

Bogotá, 8 de agosto de 2024

# Doctora

**Edna Marcela Ramírez Orozco**

## Directora Nuevas Creaciones

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO -SIC-**

E.

**S.**

D.

REF.: SOLICITANTES : YARA INTERNATIONAL ASA

**TITULO :** UNA MEZCLA DE UREA, BIURET Y SUBPRODUCTOS QUE CONTIENEN N CREADOS DURANTE LA PRODUCCIÓN DE BIURET COMO UNA FUENTE ALIMENTICIA DE NITRÓGENO NO PROTEICO PARA RUMIANTES Y USOS DE ESTA

**No. de Solicitud :** PCT/EP2023/054080  
**Internacional**

**ENTRADA A FASE NACIONAL EN COLOMBIA CON LAS MODIFICACIONES QUE  
SE LLEVARON A CABO EN FASE INTERNACIONAL BAJO ARTÍCULO 34  
(CAPÍTULO II)**

**HELENA CAMARGO WILLIAMSON**, mayor de edad y vecina de Bogotá D.C., identificada como aparece al pie de mi firma, en mi condición de apoderada de la sociedad **YARA INTERNATIONAL ASA**, adjunto este memorial con el fin de brindar mayor claridad al Examinador a la hora de revisar los documentos radicados. Así, me permito señalar que la presente solicitud entrara a fase nacional en Colombia con las modificaciones que se llevaron a cabo en fase internacional presentadas en Capítulo II bajo le artículo 34.

Así las cosas, me permito adjuntar la traducción de la solicitud con modificaciones bajo artículo 34 (Capítulo II), el cual comprende las reivindicaciones modificadas, además, de la traducción de la descripción, reivindicaciones y resumen originalmente presentados en fase internacional. Y, por último, adjuntamos algunos de los documentos que se han presentado y emitido durante la fase internacional, tales como la publicación internacional, el reporte de búsqueda y opinión escrita, el reporte preliminar internacional de patentabilidad (IPRP), entre otros.

De acuerdo con todo lo anterior, amablemente solicitamos que proceda a llevar a cabo el trámite de la solicitud y el estudio de patentabilidad a las reivindicaciones radicadas y basadas en las modificaciones bajo el artículo 34 (capítulo II) de la solicitud internacional. Así las cosas, considero que la presente invención cumple con todos los requisitos formales de acuerdo con el numeral 5.2.5. del Título X de la Circular Única de la Superintendencia y la Decisión 486.

De la Señora directora,

**POSSE HERRERA RUIZ**



**HELENA CAMARGO WILLIAMSON**

**C.C 35.455.268 de Bogotá**

**T.P. 76.985**

**UNA MEZCLA DE UREA, BIURET Y SUBPRODUCTOS QUE CONTIENEN N  
CREADOS DURANTE LA PRODUCCIÓN DE BIURET COMO UNA FUENTE  
ALIMENTICIA DE NITRÓGENO NO PROTEICO PARA RUMIANTES Y USOS DE  
ESTA**

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**CAMPO TÉCNICO**

La presente divulgación se refiere al campo de fuentes alimenticias de nitrógeno no proteico (NNP), más en particular, una mezcla de urea, biuret y subproductos que contienen N creados durante el proceso de condensación de urea, o dicho de otro modo, durante la producción de biuret, en un suplemento alimenticio para rumiantes.

**ANTECEDENTES**

Los rumiantes se definen como mamíferos herbívoros ungulados grandes que pastorean o ramonean que son capaces de adquirir nutrientes de pienso de origen vegetal al digerirlos en un estómago especializado que tiene cuatro compartimentos, uno de los cuales es el rumen. Gracias a las acciones microbianas en el rumen antes de la digestión, los rumiantes tienen la capacidad única de usar fuentes de proteína (de baja calidad) y también fuentes de nitrógeno no proteico (NNP). Las fuentes de NNP proporcionan nitrógeno para rumiantes, especialmente en dietas bajas en proteínas. Esto les da a los animales rumiantes una ventaja competitiva por sobre otros animales domésticos. La relación simbólica con la microflora del rumen les permite a los rumiantes suprareciclar nutrientes para producir proteínas animales altamente nutritivas, mediante una fermentación pregástrica eficiente del pienso, lo que ocurre en el reticulorumen, el compartimento más grande de su tubo gastrointestinal. Los rumiantes proporcionan un ambiente óptimo para que los microorganismos se desarrollen, y estos, a su vez, le suministran al huésped rumiante proteína cruda microbiana, que es de alta calidad en términos de suplemento de aminoácidos. En sistemas de producción de carne vacuna y lácteos, la proteína cruda microbiana sintetizada en el rumen proporciona la mayoría de la proteína metabolizable que necesita el animal. Una mayor utilización de nitrógeno (N) para la síntesis bacteriana y la captura de N alimenticio en productos anabólicos, en particular, aminoácidos absorbidos, puede verse influenciada por la manipulación alimenticia, principalmente por los niveles de energía en la ración. Sin embargo, existe un límite máximo para la eficiencia general del proceso: un máximo de 50 a 60 % de N alimenticio, o 70 a 90 % de N digerido aparente se convertirá en aminoácidos y se liberará a la vena porta. Por esta razón, en particular

en dietas para ganado vacuno, es extremadamente importante evaluar la cinética óptima de la liberación de N en fuentes de NNP para cada fuente de energía (por ejemplo, dieta de forraje basal, subproductos suplementarios, etc.) para maximizar la utilización de N y minimizar la excreción.

- 5 En la mayoría de los sistemas de producción de carne vacuna, hay disponibles varios granos y subproductos de granos como fuentes de proteína suplementarias. Sin embargo, por cada kg de N suministrado, es casi imposible competir con los costos menores de proteína cruda suplementaria en forma de NNP.

- 10 En efecto, el nitrógeno no proteico, principalmente en forma de urea, se conoce como una fuente rentable de nitrógeno suplementario (N) en la producción para rumiantes. La urea comercial/de calidad para pienso en general contiene solo rastros de biuret, en donde el contenido de biuret no excede 1,0 % en peso. No obstante, la urea generalmente no se incluye en más de 1 % en peso de la dieta. Toda carencia de proteína en una dieta o programa de suplementos se cubre con suplementos a base de
- 15 urea en forma de cubos de pastura, suplementos líquidos o en pelets y cubetas. Aparte de urea, otros compuestos químicos que forman amoníaco tales como sulfato de amonio se han usado en la formulación de dietas ruminales para reemplazar parte de las fuentes de proteína vegetal y para proporcionar cantidades adecuadas de proteína degradable ruminal para una mejor eficiencia en la digestión de fibra y la síntesis de proteína
- 20 microbiana. Sin embargo, el uso de NNP habitualmente se ve limitado por la veloz tasa de liberación de N en el rumen de un (animal) rumiante en forma de amoníaco. Si la liberación de amoníaco en el rumen excede la capacidad de uso de la microbiota ruminal, este exceso de N se excretará, con la consiguiente pérdida de energía y eficiencia de N. Además, si la concentración de amoníaco sobrepasa la capacidad de
- 25 excreción del hígado del rumiante, puede ocurrir intoxicación por amoníaco o la muerte. En consecuencia, en el arte previo se apuntó a mejorar el uso de NNP para reducir la tasa de liberación de N en el rumen. En los últimos años, se desarrollaron y se probaron varios productos de urea de liberación lenta en la nutrición de rumiantes, por ejemplo, a través de medios físicos, tal como mediante la aplicación de un recubrimiento, con
- 30 resultados variables. La aplicación de un recubrimiento con el objetivo de obtener liberación lenta significa agregar grasa, cera o similares a la fuente de NNP. Esto tiene la desventaja de que el contenido de NNP está diluido. Asimismo, un recubrimiento aumenta el costo de producción de una fuente alimenticia de N en dietas para ganado que deberían ser rentables. Otra solución es aplicar medios químicos, tales como usar
- 35 biuret puro. La desventaja del biuret puro es que la producción de este es muy costosa,

ya que el tiempo de calentamiento para obtener el biuret puro a partir de urea se tiene que extender mucho.

Además, actualmente existe un interés creciente en disminuir el impacto ambiental de la producción de rumiantes, que incluye carne vacuna y lácteos, y se aplican muchas estrategias para alcanzar este objetivo.

En los sistemas de alimentación de rumiantes actuales, la producción de metano ( $\text{CH}_4$ ) es una consecuencia inevitable de la digestión fermentativa. El consorcio microbiano del rumen debe desechar el hidrógeno que se produce durante la fermentación. De lo contrario, el hidrógeno se acumularía en el rumen y de esta manera inhibiría la fermentación de piensos ingeridos. Por lo tanto, al sistema microbiano del rumen se le proporcionan metanógenos que reducen el dióxido de carbono ( $\text{CO}_2$ ) a metano.

El mayor sumidero (de electrones) de hidrógeno en el rumen es metano producido por la reducción de dióxido de carbono mediante el uso de coenzimas reducidas, tales como NADH como la fuente de electrones. Sin embargo, el metano es una pérdida de energía para los rumiantes y un gas de efecto invernadero potente, que tiene un potencial de calentamiento global 28 veces mayor que el dióxido de carbono.

A nivel mundial, los rumiantes producen alrededor de 80 millones de toneladas de metano anualmente, lo que representa alrededor de 28 % de las emisiones de metano antropogénico. En consecuencia, es altamente prioritaria la adopción de prácticas de manejo de rumiantes que producen mayores reducciones de liberación de metano.

Un objetivo de la presente divulgación es proporcionar fuentes de NNP para rumiantes, tales como una composición de suplemento alimenticio para rumiantes que supera las limitaciones del arte previo conocido, útil para mejorar la fermentación ruminal, la productividad y el rendimiento de los animales rumiantes, y la emisión de metano entérico, especialmente en dietas baja en proteínas crudas, que proporciona N oportunamente, de manera tal que se mejore el crecimiento microbiano en el rumen y se mejore la producción de energía que genera compuestos.

## SÍNTESIS

En general, la presente divulgación se refiere a una composición o mezcla que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea, y usos de estos y métodos relacionados con estos. Las expresiones “composición de NNP”, “mezcla de NNP” y “composición que comprende urea, biuret y subproductos que contienen N de la producción de biuret a partir de urea” generalmente se utilizan de manera indistinta en la presente, y se refieren a una composición o

composición de NNP de acuerdo con la presente divulgación, como se indica más en detalle en la presente.

De acuerdo con un primer aspecto de la presente divulgación, se divulga el uso de una mezcla o composición de biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para reducir la emisión de metano producida por un animal rumiante. Se ha observado que la suplementación de un pienso para rumiantes con urea, biuret y subproductos que contienen N de la producción de biuret a partir de urea como una fuente de NNP, reduce la producción de metano entérico y la emisión de animales rumiantes sin comprometer o incluso mejorar su rendimiento, en particular, en comparación con una dieta o pienso para rumiantes suplementados con urea como fuente de NNP.

De acuerdo con un segundo aspecto de la presente divulgación, se divulgó el uso de una mezcla de biuret, urea y compuestos menores que contienen N creados durante el proceso de condensación de urea como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante. Dicho de otro modo, se divulga el uso de una composición que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante, en particular, en comparación con una dieta o pienso para rumiantes suplementados con urea como fuente de NNP.

De acuerdo con un tercer aspecto de la presente divulgación, se divulgó el uso de una mezcla de biuret, urea y compuestos menores que contienen N creados durante el proceso de condensación de urea como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para mejorar la eficiencia de la síntesis de proteína microbiana (EMPS). Dicho de otro modo, se divulga el uso de una composición que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para mejorar la eficiencia de la síntesis de proteína microbiana (EMPS), en particular, en comparación con una dieta o pienso para rumiantes suplementados con urea como fuente de NNP.

De acuerdo con un cuarto aspecto de la presente divulgación, se divulgó el uso de una mezcla de biuret, urea y compuestos menores que contienen N creados durante el

- proceso de condensación de urea como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para aumentar la digestibilidad de la materia seca (DM) y la materia orgánica (OM). Dicho de otro modo, se divulga el uso de una composición que comprende biuret, urea y subproductos que contienen N de la
- 5 producción de biuret a partir de urea como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para aumentar la digestibilidad de la materia seca (DM) y la materia orgánica (OM), en particular, en comparación con una dieta o pienso para rumiantes suplementados con urea como fuente de NNP.
- De acuerdo con un aspecto adicional de la presente divulgación, se divulga el uso de
- 10 una composición de suplemento alimenticio para rumiantes que comprende urea, biuret y subproductos que contienen N de la producción de biuret a partir de urea como una fuente de NNP para mejorar la ingesta de materia seca (DMI) de animales rumiantes, en particular, en comparación con una dieta o pienso para rumiantes suplementados con urea como fuente de NNP.
- 15 El solicitante descubrió inesperadamente que al usar una combinación de urea, biuret y compuestos menores que contienen N creados durante el proceso de condensación de urea para formar el biuret (también denominado “subproductos de la producción de biuret basada en urea” o “subproductos que contienen N de la producción de biuret a partir de urea”), la fermentación ruminal se altera directamente de manera que se
- 20 disminuye la producción de metano entérico, disminuye la relación de acetato con respecto a propionato, así como también mejora la eficiencia de la síntesis de proteína microbiana. También se descubrió que aumentó la digestibilidad de DM y OM, y que aumentó la ingesta de materia seca del animal rumiante, en particular, en comparación con una dieta o pienso para rumiantes suplementados con urea como fuente de NNP.
- 25 De acuerdo con un posible uso de acuerdo con la presente divulgación, la mezcla o composición comprende entre 30 – 60 % en peso de urea y entre 30 y 60 % en peso de biuret, en donde el % en peso es en función del peso total de la mezcla o composición. Más en particular, la mezcla o composición comprende entre 35 y 55 % en peso de urea y entre 35 y 50 % en peso de biuret, en donde el % en peso es en función del peso total
- 30 de la composición. Incluso más en particular, la mezcla o composición comprende entre 37 y 52 % en peso de urea y entre 39 y 46 % en peso de biuret, en donde el % en peso es en función del peso total de la composición.
- En un uso particular de acuerdo con la presente divulgación, la composición comprende entre 5 y 30 % en peso de subproductos que contienen N de la producción de biuret a
- 35 partir de urea, en función del peso total de la composición.

En un uso específico de acuerdo con la presente divulgación, la composición de suplemento alimenticio para rumiantes comprende además:

- entre 2,5 y 8 % en peso de triuret y/o
  - entre 0,1 y 5 % en peso de amelida y/o
  - 5 - entre 2,5 y 15 % en peso de ácido cianúrico y
  - entre 0,1 y 2,0 % en peso de humedad,
- en función del peso total de la composición.

De acuerdo con un uso específico adicional de acuerdo con la presente divulgación, la mezcla comprende además

- 10 - entre 3 y 6 % en peso de triuret y/o
- entre 0,5 y 3 % en peso de amelida y/o
- entre 3 y 10 % en peso de ácido cianúrico y
- entre 0,1 y 1,5 % en peso de humedad.

- Otro aspecto de la presente divulgación proporciona una composición de pienso para rumiantes que comprende uno o más ingredientes nutricionales y un NNP que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea. En algunas formas de realización, la composición de NNP comprende (a) entre 30 y 60 % en peso de urea; (b) entre 30 y 60 % en peso de biuret; y (c) entre 5 % en peso y 30 % en peso de subproductos que contienen N de la producción de biuret a partir de urea, en función del peso total de la composición de NNP. Más en particular, la composición de NNP comprende (a) entre 37 y 52 % en peso de urea; (b) entre 39 y 46 % en peso de biuret; (c) entre 3 % en peso y 6 % en peso de triuret; (d) entre 0,5 y 3 % en peso de amelida; y (e) entre 3 y 10 % en peso de ácido cianúrico, en función del peso total de la composición de NNP. En algunas formas de realización, el uno o más
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- ingredientes nutricionales forman una ración nutricionalmente equilibrada o una ración basal para un rumiante.

- De acuerdo con un aspecto relacionado adicional de la presente divulgación, se divulga un método para reducir las emisiones de metano entérico de un animal rumiante, en donde dicho método comprende administrar al animal rumiante una composición de NNP de acuerdo con la presente divulgación, que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea, en donde la composición comprende entre 30 y 60 % en peso de urea y entre 30 y 60 % en peso de biuret. Se descubrió que las emisiones de metano entérico del animal rumiante, expresado como g de metano/kg de ingesta de materia seca o g de metano/kg de ingesta de materia orgánica, se reduce en al menos 5 %, en particular, en al menos 10
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%, tal como en alrededor de 15 %, cuando la composición de NNP se administra a un rumiante como fuente de NNP en comparación con una dieta que comprende urea como fuente de NNP.

De acuerdo con un aspecto relacionado adicional de la presente divulgación, se divulga un método para disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un animal rumiante, en donde dicho método comprende administrar al animal rumiante una composición de NNP de acuerdo con la presente divulgación, que comprende biuret, urea y subproductos que contienen N de la producción de biuret, en donde la composición de NNP comprende entre 30 y 60 % en peso de urea y entre 30 y 60 % en peso de biuret. Se descubrió que la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante, particularmente calculada mediante una fermentación *in vitro*, tal como una fermentación de flujo continuo con fluido del rumen para simular la fermentación ruminal, se disminuye en al menos 5 %, en particular, en al menos 10 %, tal como en alrededor de 20 %, cuando la composición de NNP se administra a un rumiante como fuente de NNP en comparación con una dieta que comprende urea como fuente de NNP.

De acuerdo con un aspecto relacionado adicional de la presente divulgación, se divulga un método para mejorar la eficiencia de la síntesis de proteína microbiana (EMPS) por microbios del rumen en un animal rumiante, en donde dicho método comprende administrar al animal rumiante una composición de NNP de acuerdo con la presente divulgación que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea, en donde la composición de NNP comprende entre 30 y 60 % en peso de urea y entre 30 y 60 % en peso de biuret. Se descubrió que la EMPS, particularmente calculada mediante una fermentación *in vitro* con fluido del rumen para simular la fermentación ruminal aumentó en al menos 5 %, en particular, en al menos 10 %, cuando la composición de NNP se administra a un rumiante como fuente de NNP en comparación con una dieta que comprende urea como fuente de NNP.

De acuerdo con un aspecto relacionado adicional de la presente divulgación, se divulga un método para aumentar la digestibilidad de la materia seca (DM) y la materia orgánica (OM), en donde dicho método que comprende administrar al animal rumiante una composición de NNP de acuerdo con la presente divulgación que comprende biuret, urea y subproductos que contienen N de la producción de biuret, en donde la composición de NNP comprende entre 30 y 60 % en peso de urea y entre 30 y 60 % en peso de biuret.

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- De acuerdo con un aspecto relacionado adicional de la presente divulgación, se divulga un método para aumentar la ingesta de materia seca (DMI) de un animal rumiante, en donde dicho método comprende administrar al animal rumiante una composición de NNP de acuerdo con la presente divulgación que comprende biuret, urea y subproductos que contienen N de la producción de biuret, en donde la composición de NNP comprende entre 30 y 60 % en peso de urea y entre 30 y 60 % en peso de biuret. Se descubrió que la DMI, expresada en kg de DM/día, aumenta en al menos 5 % cuando la composición (de NNP) se administra a un rumiante como fuente de NNP en comparación con una dieta que comprende urea como fuente de NNP.
- 5 En formas de realización particulares, la composición de NNP de acuerdo con la presente divulgación y administrada en los métodos de acuerdo con la presente divulgación comprende entre 35 y 55 % en peso de urea y entre 35 y 50 % en peso de biuret, en función del peso total de la composición, más en particular, comprende entre 37 y 52 % en peso de urea y entre 39 y 46 % en peso de biuret, en función del peso
- 10 total de la composición.
- En formas de realización particulares, la composición de NNP de acuerdo con la presente divulgación y administrada en los métodos de acuerdo con la presente divulgación comprende además entre 5 y 30 % en peso de subproductos que contienen N de la producción de biuret a partir de urea, en función del peso total de la composición.
- 15 En formas de realización particulares, la composición de NNP de acuerdo con la presente divulgación y administrada en los métodos de acuerdo con la presente divulgación comprende entre 2,5 y 8 % en peso de triuret, y/o entre 0,1 y 5 % en peso de amelida y/o entre 2,5 y 15 % en peso de ácido cianúrico, y entre 0,1 y 2,0 % en peso de humedad, en función del peso total de la composición, más en particular, en donde
- 20 la composición comprende entre 3 y 6 % en peso de triuret, y/o entre 0,5 y 3 % en peso de amelida, y/o entre 3 y 10 % en peso de ácido cianúrico, y entre 0,1 y 1,5 % en peso de humedad, en función del peso total de la composición.
- En algunas formas de realización, los métodos de acuerdo con la presente divulgación comprenden además las etapas de preparar o proporcionar una composición de suplemento alimenticio para rumiantes que comprende la composición de NNP, o preparar o proporcionar una composición de pienso para rumiantes que comprende la composición de NNP, y administrar el suplemento alimenticio para rumiantes o la
- 25 composición de pienso para rumiantes al animal rumiante. Más en particular, la composición de NNP, que comprende biuret, urea y subproductos que contienen N de la producción de biuret de acuerdo con la presente invención está presente en la
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composición de pienso para rumiantes en una concentración entre 0,5 y 4 % en peso, más en particular, entre 0,5 y 3,5 % en peso o entre 0,5 y 3 % en peso, incluso más en particular, entre 1 y 2,5 % en peso, en donde el % en peso es en función de la base de materia seca total de la composición de pienso para rumiantes. En particular, la  
5 composición de pienso para rumiantes comprende además uno o más ingredientes nutricionales, en donde el uno o más ingredientes nutricionales forman una ración nutricionalmente equilibrada o una ración basal para un animal rumiante. Cabe destacar que los diferentes métodos de acuerdo con la presente divulgación son métodos no terapéuticos.

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### DESCRIPCIÓN DE LAS FIGURAS

La figura 1 muestra un gráfico de la producción de gas acumulado de 6 productos originales diferentes que contienen nitrógeno, en particular, urea sin recubrir (A), urea recubierta (B), biuret (D), triuret (E), amelida (F) y ácido cianúrico (G), y una mezcla de  
15 acuerdo con la presente divulgación (C) sobre una base isonitrogenada con respecto a un blanco (H) durante 72 horas de incubación *in vitro*.

La figura 2 muestra una descripción general de las reacciones involucradas en la producción de biuret a partir de urea, lo cual incluye la formación de subproductos que contienen N.

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### DESCRIPCIÓN DETALLADA

Los inventores de la presente descubrieron que una composición (o mezcla) de NNP específica para rumiantes, que comprende urea, biuret y uno o más subproductos que contienen N de un proceso de condensación de urea o proceso de producción de biuret,  
25 tiene muchas ventajas al alimentar a un rumiante como una fuente de NNP, particularmente en comparación con un rumiante alimentado con urea como una fuente de NNP. Además, para las diferentes formas de realización y aspectos previstos en la presente, la composición de NNP o mezcla de NNP de acuerdo con la presente divulgación supera significativamente a la urea como una fuente de NNP (en  
30 comparación con un rumiante alimentado con una dieta sin fuente de NNP).

Las ventajas de una composición de NNP de acuerdo con la presente divulgación son en particular:

(1) reducción de las emisiones de metano entérico de animales rumiantes. La reducción de la producción o emisiones de metano de animales rumiantes se puede evaluar *in vivo*  
35 al determinar las emisiones de metano de un rumiante alimentado con una dieta

particular, por ejemplo, usando la técnica del trazador SF<sub>6</sub>, como se describe, por ejemplo, en el ejemplo 3, en donde las emisiones de metano se pueden expresar como g de metano emitido por kg de materia seca ingerida o por kg de materia orgánica ingerida. De manera alternativa, las emisiones de metano se pueden analizar al evaluar la producción de metano en incubaciones de cultivo *in vitro* de fluido ruminal, como se describe, por ejemplo, en el ejemplo 4, en donde la producción de metano se puede expresar como mM, ml o mg de metano producido por g de materia orgánica incubada o digerida;

(2) disminuyó la relación de acetato con respecto a propionato producida por los microbios del rumen. El contenido de acetato y propionato se puede determinar mediante cromatografía de gases de los ácidos grasos volátiles (VFA) en una muestra, como entiende una persona del oficio de nivel medio. En algunas formas de realización, la muestra se obtiene mediante una fermentación *in vitro* con fluido de rumiante, para simular la fermentación ruminal, en particular, en donde una incubadora o fermentador adecuado se inocula con fluido ruminal y se le suministra una composición de pienso para rumiantes para simular la fermentación ruminal;

(3) aumentó la eficiencia de la síntesis de proteína microbiana (EMPS). Como entenderán las personas del oficio de nivel medio, la EMPS es una medida para determinar cuánta proteína microbiana se produce por la actividad microbiana para cada unidad de sustrato fermentable que se suministra al rumiante. Puede determinarse mediante un ensayo *in vitro* que comprende fermentaciones de flujo continuo o en lote con fluido del rumen, en particular, en donde una incubadora o fermentador adecuado se inocula con fluido ruminal y se le suministra una composición de pienso para rumiantes para simular la fermentación ruminal; como se explica, por ejemplo, en el ejemplo 2 o 4;

(4) aumentó la digestibilidad de la materia seca (DM) y la materia orgánica (OM). La digestibilidad de DM y OM puede evaluarse mediante la diferencia en la materia seca y la materia orgánica (es decir, materia seca menos el contenido de ceniza) del pienso en comparación con la muestra después de la fermentación (*in vitro*), como entiende una persona del oficio de nivel medio;

(5) aumentó la fermentación ruminal después de 72 h en un ensayo *in vitro*;

(6) mejoró la ingesta de materia seca (DMI) de animales rumiantes.

