia, cuando su concentración en la dieta total no es muy elevada (Hammond, 1995, Carmona Agudelo, 2007).

Otro metabolito secundario presente en algunas arbóreas y arbustivas forrajeras tropicales son las saponinas, las cuales también tienen efectos marcados en la nutrición animal (Francis et al., 2002). Además de tener propiedades farmacológicas importantes. Las saponinas del latín sapo = jabón, son detergentes naturales (sulfactantes) ya que tienen un componente soluble en agua y otro soluble en grasa normalmente se unen a azúcares conjugados de triterpenoides o esteroides formando espumas estables al entrar en contacto con el agua, confiriéndole potencialidad como factor timpánico (Cheeke, 2000: Carmona Agudelo, 2007; Augustin et al., 2011).

Las saponinas tienen un característico sabor amargo. Este aspecto, además de su efecto irritante a nivel de la boca y garganta le confieren una baja palatabilidad, lo

cual redundando en un bajo consumo voluntario. Además, estas forman complejos insolubles con algunos minerales como Ca, Fe, y Zn, haciéndolos no disponibles para el animal, estas sustancias pueden tener efectos negativos sobre las bacterias celulolíticas, pero los rumiantes en pastoreo pueden adaptarse a ellas y detoxificarlas (Cheeke, 2000).

Los anteriores conceptos señalan a las saponinas como metabolitos secundarios generalmente con efectos negativos en la alimentación animal, pero diversos trabajos reportan sus bondades como sustancias defaunantes a nivel ruminal, en otras palabras, disminuyen la cantidad de protozoarios, obteniéndose importantes beneficios nutricionales (Cheeke, 2000, Singer et al., 2008).

Cuando el rumen es defaunado, la degradabilidad de proteínas del alimento es más bajo que en el rumen sin defaunar, sobre todo cuando las fuentes de proteína insoluble predominan. Así, cuando el rumen es defaunado puede esperarse que mayor cantidad de proteína escape de la digestión y fluya al intestino. Esto se considera un efecto positivo teniendo en cuenta, que frecuentemente en los sistemas de alimentación de nuestro medio, el suministro de proteína al intestino delgado es deficiente. El impacto positivo que esto pueda representar depende de la calidad de la proteína que escapa a la degradación ruminal, hacia el tracto posterior (Carmona Agudelo, 2007).

La actividad defaunadora atribuida a las saponinas se justifica debido a su actividad citotóxica sobre los mismos; en pruebas *in vitro*, se ha determinado que con niveles de saponinas de 1,2 g/L su efecto defaunador se puede observar sobre el 100% sobre los Holotrichos y 95% sobre los Entodinoformas, con un aumento en la digestibilidad de la materia seca (Cheeke, 2000).

En un estudio realizado por Navas et al.(1992), concluyó que el efecto de *Enterolobium cyclocarpum* y *Sapindus saponaria* como defaunantes se debe a la presencia de sustancias detergentes, como las saponinas en sus hojas. En

estudios con ovejas, la administración de *Enterolobium cyclocarpum* promovió una mayor disminución de protozoos (43% menos) comparado con aquellas ovejas que no recibieron este forraje en su dieta (Navas et al., 1992).

Así mismo, los más de 25 tipos glucósidos cianogénicos representan otro grupo de compuesto secundario el cual está presente en más de 2000 plantas, estos compuestos a nivel ruminal por acción de una glicosidasa de origen ruminal o vegetal liberan la agliocona que por acción enzimática se transforma en ácido cianhídrico el cual es absorbido y puede causar serios problemas de toxicidad, este tipo de metabolito limita el consumo voluntario la digestibilidad de la proteína y la disponibilidad de metionina. En ausencia de hidrólisis, los glucósidos son relativamente inocuos ya que pueden ser excretados como tal (Roige and Tapia, 1996). Así mismo, se ha señalado que a pesar de su potencial tóxico, es poco el efecto que se da en bacterias anaeróbicas del rumen, las cuales pueden utilizarlo incluso como fuentes de N y el solo secado al sol reduce estas sustancias.

Se han realizado estudios para disminuir el contenido de glucósidos cianogénicos que van desde secado hasta ensilado, en los que se indica que al parecer a partir de la sexta semana del período de ensilaje, el nivel de esta sustancia tóxica disminuye a un nivel que no es tóxico para los animales, incluso para los monogástricos los cuales al tener pH ácido en el estómago inhiben la acción de la glicosidasa. En el caso de los rumiantes, el proceso de digestión fermentativa en el rumen neutraliza el efecto del ácido cianhídrico y no se reportan problemas aun suministrando el follaje en forma fresca (Preston et al., 2005).

Finalmente Jiménez et al. (2011) reportó efectos positivos de los metabolitos secundarios presentes en dos arbóreas forrajeras *Leucaena leucocephala* y *Salix babylonica* (SB) sobre la producción de gas *in vitro* observándose mayor producción de gas en aquellos animales tratados con *Salix babylonica*, este extracto además mejoro los parámetros de fermentación ruminal (Jiménez-Peralta et al., 2011).

## **2.6. Efecto de los metabolitos secundarios sobre el estado de salud de los animales**

Los metabolitos secundarios presentes en las plantas son fuentes únicas de productos farmacéuticos, cosméticos, aditivos alimenticios, saborizantes y otros materiales industriales, por lo que su uso se ha extendido alrededor de todo el mundo e incluso se han hecho cultivos celulares de plantas para poder obtener los metabolitos secundarios que no se pueden sintetizar de forma química o cuya extracción es muy difícil (Cheeke, 2000; Zhao et al., 2005; Augustin et al., 2011).

En estudios recientes se ha reportado que los metabolitos secundarios presentes en la mayoría de las plantas forrajeras no solo tiene efectos a nivel productivo, sino que también ejercen cierta influencia a nivel salud ya que se ha visto que son muchas las plantas que poseen metabolitos secundarios que tienen efectos bactericidas o bacteriostáticos (Waterman et al., 1980).

Por otro lado, las plantas ricas en taninos han atraído la mayor atención por su efecto sobre los nematodos gastrointestinales de los rumiantes. Las plantas taniníferas pueden tener una actividad antiparasitaria directa pero también podrían tener un efecto indirecto a través de mejorar la respuesta inmune de los animales contra los nematodos gastrointestinales (Hoste et al., 2005). Hay reportes de que los taninos pueden mejorar la resiliencia (menos signos clínicos, mejor crecimiento y producción de lana) y resistencia (menor cantidad de huevos de nematodos en heces, menor carga parasitaria y menor fertilidad de hembras parásitas) de los caprinos y ovinos infectados con nematodos gastrointestinales (Torres-Acosta et al., 2008).

La propiedad antiparasitaria depende de la estructura del compuesto secundario, del nivel de ingestión y de su disponibilidad en el tracto gastrointestinal de los animales (Athanasidou and Kyriazakis, 2004).

Aparentemente el mecanismo de acción de los taninos sobre las larvas infectantes (L3) de *H. contortus* y *T. colubriformis* consiste en evitar que estos parásitos desenvainen. Esto evita que los nematodos gastrointestinales puedan establecerse en su sitio de acción y puedan continuar con su ciclo evolutivo, mientras que en los parásitos adultos los taninos aparentemente se unen a la boca y posiblemente al aparato reproductor de los parásitos (por la afinidad de los taninos a las proteínas ricas en prolina de la cutícula del nematodo); sin embargo, se deberá determinar qué cantidad se requiere para ocasionar el efecto antihelmíntico pero sin perjuicio para el animal o para el consumidor de dicho animal (Torres-Acosta et al., 2008).

Además de su propiedad antiparasitaria también se ha visto que los polifenoles pueden ser considerados antioxidantes naturales, disminuyen el riesgo de enfermedades cardiovasculares, poseen propiedades antimutagénicas y antigenóticas e incluso se dice que los flavonoides previenen alteraciones genéticas producidas por mutágenos químicos, esto en modelos animales de tal modo que pueden ser considerados como anti-cancerígenos naturales (Akond et al., 2011; Veloz-García et al., 2004).

Otro compuesto secundario que parece tener efectos directos sobre la salud animal y la respuesta inmune del mismo son las saponinas detergentes naturales en plantas tales como *Yucca schidigera*. Las saponinas tienen propiedades antimicrobianas, fungicidas, insecticidas, molusquicidas, anticancerígena, antiinflamatoria y alelopáticas (Augustin et al., 2011).

Sobre los protozoarios las saponinas ejercen una actividad membranolítica, al formarse un complejo entre saponina – colesterol de la membrana celular del protozoario causando la lisis celular. Además poseen una actividad antibacteriana y pueden modificar la fermentación ruminal suprimiendo protozoarios e inhibiendo selectivamente bacterias ruminales e intestinales (*Lactobacillus plantarum* y *Enterococcus faecium* en borregos) (Salem et al., 2010); estas propiedades han

sido explotadas sobre todo a nivel salud humana y animal contra Giardiadisis, Leishmaniasis y Coccidiosis.

Por otro lado, se ha reportado que las saponinas favorecen la absorción de los lípidos, mediante la formación de micelas con las sales biliares y colesterol en el intestino, reduce la absorción de ácidos grasos al secuestrar las ácidos biliares importantes para la formación de micelios y absorción de grasas (Cheeke, 2000). También se conoce que el consumo excesivo de los metabolitos secundarios puede afectar el bienestar y la salud del animal e incluso causar la muerte, dependiendo del tipo de compuesto de secundario y de la cantidad consumida del mismo (Roige and Tapia, 1996). Por ejemplo, las cumarinas pueden ser hepatotóxicos además de ejercer un efecto hemorrágico atribuido a su actividad específica anti-vitamina K (Carmona Agudelo, 2007).

Los taninos condensados disminuyen la absorción intestinal al causar toxicidad a la mucosa que lo reviste además de causar necrosis renal, pancreática y de piel (Reed, 1995). Las saponinas (terpenoides) son considerados responsables de una disminución en el consumo, causando carencias alimenticias, hemólisis y hasta la muerte (Athanasiadou and Kyriazakis, 2004; Sun et al., 2009).

El consumo de plantas conteniendo simultáneamente glucósidos cianogénicos, terpenos o alcaloides puede provocar problemas neurológicos, los taninos disminuyen el consumo voluntario, reducen la digestibilidad y producen un funcionamiento inadecuado del rumen. Se ha observado que los taninos hidrolizados son potencialmente tóxicos, afectan hígado y riñones lo que puede resultar en muerte.

El consumo excesivo de alcaloides, glicósidos y terpenoides ocasionan lesiones en el sistema nervioso central (Athanasiadou and Kyriazakis, 2004). Reed (2001) señala que las proantocianidinas, presentes en algunas leguminosas tropicales no son absorbidas pero pueden afectar la mucosa del tracto digestivo, disminuyendo

la absorción de nutrientes (principalmente aminoácidos esenciales, tipo metionina y lisina) (Reed, 1995).

En estudios realizados tanto en monogástricos como en rumiantes se ha observado que los metabolitos secundarios presentes en su dieta por periodos de tiempo más o menos prolongados, afectan de manera negativa las células blancas y rojas de la sangre, lo que disminuye sus concentraciones y causa en el animal anemias, inmunodeficiencias e incluso la muerte (Adedapo et al., 2005; 2007b; Mahgoub et al., 2008b).

Se ha establecido que el efecto de los metabolitos secundarios sobre los linfocitos es indirecto causando anemia no regenerativa o hipoproliferativa-, que es un trastorno de células madre caracterizado por una producción reducida en médula ósea de todos los componentes de la sangre o la supresión de la hematopoyesis, por lo que se considera que este proceso tiene un efecto negativo sobre los neutrófilos, linfocitos, eosinófilos, y monocitos (Adedapo et al., 2007b).

## **2.7. Efecto de los metabolitos secundarios sobre la respuesta inmune**

Otro punto de importancia con respecto a las saponinas es que dichos compuestos tienen efecto directo sobre el sistema inmune ya que producen una gran variedad de antígenos específicos además de estimular la respuesta inmune no específica (Friedrich, 1997; Cheeke, 2000; Weng et al., 2010). Se ha propuesto que las saponinas pueden ejercer un importante efecto anti-cancerígeno, los mecanismos propuestos para explicar esta propiedad son: su efecto citotoxicidad directa y su efecto inmunomodulador estimulando a las células T (colaboradores y citotóxico), B y NK y la producción de interferón aunando a esto estimulan la proliferación celular tanto en bazo como en nódulos linfáticos (Wu et al., 1990; Rao and Sung, 1995).

En general, las saponinas estimulan la respuesta inmune tanto celular como humoral (Athanasiadou and Kyriazakis, 2004, Harikrishnan et al., 2011; Liu et al., 2011). Aunque hay estudios que proponen su principal blanco son los linfocitos T citotóxicos sobre los cuales ejerce un efecto estimulador directo gracias a la presencia de un grupo acyl en C-28 (Sun et al., 2009; 2011).

La acción inmunoestimuladora de las saponinas se ha asociado a su estructura química, por lo que las clasificadas como triterpénicas (glucósidos de triterpenoide) son más inmunogénicas que las esteroideas (glucósidos de esteroide) la razón es la presencia de un grupo aldehído en C-4, la cadena de oligosacáridos unidos a la posición 3 y 28 y principalmente por la de un grupo carboxilo formador de amina que es el que le confiere la capacidad inmunogénica. Un ejemplo claro de este tipo de saponina es la extraída a partir de la corteza de *Quillaja saponaria* Molina la cual ha sido muy utilizada como adyuvante (Press et al., 2000; Sun et al., 2009, 2011).

La saponina Quil A se ha utilizado en vacunas tanto inyectadas como orales, presentando igual eficacia en ambas vías. Hay pruebas de que la administración oral de saponinas puede estimular el sistema inmune TCD4 y CD8 y aumentar la resistencia a un desafío de la enfermedad (Weng et al., 2010; Sjolander et al., 1998).

De acuerdo a algunos estudios, los flavonoides ejercen un efecto anti-cancerígeno debido a que son capaces de inhibir la proliferación celular de linfocitos T citotóxicos y células NK, mientras que algunos tipos de alcaloides al parecer ejercen un efecto inmunoestimulador sobre la respuesta inmune adquirida por lo que son usados en pacientes con VIH y herpes (Reinhard, 1998; Provenza and Villalba, 2010).

Por otro lado, los taninos condensados estimulan al sistema inmune de forma indirecta al disminuir la degradación de aminoácidos como arginina, glutamina y

cisteína, aminoácidos que regulan la activación de linfocitos T y B, células NK, macrófagos, la producción de anticuerpos y citoquinas en el intestino funcionan como pro bióticos (Provenza and Villalba, 2010).

Los alcaloides presentes en algunas plantas estimulan la función de las células NK, la citotoxicidad dependiente de anticuerpos, la citotoxicidad mediada por componentes del complemento y reducen la producción de citoquinas pro inflamatorias (IL 1, IL 10 y TNF-  $\alpha$ ). (Manu and Kuttan, 2007). Los terpenos estimulan la citotoxicidad mediada por células NK y reducen la producción de algunas citoquinas preinflamatorias (Provenza and Villalba, 2010).

Con todo lo anterior podemos pensar que los metabolitos secundarios presentes en las plantas pueden ser utilizados como aditivos alimentarios mejorando parámetros productivos y ejerciendo un efecto inmunoestimulador o inmunosupresor directo o indirecto dependiendo del tipo de compuestos.

## **2.8. Uso de enzimas exógenas en la alimentación de rumiantes**

Debido al constante aumento en los precios del maíz y otros insumos utilizados en la alimentación animal, constantemente se están buscando alternativas que sirvan como fuentes de energía, que optimicen el metabolismo de los nutrientes consumidos y que permitan disminuir los costos por alimentación, por lo que se han empleado pre y prebióticos, arbustivas y arbóreas forrajeras, así como enzimas exógenas las cuales brindan los beneficios de disminuir el efecto de nocivo de factores antinutricionales presentes en los componentes de la dieta, mejoran la digestibilidad y permiten aprovechar todo el potencial energético del alimento (Pinos-Rodríguez et al., 2002; Rojo-Rubio et al., 2007).

Las enzimas son proteínas que catalizan reacciones bioquímicas, éstas se conocen desde la segunda mitad del siglo XII, se empezaron a emplear en la alimentación animal a partir de los años 70s primordialmente en aves y cerdos y

muy poco en rumiantes con la finalidad de mejorar la digestión, el aprovechamiento de alimento y por ende aumentar la producción (Bhat, 2000).

El mecanismo de acción de las enzimas a nivel intestinal consiste en unirse de forma específica a las diferentes moléculas presentes en el alimento, provocar su ruptura de tal forma que estas moléculas más pequeñas pueden ser absorbidas a través de la membrana intestinal. Estas pueden ser de naturaleza endógenas, es decir que el propio animal las produce en función de sus necesidades y del tipo de dieta que ingiera, o exógenas, es decir, que se suplementan en el alimento para cumplir con una función específica.

Las enzimas pueden ser de origen fúngico o bacteriano, ejercen su mecanismo de acción a un pH que va de 4 a 9 °C y a una temperatura que oscila entre los 30 a 90 °C generalmente son bien aceptadas tanto por el animal como por el consumidor ya que carecen de sabor y olor, no se absorben ni dejan residuos en los productos animales (Rojo-Rubio et al., 2007). En el caso de los rumiantes las enzimas exógenas trabajan de forma sinérgica con las enzimas microbianas del rumen, con la finalidad de incrementar el potencial hidrolítico dentro del mismo (Salem et al., 2011a).

Se pueden clasificar de acuerdo al sustrato sobre el que actúan, las carbohidrasas (amilasa,  $\beta$  glucanasas y xilasas) son utilizadas para mejorar la digestibilidad de los almidones y polisacáridos no amiláceos de los cereales, mientras que las proteasas (subtilisina), mejoran la digestibilidad de las proteínas.

Algunas ventajas del uso de las enzimas exógenas son: favorece la digestión de la fibra (polisacáridos no amiláceos), reduce la viscosidad del bolo alimenticio, incrementa la biodisponibilidad de los nutrientes, facilita el ataque de las enzimas endógenas a los nutrientes, inhiben el crecimiento de bacterias patógenas, favorece el crecimiento de flora intestinal prebiótica por la hidrólisis de los

polisacáridos no amiláceos (oligosacáridos) (Pinos-Rodríguez et al., 2002; Malik and Bandla, 2010.).

Además permiten utilizar mejor el valor energético de los cereales lo que supone un ahorro económico, reduce la excreción y las pérdidas de ciertos compuestos como el fósforo y el nitrógeno, y su liberación al medio ambiente por lo que disminuye el impacto ambiental de las explotaciones (Bedford and Partridge, 2010).

Previenen alteraciones digestivas y diarreas y como consecuencia mejora parámetros productivos, reproductivos, sanitarios e inmunológicos dado el mejor aprovechamiento de nutrientes en cantidad y calidad (Pinos-Rodríguez et al., 2002; Ghorai et al., 2009; Malik and Bandla, 2010; Bedford and Partridge, 2010). Además de que reduce costos por alimentación ya que con su uso se puede disminuir el uso de granos debido al mayor aporte de energía que realizan los sustratos fibrosos en rumiantes (Rojo-Rubio et al., 2007; Bedford and Partridge, 2010).

Las enzimas exógenas más utilizadas en la alimentación de los rumiantes son las fibrolíticas (Yang et al., 2000). Esto debido a su hábitos alimenticios que se basan en el consumo de fibra la cual tiene altos contenidos de celulosa y hemicelulosa, polisacáridos estructurales de las plantas, por lo las celulasas y las hemicelulasas son enzimas exógenas básicas en la alimentación de rumiantes (Bhat, 2000, Bedford and Partridge, 2010). El uso de este tipo de enzimas en rumiantes depende de su estabilidad en el alimento y en rumen, la habilidad de los componentes de la enzima para hidrolizar los polisacáridos de la pared celular y de la habilidad del animal para utilizar eficientemente los productos de la reacción (Bhat, 2000).

Endoglucanasa, exoglucanasa y  $\beta$ -glucosidasas hidrolizan celulosa, mientras que el xilan componente principal de la hemicelulosa es hidrolizado por endo  $\beta$ - 1,4

xilanasa (xilan) y  $\beta$ - 1,4- xilosidasa (xilosa) (Bedford and Partridge, 2010). Aunque también se incluyen en los complejos enzimáticos para rumiantes proteasas y  $\alpha$ -amilasa ya que se ha observado que las proteasas tiene la capacidad de atacar algunos componentes de la pared celular que actúan como barreras físicas para la degradación de la fibra, mientras que las amilasas degradan el almidón presente en los granos (Bedford and Partridge, 2010).