En este contexto, y sin pretender limitarse a la teoría, se cree que hubo una presión selectiva sobre los microbios en el rumen que producen acetato y propionato de manera tal que se produce más propionato y menos acetato, en vista de las composiciones de

suplemento alimenticio para rumiantes sin la mezcla de acuerdo con la presente divulgación. El propionato es el precursor glucogénico más importante en los rumiantes. Esto significa que disminuir la relación de acetato con respecto a propionato mejora el suministro de energía para uso metabólico, a través del cual hay más energía disponible para que el rumiante use, lo que mejora su producción de proteínas. En otras palabras, la fermentación ruminal se volvió más eficiente en cuanto a energía, lo que favorece la producción de carne, leche, etc.

El biuret generalmente se forma a través de una descomposición térmica no catalítica de urea. En la presente, este proceso generalmente se denomina “producción de biuret”, “producción de biuret a partir de urea” o “proceso de condensación de urea”. La reacción principal y las reacciones secundarias en la producción de biuret a partir de urea son conocidas por las personas del oficio de nivel medio y se representan en la figura 2. Cuando la urea se calienta hasta temperaturas por encima de su punto de fusión, se produce lentamente amoníaco. Si el rango de temperatura se limita ligeramente por encima del punto de fusión normal de la urea, es decir, 132 °C, se forma biuret. Cuando se calienta la fusión de urea adicionalmente, se forman varios otros “compuestos que contienen N” o “subproductos que contienen N” diferentes en cantidades muy pequeñas (por ello se usa el término “menor”). Estos compuestos que contienen N que se generan a partir de la descomposición térmica de la urea comprenden, entre otros, ácido cianúrico, triuret, amelida, etc. En la presente, estos compuestos se denominan “subproductos que contienen N de la producción de biuret” o “compuestos menores que contienen N creados durante el proceso de condensación de urea”. En formas de realización particulares, dichos subproductos que contienen N de la producción de biuret comprenden triuret, amelida y ácido cianúrico. Estos subproductos se hidrolizan deficientemente en el rumen. El ácido cianúrico y el triuret, así como el biuret, no son tóxicos para los rumiantes y tienen tan buenas cifras de retención de nitrógeno como el biuret. La amelida es un subproducto inevitable en la producción de la cantidad necesaria de biuret. Sin embargo, la cantidad de amelida debe limitarse ya que puede ser tóxica en dosis más altas.

Como se usa en la presente, la composición o mezcla de acuerdo con la presente divulgación, en particular, la composición o mezcla de NNP de acuerdo con la presente divulgación, generalmente comprende urea, biuret y uno o más subproductos de un proceso de producción de biuret. Más en particular, la composición o mezcla de NNP de acuerdo con la presente divulgación, comprende entre 60 % en peso y 95 % en peso de urea y biuret, y entre 5 % en peso y 40 % en peso de subproductos que contienen N de

un proceso de producción de biuret, en función del peso total de la composición o mezcla. Incluso más en particular, la composición o mezcla de NNP de acuerdo con la presente divulgación comprende entre 70 % en peso y 95 % en peso o entre 75 % en peso y 95 % en peso de urea y biuret, y entre 5 % en peso y 30 % en peso o entre 5 %  
5 en peso y 25 % en peso de subproductos que contienen N de un proceso de producción de biuret, en función del peso total de la composición o mezcla.

En formas de realización particulares, la composición o mezcla de NNP de acuerdo con la presente divulgación comprende entre 30 y 60 % en peso de urea y entre 30 y 60 % en peso de biuret, más en particular, entre 35 y 55 % en peso de urea y entre 35 % en  
10 peso y 50 % en peso de biuret, incluso más en particular, entre 37 y 52 % en peso o entre 40 y 50 % en peso de urea y entre 39 y 46 % en peso o entre 40 y 45 % en peso de biuret, en función del peso total de la composición o mezcla. En algunas formas de realización, la composición o mezcla de NNP de acuerdo con la presente divulgación, comprende además entre 5 % en peso y 30 % en peso de subproductos que contienen  
15 N de un proceso de producción de biuret a partir de urea, en particular, entre 5 % en peso y 25 % en peso de subproductos que contienen N de un proceso de producción de biuret a partir de urea, en función del peso total de la composición o mezcla. En algunas formas de realización, los subproductos que contienen N de un proceso de producción de biuret a partir de urea comprenden uno o más de triuret, amelida y ácido cianúrico.

En algunas formas de realización, la composición de NNP o mezcla de NNP de acuerdo con la presente divulgación comprende, como subproductos que contienen N de un proceso de producción de biuret, (a) entre 2,5 % en peso y 8 % en peso de triuret, más en particular, entre 3 y 6 % en peso de triuret; (b) entre 0,1 y 5 % en peso de amelida, más en particular, entre 0,5 y 3 % en peso de amelida; y/o (c) entre 2,5 y 15 % en peso  
20 de ácido cianúrico, más en particular, entre 3 y 10 % en peso de ácido cianúrico, en función del peso total de la composición o mezcla.

En algunas formas de realización, la composición o mezcla de NNP de acuerdo con la presente divulgación comprende además entre 0,1 y 2 % en peso de humedad (agua), más en particular, entre 0,1 y 1,5 % en peso de humedad, en función del peso total de  
30 la composición o mezcla.

En formas de realización particulares, la composición o mezcla de NNP de acuerdo con la presente divulgación es una composición de suplemento alimenticio para rumiantes.

Las formas de realización particulares de la composición de NNP o la composición de suplemento alimenticio para rumiantes de acuerdo con la presente divulgación  
35 comprende

- entre 30 y 60 % en peso de urea, más en particular, entre 35 y 55 % en peso de urea, incluso más en particular, entre 37 y 52 % en peso de urea,
  - entre 30 y 60 % en peso de biuret, más en particular, entre 35 y 50 % en peso de biuret, incluso más en particular, entre 39 y 46 % en peso de biuret,
- 5    - entre 3 y 6 % en peso de triuret,
- entre 0,5 y 3 % en peso de amelida,
  - entre 3 y 10 % en peso de ácido cianúrico,
  - entre 0,1 y 1,5 % en peso de humedad, en donde el % en peso es en función del peso total de la composición.
- 10    Opcionalmente, para todas las formas de realización de la composición de NNP de acuerdo con la presente divulgación, uno o más agentes antiaglomerantes, como se conocen generalmente en el estado de la técnica, se pueden agregar a la composición de suplemento alimenticio para rumiantes, incorporadas en el suplemento alimenticio, o bien como una cobertura, para mejorar las propiedades de manipulación y para mejorar
- 15    la calidad de la composición de suplemento alimenticio para rumiantes.
- Además, también es posible agregar cualquier prebiótico, probiótico, posbiótico, antibiótico, bioestimulantes conocidos en el estado de la técnica por potenciar / favorecer la asimilación de nitrógeno y fermentación ruminal.
- Un aspecto de la presente divulgación se refiere al uso de una composición o mezcla
- 20    de NNP de acuerdo con la presente divulgación, o dicho de otro modo una mezcla o composición de urea, biuret y compuestos menores que contienen N creados durante el proceso de condensación de urea para obtener el biuret, como una fuente de NNP en una composición de suplemento alimenticio para rumiantes, en particular, al mismo tiempo o simultáneamente, para
- 25    -            disminuir las emisiones de metano entérico de un animal rumiante,
- mejorar la ingesta de materia seca de un animal rumiante,
  - disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante,
  - mejorar la EMPS (eficiencia de la síntesis de proteína microbiana), y
- 30    -            aumentar la digestibilidad de DM y OM.
- De acuerdo con un aspecto relacionado de la presente divulgación, se divulga una composición que comprende urea, biuret y subproductos que contienen N de la producción de biuret a partir de urea, para su uso como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para disminuir la producción de

metano entérico o las emisiones de un animal rumiante o para disminuir la producción de metano entérico de los microbios del rumen en un rumiante.

De acuerdo con un aspecto relacionado de la presente divulgación, se divulga una composición que comprende urea, biuret y subproductos que contienen N de la  
5 producción de biuret a partir de urea, para su uso como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante.

De acuerdo con un aspecto relacionado adicional de la presente divulgación, se divulga una composición que comprende urea, biuret y subproductos que contienen N de la  
10 producción de biuret a partir de urea, para su uso como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para mejorar la EMPS en un rumiante.

De acuerdo con un aspecto relacionado adicional de la presente divulgación, una composición que comprende urea, biuret y subproductos que contienen N de la  
15 producción de biuret a partir de urea, para su uso como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para aumentar la digestibilidad de DM y OM en un rumiante.

De acuerdo con un aspecto relacionado de la presente divulgación, se divulga una composición que comprende urea, biuret y subproductos que contienen N de la  
20 producción de biuret a partir de urea, para su uso como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para aumentar la ingesta de materia seca de un animal rumiante.

Un aspecto relacionado de la presente divulgación proporciona un método para reducir la producción de metano entérico de un animal rumiante, que comprende las etapas de  
25 - preparar una composición de pienso para rumiantes que comprende una composición de NNP de acuerdo con la presente divulgación, como una fuente de NNP, particularmente una composición de NNP que comprende biuret, urea y subproductos que contienen N de la producción de biuret tal como se divulga en la presente; y  
- alimentar a un rumiante con la composición de pienso para rumiantes.

30 Un aspecto relacionado de la presente divulgación proporciona un método para disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante, que comprende las etapas de:

- preparar una composición de pienso para rumiantes que comprende una composición de NNP de acuerdo con la presente divulgación, como una fuente de NNP,

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particularmente una composición de NNP que comprende biuret, urea y subproductos que contienen N de la producción de biuret tal como se divulga en la presente; y

- alimentar a un rumiante con la composición de pienso para rumiantes.

Un aspecto relacionado adicional de la presente divulgación proporciona un método para mejorar la EMPS en un rumiante, que comprende las etapas de

- preparar una composición de pienso para rumiantes que comprende una composición de NNP de acuerdo con la presente divulgación, como una fuente de NNP, particularmente una composición de NNP que comprende biuret, urea y subproductos que contienen N de la producción de biuret tal como se divulga en la presente; y

- alimentar a un rumiante con la composición de pienso para rumiantes.

Un aspecto relacionado adicional de la presente divulgación se refiere a un método para aumentar la digestibilidad de DM y OM en un rumiante, que comprende las etapas de

- preparar una composición de pienso para rumiantes que comprende una composición de NNP de acuerdo con la presente divulgación, como una fuente de NNP, particularmente una composición de NNP que comprende biuret, urea y subproductos que contienen N de la producción de biuret tal como se divulga en la presente; y

- alimentar a un rumiante con la composición de pienso para rumiantes.

En algunas formas de realización, las emisiones de metano entérico del animal rumiante se reducen en al menos 5 % o en al menos 10 %, tal como en alrededor de 15 %, cuando se expresan en g/kg de DMI (ingesta de materia seca) o en g/kg de OMI (ingesta de materia orgánica), cuando la composición de NNP de acuerdo con la presente divulgación se administra a un rumiante como fuente de NNP en comparación con la dieta que comprende urea como fuente de NNP.

En algunas formas de realización, la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante disminuye en al menos 5 %, en particular, en al menos 10 %, tal como en alrededor de 20 %, cuando la composición de NNP de acuerdo con la presente divulgación se administra a un rumiante como fuente de NNP en comparación con una dieta que comprende urea como fuente de NNP. En particular, la relación de acetato con respecto a propionato se puede determinar en una muestra obtenida de una fermentación *in vitro* con fluido de rumiante, para simular la fermentación ruminal, particularmente en donde una incubadora o fermentador adecuado, tal como un fermentador o incubadora de flujo continuo o en lote, se inocula con fluido ruminal y se le suministra una composición de pienso para rumiantes para simular la fermentación ruminal.

En algunas formas de realización, la eficiencia de la síntesis de proteína microbiana (EMPS) en un rumiante aumenta en al menos 5 %, en particular, en al menos 10 %, tal como en alrededor de 20 %, cuando la composición de NNP de acuerdo con la presente divulgación se administra a un rumiante como fuente de NNP en comparación con una dieta que comprende urea como fuente de NNP. En particular, la EMPS se puede determinar en una muestra obtenida de una fermentación *in vitro* con fluido de rumiante, para simular la fermentación rumiante, particularmente en donde una incubadora o fermentador adecuado, tal como un fermentador o incubadora de flujo continuo o en lote, se inocula con fluido ruminal y se le suministra una composición de pienso para rumiantes para simular la fermentación ruminal.

En algunas formas de realización, el método para disminuir la producción de metano entérico de un animal rumiante, para disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante, para mejorar la EMPS en un rumiante, para aumentar la digestibilidad de DM u OM en un rumiante, y/o para aumentar la ingesta de materia seca (DMI) de un rumiante comprende además la etapa de reemplazar una porción de proteínas vegetales en la composición de pienso con la composición de NNP de nitrato de acuerdo con la presente divulgación.

También cabe destacar que los diferentes métodos de acuerdo con la presente divulgación son métodos no terapéuticos.

Como se considera en la presente, un rumiante o animal rumiante es un animal bovino, ovino o caprino, particularmente un animal domesticado con un rumen funcional. En formas de realización particulares, el rumiante es una oveja o un ganado, más en particular, el rumiante es un ganado vacuno o ganado lechero.

La presente divulgación proporciona además un pienso, material de pienso, premezcla o aditivo para pienso para alimentar a un rumiante, que comprende la composición de NNP de acuerdo con la presente divulgación. La composición de NNP de acuerdo con la presente divulgación se puede combinar con un pienso para rumiantes o material de pienso, o con una premezcla para alimentar a un rumiante. En el contexto de la presente divulgación, la expresión premezcla o premezcla de nutrientes se usa como es conocido para una persona del oficio de nivel medio y denota una mezcla que comprende uno o más ingredientes tales como vitaminas, oligominerales, medicamentos, suplementos para pienso y diluyentes. Un pienso para rumiantes o material de pienso para rumiantes generalmente se refiere a composiciones nutricionales adecuadas para alimentar a rumiantes, como las conoce la persona del oficio de nivel medio, e incluye los piensos compuestos (es decir, generalmente una mezcla de piensos producida de manera

industrial de al menos dos materiales de pienso), los piensos completos (es decir, un pienso compuesto que contiene todos los nutrientes principales necesarios para una ración diaria, que incluye también todos los forrajes necesarios para el rumiante), o un pienso concentrado (es decir, un pienso compuesto que tiene un alto contenido de ciertas sustancias pero que es suficiente para una ración diaria solo si se usa en combinación con otro pienso).

En formas de realización particulares, se divulga una composición de pienso para rumiantes que comprende uno o más ingredientes nutricionales y una composición de NNP que comprende (a) entre 30 y 60 % en peso o entre 35 y 55 % en peso de urea; (b) entre 30 y 60 % en peso o entre 35 y 50 % en peso de biuret; y (c) entre 5 % en peso y 30 % en peso de subproductos que contienen N de la producción de biuret a partir de urea, en función del peso total de la composición de NNP. Más en particular, la composición de NNP comprende (a) entre 37 y 52 % en peso de urea; (b) entre 39 y 46 % en peso de biuret; (c) entre 3 % en peso y 6 % en peso de triuret; (d) entre 0,5 y 3 % en peso de amelida; y (e) entre 3 y 10 % en peso de ácido cianúrico, en función del peso total de la composición de NNP. En algunas formas de realización, el uno o más ingredientes nutricionales forman una ración nutricionalmente equilibrada o una ración basal para un rumiante, como entiende una persona del oficio de nivel medio.

En algunas formas de realización, se divulga una composición de pienso para rumiantes o material de pienso que comprende entre 0,5 y 4 % en peso, más en particular, entre 0,5 y 3,5 % en peso o entre 0,5 y 3 % en peso, incluso más en particular, entre 1 y 2,5 % en peso de la composición de NNP de acuerdo con la presente divulgación, en donde el % en peso es en función de la materia seca total de la composición de pienso o material de pienso,

La presente divulgación se refiere además a un método para alimentar o criar a un rumiante, que comprende administrar la composición de NNP de acuerdo con la presente divulgación al rumiante, particularmente durante el régimen de alimentación, y/o alimentar al rumiante con la composición de pienso para rumiantes de acuerdo con la presente divulgación.

La divulgación tal como se definió anteriormente se ilustrará y explicará con más detalle en la siguiente parte experimental, la cual no pretende limitar el alcance de esta divulgación de ninguna manera.

## **EJEMPLOS**

### **EJEMPLO 1**

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Cada fuente de NNP tiene características específicas, que determinan la liberación de N después de entrar en el rumen. Sin embargo, la eficiencia del uso de N que haga la microbiota del rumen dependerá de la cantidad y la tasa de liberación de la fuente de NNP. Esto puede dar como resultado una diferencia en la eficiencia de la producción de proteína microbiana en el rumen. En este ejemplo, se evaluó la utilización de N *in vitro* de 3 productos de NNP diferentes y la mezcla de acuerdo con la presente divulgación en comparación con urea recubierta y sin recubrir como fuentes de NNP para la fermentación ruminal.

### ***Materiales de prueba***

- Se seleccionó un total de 6 productos de NNP diferentes y una mezcla de acuerdo con la presente divulgación para evaluar en un modelo de fermentación ruminal *in vitro* (ver la tabla 1) más adelante. La mezcla de acuerdo con la presente divulgación, en los diferentes ejemplos a la que en general se hace referencia mediante el código UB, en general comprende entre 37 y 52 % en peso de urea, entre 39 y 46 % en peso de biuret, entre 3 y 6 % en peso de triuret, entre 0,5 y 3 % en peso de amelida, entre 3 y 10 % en peso de ácido cianúrico, y entre 0,1 y 1,5 % en peso de humedad, en función del peso total de la mezcla.

*Tabla 1: Descripción general de productos evaluados, su proveedor, forma física, pureza y contenido de nitrógeno*

| Producto                                      | Proporcionado por | Forma física | Pureza (%) | Contenido de nitrógeno (%) |
|-----------------------------------------------|-------------------|--------------|------------|----------------------------|
| Urea sin recubrir                             | Yara              | Perlas       | 99         | 46,2                       |
| Urea                                          | Yara              | Perlas       | 89         | 41,5                       |
| Biuret                                        | Alfa Aesar        | Polvo        | 97         | 39,5                       |
| Ácido cianúrico                               | Yara              | Polvo        | 100        | 32,6                       |
| Triuret                                       | Enamina           | Polvo        | 95         | 36,4                       |
| Amelida                                       | Dr. Ehrenstorfer  | Polvo        | 99         | 43,3                       |
| Mezcla de acuerdo con la presente divulgación | Solicitante       | Polvo        | 100        | 41,0                       |

### ***Protocolo in vitro***

El modelo *in vitro* usado para simular la digestión en el rumen y la utilización de los productos es el protocolo de Prueba de Producción de Gas, adaptado para la digestibilidad de proteínas, como se describe con más detalle en el siguiente párrafo.

Por la mañana se recolectó aproximadamente 1 litro de fluido del rumen de dos animales diferentes fistulados ruminalmente. El fluido del rumen se coló a través de una estameña, se mantuvo a 39 °C en CO<sub>2</sub> y luego se diluyó 1:19 con un amortiguador libre de nitrógeno y solución mineral que contenía 10,03 g de NaHCO<sub>3</sub>, 1,43 g de Na<sub>2</sub>HPO<sub>4</sub>, 1,55 g de KH<sub>2</sub>PO<sub>4</sub>, 0,15 g de MgSO<sub>4</sub>·7H<sub>2</sub>O, 0,52 g de Na<sub>2</sub>S, 0,017 g de CaCl<sub>2</sub>·2H<sub>2</sub>O, 0,015 g de MnCl<sub>2</sub>·4H<sub>2</sub>O, 0,002 g de CoCl<sub>3</sub>·6H<sub>2</sub>O, 0,012 g de FeCl<sub>3</sub>·6H<sub>2</sub>O y 0,125 mg de resazurina por litro.

Para garantizar que el fluido del rumen se drene de nitrógeno libre y para garantizar que el nitrógeno sería el factor limitante para el crecimiento microbiano al inicio del experimento, el fluido del rumen amortiguado se incubó previamente durante 4 horas a 39 °C con una sobrecarga de carbohidratos de glucosa, xilosa y almidón soluble, cada uno a 3,33 g/l. Durante esta incubación previa, todo el nitrógeno disponible en el fluido del rumen se incorporaría a la proteína bacteriana y otros componentes que contienen nitrógeno bacteriano.

Cada producto se pesó en diferentes botellas de vidrio sobre una base isonitrogenada, proporcionando 15 mg de N por matraz. Luego, se agregaron 60 ml del fluido del rumen amortiguado incubado previamente, en purga continua con CO<sub>2</sub>. Los matraces se cerraron con tapones de caucho y se colocaron en un baño de agua con agitación con 50 movimientos por minuto a 39 °C para iniciar el período de incubación. La producción de gas de cada matraz se registró continuamente durante 72 horas usando un sistema totalmente automatizado. Todos los productos de prueba que incluyen un blanco (es decir, sin producto añadido al matraz de fluido del rumen) se evaluaron en una ejecución por triplicado.

La producción de gas acumulado se midió continuamente durante 72 horas. Los resultados promedio por cada triplicado se muestran en la figura 1.

### **Resultados**

Como se puede observar en la figura 1, las curvas de producción de gas acumulado de amelida y ácido cianúrico fueron comparables a la muestra en blanco. En general, los productos son relativamente poco solubles en agua en comparación con los otros productos evaluados. Además, el nitrógeno en estos productos se incorpora a una estructura de anillo que es menos accesible. La solubilidad de amelida y ácido cianúrico en nitrógeno parece ser demasiado baja para favorecer el crecimiento bacteriano. Para

amelida, el ácido cianúrico así como el blanco, la producción de gas sin embargo no es igual a cero, dado que el intercambio microbiano libera pequeñas cantidades de nitrógeno a la solución, que pueden usar los microorganismos restantes. La producción de gas acumulado después de 72 horas no fue significativamente diferente entre el  
5 blanco, amelida y ácido cianúrico.

Como también se puede observar en la figura 1, los perfiles de producción de gas acumulado de la urea sin recubrir y la urea recubierta mostraron un período de desfase de aproximadamente 5 horas, después del cual la producción de gas acumulado aumentó rápidamente a un máximo de 17 ml/hora para la urea sin recubrir y 19 ml/hora  
10 para la urea recubierta. Después de aproximadamente 15 horas de incubación, la producción de gas acumulado se ralentizó rápidamente y se detuvo después de 30 horas. La solubilidad de urea en agua demostró ser muy rápida, dentro de minutos. El período de desfase más prolongado de 5 horas para productos de urea se puede explicar a partir del efecto de la rápida liberación de amoníaco sobre el fluido del rumen  
15 amortiguado incubado previamente, evitando la liberación de  $\text{CO}_2$  del fluido. Se esperaba que la urea recubierta tuviera una tasa de liberación de nitrógeno más lenta que la urea sin recubrir, que no resultó en una diferencia en producción de gas acumulado durante las 72 horas de incubación. La producción de gas acumulado de la urea sin recubrir fue numéricamente menor que la urea recubierta entre 10 y 20 horas  
20 de incubación, lo cual puede estar relacionado con la liberación más lenta del nitrógeno de la urea recubierta. El Biuret y triuret mostraron una curva de producción de gas acumulado por debajo de la urea recubierta y sin recubrir. El biuret tuvo un período de desfase comparable de 5 horas, pero una tasa de producción de gas más lenta en las primeras 15 horas de incubación. La producción de gas acumulado fue la más alta en el  
25 período de entre 15 y 25 horas de incubación, y se ralentizó luego hasta que se alcanzó la máxima producción de gas acumulado después de 40 horas. El triuret mostró un tiempo de desfase más prolongado de 9 horas, lo que sugiere una liberación de nitrógeno más lenta que se esperaba en función de las características de triuret con respecto a biuret que es menos soluble. Después de 9 horas, la tasa de producción de  
30 gas comenzó de manera relativamente lenta, y alcanzó el pico a las 20 horas de incubación. La producción de gas acumulado fue más bien estable a un nivel bajo de aproximadamente 4 ml/hora de 20 a 50 horas y disminuyó luego hasta que alcanzó el nivel final después de 60 horas.

Como también se puede observar en la figura 1, la producción de gas acumulado de la  
35 mezcla de acuerdo con la presente divulgación fue más alta en comparación con urea

recubierta durante las 72 horas de incubación. En el período de entre 20 y 40 horas de incubación, la producción de gas acumulado para la mezcla de acuerdo con la presente divulgación permaneció más lenta que la producción de gas acumulado de urea recubierta, que probablemente está relacionada con la liberación más lenta de N del biuret y otros compuestos de NNP presentes en esta mezcla. No obstante, la producción de gas acumulado de la mezcla de acuerdo con la presente divulgación siguió aumentando después de 40 h, y así se obtuvo la mayor producción de gas acumulado entre todas las fuentes de NNP evaluadas.

### **Conclusión**

- 10 La tasa de liberación de amoníaco rápida de urea sin recubrir limita la cantidad de urea que se puede usar en una formulación de dieta para rumiantes. Los altos niveles de inclusión de urea sin recubrir en una formulación de dieta para rumiantes aumentará la concentración de amoníaco en el rumen rápidamente, lo cual conlleva a acumulación y absorción ruminal de amoníaco y la posterior excreción de urea en la orina.
- 15 Como se esperaba, los compuestos puros de N, es decir, amelida, triuret y ácido cianúrico, que son subproductos que contienen N conocidos de la producción de biuret mediante descomposición térmica de urea y que también están presentes en menores cantidades en la mezcla de acuerdo con la presente divulgación, generaron una producción de gas acumulado mucho menor que urea sin recubrir y recubierta. La amelida y cianúrico parecen no ser adecuados como fuentes de nitrógeno digeribles en el rumen en nutrición para ganado lechero. Su solubilidad y la tasa de liberación de nitrógeno son demasiado lentas para favorecer el crecimiento microbiano. Las bacterias del rumen pueden usar el nitrógeno de biuret y triuret, pero este se libera más lento que la urea recubierta o sin recubrir. La lenta liberación de nitrógeno del triuret y biuret puede mejorar la utilización del nitrógeno. Sin embargo, se puede esperar que escape del rumen aproximadamente 12 a 24 horas después de la ingesta (según las características de la dieta, tales como digestibilidad y tamaño de partículas). Una parte del biuret y triuret (aproximadamente 60 %) no será usada por los microbios del rumen aun después de 24 horas, en función de los perfiles de producción de gas acumulado. Esta cantidad de nitrógeno sin usar probablemente pase el rumen y sea excretado después de pasar a través del tubo gastrointestinal.