Las investigaciones relacionadas con del uso de este tipo de enzimas en rumiantes se han centrado en mejorar la digestibilidad de la fibra para favorecer el consumo de energía digestible consumida por el animal, lo cual disminuye la cantidad de alimento requerido para ganar un kilo de carne o para producir un kg de leche, además de favorecer el rendimiento de los animales aumentando la ganancia de peso o la producción de leche por día, lo que a su vez abarata los costos de producción (Bedford and Partridge, 2010).

El modo en el cual actúan las enzimas exógenas a nivel de rumen es un poco incierto pero se considera que al ser adicionadas a la dieta permiten una mejor unión al sustrato, incrementan la colonización microbiana del alimento ya que actúan de forma sinérgica con las enzimas de origen microbiano en el rumen, aumentan la resistencia de estas enzimas a proteólisis ruminal microbial, prolonga el tiempo de vida media en el rumen e incrementa la síntesis de proteína microbiana en el rumen (Yang et al., 2000; Bedford and Partridge, 2010; Salem et al., 2013 ).

Generalmente las enzimas exógenas fibrolíticas que se utilizan en los rumiantes son producto de la fermentación ruminal por lo que son muy estables a pH (5.5 – 6.8) y temperatura ruminal ( $39\pm 1^{\circ}\text{C}$ ), dichas enzimas se sintetizan a partir de hongos y bacterias ruminales, pero a diferencia de los extractos de la fermentación ruminal estos productos enzimáticos son más concentrados y purificados, proveen una actividad enzimática controlada además de no contener células vivas (Pinos-Rodríguez et al., 2002; Beauchemin et al., 1995; Bedford and Partridge, 2010).

Existen múltiples estudios en los cuales se ha demostrado que el uso de enzimas exógenas en la nutrición de rumiantes favorece el consumo de materia seca, la digestibilidad *in vitro* de la fibra (Lewis et al., 1996; Pinos-Rodríguez et al., 2002; Salem et al., 2013), la ganancia diaria de peso, la producción de leche (Beauchemin et al., 1995; Arriola et al., 2011; Baal et al., 2005), aunque la presencia de estos efectos depende del tipo de enzima utilizada y de su actividad, de su origen, del tipo de animal ya que hay efectos que solo se han observado en bovinos pero no en borregos, de la etapa productiva del animal, método de suplementación, entre otros. (Bedford and Partridge, 2010; Arriola et al., 2011; Salem et al., 2013).

En un estudio realizado por Feng et al. (1996) se demostró que el uso de enzimas exógenas fibrolíticas favorece la degradación ruminal de los forrajes (Feng et al., 1996). En ganado productor de leche se observó un incremento en la producción, esto debido al mejor aprovechamiento de la energía proveniente de la fibra potencialmente degradable en rumen, es posible reducir la cantidad de granos incorporados a la dieta de este tipo de animales, efecto que no se ha observado en el ganado productor de carne debido a la alta proporción de granos y la baja proporción de fibra presente en la dieta (Beauchemin et al., 1995; Lewis et al., 1996; Shekhar et al., 2010).

Por otro lado, El-Adawy et al. (2008) demostraron que al adicionar ZADO®, complejo enzimático a base de celulasas (7.1 unit/g), xilanasas (2.3 unit/g),  $\alpha$  amilasa (61.5 unit/g) y proteasas (29.2 unit/g) de origen bacteriano, a hojas de cuatro especies forrajeras como *Acacia saligna*, *Atriplex halimus*, *Lucena leucocephala* y *Eucalyptus camaldulensis* se mejora la producción de gas *in vitro*. Este producto ejerció un efecto positivo sobre la degradación de los metabolitos secundarios presentes en las hojas de los árboles en estudios realizados en conejos (El-Adawy et al., 2008; Salem et al., 2011a; 2011b). ZADO® ejerce un efecto positivo al ser utilizado en rumiantes ya que favorece la

utilización de nutrientes en forrajes de baja calidad *in vivo* e *in vitro* (El-Adawy et al., 2008).

Gado et al. (2011) al adicionar ZADO® (5g/kg) a ensilado pulpa de naranja y proporcionárselo a corderos en crecimiento y evaluar su efecto sobre la digestión y algunos parámetros de crecimiento observo que aquellos corderos que consumieron ensilado de pulpa de naranja tratado con enzimas se favoreció la producción ácidos grasos volátiles, el consumo voluntario, digestibilidad de la materia seca, y la ganancia de peso (Gado et al., 2011).

Salem et al. (2011) administraron ZADO® (10 g/animal/día) a corderos y cabras con la finalidad de estimar el efecto que esta enzima sobre la digestibilidad de los nutrientes y el comportamiento productivo de los animales. Estos autores concluyeron que en aquellos animales que consumieron enzimas exógenas, la digestibilidad de los nutrientes se incrementó más en las cabras que en los ovinos, además de que estas presentaron el consumo más bajo de materia seca, que estuvo asociado con el incremento en la digestibilidad y en la ganancia diaria de peso. Se puede comentar que las cabras son más eficientes en el uso de enzimas exógenas fibrolíticas que los borregos, adicionalmente observó que al usar esta dosis de enzimas no producía efectos detrimentales en la salud de los animales (Salem et al., 2011a).

En 2013, se realizó un experimento con la misma enzima en ganado productor de carne a una dosis de 40g/animal/día y se observó un incremento en la digestibilidad de la FDN, FDA, la concentración de amoníaco en rumen y de ácidos grasos de cadena corta lo que favoreció la ganancia de peso en los animales que fueron dosificados (Salem et al., 2013). Arriola et al. (2011) demostró que al utilizar enzimas exógenas (Dyadic International Inc. Jupiter, FL.) en vacas lactantes que consumieron dietas bajas en concentrado (33% materia seca de la dieta) asperjadas con una dosis de 3.4 mg enzima/g MS de TMR se disminuyó la producción de metano, favoreció la concentración de ácidos grasos volátiles en el

rumen, digestibilidad de la materia seca, proteína cruda, fibra neutro detergente, fibra ácido detergente y la eficiencia en la producción de leche (Arriola et al., 2011).

### III.- JUSTIFICACIÓN

Las repercusiones del uso de aditivos alimenticios como las enzimas exógenas y el extracto de *Salix babylonica* sobre la salud de animal así como su acción directa e indirecta sobre la respuesta inmune no han sido determinadas.

Sin embargo hay una cantidad considerable de estudios que sugieren que los metabolitos secundarios presentes en el extracto de *Salix babylonica* pueden producir efectos negativos sobre la salud de los animales e incluso la muerte, por otro lado se ha demostrado que estos mismos metabolitos a dosis óptimas ejercen una acción inmunomoduladora directa e indirecta.

En lo referente al uso de enzimas exógenas en la nutrición de rumiantes, se ha supuesto que son inocuas y que no causan alteraciones en la salud de los animales que las consumen sin que esto haya sido demostrado, además el efecto que de forma indirecta este aditivo alimenticio puede tener sobre el sistema inmune tampoco ha sido documentado.

#### **IV.- HIPÓTESIS**

El uso de enzimas exógenas y extracto de *Salix babylonica* como aditivos alimenticios en las dietas de corderos en crecimiento produce efectos positivos a nivel hematológico, inmunológico y productivo.

Es posible que exista una correlación entre la respuesta productiva y la respuesta inmune celular en corderos que consumen enzimas exógenas y extracto de *Salix babylonica* como aditivos alimenticios.

## V.-OBJETIVOS

### 5.1. GENERAL

Evaluar el efecto de las enzimas exógenas y el extracto de *Salix babylonica* usados como aditivos alimenticios sobre parámetros hematológicos e inmunológicos y determinar la correlación respuesta productiva-respuesta inmune celular en corderos en crecimiento.

### 5.2. ESPECÍFICOS

- Evaluar el efecto de las enzimas exógenas y el extracto de *Salix babylonica* sobre parámetros hematológicos de corderos en crecimiento.
- Evaluar el efecto de las enzimas exógenas y el extracto de *Salix babylonica* sobre la respuesta inmune celular de corderos en crecimiento.
- Evaluar el efecto de las enzimas exógenas y el extracto de *Salix babylonica* sobre la ganancia diaria de peso y consumo alimenticio de los corderos.
- Estimarla correlación entre la ganancia diaria de peso y la respuesta inmune celular en corderos en crecimiento.
- Identificar si la especie (ovinos o caprinos) es un factor que modifica los efectos de los tratamientos.

## VI. MATERIALES Y MÉTODOS

El trabajo experimental se llevó a cabo en las instalaciones de la posta zootécnica de la Facultad de Medicina Veterinaria y Zootecnia de la Universidad Autónoma del Estado de México, que se encuentra situada en las coordenadas geográficas latitud norte 19° 24'47'' y longitud oeste 099° 40'44'' a 2600 m sobre el nivel del mar (IGECEM, 2012), en el que predomina el clima templado húmedo con una precipitación media anual de 790 mm (González Huerta et al., 2007).

Con la finalidad de evaluar dos tipos de enzimas exógenas comerciales y de poder hacer una comparación por especie, el trabajo experimental se dividió en dos sub-experimentos, el primero se realizó con 20 corderos en crecimiento utilizando como tratamientos el complejo enzimático ZADO® y el extracto de *Salix babylonica*.

Mientras que en segundo experimento se realizó con 4 corderos en crecimiento y 4 cabritos en crecimiento en este caso los tratamientos a evaluar fueron la enzima exógena xilanas (Dyadic International Inc. Jupiter, FL) y el extracto de *Salix babylonica*.

### 6.1. Experimento 1

#### 6.1. 1. Animales

Se utilizaron 20 ovinos machos de raza Suffolk entre 6 y 8 meses de edad con un peso de  $24 \pm 0.3$  kg, los cuales fueron alojados en jaulas individuales de 1.5 x 1.5 m, alimentados *ad libitum* de forma individual con una dieta basal de 70% ensilado de maíz, 30% concentrado comercial (Purina®, Cuautitlán, México), balanceada para cubrir los requerimientos de corderos de su edad y peso (NRC, 2007). Los animales tuvieron acceso a agua fresca durante todo el periodo experimental.

### **6.1.2. Grupos experimentales**

Los corderos fueron pesados y asignados de forma aleatoria en cuatro grupos experimentales de cinco animales cada uno: Control: los animales fueron alimentados únicamente con la dieta basal, SB: dieta basal más 30ml de extracto de *Salix babylonica*, EZ: dieta basal más 10g de enzimas exógenas (ZADO®, Cairo, Egypt) y SBEZ: dieta basal más 30ml de extracto de *Salix babylonica* y 10g de enzimas exógenas.

Las enzimas y el extracto fueron proporcionados por un periodo de 60 días, vía oral antes de administrar el alimento, las enzimas se mezclaron con 200g de concentrado y se ofrecieron de forma individual por 20 minutos. Una vez transcurrido este tiempo el concentrado y las enzimas no consumidas se mezclaron con el resto del alimento, mientras que el extracto se ofreció vía oral con una jeringa.

### **6.1.3. Preparación del extracto**

El extracto se preparó de acuerdo a la técnica propuesta por Salem et al. (2011), la cual consiste en coleccionar de forma aleatoria hojas frescas de por lo menos 5 árboles de *Salix babylonica* en diferentes estados fenológicos, una vez coleccionadas las hojas se molieron y mezclaron con una solución previamente preparada (1kg de hojas previamente coleccionadas en 8L de solución) a base de agua destilada, etanol (99/100, analytical grade, Fermont®, Monterrey, México) y metanol (99.8/100, analytical grade, Fermont®, Monterrey, México) en una proporción 80:10:10 (Salem et al., 2011b).

La mezcla de hojas y solución se incubó por 48 a 72 h a una temperatura de 25 a 30 °C; una vez transcurrido este tiempo la mezcla se incubó por 1 h en baño de agua a 39 °C, para posteriormente, filtrarse y almacenarse a 4 °C.

#### **6.1. 4. Determinación de metabolitos secundarios en el extracto**

Para la determinación de los metabolitos secundarios presentes en el extracto, se utilizaron 10 ml de extracto de *Salix babylonica* el cual se mezcló en un embudo de separación con un volumen doble de acetato de etilo (99.7/100, analytical grade, Fermont®, Monterrey, México) para determinar el contenido de compuestos fenólicos totales, una vez separados los fenólicos totales se agregó un volumen doble de n- butanol (99.9/100, analytical grade, Fermont®, Monterrey, México) para determinar el contenido de saponinas (Ahmed et al., 1990). La fracción restante, se consideró como la fracción acuosa del extracto la cual contiene lectinas, polipéptidos y almidón (Cowan, 1999).

#### **6.1.5. Muestreo**

##### **6.1.5.1. Pesaje de animales**

Todas las unidades experimentales se pesaron al inicio del experimento y cada 15 días, antes de proporcionarles el alimento, con ayuda de una báscula (Báscula de recibo TOROREY, EQB 100kg/200lb).

##### **6.1.5.2. Colecta de muestras**

Se colectaron 3 muestras de sangre vía venopunción yugular de cada uno de los animales los días 0, 15, 30, 45 y 60 del experimento, haciendo uso de tubos vacutainer (BD tube, Monterrey, México), sin anticoagulante para obtener suero y hacer una bioquímica sanguínea, con EDTA para la realización de un hemograma completo y con heparina para desarrollar un análisis de inmunofenotipo por citometría de flujo.

## **6.1.6. Análisis de las muestras**

### **6.1.6.1. Bioquímica sanguínea**

Para la realización de este análisis, las muestras de sangre fueron mantenidas a temperatura ambiente durante un periodo de 1h; posteriormente, fueron centrifugadas a 1500 rpm por 10 minutos, y posteriormente extraer el suero, el cual fue congelado a  $-20^{\circ}\text{C}$  para su análisis ser analizadas después.

Una vez colectadas las 100 muestras de suero, estas se analizaron en un espectrofotómetro semiautomático BTS350 (Instrumentation Laboratory®), para determinar las concentraciones de proteína total, glucosa, creatinina, urea, ALP (fosfatasa alcalina), AST (aspartato aminotransferasa), ALT (alanino aminotransferasa), fósforo, calcio y magnesio (mmol/L).

### **6.1.6.2. Hemograma Completo**

Las muestras de sangre colectadas en tubos con EDTA se mantuvieron y transportaron a una temperatura de  $4^{\circ}\text{C}$ , para posteriormente determinar concentraciones de eritrocitos ( $10^{12}/\text{L}$ ), hemoglobina (g/L), hematocrito (L/L), conteo total ( $10^9/\text{L}$ ) y diferencial de leucocitos ( $10^9/\text{L}$ ), volumen corpuscular medio (VCM en fL), concentración de hemoglobina corpuscular media (CHCM en g/L) y proteína plasmática (g/dl).

Para el conteo de eritrocitos, así como para el análisis total y diferencial de leucocitos se utilizó un hemocitómetro (Cámara de Recuento BLAUBRAND®), la concentración de hemoglobina se determinó con el método de la cianometahemoglobina, el hematocrito se obtuvo mediante la técnica del microhematocrito, la proteína plasmática se determinó con un refractómetro. Se determinaron los índices de Wintrobe VCM y CHCM para diagnosticar algún tipo

de anemia teniendo en cuenta los valores de eritrocitos, hematocrito y hemoglobina (Coffin et al., 1959; Archer and Jeffcott, 1977; Smith, 2007).

Las siguientes formulas se utilizaron para para calcular los índices de Wintrobe de acuerdo con (Smith, 2007).

$$VCM = \frac{\text{Hematocrito } \%}{\text{Total de eritrocitos}} \times 10$$

$$CHCM = \frac{\text{Hemoglobina g/dL}}{\text{Hematocrito}} \times 100$$

#### 6.1.6.3. Inmunofenotipo por citometría de flujo

Para este análisis se utilizaron las muestras de sangre colectadas en tubos con heparina, las cuales de igual manera se mantuvieron a una temperatura de 4°C.

Los inmunofenotipos determinados en cada una de las muestras fueron linfocitos T colaboradores (Ab mouse anti sheep CD4RPE/MCA2213PE), linfocitos T citotóxicos (Ab mouse anti sheep CD8FITC/MCA2216F, D.F, México), granulocitos (Ab mouse anti bovine CD11bFITC/MCA1425F,.D.F, México) y monocitos (Ab mouse anti human CD14RPE/ MCA1568PE, D.F, México) usando anticuerpos de AbDSerotec® (Macey, 2007).

La muestras de sangre se sometieron a un gradiente celular con Ficoll (Lymphoprep® BD) para separar únicamente linfocitos (colaboradores y citotóxicos), granulocitos (neutrófilos, eosinófilos, basófilos) y macrófagos, este proceso consistió en hacer dilución 1:1 con sangre y PBS 1x, la cual fue colocada en 3ml de Ficoll, para posteriormente centrifugar a 1500rpm (centrifuga análoga para 12 tubos 3500 rpm), 20 min. a temperatura ambiente. Para la segunda fase se colectaron las células, de interés las cuales se mezclaron con 2ml PBS 1x, y se centrifugaron a 1500 rpm x 5 minutos.

Una vez obtenida la suspensión de células blancas, se colocaron 20µl de la suspensión en placas de 96 pozos. Además de la suspensión, se le agrego a cada pozo 100 µl de PBS 1x y se centrifugo a 1500 rpm, 5 minutos. Posteriormente se elimino al sobrenadante y se dejo únicamente las células, a las cuales se les agrego 50µl del anticuerpo correspondiente (CD4RPE, CD8FITC, CD11FITC o CD14RPE). La mezcla se incubo por 30 minutos a 4 °C y posteriormente se lavo con PBS 1x. Se agrego el segundo anticuerpo, se incubo de nuevo por 30 minutos a 4°C y finalmente se lavo con PBX 1x se leyó en el citómetro de flujo BD FACS (Calibur™ USA).

#### **6.1.7. Análisis estadístico**

Los resultados de los análisis realizados en la muestras de sangre (bioquímica sanguínea, hemograma completo e inmunofenotipos) de cada animal fueron analizadas utilizando el procedimiento MIXED de SAS (2002) con medidas repetidas, el modelo incluye 4 tratamientos (Control, EZ, SB y EZSB) y cinco días de muestreo (0, 15, 30, 45 y 60) a los cuales se les determinaron los efectos lineales, cuadráticos y cúbicos. A los resultados de los cuatro inmunofenotipos (células T CD4, células T CD8, granulocitos y macrófagos) además se les realizó un análisis de correlación con la ganancia diaria de peso por tratamiento y día de muestreo en PROC CORR de SAS 2002 (SAS, 2002).

### **6.2. Experimento 2**

#### **6.2.1. Animales**

Para la realización de este experimento se utilizaron 4 corderos raza Suffolk de entre 3 y 4 meses de edad con un peso de 24kg ( $\pm 2.27$ ) y 4 cabras raza Saanen de entre 5 a 8 meses de edad con un peso de 20kg ( $\pm 2.5$ ). Los animales se sometieron a un periodo de adaptación a la dieta por 2 semanas, la cual fue balanceada de acuerdo a los requerimientos de animales de su edad y peso

(NRC, 2007). Trascurridas las dos semanas, cada uno de los ocho animales se colocaron en jaulas metabólicas. El alimento y el agua se les ofreció forma individual *ad libitum*.

### **6.2.2. Grupos experimentales**

Después de adaptarse a la dieta, los animales se asignaron de forma aleatoria a uno de cuatro tratamientos i) Control: administración de la dieta basal más 30ml de agua destilada, SB: dieta basal más 30ml de extracto de *Salix babylonica*, EZ: dieta basal más 1µl/g/MS ingerida de enzimas exógenas (Dyadic International Inc. Jupiter, FL) diluido en 30ml de agua destilada y SBEZ: dieta basal más 30ml de extracto de *Salix babylonica* y 1µl/g/MS ingerida de enzimas exógenas diluida en 30ml de agua destilada.

Los tratamientos se administraron via oral mediante jeringa antes de proporcionarles el alimento. Cada tratamiento se ofrecio por un periodo de 15 días, por lo que el experimento completo duro 60 días.

### **6.2.3. Preparación del extracto**

El extracto fue preparado de acuerdo a la técnica propuesta por Salem et al. (2011), la cual consiste en coleccionar de forma aleatoria hojas frescas de por lo menos 5 árboles de *Salix babylonica* en diferentes estados fenológicos, una vez colectadas las hojas se molieron y mezclaron con una solución preparada con anterioridad (1kg de hojas de *Salix babylonica* previamente colectadas en 8L) a base de agua destilada, etanol (99/100, analytical grade, Fermont®, Monterrey, México) y metanol (99.8/100, analytical grade, Fermont®, Monterrey, México) en una proporción 80:10:10 (Salem et al., 2011b).

La mezcla de hojas y solución se incubo por 48 a 72 h a una temperatura de 25 a 30 °C, una vez transcurrido este tiempo, la mezcla se incubo por 1 h en baño de

agua a 39 °C. Transcurrido este periodo se filtro y se almaceno a temperatura de refrigeración.

#### **6.2.4. Determinación de metabolitos secundarios en el extracto**

Para la determinación de los metabolitos secundarios en el extracto se utilizaron 10 ml de extracto de *Salix babylonica* el cual se mezclo en un embudo de separación con un volumen doble de acetato de etilo (99.7/100, analytical grade, Fermont®, Monterrey, México) para determinar el contenido de compuestos fenólicos totales, una vez separados los fenólicos totales se agregó un volumen doble de n- butanol (99.9/100, analytical grade, Fermont®, Monterrey, México) para determinar el contenido de saponinas (Ahmed et al., 1990) La fracción restante, se consideró como la fracción acuosa del extracto la cual contiene lectinas, polipéptidos y almidón (Cowan, 1999).

#### **6.2.5. Muestreo**

##### 6.2.5.1. Colecta de muestras.

Se colectaron 2 muestras de sangre vía venopunción yugular de cada uno de los animales el día 15 de cada periodo experimental, mediante la utilización de tubos vacutainer (BD tube, Monterrey, México), sin anticoagulante para obtener suero y hacer una bioquímica sanguínea y con EDTA para la realización del hemograma completo.

## 6.2.6. Análisis de las muestras

### 6.2.6.1. Bioquímica sanguínea

Para la realización de este análisis, las muestras de sangre se mantuvieron a temperatura ambiente durante un periodo de 1h, posteriormente se centrifugaron a 1500 rpm por 10 minutos. El suero se refrigeró a  $-20^{\circ}\text{C}$  para su análisis posterior. Una vez colectadas todas las muestras de suero, se analizaron en un espectrofotómetro semiautomático BTS350 (Instrumentation Laboratory®), para determinar las concentraciones de proteína total, glucosa, creatinina, urea, ALP (fosfatasa alcalina), AST (aspartato aminotransferasa), ALT (alanino aminotransferasa), fósforo, calcio y magnesio (mmol/L).

### 6.2.6.2. Hemograma completo

Las muestras de sangre colectadas en tubos con EDTA, se mantuvieron y transportaron con un ambiente de  $4^{\circ}\text{C}$ . Se determinaron los perfiles de eritrocitos ( $10^{12}/\text{L}$ ), hemoglobina (g/L), hematocrito (L/L), conteo total ( $10^9/\text{L}$ ) y diferencial de leucocitos ( $10^9/\text{L}$ ), volumen corpuscular medio (VCM en fL), concentración de hemoglobina corpuscular media (CHCM en g/L) y proteína plasmática (g/DL).

Para el conteo de eritrocitos, así como para el análisis total y diferencial de leucocitos se utilizó un hemocitómetro (Cámara de Recuento BLAUBRAND®), la concentración de hemoglobina se determinó con el método de la cianometahemoglobina, el hematocrito se obtuvo utilizando la técnica del microhematocrito, la proteína plasmática se determinó con un refractómetro. Se determinaron los índices de Wintrobe VCM y CHCM para diagnosticar algún tipo de anemia teniendo en cuenta los valores de eritrocitos, hematocrito y hemoglobina (Coffin et al., 1959, Archer and Jeffcott, 1977, Smith, 2007).

Se utilizaron las siguientes formulas para calcular los índices de Wintrobe de acuerdo con la metodología de Smith, (2007).

$$VCM = \frac{\text{Hematocrito \%}}{\text{Total de eritrocitos}} \times 10$$

$$CHCM = \frac{\text{Hemoglobina g/dL}}{\text{Hematocrito}} \times 100$$

### **6.2.7. Análisis estadístico**

Los resultados de los análisis realizados en la muestras de sangre (bioquímica sanguínea y hemograma completo) de cada animal en cada tratamiento en cada periodo fueron analizados utilizando el PROC GLM de SAS (2002) en un diseño de cuadrado latino 4x4 donde se consideraron 4 animales corderos o cabras, 4 tratamientos y 4 periodos experimentales de 15 días cada uno (SAS, 2002).

## VII. RESULTADOS

### 7. 1. Artículo Effect of exogenous enzyme and *Salix babylonica* extract or their combination on haematological parameters in growing lambs.

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#### Effect of exogenous enzymes and *Salix babylonica* extract or their combination on haematological parameters in growing lambs

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#### ABSTRACT

The aim of this study was to compare the use of exogenous enzyme preparations (EZ) and/or *Salix babylonica* extract (SB) or their combination as feed additives on some haematological parameters in growing lambs. Twenty Suffolk lambs of 6 to 8-months-old with  $24 \pm 0.3$  kg body weight were used in the study. Lambs were divided into 4 groups of 5 animals each in a completely randomized design and the treatments were: 1. control: fed a basal diet of concentrate (30%) and maize silage (70%); 2. EZ: fed the basal diet plus 10 g of enzyme; 3. SB: fed the basal diet plus 30 ml of *S. babylonica* extract, and 4. EZSB: fed the basal diet plus 10 g enzyme and 30 ml of *S. babylonica* extract. Lambs were housed in individual cages and the experiment was conducted for 60 days. The SB was given orally while the EZ was mixed with a small amount of the concentrate and maize silage and was offered *ad libitum*. Blood samples were collected from each animal on days 0, 15, 30, 45 and 60 of

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## Effect of exogenous enzymes and *Salix babylonica* extract or their combination on haematological parameters in growing lambs

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experiment and analysed for haematological parameters. The treatments of EZ, SB or EZSB did not affect any of the measured blood parameters. Day of sampling modified concentrations of red blood cells (P=0.001; linear effect), haematocrit (P=0.01; quadratic effect), haemoglobin (P=0.01; linear effect), mean corpuscular volume (P=0.01; linear effect), monocytes (P=0.004; quadratic effect) and plasma protein (P=0.0002, linear effect). It could be concluded that *Salix babylonica* extract, exogenous enzymes and their combination as feed additives had not a negative effects on the blood parameters measured and therefore on the health of the lambs.

KEY WORDS: *Salix babylonica* extract, exogenous enzymes, haematological parameters, lambs

## INTRODUCTION

The cost of feeding livestock accounts for more than 50% of the total production costs. Use of feed additives such as pre- and probiotics, ionophores, enzymes, and fodder shrubs and trees extracts have been used recently mainly in ruminant nutrition (Salem et al., 2006, 2010; Chung et al., 2012). Commercial exogenous enzymes provide the benefits of reducing the antinutritional factors in dietary components to improve digestibility (Salem et al., 2012). Some enzyme formulations increased dry matter intake, *in vivo* fibredigestibility, and milk production of dairy cows (Gado et al., 2009, 2011). However, effects of enzyme additives vary depending on the type of animals (energy requirement of the animal and level of feeding), diet composition, enzyme formulation, dose rate, and delivery method of enzyme products (Chung et al., 2012).

Plant extracts rich in secondary compounds have been shown to have positive effects on daily gain, voluntary intake, milk production and ruminal fermentation parameters (Salem et al., 2011b), besides having a protective effect on the protein in the rumen to promote duodenal absorption, minimizes the excretion of nitrogen, modify the acetate to propionate ratio in rumen fluid and decrease parasitic load (Athanasidou and Kyriazakis, 2004; Salem et al., 2010; Jiménez et al., 2011). Although, the use of plant extract could be limit by their secondary compound concentrations, consumption of large amounts of tannins or saponins (Athanasidou and Kyriazakis, 2004) may have a direct haemolytic effect and may even cause death. Moreover, long-term feeding of plants rich in secondary compounds may have detrimental effects on animal health and even cause death (Mahgoub et al., 2008). Increasing the cost of commercial feed additives (i.e., exogenous enzymes) provide the opportunity for researchers to search for other alternative feed additive such as plant extract.

It was hypothesized that using the exogenous enzymes alone or in combination with tree extracts would have a synergetic effect and infer health benefits to the lambs. Therefore, the aim of this study was to compare the use of exogenous enzymes and *S. babylonica* extract or their combination as feed additives on haematological parameters in growing lambs.

## MATERIAL AND METHODS

This study was conducted at the experimental farm of the Faculty of Veterinary Medicine of the Autonomous University of Mexico State. The handling of animals was done according to international bioethical standards and NOM-062 - ZOO1999.

### *Animals and treatments*

Twenty Suffolk lambs of 6 to 8-months-old with  $24 \pm 0.3$  kg body weight (BW) were used in the study. The lambs were housed in individual cages (1.5×1.5 m) in a completely randomized design and the experiment was conducted for 60 days. After 2 weeks of adaptation to the basal diet of 70% maize silage and 30% commercial concentrate (Purina<sup>®</sup>, Cuautitlan, Mexico) formulated according to NRC (1985) nutrient requirements; the lambs were weighed and randomly distributed into 4 groups of 5 animals each. The treatments comprised: 1. control: lambs were fed the basal diet of concentrate and maize silage only, 2. EZ: lambs were fed the basal diet plus 10 g of exogenous enzyme preparations (ZADO<sup>®</sup>, Cairo, Egypt), 3. SB: lambs were fed the basal diet plus 30 ml of *Salix babylonica* extract, and 4. EZSB: lambs were fed the basal diet plus 10 g exogenous enzymes and 30 ml of *S. babylonica* extract. The daily dose of SB was given orally before the morning feeding, while the EZ was fed to the lambs mixed with a small amount of the concentrate (200 g) for 20 min and then mixed with the rest of the concentrate and was offered for 1 h before feeding the maize silage *ad libitum* for the rest of the day. The chemical composition of the basal diets is presented in Table 1.

Table 1. Chemical composition of the basal diet (commercial concentrate and maize silage) and levels of secondary metabolites in the *Salix babylonica* extract, g/kg DM

Indices	Concentrate	Maize silage	<i>Salix babylonica</i> extract
<i>Chemical composition crude protein</i>			
	147.3	106.2	
ether extract	119.8	85.2	
neutral detergent fibre	160.4	444.8	
acid detergent fibre	27.7	111.2	
acid detergent lignin	7.6	18.0	
<i>Secondary metabolites total</i>			
phenolics			16.4
saponins			5.4
aqueous fraction <sup>1</sup>			76.3

<sup>1</sup>aqueous fraction: lectins, polypeptides, starch (Cowan, 1999)

ZADO<sup>®</sup> is a powdered multi-enzyme commercially available feed additive product produced from *Ruminococcusflavefaciens* by the Academy of Scientific Research and Technology in Egypt (Patent No. 22155, Cairo, Egypt). Prior to this work, the enzyme mixture was assayed for several enzymatic activities, and it was found to contain (per gram of enzyme preparation) 7.1 unit of endoglucanase, 2.3 unit of xylanase, 61.5 unit of  $\alpha$ -amylase and 29.2 unit of protease activity.

To make the silage, whole maize plants (at medium stage i.e., 70% moisture content) were chopped into 1-2 cm pieces using a forage chopper and the silage made immediately in a flat 10-ton silo. After two months, the use of the silage was initiated for feeding experimental animals as well as the other animals of the experimental farm of the faculty.

#### *Preparation of extract*

The *S. babylonica* extract was prepared as described by Salem et al. (2011b). Briefly, fresh leaves of *S. babylonica* were collected randomly from several young and mature trees (minimum 5 different trees) in autumn and were chopped (1-2 cm) and immediately extracted in the proportion of 1 g leaf per 8 ml of solvent mixture. The mixture of solvents contained 10 ml methanol (99.8/100, analytical grade, Fermont<sup>®</sup>, Monterrey, Mexico), 10 ethanol ml (99/100, analytical grade, Fermont<sup>®</sup>, Monterrey, Mexico) and 80 ml distilled water. Leaves were soaked and incubated in solvent in the laboratory at 25-30°C for 48-72 h in closed flasks. After incubation, all flasks were incubated in a water bath at 39°C for one h and then immediately filtered and the filtrate collected and stored at 4°C for further use.

#### *Sampling and measurements*

Five millilitres of peripheral blood was collected from each lamb by jugular venipuncture into EDTA vacutainer tubes (BD tube, Monterrey, Mexico) on days 0, 15, 30, 45 and 60 of the experiment. Samples of both concentrate and silage were also collected during the experimental period and stored at -20 C for later chemical analysis.

Two samples of the concentrate, silage and SB extract were collected weekly during the 60 days of experiment. Each sample type (i.e., concentrate, silage or extract) were pooled and stored for further analysis.

#### *Sample analysis*

Blood samples were analysed for red blood cell count (RBC), the differential white cell count: neutrophils, lymphocytes, basophils, eosinophils and monocytes,

packed cell volume (PCV), haemoglobin (HGB), mean corpuscular volume (MCV), mean cell haemoglobin concentration (MHC), white blood cell count (WBC), and plasma protein concentration. Haemoglobin concentration was determined using the cyanmethaemoglobin method. Haematocrit was determined by micro haematocrit technique. Erythrocyte and total leucocytes and differential leucocyte counts were determined using the haemocytometer method. Total plasma proteins were determined using a refractometer (Archer and Jeffcott, 1977). Mean corpuscular volume (MCV) and MHC were calculated to diagnose any type of anaemia by taking into account the values of erythrocytes, haemoglobin and haematocrit predetermined:

$$\text{MCV } (\mu\text{ m}^3/\text{erythrocyte}) = \frac{\text{Hematocrit } (\%) \times 10}{\text{Total de erythrocyte}}$$

$$\text{MHC } (\%) = \frac{\text{Hemoglobin } (\text{g/dl}) \times 10}{\text{Hematocrit}}$$

Samples of concentrate and silage were analysed for DM, ash, nitrogen (N), and ether extract (EE) according to AOAC (1997). The neutral detergent fibre (NDF; Van Soest et al., 1991), acid detergent fibre (ADF) and lignin (AOAC, 1997) were analysed using an ANKOM 200 FibreAnalyser Unit (ANKOM Technology Corporation, Macedon, NY, USA). The NDF was assayed without use of  $\alpha$ -amylase but with sodium sulphite in the NDF. Both NDF and ADF are expressed without residual ash.

Plant secondary metabolites were determined using ten millilitres of extract liquor and fractionated by funnel separation with a double volume of ethyl acetate (99.7/100, analytical grade, Fermont<sup>®</sup>, Monterrey, Mexico) to determine total phenolics by drying and quantifying the total phenolics layer in the funnel. After total phenolics separation, a double volume of n-butanol (99.9/100, analytical grade, Fermont<sup>®</sup>, Monterrey, Mexico) was added to fractionate the saponins (Ahmed et al., 1990). The remaining solution was considered to be the aqueous fraction that has the other secondary metabolites such lectins, polypeptides and starch (Cowan, 1999) (Table 1).

Endoglucanase activity was assayed by liberating glucose from carboxymethyl cellulose, which was determined calorimetrically using alkaline copper reagent as described by Robyt and Whelan (1972). One unit of endoglucanase catalyzes the liberation of 1 mmol of glucose per min from sodium carboxymethylcellulose at 40°C and pH 4.5. Furthermore,  $\alpha$ -amylase was assayed by its ability to produce reducing groups from starch, which were measured by the

reduction of 3,5-dinitrosalicylic acid (Bernfeld, 1955). One unit of  $\alpha$ -amylase catalyzes the liberation of 1 mmol of reducing groups per min from soluble starch at 25°C and pH 6.0, calculated as maltose equivalents. Protease activity was determined by the hydrolysis of dimethyl casein (DMC) and the liberated amino acids were determined using 2,4,6-trinitrobenzene sulphonic acid (Lin et al., 1969). One DMC-U catalyzes the cleavage of 1 mmol of peptide bond per min from DMC at 25°C and pH 7.0 expressed in terms of newly formed terminal amino groups. Xylanase catalyzes the hydrolysis of xylan from oat spelt, and the reducing groups liberated were determined using alkaline copper reagent (Robyt and Whelan, 1972). One unit catalyzes the liberation of 1 mmol reducing groups per h from xylan at 37°C and pH 5.5, expressed as xylose equivalents.

### *Statistical analyses*

Data of the haematological parameters was analysed using the MIXED procedure of SAS (2002) with repeated measures (Littell et al., 1998). The structure of the variance-covariance error matrix employed was unstructured, based on Bayesian criteria observed with several alternative structures. Terms in the model were diet (i.e., control, SB, EZ, EZSB), days of sampling (i.e., 0, 15, 30, 45 and 60 of the experiment) and linear, quadratic and cubic effects were determined. The repeated term was sampling days, with lamb within diet the subject. Results reported in Tables and in text are least square means of fixed effects with their corresponding standard errors. Test of simple effects were used to partition (slice) interaction effects by diet in order to test effects of period separately for each diet (SAS, 2002). Significant differences between treatment means and time were assessed using the Tukey procedure at  $P < 0.05$  level.

## RESULTS

The treatments of EZ, SB or EZSB did not affect any of the measured blood parameters while the day of sampling had a significant impact on some parameters (Table 2). However, red blood cells were increased by 20% ( $P=0.001$ ; linear effect) on day 60, while haemoglobin was increased ( $P=0.01$ ; linear effect) on days 0, 30 and 45 compared with the days 15 and 60 of the experiment. The values of MCV were decreased ( $P=0.015$ ; linear effect) on day 60, while monocytes were increased at days 30 and 45 ( $P=0.004$ ; quadratic effect) compared with others days. Haematocrit was increased ( $P=0.02$ ; quadratic effect) on day 30 and 45.

Plasma protein was increased ( $P=0.001$ ; linear effect) on days 0 and 60  
 Table 2. Effect of exogenous enzyme preparation (EZ) and *S. babingtonia* (SB) extract and their combination (EZSB) on haematological parameters in growing lambs after 0, 15, 30, 45, and 60 days of the experiment (n = 5 lambs)

Indices	RBC	HMT	IMG	MCV	MIIC	WBC	SGN	LYM	MON	BAS	EOS	PSP
<i>Treatment</i>												
control	8.17	0.296	98.4	37.7	331.8	8.71	3.23	5.22	0.027	0.018	0.11	67.12
EZ	8.03	0.286	95.1	35.7	331.9	7.91	2.79	4.94	0.100	0.017	0.08	63.09
SB	8.06	0.296	98.2	37.0	331.8	9.50	3.37	5.82	0.000	0.015	0.10	65.48
EZSB	7.84	0.288	95.4	37.2	331.6	9.28	3.43	5.56	0.022	0.029	0.12	62.84
SEM	0.343	0.0090	3.00	1.26	0.22	0.493	0.301	0.408	0.0180	0.010	0.016	1.293
P value	0.924	0.795	0.790	0.793	0.718	0.146	0.477	0.469	0.220	0.861	0.792	0.097
<i>Day of experiment</i>												
D0	7.77 <sup>c</sup>	0.295 <sup>b</sup>	98.0 <sup>a</sup>	38.4 <sup>a</sup>	331.7	8.84	3.10	5.56	0.006 <sup>c</sup>	0.020	0.11	66.71 <sup>a</sup>
D15	7.24 <sup>f</sup>	0.282 <sup>c</sup>	93.5 <sup>b</sup>	38.9 <sup>a</sup>	331.7	9.44	3.86	5.24	0.000 <sup>c</sup>	0.036	0.17	63.00 <sup>c</sup>
D30	7.56 <sup>e</sup>	0.296 <sup>f</sup>	98.3 <sup>a</sup>	39.3 <sup>a</sup>	331.9	9.03	3.07	5.73	0.070 <sup>a</sup>	0.004	0.13	64.05 <sup>b</sup>
D45	8.19 <sup>b</sup>	0.300 <sup>e</sup>	99.6 <sup>a</sup>	36.6 <sup>b</sup>	331.5	8.33	2.99	5.15	0.120 <sup>a</sup>	0.005	0.09	64.25 <sup>b</sup>
D60	9.36 <sup>a</sup>	0.285 <sup>b</sup>	94.5 <sup>b</sup>	31.0 <sup>c</sup>	332.1	8.62	3.01	5.26	0.000 <sup>c</sup>	0.023	0.09	65.15 <sup>a</sup>
SEM	0.264	0.0056	1.87	1.25	0.23	0.377	0.244	0.286	0.0191	0.010	0.017	0.834
<i>P values</i>												
linear	0.001	0.415	0.013	0.015	0.526	0.076	0.062	0.859	0.688	0.558	0.582	0.001
quadratic	0.893	0.021	0.348	0.649	0.493	0.001	0.102	0.005	0.004	0.739	0.088	0.385
cubic	0.926	0.501	0.841	0.633	0.266	0.334	0.329	0.926	0.583	0.557	0.410	0.350

RBC - red blood cells ( $\times 10^{12}/l$ ); HMT - haematocrit ( $l/l$ ); IMG - haemoglobin (g/l); MCV - mean corpuscular volume (fl); MIIC - mean cell haemoglobin concentration (g/l); WBC - white blood cells ( $\times 10^9/l$ ); SGN - segmented neutrophils ( $\times 10^9/l$ ); LYM - lymphocytes ( $\times 10^9/l$ ); MON - monocytes ( $\times 10^9/l$ ); BAS - basophils; EOS - eosinophils; PSP - plasma protein (g/dl); <sup>a,b,c</sup> different superscripts following means within the same column indicate differences at  $P<0.05$ ; SEM - standard error of the mean

Plasma protein was increased ( $P=0.001$ ; linear effect) on days 0 and 60 continued to days 30 and 45, but it was decreased on day 30 of the experiment. No significant differences were observed in MHC, blood cells, segmented neutrophils, lymphocytes, basophils and eosinophils among days of sampling during the experiment.