La mezcla de acuerdo con la presente divulgación, particularmente la proporción de urea, biuret y los compuestos menores de N creados mediante la producción de biuret que parte desde urea en la presente, dio como resultado un patrón de fermentación inesperado como lo muestra la dinámica de la producción de gas acumulado,

alcanzando la mayor producción de gas total de entre todas las fuentes de NNP evaluadas. Como se puede ver en la figura 1, al comienzo, la pendiente de la curva de producción de gas acumulado es menos pronunciada en comparación con la de urea y urea sin recubrir, supuestamente debido a una cantidad menor de urea rápidamente disponible, pero luego, cuando los microorganismos usan el biuret que se fermentó de manera más lenta, la producción de gas acumulado es la más alta de todos los productos evaluados. Cuanto más gas acumulado se produce, mejor, dado que esto significa que la fermentación ruminal se maximiza. Esto indica que la mezcla de acuerdo con la presente divulgación, que comprende urea, biuret y subproductos que contienen N de la producción de biuret a partir de urea tiene un efecto sinérgico sobre la fermentación ruminal en comparación con la fermentación de los componentes individuales (tomados individualmente).

## **EJEMPLO 2**

En este ejemplo, se llevó a cabo un ensayo *in vitro* usando fermentadores de cultivo de doble flujo continuo, con el objetivo de simular la fermentación ruminal.

### **Fermentadores**

Se usaron ocho fermentadores de cultivo de doble flujo continuo de 1320 ml en dos períodos replicados. Cada período consistió en 5 días para la adaptación del fluido ruminal a los tratamientos y 3 días para el muestreo. El primer día de cada período, todos los fermentadores se inocularon con fluido ruminal sin diluir filtrado a través de dos capas de estameña para quitar las partículas del pienso. Las condiciones de fermentación fueron constantes, es decir,

- una temperatura de 39 °C,
- un pH de  $6,4 \pm 0,05$  mediante infusión de 3 N de HCl o 5 N de NaOH,
- anaerobiosis con infusión continua de gas N<sub>2</sub> a una tasa de 40 ml/min, y
- Infusión continua de saliva artificial para obtener tasas de dilución de líquidos y sólidos de 10 %/h y 5 %/h, respectivamente.

A los fermentadores se les suministraron 95 gramos/día de materia seca de una dieta formulada para cumplir o exceder las recomendaciones actuales de nutrientes para vacas lecheras en período de lactancia, es decir,

- 166 g/kg de proteína cruda (CP),
- 323 g/kg de fibra detergente neutra (NDF), y
- 201 g/kg de fibra detergente ácida (ADF),

Y se les suministró en tres administraciones diarias, es decir, a las 7, 15 y 23 en punto con una dieta de forraje : concentrado en un rango de 53 : 47.

### **Animales y dietas del tratamiento**

El fluido ruminal se tomó de dos vacas lecheras Holstein provistas de cánulas ruminales alimentadas con una dieta de 60 : 40 forraje : concentrado con alfalfa y grano molido (11 kg de materia seca/vaca/día). La tarde antes de la extracción ruminal, no se alimentó a los animales rumiantes.

En consecuencia, los tratamientos nutricionales con NNP fueron de la siguiente manera:

- Control: dieta basal a la que se agregó 0,51 % en peso de urea, en donde el % en peso es sobre la base de materia seca,

- Dieta: dieta basal a la que se agregó 0,55 % en peso de la mezcla de acuerdo con la presente divulgación, con la composición como en el ejemplo 1, en donde el % en peso es sobre la base de materia seca.

Para obtener dietas isoenergéticas e isonitrogenadas usando diferentes fuentes de NNP, se usó una determinación del contenido de nitrógeno mediante el método Kjeldahl para determinar el contenido de nitrógeno de cada producto de NNP (ver la tabla 2 a continuación).

*Tabla 2: Contenido de nitrógeno de fuentes de nitrógeno no proteico*

| Ejemplo experimental | % en peso de N |
|----------------------|----------------|
| Control              | 44,37          |
| Dieta                | 40,94          |

Dadas las diferencias en el porcentaje de N entre los productos, el nivel de inclusión de cada fuente de NNP fue diferente (ver la tabla 3 a continuación).

*Tabla 3. Composición del ingrediente de los tratamientos nutricionales*

| Ingrediente (% en peso de materia seca) | Tratamiento nutricional con NNP |      |
|-----------------------------------------|---------------------------------|------|
|                                         | Control                         | D1   |
| Grano de maíz, seco molido              | 40,6                            | 40,6 |
| Heno de legumbres < 40 % en peso de NDF | 26,7                            | 26,7 |
| Césped, heno, maduro                    | 26,3                            | 26,3 |
| Harina de soja, 48 % en peso de CP      | 4,61                            | 4,61 |
| Urea                                    | 0,51                            | -    |

|                                                              |      |      |
|--------------------------------------------------------------|------|------|
| Mezcla de acuerdo con la presente divulgación (ver el ej. 1) | -    | 0,55 |
| Carbonato de calcio (CaCO <sub>3</sub> )                     | 0,46 | 0,46 |

## 5 **Recolección de muestras**

El segundo día de muestreo, se tomaron 4 ml de fluido del fermentador filtrado 2 horas después de la alimentación matutina para determinar las concentraciones de VFA.

Durante los días de muestreo, los recipientes de recolección se mantuvieron a 4 °C para evitar la actividad microbiana. Los efluentes sólidos y líquidos de 3 días de muestreo formaron un compuesto, se mezclaron dentro del fermentador y se homogeneizaron. Se tomaron submuestras para los análisis de los ácidos grasos volátiles (VFA). El resto de la muestra se liofilizó (secado por congelación) y las muestras secas se analizaron para determinar DM, cenizas, N y purinas para calcular nitrógeno bacteriano y contenido de materia orgánica (OM).

Las células bacterianas se obtuvieron de los matraces del fermentador el último día de cada período experimental. Las bacterias sólidas y líquidas asociadas se aislaron usando una combinación de varios procedimientos de desprendimiento seleccionados para obtener el máximo desprendimiento sin afectar la integridad de la célula. Las células bacterianas se liofilizaron y las muestras secas se analizaron para determinar materia seca (DM), cenizas, N y purinas para calcular el nitrógeno bacteriano.

## **Análisis químico**

La materia seca (DM) del efluente se determinó mediante la liofilización de alícuotas de 300 ml por triplicado. El contenido de DM de las dietas y muestras bacterianas se determinaron al secar muestras durante 24 horas en un horno de aire forzado a 103 °C (ver AOAC, 1990; método 950.01).

Las muestras secas de dietas, efluentes y bacterias se incineraron durante la noche a 550 °C en un horno de mufla (ver AOAC, 1990, método 976.05) y la materia orgánica (OM) se determinó por diferencia. El N total de dietas, efluentes y muestras bacterianas se determinaron mediante un método Kjeldhal (AOAC, 1990; método 976.05). La proteína cruda (CP) de la muestra se calculó como  $N \times 6,25$ . La CP del efluente se determinó en muestras líquidas. La degradación de proteína cruda se calculó como la diferencia entre CP en la dieta menos CP en el efluente y se expresó como % en peso de DM. La EMPS se determinó al dividir el N bacteriano por la materia orgánica fermentada.

Los componentes de la fibra de las dietas y los efluentes se analizaron de manera secuencial usando una alfa-amilasa termoestable y sulfito de sodio y se presentaron sin cenizas residuales.

Las muestras para el análisis de VFA se prepararon tal como se describe en Jouany, J. P., y J. Senaud. "Influence des ciliés du rumen sur la digestion de différents glucides chez le mouton. I.—Utilisation des glucides pariétaux (cellulose et hémicelluloses) et de l'amidon." *Reproduction Nutrition Développement* 22.5 (1982): 735-752. y se analizaron mediante cromatografía de gases.

### Resultados

En la tabla 4 a continuación, se muestra el efecto de las dietas con NNP sobre el metabolismo del nitrógeno de microorganismos ruminales, la verdadera digestibilidad de DM y OM, la degradación de CP, la eficiencia de la síntesis de proteína microbiana (EMPS) en el rumen (o en otras palabras, cuánta proteína microbiana se produce debido a la actividad microbiana para cada unidad de sustrato que se suministra al rumiante), en la dieta de control ("Control" – que comprende urea) y una dieta ("Dieta" – que comprende UB) que comprende una mezcla de acuerdo con la presente divulgación.

*Tabla 4: Efecto del tratamiento nutricional con NNP sobre el metabolismo del nitrógeno de microorganismos ruminales*

|                                                            | Tratamiento nutricional con NNP |            |
|------------------------------------------------------------|---------------------------------|------------|
|                                                            | Control (urea)                  | Dieta (UB) |
| Verdadera digestibilidad, % en peso de DM                  |                                 |            |
| DM                                                         | 53,53                           | 56,79      |
| OM                                                         | 52,95                           | 55,90      |
| Degradación de CP (% de materia seca)                      | 47,7                            | 53,2       |
| EMPS (g de N bacteriano/kg de materia orgánica fermentada) | 35,7                            | 39,4       |
| VFA                                                        |                                 |            |
| Acetato                                                    | 62,92                           | 60,06      |
| Propionato                                                 | 18,18                           | 23,29      |

|                                               |     |      |
|-----------------------------------------------|-----|------|
| Relación de acetato con respecto a propionato | 3,5 | 2,76 |
|-----------------------------------------------|-----|------|

### **Conclusión**

- Según la tabla 4, se puede concluir que la dieta que comprende una mezcla de urea, biuret y la cantidad menor de compuestos que contienen N que derivan de la producción de biuret a partir de urea de acuerdo con la presente divulgación tienen
- una mayor digestibilidad verdadera de DM y OM, con un aumento en la digestibilidad de alrededor de 5 %-6 % en comparación con una dieta que comprende urea como fuente de NNP,
  - un aumento de la EMPS, lo que significa que la microbiota del rumen maximizó la utilización del N, con un aumento de EMPS de alrededor de 10 % en comparación con una dieta que comprende urea como fuente de NNP, y
  - una mayor cantidad de propionato y una menor cantidad de acetato, mediante la cual disminuyó la relación de acetato con respecto a propionato, lo que sugiere que esta mezcla de NNP de acuerdo con la presente divulgación mejora la eficiencia de la utilización de energía ruminal, con una disminución en la relación de acetato con respecto a propionato de alrededor de 20 % en comparación con una dieta que comprende urea como una fuente de NNP.

### **EJEMPLO 3**

#### ***Animales, diseño experimental y tratamientos***

Un total de 72 novillos de un año cruzados [303 kg  $\pm$  29 kg de peso corporal (BW) inicial se estratificaron por peso corporal (BW) y se asignaron aleatoriamente a tres tratamientos. Todos los novillos consumían una dieta basal de ensilaje de maíz, residuos de desmote de algodón y una premezcla de vitaminas y minerales.

Los novillos se alojaron en establos (8 novillos / establo, 3 establos / tratamiento). Los novillos se enriquecieron con dos fuentes de NNP diferentes sobre una base isonitrogenada, considerando como referencia el N proporcionado por la urea cuando se incluyó a 1 % en peso de la dieta sobre la base de materia seca (DM).

En la tabla 5, se muestra la composición de la dieta basal suplementada con dos tipos de NNP diferentes, es decir, urea (Urea) (46 % de N) y una mezcla de urea y biuret de acuerdo con la presente divulgación (UB), con una composición como en el ejemplo 1 (41 % de N).

A fin de determinar la DM del pienso, aproximadamente 0,5 g de la muestra se pesaron por duplicado, se secaron en un horno de aire forzado a 100 °C durante 24 horas, y se incineraron a 550 °C durante seis horas.

Para la determinación del componente fibroso, las muestras se pesaron por duplicado en bolsas F56 (Ankom Technology Crop., Macedon, NY) y se analizaron para determinar la fibra detergente neutra (FDN), usando  $\alpha$ -amilasa termoestable y sulfito de sodio, y posteriormente para determinar la fibra detergente ácida (FDA) como se describió en Van Soest *et al.* (1991) en el analizador de fibra Ankom 200 (Ankom Technology Corp., Macedon, NY).

La concentración de N se analizó mediante el método de combustión seca Dumas usando un Vario Micro Cube (Elementar, Manchester, Reino Unido) después de que las muestras se sometieran a molino de bolas usando un Mixer Mill MM 400 (Retsch) a 25 Hz durante 9 minutos.

Las muestras de pienso se analizaron para determinar la concentración de almidón mediante el método enzimático-colorimétrico de Hall (2015) con las siguientes modificaciones: la glucosa se analizó usando un kit colorimétrico cuantitativo (G7521-11, Pointe Scientific Inc., Canton, MI), la absorbancia se leyó en muestras de 200  $\mu$ l a DO520 en placas de fondo plano de 96 cavidades (Corning Costar 3361, Thermo Fisher Scientific Inc., Waltham, MA) usando un lector de placas (Fisherbrand UV / VIS AccuSkan GO Spectrophotometer, Thermo Fisher Scientific Inc., Hampton, NH).

*Tabla 5: Composición de la dieta basal suplementada con diferentes tipos de NNP*

| Ítem                                        | Tratamiento |       |
|---------------------------------------------|-------------|-------|
|                                             | +Urea       | +UB   |
| Ingrediente, % en peso de materia seca      |             |       |
| Ensilaje de maíz                            | 77,00       | 76,88 |
| Residuos de desmote de algodón              | 20,00       | 20,00 |
| Mineral                                     | 2,00        | 2,00  |
| Fuente de NNP                               | 1,00        | 1,12  |
| Composición                                 |             |       |
| DM (materia seca), % en peso suministrado   | 37,13       | 36,17 |
| CP (proteína cruda), % en peso de DM        | 13,20       | 12,70 |
| CP soluble, % en peso de CP                 | 49,00       | 53,00 |
| iNDF (fibra detergente neutra no digerible) | 41,40       | 38,30 |
| ADF (fibra detergente ácida)                | 30,10       | 29,40 |

|                          |       |       |
|--------------------------|-------|-------|
| Almidón, % en peso de DM | 27,70 | 29,80 |
| Ceniza, % en peso de DM  | 5,91  | 5,78  |

### ***Adaptación animal***

Desde el día -17 hasta el día -16 con respecto al inicio del período experimental, los novillos recibieron 20 % de su NNP suplementario total.

Desde el día -15 hasta el día -12 con respecto al inicio del período experimental, los novillos recibieron 40 % de su NNP suplementario total.

Desde el día -11 hasta el día -8 con respecto al inicio del período experimental, los novillos recibieron 60 % de su NNP suplementario total.

Desde el día -7 hasta el día -3 con respecto al inicio del período experimental, los novillos recibieron 80 % de su NNP suplementario total.

Desde el día -2 hasta el inicio del período experimental (día 0), los novillos recibieron 100 % de su NNP suplementario total.

### ***Mediciones del perfil sanguíneo***

Las muestras de sangre se recolectaron cada 14 días durante la evaluación del rendimiento mediante punción en la vena yugular en tubos evacuados de 10 ml que contenían heparina Na (BD Vacutainer, Franklin Lakes, NJ) y se colocaron en hielo y se centrifugaron a 1500 x g durante 15 minutos a 4 °C. El plasma se transfirió a tubos de polipropileno (12 x 75 mm, Fisherbrand, Thermo Fisher Scientific Inc, Waltham, MA) y se almacenó a – 20 °C para análisis adicional.

### ***Mediciones de emisión de metano entérico***

Cuando un rumiante come pienso, los nutrientes están inicialmente en forma de carbohidratos, proteínas y grasas (o lípidos). Durante la fermentación de los carbohidratos, se produce dióxido de carbono y metano.

La emisión de metano se midió en todos los novillos durante cinco días consecutivos usando la técnica del marcador SF<sub>6</sub> (Johnson *et al.*, 1994; Henry *et al.*, 2020). En ésta técnica, los novillos recibieron un tubo de permeación calibrado cargado con SF<sub>6</sub> (3,1 g ± 0,2 g) antes del período de medición. La tasa promedio de liberación de SF<sub>6</sub> fue 5,2 g ± 1,1 mg/d. Después de cinco días de entrenamiento, un recipiente recolector de gas al vacío se unió a cada animal para recolectar una muestra de la producción diaria de gas. Cada día, a la misma hora, se reemplazó un nuevo recipiente al vacío durante los cinco días del período de medición. Se usaron cinco recipientes de recolección para determinar las concentraciones ambientales de CH<sub>4</sub> y SF<sub>6</sub>. Se tomó una muestra de gas de cada recipiente y se conservó en botellas de 120 ml para su posterior análisis de CH<sub>4</sub> y SF<sub>6</sub> usando cromatografía de gases. Para el experimento actual, solo los novillos con

al menos tres días de recolección y medición exitosos se consideraron en el análisis final. Las muestras de gas se analizaron mediante cromatografía de gases (Agilent 7820A GC, Agilent Technologies, Palo Alto, CA). Se usó un detector de ionización de llama y de captura de electrones para el análisis de CH<sub>4</sub> y SF<sub>6</sub>, respectivamente, con una columna capilar (sílice de trama fusionada 25 m x 0,32 mm, recubrimiento Molsieve 5A, Varian CP7536, Varian Inc., Lake Forest, CA). Las temperaturas del inyector, la columna y el detector para el análisis de CH<sub>4</sub> fueron 80, 160 y 200 °C, respectivamente. Para SF<sub>6</sub>, las temperaturas fueron 50, 30 y 300 °C para el inyector, la columna y el detector, respectivamente. El gas portador de CH<sub>4</sub> y SF<sub>6</sub> fue N<sub>2</sub>. Las emisiones de CH<sub>4</sub> se calcularon de acuerdo con la siguiente ecuación:

$$QCH_4 = QSF_6 \times \frac{([CH_4]_{\gamma} - [CH_4]_{\beta})}{([SF_6]_{\gamma} - [SF_6]_{\beta})}$$

QCH<sub>4</sub> = emisiones de CH<sub>4</sub> por animal (g / día)

QSF<sub>6</sub> = tasa de liberación de SF<sub>6</sub> (mg / día)

[CH<sub>4</sub>]<sub>γ</sub> = la concentración de CH<sub>4</sub> en el recipiente de recolección de los animales

[CH<sub>4</sub>]<sub>β</sub> = la concentración de CH<sub>4</sub> en el recipiente de recolección ambiental

[SF<sub>6</sub>]<sub>γ</sub> = la concentración de SF<sub>6</sub> en el recipiente de recolección de los animales

[SF<sub>6</sub>]<sub>β</sub> = la concentración de SF<sub>6</sub> en el recipiente de recolección ambiental

### ***Relación del aumento de peso con respecto al pienso***

La relación del aumento de peso con respecto al pienso (G:F) se calculó como la relación del aumento de peso promedio diario (ADG) con respecto a la ingesta diaria de materia seca (DMI). La ingesta del pienso se registró diariamente. La ingesta individual se determinó mediante el sistema de monitoreo de ingesta GrowSafe®. En el control del ganado, GrowSafe Systems es una herramienta usada comúnmente para monitorear de cerca los datos de alimentación de cada animal usando la tecnología de etiquetas de identificación por radiofrecuencia (RFID). Los animales están equipados con una etiqueta RFID única que los comederos leen cada vez que el animal baja la cabeza al comedero para consumir el pienso. El aumento de peso promedio diario (ADG) y la eficiencia del pienso se calcularon durante la evaluación del rendimiento. El ADG se determinó mediante la diferencia entre el BW final y el BW inicial dividido por el número de días de la evaluación del rendimiento (56 días). El BW inicial se calculó como el BW promedio sin reducir de vaquillonas los días 16 y 17. El BW final fue el BW promedio sin reducir medido los días 72 y 73. El BW sin reducir se obtuvo cada 14 días.

### ***Efecto de la suplementación de NNP sobre el rendimiento animal y el nitrógeno ureico en plasma (PUN)***

En la tabla 6, se muestra el efecto de la suplementación de NNP con urea y UB sobre el rendimiento del crecimiento. Más en particular, se muestran el BW inicial y final y el ADG, todos expresados en kg, la ingesta de materia seca (DMI) expresada en kg / día y en porcentaje de BW, la relación de aumento con respecto al pienso (G:F) expresada en kg / kg, la energía neta de aumento (NEg) expresada en Mcal / kg de DM y el nitrógeno ureico en plasma (PUN) expresado en mg / dl.

*Tabla 6: Efecto de la suplementación de NNP sobre el rendimiento animal y el nitrógeno ureico en plasma (PUN)*

| Ítem                  | Tratamiento |        |
|-----------------------|-------------|--------|
|                       | +Urea       | +UB    |
| BW inicial (kg)       | 288,69      | 283,18 |
| BW final (kg)         | 343,69      | 342,11 |
| ADG (kg)              |             |        |
| 0 – 14                | 0,78        | 0,72   |
| 0 – 28                | 0,81        | 0,72   |
| 0 – 42                | 1,02        | 1,04   |
| 0 – 56                | 1,01        | 1,05   |
| DMI (kg / día)        |             |        |
| 0 – 14                | 6,82        | 6,91   |
| 0 – 28                | 7,03        | 7,40   |
| 0 – 42                | 7,24        | 7,85   |
| 0 – 56                | 7,29        | 7,98   |
| DMI (% de BW)         | 2,31        | 2,54   |
| G:F (kg / kg)         |             |        |
| 0 – 14                | 0,10        | 0,10   |
| 0 – 28                | 0,11        | 0,10   |
| 0 – 42                | 0,14        | 0,13   |
| 0 – 56                | 0,14        | 0,13   |
| NEg (Mcal / kg de DM) | 0,55        | 0,52   |
| PUN (mg / dl)         |             |        |
| 0                     | 7,80        | 7,59   |
| 14                    | 6,76        | 7,52   |
| 28                    | 10,41       | 8,78   |
| 42                    | 9,25        | 7,55   |

|    |      |      |
|----|------|------|
| 56 | 8,20 | 8,60 |
|----|------|------|

La tabla 6 demuestra que, en comparación con la suplementación con urea, la suplementación con la fuente de NNP de UB:

- aumentó el ADG, en particular, durante la segunda mitad del período de prueba;
- 5 - aumentó la DMI durante la duración completa del experimento, con un aumento en DMI de al menos 5 % durante los distintos períodos de tiempo considerados, en comparación con la dieta que contenía urea como fuente de NNP;
- aumentó la DMI por unidad de BW, con un aumento de alrededor de 10 % en comparación con la dieta que contenía urea como fuente de NNP.

#### 10 **Efecto de la suplementación de NNP sobre emisiones entéricas de novillos**

El metano es una pérdida de energía que representa entre 2 % y 12 % de la ingesta de energía bruta en rumiantes.

En la tabla 7, se muestra el efecto de la suplementación de NNP con urea y UB sobre la emisión entérica de metano de novillos. Más en particular, se muestra la ingesta expresada en kg / día, y la emisión de metano expresada en g / kg de DMI, g / kg de OMI y g / kg de ADG.

*Tabla 7: Efecto de la suplementación de NNP sobre la emisión de metano entérico de novillos*

| Ítem                     | Tratamiento |        |
|--------------------------|-------------|--------|
|                          | +Urea       | +UB    |
| Ingesta de DM (kg / día) | 6,08        | 7,11   |
| Emisión de metano        |             |        |
| (g / kg de DMI)          | 36,59       | 31,21  |
| (g / kg de OMI)          | 38,68       | 32,94  |
| (g / kg de ADG)          | 213,79      | 212,31 |

Según la tabla 7, se puede concluir que suplementar la dieta de novillos de un año con UB redujo la emisión de metano sin comprometer o incluso mejorar su rendimiento. En particular, se observó una disminución en las emisiones de metano, cuando se expresa como g/kg de DMI o g/kg de OMI, de alrededor de 15 %, en comparación con una dieta que contenía urea como fuente de NNP. De manera simultánea, se observó que la DMI considerada durante la duración completa del experimento aumentó alrededor de 17 % cuando se usó UB como fuente de NNP, en comparación con la dieta que contenía urea como fuente de NNP.

#### 35 **EJEMPLO 4**

En este ejemplo, se llevó a cabo un ensayo *in vitro* usando incubaciones de cultivo en lote, con el objetivo de simular la fermentación ruminal.

### ***Adaptación animal***

5 Dos novillos cruzados Angus canulizados ruminalmente ( $808,8 \pm 36,3$  kg de peso corporal) se usaron como donantes de fluido ruminal para las incubaciones de cultivo *in vitro* en lote. Los novillos se alimentaron con una dieta de ensilaje de maíz y residuos de desmote (70 % y 30 % sobre la base de materia seca, respectivamente) *ad libitum* al menos 35 días antes de recolectar el fluido ruminal para llevar a cabo las incubaciones *in vitro*. Cada novillo recibió una mezcla de NNP equivalente a 100 g de urea por día  
10 compuesta por una cantidad igual de N de las diferentes fuentes de NNP para adaptar la comunidad microbiana del rumen. De este modo, cada novillo se alimentó diariamente con 33 g de urea (46 % en peso de N) o 37 g de una mezcla de urea-biuret de acuerdo con la presente divulgación (UB) que comprendía 41 % en peso de N. Estas cantidades se mezclaron con la dieta de pienso cada dos días.

### ***Preparación del pienso y análisis químico***

El pienso se preparó mediante el secado de ensilaje de maíz y residuos de desmote durante 45 horas a 55 °C y se molió para pasar una rejilla de 2 mm en un molino Wiley.