## DISCUSSION

In this study, none of the treatments of EZ, SB or EZSB had any effect on blood parameters measured as an indicator for animal health. It has been shown that the use of EZ improves utilization of nutrients in the feed through its effect on their degradation and absorption. This effect was not demonstrated on blood parameters and is consistent with Salem et al. (2011a) who showed that the lambs that consumed EZ treatment did not have signs of disease with the daily addition of 10 g/lamb/day of ZADO<sup>®</sup> and increased nutrient digestibilities and average daily gain in sheep and goat. In the present study, white blood cells did not differ ( $P=0.146$ ) among treatments that could be very important results for the role of these cells in immune response innate and acquired as phagocytic cells, antigen presenting cells and cytokine-producing cells (Provenza and Villalba, 2010). These results may confirm the validity to use the SB extract as feed additive instead of the EZ preparation taking in account the other effects on animal performance. The present results of no impact of treatments on blood parameters is an important finding in the case of those lambs that consumed extract of *S. babylonica* because they contain secondary compounds which could be expected to have a detrimental effect on cellular components and protein in blood mainly because of the presence of tannins and saponins (Khalil and El-Adawy, 1994). It is known that saponins have haemolytic activity when it was used in high concentration and may cause death to animals by the affinity of the aglyconesaponins with cell membrane cholesterol (sterol) which forms a complex and increase in cellular permeability with subsequent loss of haemoglobin (Wang et al., 2007). In the present study, the dose used of secondary compounds in the extract was low therefore we expected that animals consuming extract of *S. babylonica* would increase red blood cells and also have a high amount of circulating haemoglobin in blood which did not happen and the haemoglobin values are within the range for lamb of similar age. Mahgoub et al. (2008) used unconventional feed high in phenolics and condensed tannins in sheep to evaluate their effect on the clinical profile of the animals, and they noted that a negative effect on haematocrit (Mahgoub et al., 2008). These authors also assumed that the detrimental effect of the secondary compound was dependent on the animal species that consume secondary metabolites (Provenza and Villalba, 2010).

Time of sampling had effects on red blood cells, haematocrit, haemoglobin, MCV, monocytes and plasma protein. This may be because ruminants have the ability to adapt to the consumption of plants with high content of secondary compounds such as *S. babylonica*, or EZ addition that improved ruminal microorganisms activities with increasing the time of feeding diets with EZ until 60 days (Salem et al., 2011b). Other haematological parameters such as white blood cells, segmented neutrophils, lymphocytes, basophils and eosinophils were not affected by the sampling time. This result is contradictory because *S. babylonica* secondary compounds have been shown to have effect on white blood cells and their immunostimulatory activity (Provenza and Villalba, 2010). Some studies revealed that ginsenosides and astragalussaponins strengthen the phagocytic function of the reticuloendothelial system, elevate the lymphocyte transformation rate and promote antibody formation (Liu et al., 2011). Saponin have direct effect on the immune system produced a variety of specific antigens and stimulate specific immune response (Weng et al., 2010). Addition of EZ also may be affect on the animal immune response.

## CONCLUSIONS

The exogenous enzymes and *Salix babylonica* extract, either individually or in combination had no effect on haematological parameters of lamb as an indicator health status during the 60-day experiment.

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## 7. 2. Artículo Effects of exogenous enzyme and *Salix babylonica* L. extract on Cellular immune response and its correlation with average daily weight gain in growing lambs.



Animal Nutrition and Feed Technology (2013) 13: 411-422

### Effects of Exogenous Enzymes and *Salix babylonica* L. Extract on Cellular Immune Response and its Correlation with Average Daily Weight Gain in Growing Lambs<sup>‡</sup>

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#### ABSTRACT

Rivero, N., Salem, A.Z.M., Ronquillo, M.G., Cerrillo-Soto, M.A., Camacho, L.M., Gado, H. and Peñuelas, C.G. 2013. Effects of exogenous enzymes and *Salix babylonica* L. extract on cellular immune response and its correlation with average daily weight gain in growing lambs. *Animal Nutrition and Feed Technology*, 13: 411-422.

The aim of this study was to determine the effects of exogenous enzyme (EZ) and *Salix babylonica* L. (SB) extract on cellular immune response, and its correlation with average daily weight gain (ADG). Twenty Suffolk lambs, 6-8 months of age and average live weight of  $24 \pm 0.3$  kg, were used in a trial which lasted 60 days. The lambs were distributed into 4 groups of 5 lambs each and housed in individual 1.5x1.5 m cages in a completely randomized design. The treatments were: (i) Control; lambs consuming basal diet (BD) only; (ii) EZ; lambs consuming BD plus 10g of EZ (ZADO<sup>®</sup>); (iii) SB; lambs consuming BD plus 30 mL of SB, and (iv) EZSB; lambs consuming BD plus 10g EZ and 30 mL of SB. Blood samples were collected on days 0, 15, 30, 45 and 60 and analysed for helper T lymphocytes, cytotoxic T lymphocytes, granulocytes and monocytes by flow cytometry. Treatments had no effect on parameters measured, but day of sampling had linear and cubic effects on helper T lymphocytes, granulocytes and monocytes ( $P < 0.01$ ) and cubic effects on cytotoxic T lymphocytes ( $P < 0.01$ ). The results suggest that EZ and SB have immunosuppressive effects in the first 15 days, after this effect were immunosuppressive on cytotoxic T lymphocytes and granulocytes, for monocytes the effect was immunostimulant. No there were correlation between ADG and cellular immune response in this experiment.

**Key words:** Exogenous enzyme, *Salix babylonica* L. extract, Immune response, Performance Lambs.

<sup>\*</sup>This paper is part of the special issue entitled: Exogenous Enzymes in Animal Nutrition - Benefits and Limitations, Guest Edited by A.Z.M. Salem and N. Delongue, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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**Effects of Exogenous Enzymes and *Salix babylonica* L.  
Extract on Cellular Immune Response and its Correlation with Average Daily Weight Gain in Growing Lambs<sup>2</sup>**

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**ABSTRACT**

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**Key words:** Exogenous enzyme, *Salix babylonica* L. extract, Immune response, Performance, Lambs.

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## INTRODUCTION

There is a complex tripartite interaction between malnutrition, immune system and infectious diseases (Mitchell *et al.*, 2003). Deficiency of macro- and micronutrients causes dysfunction of the immune system (Mitchell *et al.*, 2003; Chandra, 2004; Wintergerst *et al.*, 2007). These deficiencies increase the vulnerability of animals to disease which is exacerbated by malnutrition both altering defense mechanisms such as anatomical barriers, cellular and humoral immune responses against antigens (Calder and Kew, 2002; Maggini *et al.*, 2007). Thus, the animal is not capable to initiate an immune response and the infection might not be controlled (Erickson *et al.*, 2000; Enwonwu, 2006) leading the animal to under-express their productive potential.

In order to promote a more efficient use of macro and micro-nutrients contained in the diet, feed additives such as pre- and probiotics, enzymes and exogenous ionophores and fodder tree extracts have been examined in previous studies (Salem *et al.*, 2010; Salem *et al.*, 2011a; Chung *et al.*, 2012). Although there is evidence that such feed additives can directly or indirectly maintain the immunocompetence of the animal, namely by stimulating cells of the immune system or providing nutrients for these cells to function normally (Nayak, 2010; Owusu-Asiedu *et al.*, 2010).

The activity of ZADO® which is a commercial endogenous enzyme (EZ), has proved to increase the digestibility of DM, CP, NDF, ADF and the concentration of volatile fatty acids in rumen, and thus, resulting in improvements in animal productivity by increasing feed intake, ADG, feed efficiency and milk production (Arriola *et al.*, 2011; Gado *et al.*, 2011). Addition of EZ increased forage energy utilization which may have an indirect effect on the immune system under the assumptions of immune nutrition which are based in adequate utilization of dietary nutrients to promote maintenance of immunocompetence (Keith and Jeejeebhoy, 1997).

There are different types of possible measurements to investigate relationships between nutrition and immune response. Phenotyping and enumeration of immune cells by flow cytometry has proven to be accurate allowing the comparison of cell ratios and analysis of cell counts (Mitchell *et al.*, 2003). The objective was to determine effects of exogenous enzyme and *S. babylonica* L. extract on cellular immune response and to evaluate if it correlates with average daily weight gain in lambs.

## MATERIALS AND METHODS

This study was conducted at the experimental farm unit of the Faculty of Veterinary Medicine of the Autonomous University of Mexico State. The handling of animals was performed according to international bioethical standards and NOM-062ZOO-1999 (SAGARPA, 1999).

### *Animals and treatments*

Twenty Suffolk lambs, 6-8 months of age and average live weight of 24±0.3 kg, were used. Lambs were housed in individual 1.5×1.5 m cages in a completely

randomized design and the experiment was conducted for a period of 60 days. After 2 weeks of adaptation to the basal diet of 70% corn silage and 30% commercial concentrate (Purina®, Cuautitlan, Mexico) which was formulated to meet nutrient requirements (NRC, 1985), the lambs were weighed and randomly distributed into 4 groups of 5 lambs. The treatments were: (i) Control: basal diet of concentrate and corn silage; (ii) EZ: basal diet plus 10 g/day of exogenous enzyme preparations (ZADO®, Cairo, Egypt); (iii) SB: basal diet plus 30 mL/day of *Salix babylonica* L. extract, and (iv) EZSB: basal diet plus 10 g/day exogenous enzymes and 30 mL of *S. babylonica* L. extract. The daily dose of SB was given orally before the morning feeding; the EZ was mixed with 200 g of concentrate and offered 1 h before the rest to corn silage and concentrate was provided. The chemical composition of the basal diets is shown in Table 1.

Table 1. Chemical composition of the basal diet (commercial concentrate and corn silage) and levels of secondary metabolites (g/kg DM) in the *S. babylonica* L. extract

	Concentrate	Corn silage	<i>S. babylonica</i> L. extract
Chemical composition			
Dry matter <sup>7</sup>	880	360	
Organic matter	325	684	
Crude protein	157.1	106.2	
Ether extract	119.8	85.2	
Neutral detergent fiber	160.4	444.8	
Acid detergent fiber	27.7	111.2	
Lignin	7.6	18.0	
Ingredients of concentrate g/kg			
Corn grain flaked	200		
Corn grain cracked	260		
Sorghum grain	154		
Molasses sugar cane	100		
DDG	100		
Soya bean meal	96		
Weath bran	70		
NaCOO <sub>3</sub>	10		
Mineral mixture <sup>2</sup>	10		
Secondary metabolites			
Total phenolics	-	-	16.4
Saponins	-	-	5.4
Aqueous fraction <sup>3</sup>	-	-	76.3

<sup>1</sup> DM expressed as g/kg fresh silage; <sup>2</sup>Mineral mixture: Ca, 190 g/d; P, 115 g/d; Mg, 63 g/d; Cl, 167 g/d; K, 380 g/d; Na, 70 g/d; S, 53 g/d; Co, 3.3 mg/d; Cu, 197 mg/d; Fe, 360 mg/d; Mn, 900 mg/d; Se, 2 mg/d; Zn, 810 mg/d; vitamin A, 940 (1000 IU/d); vitamin D, 165 (1000 IU/d); vitamin E, 374 (1000 IU/d); <sup>3</sup>The aqueous fraction contains lectins, polypeptides and starch (Cowan, 1999).

ZADO® is a powdered multi-enzyme commercially available feed additive produced from *Ruminococcus flavefaciens* by the Academy of Scientific Research and Technology in Egypt (Patent No.: 22155, Cairo, Egypt). Prior to the study, the mixture was assayed for several enzymatic activities and found to contain (/g of enzyme preparation) 7.1 units of endoglucanase, 2.3 units of xylanase, 61.5 units of  $\alpha$ -amylase and 29.2 units of protease activity (Gado *et al.*, 2011).

In order to prepare silage, whole corn plants (at ~70% moisture) were chopped into 1 to 2 cm pieces using a forage chopper. Silage was accomplished in a flat 10 t silo and after 2 months, it was offered for feeding.

#### *Preparation of extract*

The *S. babylonica* L. extract was prepared as described by Salem *et al.* (2011b). Briefly, fresh leaves of *S. babylonica* L. were collected randomly from several young and mature trees (minimum 5 different trees) in autumn and chopped (1 to 2 cm) and immediately extracted in the proportion of 1 g leaf per 8 mL of solvent mixture, which contained 10 mL methanol (99.8/100, analytical grade, Fermont®, Monterrey, Mexico), 10 mL ethanol (99/100, analytical grade, Fermont®, Monterrey, Mexico) and 80 mL distilled water. Leaves were soaked and incubated in this solvent in the laboratory at 25 to 30°C for 48 to 72 h in closed flasks. After incubation, all flasks were incubated in a water bath at 39°C for 1 h and then immediately filtered and the filtrate collected and stored at 4°C for further use.

#### *Sampling and measurements*

Five ml of peripheral blood were withdrawn from each animal via jugular venipuncture into Heparin vacutainer tubes (BD tube, Monterrey, Mexico) kept in ice on days 0, 15, 30, 45 and 60 of the experiment. Blood was analysed to determine immune phenotypes by flow cytometry. The immune phenotypes determined were helper T lymphocytes (Ab mouse anti sheep CD4RPE/MCA2213PE), cytotoxic T lymphocytes (Ab mouse anti sheep CD8FITC/MCA2216F, D.F, Mexico), granulocytes (Ab mouse anti bovine CD11bFITC/MCA1425F, D.F, Mexico) and monocytes (Ab mouse anti human CD14RPE/MCA1568PE, D.F, Mexico) with antibodies AbDSerotec®.

The animals were weighed on the day of sampling, every 15 days. The feed offered and rejected was weighed every day. Two samples of concentrate, silage and SB extract were collected weekly and stored at -20°C for later chemical analysis. Additional samples by type (*i.e.*, concentrate, silage, extract) were pooled and stored for further analysis.

#### *Processing of samples*

Each sample was processed to determine the four immune phenotypes, initially the cellular gradient was obtained using Ficoll (Lymphoprep® BD) to recover white

blood cells as lymphocytes (T helper and cytotoxic), granulocytes (neutrophils, eosinophils and basophils) and monocytes (macrophages) (Macey, 2007).

#### *Gradient*

Blood sample and PBS 1 x were used to make a 1: 1 dilution. This mixture was placed in 3 mL of cold lymphoprep®, centrifuged at 1200 rpm at 4°C for 20 min, then the collected cells were placed in 2 mL of PBS 1x and centrifuged again at 1500 rpm at 4°C for 5 min (Macey, 2007).

#### *Sample staining*

After the cell gradient was processed, 20 µL of each sample was placed in a 96 well plate and centrifuged at 1500 rpm for 5 min. The supernatant was removed and 50 µL of the adequate antibody was incorporated (helper T lymphocytes, cytotoxic T lymphocytes, granulocytes, monocytes). After 30 min at 4°C incubation, 100 µL of PBS 1x was added, centrifuged again and the supernatant removed, while a secondary antibody was added and incubated for 30 min at 4°C. Then a final wash with PBS 1x was performed. The readings were conducted in a flow cytometer (FacsCalibur) (Macey, 2007).

#### *Chemical analyses and assays*

Samples of concentrate and silage were analysed for DM (#934.01), ash (#942.05), N (#954.01) and EE (#920.39) according to AOAC (1997) and Van Soest *et al.* (1991). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin (sa) (AOAC, 1997; #973.18) were analysed using an ANKOM 200 Fibre Analyzer Unit (ANKOM Technology Inc., Macedon, NY, USA). The NDF was assayed without use of an alpha amylase but with sodium sulphite. Both NDF and ADF are expressed without residual ash.

Plant secondary metabolites were determined using 10 mL of extract liquor and fractionated by funnel separation with a double volume of ethyl acetate (99.7/100, analytical grade, Fermont®, Monterrey, Mexico) to determine total phenolics by drying and to quantify the total phenolics layer in the funnel. After total phenolics separation, a double volume of n-butanol (99.9/100, analytical grade, Fermont®, Monterrey, Mexico) was added to fractionate saponins (Ahmed *et al.*, 1990). The remaining solution was considered to be the aqueous fraction which contains the other secondary metabolites, lectins, polypeptides and starch (Cowan, 1999; Table 1).

Endoglucanase activity was assayed by liberating glucose from carboxymethyl cellulose, which was determined calorimetrically using alkaline copper reagent as described by Robyt and Whelan (1972). One unit of endoglucanase catalyzes liberation of one mmol of glucose/min from sodium carboxymethyl cellulose at 40°C and pH 4.5. The  $\alpha$ -amylase was assayed by its ability to produce reducing groups from starch, which were measured by reduction of 3,5-dinitrosalicylic acid (Bernfeld, 1955). One unit of

$\alpha$ -amylase catalyzes liberation of one mmol of reducing groups/min from soluble starch at 25°C and pH 6.0, calculated as maltose equivalents. Protease activity was determined by hydrolysis of dimethyl casein (DMC) and liberated amino acids were determined using 2,4,6-trinitrobenzene sulfonic acid (Lin *et al.*, 1969). One DMC-U catalyzes cleavage of one mmol of peptide bond/min from DMC at 25°C and pH 7.0 expressed in terms of newly formed terminal amino groups. Xylanase catalyzes hydrolysis of xylan from oat spelt, and the reducing groups liberated were determined using alkaline copper reagent (Robyt and Whelan, 1972). One unit catalyzes liberation of one mmol reducing groups per hour from xylan at 37°C and pH 5.5, expressed as xylose equivalents.

#### *Statistical analyses*

Data related to immunological parameters were analysed using the MIXED procedure of SAS (2002) with repeated measures (Littell *et al.*, 1998). The structure of the variance-covariance error matrix employed was unstructured, based on Bayesian criteria observed with several alternative structures. Terms in the model were diet (*i.e.*, control, SB, EZ, EZSB), days of sampling (*i.e.*, 0, 15, 30, 45 and 60 of the experiment) and its linear, quadratic and cubic effects. The repeated term was sampling days, with lamb within diet as the subject. Results are reported in Tables and in text with their respective standard error of the mean. Tests of simple effects were used to partition interaction effects by diet in order to test effects of period separately for each diet using SAS. Significant differences between treatment means and time were assessed using Tukey's test at  $P < 0.05$  level. Additionally, correlation analyses between ADG and each of the four immunological parameters (*i.e.*, helper T lymphocytes, cytotoxic T lymphocytes, granulocytes and monocytes) for treatment and day of sample were performed using PROC CORR of SAS.

## **RESULTS**

#### *Effect of EZ on immunological parameters*

Treatment resulted in no differences on the four immunological parameters measured (Table 2). Addition of this additive resulted in no effects on DMI and ADG (Table 2). Similarly, no correlations between ADG and each of the immunological parameters such as helper T lymphocytes, cytotoxic T lymphocytes, granulocytes and monocytes were registered.

#### *Effect of SB extract on immunological parameters*

There were no differences among treatments due to incorporation of SB to the diets of lambs. The values of helper T lymphocytes and cytotoxic T lymphocytes increased in lambs offered SB compared to the control. Granulocytes and monocytes, however, decreased in lambs fed SB compared to the control (Table 2). The DMI and ADG were unaffected by the intake of *S. babylonica* extract (Table 2). The ADG was not correlated with helper T lymphocytes or cytotoxic T lymphocytes. The same trend

Table 2. Effects of exogenous enzyme preparation (EZ) and *S. babingtonia* L. (SB) extract as well as their mixture (EZSB) on cellular immune response parameters, dry matter intake (DMI) and average daily gain (ADG) in growing lambs after 0, 15, 30, 45 and 60 days of the experiment ( $n=5$  lambs).

	Treatment										SEM			P							
	EZ		SB		EZSB		SEM		F		Day of Experiment			SEM			P				
	Control	0	15	30	45	60	0	15	30	45	60	DM	DM	DM	DM	DM	DM	DM	DM	DM	
Helper T lymphocytes	7.54	7.97	7.75	7.65	0.323	0.81	6.59 <sup>a</sup>	5.63 <sup>a</sup>	9.30 <sup>a</sup>	9.55 <sup>a</sup>	7.55 <sup>a</sup>	9.55 <sup>a</sup>	41.0 <sup>a</sup>	44.3 <sup>a</sup>	1.04	0.89	0.42	<0.01	<0.01	<0.01	<0.01
Cytotoxic T lymphocytes	40.20	38.47	40.47	41.01	0.934	0.28	37.8 <sup>a</sup>	43.7 <sup>a</sup>	33.4 <sup>a</sup>	41.0 <sup>a</sup>	44.3 <sup>a</sup>	41.0 <sup>a</sup>	44.3 <sup>a</sup>	1.04	0.89	0.42	<0.01	<0.01	<0.01	<0.01	<0.01
Granulocytes	38.59	38.18	37.79	38.23	0.596	0.82	38.50 <sup>a</sup>	43.50 <sup>a</sup>	31.41 <sup>a</sup>	38.42 <sup>a</sup>	39.51 <sup>a</sup>	38.42 <sup>a</sup>	39.51 <sup>a</sup>	0.568	<0.01	0.06	<0.01	<0.01	<0.01	<0.01	<0.01
Monocytes	9.87	9.8	9.2	9.48	0.366	0.55	5.80 <sup>a</sup>	6.16 <sup>a</sup>	9.53 <sup>a</sup>	13.20 <sup>a</sup>	13.14 <sup>a</sup>	9.53 <sup>a</sup>	13.14 <sup>a</sup>	0.388	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.18
DMI g/d	490.4	491.61	528.84	494.04	1.65	0.96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ADG	134.91	135.72	160.54	122.31	0.4357	0.7108	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

<sup>a</sup>Different superscripts following means within the same row and experimental factor indicate differences at  $P < 0.05$ ; SB, *S. babingtonia* L. extract; EZ, exogenous enzyme; EZSB, *S. babingtonia* L. extract + exogenous enzyme.

Table 3. Pearson's correlation coefficient ( $r$ ) between average daily weight gain and cellular immune response parameters of growing lambs fed diets with addition of exogenous enzyme preparation (EZ) and *S. babingtonia* L. (SB) extract as well as their mixture (EZSB) after 15, 30, 45, and 60 days of the experiment ( $n=5$  lambs).

	Treatment										Day of experiment												
	SB		EZSB		EZ		F		T		15			30			45			60			
	r	F	r	F	r	F	r	F	r	F	r	F	r	F	r	F	r	F	r	F	r	F	
Helper T lymphocytes	0.73	0.27	-0.12	0.58	0.77	0.25	0.58	0.42	-0.59	0.94	0.94	-0.87	0.13	-0.33	0.67	0.37	0.63	0.63	0.63	0.63	0.63	0.63	0.63
Cytotoxic T lymphocytes	-0.85	0.15	-0.60	0.40	-0.30	0.70	-0.97	0.03	0.45	0.55	0.55	-0.23	0.77	0.75	0.25	-0.11	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Granulocytes	0.88	0.12	-0.73	0.27	-0.38	0.62	-0.91	0.09	-0.64	0.37	0.37	0.30	0.70	0.39	0.61	0.04	0.94	0.94	0.94	0.94	0.94	0.94	0.94
Monocytes	0.12	0.86	-0.88	0.12	0.84	0.16	-0.04	0.94	-0.71	0.29	0.29	-0.24	0.76	0.58	0.42	-0.03	0.97	0.97	0.97	0.97	0.97	0.97	0.97

SB, *S. babingtonia* L. extract; EZ, exogenous enzyme; EZSB, *S. babingtonia* L. extract+ exogenous enzyme.

was observed for granulocytes ( $r=-0.73$ ,  $P=0.27$ ) and monocytes ( $r=-0.88$ ,  $P=0.12$ ) as shown in Table 3. Helper T lymphocytes values were also not correlated to treatment EZSB ( $r=0.58$ ,  $P=0.42$ ), while they were negatively correlated to cytotoxic T lymphocytes ( $r=-0.97$ ,  $P=0.03$ ). Granulocytes tended to be negatively correlated ( $r= -0.91$ ,  $P=0.09$ ) to the same treatment.

#### *Effect of the time of sampling on immunological parameters*

The effect of time of sampling was different among treatments. Helper T lymphocytes values increased ( $P=0.01$ , linear and cubic effect), on day 30 and 45 compared with the day 15 when this value decreased. In the case of cytotoxic T lymphocytes, the values increased ( $P=0.01$ , cubic effect) on day 15, 45 and 60 and decreased on day 0 and 30. The values of granulocytes increased ( $P=0.01$ , linear and cubic effect), on day 15 compared to days 0, 30, 45 and 60 of the experiment. In accordance, monocytes values also increased ( $P=0.01$ , linear and quadratic effect) on day 45 and 60 and decreased on day 0 and 15 and 30 (Table 2). There were no correlations between some immunity parameters and ADG.

## **DISCUSSION**

#### *Effect of EZ on immunological parameters*

There is no available literature on the impact of EZ on the immunological status in ruminants and in this study addition of EZ did not produce negative effects on immunological parameters. In a previous study, researchers did not observe adverse effects on animal health when they use 10 g of EZ per lamb/day (Salem *et al.*, 2011a).

However, enzymes are not routinely used in ruminant diets because it is generally assumed the enzyme proteins would be rapidly degraded by ruminal microbes (Beauchemin *et al.*, 1995; Beauchemin *et al.*, 2003). Addition of EZ to lamb diets did not result in correlations between ADG and the helper T lymphocytes and monocytes which suggests that when the animals are able to meet their nutrient requirements, they are capable of initiating immune response against antigens (Erickson *et al.*, 2000; Mitchell *et al.*, 2003; Enwonwu, 2006).

#### *Effect of SB extract on immunological parameters*

Extract of *S. babylonica* L. did not result in a detrimental effect on immunological parameters in the present study, which was probably due to the low concentrations of secondary compounds (principally tannins and saponins) which were dosed daily to the lambs (Khalil and El-Adawy, 1994). Other studies have indicated negative effects of tannins and saponins on red and white blood cells counts resulting in anemia and immune deficiency (Adedapo *et al.*, 2005; Adedapo *et al.*, 2007; Mahgoub *et al.*, 2008). The absence of a negative effect on white blood cells in our study indicates that the SB extract is innocuous to this kind of cells. However, the same dose of SB extract used in other experiments showed anti-helminthic properties reducing intestinal worm

loads up to 40%, which represent an important effect for animal health, moreover, feed intake was not affected by extract addition as occurred in the present study (Salem *et al.*, 2011b).

We did not observe negative effects of ethanol present in the extract which in others studies has shown immunosuppressive effects by inhibition of enzymatic pathways (Hote *et al.*, 2008), therefore demonstrating that the ethanol content in the extract of *S. babylonica* L. is safe to lambs. Our results are not in accordance to those reported by Mahgoub *et al.* (2008) and Adedapo *et al.* (2007) who observed negative effects on white blood cells in sheep consuming diets rich in secondary compounds or extracts from different plants.

There were no correlations between ADG and the cytotoxic T lymphocytes, granulocytes and monocytes in SB lambs. These results suggested that when the animal presents increased immunological parameters, it cannot express its potential in production because the engulfing, presence or processing of the antigen may cause fever, emesis, diarrhea and anorexia (Grimble, 1998; Maggini *et al.*, 2007). This scenario promotes decrease in feed intake, ADG, milk production and other productive parameters (Maggini *et al.*, 2007).

There were negative ( $r=-0.97$ ,  $P=0.03$ ) correlations between ADG and cytotoxic T lymphocytes in EZSB group. This result suggests that when the animal present increased levels of such types of cells, it is processing an antigen which promotes a detrimental effect on ADG for the mechanism mentioned above. On the contrary, when the levels the cytotoxic T lymphocytes are low, the animal seems healthy which contribute to reach its maximal performance (Grimble, 1998; Maggini *et al.*, 2007).

#### *Effect of the time of sampling on immunological parameters*

The level of helper T lymphocytes in peripheral blood was linearly increased as the time of sampling advanced. These results indicate that treatments had negative effect (immunosuppressant) on helper T lymphocytes for the first fifteen days and then it had an immunostimulant effect on these cells, which remains for 30 days and then this effect began to diminish. Similar immunostimulant effects have been observed in fish treated with plant extracts and their products (Harikrishnan *et al.*, 2011). In ruminants, a period for adaptation while conducting nutrition experiments is very important, due to the fact that rumen micro-organisms require a minimum of 7 days to adapt to a diet. Thereafter, the micro-organisms begin to metabolize the dietary treatments (Van Soest, 1991). In this experiment, this time was evaluated in order to know the time it took to observe immunostimulant or immunosuppressant effects and also how long these effects remained. Mahgoub *et al.* (2008) evaluated the effect of secondary compounds at the beginning and at the end of the experiment, and they observed a decrement in the counts of this type of cells, nonetheless, this effect was weak at the end of the experiment.

The cytotoxic T lymphocytes augmented with time, and this increase was more

evident in the first 15 days. There was a more prolonged immunostimulant effect which suggests that the treatments had a positive effect on cytotoxic T lymphocytes. The effect on white blood cells is indirect although excessive ingestion of a wide variety of plants or their products (extracts) has been found to cause hypoproliferative or nonregenerative anemia. This cell disorder is characterized by reduced bone marrow production of all blood components in the absence of a primary disease process infiltrating the bone marrow or suppressing haematopoiesis. This process had a negative effect on neutrophils, lymphocytes, eosinophils, and monocytes (Adedapo *et al.*, 2007).

The time effect on granulocytes (neutrophils, eosinophils and basophils) was similar to cytotoxic T lymphocytes, with a stimulating effect on this cell type. These results may be due to the effect of extract saponins on cells and immune response (Erickson *et al.*, 2000; Harikrishnan *et al.*, 2011). Reports indicate that the function of neutrophils, eosinophils and basophils depends upon adequate consumption of macro and micronutrients; thus, malnutrition contributes to immune suppression (Mitchell *et al.*, 2003).

Regarding monocytes, our results suggest an immunostimulator effect because the animals them to 14 days have the capacity to metabolize and use any compounds presents in the diet. These results confirm that time is an important issue while feed additives are used and the period of adaptation to a particular diet as well (Van Soest, 1994).

## **CONCLUSIONS**

The results suggest that exogenous enzyme and *S. babylonica* L. extract have immunostimulant effect the first 15 days, after these feed additives have immunosuppressive effects principally on cytotoxic T lymphocytes and granulocytes. The results indicate that EZ and SB as used in this experiment did not affect cellular immune response, thus, they might be used in lamb nutrition practices promoting growth performance without altering animal health. Both feed additives produced similar results supporting the use of *Salix babylonica* L extract as a suitable alternative when people cannot access to EZ.

The effect of the treatments throughout time ought to be considered in animal nutrition experiments. Studies evaluating the immune response might favour estimations of the effect of feed additives after the initial fifteen days.

## **ACKNOWLEDGMENTS**

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**7. 3. Articulo Biochemical blood parameters in lambs fed diet in presence of mixed *Salix babylonica* extract and exogenous enzyme as feed additives in subtropical environments of Mexico.**

On Tuesday, 8 October 2013, 0:22, Tropical Animal Health and Production  
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**Biochemical blood parameters in lambs fed diet in presence of mixed *Salix babylonica* extract and exogenous enzyme as feed additives in subtropical environments of Mexico**

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Title head: *Salix babylonica* extract and exogenous enzyme as feed additives

## Abstract

The aim of this study was to evaluate the interaction impacts of *Salix babylonica* L. (SB) extract with exogenous enzyme (EZ) as feed additives on some serum biochemistry parameters in lambs in subtropical conditions of Mexico. Twenty Suffolk lambs ( $24\pm 0.3$  kg live weight and 6-8 months of age) were used during 60 days. The lambs were distributed in individual cages of 1.5x1.5 m cages into 4 treatments of 5 lambs each of completely randomized design. All animals were fed a basal diet (BD) of 70% maize silage and 30% commercial concentrate. Treatments were: (i) Control; PD without any additives; (ii) EZ; PD plus 10g of EZ; (iii) SB; BD plus 30 ml of *S. babylonica* (SB) extract; and (iv) EZSB; BD plus 10g EZ and 30 ml of SB extract. Blood samples were collected on days 0, 15, 30, 45 and 60 and analyzed for total proteins, glucose, creatinine, blood urea N (BUN), alanin aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), calcium, phosphorus and magnesium. Interactions between treatmentxtime were occurred in creatinine ( $P=0.040$ ), BUN ( $P=0.004$ ) and magnesium ( $P=0.013$ ). The highest values of total proteins were obtained with Control and SB animals *versus* EZ or EZSB. The SB treatment tended to increased ( $P=0.057$ ) the levels of creatinine *versus* other treatments. The ALP level was highest ( $P=0.043$ ) in SB animals. In conclusion, supplementation with *S. babylonica* extract, exogenous enzymes and/or their combination did not cause any major health disorders in lambs within 60 days of experiment under the subtropical conditions of Mexico.

**Key words:** *Salix babylonica* extract; exogenous enzyme; biochemical parameters; lambs.

## Introduction

The concept of clean green and ethical' animal production has been promoted to address societal demands for agricultural products which are produced with fewer chemical inputs ('clean'), less negative impacts on the environment ('green') and greater care of the animals ('ethical'), these concepts had modified the livestock production systems (Durmic and Blache, 2012). This situation compiled nutritionists to search for natural strategies of low cost and easy to apply; plants and plant extracts are the best solution for alternatives to synthetic drugs based on their potent properties and complex bioactivity (Lu, 2011; Durmic and Blache, 2012). The use of plants or its extracts in herbivores is restricted by its secondary compounds content (simple phenolic compounds or condensed tannins, saponins or essential oils rich in terpene) as there are inverse relationships between secondary compounds level in forage and palatability, voluntary intake, digestibility and N retention. Although it can cause damage in liver, kidney and digestive tract, these secondary compounds may benefit animal health and production when they are fed in the correct form and dose (Silanikove *et al.*, 1996; Acamovic and Brooker, 2005; Valizadeh *et al.*, 2010; Lu, 2011; Vasta and Luciano, 2011; Salem *et al.*, 2011b; Durmic and Blache, 2012). If the animals were fed some plants containing high levels of antinutritional compounds for extended periods, detrimental effects on animal health and reduced ability to withstand been reported (Mahgoub *et al.*, 2008a; Olafadehan, 2011).

The use to exogenous enzymes extracted from ruminal microorganisms in ruminant feeds as by-products, forage, low quality fibrous feeds with a poor nutritive value, reduced the use of antibiotics in the diets and may decrease the pollution of atmosphere because decrease the CH<sub>4</sub> production (Arriola *et al.*, 2011; Bedford and Cowieson, 2012).

On the other hand, the use of exogenous enzymes has positive relation with palatability, feed intake, rumen microbial N synthesis, digestibility, and improve animal performance as milk production, live weight gain, feed efficiency and immunity (Arriola *et al.*, 2011; Gado *et al.*, 2011; Salem *et al.*, 2011a; Bedford and Cowieson, 2012).

It's well known that normal physiological processes are affected long before the death of an organism; hence there is a need to check physiological and biochemical indicators of health. One of the fastest means of ascertaining toxicity of ingested feed in animals is by the assessment of their blood. Blood contains diagnostically relevant parameters which act as a pathological reflector of the status of animals exposed to toxicants (Olafadehan, 2011). The objective of this study was to evaluate the effects of *Salix babylonica* L. (SB) extract, exogenous enzyme (EZ) and their combination on some serum biochemistry parameters in sheep.

## **Material and methods**

This study was conducted at the experimental farm of the Faculty of Veterinary Medicine and livestock of the Autonomous University of Mexico State. The handling of animals was done according to international bioethical standards and NOM-062 - ZOO-1999.

### **Animals and treatments**

Twenty Suffolk lambs of 6 to 8-months-old and  $24 \pm 0.3$  kg body weight (BW) were used in this study. The lambs were housed in individual cages (1.5×1.5 m) in a completely randomized design for 60 days. After 2 weeks of adaptation to the basal diet (BD) of 70% maize silage and 30% commercial concentrate (Purina<sup>®</sup>, Cuautitlan, Mexico) formulated according to NRC (2007) nutrient requirements; the lambs were weighed and randomly distributed into 4 groups of 5 animals each. The treatments were: (i) Control; BD only without any additive; (ii) EZ; BD plus 10g of EZ (ZADO<sup>®</sup> Cairo, Egypt); (iii) SB; BD plus 30ml of *S. babylonica* extract, and (iv)

EZSB; BD plus 10g EZ and 30 ml of SB extract. The daily dose of SB extract was given orally before the morning feeding; the EZ was mixed with a 200g of concentrate and offered 1h before of provide the rest to corn silage and concentrate the rest of the day. The chemical composition of the basal diets is presented in Table 1.

ZADO<sup>®</sup> is a powdered multi-enzyme commercially available feed additive product produced from *Ruminococcus flavefaciens* by the Academy of Scientific Research and Technology in Egypt (Patent No. 22155, Cairo, Egypt). Prior to this work, the enzyme mixture was assayed for several enzymatic activities, and it was found to contain (per gram of enzyme preparation) 7.1 unit of endoglucanase, 2.3 unit of xylanase, 61.5 unit of  $\alpha$ -amylase and 29.2 unit of protease activity.

#### Preparation of silage

Whole maize plants (at medium stage with about 70% moisture content) were chopped into 1-2 cm lengths using a forage chopper. Chopped maize was immediately filled in a flat 10-ton silo. After two months, experimental animals were initiated to be fed the silage as well as the other animals of the experimental farm of the faculty.

#### Preparation of extract

The *S. babylonica* extract was prepared as described by Salem et al. (2011b). Briefly, fresh leaves of *S. babylonica* were collected randomly from several young and mature trees (minimum 5 different trees) in autumn and were chopped (1-2 cm) and immediately extracted in the proportion of 1g leaf per 8ml of solvent mixture. The mixture of solvents contained 10ml methanol, 10 ethanol ml and 80 ml distilled water. Leaves were soaked and incubated in solvent in the laboratory at 25-30°C for 48-72 h in closed flasks. After incubation, all flasks were incubated in a

water bath at 39°C for one h and then immediately filtered, and the filtrate collected and stored at 4°C for further use.

### Sampling and measurements

Five mL of peripheral blood was collected from each lamb by jugular venipuncture in red vacutainer tubes (BD tube, Monterrey, Mexico) on days 0, 15, 30, 45 and 60 of the experiment. The samples were conserved at room temperature until processing, after were centrifuged at 1500 rpm for 10 min for serum extraction which was stored in 1.5 ml eppendorf tubes at -20°C according to groups for further biochemical analysis.

Samples of both concentrate and silage were also collected during the experimental period and stored at -20 C for later chemical analysis.

Two samples of the concentrate, silage and SB extract were collected weekly during the 60 days of experiment. Each sample type (*i.e.*, concentrate, silage or extract) were pooled and stored for further analysis.