### ***Diseño experimental y tratamientos***

Las incubaciones de cultivo *in vitro* en lote se llevaron a cabo como un diseño en bloque  
20 completo aleatorizado usando 70 % en peso de ensilaje de maíz y 30 % en peso de residuos de desmote sobre la base de materia seca conservando las características de la dieta usadas durante el período de adaptación animal. Los tratamientos se diseñaron para ser isonitrogenados (= que tienen la misma cantidad de nitrógeno alimenticio) y equivalentes a 1 % en peso de inclusión de urea. En consecuencia, los tratamientos con  
25 NNP fueron de la siguiente manera:

- Control sin suplementación de NNP (“control”);
- 100 % en peso de urea (Rumisan ®) (“urea”);
- 100 % en peso de una mezcla de urea/biuret de acuerdo con la presente solicitud (“UB”).

### ***Recolección de fluido del rumen e incubaciones in vitro***

Las incubaciones *in vitro* se llevaron a cabo en tres días separados (réplicas). Una muestra representativa de lo digerido se recolectó de diferentes lugares del rumen de los novillos canulizados ruminalmente y se coló a través de 4 capas de estameña, se colocó en recipientes térmicos precalentados y se transportó al laboratorio dentro de los  
35 30 minutos después de la recolección. En el laboratorio, el fluido ruminal de los dos

novillos se mantuvo en flujo constante de CO<sub>2</sub> y se combinó en proporciones iguales. El fluido ruminal combinado se mezcló con amortiguador de McDougall (McDougall, 1948) con y sin 0,077 g/litro de 10 % en peso de sulfato de amonio enriquecido con <sup>15</sup>N en una relación 1:4, y se usó como un inóculo. Se incubaron dos botellas de 500 ml provistas de un brazo lateral y un *septum* de caucho por tratamiento. Las botellas contenían 5,6 g de materia seca de sustrato y 400 ml de inóculo enriquecido con <sup>15</sup>N, y se incubaron durante 24 horas a 39 °C con una agitación de 60 rpm para monitorear la cinética de producción de gas usando un sistema de monitoreo de gas Ankom de Ankom Technologies, Fairport, Nueva York. Asimismo, una botella de 500 ml sin inóculo enriquecido con <sup>15</sup>N se incubó para determinar el enriquecimiento de <sup>15</sup>N basal. A las 12 horas de incubación, se recolectaron dos muestras de 10 ml de fluido del rumen de cada botella a través del puerto del *septum* usando una jeringa de 20 ml. Estas muestras se acidificaron mediante la adición de 10 µL de una solución de 20 % v/v de H<sub>2</sub>SO<sub>4</sub> a cada muestra de 10 ml, y se congelaron a menos 20 °C hasta análisis adicional. Al final de las 24 horas de incubación, se registró el pH final, las botellas se colocaron en hielo para evitar fermentación microbiana adicional y se recolectaron dos muestras de 10 ml y se acidificaron mediante la adición de 20 µL de una solución de 20 % v/v de H<sub>2</sub>SO<sub>4</sub> y se congelaron a menos 20 °C hasta análisis adicional. El contenido de fermentación restante se agitó luego a 60 rpm durante 30 segundos y se dividió en dos submuestras. De la primera submuestra, compuesta de contenido total, un volumen de 180 ml se congeló a menos 20 °C hasta análisis adicional y se usaron 180 ml de la segunda submuestra para recuperar el pelet microbiano. Además, dos tubos de 100 ml por tratamiento que contenían 0,7 g del sustrato sobre la base de materia seca y 50 ml de inóculo sin enriquecimiento de <sup>15</sup>N y dos blancos se incubaron durante 24 horas a 39 °C con agitación a una velocidad de 60 rpm. Después de las 24 horas de incubación con fluido ruminal, se agregaron 6 ml de HCl y 2 ml de una solución de pepsina al 5 %. Los tubos se incubaron durante otras 48 horas a 39 °C. Después del período de incubación, los tubos se mantuvieron en hielo y luego se almacenaron para análisis adicional.

#### ***Concentración de ácidos grasos volátiles (VFA)***

Los VFA producidos mediante fermentación microbiana en el rumen son la principal fuente de energía absorbida por el tubo digestivo de rumiantes. La cantidad y el perfil de VFA en el rumen tienen consecuencias para la eficiencia del uso de energía, producción de metano, riesgos de acidosis ruminal (caracterizada por una disminución en el pH causada por una acumulación de ácidos orgánicos en el rumen y un cambio en el delicado equilibrio de los microorganismos del rumen). Se usó una solución a base

de agua usando una extracción de acetato de etilo para determinar las concentraciones de VFA a las 12 y 24 horas. Las muestras se centrifugaron a  $10,000 \times g$  durante 15 min a  $4^\circ\text{C}$ , 2 ml del sobrenadante se mezclaron con 0,4 ml (relación 5:1) de una solución de ácido metafosfórico-crotónico, y las muestras se congelaron durante la noche. Luego, las muestras se descongelaron y se centrifugaron nuevamente a  $10,000 \times g$  durante 15 min a  $4^\circ\text{C}$ . El sobrenadante se transfirió a tubos de cultivo desechables de borosilicato de  $12 \times 75$  mm (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) y se mezcló con acetato de etilo para formar una mezcla de acetato de etilo : sobrenadante 2:1. Las muestras se analizaron con un cromatógrafo de gases (Agilent 7820A GC, Agilent Technologies) usando un detector de ionización de llama y una columna capilar (CP-WAX 58 FFAP  $25 \text{ m} \times 0,53 \text{ mm}$ , Varian CP7767; Varian Inc.).

### ***Síntesis de proteína microbiana***

El ecosistema microbiano del rumen está formado principalmente por especies de bacterias, hongos y protozoos. Los microbios fermentan los alimentos ingeridos por el rumiante y, mediante fermentación, producen productos finales que son utilizados por el rumiante así como los microbios en sí mismos para su propia proliferación y crecimiento. La diversidad de especies y sus proporciones relativas en la comunidad microbiana dependen principalmente de la dieta que consume el animal. Por ende, es importante que se alimente al animal con una mezcla saludable de diferentes productos alimenticios para que el rumen y su microflora sigan funcionando de manera eficiente. La velocidad de digestión del pienso depende de la calidad y del suplemento del pienso y se ve afectada por la cantidad y tipo de microbios, el pH del rumen, los nutrientes que limitan el crecimiento de los microbios y la eliminación de microbios del rumen. La energía y las proteínas son los nutrientes principales que limitan el crecimiento microbiano, y, por lo tanto, la fermentación ruminal. La población microbiana necesita energía y proteínas para su crecimiento y multiplicación. Las proteínas, cuando se digieren, se descomponen en péptidos, que son cadenas cortas de aminoácidos. La digestión adicional de los péptidos produce aminoácidos individuales y eventualmente amoníaco. Los microbios del rumen son la fuente principal de proteínas del animal rumiante. Si hay suficiente energía disponible, los microbios del rumen descomponen la proteína degradable del rumen en aminoácidos y luego en amoníaco que es una fuente principal de N para el crecimiento microbiano. Los microbios también convierten el NNP en amoníaco, lo que contribuye a la reserva de amoníaco disponible para el crecimiento microbiano. La proteína microbiana se expulsa continuamente del rumen al intestino donde se somete a digestión enzimática. Los aminoácidos producidos a partir de la

proteína microbiana digerida se absorben a través del intestino delgado. Por lo tanto, la cantidad de proteína microbiana que fluye hacia los intestinos depende, en gran medida, de la disponibilidad de energía fermentable y N en la dieta consumida por los rumiantes. El nitrógeno (N) total y el suplemento isotópico se midieron en todo el contenido y en el pelet bacteriano mediante una espectrometría de masas de relación isotópica usando un analizador Vario Micro Cube de Elementar Analyzer System GmbH. La submuestra de pelet de bacterias se centrifugó lentamente a 1,000 x g durante 10 minutos a 4 °C. El sobrenadante resultante se centrifugó rápidamente a 20,000 x g durante 20 minutos a 4 °C para obtener el pelet de bacterias. Luego, este pelet de bacterias se lavó con una solución salina al 0,9 % y se centrifugó a 20,000 x g durante 20 minutos a 4 °C, descartando el sobrenadante. El procedimiento de lavado con solución salina se repitió dos veces. Finalmente, el pelet bacteriano se liofilizó. Por separado, la submuestra de 180 ml de contenido total se liofilizó completamente. Las muestras secas del contenido total y del pelet bacteriano se molieron manualmente y se pesaron en una cápsula de estaño de peso estándar presionada de 8 x 5 mm usando una microbalanza Mettler-Toledo Excellence Plus XP de Mettler-Toledo GmbH, Laboratory and Weighing Technologies. Luego, se agregaron 35 µL de una solución de 10 g/L de K<sub>2</sub>CO<sub>3</sub> a cada muestra y se secaron durante la noche en un horno de aire forzado a 60 °C para evaporación completa de NH<sub>3</sub>-N. El porcentaje de átomo <sup>15</sup>N en las muestras secas se analizó en un espectrómetro de masas de relación isotópica (IsoPrime 100 de IsoPrime) y se expresó como la abundancia fraccional de fracciones isotópicas (<sup>15</sup>N / <sup>14</sup>N) multiplicada por 10.

#### ***Concentración de amoníaco-N***

La producción de amoníaco ruminal refleja, en gran medida, la cantidad de proteína degradable en el rumen. El nitrógeno amoniacal (amoníaco-N) ruminal es un nutriente crítico para favorecer el crecimiento microbiano y la fermentación. Asegurar la disponibilidad de amoníaco-N en el rumen de manera cuantitativa y oportuna es fundamental para lograr la tasa máxima de fermentación y digestibilidad del pienso.

La concentración de amoníaco-N a 12 y 24 horas en el fluido de incubación se midió después de centrifugar a 10,000 x g durante 15 minutos a 4 °C usando una centrifugadora Avanti J-E de Beckman Coulter® Incorporated. Un ml de reactivo de fenol se colocó con pipetas en tubos de cultivo desechables de borosilicato de 12 x 75 mm (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA). Una alícuota de 20 µL del sobrenadante de la muestra centrifugada se transfirió a los tubos de cultivo que contenían fenol. Después de agitar en vórtice, se agregaron 0,8 ml de solución de

hipoclorito a la mezcla y se agitó en vórtice nuevamente. Los tubos de cultivo se cubrieron con canicas de vidrio y se colocaron en un baño de agua a 95 °C durante 5 minutos. La absorbancia se leyó en placas de 96 cavidades de fondo plano a una longitud de onda de 665 nm usando un lector de placas DU-500 de Beckman Coulter® Inc. El residuo de fermentación reservado en los tubos de 100 ml se filtró, se secó a 105 °C en un horno de aire forzado durante 24 horas y se incineró a 550 °C durante 6 horas para determinar la materia orgánica sin digerir.

### **Desaparición de nutrientes**

La digestibilidad representa la proporción de nutrientes ingeridos que son absorbidos por el animal. La materia seca se define como la pérdida de peso de las muestras cuando se secan en un horno por sobre 100 °C durante 12 – 24 horas, mientras que la materia orgánica define la pérdida de peso de la materia seca cuando se combustiona (la materia seca menos el contenido de ceniza). La materia seca consiste en todos los nutrientes, mientras que la materia orgánica consiste en todos los nutrientes con excepción de las cenizas. La digestibilidad de la materia orgánica se define como la proporción de materia orgánica en el pienso que se digiere en el rumen o en el tubo digestivo total del rumiante. La digestibilidad ruminal de la materia orgánica puede usarse para estimar la energía disponible para estimular el crecimiento microbiano y la síntesis de proteína microbiana resultante en el rumen.

La digestibilidad *in vitro* de la materia orgánica seca (IVDMD) y la digestibilidad *in vitro* de la materia orgánica (IVOMD) se calcularon con las siguientes fórmulas:

$$IVDMD (\%) = \frac{\text{materia seca incubada} - \text{materia seca residual}}{\text{materia seca incubada}} \times 100$$

$$IVOMD (\%) = \frac{\text{materia orgánica incubada} - \text{materia orgánica residual}}{\text{materia orgánica incubada}} \times 100$$

### **Resultados**

En la tabla 8 a continuación, se muestra la concentración de VFA total y la concentración de amoníaco-N en: una dieta de control sin suplementación de NNP (“control”), una dieta a base de ensilaje de maíz con suplementación de urea (“urea”) o con suplementación de una mezcla de urea-biuret de acuerdo con la presente divulgación (“UB”) después de 12 horas de incubación. En la tabla 9 a continuación, se muestra lo mismo después de 24 horas de incubación. En la tabla 9, también se muestran el pH del fluido, la degradabilidad *in vitro* de la materia seca (IVDMD) y la degradabilidad *in vitro* de la materia orgánica (IVOMD) después de 24 horas de incubación. El pH del rumen tiene profundos efectos sobre el crecimiento de los microbios ruminales y la digestión que se lleva a cabo en este preestómago. El pH del rumen puede afectar el grado de

fermentación ruminal al alterar las poblaciones microbianas y la capacidad de absorción de los tejidos. Un pH ruminal bajo durante períodos prolongados cada día, por ejemplo, puede afectar a la ingesta de pienso, el metabolismo microbiano y la digestión del pienso, y también se relacionó con la inflamación, la diarrea y la depresión de la grasa láctea.

*Tabla 8: Efecto de la suplementación de NNP sobre VFA y amoníaco-N a las 12 horas de incubación en una dieta a base de ensilaje de maíz*

| Variable   | Control | Urea  | UB    |
|------------|---------|-------|-------|
|            | mM      |       |       |
| VFA total  | 30,95   | 31,77 | 32,55 |
| Amoníaco-N | 0,35    | 2,74  | 2,61  |

*Tabla 9: Efecto de la suplementación de NNP sobre VFA y amoníaco-N, pH y digestibilidad in vitro o materia orgánica y seca a las 24 horas de incubación en una dieta a base de ensilaje de maíz*

| Variable   | Control | Urea  | UB    |
|------------|---------|-------|-------|
|            | mM      |       |       |
| VFA total  | 41,39   | 40,96 | 42,85 |
| Amoníaco-N | 2,48    | 6,55  | 7,34  |
| pH         | 6,34    | 6,38  | 6,37  |
| IVDMD, %   | 45,02   | 45,08 | 45,78 |
| IVOMD, %   | 64,13   | 65,38 | 64,17 |

Según las tablas 8 y 9, se puede concluir que

- Con respecto a VFA total, la mezcla de UB de acuerdo con la presente solicitud generó una mayor cantidad de VFA en comparación con el control sin suplementación de NNP o con suplementación de urea. Estos descubrimientos indican que la mezcla de urea y biuret de acuerdo con la presente divulgación es más efectiva para favorecer la fermentación *in vitro* que la suplementación de urea.
- Con respecto al amoníaco-N, como se esperaba, la suplementación con NNP, es decir, urea o la mezcla de UBN aumentó su concentración.
- Con respecto a la IVDMD, la suplementación con UB, pero no con urea, mejoró el grado de degradación de materia seca. Estos descubrimientos respaldan además la

proposición de que la suplementación con UB es más efectiva para favorecer la fermentación ruminal que la urea.

- En general, estos resultados demuestran que la mezcla de UB de acuerdo con la presente solicitud es una fuente efectiva de NNP que supera a la urea para favorecer la fermentación *in vitro*.

En la tabla 10, se muestra la concentración de N bacteriano, la eficiencia de las bacterias (BE) o, dicho de otro modo, la eficiencia de la síntesis de proteína microbiana (EMPS), es decir, g de N bacteriano/ kg de OM realmente digerido para (i) una dieta de control sin suplementación de NNP ("control"), (ii) una dieta a base de ensilaje de maíz con suplementación de urea ("urea") o (iii) una dieta a base de ensilaje de maíz con suplementación con una mezcla de urea-biuret de acuerdo con la presente solicitud ("UB"), después de 24 horas de incubación. Estos parámetros definen la capacidad de los microbios del rumen para usar N.

*Tabla 10: Efecto de la suplementación de NNP sobre la síntesis microbiana y la producción de eficiencia microbiana en una dieta a base de maíz*

| Variable         | Control | Urea  | UB    |
|------------------|---------|-------|-------|
| N bacteriano, mg | 55,66   | 67,34 | 59,55 |
| EMPS             | 27,07   | 34,46 | 41,98 |

Según la tabla 10, se puede concluir que la suplementación de la dieta de control con NNP aumenta la cantidad de N asimilado por las bacterias. La suplementación con la mezcla de urea/biuret de acuerdo con la presente solicitud dio como resultado, en particular, una mayor eficiencia bacteriana/mayor eficiencia de la síntesis de proteína microbiana. Estos resultados coinciden con los resultados del ejemplo 2.

### **Producción de metano**

Cuando un rumiante come pienso, los nutrientes están inicialmente en forma de carbohidratos, proteínas y grasas (o lípidos). Durante la fermentación de los carbohidratos, se produce dióxido de carbono y metano.

La cantidad de metano producido se determinó al medir la producción total de gas y la contribución de metano a esta. La producción de gas se registró usando un sistema de monitoreo de gas Ankom de Ankom Technologies. Este sistema se adaptó a botellas de 500 ml durante todo el proceso de fermentación. La cinética de la producción se ajustó a un modelo de Gompertz para determinar la tasa de producción máxima total de gas

(M), la tasa fraccionaria de gas producido (kf) y la fase de desfase (L). Una submuestra de gas se analizó para medir la concentración de metano y calcular la producción total de metano durante la fermentación. La concentración de metano se determinó usando un cromatógrafo de gases Agilent 7820 GC de Agilent Technologies con ionización de llama y una columna capilar (sílice de trama fusionada 25 m x 0,32 mm, recubrimiento Molsieve 5A, Varian CP 7536 de Varian Inc.).

En la tabla 11, se muestra el efecto de la suplementación de NNP sobre la producción de metano en una dieta a base de ensilaje de maíz. Más en particular, se muestra la producción de gas metano expresada en mM/g de materia orgánica incubada (OMi) y expresada en mM/g de materia orgánica digerida (OMd), en una dieta de control sin suplementación de NNP (“control”), una dieta a base de ensilaje de maíz con suplementación de urea (“urea”) o bien con una mezcla de urea/biuret de acuerdo con la presente solicitud (“UB”).

La tabla 11 muestra que la suplementación con una mezcla de UB de acuerdo con la presente divulgación dio como resultado una menor producción de metano en comparación con la dieta de control o la dieta con urea como NNP. La producción de metano disminuyó en alrededor de 4-5 % cuando está expresada por g de OMi o por g de OMd, en comparación con la urea como NNP.

*Tabla 11: Efecto de la suplementación de NNP sobre la producción de metano en una dieta a base de ensilaje de maíz*

| Variable    | Control | Urea | UB   |
|-------------|---------|------|------|
| mM/g de OMi | 1,24    | 1,23 | 1,18 |
| mM/g de OMd | 6,93    | 6,86 | 6,58 |

#### **EJEMPLO 5 – dosis en aumento de UB en fermentación y producción de metano *in vitro***

El objetivo principal del ejemplo 5 fue evaluar el impacto de dosis en aumento de una mezcla de urea y biuret de acuerdo con la presente divulgación, en adelante UB, con la composición como en el ejemplo 1 (41 % de N, YARA International) sobre la fermentación *in vitro*, la digestibilidad de nutrientes y la producción de metano en una dieta a base de ensilaje de maíz.

#### ***Enfoque y procedimientos de investigación***

Se usaron dos novillos canulizados ruminalmente como donantes de fluido ruminal para las incubaciones de cultivo *in vitro* en lote. Los novillos se alimentaron con ensilaje de

maíz, residuos de desmote de algodón y una premezcla de vitaminas y minerales (70, 28 y 2 % sobre la base de materia seca, respectivamente) *ad libitum* al menos 35 d antes de recolectar el fluido ruminal para llevar a cabo las incubaciones *in vitro*. Para adaptar los novillos a la presencia de NNP, cada novillo recibió el N equivalente a 100 g de urea por día (46 g de N diarios/novillo) pero dividido en las dos fuentes de NNP. Por ende, cada novillo se alimentó diariamente con 33 g de urea o 37 g de una mezcla de urea/biuret de acuerdo con la presente solicitud. Estas cantidades se mezclaron diariamente con la dieta. Las incubaciones *in vitro* se llevaron a cabo en tres días separados (réplicas) usando el mismo ensilaje suministrado a los novillos como sustrato de incubación. Las fuentes de NNP a evaluar se analizaron para determinar el contenido total de N y se incubaron sobre una base isonitrogenada.

#### **Tratamientos**

Los sustratos de incubación (tratamientos) a evaluar fueron el control (CON, sin suplementación de NNP) y cuatro inclusiones en aumento (de 1× a 4×) de la mezcla de urea-biuret de acuerdo con la presente solicitud (UB). Los tratamientos a 1× se diseñaron para ser isonitrogenados y equivalentes a 1 % de la inclusión sobre la base de materia seca de urea (Yara International) en la dieta, excepto por el tratamiento con CON.

#### **Incubaciones de cultivo en lote**

Las incubaciones *in vitro* se llevaron a cabo en tres días separados (réplicas) usando el mismo ensilaje suministrado a los novillos como sustrato de incubación. Una muestra representativa de lo digerido del rumen se recolectó de diferentes lugares del rumen de los novillos canulizados ruminalmente y se coló a través de 4 capas de estameña, se colocó en recipientes térmicos precalentados y se transportó al laboratorio antes de los 30 minutos de la recolección. En el laboratorio, el fluido ruminal de los dos novillos se mantuvo en flujo constante de CO<sub>2</sub> y se combinó en proporciones iguales. El flujo ruminal combinado se mezcló con el amortiguador de McDougall a una relación 1:4 (es decir, inóculo).

Dos botellas de 120 ml por tratamiento que contenían 0,7 g y 50 ml de inóculo se incubaron durante 24 h a 39 °C con agitación suave (60 rpm). Además, se incubaron dos botellas sin sustrato como botellas vacías. La presión de gas se registró a las 24 h y se almacenó una muestra de gas para análisis adicional. Al final de las 24 h de incubación, se registró el pH final del fluido de fermentación y se recolectaron dos muestras de 10 ml y se acidificaron al agregar 20 µL de una solución al 20 % (v/v) de H<sub>2</sub>SO<sub>4</sub> y se congelaron a -20 °C hasta análisis adicional.

La medición de la digestibilidad *in vitro* de la materia orgánica (IVOMD) se llevó a cabo al incubar un conjunto separado de tubos duplicados de polipropileno de 100 ml durante 24 h a 39 °C en cada uno de los días de réplica. Estos matraces contenían 0,7 g del sustrato y se inocularon con 50 ml de una mezcla 4:1 de amortiguador McDougall:fluido ruminal. Después de 24 h de incubación con fluido ruminal, se agregó una solución de pepsina-HCl, y se incubaron tubos durante otras 48 h más antes de filtrarlos, secarlos a 105 °C en un horno de aire forzado durante 24 h, e incinerarlos a 550 °C durante 6 h para determinar la materia orgánica sin digerir.

#### **Análisis de muestras**

- 10 Las concentraciones de NH<sub>3</sub>-N en el fluido de incubación se midieron después de centrifugar a 10,000 x *g* durante 15 min a 4 °C (Avanti J-E, Beckman Coulter Inc., Palo Alto, CA) siguiendo la metodología descrita en Broderick y Kang (1980). Brevemente, se colocó en pipetas 1 ml de un reactivo de fenol en tubos de cultivo desechables de borosilicato de 12 × 75 mm (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA).
- 15 Una alícuota de 20 µL del sobrenadante de la muestra centrifugada se transfirió a los tubos de cultivo que contienen fenol. Después de agitar en vórtice, se agregaron 0,8 ml de una solución de hipoclorito a la mezcla y se agitó en vórtice nuevamente. Los tubos de cultivo se cubrieron con canicas de vidrio y se colocaron en un baño de agua a 95 °C durante 5 min. La absorbancia se leyó en muestras de 200 µL a OD<sub>620</sub> en placas de 20 96 cavidades de fondo plano (Corning Costar 3361, Thermo Fisher Scientific Inc., Waltham, MA) usando un lector de placas (Fisherbrand UV/VIS AccuSkan GO Spectrophotometer, Thermo Fisher Scientific Inc., Hampton, NH). Todos los ensayos se llevaron a cabo en determinaciones duplicadas con análisis posteriores realizados cuando los CV estaban por encima del 5 %.
- 25 La producción total de gas se midió usando un transductor manual (Digital Test Gauge, Ashcroft Inc., Stratford, CT, EE.UU.), y se analizó una submuestra para medir la concentración de CH<sub>4</sub> mediante cromatografía de gases para calcular la producción total de CH<sub>4</sub> durante el transcurso de la fermentación. Para determinar las concentraciones de CH<sub>4</sub>, se usó un cromatógrafo de gases (Agilent 7820A GC, Agilent Technologies, 30 Palo Alto, CA) que usa ionización de llama y una columna capilar (sílice de trama fusionada 25 m × 0,32 mm, recubrimiento Molsieve 5A, Varian CP7536; Varian Inc., Lake Forest, CA). Las temperaturas del inyector, la columna, y el detector fueron 80, 160, y 200 °C, respectivamente, y N<sub>2</sub> fue el gas portador que fluyó a 3,3 ml/min. La relación de separación para la muestra de CH<sub>4</sub> inyectada fue 100:1.

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Además, las concentraciones totales de ácidos grasos volátiles (VFA) se midieron mediante cromatografía de gases en el fluido de incubación al final de la fermentación para evaluar los cambios potenciales en el perfil de VFA que se producen, siguiendo la metodología descrita en Ruiz-Moreno *et al.* (2015). Brevemente, las muestras se centrifugaron a  $10,000 \times g$  durante 15 min a  $4^\circ\text{C}$ . Dos milímetros del sobrenadante se mezclaron con 0,4 ml (relación 5:1) de una solución de ácido metafosfórico:crotónico (estándar interno), y las muestras se congelaron durante la noche. Las muestras se descongelaron y se centrifugaron nuevamente a  $10,000 \times g$  durante 15 min a  $4^\circ\text{C}$ . El sobrenadante se transfirió a tubos de cultivo desechables de borosilicato de 12 mm  $\times$  75 mm (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) y se mezcló con acetato de etilo para formar una mezcla de acetato de etilo:sobrenadante 2:1. Los tubos de cultivo se agitaron vigorosamente y luego se dejaron reposar 5 min para permitir la separación del acetato de etilo. Una submuestra del acetato de etilo se transfirió a pequeños viales antes del análisis. Las muestras se analizaron con un cromatógrafo de gases (Agilent 7820A GC, Agilent Technologies) usando un detector de ionización de llama y una columna capilar (CP-WAX 58 FFAP 25 m  $\times$  0,53 mm, Varian CP7767; Varian Inc.). La temperatura de columna se mantuvo a  $110^\circ\text{C}$ , y las temperaturas del inyector y detector fueron  $200^\circ\text{C}$  y  $220^\circ\text{C}$ , respectivamente.

#### **Dosis en aumento de urea/biuret en fermentación in vitro y emisiones de $\text{CH}_4$ - resultados**

Los resultados se representan en la tabla 12.

*Tabla 12. Efecto de aumentar la inclusión de UB sobre el pH de la fermentación fluida, las concentraciones de ácidos grasos volátiles y amoníaco-N, la digestibilidad in vitro de la materia orgánica, y la producción de gas y metano en una dieta a base de ensilaje de maíz.*

| Variable <sup>1</sup>               | Inclusión de UB |        |        |       |       |
|-------------------------------------|-----------------|--------|--------|-------|-------|
|                                     | 0x              | 1x     | 2x     | 3x    | 4x    |
| pH                                  | 6,50            | 6,49   | 6,54   | 6,59  | 6,63  |
| Ácidos grasos volátiles totales, mM | 88,72           | 105,78 | 101,83 | 99,92 | 96,03 |
| Acetato, mM                         | 55,57           | 65,41  | 52,45  | 61,03 | 59,15 |
| Propionato, mM                      | 18,19           | 22,12  | 21,14  | 21,09 | 19,68 |
| Butirato, mM                        | 10,79           | 13,21  | 13,36  | 13,02 | 12,69 |
| Acetato, mol/100 mol                | 62,64           | 61,76  | 61,32  | 61,00 | 61,56 |
| Propionato, mol/100 mol             | 20,52           | 20,89  | 20,77  | 21,07 | 20,52 |

|   |                                   |        |        |        |        |        |
|---|-----------------------------------|--------|--------|--------|--------|--------|
|   | Butirato, mol/100 mol             | 12,15  | 12,57  | 13,11  | 13,13  | 13,25  |
|   | Acetato:Propionato                | 3,05   | 2,97   | 2,97   | 2,90   | 3,01   |
|   | Amoníaco-N, mM                    | 3,25   | 6,96   | 9,96   | 13,88  | 15,39  |
|   | IVOMD, %                          | 54,40  | 54,96  | 55,13  | 58,77  | 57,48  |
| 5 | Producción de gas, ml/g de OMD    | 300,26 | 301,34 | 297,04 | 263,94 | 262,43 |
|   | Producción de metano, mM/g de OMD | 33,73  | 32,74  | 32,43  | 28,84  | 23,02  |

<sup>1</sup> IVOMD: Digestibilidad *in vitro* de materia orgánica; OMD: Materia orgánica degradada.

10 Las dosis en aumento de UB aumentaron linealmente la concentración de NH<sub>3</sub>-N, el pH del fluido del rumen y la IVOMD. Asimismo, la concentración de VFA total, acetato, propionato y butirato tuvieron un aumento cuadrático cuando se aumentó la inclusión de UB. Adicionalmente, el aumento de la inclusión de UB redujo linealmente la relación de acetato:propionato y la producción de gas y CH<sub>4</sub> (tabla 12).

15 En el presente experimento, la inclusión de UB aumentó la concentración de NH<sub>3</sub>-N que dio como resultado una mayor IVOMD y concentración de VFA total e individual, lo que indica una mayor fermentación. Los microbios del rumen pueden usar NH<sub>3</sub>-N para sintetizar la proteína microbiana promoviendo una mayor fermentación. Además, la mayor concentración de NH<sub>3</sub>-N concuerda con el aumento lineal del pH del fluido de rumen.

20 El aumento de la inclusión de UB promovió otras vías metabólicas distintas de la formación de acetato, con una disminución lineal en la proporción de acetato al aumentar la suplementación de UB.

25 Además, el aumento de la suplementación de UB disminuyó linealmente la producción de gas y CH<sub>4</sub>, pero aumentó los VFA y la IVOMD, lo que sugiere cambios en las comunidades microbianas del rumen, posiblemente Archaeas y bacterias fibrolíticas, que explican la menor producción de CH<sub>4</sub> y la menor proporción de acetato. Sin pretender limitarse a la teoría, la mayor concentración de NH<sub>3</sub>-N durante la incubación da como resultado una mayor tasa de fermentación, lo cual aumenta la concentración de VFA e IVOMD. Sin embargo, la acumulación más rápida de NH<sub>3</sub>-N puede tener como resultado un efecto tóxico sobre algunas comunidades microbianas del rumen, lo que afecta la producción de gas y CH<sub>4</sub>.

30 A continuación, en la presente se establecen declaraciones particulares adicionales (características) y formas de realización y usos de una composición de NNP de acuerdo con la presente divulgación.

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- Declaración 1. Uso de una composición que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante.
- 5 Declaración 2. Uso de una composición que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para mejorar la eficiencia de la síntesis de proteína microbiana (EMPS).
- 10 Declaración 3. Uso de una composición que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea como una fuente de NNP en una composición de suplemento alimenticio para rumiantes para aumentar la digestibilidad de la materia seca (DM) y la materia orgánica (OM).
- 15 Declaración 4. Uso de una composición de suplemento alimenticio para rumiantes que comprende urea, biuret y subproductos que contienen N de la producción de biuret a partir de urea, como una fuente de NNP para reducir la emisión de metano producida por animales rumiantes.
- 20 Declaración 5. Uso de una composición de suplemento alimenticio para rumiantes que comprende urea, biuret y subproductos que contienen N de la producción de biuret a partir de urea, como una fuente de NNP para mejorar la ingesta de materia seca (DMI) de animales rumiantes.
- 25 Declaración 6. Uso de acuerdo con cualquiera de las declaraciones 1 a 5, en donde la composición comprende entre 30 y 60 % en peso de urea y entre 30 y 60 % en peso de biuret, en particular, entre 35 y 55 % en peso de urea y entre 35 y 50 % en peso de biuret, en función del peso total de la composición.
- 30 Declaración 7. Uso de acuerdo con la declaración 6, en donde la composición comprende entre 37 y 52 % en peso de urea y entre 39 y 46 % en peso de biuret, en función del peso total de la composición.
- Declaración 8. Uso de acuerdo con cualquiera de las declaraciones 1 a 7, en donde la composición comprende además entre 5 y 30 % en peso de subproductos que contienen N de la producción de biuret a partir de urea, en función del peso total de la composición.
- Declaración 9. Uso de acuerdo con la declaración 8, en donde la composición comprende además entre 2,5 y 8 % en peso de triuret, y/o entre 0,1 y 5 % en peso de amelida, y/o entre 2,5 y 15 % en peso de ácido cianúrico, y entre 0,1 y 2,0 % en peso de humedad, en función del peso total de la composición.

Declaración 10. Uso de acuerdo con la declaración 8, en donde la composición comprende además: entre 3 y 6 % en peso de triuret, y/o entre 0,5 y 3 % en peso de amelida, y/o entre 3 y 10 % en peso de ácido cianúrico, y entre 0,1 y 1,5 % en peso de humedad.

- 5 Declaración 11. Composición de pienso para rumiantes que comprende uno o más ingredientes nutricionales y una composición de NNP, en donde la composición de NNP comprende (a) entre 35 y 55 % en peso de urea; (b) entre 35 y 50 % en peso de biuret; y (c) entre 5 % en peso y 30 % en peso de subproductos que contienen N de la producción de biuret a partir de urea, en función del peso total de la composición de  
10 NNP, en particular, en donde los subproductos que contienen N son triuret, amelida y/o ácido cianúrico.

- Declaración 12. Composición de pienso para rumiantes de acuerdo con la declaración 11, en donde la composición de NNP comprende (a) entre 37 y 52 % en peso de urea; (b) entre 39 y 46 % en peso de biuret; (c) entre 3 % en peso y 6 % en peso de triuret;  
15 (d) entre 0,5 y 3 % en peso de amelida; y (e) entre 3 y 10 % en peso de ácido cianúrico, en función del peso total de la composición de NNP.

- Declaración 13. Composición de pienso para rumiantes de acuerdo con la declaración 11 o 12, en donde el uno o más ingredientes nutricionales forman una ración  
20 nutricionalmente equilibrada o una ración basal para un rumiante.

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## REIVINDICACIONES

1. Uso de una composición que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea como una fuente de NNP en un pienso para rumiantes o en una composición de suplemento alimenticio para rumiantes para  
5 disminuir las emisiones de metano entérico de un animal rumiante, en particular, en comparación con un pienso para rumiantes o una composición de suplemento alimenticio para rumiantes que comprende urea como una fuente de NNP, en donde la composición comprende entre 30 y 60 % en peso de urea y entre 30 y 60 % en peso de biuret.
- 10 2. Uso de acuerdo con la reivindicación 1, para disminuir además y simultáneamente la relación de acetato con respecto a propionato producida por microbios del rumen en un animal rumiante, en particular, en comparación con un pienso para rumiantes o una composición de suplemento alimenticio para rumiantes que comprende urea como una fuente de NNP.
- 15 3. Uso de acuerdo con la reivindicación 1 o 2, para mejorar además y simultáneamente la eficiencia de la síntesis de proteína microbiana (EMPS) por microbios del rumen en un animal rumiante, en particular, en comparación con un pienso para rumiantes o una composición de suplemento alimenticio para rumiantes que comprende urea como una fuente de NNP.
- 20 4. Uso de acuerdo con cualquiera de las reivindicaciones 1 a 3, para aumentar además y simultáneamente la digestibilidad de la materia seca (DM) y la materia orgánica (OM), en particular, en comparación con un pienso para rumiantes o una composición de suplemento alimenticio para rumiantes que comprende urea como una fuente de NNP.
- 25 5. Uso de acuerdo con cualquiera de las reivindicaciones 1 a 4, para aumentar además y simultáneamente la ingesta de materia seca (DMI) de un animal rumiante, en particular, en comparación con un pienso para rumiantes o una composición de suplemento alimenticio para rumiantes que comprende urea como una fuente de NNP.
- 30 6. Uso de una composición que comprende biuret, urea y subproductos que contienen N de la producción de biuret a partir de urea como una fuente de NNP en un pienso para rumiantes o en una composición de suplemento alimenticio para rumiantes para:  
- disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un animal rumiante, en particular, en comparación con un pienso para rumiantes o una composición de suplemento alimenticio para rumiantes que comprende urea como una fuente de NNP;

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- mejorar la eficiencia de la síntesis de proteína microbiana (EMPS) por microbios del rumen en un animal rumiante, en particular, en comparación con un pienso para rumiantes o una composición de suplemento alimenticio para rumiantes que comprende urea como una fuente de NNP;
- 5    - aumentar la digestibilidad de la materia seca (DM) y la materia orgánica (OM), en particular, en comparación con un pienso para rumiantes o una composición de suplemento alimenticio para rumiantes que comprende urea como una fuente de NNP; y/o
- 10   - aumentar la ingesta de materia seca (DMI) de un animal rumiante, en particular, en comparación con un pienso para rumiantes o una composición de suplemento alimenticio para rumiantes que comprende urea como una fuente de NNP, en donde la composición comprende entre 30 y 60 % en peso de urea y entre 30 y 60 % en peso de biuret.
- 15   7. Uso de acuerdo con cualquiera de las reivindicaciones 1 a 6, en donde la composición comprende entre 35 y 55 % en peso de urea y entre 35 y 50 % en peso de biuret, en función del peso total de la composición.
- 20   8. Uso de acuerdo con la reivindicación 7, en donde la composición comprende entre 37 y 52 % en peso de urea y entre 39 y 46 % en peso de biuret, en función del peso total de la composición.
- 25   9. Uso de acuerdo con cualquiera de las reivindicaciones 1 a 8, en donde la composición comprende además entre 5 y 30 % en peso de subproductos que contienen N de la producción de biuret a partir de urea, en función del peso total de la composición.
- 30   10. Uso de acuerdo con la reivindicación 9, en donde la composición comprende entre 2,5 y 8 % en peso de triuret, y/o entre 0,1 y 5 % en peso de amelida, y/o entre 2,5 y 15 % en peso de ácido cianúrico, y entre 0,1 y 2,0 % en peso de humedad, en función del peso total de la composición.
- 35   11. Uso de acuerdo con la reivindicación 10, en donde la composición comprende entre 3 y 6 % en peso de triuret, y/o entre 0,5 y 3 % en peso de amelida, y/o entre 3 y 10 % en peso de ácido cianúrico, y entre 0,1 y 1,5 % en peso de humedad, en función del peso total de la composición.
- 12. Un método para reducir las emisiones de metano entérico de un animal rumiante, que comprende administrar al animal rumiante una composición de NNP que comprende biuret, urea y subproductos que contienen N de la producción de biuret, en donde la composición de NNP comprende entre 30 y 60 % en peso de urea y entre 30 y 60 % en peso de biuret, en particular, en donde la composición de NNP comprende entre 35 y

55 % en peso de urea, entre 35 y 50 % en peso de biuret, y entre 5 y 30 % en peso de subproductos que contienen N de la producción de biuret a partir de urea, en función del peso total de la composición.

5 13. El método de acuerdo con la reivindicación 12, en donde las emisiones de metano entérico del animal rumiante, cuando se expresan como g de metano/kg de ingesta de materia seca (DMI) o como g de metano/kg de ingesta de materia orgánica, se reducen en al menos 5 %, en particular, en al menos 10 %, cuando la composición de NNP se administra a un rumiante como fuente de NNP, en comparación con una dieta que comprende urea como fuente de NNP.

10 14. El método de acuerdo con la reivindicación 12 o 13, en donde dicho método es además, simultáneamente, un método para:

- disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un animal rumiante, en particular, en donde la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante, en particular,  
15 determinada mediante una fermentación de flujo continuo *in vitro* con fluido del rumen para simular la fermentación ruminal, se disminuye en al menos 5 %, en particular, en al menos 10 %, cuando la composición de NNP se administra a un rumiante como fuente de NNP en comparación con una dieta que comprende urea como fuente de NNP,

- mejorar la eficiencia de la síntesis de proteína microbiana (EMPS) por microbios del  
20 rumen en un animal rumiante, en donde la EMPS, en particular, determinada mediante una incubación *in vitro* de fluido del rumen continua o en lote para simular la fermentación ruminal, se aumentó en al menos 5 %, en particular, en al menos 10 %, cuando la composición de NNP se administra a un rumiante como fuente de NNP en comparación con una dieta que comprende urea como fuente de NNP;

25 - aumentar la digestibilidad de la materia seca (DM) y la materia orgánica (OM); y/o  
- aumentar la ingesta de materia seca (DMI) de un animal rumiante, en particular, en donde la DMI, expresada en kg/día aumenta en al menos 5 % cuando la composición de NNP se administra a un rumiante como fuente de NNP en comparación con una dieta que comprende urea como fuente de NNP.

30 15. El método de acuerdo con cualquiera de las reivindicaciones 12 a 14, en donde la composición de NNP comprende entre 35 y 55 % en peso de urea y entre 35 y 50 % en peso de biuret, en función del peso total de la composición, más en particular, comprende entre 37 y 52 % en peso de urea y entre 39 y 46 % en peso de biuret, en función del peso total de la composición.

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16. El método de acuerdo con cualquiera de las reivindicaciones 12 a 15, en donde la composición de NNP comprende además entre 5 y 30 % en peso de subproductos que contienen N de la producción de biuret a partir de urea, en función del peso total de la composición.

5 17. El método de acuerdo con la reivindicación 16, en donde la composición de NNP comprende entre 2,5 y 8 % en peso de triuret, y/o entre 0,1 y 5 % en peso de amelida, y/o entre 2,5 y 15 % en peso de ácido cianúrico, y entre 0,1 y 2,0 % en peso de humedad, en función del peso total de la composición, más en particular, en donde la composición  
10 comprende entre 3 y 6 % en peso de triuret, y/o entre 0,5 y 3 % en peso de amelida, y/o entre 3 y 10 % en peso de ácido cianúrico, y entre 0,1 y 1,5 % en peso de humedad, en función del peso total de la composición.

18. El método de acuerdo con cualquiera de las reivindicaciones 12 a 17, que comprende las etapas de preparar o proporcionar una composición de suplemento alimenticio para rumiantes que comprende la composición de NNP, o preparar o  
15 proporcionar una composición de pienso para rumiantes que comprende la composición de NNP, y administrar el suplemento alimenticio para rumiantes o la composición de pienso para rumiantes al animal rumiante.

19. El método de acuerdo con la reivindicación 18, en donde la composición de NNP está presente en la composición de pienso para rumiantes en una concentración entre  
20 0,5 y 4 % en peso, más en particular, entre 0,5 y 3,5 % en peso o entre 0,5 y 3 % en peso, aún más en particular, entre 1 y 2,5 % en peso, en donde el % en peso es en función de la materia seca total de la composición de pienso para rumiantes.

20. El método de acuerdo con la reivindicación 18 o 19, en donde la composición de pienso para rumiantes comprende además uno o más ingredientes nutricionales, en  
25 donde el uno o más ingredientes nutricionales forman una ración nutricionalmente equilibrada o una ración basal para un rumiante.

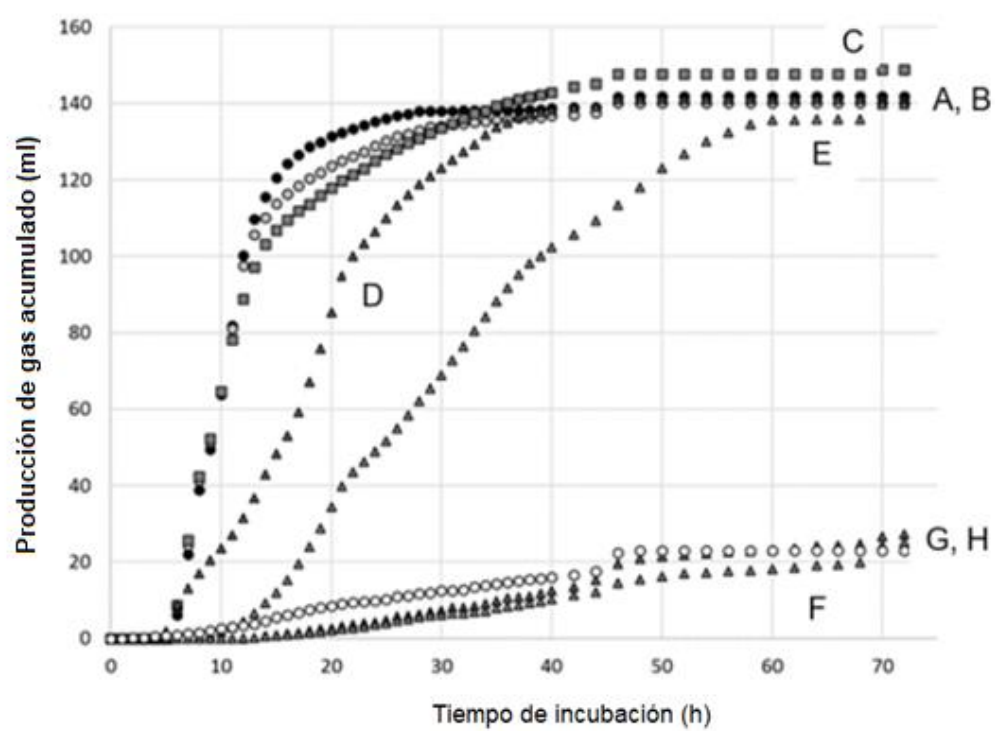
30

35

## RESUMEN

### UNA MEZCLA DE UREA, BIURET Y SUBPRODUCTOS QUE CONTIENEN N CREADOS DURANTE LA PRODUCCIÓN DE BIURET COMO UNA FUENTE ALIMENTICIA DE NITRÓGENO NO PROTEICO PARA RUMIANTES Y USOS DE ESTA

La presente divulgación se refiere al uso de una mezcla de urea, biuret y subproductos que contienen N de la producción de biuret a partir de urea, como una fuente de NNP en una composición de suplemento alimenticio para rumiantes (i) para reducir la emisión de metano producida por animales rumiantes, (ii) para disminuir la relación de acetato con respecto a propionato producida por microbios del rumen en un rumiante, (iii) para mejorar la EMPS, (iv) para aumentar la digestibilidad de DM y OM, y/o (v) para mejorar la ingesta de materia seca (DMI) de un animal rumiante. La presente divulgación se refiere además a una composición de suplemento alimenticio para rumiantes que comprende urea, biuret y subproductos que contienen N de la producción de biuret a partir de urea, así como también métodos para alimentar a un rumiante con pienso que comprende dicha composición de suplemento alimenticio para rumiantes.



- Urea sin recubrir
- Urea recubierta
- mezcla de acuerdo con la presente divulgación
- ▲ Biuret
- △ Triuret
- ▲ Amelida
- ▲ Ácido cianúrico
- Blanco

**Figura 1**

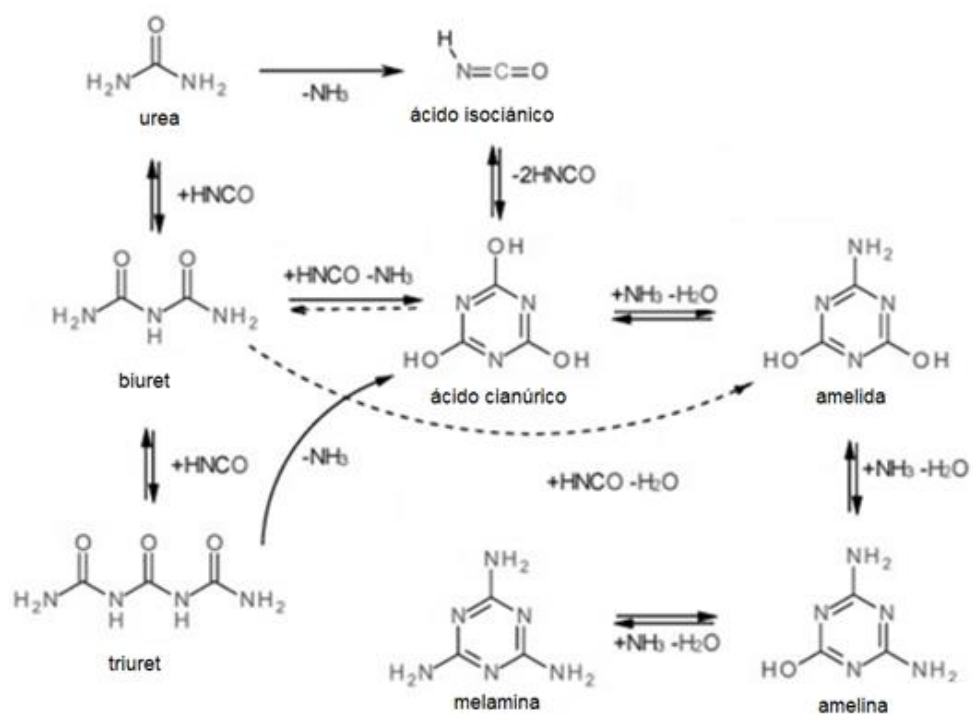


Figura 2

**CLAIMS (amendments indicated)**

1. Use of a composition comprising biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed or in a ruminant feed supplement composition for decreasing the enteric methane emissions of a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source, wherein the composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret.
2. Use according to claim 1, for further and simultaneously decreasing the acetate-to-propionate ratio produced by rumen microbes in a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
3. Use according to claim 1 or 2, for further and simultaneously improving the efficiency of microbial protein synthesis (EMPS) by rumen microbes in a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
4. Use according to any one of claims 1 to 3, for further and simultaneously increasing the dry matter (DM) and organic matter (OM) digestibility, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
5. Use according to any one of claims 1 to 4, for further and simultaneously increasing the dry matter intake (DMI) of a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
6. Use of a composition comprising biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed or in a ruminant feed supplement composition for improving rumen fermentation, wherein:

~~—decreasing—~~ the acetate-to-propionate ratio produced by rumen microbes in a ruminant animal is decreased, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source; and/or wherein  
~~—improving—~~ the efficiency of microbial protein synthesis (EMPS) by rumen microbes in  
 5 a ruminant animal is improved, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source;  
 wherein the composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret.

10 7. Use according to claim 6, for further and simultaneously ;

- increasing the dry matter (DM) and organic matter (OM) digestibility particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source; and/or
- increasing the dry matter intake (DMI) of a ruminant animal, particularly when  
 15 compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source,

87. Use according to any one of claims 1 to 76, wherein the composition comprises between 35 and 55 wt.% of urea and between 35 and 50 wt.% of biuret, based on the  
 20 total weight of the composition.

98. Use according to claim 87, wherein the composition comprises between 37 and 52 wt.% of urea and between 39 and 46 wt.% of biuret, based on the total weight of the composition.

25

109. Use according to any one of claims 1 to 98, wherein the composition further comprises between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.