### Sample analysis

The choice of biochemical measurements is that conventionally used for diagnosing human and domestic animal hepatic and kidney damage, and general metabolic disorders (Silanikove et al., 1996). The serum samples were analyzed using specific kits of IL<sup>TM</sup> tests for total proteins (#0018481300), glucose (#0018480000), creatinine (#0018480900), blood urea N (BUN;#0018480400), alanine aminotransferase or glutamic pyruvate transaminase (ALT/GPT; #0018480700), aspartate aminotransferase or glutamic oxalacetic transaminase (AST/GOT) (#0018480800), alkaline phosphatase (ALP) (#0018480600), calcium (#0018258840), phosphorus (#0018481900) and magnesium (cat. #0018481600).

All metabolites were determined using spectrophotometry analysis using a BTS 350 Chemistry System analyzer (Instrumentation Laboratory, Mexico).

Samples of concentrate and silage were analyzed for DM (#934.01), ash (#942.05), N (#954.01), and ether extract (EE; #920.39) according to AOAC (1997). The neutral detergent fiber (NDF; Van Soest *et al.*, 1991), acid detergent fiber (ADF) and lignin (AOAC, 1997; #973.18) were analyzed using an ANKOM 200 FibreAnalyser Unit (ANKOM Technology Corporation, Macedon, NY, USA). The NDF was assayed without use of  $\alpha$ -amylase but with sodium sulphite in the NDF. Both NDF and ADF are expressed without residual ash.

Plant secondary metabolites of extract were determined using 10 mL of extract liquor and fractionated by funnel separation with a double volume of ethyl acetate (99.7/100, analytical grade, Fermont<sup>®</sup>, Monterrey, Mexico) to determine total phenolics by drying and to quantify the total phenolics layer in the funnel. After total phenolics separation, a double volume of n-butanol (99.9/100, analytical grade, Fermont<sup>®</sup>, Monterrey, Mexico) was added to fractionate saponins (Ahmed *et al.*, 1990). The remaining solution was considered to be the aqueous fraction which contains the other secondary metabolites lectins, polypeptides and starch (Cowan, 1999) Table 1.

Endoglucanase activity was assayed by liberating glucose from carboxymethyl cellulose, which was determined calorimetrically using alkaline copper reagent as described by Robyt and Whelan (1972). One unit of endoglucanase catalyzes the liberation of 1 mmol of glucose per min from sodium carboxymethyl cellulose at 40°C and pH 4.5. Furthermore,  $\alpha$ -amylase was assayed by its ability to produce reducing groups from starch, which were measured by the reduction of 3,5-dinitrosalicylic acid (Bernfeld, 1955). One unit of  $\alpha$ -amylase catalyzes the liberation of 1 mmol of reducing groups per min from soluble starch at 25°C and pH 6.0, calculated as maltose equivalents. Protease activity was determined by the hydrolysis of dimethyl casein (DMC) and the liberated amino acids were determined using 2,4,6-trinitrobenzene sulphonic acid (Lin *et al.*, 1969). One DMC-U catalyzes

the cleavage of 1 mmol of peptide bond per min from DMC at 25°C and pH 7.0 expressed in terms of newly formed terminal amino groups. Xylanase catalyzes the hydrolysis of xylan from oat spelt, and the reducing groups liberated were determined using alkaline copper reagent (Robyt and Whelan, 1972). One unit catalyzes the liberation of 1 mmol reducing groups per h from xylan at 37°C and pH 5.5, expressed as xylose equivalents.

### Statistical analyses

Data were analyzed using the MIXED procedure of SAS (2002) with repeated measures (Littell *et al.*, 1998). Terms in the model were diet (*i.e.*, control, SB, EZ, EZSB), days of sampling (*i.e.*, 0, 15, 30, 45 and 60 d of the experiment). Results reported in tables and in text with their corresponding standard errors of the mean. Tests of simple effects were used to partition (slice) interaction effects by treatment in order to test effects of period separately for each diet and the interaction treatment  $\times$  time (SAS, 2002). The significant differences between treatment means, time and interaction treatment  $\times$  time were determined with a value to significance of  $P < 0.05$ .

### Results

Interactions between treatment  $\times$  time were observed on the levels of creatinine ( $P = 0.0396$ ), BUN ( $P = 0.004$ ) and magnesium ( $P = 0.013$ ). No interactions were observed for the other measured parameters. Treatments of SB, EZ, and SBEZ had not affected blood glucose, creatinine, BUN, AST, ALP, and phosphorus content, while the levels of total proteins were modified. The highest values total proteins were obtained with control and SB animals. The SB treatment tended to increase ( $P = 0.057$ ) the levels of creatinine *versus* other treatments. For ALT, the highest ( $P = 0.043$ ) value was observed in SB animals. Control and SB had increased ( $P = 0.007$ ) blood calcium content, while EZ treatment increased ( $P = 0.023$ ) the level of magnesium *versus* other groups.

The sampling time produced significant differences in all measured parameters. At day 0, the lowest concentrations of glucose, ALT, AST, ALP, calcium, phosphorus and magnesium were observed in control animals *versus* other group. The same results were observed with total proteins, ALT, and calcium for SB groups; BUN, and ALT for EZ group; ALP for SBEZ group. Total proteins, glucose, Creatinine and urea had the highest concentrations in days 0 and 15 (P=0.018), 15 and 30 (P=0.001), 30 and 45 (P=0.001), 30 and 60 (P=0.001). Enzymes of kidney and liver functions were also had the highest values in 30 and 60 (ALT); 0 and 30 (AST); 30 and 45 (ALP). Serum calcium, phosphorus and magnesium were highest in days 60; 45 and 60; 30 and 60, respectively.

## **Discussion**

The objective of our study was analyzed the effect of *S. babylonica* extract, exogenous enzymes preparation (ZADO<sup>®</sup>) and their combination on some serum parameters, and that all experiment the animals were in good conditions and had no signs of ill health. Serological data were used as an indication of health status of the experimental animals.

For total proteins, the reference values are between 60- 75 g/L a hypoproteinemia was observed in all treatments. Values lower than reference range is dangerous because the role of protein in numerous physiologic process as integrity of body tissues, enzymes, hormones, carriers for others plasma constituents, etc. (Smith, 2007), there are reports to indicate that total proteins and albumin can be lowered in the case of infection by blood-spoiling parasites, such as *Haemonchus contortus*, but in this case the values decreasing until 15g/L (Braun *et al.*, 2010) and our values no were lower to about 50 g/L. Serum total proteins reflect the nutritional status of the animal, and it has a positive correlation with dietary protein (Kumar *et al.*, 1980). So, the observed decreased blood total proteins may be due to the decreased protein intake as the dietary protein affected the concentration of

plasma protein especially when animals were fed low protein diets (Allam *et al.*, 2009; Rowlands, 1980).

The level of total proteins is the highest in the group SB this results indicate that tannins presents in the extract has a protector effect on protein. Tannins have an ability of to form strong insoluble complexes with proteins that are resistant to stomach pH, where tannins have a large number of free phenolic hydroxyl groups that form strong hydrogen bonds with proteins and carbohydrates. When animals fed on tannin-rich forages, tannin-protein complexes can reduce the digestion of forage protein (Imik *et al.*, 2008; Mahgoub *et al.*, 2008b; Lu, 2011; Durmic and Blache, 2012).

In the others two groups of EZ and SBEZ, the levels of serum total proteins were statistically lower. ZADO<sup>®</sup> contained about 29.2 unit/g to proteases (Gado *et al.*, 2011, Salem *et al.*, 2011a) that was not sufficient to increase the quantity of crude protein ingested by the lambs of this group as the quantity of protein in the diet is relation with the plasma protein concentration (Smith, 2007) we didn't observed any increase in this parameter. However, in the group SBEZ the lowered value of serum total proteins may be due to the antagonist effect between exogenous enzyme and secondary compounds, because the enzyme decreasing the activity of secondary compounds as shown the study of Gado *et al.* (2011) in which all secondary metabolites in the orange pulp were reduced with the addition of ZADO<sup>®</sup> during its ensiling. Salem *et al.* (2011) add 10 g of ZADO<sup>®</sup>/animal/day to the diet of lambs as a feed additives and observed an increased diet crude protein content but did not determined the total proteins concentration in serum (Salem *et al.*, 2011a).

Results of serum total proteins levels were different to other studies in which the effect of secondary compounds had been evaluated, and in the which the authors didn't observed any increase in this parameter when sheep or goats were feed with diets with high levels of secondary compounds or when the animals ingest this

compounds as a feed additives (Silanikove *et al.*, 1996; Imik *et al.*, 2008; Mahgoub *et al.*, 2008a; Valizadeh *et al.*, 2010; Olafadehan, 2011). It may be due to the kind of forage used, concentration of secondary compounds, age of the animals and adaptation time, etc. Ramirez- Restrepo (2010a,b) conducted an experiment with *Salix* spp. as a fodder blocks in sheep and didn't observed changes in the levels of serum total proteins but he demonstrated the stimulate effect of condensed tannins in this forage on cell-mediated immune response.

The serum creatinine is no conditioner by the diet. Its concentration in serum provides a measure of glomerular filtration rate in ruminants. This parameter is an indicator of renal failure; small increases in creatinine may be see with progressively compromised renal function (Turner *et al.*, 2005; Ford and Mazzaferro, 2007; Smith, 2007). In the present study, the levels of creatinine were within the normal range (76-176.8  $\mu\text{mol/L}$ ) reported for healthy sheep (Ford and Mazzaferro, 2007; Smith, 2007), but were higher in SB group which indicate that secondary compounds (phenols and condensed tannins) presents in the extract were negatively affected glomerular filtration, causing renal damage (Silanikove *etal.*, 1996, Mahgoub *et al.*, 2008b, Olafadehan, 2011, Durmic and Blache, 2012). Increasing in protein intake had no effect on plasma creatinine concentration but it can be positively related to the turnover of the protein pool in ruminants (Turner *et al.*, 2005). Mahgoub *et al.* (2011) didn't observed any changes in this parameter when provided feeds that contained phenols and condensed tannins to sheep, but they observed a decrease in blood urea nitrogen. This is associated with liver failure. The same author observed a damage functional and structural in gut, liver and kidney of sheep fed non- conventional feeds containing phenols and condensed tannins (Mahgoub *et al.*, 2008a; Mahgoub *et al.*, 2008b).

In our experiment, BUN was not modified with the treatments. It may be reduced slightly in animals given anabolic steroids or fed diets with low protein content but adequate caloric content. Changes in both BUN and creatinine can be associate to renal failure (Smith, 2007; Mahgoub *et al.*, 2008a; Braun *et al.*, 2010). Although

generally BUN tends to be decreased in ruminants fed diets with low protein content or with severe liver disease (Mahgoub *et al.*, 2008a).

Liver ALT enzyme are used to assess liver damage (Mahgoub *et al.*, 2008a). In the present study, its levels were in normal range (15-40 u/L) as reported by Ford and Mazzaferro (2007) and Smith (2007). The higher levels of ALT in SB group indicating that secondary compound presented in SB caused moderate damage in liver since high values in serum indicate hepatocyte injury and intracellular enzymes output (Ford and Mazzaferro, 2007; Mahgoub *et al.*, 2008a,b; Olafadehan, 2011). It's possible that this negative effect can be caused by the ethanol and methanol in the extract (Lieber, 1990).

For mineral as calcium, phosphorus and magnesium, there are a little investigates in sheep. In our experiment, calcium values were in the normal range (2.3-2.9mmol/L) for healthy sheep. This electrolyte maintain neuromuscular excitability, permeability of cells membranes, conduction of nerve impulses muscle contraction and blood clotting (Ford and Mazzaferro, 2007; Smith, 2007). Renal excretion, intestinal absorption, mobilization from large bones and its concentration in the diet, all regulate its concentration in serum (Ford and Mazzaferro, 2007; Smith, 2007; Braun *et al.*, 2010). In the present study, calcium was higher in Control group with no significant differences with SB group. It is a good results as secondary compounds in the extract did not produced negative effects on calcium metabolism as occur in the experiment of Olafadehan (2011) who reported a decreased calcium in serum when the goats were fed with *Pterocarpus erinaceus* rich in tannins.

In the case of magnesium, its known that its concentration in serum are influenced by food supply (Ford and Mazzaferro, 2007; Smith, 2007). The reported that the normal range is about 0.8 – 1.15 mmol/L. In the present study, its levels in blood were higher compared with the normal range in the treatment EZ but not in the treatments of Control, SB and SBEZ. These results are apposite to results of

Olafadehan (2011), who observed an increase in serum concentration of magnesium in goats fed on *Pterocarpus erinaceus*. In our study SB did not produced a significant increase or decrease in serum magnesium concentration. Our results of serum Ca and magnesium content confirm that tannins presents in *S. babylonica* extract did not produced any changes in absorption of Ca and magnesium from the gastrointestinal tract with no signs of mineral depletion (Silanikove *et al.*, 1996; Mahgoub *et al.*, 2008b; Olafadehan, 2011). The differences between our results and results of other studies fed diets rich in secondary compounds for serum concentration of Ca and Mg maybe due to kind of plant used, concentration of secondary compound, age of animals, etc (Mahgoub *et al.*, 2008b).

The time produced significant differences in all measured parameters for all treatments; but there are no studies in which the time had been consider. It's possible that these differences can be associated with the time of adaptation required for ruminant microorganism and cells for detoxifying, metabolizing and using the diet compounds and drugs (Durmic and Blache, 2012).

There were interaction between time and treatment for creatinine, BUN and magnesium. These differences indicated that treatments can cause a damaged renal if used for long time. Damage of kidney would most likely lead to renal failure and to changes in serum urea and creatinine and minerals concentration (Silanikove *et al.*, 1996; Olafadehan, 2011).

## **Conclusions**

The absence of clinical signs of ill health, toxicity symptoms suggest that secondary compounds presented in *Salix babylonica* extract were well tolerated by the sheep. The results indicated than exogenous enzyme had no negative effects on serum parameters. Although, *Salix babylonica* extract produced some significant negative effects, the parameters in which significantly differed were in

normal range. It was thus concluded that the supplementation with *Salix babylonica* extract, exogenous enzymes and their mix did not cause any major health disorders when these fed for more than 60 days in lambs under the hot environment of Mexico.

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**Table 1.** Chemical composition of the basal diet (commercial concentrate and corn silage) and levels of secondary metabolites (g/kg DM) in the *S. babylonica* extract.

	Concentrate	Corn silage	<i>S. babylonica</i> L. extract
<b>Chemical composition</b>			
Dry matter <sup>1</sup>	880	360	
Organic matter	325	684	
Crude protein	157	106	
Ether extract	120	85	
Neutral detergent fiber	160	445	
Acid detergent fiber	28	111	
Lignin	8	18	
<b>Ingredient of concentrate g/kg</b>			
Corn grain flacked		200	
Corn grain cracked		260	
Sorghum grain		154	
Molasses		100	
Dried distillers grains with solubles		100	
Soya bean meal		96	
Wheat bran		70	
NaCO <sub>3</sub>		10	
Mineral mix <sup>2</sup>		10	
<b>Secondary metabolites</b>			
Total phenolics			16.4
Saponins			5.4
Aqueous fraction <sup>3</sup>			76.3

<sup>1</sup>DM expressed as g/kg fresh silage.<sup>2</sup>Mineral mixture: Ca, 190 g/d; P, 115 g/d; Mg, 63 g/d; Cl, 167 g/d; K, 380 g/d; Na, 70 g/d; S, 53 g/d; Co, 3.3 mg/d; Cu, 197 mg/d; Fe, 360 mg/d; Mn, 900 mg/d; Se, 2 mg/d; Zn, 810 mg/d; Vit.A, 940(1000 IU/d); Vit.D, 165 (1000 IU/d); Vit.E, 374 (1000 IU/d).<sup>3</sup> The aqueous fraction contains lectins, polypeptides and starch (Cowan, 1999)

**Table 2.** Serum biochemistry parameters in growing lambs treated with *S. babylonica* L. (SB), exogenous enzyme preparation (EZ) and their mixture (EZSB) sampled the days 0, 15, 40, 45 and 60 of the experiment

Treatment	Time	Protein, g/L	Glucose, mmol/L	Creatinine, μmol/L	BUN, mmol/L	ALT, U/L	AST, U/L	ALP, U/L	Calcium, mmol/L	Phosphorus, mmol/L	Magnesium mmol/L
<b>Control</b>	0	64.38	0.6	100.0	3.3	13.2	52.4	179.2	2.48	1.97	0.99
	15	61.10	2.6	88.18	2.6	14.4	59.6	224.4	2.86	2.33	1.10
	30	58.92	2.6	107.40	3.1	15.8	65.4	295.0	2.48	2.26	1.21
	45	55.72	2.8	104.20	2.3	15.6	59.2	310.0	2.78	2.66	1.24
	60	53.14	1.4	107.52	3.2	20.4	53.0	224.4	2.66	2.63	1.28
<b>Mean</b>		58.65 <sup>a</sup>	2.0	101.48 <sup>b</sup>	2.93	15.88 <sup>a</sup>	57.92	246.6	2.65 <sup>a</sup>	2.37	1.16 <sup>b</sup>
<b>SB</b>	0	57.88	1.4	118.84	3.2	14.8	55.8	220.6	2.44	2.39	1.04
	15	60.88	2.8	101.40	2.7	15.8	59.2	248.0	2.64	2.4.0	1.07
	30	61.74	3.0	120.98	3.7	19.2	71.0	328.4	2.44	2.28	1.46
	45	58.78	2.6	120.56	2.8	18.6	65.4	309.6	2.58	2.72	1.25
	60	59.38	0.4	110.66	6.7	20.8	55.4	261.8	2.74	2.57	1.09
<b>Mean</b>		59.70 <sup>a</sup>	2.04	114.49 <sup>a</sup>	3.86	17.84 <sup>a</sup>	61.33	273.68	2.56 <sup>a</sup>	2.47	1.18 <sup>b</sup>
<b>EZ</b>	0	55.08	1.4	104.78	2.6	12.6	55.4	209.8	2.22	2.10	1.00
	15	59.92	2.8	97.42	4.4	14.8	54.2	232.4	2.34	2.51	1.50
	30	54.26	2.6	112.06	4.0	15.8	64.2	277.8	2.34	2.23	1.35
	45	50.96	2.2	112.72	3.2	15.0	55.4	296.2	2.38	3.02	1.41
	60	47.56	0.8	101.2	4.3	16.4	58.0	301.6	2.62	3.06	1.61
<b>Mean</b>		53.55 <sup>b</sup>	1.96	105.64 <sup>b</sup>	3.74	14.92 <sup>b</sup>	57.44	263.56	2.38 <sup>b</sup>	2.58	1.37 <sup>a</sup>
<b>SBEZ</b>	0	55.88	1.2	91.40	2.4	17.0	97.6	200	2.16	2.03	0.92
	15	54.3	2.8	94.36	3.8	9.8	41.8	234.4	2.34	2.52	1.14
	30	54.74	2.8	103.44	3.7	13.6	58.2	308.6	2.14	2.73	1.31
	45	52.16	2.6	107.78	2.7	11.8	49.4	341.0	2.40	2.78	1.12

	60	54.92	1.0	110.78	4.0	12.8	49.8	333.2	4.60	2.98	1.31
<b>Mean</b>		54.4 <sup>b</sup>	2.08	101.47 <sup>b</sup>	3.36	13 <sup>b</sup>	59.36	283.44	2.32 <sup>b</sup>	2.61	1.16 <sup>b</sup>
<b>SEM Pooled</b>		0.168	0.0478	0.388	0.0499	0.1191	0.6937	2.56	0.0254	0.0165	0.0086
<b>P-value</b>											
<b>Treatment</b>		0.0456	0.9623	0.0572	0.2804	0.0426	0.9806	0.8041	0.0066	0.3983	0.0227
<b>Time</b>		0.0177	0.0001	0.0001	0.0001	0.0082	0.0213	0.0001	0.0002	0.0001	0.0001

SB, *S. babylonica* L. extract; EZ, exogenous enzyme; EZSB, *S. babylonica* L. extract + exogenous enzyme; BUN; blood urea nitrogen, ALT, alanine aminotransferase or glutamic pyruvate transaminase; AST, aspartate aminotransferase or glutamic oxalacetic transaminase; ALP, alkaline phosphatase; SEM, Standard error of the mean.<sup>a, b</sup>Different superscripts following treatments mean in the column indicate differences at  $P < 0.05$ .