30 1149. Use according to claim 109, wherein the composition comprises between 2.5 and 8 wt.% of triuret, and/or between 0.1 and 5 wt.% of ammeline, and/or between 2.5 and 15 wt.% of cyanuric acid, and between 0.1 and 2.0 wt.% of moisture, based on the total weight of the composition.

~~11~~12. Use according to claim ~~10~~11, wherein the composition comprises between 3 and 6 wt.% of triuret, and/or between 0.5 and 3 wt.% of ammelide, and/or between 3 and 10 wt.% of cyanuric acid, and between 0.1 and 1.5 wt.% of moisture, based on the total weight of the composition.

13. Use according to any one of claims 1 to 12, wherein the NPN composition is present in the ruminant feed composition in a concentration between 0.5 and 4 wt.%, more in particular between 0.5 and 3.5 wt.% or between 0.5 and 3 wt.%, even more in particular between 1 and 2.5 wt.%, with wt% based on the total dry matter basis of the ruminant feed composition.

~~12~~14. A method for reducing the enteric methane emissions of a ruminant animal, comprising administering to the ruminant animal a NPN composition comprising biuret, urea and N-containing by-products of the biuret production, wherein the NPN composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret, particularly wherein the NPN composition comprises between 35 and 55 wt.% of urea, between 35 and 50 wt.% of biuret, and between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.

~~13~~15. The method according to claim ~~12~~14, wherein the enteric methane emissions of the ruminant animal, when expressed as g methane/kg dry matter intake (DMI) or as g methane/kg organic matter intake, are reduced by at least 5%, particularly by at least 10%, when the NPN composition is administered to a ruminant as NPN source, compared to a diet comprising urea as NPN source.

~~14~~16. The method according to claim ~~12 or 13~~14 or 15, wherein said method is further simultaneously a method for:

- decreasing the acetate-to-propionate ratio produced by rumen microbes in a ruminant animal, particularly wherein the acetate-to-propionate ratio produced by rumen microbes in a ruminant, particularly determined via an *in vitro* continuous flow fermentation with rumen fluid to simulate rumen fermentation, is decreased by at least

5%, in particular by at least 10%, when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source,

- improving the efficiency of microbial protein synthesis (EMPS) by rumen microbes in a ruminant animal, wherein the EMPS, particularly determined via an *in vitro* continuous or batch rumen fluid incubation to simulate rumen fermentation, was increased by at least 5%, in particular by at least 10%, when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source;

- increasing the dry matter (DM) and organic matter (OM) digestibility; and/or

- increasing the dry matter intake (DMI) of a ruminant animal, particularly wherein the DMI, expressed in kg/day is increased by at least 5% when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source.

1517. The method according to any one of claims ~~12 to 14~~14 to 16, wherein the NPN composition comprises between 35 and 55 wt.% of urea and between 35 and 50 wt.% of biuret, based on the total weight of the composition, more in particular comprises between 37 and 52 wt.% of urea and between 39 and 46 wt.% of biuret, based on the total weight of the composition.

1618. The method according to any one of claims ~~12 to 15~~14 to 17, wherein the NPN composition further comprises between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.

1719. The method according to claim 1618, wherein the NPN composition comprises between 2.5 and 8 wt.% of triuret, and/or between 0.1 and 5 wt.% of ammeline, and/or between 2.5 and 15 wt.% of cyanuric acid, and between 0.1 and 2.0 wt.% of moisture, based on the total weight of the composition, more in particular, wherein the composition comprises between 3 and 6 wt.% of triuret, and/or between 0.5 and 3 wt.% of ammeline, and/or between 3 and 10 wt.% of cyanuric acid, and between 0.1 and 1.5 wt.% of moisture, based on the total weight of the composition.

1820. The method according to any one of claims ~~12 to 17~~14 to 19, comprising the steps of preparing or providing a ruminant feed supplement composition comprising the NPN

composition, or preparing or providing a ruminant feed composition comprising the NPN composition, and administering the ruminant feed supplement or the ruminant feed composition to the ruminant animal.

5 ~~19~~21. The method according to claim ~~18~~20, wherein the NPN composition is present in the ruminant feed composition in a concentration between 0.5 and 4 wt.%, more in particular between 0.5 and 3.5 wt.% or between 0.5 and 3 wt.%, even more in particular between 1 and 2.5 wt.%, with wt% based on the total dry matter basis of the ruminant feed composition.

10

~~20~~22. The method according to claim ~~18 or 19~~20 or 21, wherein the ruminant feed composition further comprises one or more nutritional ingredients, wherein the one or more nutritional ingredients form a nutritionally balanced ration or a basal ration for a ruminant.

15



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## (54) Title: A MIXTURE OF UREA, BIURET AND N-CONTAINING BY-PRODUCTS CREATED DURING BIURET PRODUCTION AS A DIETARY NON-PROTEIN NITROGEN-SOURCE FOR RUMINANTS AND USES THEREOF

(57) Abstract: The present disclosure relates to the use of a mixture of urea, biuret and N-containing by-products of the biuret production out of urea, as a NPN-source in a ruminant feed supplement composition (i) to reduce methane emission produced by ruminant animals, (ii) to decrease the acetate to propionate ratio produced by rumen microbes in a ruminant, (iii) to improve the EMPS, (iv) to increase the DM and OM digestibility, and/or (v) to improve the dry matter intake (DMI) of ruminant animal. The present disclosure further relates to a ruminant feed supplement composition comprising urea, biuret and N-containing by-products of the biuret production out of urea, as well as to methods of feeding a ruminant with a feed comprising said ruminant feed supplement composition.



## **A MIXTURE OF UREA, BIURET AND N-CONTAINING BY-PRODUCTS CREATED DURING BIURET PRODUCTION AS A DIETARY NON-PROTEIN NITROGEN-SOURCE FOR RUMINANTS AND USES THEREOF**

### **5 Technical field**

The present disclosure relates to the field of dietary non-protein nitrogen (NPN)-sources, more in particular a mixture of urea, biuret and N-containing by-products created during the urea condensation process, or stated differently, during biuret production, in a ruminant feed supplement.

10

### **Background**

Ruminants are defined as large hoofed herbivorous grazing or browsing mammals that are able to acquire nutrients from plant-based feed by digesting it in a specialized stomach having four compartments, one of which is the rumen. Thanks to the microbial actions in the rumen prior to digestion, ruminants have the unique ability to use (low quality) protein sources and also non-protein nitrogen (NPN) sources. NPN sources provide nitrogen for ruminants, especially in low-protein diets. This gives ruminant animals a competitive advantage over other domesticated animals. The symbiotic relationship with the rumen microflora allows ruminants to upcycle nutrients to produce highly nutritious animal proteins, by means of an efficient pre-gastric fermentation of feeds, which takes place in the reticulorumen, the largest compartment of their gastrointestinal tract. Ruminants provide an optimum environment for microorganisms to thrive, and they, in turn, supply the ruminant host with microbial crude protein, which is of high quality in terms of amino acids supplement. In beef and dairy production systems, the microbial crude protein synthesized in the rumen provides the majority of the metabolizable protein required by the animal.

Increased utilization of nitrogen (N) for bacterial synthesis and capture of dietary N into anabolic products, particularly absorbed amino acids, may be influenced by dietary manipulation, mainly by energy levels in the ration. However, there is an upper limit to the overall efficiency of the process: a maximum of 50 to 60% of dietary N, or 70 to 90% of apparent digested N will be converted into amino acids and released into the portal vein. For this reason, particularly in beef cattle diets, it is extremely important to assess the optimum kinetics of N release in NPN sources for each energy source (e.g.,

basal forage diet, supplemental by-products, etc.) to maximize N utilization and minimize excretion.

In most beef production systems, several grains and grain byproducts are available as supplemental protein sources. However, on a per kg of N supplied, it is nearly impossible to compete with the lower costs of supplemental crude protein in the form of NPN.

Indeed, non-protein nitrogen, mainly in the form of urea, is known as a cost-effective source of supplemental nitrogen (N) in ruminant production. Commercial/feed-grade urea typically contains only traces of biuret, with a biuret content not exceeding 1.0 wt%.

Urea is however usually not included in more than 1 wt.% of the diet. Any shortage of protein in a diet or supplementation program is frequently covered by the use of urea-based supplements in the form of range cubes, liquid or pelleted supplements and tubs.

Apart from urea, other ammonia forming chemical compounds such as ammonium sulfate have been used in the formulation of ruminal diets to replace part of plant protein sources and providing adequate amounts of ruminal degradable protein for a better efficiency of fiber digestion and the synthesis of microbial protein. The use of NPN, however, has been traditionally limited by the fast rate of N-release in the rumen of a ruminant (animal) in the form of ammonia. If the release of ammonia in the rumen exceeds the use capacity by ruminal microbiota, this N-excess will be excreted, with a consequent loss of energy and N-efficiency. Furthermore, if the ammonia concentration overpasses the liver excretion capacity of the ruminant, ammonia toxicity or death may occur.

As a result, in the prior art, it has been aimed at improving the use of NPN to reduce the rate of N-release in the rumen. In recent years, several slow-release urea products have been developed and tested in ruminant nutrition, for instance by physical means, such as by applying a coating, with variable results. Applying a coating with the aim to obtain slow release means adding fat, wax or the like to the NPN-source. This has the disadvantage that the NPN-content is diluted. A coating furthermore increases the production cost of a dietary N-source in cattle diets that should be cost effective. Another solution is to apply chemical means such as using pure biuret. The disadvantage of pure biuret is that the production thereof is very expensive, because the heating time to obtain the pure biuret out of urea has to be extended a lot.

In addition, currently, there is growing interest in decreasing the environmental impact of ruminant production, including beef and dairy, and many strategies are being pursued to achieve this goal.

5 In the current ruminant feeding systems, methane (CH<sub>4</sub>) production is an inevitable consequence of fermentative digestion. The microbial consortium of the rumen must dispose hydrogen that is produced during fermentation. Otherwise, hydrogen would accumulate in the rumen and thereby inhibit fermentation of ingested feeds. Therefore, the rumen microbial system is provided with methanogens that reduce carbon dioxide (CO<sub>2</sub>) to methane.

10 The major hydrogen (electron) sink in the rumen is methane produced by the reduction of carbon dioxide using reduced co-enzymes such as NADH as the electron source. Methane is however an energy loss for ruminants and a potent greenhouse gas, which has a 28-times greater global warming potential than carbon dioxide.

Globally, ruminants produce some 80 million tons of methane annually, accounting for  
15 some 28% of manmade methane emission. The adoption of ruminant management practices which result in major reductions of release of methane are consequently high priority.

It is a goal of the present disclosure to provide NPN-sources for ruminants, such as for a ruminant feed supplement composition which overcomes the limitations of the known  
20 prior art, useful for improving the rumen fermentation, the productivity and the performance of the ruminant animals, and the enteric methane emission, especially in low crude protein diets, which provides N in a timely manner such that the microbial growth in the rumen is enhanced and the production of energy yielding compounds is improved.

25

### Summary

The present disclosure generally relates to a composition or mixture comprising biuret, urea, and N-containing by-products of the biuret production out of urea, and uses thereof and methods related thereto. The terms “NPN composition”, “NPN mixture”, and  
30 “composition comprising urea, biuret and N-containing by-products of the biuret production out of urea” are generally used interchangeably herein, and refer to a composition or NPN composition according to the present disclosure, as further detailed herein.

According to a first aspect of the present disclosure, the use of a mixture or composition of biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed supplement composition to reduce the methane emission produced by a ruminant animal is disclosed. It has been observed that the supplementation of a ruminant feed with urea, biuret and N-containing by-products of the biuret production out of urea as a NPN source, reduces enteric methane production and emission of ruminant animals without compromising or even improving their performance, in particular when compared to a diet or ruminant feed supplemented with urea as NPN source.

According to a second aspect of the present disclosure, the use of a mixture of biuret, urea and minor N-containing compounds created during the urea condensation process as a NPN-source in a ruminant feed supplement composition to decrease the acetate to propionate ratio produced by rumen microbes in a ruminant has been disclosed. Stated differently, the use of a composition comprising biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed supplement composition to decrease the acetate to propionate ratio produced by rumen microbes in a ruminant is disclosed, in particular when compared to a diet or ruminant feed supplemented with urea as NPN source.

According to a third aspect of the present disclosure, the use of a mixture of biuret, urea and minor N-containing compounds created during the urea condensation process as a NPN-source in a ruminant feed supplement composition to improve the efficiency of microbial protein synthesis (EMPS) has been disclosed. Stated differently, the use of a composition comprising biuret, urea and N-containing by-product of the biuret production out of urea as a NPN-source in a ruminant feed supplement composition to improve the efficiency of microbial protein synthesis (EMPS) is disclosed, in particular when compared to a diet or ruminant feed supplemented with urea as NPN source.

According to a fourth aspect of the present disclosure, the use of a mixture of biuret, urea and minor N-containing compounds created during the urea condensation process as a NPN-source in a ruminant feed supplement composition to increase the dry matter (DM) and organic matter (OM) digestibility has been disclosed. Stated differently, the use of a composition comprising biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed supplement composition to increase the dry matter (DM) and organic matter (OM) digestibility is disclosed, in

particular when compared to a diet or ruminant feed supplemented with urea as NPN source.

According to a further aspect of the present disclosure, the use of a ruminant feed supplement composition comprising urea, biuret and N-containing by-products of the biuret production out of urea as a NPN-source to improve the dry matter intake (DMI) of ruminant animals is disclosed, in particular when compared to a diet or ruminant feed supplemented with urea as NPN source.

It has now unexpectedly been discovered by the applicant that by using a combination of urea, biuret and minor N-containing compounds created during the urea condensation process to form the biuret (also referred to as “by-products of the biuret production based on urea” or “N-containing by-products of the biuret production out of urea”), the rumen fermentation is altered directly in a way that the enteric methane production is decreased, the acetate to propionate ratio is decreased, as well as that the efficiency of microbial protein synthesis is improved. It has furthermore been found that the DM and OM digestibility is increased, and that the dry matter intake of the ruminant animal is increased, in particular when compared to a diet or ruminant feed supplemented with urea as NPN source.

According to a possible use according to the present disclosure, the mixture or composition comprises between 30 – 60 wt.% of urea and between 30 and 60 wt.% biuret, with wt% based on the total weight of the mixture or composition. More in particular, the mixture or composition comprises between 35 and 55 wt.% of urea and between 35 and 50 wt.% of biuret, with wt% based on the total weight of the composition. Even more in particular, the mixture or composition comprises between 37 and 52 wt.% of urea and between 39 and 46 wt.% of biuret, with wt% based on the total weight of the composition.

In a particular use according to the present disclosure, the composition comprises between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.

In a specific use according to the present disclosure, the ruminant feed supplement composition further comprises:

- between 2.5 and 8 wt.% of triuret, and/or
- between 0.1 and 5 wt.% of ammeline, and/or
- between 2.5 and 15 wt.% of cyanuric acid, and

- between 0.1 and 2.0 wt.% of moisture,  
based on the total weight of the composition.

According to a further specific use according to the present disclosure, the mixture further comprises

- 5 - between 3 and 6 wt.% of triuret, and/or
- between 0.5 and 3 wt.% of ammelide, and/or
- between 3 and 10 wt.% of cyanuric acid, and
- between 0.1 and 1.5 wt.% of moisture.

Another aspect of the present disclosure provides a ruminant feed composition  
10 comprising one or more nutritional ingredients and an NPN comprising biuret, urea and N-containing by-products of the biuret production out of urea. In certain embodiments, the NPN composition comprises (a) between 30 and 60 wt.% of urea; (b) between 30 and 60 wt.% of biuret; and (c) between 5 wt.% and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the NPN composition.

15 More in particular, the NPN composition comprises (a) between 37 and 52 wt.% of urea; (b) between 39 and 46 wt.% of biuret; (c) between 3 wt.% and 6 wt.% of triuret; (d) between 0.5 and 3 wt.% of ammelide; and (e) between 3 and 10 wt.% of cyanuric acid, based on the total weight of the NPN composition. In certain embodiments, the one or more nutritional ingredients form a nutritionally balanced ration or a basal ration for a  
20 ruminant.

According to a further related aspect of the present disclosure, a method for reducing the enteric methane emissions of a ruminant animal is disclosed, said method comprising administering to the ruminant animal a NPN composition according to the present disclosure, comprising biuret, urea and N-containing by-products of the biuret  
25 production out of urea, wherein the composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret. It has been found that the enteric methane emissions of the ruminant animal, expressed as g methane/kg dry matter intake or g methane/kg organic matter intake, is reduced by at least 5%, particularly by at least 10%, such as by about 15%, when the NPN composition is administered to a ruminant as NPN  
30 source compared to a diet comprising urea as NPN source.

According to a further related aspect of the present disclosure, a method for decreasing the acetate-to-propionate ratio produced by rumen microbes in a ruminant animal is disclosed, said method comprising administering to the ruminant animal a NPN

composition according to the present disclosure, comprising biuret, urea and N-containing by-products of the biuret production, wherein the NPN composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret. It has been found that the acetate-to-propionate ratio produced by rumen microbes in a ruminant, particularly estimated via an *in vitro* fermentation, such as a continuous flow fermentation with rumen fluid to simulate rumen fermentation, is decreased by at least 5%, in particular by at least 10%, such as by about 20%, when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source.

10 According to a further related aspect of the present disclosure, a method for improving the efficiency of microbial protein synthesis (EMPS) by rumen microbes in a ruminant animal is disclosed, said method comprising administering to the ruminant animal a NPN composition according to the present disclosure comprising biuret, urea and N-containing by-products of the biuret production out of urea, wherein the NPN  
15 composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret. It has been found that the EMPS, particularly estimated via an *in vitro* fermentation with rumen fluid to simulate rumen fermentation was increased by at least 5%, in particular by at least 10%, when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source.

20 According to a further related aspect of the present disclosure, a method for increasing the dry matter (DM) and organic matter (OM) digestibility is disclosed, said method comprising administering to the ruminant animal a NPN composition according to the present disclosure comprising biuret, urea and N-containing by-products of the biuret production, wherein the NPN composition comprises between 30 and 60 wt.% of urea  
25 and between 30 and 60 wt.% biuret.

According to a further related aspect of the present disclosure, a method for increasing the dry matter intake (DMI) of a ruminant animal, said method comprising administering to the ruminant animal a NPN composition according to the present disclosure comprising biuret, urea and N-containing by-products of the biuret production, wherein  
30 the NPN composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret. It has been found that the DMI, expressed in kg DM/day, is increased by at least 5% when the (NPN) composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source.

In particular embodiments, the NPN composition according to the present disclosure and administered in the methods according to the present disclosure comprises between 35 and 55 wt.% of urea and between 35 and 50 wt.% of biuret, based on the total weight of the composition, more in particular comprises between 37 and 52 wt.% of urea and  
5 between 39 and 46 wt.% of biuret, based on the total weight of the composition.

In particular embodiments, the NPN composition according to the present disclosure and administered in the methods according to the present disclosure further comprises between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.

10 In particular embodiments, the NPN composition according to the present disclosure and administered in the methods according to the present disclosure comprises between 2.5 and 8 wt.% of triuret, and/or between 0.1 and 5 wt.% of ammelide, and/or between 2.5 and 15 wt.% of cyanuric acid, and between 0.1 and 2.0 wt.% of moisture, based on the total weight of the composition, more in particular, wherein the composition comprises  
15 between 3 and 6 wt.% of triuret, and/or between 0.5 and 3 wt.% of ammelide, and/or between 3 and 10 wt.% of cyanuric acid, and between 0.1 and 1.5 wt.% of moisture, based on the total weight of the composition.

In certain embodiments, the methods according to the present disclosure further comprises the steps of preparing or providing a ruminant feed supplement composition  
20 comprising the NPN composition, or preparing or providing a ruminant feed composition comprising the NPN composition, and administering the ruminant feed supplement or the ruminant feed composition to the ruminant animal. More in particular, the NPN composition, comprising biuret, urea and N-containing by-products of the biuret production according to the present invention is present in the ruminant feed  
25 composition in a concentration between 0.5 and 4 wt.%, more in particular between 0.5 and 3.5 wt.% or between 0.5 and 3 wt.%, even more in particular between 1 and 2.5 wt.%, with wt% based on the total dry matter basis of the ruminant feed composition. In particular, the ruminant feed composition further comprises one or more nutritional ingredients, wherein the one or more nutritional ingredients form a nutritionally  
30 balanced ration or a basal ration for a ruminant animal.

It is understood that the different methods according to the present disclosure are non-therapeutic methods.

### Description of the figures

FIG. 1 shows a graph of the cumulative gas production of 6 different nitrogen-containing original products, in particular uncoated urea (A), coated urea (B), biuret (D), triuret (E), ammelide (F) and cyanuric acid (G), and a mixture according to the present disclosure (C) on an isonitrogenous base against a blank (H) during 72 hours of *in vitro* incubation.

FIG. 2 shows an overview of the reactions involved in the production of biuret from urea, including the formation of N-containing by-products.

### Detailed description

The present inventors have surprisingly found that a specific NPN composition (or mixture) for ruminants, comprising urea, biuret and one or more N-containing by-products of a urea condensation process or biuret production process, has many advantages when fed to a ruminant as a NPN source, particularly when compared to a ruminant fed with urea as a NPN source. Moreover, for the different embodiments and aspects envisaged herein, the NPN composition or NPN mixture according to the present disclosure significantly outperforms urea as a NPN source (when compared to a ruminant fed with a diet without NPN source).

The advantages of a NPN composition according to the present disclosure are in particular:

- (1) reduction of the enteric methane emissions by ruminant animals. The reduction of the methane production or emissions by ruminant animals can be assessed *in vivo*, by determining the methane emissions of a ruminant fed with a particular diet, for instance using the SF<sub>6</sub> tracer technique as e.g. described in example 3, wherein the methane emissions may be expressed as g methane emitted per kg dry matter intake or per kg organic matter intake. Alternatively, the methane emissions may be assessed by evaluating the methane production in *in vitro* culture incubations of ruminal fluid, as e.g. described in example 4, wherein the methane production may be expressed as mM, ml or mg methane produced per g of organic matter incubated or digested;
- (2) the acetate to propionate ratio produced by the rumen microbes was decreased. The acetate and propionate content can be determined by gas chromatography determination of the volatile fatty acids (VFA) in a sample, as known by the skilled person. In certain embodiments, the sample is obtained via an *in vitro* fermentation with ruminant fluid, to simulate rumen fermentation, particularly wherein a suitable fermenter or incubator is

inoculated with ruminal fluid and fed with a ruminant feed composition to simulate rumen fermentation;

(3) the efficiency of microbial protein synthesis (EMPS) was increased. As understood by the skilled person, the EMPS is a measure for how much microbial protein is produced due to the microbial activity for every unit of fermentable substrate which is fed to the ruminant. It may be determined by an *in vitro* assay comprising continuous flow or batch fermentations with rumen fluid, particularly wherein a suitable fermenter or incubator is inoculated with ruminal fluid and fed with a ruminant feed composition to simulate rumen fermentation, as for instance explained in example 2 or 4;

(4) the dry matter (DM) and organic matter (OM) digestibility was increased. The DM and OM digestibility may be assessed by the difference in dry matter and organic matter (i.e. dry matter minus the ash content) of the feed vs the sample after (in vitro) fermentation, as known by the skilled person;

(5) the rumen fermentation was increased after 72h in an *in vitro* assay;

(6) the dry matter intake (DMI) of ruminant animals was increased.

In this context, and without wishing to be bound by theory, it is believed that there has been a selective pressure on the microbes in the rumen that produce acetate and propionate in such a way that more propionate and less acetate, in view of ruminant feed supplement compositions without the mixture according to the present disclosure, are produced. Propionate is the most important glucogenic precursor in ruminants. This means that lowering the acetate to propionate ratio enhances the supply of energy for metabolic use, through which more energy is available to be used by the ruminant enhancing its production of proteins. In other words, the rumen fermentation has been made more efficient in an energetic way, supporting the production of meat, milk, etc.

25

Biuret is typically formed through a non-catalytic thermal decomposition of urea. This process is generally referred to herein as “biuret production”, “biuret production out of urea” or “urea condensation process”. The main reaction and the side reactions in biuret production out of urea are known to the skilled person and they are represented in FIG.

2. When urea is heated to temperatures above its melting point, ammonia is slowly evolved. If the temperature range is limited slightly above the normal melting point of urea, i.e. 132 °C, biuret is formed. When heating the urea melt further, several other different “N-containing compounds” or “N-containing by-products” are formed in very

small amounts – hence use of the term “minor”. These N-containing compounds resulting out of the thermal decomposition of urea encompass amongst others cyanuric acid, triuret, ammelide, etc. These compounds are referred to herein as “N-containing by-products of biuret production” or as “minor N-containing compounds created during the urea condensation process”. In particular embodiments, said N-containing by-products of biuret production comprise triuret, ammelide and cyanuric acid. These by-products are poorly hydrolysed in the rumen. Cyanuric acid and triuret are, just like biuret, non-toxic for the ruminants and give as good nitrogen retention figures as biuret does. Ammelide is an unavoidable by-product in the production of the necessary amount of biuret. The amount of ammelide however needs to be limited since it can be toxic in higher doses.

As used herein, the composition or mixture according to the present disclosure, in particular the NPN composition or mixture according to the present disclosure, generally comprises urea, biuret and one or more by-products of a biuret production process. More in particular, the NPN composition or mixture according to the present disclosure, comprises between 60 wt.% and 95 wt.% of urea and biuret, and between 5 wt.% and 40 wt.% of N-containing by-products of a biuret production process, based on the total weight of the composition or mixture. Even more in particular, the NPN composition or mixture according to the present disclosure comprises between 70 wt.% and 95 wt.% or between 75 wt.% and 95 wt.% of urea and biuret, and between 5 wt.% and 30 wt.% or between 5 wt.% and 25 wt.% of N-containing by-products of a biuret production process, based on the total weight of the composition or mixture.

In particular embodiments, the NPN composition or mixture according to the present disclosure comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% of biuret, more in particular between 35 and 55 wt.% of urea and between 35 wt.% and 50 wt.% of biuret, even more in particular between 37 and 52 wt.% or between 40 and 50 wt.% of urea and between 39 and 46 wt.% or between 40 and 45 wt.% of biuret, based on the total weight of the composition or mixture. In certain embodiments, the NPN composition or mixture according to the present disclosure, further comprises between 5 wt.% and 30 wt.% of N-containing by-products of a biuret production process out of urea, in particular between 5 wt.% and 25 wt.% of N-containing by-products of a biuret production process out of urea, based on the total weight of the composition or mixture.

In certain embodiments the N-containing by-products of a biuret production process out of urea comprises one or more of triuret, ammelide and cyanuric acid.

In certain embodiments, the NPN composition or NPN mixture according to the present disclosure comprises, as N-containing by-products of a biuret production process, (a) 5 between 2.5 wt.% and 8 wt.% of triuret, more in particular between 3 and 6 wt.% of triuret; (b) between 0.1 and 5 wt.% of ammelide, more in particular between 0.5 and 3 wt.% of ammelide; and/or (c) between 2.5 and 15 wt.% of cyanuric acid, more in particular between 3 and 10 wt.% of cyanuric acid, based on the total weight of the composition or mixture.

10 In certain embodiments, the NPN composition or mixture according to the present disclosure further comprises between 0.1 and 2 wt.% moisture (water), more in particular between 0.1 and 1.5 wt.% moisture, based on the total weight of the composition or mixture.

In particular embodiments, the NPN composition or mixture according to the present 15 disclosure is a ruminant feed supplement composition.

Particular embodiments of the NPN composition or mixture or ruminant feed supplement composition according to the present disclosure comprises

- between 30 and 60 wt.% of urea, more in particular between 35 and 55 wt.% of urea, even more in particular between 37 and 52 wt.% of urea,
- 20 - between 30 and 60 wt.% of biuret, more in particular between 35 and 50 wt.% of biuret, even more in particular between 39 and 46 wt.% of biuret,
- between 3 and 6 wt.% of triuret,
- between 0.5 and 3 wt.% of ammelide,
- between 3 and 10 wt.% of cyanuric acid,
- 25 - between 0.1 and 1.5 wt.% of moisture, with wt.% based on the total weight of the composition.

Optionally, for all embodiments of the NPN composition according to the present disclosure, one or more anti-caking agents as generally known in the art can be added to the ruminant feed supplement composition, either incorporated in the feed supplement, 30 either as a coating, to improve handling properties and to improve the quality of the ruminant feed supplement composition.

Furthermore, it is also possible to add any pre-biotics, pro-biotics, post-biotics, anti-biotics, bio stimulants known in the art for fostering/supporting the nitrogen assimilation and rumen fermentation.

- 5 An aspect of the present disclosure relates to the use of a NPN composition or mixture according to the present disclosure, or stated differently a mixture or composition of urea, biuret and minor N-containing compounds created during the urea condensation process to obtain the biuret, as a NPN-source in a ruminant feed supplement composition to, particularly at the same time or simultaneously,
- 10 - decrease the enteric methane emissions of a ruminant animal,  
- improve the dry matter intake of a ruminant animal,  
- decrease the acetate to propionate ratio produced by rumen microbes in a ruminant,  
- improve the EMPS (efficiency of microbial protein synthesis), and  
15 - increase the DM and OM digestibility.

According to a related aspect of the present disclosure, a composition comprising urea, biuret and N-containing by-products of biuret production out of urea, for use as an NPN-source in a ruminant feed supplement composition to decrease the enteric methane production or emissions of a ruminant animal or to decrease the enteric methane production by rumen microbes in a ruminant is disclosed.

20

According to a related aspect of the present disclosure, a composition comprising urea, biuret and N-containing by-products of biuret production out of urea, for use as an NPN-source in a ruminant feed supplement composition to decrease the ratio of acetate to propionate produced by rumen microbes in a ruminant is disclosed.

25 According to a further related aspect of the present disclosure, a composition comprising urea, biuret and N-containing by-products of biuret production out of urea, for use as an NPN-source in a ruminant feed supplement composition to improve the EMPS in a ruminant is disclosed.

According to a further related aspect of the present disclosure, a composition comprising  
30 urea, biuret and N-containing by-products of biuret production out of urea, for use as an NPN-source in a ruminant feed supplement composition to increase the DM and OM digestibility in a ruminant.

According to a related aspect of the present disclosure, a composition comprising urea, biuret and N-containing by-products of biuret production out of urea, for use as an NPN-source in a ruminant feed supplement composition to increase the dry matter intake of a ruminant animal is disclosed.

5

A related aspect of the present disclosure provides a method for reducing the enteric methane production of a ruminant animal, comprising the steps of

- preparing a ruminant feed composition comprising a NPN composition according to the present disclosure, as a NPN source, particularly a NPN composition comprising biuret, urea and N-containing by-products of the biuret production as disclosed herein;
- 10 and
- feeding a ruminant with the ruminant feed composition.

A related aspect of the present disclosure provides a method to decrease the acetate to propionate ratio produced by rumen microbes in a ruminant, comprising the steps of:

- 15 - preparing a ruminant feed composition comprising a NPN composition according to the present disclosure, as a NPN source, particularly a NPN composition comprising biuret, urea and N-containing by-products of the biuret production as disclosed herein;
- and
- feeding a ruminant with the ruminant feed composition.

20 A further related aspect of the present disclosure provides a method to improve the EMPS in a ruminant, comprising the steps of

- preparing a ruminant feed composition comprising a NPN composition according to the present disclosure, as a NPN source, particularly a NPN composition comprising biuret, urea and N-containing by-products of the biuret production as disclosed herein;
- 25 and
- feeding a ruminant with the ruminant feed composition.

A further related aspect of the present disclosure relates to a method to increase the DM and OM digestibility in a ruminant, comprising the steps of

- preparing a ruminant feed composition comprising a NPN composition according to the present disclosure, as a NPN source, particularly a NPN composition comprising biuret, urea and N-containing by-products of the biuret production as disclosed herein;
- 30 and
- feeding a ruminant with the ruminant feed composition.

In certain embodiments, the enteric methane emissions of the ruminant animal are reduced by at least 5% or by at least 10%, such as by about 15%, when expressed in g/kg DMI (dry matter intake) or g/kg OMI (organic matter intake), when the NPN composition according to the present disclosure is administered to a ruminant as NPN source compared to diet comprising urea as NPN source.

In certain embodiments the acetate to propionate ratio produced by rumen microbes in a ruminant is decreased by at least 5%, in particular by at least 10%, such as by about 20%, when the NPN composition according to the present disclosure is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source. In particular, the acetate-to-propionate ratio may be determined on a sample obtained from an *in vitro* fermentation with ruminant fluid, to simulate rumen fermentation, particularly wherein a suitable fermenter or incubator, such as a continuous flow or batch fermenter or incubator, is inoculated with ruminal fluid and fed with a ruminant feed composition to simulate rumen fermentation.

In certain embodiments, the efficiency of microbial protein synthesis (EMPS) in a ruminant is increased by at least 5%, in particular by at least 10%, such as by about 20%, when the NPN composition according to the present disclosure is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source. In particular, the EMPS may be determined on a sample obtained from an *in vitro* fermentation with ruminant fluid, to simulate rumen fermentation, particularly wherein a suitable fermenter or incubator, such as a continuous flow or batch fermenter or incubator, is inoculated with ruminal fluid and fed with a ruminant feed composition to simulate rumen fermentation.

In certain embodiments, the method for decreasing the enteric methane production of a ruminant animal, for decreasing the acetate to propionate ratio produced by rumen microbes in a ruminant, for improving the EMPS in a ruminant, for increasing the DM or OM digestibility in a ruminant, and/or for increasing the dry matter intake (DMI) of a ruminant further comprises the step of replacing a portion of vegetable proteins in the feed composition with the NPN composition according to the present disclosure.

It is further understood that the different methods according to the present disclosure are non-therapeutic methods.

As considered herein a ruminant or ruminant animal is a bovine, ovine or caprine animal, particularly a domesticated animal with a functional rumen. In particular embodiments, the ruminant is a sheep or a cattle, more in particular the ruminant is a beef cattle or dairy cattle.

5

The present disclosure further provides a feed, feed material, premix or feed additive for feeding a ruminant comprising the NPN composition according to the present disclosure. The NPN composition according to the present disclosure may be combined with a ruminant feed or feed material, or with a premix for feeding a ruminant. In context of the present disclosure the term premix or nutrient premix is used as known to the person skilled in the art and denotes a mixture comprising one or more ingredients such as vitamins, trace minerals, medicaments, feed supplements and diluents. A ruminant feed or ruminant feed material generally refers to nutritional compositions suitable for feeding to ruminants, as known to the skilled person, and includes compound feeds (i.e. generally an industrially produced feed mixture of at least two feed materials), complete feeds (i.e. a compound feed containing all main nutrients needed for a daily ration, including also all the roughage needed by the ruminant), or a concentrate feed (i.e. a compound feed which has a high content of certain substances but which is sufficient for a daily ration only if used in combination with other feed).

20 In particular embodiments, a ruminant feed composition comprising one or more nutritional ingredients and a NPN composition comprising (a) between 30 and 60 wt.% or between 35 and 55 wt.% of urea; (b) between 30 and 60 wt.% or between 35 and 50 wt.% of biuret; and (c) between 5 wt.% and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the NPN composition, is disclosed. More in particular, the NPN composition comprises (a) between 37 and 52 wt.% of urea; (b) between 39 and 46 wt.% of biuret; (c) between 3 wt.% and 6 wt.% of triuret; (d) between 0.5 and 3 wt.% of ammeline; and (e) between 3 and 10 wt.% of cyanuric acid, based on the total weight of the NPN composition. In certain embodiments, the one or more nutritional ingredients form a nutritionally balanced ration or a basal ration for a ruminant, as known by the skilled person.

25

In certain embodiments, a ruminant feed composition or feed material comprising between 0.5 and 4 wt.%, more in particular between 0.5 and 3.5 wt.% or between 0.5 and 3 wt.%, even more in particular between 1 and 2.5 wt.% of the NPN composition

according to the present disclosure, is disclosed, with wt.% based on the total dry matter of the feed composition or feed material,

The present disclosure further relates to a method for feeding or rearing a ruminant comprising administering the NPN composition according to the present disclosure to the ruminant, particularly during the feeding regime, and/or feeding the ruminant with the ruminant feed composition according to the present disclosure.

The disclosure as defined here above will now be illustrated and explained in more detail in the following experimental part, which is not intended to limit the scope of this disclosure anyway.

### Examples

#### **Example 1**

Each NPN-source has specific characteristics, determining the release of N after entrance into the rumen. The efficiency of N-use by the rumen microbiota will however depend on the amount and the release rate of the NPN-source. This may result in a difference in the efficiency of microbial protein production in the rumen. In this example, the N-utilization *in vitro* of 3 different NPN-products and the mixture according to the present disclosure compared to coated and uncoated urea as NPN-sources for rumen fermentation was assessed.

#### ***Test materials***

A total of 6 different NPN-products and a mixture according to the present disclosure were selected to be tested in an *in vitro* rumen fermentation model (see Table 1) below. The mixture according to the present disclosure, in the different examples typically referred to by the code UB, generally comprises between 37 and 52 wt.% of urea, between 39 and 46 wt.% of biuret, between 3 and 6 wt.% of triuret, between 0.5 and 3 wt.% of ammelide, between 3 and 10 wt.% of cyanuric acid, and between 0.1 and 1.5 wt.% of moisture, based on the total weight of the mixture.

*Table 1: Overview of tested products, their supplier, physical form, purity and nitrogen content*

| Product                                     | Supplied by      | Physical form | Purity (%) | Nitrogen content (%) |
|---------------------------------------------|------------------|---------------|------------|----------------------|
| Uncoated urea                               | Yara             | Pearls        | 99         | 46.2                 |
| Urea                                        | Yara             | Pearls        | 89         | 41.5                 |
| Biuret                                      | Alfa Aesar       | Powder        | 97         | 39.5                 |
| Cyanuric acid                               | Yara             | Powder        | 100        | 32.6                 |
| Triuret                                     | Enamine          | Powder        | 95         | 36.4                 |
| Ammelide                                    | Dr. Ehrenstorfer | Powder        | 99         | 43.3                 |
| Mixture according to the present disclosure | Applicant        | Powder        | 100        | 41.0                 |

### ***In vitro protocol***

- 5 The *in vitro* model used to simulate rumen digestion and utilization of the products is the Gas Production Test protocol, adapted for protein digestibility, as described in more detail in the following paragraph.

Approximately 1 liter of rumen fluid was collected from two different rumen-fistulated animals in the morning. Rumen fluid was strained through a cheesecloth, kept at 39°C under CO<sub>2</sub> and then diluted 1:19 with a nitrogen-free buffer and mineral solution  
 10 containing 10.03 g NaHCO<sub>3</sub>, 1.43 g Na<sub>2</sub>HPO<sub>4</sub>, 1.55 g KH<sub>2</sub>PO<sub>4</sub>, 0.15 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.52 g Na<sub>2</sub>S, 0.017 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.015 g MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.002 g CoCl<sub>3</sub>·6H<sub>2</sub>O, 0.012 g FeCl<sub>3</sub>·6H<sub>2</sub>O and 0.125 mg resazurin per liter.

To ensure the rumen fluid was depleted of free nitrogen and to ensure nitrogen would  
 15 be the limiting factor for the microbial growth at the start of the experiment, the buffered rumen fluid was pre-incubated for 4 hours at 39°C with a carbohydrate overload of glucose, xylose and soluble starch, each at 3.33 g/l. During this pre-incubation, all nitrogen available in the rumen fluid would be incorporated into bacterial protein and other bacterial nitrogen-containing components.

- 20 Each product was weighed into different glass bottles on an isonitrogenous base, providing 15 mg N per flask. Then, 60 ml of the pre-incubated buffered rumen fluid was

added, under continuous flushing with CO<sub>2</sub>. Flasks were closed with rubber stoppers and placed in a shaking water bath with 50 movements per minute at 39 °C to start the incubation period. The gas production of each flask was recorded continuously for 72 hours using a fully automated system. All test products including a blank (i.e. no product added to the flask of rumen fluid) were tested in one run in triplicate.

The cumulative gas production was measured continuously for 72 hours. The results averaged per triplicate are shown in Figure 1.

### ***Results***

As can be seen in Figure 1, the accumulated gas production curves of ammelide and cyanuric acid were comparable to the blank sample. In general, the products are relatively low soluble in water compared to the other tested products. Moreover, the nitrogen in these products is incorporated in a ring structure which is less accessible. The nitrogen solubility of ammelide and cyanuric acid appeared to be too low to support bacterial growth. For ammelide, cyanuric acid as well as the blank, the gas production is however not equal to zero since microbial turnover release small amounts of nitrogen into the solution which can be used by the remaining microorganisms. The cumulative gas production after 72 hours was not significantly different between the blank, ammelide and cyanuric acid.

As can furthermore be seen in Figure 1, the cumulative gas production profiles of the uncoated urea and the coated urea showed a lag period of approximately 5 hours, after which the cumulative gas production rapidly increased to a maximum of 17 ml/hour for the uncoated urea and 19 ml/hour for the coated urea. After approximately 15 hours of incubation, the cumulative gas production slowed down rapidly and stopped after 30 hours. The solubility of urea in water has been shown to be very quick, within minutes. The longer lag time of 5 hours for urea products can be explained from the effect of the fast release of ammonia on the pre-incubated buffered rumen fluid, preventing the release of CO<sub>2</sub> from the fluid. Coated urea was expected to have a slower nitrogen release rate than uncoated urea, which did not result in a difference in cumulative gas production over the 72 hours of incubation. The cumulative gas production of the uncoated urea was numerically lower than the coated urea between 10 and 20 hours of incubation, which may be related to the slower release of the nitrogen from the coated urea. Biuret and triuret showed a cumulative gas production curve below the coated and

uncoated urea. Biuret had a comparable lag time of 5 hours, but a slower gas production rate in the first 15 hours of incubation. The cumulative gas production was the highest in the period of between 15 and 25 hours of incubation, and slowed down afterwards until the maximum cumulative gas production was reached after 40 hours. Triuret showed a longer lag time of 9 hours, suggesting a slower release of nitrogen which was expected based on the characteristics of triuret relative to biuret which is less soluble. After 9 hours, the gas production rate started relatively slowly, peaking at 20 hours of incubation. The cumulative gas production was rather stable at a low level of approximately 4 ml/hour from 20 to 50 hours and decreased afterwards until the end level was reached after 60 hours.

As further can be seen in Figure 1, the cumulative gas production of the mixture according to the present disclosure was higher in comparison with coated urea during the 72 hours of incubation. In the period between 20 and 40 hours of incubation, the cumulative gas production for the mixture according to the present disclosure remained lower than the cumulative gas production of coated urea, which is likely related to the slower release of N from the biuret and other NPN compounds present in this mixture. Nevertheless, the cumulative gas production from the mixture according to the present disclosure kept increasing after 40 h, obtaining the greatest cumulative gas production amongst all the NPN sources tested.

20

### ***Conclusion***

The rapid ammonia release rate of uncoated urea limits the amount of urea that may be used in a ruminant diet formulation. High inclusion levels of uncoated urea in a ruminant diet formulation will increase the ammonia concentration in the rumen rapidly, leading to ruminal accumulation and absorption of ammonia and subsequent excretion of urea in the urine.

As expected, the pure N-compounds, i.e. ammelide, triuret and cyanuric acid, which are well-known N-containing by-products of the biuret production by thermal decomposition of urea and which are also present in minor amounts in the mixture according to the present disclosure, generated a much lower cumulated gas production than uncoated and coated urea. Ammelide and cyanuric seem not to be suitable as rumen digestible nitrogen sources in dairy cattle nutrition. Their solubility and nitrogen release rate are too slow to support microbial growth. Nitrogen from biuret and triuret can be

used by rumen bacteria, but it is released more slowly than coated or uncoated urea. The slow release of nitrogen from triuret and biuret may improve nitrogen utilization. However, it can be expected to escape from the rumen approximately 12 to 24 hours after ingestion (depending on the diet characteristics such as digestibility and particle size). Part of the biuret and triuret (approximately 60%) will still be unused by the rumen microbes after 24 hours, based on the cumulative gas production profiles. This amount of unused nitrogen is likely to pass the rumen and be excreted after passage through the gastrointestinal tract.

The mixture according to the present disclosure, particularly the proportion of urea, biuret and the minor N-compounds created by the production of biuret starting from urea therein, resulted in an unexpected fermentation pattern as shown by the cumulative gas production dynamics, reaching the greatest total gas production amongst all the NPN sources assessed. As can be seen in Figure 1, in the beginning, the slope of the cumulative gas production curve is less steep in comparison with the one of urea and uncoated urea, presumably due to a lower amount of rapidly available urea, but afterwards, when the more slowly fermented biuret is used by the microorganisms, the cumulative gas production is the highest of all tested products. The more cumulative gas is produced, the better, since this means that rumen fermentation is maximized. This indicates that the mixture according to the present disclosure, comprising urea, biuret and N-containing by-products of the biuret production out of urea has a synergistic effect on the rumen fermentation compared to the fermentation of the individual components (taken individually).

### **Example 2**

In this example, an *in vitro* assay was performed using dual-flow continuous culture fermenters, aiming to simulate rumen fermentation.

#### ***Fermenters***

Eight 1320 ml dual-flow continuous culture fermenters were used in two replicated periods. Each period consisted out of 5 days for adaptation of the ruminal fluid to the treatments and 3 days for sampling. On the first day of each period, all fermenters were inoculated with undiluted ruminal fluid filtered through two layers of cheesecloth to remove the feed particles. The fermentation conditions were constant, i.e.

- a temperature of 39 °C,
  - a pH of  $6.4 \pm 0.05$  by infusion of 3 N HCl or 5 N NaOH,
  - anaerobiosis with continued infusion of N<sub>2</sub> gas at a rate of 40 ml/min, and
  - continued infusion of artificial saliva to obtain liquid and solid dilution rates of
- 5      10 %/h and 5 %/h, respectively.

The fermenters were fed 95 grams/day of dry matter of a diet formulated to meet or exceed current nutrients recommendations for lactating dairy cows, i.e.

- 166 g/kg crude protein (CP),
- 323 g/kg neutral detergent fiber (NDF), and
- 10 - 201 g/kg acid detergent fiber (ADF),

And were fed in three daily administrations, i.e. at 7, 15 and 23 o'clock with a forage : concentrate diet in a range of 53 : 47.

#### *Animals and treatment diets*

- 15      Ruminal fluid was taken from two Holstein dairy cows fitted with ruminal cannulas fed with a 60 : 40 forage : concentrate diet with alfalfa and cracked grain (11 kg dry matter/cow/day). The afternoon before the ruminal extraction, the ruminant animals were not fed.

The NPN-dietary treatments were as follows:

- 20      - Control: basal diet to which 0.51 wt.% urea was added, with wt.% on dry matter basis,
- Diet: basal diet to which 0.55 wt.% of the mixture according to the present disclosure, with composition as in example 1, was added, with wt.% on dry matter basis.
- 25      To obtain isoenergetic and isonitrogenous diets using different NPN sources, a nitrogen content determination using the Kjeldahl method was used to determine the nitrogen content of each NPN product (see Table 2 below).

*Table 2: Nitrogen content of non-protein nitrogen sources*

| Experimental example | N wt.% |
|----------------------|--------|
| Control              | 44.37  |
| Diet                 | 40.94  |

Due to the N-percentage differences among the products, the inclusion level of each NPN-source was different (see Table 3 below).

*Table 3. Ingredient composition of the dietary treatments*

| Ingredient (wt.% of dry matter)                         | NPN dietary treatment |      |
|---------------------------------------------------------|-----------------------|------|
|                                                         | Control               | D1   |
| Corn grain, craked dry                                  | 40.6                  | 40.6 |
| Legume hay < 40 wt.% NDF                                | 26.7                  | 26.7 |
| Grass, hay, mature                                      | 26.3                  | 26.3 |
| Soybean meal, 48 wt.% CP                                | 4.61                  | 4.61 |
| Urea                                                    | 0.51                  | -    |
| Mixture according to the present disclosure (see ex. 1) | -                     | 0.55 |
| Calcium carbonate (CaCO <sub>3</sub> )                  | 0.46                  | 0.46 |

5

### ***Sample collection***

On the second day of sampling, 4 ml of filtered fermenter fluid was taken 2 hours after the morning feeding to determine VFA-concentrations.

During sampling days, the collection vessels were maintained at 4 °C to prevent microbial activity. Solid and liquid effluents from 3 sampling days were composited and mixed within the fermenter and homogenized. Subsamples were taken for the analyses of the volatile fatty acids (VFA). The remainder of the sample was lyophilized (freeze drying) and the dry samples were analyzed for DM, ash, N and purines to calculate bacterial nitrogen and organic matter content (OM).

Bacterial cells were obtained from the fermenter flasks the last day of each experimental period. Solid and liquid associated bacteria were isolated using a combination several detachment procedures selected to obtain the maximum detachment without affecting the cell integrity. The bacterial cells were lyophilized and the dry samples were analyzed for dry matter (DM), ash, N and purines to calculate bacterial nitrogen.

20

### ***Chemical analyses***

Effluent dry matter (DM) was determined by lyophilizing 300 ml aliquots in triplicate. The DM content of the diets and bacterial samples were determined by drying samples for 24 hours in an 103 °C forced air oven (see AOAC, 1990; method 950.01).

- 5 Dry samples of diets, effluents and bacteria were ashed overnight at 550 °C in a muffle furnace (see AOAC, 1990, method 976.05) and organic matter (OM) was determined by difference. Total N of diets, effluents and bacterial samples were determined by a Kjeldhal method (AOAC, 1990; method 976.05). Sample crude protein (CP) was calculated as  $N \times 6.25$ . Effluent CP was determined in liquid samples. Crude protein
- 10 degradation was calculated as the difference between CP in the diet minus CP in the effluent and expressed as wt.% of DM. The EMPS was determined by dividing bacterial N by fermented organic matter.

The fiber components of the diets and the effluents were analyzed sequentially using a thermostable alpha-amylase and sodium sulfite and expressed without residual ash.

- 15 The samples for the VFA-analysis were prepared as described by Jouany, J. P., and J. Senaud. "Influence des ciliés du rumen sur la digestion de différents glucides chez le mouton. I.—Utilisation des glucides pariétaux (cellulose et hémicelluloses) et de l'amidon." *Reproduction Nutrition Développement* 22.5 (1982): 735-752. and analyzed by gas chromatography.

20

### ***Results***

- In Table 4 below, the effect of the NPN-diets on the nitrogen metabolism of ruminal microorganisms, true digestibility of DM and OM, CP degradation, the efficiency of microbial protein synthesis (EMPS) in the rumen (or in other words, how much
- 25 microbial protein is produced due to the microbial activity for every unit of substrate which is fed to the ruminant), in the control diet ("Control" – comprising urea) and a diet ("Diet" – comprising UB) comprising a mixture according to the present disclosure is shown.