#### **7.4. Artículo Haematological and biochemical profile in lambs and goats that consumed *Salix babylonica* extract, exogenous enzyme and their combination as feed additives.**

##### **Haematological and biochemical profile in lambs and goats that consumed *Salix babylonica* extract, exogenous enzyme and their combination as feed additives.**

#### **ABSTRACT**

A study was conducted to investigate the effect of exogenous enzyme (EZ), *Salix babylonica* (SB) extract and their combination as feed additives on haematological and biochemical profile in lambs and goats. Four suffolk lambs of 3 to 4-months-old with 24 kg body weight (BW) and four saanen goats of 5 to 8-months-old with 20 BW were used. After 2 weeks of adaptation to the basal diet; the animals were weighed and randomly distributed into 4 groups of one animal (lambs or goats). The animals were housed in metabolic cages in a completely randomized design and the experiment was conducted in four periods of 15 days (60 days). The treatments were: 1. control: animals were fed the basal diet plus 30ml of distilled water, 2. EZ: lambs were fed the basal diet plus 1 $\mu$ l/g/DM intake of xylanase (Dyadic International Inc. Jupiter, FL) in 30ml of distilled water 3. SB: lambs were fed the basal diet plus 30 ml of *Salix babylonica* extract, and 4. EZSB: lambs were fed the basal diet plus 1 $\mu$ l/g/DM intake of xylanase (Dyadic International Inc. Jupiter, FL) in 30ml of distilled water and 30 ml of *S. babylonica* extract. The daily dose of treatments was given orally before the morning feeding. Five milliliters of blood samples were collected on day 15 of each period and analyzed for haematological and biochemical profile. The treatments has effects on HMT ( $p = 0.0259$ ) in lambs and eosinophils ( $p = 0.0191$ ) and glucose ( $p = 0.0334$ ) in goats. Since all the studied blood parameters were within the normal range for healthy goats and there wereno signs of disease, it is concluded that EZ, SB and their combination did not pose any threat to the health of the animals under the conditions of the experiment both feed additives are innocuous.

**Key words:** haematological and biochemical profile, lambs, goats, exogenous enzyme, *S. babylonica* extract.

## **INTRODUCTION**

In recent years the use of feed additives has been increased because with its use i) decrease the cost of alimentation, which upon more than 50% the cost of production, 2) enhance productive parameters 3) improve health and well-being animal (Salem et al., 2006, Salem et al., 2010, Provenza and Villalba, 2010, Arriola et al., 2011, Lu, 2011, Chung et al., 2012).

Exogenous enzyme has positive effects improve feed utilization and animal performance by enhancing fiber degradation, increase live weight gain (16%) by increase in to nutrients digestibility, fibrolytic enzyme increased the digestibility of DM, CP, neutral detergent fiber, and acid detergent fiber and the efficiency of milk production in dairy cows (Arriola et al., 2011, Salem et al., 2013 ). The exogenous enzyme has positive relation with palatability, feed intake, rumen microbial N synthesis, digestibility and improve animal performance as milk production, live weight gain, feed efficiency and some enzyme products has potential immunostimulatory (Arriola et al., 2011, Gado et al., 2011, Salem et al., 2011a, Bedford and Cowieson, 2012). Its function is dependent the specific activity of the enzymes, their mode and level of application, as well as the type of animal and its diet, may affect enzyme efficacy (Salem et al., 2013 )

Secondary metabolites presents in *Salix babylonica* extract at low doses and in appropriate mixtures can have beneficial effects on animal nutrition and health, at high doses can negative effects on metabolic and cellular process, reduce forage intake cause weight loss and in some cases the death in animals intolerance or with any physiological damage (Provenza and Villalba, 2010).

In animal production secondary metabolites have positive effects on daily gain, voluntary intake, milk production and ruminal fermentation parameters, have a

protective effect on the protein in the rumen to promote duodenal absorption, minimizes the excretion of nitrogen, modify the acetate to propionate ratio in rumen fluid and decrease parasitic load (Athanasiadou and Kyriazakis, 2004, Rivero et al., 2012).

There are studies that indicate that secondary metabolites can cause anemia by destruction of erythrocytes, decrease in white blood cells, damage in liver and kidney even death if consumed for a considerable time at high doses (Mahgoub et al., 2008a, Mahgoub et al., 2008b).

On the other hand there are jobs that indicate that secondary metabolites present in a big diversity of plants do not produce negative effects on animal health principally in goats because it is possible that these have the ability to avoid consuming browse in amounts exceeding their capacity to detoxify and it has enhanced capacity to detoxify tannins in comparison with other ruminant species (Silanikove et al., 1996, Olafadehan, 2011).

Blood contains diagnostically relevant parameters which act as a pathological reflector of the status of animals exposed to toxicants and is the fastest means for ascertaining toxicity of ingested feed in animals (Olafadehan, 2011). In the present experiment haematological and biochemical data were used as an indication of health status of the experimental animals.

## **MATERIAL AND METHODS**

This study was conducted at the experimental farm and Research and Advanced Studies in Animal Health of the Faculty of Veterinary Medicine of the Autonomous University of Mexico State. The handling of animals was done according to international bioethical standards and NOM-062 - ZOO-1999.

## Animals and treatments

The animals used in this study were four suffolk lambs of 3 to 4-months-old with 24 kg body weight (BW) and four saanen goats of 5 to 8-months-old with 20 BW. After 2 weeks of adaptation to the basal diet, formulated according to NRC (National Research Council. Committee on Nutrient Requirements of Small, 2007) nutrient requirements; the animals were weighed and randomly distributed into 4 groups of one animal (lambs or goats). The animals were housed in metabolic cages in a completely randomized design and the experiment was conducted in four periods of 15 days (60 days). The treatments were: 1. control: animals were fed the basal diet plus 30ml of distilled water, 2. EZ: lambs were fed the basal diet plus 1µl/g/DM intake of Dyadic (International Inc. Jupiter, FL) in 30ml of distilled water, 3. SB: lambs were fed the basal diet plus 30 ml of *Salix babylonica* extract, and 4. EZSB: lambs were fed the basal diet plus 30 ml of exogenous enzyme preparation and 30 ml of *S. babylonica* extract. The daily dose of treatments was given orally before the morning feeding. The chemical composition of the basal diets is presented in (Table 1).

## Preparation of extract

The *S. babylonica* extract was prepared as described by Salem et al. 2011(Salem et al., 2011b). Briefly, fresh leaves of *S. babylonica* were collected randomly from several young and mature trees (minimum 5 different trees) in autumn and were chopped (1-2 cm) and immediately extracted in the proportion of 1g leaf per 8 ml of solvent mixture. The mixture of solvents contained 10ml methanol (99.8/100, analytical grade, Fermont®, Monterrey, Mexico), 10 ethanol ml (99/100, analytical grade, Fermont®, Monterrey, Mexico) and 80ml distilled water. Leaves were soaked and incubated in solvent in the laboratory at 25-30°C for 48-72 h in closed flasks. After incubation, all flasks were incubated in a water bath at 39°C for one h

and then immediately filtered and the filtrate collected and stored at 4°C for further use.

#### Sampling and measurements

Five milliliters of blood samples were collected from each animal in plain glass vacutainers or with EDTA (BD tube, Monterrey, Mexico) by jugular venipuncture on day 15 of each period. The samples collected in glasses with EDTA were maintained to cooling temperature and the collected in plain vacutainers were conserved at room temperature until it processing, after were centrifuged to 1500 rpm for 10 min and extracted the serum which was stored in eppendorf tubes the 1.5ml to -20°C according to groups for further biochemical analysis.

Samples of diet were also collected during the experimental period and stored at -20 C for later chemical analysis.

Two samples of the diet and SB extract were collected weekly during the 60 days of experiment. Each sample of diet or extract were pooled and stored for further analysis.

#### Sample analysis

The samples collected in glasses with EDTA were analyzed for red blood cell count (RBC), the differential white cell count: neutrophils, lymphocytes, basophils, eosinophils and monocytes, packed cell volume (PCV), hemoglobin (HMG), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC), and plasma protein concentration. Haemoglobin concentration was determined using the cyanmethaemoglobin method. Haematocrit was determined by microhaematocrit technique. Erythrocyte and total leucocytes and differential leucocyte counts were determined using the haemocytometer method. Total plasma proteins were determined using a refractometer (Archer and Jeffcott, 1977). Mean corpuscular volume (MCV) and

MCHC (Mean Corpuscular Hemoglobin Concentration) were calculated to diagnose any type of anaemia by taking into account the values of erythrocytes, haemoglobin and haematocrit predetermined (Coffin et al., 1959, Smith, 2007).

$$MCV = \frac{\text{Hematocrit}\% \times 10}{\text{Erythrocyte count}}$$

$$MCHC = \frac{\text{Hemoglobin g/dL} \times 100}{\text{Hematocrit}\%}$$

(Smith, 2007)

The serum samples were analysed for total protein, glucose, creatinine, urea, alanine aminotransferase or glutamic pyruvate transaminase (ALT/ GPT), aspartate aminotransferase or glutamic oxalacetic transaminase (AST / GOT), calcium, phosphorus and magnesium.

All metabolites were determined using IL test's Total protein (Cat. 0018481300), Glucose (Cat. 0018480000), Creatinine (Cat. 0018480900), Urea (Cat. 0018480400), ALT (Cat. 0018480700), AST (Cat. 0018480800), Calcium (Cat. 0018258840), Phosphorus (Cat. 0018481900) and Magnesium (Cat. 0018481600) this analysis was made with spectrophotometry analysis using a BTS 350 Chemistry System analyser (Instrumentation Laboratory, Mexico).

Samples of diet were analysed for DM, ash, nitrogen (N), according to AOAC (1997) (AOAC, 1997). The neutral detergent fibre (NDF) (Van Soest et al., 1991), acid detergent fibre (ADF) and lignin (AOAC, 1997) were analysed using an ANKOM 200 Fibre Analyser Unit (ANKOM Technology Corporation, Macedon, NY, USA). The NDF was assayed without use of  $\alpha$ -amylase but with sodium sulphite in the NDF. Both NDF and ADF are expressed without residual ash.

Plant secondary metabolites were determined using 10ml of extract liquor and fractionated by funnel separation with a double volume of ethyl acetate (99.7/100, analytical grade, Fermont<sup>®</sup>, Monterrey, Mexico) to determine total phenolics by drying and to quantify the total phenolics layer in the funnel. After total phenolics separation, a double volume of n-butanol (99.9/100, analytical grade, Fermont<sup>®</sup>, Monterrey, Mexico) was added to fractionate saponins (Ahmed et al., 1990). The remaining solution was considered to be the aqueous fraction which contains the other secondary metabolites lectins, polypeptides and starch (Cowan, 1999) (Table 1).

### Statistical analyses

Data were analyzed using the PROC GLM procedure of SAS (2002) with Latin square design (four animals, four treatments and four periods) (Littell et al., 1998). Results reported in tables and in text with their corresponding standard errors of the mean (SAS, 2002). The significant differences between treatment means, were determined with a value to significance of  $P < 0.05$  with Tukey.

## RESULTS

In the case of lambs the treatments CX, EZ, SB and EZSB didn't produced differences in RBC, HMG, MCV, MCHC, WBC, SGN, LYM, BAS, EOS, but in HMT there are significant differences ( $P = 0.0259$ ), the groups SB and EZSB had the highest values compared with CX and EZ. For serological parameters no there were significant statistical differences in all parameters measured (Table 2).

For goats the results were similar but in this case there were statistical differences in eosinophils ( $P = 0.01901$ ) the highest values were observed in CX and EZ compared with the others two treatments. In the serological analysis there were significant statistical differences only in Glucose ( $P = 0.0334$ ) obtaining the lowest values of this parameter in the groups EZSB compared with the others three

groups, for the rest of parameters no there were statistical differences between the four treatments (Table 3).

In this experiment observed that both lambs and goats were a similar behavior in the parameters measured and the animals never showed sign of disease (Table 2 and 3)

## **DISCUSSION**

The objective of this experiment was analyze the effect of *Salix babylonica* extract, exogenous enzyme and their combination on haematological and serum biochemical blood parameters in growing lambs and goats, the results suggest that treatments didn't produced negative effects on parameter measured as an indicators for animal health.

For lambs the values to PCV were modified by treatment observed the highest values in SB and EZSB compared with the others two groups, this parameter is a rapid screening technique for seeking anemia and is used as indicator to animal hydration. The anemia its cause by blood loss, inadequate erythrocyte production and increased erythrocyte destruction or haemolysis (Archer and Jeffcott, 1977, Smith, 2007).

In the present study the modified in the values to PCV by treatments are not considered as indicator to anemia or dehydration because this values were within references values for sheep's (0.27 – 0.45 L/L) (Archer and Jeffcott, 1977, Smith, 2007).

Mahgoub et al 2008 reported decrease in PCV in sheep after to fed with no - conventional feeds containing phenols and condensed tannins (Mahgoub et al., 2008a) but this decrease were within normal range for sheep healthy and can't be

considered as an indicator of injury or disease (Smith, 2007). In contrast to this study in our study we didn't observed decrease in lymphocytes and eosinophils.

Parallel results were obtained by Olafadeha (2011) who reported low PCV in goats fed with tannins- rich forage, but equal to our study RBC were in range and reject haemolytic anaemia and depression of erythrocytogenesis (Olafadehan, 2011).

HMG MCV and MCHC were in normal range suggesting the absence of microcytic hypochromic anaemia occasioned by iron deficiency and improper utilization for the formation of Hb, the values of blood indices (MCV=28 - 40 fL and MCHC=310 – 340 g/L) confirm that the animals were not anemic (Smith, 2007, Olafadehan, 2011).

Phosphorus and magnesium levels were increased as this increase were in all treatments include CX group we conclude that it can be due to supra- nutritional levels because its levels in serum are influenced for food supply (Turner et al., 2005, Smith, 2007). In 2008 Mahgoub et al observed the same increase in both minerals but these are not explained (Mahgoub et al., 2008a).

In the case on goats there were statistical differences for eosinophils in the hematology analysis and glucose in serum biochemical analysis. Eosinophils are produced in bone marrow and are more important in regulating inflammatory and allergic reactions but principally controlling parasitic infection (Smith, 2007).

The present study the levels of eosinophils were in normal range ( $0.05 - 0.65 \times 10^9/L$ ) (Archer and Jeffcott, 1977, Smith, 2007) in the four treatments. Moreover this study demonstrate that the use to feed additives as EZ and SB in animal nutrition have positive effects on levels to eosinophils in blood because the levels of this parameter were lower in CX group in contrast to the other three groups that consuming EZ or SB. The enzyme by indirect effect and the *S. extract* by the content to secondary metabolites as saponins and tannins (Nayak, 2010, Owusu-Asiedu et al., 2010) (Harikrishnan et al., 2011, Lu, 2011).

In an experiment done in lambs grazing willow (*Salix* spp) fodders blocks the increase in the levels of eosinophils were observed but also observed increase in WBC and lymphocytes, This suggests that, in addition to the rumen escape protein benefit, feeding tannin condensed -containing forages may stimulate cell-mediated immune response (Ramirez-Restrepo et al., 2010a, Ramirez-Restrepo et al., 2010b). Moreover it is known that secondary metabolites as saponins and phenolic compounds can modulate the immune system and eosinophils play an important role as of the innate immune response (Provenza and Villalba, 2010).

These results on eosinophils were different to observed by Mahgoub et al (2008) and Olafadehan (2011) who reported lower WBC, lymphocytes and monocytes but not eosinophils in sheeps and goats fed with tannins rich non- conventional feeds and tannins- rich forage respectively but these parameters in both experiments were within the range, and confirmed that the concentration of tannins in these experiments were lower than the level that could have induced toxicity or ill health in the animals (Mahgoub et al., 2008a, Olafadehan, 2011).

The levels of glucose were modified for the treatments, were lower in EZSB and similar in CX, EZ and EZSB but this decrease is not significant because the levels are in normal range in the four treatments (2.66 – 2.49mmol/L) (Archer and Jeffcott, 1977, Smith, 2007). Mahgoub et al indicate that decrease in this metabolite can be due to food consumption with reduced nutritive value (Mahgoub et al., 2008a). In previous studies with similar results mentioned that depressed serum glucose is not due to tannic acid intoxication, but that the dietary energy was sufficiently utilized for growth and the animals were not surviving at the expense of body tissues (Olafadehan, 2011).

MCV were increased it is a reflection of mean erythrocyte size, in goats this increase is a common problem by inadequate spinning of blood which causes a spurious of MCV by trapped plasma (Smith, 2007) for this reason in all treatments

had higher values than normal range (16 – 25 fl) (Archer and Jeffcott, 1977). In a study realized in 2008 in sheep the values to MCV were lower than normal range but this increase was not produced by the treatments (Mahgoub et al., 2008a).

With respect to the others parameters evaluated the treatments didn't produced any effect and it were in normal range for both lambs and goats. Discard anemia because the levels of RBC, HMG, MCV, and MCHC were within range (Silanikove et al., 1996, Smith, 2007, Mahgoub et al., 2008a, Olafadehan, 2011). The presents results of no impact of treatments on blood parameters is an important finding in the case of those lambs and goats that consumed extract of *S. babylonica* because they contain secondary metabolites which could be expected to have a detrimental effect on cellular components and protein in blood mainly by the presence of tannins and saponins (hemolytic activity when it was used in high concentration)(Khalil and El-Adawy, 1994, Wang et al., 2007).

These results are similar to obtained by Ramirez-Restrepo 2011 who didn't observed consistent effects the grazing willow (*Salix spp.*) fodder blocks on RBC, HMG, MCV and MCHC (Ramirez-Restrepo et al., 2010a, Ramirez-Restrepo et al., 2010b).

The biochemical analysis indicates than treatments didn't produced negative effects on kidney, liver and animal health. The enzyme ALT, AST were unaffected by treatments are those used for domestic animal hepatic damage, ALT, which is a liver specific hepatocellular enzyme released by hepatocellular damage and both were within the normal ranges for sheeps and goats suggest that no damage to the liver occurred (Silanikove et al., 1996, Mahgoub et al., 2008a, Olafadehan, 2011, Mahgoub et al., 2008b).

Damage to the kidneys has been implicated for renal failure and changes in serum BUN, creatinine, uric acid and mineral concentrations. Lack of increase in these metabolites above the normal values suggests that necrotic damage to the kidney

did not occur in our experiment (Silanikove et al., 1996, Mahgoub et al., 2008a, Olafadehan, 2011).

The experimental animals, particularly those consuming *S. babylonica* extract which contained secondary metabolites, did not show clinical signs of ill health or signs of tannin toxicity such as brisket oedema, diarrhoea, constipation, anorexia, hard pelleted faeces coated with blood and mucous (Olafadehan, 2011).

In previous studies had been observed that tannins which can be consumed by goats without signs of toxic effect may be toxic for cattle and sheep because the secretion of proline rich proteins is higher during eating in goats than in sheep. The parotid saliva of goats is relatively rich in proline (6.5%), glutamine (16.5%) and glycine (6.1%), which is known to have high tannin-binding capacity, by enhancing the affinity of proteins to tannins. Moreover the ability of goats to consume large amounts of tannin-rich fodder without exhibiting toxic effects has been related to their ability to avoid consuming browse in amounts exceeding their capacity to detoxify and their enhanced capacity, in comparison with other ruminant species, to detoxify tannins (Silanikove et al., 1996, Olafadehan, 2011, Lu, 2011). In our study didn't observed signs of toxic effects in lambs or goats, and confirm that EZ and SB are innocuous (Rivero et al., 2012) in both species when are used as feed additives to 1µL g/DM and 30ml / animal/day respectively. Maybe this results is different to others studies by the kind of secondary compounds.

## **CONCLUSION**

The absence of clinical signs of ill health, tannin toxicity symptoms and the findings of all the haematological and serum metabolites within the established ranges for healthy lambs and goats suggest that EZ, EZSB and their combination were well tolerated and innocuous in lambs and goats, because did not cause any major health disorders.

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**Table 1.** Ingredients and chemical composition of basal diet (%) used in the experiment and levels of secondary metabolites in *Salix babylonica* extract, g/kgDM.

<b>Ingredient of diet</b>	<b>%</b>
Corn stover	35.0
Sorghum	35.5
Soybeans	10.0
Canola	7.0
Wheat bran	10.0
Mineral mix	2.5
<b>Chemical composition</b>	
Dry matter	97.0
NME <sup>1</sup> (Mcal/kg)	1.55
Ash	7.47
Crude protein	13.2
Neutral detergent fiber	40.6
Acid detergent fiber	14.0
Lignin	2.8
<b>Secondary metabolites g/kg DM</b>	
Total phenolics	16.4
Saponins	5.4
Aqueous faction <sup>2</sup>	76.3

<sup>1</sup> Net Metabolizable Energy.

<sup>2</sup> Aqueous fraction: lectins, polypeptides, starch (Cowan, 1999).

**Table 2.** Haematological and biochemical profile in lambs that consumed *Salix babylonica* extract, exogenous enzyme and their combination as feed additives.

Parameter	Treatments				P value	SEM
	CX	EZ	SB	EZSB		
RBC x 10 <sup>12</sup> /L	9.2	10.4	9.7	10.4	0.3228	0.68
PCV L/L	0.35 <sup>b</sup>	0.36 <sup>b</sup>	0.40 <sup>a</sup>	0.44 <sup>a</sup>	0.0259	0.02
HMG g/L	117.3	121.0	134.3	132.3	0.1864	7.91
MCV Fl	37.8	35.3	41.3	38.8	0.6263	4.45
MCHC g/L	331.5	332.0	331.2	331.5	0.2603	0.34
WBC x 10 <sup>9</sup> /L	11.0	10.0	11.1	12.1	0.3718	1.06
SGN x 10 <sup>9</sup> /L	3.6	3.7	3.9	3.8	0.9012	0.45
LYM x 10 <sup>9</sup> /L	7.2	6.2	6.6	8.0	0.2232	0.80
BAS x 10 <sup>9</sup> /L	0.00	0.02	0.01	0.00	0.6025	0.02
EOS x 10 <sup>9</sup> /L	0.13	0.09	0.16	0.12	0.8966	0.09
PSP g/Dl	65.8	70.0	67.5	67.5	0.3443	2.13
Glucose mmol/L	3.6	4.4	4.1	4.3	0.1407	0.28
Creatinine mmol/L	84.1	84.3	87.6	83.7	0.5539	2.91
Urea mmol/L	6.9	7.9	7.5	7.0	0.5667	0.77
ALT U/L	15.4	17.3	19.1	18.0	0.4125	2.11
AST U/L	65.8	67.5	72.8	73.5	0.2664	4.16
Phosphorus mmol/L	2.5	2.7	2.5	2.7	0.6351	0.17
Magnesium mmol/L	1.4	1.4	1.2	1.3	0.2667	0.10
Calcium mmol/L	2.9	2.9	3.0	2.9	0.8022	0.06

RBC - red blood cell; PCV - packed cell volume; HMG - hemoglobin; MCV - mean corpuscular volume; MCHC - mean corpuscular hemoglobin concentration; WBC - white blood cell; SGN - neutrophils segmented; LYM - lymphocytes; BAS - basophils; EOS - eosinophils; PSP - plasma protein concentration; ALT - alanine aminotransferase; AST - aspartate aminotransferase; <sup>a,b,c</sup> different superscripts following means within the same column indicate differences at P < 0.05; SEM - standard error of the mean.

**Table 3.** Haematological and biochemical profile in goats that consumed *Salix babylonica* extract, exogenous enzyme and their combination as feed additives.

Parameter	Treatments				P value	SEM
	CX	EZ	SB	EZSB		
RBC x 10 <sup>12</sup> /L	11.9	11.6	12.0	11.5	0.8491	0.63
PCV L/L	0.36	0.41	0.37	0.39	0.6186	0.04
HMG g/L	121.3	135.3	125.0	130.8	0.7087	12.63
MCV FI	30.0	34.0	30.8	33.8	0.6883	4.04
MCHC g/L	332.0	332.5	332.0	331.7	0.4547	0.44
WBC x 10 <sup>9</sup> /L	12.0	12.2	11.8	12.7	0.9738	2.06
SGN x 10 <sup>9</sup> /L	5.4	6.0	5.7	5.4	0.9660	1.27
LYM x 10 <sup>9</sup> /L	6.6	5.6	5.6	6.2	0.6779	0.93
BAS x 10 <sup>9</sup> /L	0.03	0.05	0.07	0.15	0.6566	0.11
EOS x 10 <sup>9</sup> /L	0.08 <sup>b</sup>	0.29 <sup>a</sup>	0.25 <sup>a</sup>	0.51 <sup>a</sup>	0.0191	0.09
PSP g/DI	70.3	71.3	71.3	70.5	0.9208	1.83
Glucose mmol/L	4.1 <sup>a</sup>	4.0 <sup>a</sup>	4.1 <sup>a</sup>	3.1 <sup>b</sup>	0.0334	0.28
Creatinine mmol/L	93.6	84.8	102.5	107.1	0.0512	6.45
Urea mmol/L	6.7	7.1	8.3	7.5	0.5178	1.04
ALT U/L	25.1	22.6	23.4	22.0	0.7371	2.90
AST U/L	72.6	75.1	67.5	70.0	0.5935	5.63
Phosphorus mmol/L	2.7	2.8	2.8	2.9	0.8948	0.30
Magnesium mmol/L	1.4	1.4	1.3	1.8	0.2600	0.22
Calcium mmol/L	2.5	2.3	2.5	2.4	0.8703	0.32

RBC - red blood cell; PCV - packed cell volume; HMG - hemoglobin; MCV - mean corpuscular volume; MCHC - mean corpuscular hemoglobin concentration; WBC white blood cell; SGN – neutrophils segmented; LYM – lymphocytes; BAS – basophils; EOS – eosinophils; PSP - plasma protein concentration; ALT - alanine aminotransferase; AST - aspartate aminotransferase; <sup>a,b,c</sup> different superscripts following means within the same column indicate differences at P < 0.05; SEM – standard error of the mean.

## 7.5. Resultados adicionales

### 7.5.1. Estancia de Investigación



**NORTH FLORIDA RESEARCH &  
EDUCATION CENTER - MARIANNA DE  
LA UNIVERSIDAD DE FLORIDA.**

## INTRODUCCIÓN

El Consejo Nacional de Ciencia y Tecnología con el objetivo de impulsar el desarrollo intelectual, permite a sus becarios realizar estancias en instituciones tanto nacionales como extranjeras lo extranjeras, lo cual permite a los estudiantes que solicita este tipo de recursos, interactuar con investigadores de otras instituciones, aprender metodologías nuevas y establecer vínculos interinstitucionales que puedan servir como plataformas para establecer proyectos de colaboración que beneficien a ambas instituciones.

El North Florida Research & Education Center (NFREC) es una institución muy grande y muy diversa en la cual se realizan actividades tendientes a la educación e investigación. Esta institución cuenta con 16 profesores activos que representan a 8 departamentos académicos y de la Escuela de Recursos Forestales y Conservación y 3 profesores eméritos.

Los programas de investigación y extensión en NFREC están diseñados para ayudar a los agricultores a manejar los recursos de forma eficaz, rentable y con conciencia ambiental.

El NFREC-Marianna se centra en la investigación agronómica y el ganado vacuno. Alberga el programa de eficacia de la alimentación, que es el más

grande de su tipo en investigaciones subtropicales. El programa de investigación en ganado vacuno se centra en la producción de carne, genética y reproducción.

## **OBJETIVOS**

- 1.- Reforzar las acciones tendientes a la culminación del trabajo de tesis.
- 2.- Realizar un experimento que permita complementar los resultados obtenidos en el trabajo experimental.
- 3.- Determinar el efecto de extracto de *Salix babylonica*, sobre el metabolismo y fermentación ruminal, excreción de nutrientes y emisión de metano en bovinos

## **METODOLOGÍA**

Con la finalidad de realizar un trabajo experimental en North Florida Research and Education Center (NFREC) de la Universidad de Florida el cual pudiera ser considerado como parte del trabajo de Tesis Doctoral, se consideró la posibilidad de trabajar el extracto de *Salix babylonica*, por lo que fue necesario buscar, coleccionar y procesar las hojas de dicho árbol.

Una vez que se dispuso del extracto, se procedió a aprender y aplicar diferentes técnicas estandarizadas en el laboratorio de Animal Science del NFREC-Marianna, las cuales se basaron en la obtención de muestras de líquido ruminal al cual se le aplicaron siete tratamientos 0, 100, 200, 300µl de extracto de *Salix babylonica* además de 100, 200 y 300µl de la solución con la que fue preparado el extracto y finalmente 200µl de monensina 4ppm.

Ya establecidos los tratamientos y coleccionado el líquido ruminal y preparado todo el material se aplicaron las siguientes técnicas:

## **TÉCNICAS APRENDIDAS:**

### **Batch cultura**

Preparación de la solución buffer de acuerdo a la técnica McDougall's.

Pesar 1.4 g del sustrato a utilizar y ponerlo en cada una de las botellas de Ankon

Hacer dilución 3:1 con solución Buffer y líquido ruminal.

Colocar 100 ml de la mezcla de líquido ruminal y solución buffer en cada una de las botellas Ankon.

Poner el tratamiento correspondiente.

Colocar la tapa y la bolsa de aluminio para coleccionar el gas

Poner en sistema Ankon.

Iniciar lectura

Pasadas 24hr, parar la medición en el equipo

Sacar botellas, restirar la tapa y la bolsa

Medir y registrar pH

Agregar 1ml de ácido sulfúrico para parar la fermentación.

### **Medición de Metano**

Leer en cromatógrafo el contenido de gas de cada una de las bolsas presionando por diez segundos cada bolsa con las válvulas abiertas, pasados diez segundos cerrar las válvulas y quitar la bolsa, del equipo tomar los datos de señal y área, que serán utilizados posteriormente.

### **Medición de H<sub>2</sub>S**

Preparar agua alcalina mediante la adición de NaOH 0,1 N al agua DI hasta pH 8.5-9.0.

Añadir 5 ml de agua alcalina en 18 vacutainer con tapa roja, utilizando haciendo uso de una jeringas.

Preparar los estándares diluyendo 0.5ml de RAD 171 en 24.4ml de agua destilada.

Tomar las bolsas Tedlar de aluminio que se retiran de los frascos. Homogeneizar

la muestra de gas que se encuentra dentro de la bolsa y extraer de 5 ml de gas e inyectarlo en el vacutainer. Agitar vigorosamente vacutainer después de retirar la aguja.

Bajo la campana flujo, se inyecta 0,5 ml de DPD en cada vacutainer, a continuación, 0,5 ml de reactivo de cloruro férrico. Agitar enérgicamente y esperar 30 minutos para que ocurra la reacción.

Leer cada estándar (de menor a mayor y empezando con el blanco) y cada muestra en el espectrofotómetro, registrando siempre cada valor de absorbancia.

### **NH<sub>3</sub>-N**

Centrifugar las muestras de líquido ruminal (que fueron tomadas de las botellas que se utilizaron para medir emisión de metano después de agregar 1ml de ácido sulfúrico y que fueron congeladas por día de muestreo) a 1000rpm por 15 min a 4°C

Preparar los estándares (una vez preparados se tomas como muestras pero estas no se diluyen antes de pasar las muestras a la placa).

Agregar 1ml de reactivo de fenol a dos tubos por muestra (36 tubos) de líquido ruminal.

Una vez centrifugadas las muestras de líquido ruminal, colocar 20µl de cada una de las muestras en los tubos con fenol y mezclar.

Colocar en la mezcla antes mencionada 0.8ml de hipoclorito y mezclar.

Posteriormente colocar en baño Maria a 95° C por cinco minutos.

Trascurrido este tiempo hacer una dilución 2:1 con agua destilada y las muestras que salen del baño Maria.

Colocar en una placa de 96 pozos con fondo plano, 200µl de la dilución anterior.

Leer en espectrofotómetro para placas, guardar los resultados de esta lectura, para su análisis posterior.

### **Determinación de Ácidos Grasos Volátiles con Etil Acetato**

Tomar de las muestras de líquido ruminal a las que se les agrega ácido sulfúrico, después de la incubación, de 12 a 13ml de líquido ruminal, congelarlo (-18° C).

El día que se trabajara esta técnica, sacar las muestras y descongelarlas a temperatura ambiente.

Una vez descongeladas, centrifugar a 10000rpm por 15 min a 4° C.

Tomar 2ml de líquido ruminal sobrenadante colocarla en otro tubo y agregar 0.4ml de ácido metafosfórico y congelar a -18° C.

Al día siguiente descongelar las muestras y centrifugar a 10000rpm 15 min a 4° C. Colocar 1ml del sobrenadante de cada muestra en un tubo y agregar 2ml de etil acetato.

Mezclar vigorosamente dejar reposar 1 a 2 min y coleccionar con pipetas plásticas aproximadamente 2ml de la parte superior que se separa al hacer esta mezcla y colocarlos en los viales que usa el espectrofotómetro.

## **RESULTADOS**

Procesamiento de las muestras de líquido ruminal para cada una de las técnicas anteriormente mencionadas, haciendo tres repeticiones en cada una en 3 días diferentes.

Realización de una base de datos en Excel que sirvió como base para la realización del análisis estadístico de los resultados obtenidos para cada parámetro medido.

Realización del análisis estadístico de algunos parámetros medidos.

## **CONCLUSIÓN**

Los resultados preliminares no muestran ningún efecto significativo de los tratamientos sobre los parámetros medidos.

La estancia fue muy productiva tanto académica como personalmente, considero que la disposición de las personas que se encuentran en el laboratorio es un punto que fomenta el trabajo en equipo, pero sobre todo que invita al aprendizaje.

## VIII. DISCUSIÓN GENERAL

El presente experimento se realizó con el objetivo de evaluar el efecto de las enzimas exógenas y el extracto de *Salix babylonica* como aditivos alimenticios en corderos y determinar parámetros hematológicos, inmunológicos y productivos. De la misma manera se evaluó la posible correlación entre la ganancia diaria de peso y la respuesta inmune celular.

Los resultados obtenidos en el análisis de parámetros hematológicos revelaron que ninguno de los dos tratamientos ni su combinación presentan un riesgo potencial para la salud de los animales, lo cual es un resultado relevante en el caso del extracto de *S. babylonica*, ya que se podría pensar que por el contenido de metabolitos secundarios presentes en él pudiera ejercer algún efecto negativo sobre la salud de los animales.

En estudios previos en los cuales se ha evaluado el efecto de extractos derivados de plantas sobre el estado de salud de los animales y se ha observado tanto en monogástricos como en rumiantes que en periodos que van de 15 a 90 días estos metabolitos son capaces de producir anemia, inmunosupresión, daño hepático, renal e incluso la muerte (Adedapo et al., 2007a, Adedapo et al., 2007b, Imik et al., 2008, Mahgoub et al., 2008a, Valizadeh et al., 2010, Olafadehan, 2011, Ramirez-Restrepo et al., 2010).

Probablemente la diferencia de resultados se debe al tipo de planta utilizada en cada experimento, la concentración y el tipo de compuestos secundarios presentes en ellas, el tiempo de adaptación y sobre todo al tipo de animal (Ramirez-Restrepo et al., 2010).

Por otro lado, los resultados del efecto de los tratamientos sobre la respuesta inmune demostraron que el extracto, las enzimas exógenas y su combinación son incapaces de ejercer algún tipo de efecto sobre las poblaciones de linfocitos T

colaboradores, Tcitotóxicos, granulocitos y monocitos primero que todo por la especie animal utilizada ya que se considera que los rumiantes tiene la capacidad de degradar e incluso utilizar todos aquellos compuestos que llegan al rumen y entran en contacto con los microorganismos propios de este lugar a diferencia de los monogástricos que no cuentan con esta capacidad por lo que son más susceptibles a la intoxicación por el consumo de metabolitos secundarios (Hammond, 1995).

En segundo término se conoce que el uso de compuestos secundarios como las saponinas a manera de adyuvantes en vacunas ha sido evaluada de forma parenteral, intraocular, e incluso intranasal (Sjolander et al., 1998, Weng et al., 2010). Se esperaba que los compuestos secundarios presentes en el extracto fueran capaces de inducir ambos tipos de respuesta innata y adaptativa ya que se considera que estos actúan como antígenos al presentar PAMP similares a los que presentan antígenos como bacterias, virus, hongos, etc. (Provenza and Villalba, 2010). Es probable que se requiera una dosis más alta de compuestos secundarios y otra vía de administración para poder observar la acción inmunomoduladora de los compuestos secundarios.

De igual manera, no se pudo constatar el efecto indirecto de las enzimas exógenas sobre la respuesta inmune, esto bajo los principios de inmunonutrición donde se señala que existe una fuerte interacción entre la nutrición y la respuesta inmune pues deficiencias en macro y micronutrientes se han asociado a un mal funcionamiento de los componentes de la respuesta inmune tanto innata como adaptativa (Chandra, 1997, Chandra, 2002, Calder and Kew, 2002, Maggini et al., 2007, Wintergerst et al., 2007).

En este aspecto se observó que el tiempo si es un factor que produce modificaciones en las poblaciones celulares evaluadas produciendo un efecto Inmunoestimulante; los primeros 15 días de tratamiento asocia a la fisiología propia de los microorganismos ruminales como de las células principalmente en las

células de la respuesta inmune innata las cuales son la primera línea de defensa del organismo contra un antígeno cuya función dura unos pocos días y la cual es de vital importancia para la estimulación de la respuesta inmune mediada por células (Van Soest, 1994, Provenza and Villalba, 2010).

La correlación existente entre la ganancia diaria de peso y la respuesta inmune no se pudo establecer ya que los tratamientos no produjeron diferencias en los parámetros inmunológicos; sin embargo, se sabe que esta correlación existe y deberá ser comprobada en futuras investigaciones.

En lo que respecta a la especie se determinó que no hay diferencias producidas por este factor ya que los tratamientos tuvieron un comportamiento similar tanto en ovinos como en caprinos, esto resultados contrastan con los publicados por Salem et al 2011 quienes determinaron que las cabras son más eficientes en el uso de las enzimas exógenas a diferencia de los borregos (Salem et al., 2011a); sin embargo, en este estudio no se evaluaron parámetros hematológicos como en el presente estudio.

Por otro lado, se ha establecido que las cabras son más eficientes detoxificando compuestos secundarios de las plantas, a diferencia de las vacas y los borregos por lo que se adaptan mejor este tipo de compuestos cuando forman parte de su dieta (Ramos et al., 1998, Silanikove et al., 1996, Turner et al., 2005).

Con el uso de extracto de *Salix babylonica* y enzimas exógenas, se cubren los requerimientos de limpio, verde y ético, son de origen natural fúngico o bacteriano (enzimas exógenas) o derivados de árboles (extractos de arbóreas y arbustivas forrajeras) además de disminuir los costos de producción al favorecer la utilización de la mayor cantidad de los componentes de la dieta y disminuir la emisión de algunos compuestos que tiene efecto negativos en el medio ambiente como metano, amoníaco, fosforo, etc.

Por último, con el uso de extracto de *Salix babylonica* y enzimas exógenas como aditivos alimenticios en la dieta de ovinos y caprinos su integridad y estado de salud no se ven afectados, cuando se consideran variables importantes como dosis y frecuencia (Bedford and Partridge, 2010, Provenza and Villalba, 2010, Gado et al., 2011, Salem et al., 2011b, Rivero et al., 2012).

## IX. CONCLUSIÓN.

El extracto de *Salix babylonica*, las enzimas exógenas y su combinación no producen efectos sobre los parámetros hematológicos evaluados.

El extracto de *Salix babylonica*, las enzimas exógenas y su combinación usados como aditivos alimenticios en la dieta de corderos no producen efecto sobre los parámetros inmunológicos evaluados; sin embargo, el tiempo es un factor que modifica las poblaciones celulares de linfocitos T-citotóxicos y granulocitos, produciendo un efecto inmunoestimulante los primeros 15 días posteriormente este efecto es inmunosupresor, mientras que para las linfocitos T-helper el efecto es primero inmunosupresor y posteriormente inmunoestimulante.

Existe una correlación negativa entre la ganancia diaria de peso y los linfocitos Tcitotoxicos en aquellos animales que consumieron la combinación de los tratamientos.

Las enzimas exógenas, el extracto de *Salix babylonica* y su combinación son inocuos tanto en corderos como en cabras cuando se proporcionan a manera de aditivos alimenticios a la dosis y la frecuencia empleados en los presentes experimentos.

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