30

*Table 4: Effect of NPN dietary treatment on the nitrogen metabolism of ruminal microorganisms*

|                                                           | NPN dietary treatment |              |
|-----------------------------------------------------------|-----------------------|--------------|
|                                                           | Control<br>(urea)     | Diet<br>(UB) |
| True digestibility, wt.% of DM                            |                       |              |
| DM                                                        | 53.53                 | 56.79        |
| OM                                                        | 52.95                 | 55.90        |
| CP degradation<br>(% of dry matter)                       | 47.7                  | 53.2         |
| EMPS<br>(g bacterial N/kg of fermented organic<br>matter) | 35.7                  | 39.4         |
| VFA                                                       |                       |              |
| Acetate                                                   | 62.92                 | 60.06        |
| Propionate                                                | 18.18                 | 23.29        |
| Acetate to propionate ratio                               | 3.5                   | 2.76         |

### **Conclusion**

- 5 Out of Table 4, it can be concluded that the diet comprising a mixture of urea, biuret and the minor amount of N-containing compounds originating from the biuret production out of urea according to the present disclosure have
- a higher true digestibility of DM and OM, with an increase in digestibility of about 5%-6% compared to a diet comprising urea as NPN source,
  - 10 – an increase of the EMPS, meaning that the rumen microbiota maximized N-utilization, with an increase of EMPS of about 10% compared to a diet comprising urea as NPN source, and
  - an increased amount of propionate and a decreased amount of acetate, through which acetate to propionate ratio is decreased, suggesting that this NPN-mixture
  - 15 according to the present disclosure improves the efficiency of the ruminal energy utilization, with a decrease in acetate to propionate ratio of about 20% compared to a diet comprising urea as a NPN source.

### **Example 3**

#### ***Animals, experimental design and treatments***

A total of 72 crossbred yearling steers [303 kg  $\pm$  29 kg initial body weight (BW)] were stratified by body weight (BW) and randomly allocated to three treatments. All steers consumed a basal diet of corn silage, cotton-gin trash, and a premix of vitamins and minerals.

The steers were housed in pens (8 steers / pen, 3 pens / treatment). The steers were supplemented with two different NPN-sources on an isonitrogenous basis, considering as reference the N provided by urea when included at 1 wt.% of the diet in dry matter (DM) basis.

In Table 5, the composition of the basal diet supplemented with two different types of NPN, i.e. urea (Urea) (46 % N) and a mixture of urea and biuret according to the present disclosure (UB), with composition as in example 1 (41 % N) is shown.

In order to determine DM of the feed, approximately 0.5 g of sample was weighed in duplicate, dried in a forced-air oven at 100 °C for 24 hours, and ashed at 550 °C for six hours.

For the determination of the fibrous component, samples were weighed in duplicate into F56 bags (Ankom Technology Crop., Macedon, NY) and analyzed for neutral detergent fiber (NDF), using heat-stable  $\alpha$ -amylase and sodium sulfite, and subsequently for acid detergent fiber (ADF) as described by Van Soest et al. (1991) in Ankom 200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY).

The concentration of N was analyzed through the Dumas dry combustion method using a Vario Micro Cube (Elementar, Manchester, UK) after samples were ball-milled using a Mixer Mill MM 400 (Retsch) at 25 Hz for 9 minutes.

The feed samples were analyzed for concentration of starch through the enzymatic-colorimetric method of Hall (2015) with the following modifications: glucose was analyzed using a quantitative colorimetric kit (G7521-11, Pointe Scientific Inc., Canton, MI), absorbance was read on 200  $\mu$ l samples at OD520 in flat-bottom 96-well plates (Corning Costar 3361, Thermo Fisher Scientific Inc., Waltham, MA) using a plate reader (Fisherbrand UV / VIS AccuSkan GO Spectrophotometer, Thermo Fisher Scientific Inc., Hampton, NH).

*Table 5: Composition of the basal diet supplemented with different types of NPN*

| Item                                        | Treatment |       |
|---------------------------------------------|-----------|-------|
|                                             | +Urea     | +UB   |
| Ingredient, wt.% of dry matter              |           |       |
| Corn silage                                 | 77.00     | 76.88 |
| Cotton-gin trash                            | 20.00     | 20.00 |
| Mineral                                     | 2.00      | 2.00  |
| NPN source                                  | 1.00      | 1.12  |
| Composition                                 |           |       |
| DM (dry matter), wt.% as fed                | 37.13     | 36.17 |
| CP (crude protein), wt. % of DM             | 13.20     | 12.70 |
| Soluble CP, wt. % of CP                     | 49.00     | 53.00 |
| iNDF (indigestible neutral detergent fiber) | 41.40     | 38.30 |
| ADF (acid detergent fiber)                  | 30.10     | 29.40 |
| Starch, wt.% of DM                          | 27.70     | 29.80 |
| Ash, wt.% of DM                             | 5.91      | 5.78  |

***Animal adaptation***

From day -17 to day -16 relative to the beginning of the experimental period, the steers  
 5 received 20 % of their total supplemental NPN.

From day -15 to day -12 relative to the beginning of the experimental period, the steers  
 received 40 % of their total supplemental NPN.

From day -11 to day -8 relative to the beginning of the experimental period, the steers  
 received 60% of their total supplemental NPN.

10 From day -7 to day -3 relative to the beginning of the experimental period, the steers  
 received 80 % of their total supplemental NPN.

From day -2 to the beginning of the experimental period (day 0), the steers received  
 100% of their total supplemental NPN.

***Blood profile measurements***

Blood samples were collected every 14 days during the performance evaluation via jugular venipuncture into 10 ml evacuated tubes containing Na heparin (BD Vacutainer, Franklin Lakes, NJ) and placed on ice centrifuged at 1,500 x g for 15 minutes at 4 °C.

- 5 Plasma was transferred to polypropylene tubes (12 x 75 mm, Fisherbrand, Thermo Fisher Scientific Inc, Waltham, MA) and stored at – 20 °C for further analysis.

***Enteric methane emission measurements***

- 10 When feed is eaten by a ruminant, the nutrients are initially in the form of carbohydrates, proteins and fats (or lipids). During the fermentation of the carbohydrates, carbon dioxide and methane are produced.

- Methane emission was measured in all steers for five consecutive days using the SF<sub>6</sub> tracer technique (Johnson et al., 1994; Henry et al., 2020). In this technique, the steers received a calibrated permeation tube filled with SF<sub>6</sub> (3.1 g ± 0.2 g) before the measurement period. The average SF<sub>6</sub> release rate was 5.2 g ± 1.1 mg/d. After five days of training, a vacuum gas collector canister was attached to each animal to collect a sample of daily gas production. Each day at the same hour, a new vacuum canister was replaced throughout the five days of the measurement period. Five collection canisters were used to determine environmental CH<sub>4</sub> and SF<sub>6</sub> concentrations. A gas sample was taken from each canister and conserved in 120 ml bottles for posterior analysis of CH<sub>4</sub> and SF<sub>6</sub> using gas chromatography. For the current experiment, only steers with a least three successful days of collection and measurement were considered in the final analysis. Gas samples were analyzed by gas chromatography (Agilent 7820A GC, Agilent Technologies, Palo Alto, CA). A flame ionization and electron capture detector were used for CH<sub>4</sub> and SF<sub>6</sub> analysis, respectively, with a capillary column (Plot Fused Silica 25 m x 0.32 mm, Coating Molsieve 5A, Varian CP7536, Varian Inc., Lake Forest, CA). The injector, column and detector temperatures for CH<sub>4</sub> analysis were 80, 160 and 200 °C, respectively. For SF<sub>6</sub>, temperatures were 50, 30 and 300 °C for the injector, the column and the detector, respectively. The carrier gas for CH<sub>4</sub> and SF<sub>6</sub> was N<sub>2</sub>. The emissions of CH<sub>4</sub> were calculated according to the next equation:

$$QCH_4 = QSF_6 \times \frac{([CH_4]\gamma - [CH_4]\beta)}{([SF_6]\gamma - [SF_6]\beta)}$$

QCH<sub>4</sub> = CH<sub>4</sub> emissions per animal (g / day)

$QSF_6$  =  $SF_6$  release rate (mg / day)

$[CH_4]_\gamma$  = the concentration of  $CH_4$  in the animals' collection canister

$[CH_4]_\beta$  = the concentration of  $CH_4$  in the environmental collection canister

$[SF_6]_\gamma$  = the concentration of  $SF_6$  in the animals' collection canister

5  $[SF_6]_\beta$  = the concentration of  $SF_6$  in the environmental collection canister

### ***Gain to feed ratio***

The gain to feed ratio (G:F) was computed as the ratio of the average daily gain (ADG) to daily dry matter intake (DMI). The feed intake was recorded daily. The individual  
10 intake was determined via the GrowSafe® intake monitoring system. In cattle management, GrowSafe Systems is a commonly used tool to closely monitor individual animal feeding data using radio frequency identification (RFID) tag technology. The animals are equipped with a unique RFID tag that is read by the feed bunks each time the animal lowers its head into the bunk to consume feed. Average daily weight gain  
15 (ADG) and feed efficiency were calculated during the performance evaluation. The ADG was determined by the difference between the final BW and the initial BW divided by the number of days of the performance evaluation (56 days). The initial BW was calculated as the average unshrunk BW of heifers on days 16 and 17. The final BW was the average unshrunk BW measured on days 72 and 73. The unshrunk BW was obtained  
20 every 14 days.

### ***Effect of NPN supplementation on animal performance and plasma urea nitrogen (PUN)***

In Table 6, the effect of NPN supplementation with Urea and UB on growth performance  
25 is shown. More in particular, the initial and the final BW and the ADG, all expressed in kg, the dry matter intake (DMI) expressed in kg / day and in percentage of BW, the gain to feed ratio (G:F) expressed in kg / kg, the net energy of gain (NEg) expressed in Mcal / kg of DM and the plasma urea nitrogen (PUN) expressed in mg / dL are shown.

*Table 6: Effect of NPN supplementation on animal performance and the plasma urea nitrogen (PUN)*

| Item                  | Treatment |        |
|-----------------------|-----------|--------|
|                       | +Urea     | +UB    |
| Initial BW (kg)       | 288.69    | 283.18 |
| Final BW (kg)         | 343.69    | 342.11 |
| ADG (kg)              |           |        |
| 0 – 14                | 0.78      | 0.72   |
| 0 – 28                | 0.81      | 0.72   |
| 0 – 42                | 1.02      | 1.04   |
| 0 – 56                | 1.01      | 1.05   |
| DMI (kg / day)        |           |        |
| 0 – 14                | 6.82      | 6.91   |
| 0 – 28                | 7.03      | 7.40   |
| 0 – 42                | 7.24      | 7.85   |
| 0 – 56                | 7.29      | 7.98   |
| DMI (% of BW)         | 2.31      | 2.54   |
| G:F (kg / kg)         |           |        |
| 0 – 14                | 0.10      | 0.10   |
| 0 – 28                | 0.11      | 0.10   |
| 0 – 42                | 0.14      | 0.13   |
| 0 – 56                | 0.14      | 0.13   |
| NEg (Mcal / kg of DM) | 0.55      | 0.52   |
| PUN (mg / dL)         |           |        |
| 0                     | 7.80      | 7.59   |
| 14                    | 6.76      | 7.52   |
| 28                    | 10.41     | 8.78   |
| 42                    | 9.25      | 7.55   |
| 56                    | 8.20      | 8.60   |

Table 6 demonstrates that, compared to supplementation with urea, supplementation with the UB NPN source:

- increased ADG, particularly during the second half of the test period;
  - increased DMI during the whole duration of the experiment, with an increase in DMI of at least 5% during the different time periods considered, compared to the diet containing urea as NPN source;
- 5        - increased DMI per unit of BW, with an increase of about 10% compared to the diet containing urea as NPN source.

***Effect of NPN supplementation on enteric emissions of beef steers***

10 Methane is an energy loss representing between 2 % and 12 % of the gross energy intake in ruminants.

In Table 7, the effect of the NPN supplementation with Urea and UB on enteric methane emission of beef steers is shown. More in particular, the intake expressed in kg / day, and the methane emission expressed in g / kg DMI, g / kg OMI and g/ kg ADG are shown.

15

*Table 7: Effect of NPN supplementation on enteric methane emission of beef steers*

| Item                 | Treatment |        |
|----------------------|-----------|--------|
|                      | +Urea     | +UB    |
| DM intake (kg / day) | 6.08      | 7.11   |
| Methane emission     |           |        |
| (g / kg DMI)         | 36.59     | 31.21  |
| (g / kg OMI)         | 38.68     | 32.94  |
| (g / kg ADG)         | 213.79    | 212.31 |

20 Out of Table 7, it can be concluded that supplementing the diet of yearling steers with UB reduced methane emission without compromising or even improving their performance. In particular, a decrease in methane emissions, when expressed as g/kg DMI or g/kg OMI, of about 15% was observed, compared to a diet containing urea as NPN source. Simultaneously, it was observed that the DMI considered over the whole duration of the experiment was increased by about 17% when using UB as NPN source, compared to the diet containing urea as NPN source.

25

**Example 4**

In this example, an *in vitro* assay was performed using batch culture incubations, aiming to simulate rumen fermentation.

**5     *Animal adaptation***

Two ruminally cannulated Angus crossbred steers ( $808.8 \pm 36.3$  kg body weight) were used as ruminal fluid donors for the *in vitro* batch culture incubations. The steers were fed a corn silage and gin trash diet (70% and 30% on a dry matter basis, respectively) ad libitum at least 35 days before collecting ruminal fluid to perform the *in vitro* incubations. Each steer received a NPN-mixture equivalent to 100 g of urea per day comprised of an equal amount of N from the different NPN sources to adapt the rumen microbial community. Thus, each steer was fed daily 33 g of urea (46 wt.% N) or 37 g of a urea-biuret mixture according to the present disclosure (UB) comprising 41 wt.% N. These amounts were mixed in with the feed diet every two days.

15

***Feed preparation and chemical analysis***

The feed was prepared by drying corn silage and gin trash for 45 hours at 55°C and ground to pass a 2 mm screen in a Wiley mill.

**20    *Experimental design and treatments***

The *in vitro* batch culture incubations were conducted as a randomized complete block design using 70 wt.% corn silage and 30 wt.% gin trash on a dry matter basis conserving the diet characteristics used during the animal adaptation period. The treatments were designed to be isonitrogenous (= having the same amount of dietary nitrogen) and equivalent to 1 wt.% of urea inclusion. The NPN treatments were consequently as follows:

25

- Control without NPN-supplementation (“control”);
- 100 wt% urea (Rumisan ®) (“urea”);
- 100 wt% of a urea/biuret mixture according to the present application (“UB”).

30

***Rumen fluid collection and in vitro incubations***

The *in vitro* incubations were conducted on three separate days (replicates). A representative sample of digesta was collected from different places in the rumen of

5 ruminally cannulated steers and strained through 4 layers of cheesecloth, placed in pre-warmed thermos containers, and transported to the laboratory within 30 minutes after the collection. In the laboratory, the ruminal fluid from the two steers was maintained under constant CO<sub>2</sub>-flux and was combined in equal proportions. This combined ruminal  
10 fluid was mixed with McDougall's buffer (McDougall, 1948) with and without 0.077 g/liter of 10 wt.% ammonium sulfate enriched with <sup>15</sup>N in relation 1:4, and it was used as an inoculum. Two bottles of 500 ml fitted with a side arm and a rubber septum were incubated per treatment. The bottles contained 5.6 g of substrate dry matter and 400 ml of <sup>15</sup>N enriched inoculum, and were incubated for 24 hours at 39°C with an agitation of  
15 60 rpm to monitor gas production kinetics using an Ankom Gas Monitoring System from Ankom Technologies, Fairport, New York. Also, one 500-ml bottle without <sup>15</sup>N enriched inoculum was incubated to determine the basal <sup>15</sup>N enrichment. At 12 hours of incubation, two samples of 10 ml of rumen fluid were collected from each bottle through the septa port using a 20-ml syringe. These samples were acidified by adding 10 µl of a  
20 20 vol/vol% of H<sub>2</sub>SO<sub>4</sub>-solution to each 10-ml sample, and frozen at minus 20°C until further analysis. At the end of the 24 hours incubation, the final pH was recorded, the bottles were placed on ice to prevent further microbial fermentation and two 10-ml samples were collected and acidified by adding 20 µl of a 20 vol/vol% of H<sub>2</sub>SO<sub>4</sub>-solution and were frozen at minus 20°C until further analysis. The remaining fermentation  
25 content was thereafter agitated at 60 rpm for 30 seconds and divided into two subsamples. From the first subsample, composed of whole contents, a volume of 180 ml was frozen at minus 20°C until further analysis and from the second subsample 180 ml were used to recover the microbial pellet. Furthermore, two tubes of 100 ml per treatment containing 0.7 g of the substrate on a dry matter basis and 50 ml of inoculum without <sup>15</sup>N enrichment and two blanks were incubated for 24 hours at 39 °C with  
30 agitation at a speed of 60 rpm. After the 24 hours incubation with ruminal fluid, 6 ml of HCl and 2 ml of a 5% pepsin solution were added. The tubes were incubated for another 48 hours at 39°C. After the incubation period, the tubes were maintained on ice and later stored for further analysis.

### ***Concentrations of volatile fatty acids (VFA)***

VFA produced by microbial fermentation in the rumen are the main source of energy absorbed by the digestive tract of ruminants. The amount and the profile of VFA in the

rumen has consequences for the efficiency of energy utilization, production of methane, risks of ruminal acidosis (characterized by a decrease in pH caused by an accumulation of organic acids in the rumen and a shift in the delicate balance of rumen microorganisms). A water-based solution using ethyl acetate extraction was used to  
5 determine VFA concentrations at 12 and 24 hours. Samples were centrifuged at  $10,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ , 2 ml of the supernatant were mixed with 0.4 ml (5:1 ratio) of a metaphosphoric-crotonic acid solution, and samples were frozen overnight. Samples were then thawed and centrifuged again at  $10,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The supernatant was transferred into  $12 \times 75$  mm borosilicate disposable culture tubes (Fisherbrand;  
10 Thermo Fisher Scientific Inc., Waltham, MA) and mixed with ethyl acetate to form a 2:1 ethyl acetate : supernatant mixture. The samples were analyzed with a gas chromatograph (Agilent 7820A GC, Agilent Technologies) using a flame ionization detector and a capillary column (CP-WAX 58 FFAP  $25 \text{ m} \times 0.53 \text{ mm}$ , Varian CP7767; Varian Inc.).

15

### ***Microbial protein synthesis***

The rumen microbial ecosystem is made up mainly by species of bacteria, fungi, and protozoa. The microbes ferment ingested feeds by the ruminant, and, by fermentation, produce end products that are utilized by the ruminant as well as by the microbes  
20 themselves for their own proliferation and growth. The species diversity and their relative proportions within the microbial community depend primarily on the diet consumed by the animal. It is thus important that a healthy mixture of different feedstuffs is fed to the animal to keep the rumen and its microflora functioning efficiently. The speed of digestion of the feed depends on the quality and the supplement of the feed and  
25 is affected by the number and type of microbes, the pH in the rumen, the nutrients limiting the growth of the microbes and the removal of microbes from the rumen. Energy and protein are the major nutrients that limit the microbial growth, and therefore, the rumen fermentation. The microbial population needs energy and protein for their growth and multiplication. Protein, when digested, is broken down into peptides, which are short  
30 chains of amino acids. Further digestion of peptides results in individual amino acids and eventually ammonia. Rumen microbes are the major source of protein for the ruminant animal. If there is enough energy available, the rumen microbes break down the rumen degradable protein to amino acids and then to ammonia which is a major N-

source for microbial growth. The microbes also convert NPN to ammonia, which contributes to the ammonia pool available for microbial growth. Microbial protein is continuously flushed from the rumen into the intestine where undergoes enzymatic digestion. The amino acids produced from the digested microbial protein are absorbed  
5 through the small intestine. The amount of microbial protein flowing into the intestines therefore largely depends on the availability of fermentable energy and N in the diet consumed by ruminants.

The total nitrogen (N) and the isotopic supplement were measured in the whole content and the bacterial pellet by an isotopic ratio mass spectrometry using a Vario Micro cube  
10 from Elementar Analyzer System GmbH. The bacteria pellet subsample was slowly centrifuged at 1,000 x g for 10 minutes at 4°C. The resulting supernatant was fast centrifuged at 20,000 x g for 20 minutes at 4°C to obtain the pellet of bacteria. This bacteria pellet was then washed with a 0.9% saline solution and centrifuged at 20,000 x g for 20 minutes at 4°C, discarding the supernatant. The saline wash procedure was  
15 repeated twice. Finally, the bacterial pellet was freeze-dried. Separately, the 180-ml whole content subsample was completely freeze-dried. The dried samples of the whole content and of the bacterial pellet were manually grinded and weighted into an 8 x 5-mm pressed standard-weight tin capsule using a Mettler-Toledo Excellence Plus XP Micro Balance from Mettler-Toledo GmbH, Laboratory and Weighing Technologies.  
20 Then, 35 µl of a 10 g/l solution of K<sub>2</sub>CO<sub>3</sub> were added to each sample and dried overnight in a forced-air oven at 60°C for complete NH<sub>3</sub>-N evaporation. The percentage of atom <sup>15</sup>N in the dried samples was analyzed in an isotope ratio mass spectrometer (IsoPrime 100 from IsoPrime) and expressed as the fractional abundance of isotopic fractions (<sup>15</sup>N / <sup>14</sup>N) multiplied by 10.

### ***Concentration of ammonia-N***

Ruminal ammonia production largely reflects the amount of degradable protein in the rumen. Ruminal ammonia nitrogen (ammonia-N) is a critical nutrient for supporting microbial growth and fermentation. Ensuring the availability of ammonia-N in the  
30 rumen both on a quantitative and timely manner is fundamental for achieving maximum rate of fermentation and feed digestibility.

The concentration of ammonia-N at 12 and 24 hours in the incubation fluid was measured after centrifuging at 10,000 x g for 15 minutes at 4°C using an Avanti J-E

centrifuge from Beckman Coulter® Incorporated. One ml of phenol reagent was pipetted into 12 × 75 mm borosilicate disposable culture tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA). A 20-μl aliquot of the supernatant from the centrifuged sample was transferred to the phenol-containing culture tubes. After vortexing, 0.8 ml of hypochlorite solution was added to the mixture and was vortexed again. The culture tubes were covered with glass marbles and placed in a water bath at 95°C for 5 minutes. The absorbance was read in 96-well, flat-bottom plates at a wavelength of 665 nm using a DU-500 plate reader from Beckman Coulter® Inc. The fermentation residue reserved into the tubes of 100 ml were filtered, dried at 105°C in a forced air oven for 24 hours and ashed at 550°C for 6 hours to determine the undigested organic matter.

### *Nutrient disappearance*

Digestibility represents the proportion of ingested nutrients consumed that are absorbed by the animal. Dry matter is defined as the loss of weight of samples when dried in an oven above 100°C during 12 – 24 hours, whereas organic matter defines the weight loss of the dry matter when combusted (the dry matter minus the ash content). Dry matter consists of all nutrients, whereas organic matter consists of all nutrients with the exception of ash. Organic matter digestibility is defined as the proportion of organic matter in the feed that is digested in the rumen or total ruminant digestive tract. Ruminant organic matter digestibility can be used to estimate energy available for fueling microbial growth and the resulting synthesis of microbial protein in the rumen. The in vitro dry organic matter digestibility (IVDMD) and the in vitro organic matter digestibility (IVOMD) was calculated with the following formulae:

$$IVDMD (\%) = \frac{\text{incubated dry matter} - \text{residual dry matter}}{\text{incubated dry matter}} \times 100$$

$$IVOMD (\%) = \frac{\text{incubated organic matter} - \text{residual organic matter}}{\text{incubated organic matter}} \times 100$$

### *Results*

In Table 8 below, the concentration of the total VFA and the concentration of ammonia-N in: a control diet without NPN supplementation (“control”), a corn silage-based diet

either with urea supplementation (“urea”) or with supplementation of a urea-biuret mixture according to the present disclosure (“UB”) after 12 hours of incubation is shown. In Table 9 below, the same is shown after 24 hours of incubation. In Table 9, also the pH of the fluid, the *in vitro* dry matter degradability (IVDMD) and the *in vitro* organic matter degradability (IVOMD) after 24 hours of incubation are shown. The pH of the rumen has profound effects on the growth of the ruminal microbes and the digestion that takes place in this forestomach. Rumen pH can affect the extent of rumen fermentation by altering microbial populations and tissue absorptive capacity. Low ruminal pH for prolonged periods each day for instance can affect feed intake, microbial metabolism and feed digestion, and has also been related to inflammation, diarrhea and milk fat depression.

*Table 8: Effect of NPN supplementation on VFA and ammonia-N at 12 hours of incubation in a corn silage-based diet*

| Variable  | Control | Urea  | UB    |
|-----------|---------|-------|-------|
|           | mM      |       |       |
| Total VFA | 30.95   | 31.77 | 32.55 |
| Ammonia-N | 0.35    | 2.74  | 2.61  |

*Table 9: Effect of NPN supplementation on VFA and ammonia-N, pH and in vitro digestibility or organic and dry matter at 24 hours of incubation in a corn silage-based diet*

| Variable  | Control | Urea  | UB    |
|-----------|---------|-------|-------|
|           | mM      |       |       |
| Total VFA | 41.39   | 40.96 | 42.85 |
| Ammonia-N | 2.48    | 6.55  | 7.34  |
| pH        | 6.34    | 6.38  | 6.37  |
| IVDMD, %  | 45.02   | 45.08 | 45.78 |
| IVOMD, %  | 64.13   | 65.38 | 64.17 |

Out of Tables 8 and 9, it can be concluded that

- With regards to the total VFA, the UB mixture according to the present application generated a higher amount of VFA in comparison with the control without NPN supplementation or with urea supplementation. These findings indicate that the mixture of urea and biuret according to the present disclosure is more effective in supporting in vitro fermentation than urea supplementation.
- With regards to ammonia-N, as expected, the supplementation with NPN, i.e. urea or the UB mixture increased its concentration.
- With regards to IVDMD, supplementation with UB, but not with urea, improved the extent of dry matter degradation. These findings further support the proposition that supplementation with UB is more effective in supporting rumen fermentation than urea.
- Overall, these results demonstrate that the UB mixture according to the present application is an effective source of NPN that outperforms urea in supporting in vitro fermentation.

In Table 10, the concentration of bacterial N, bacteria efficiency (BE) or, stated differently, the efficiency of microbial protein synthesis (EMPS), i.e. g of bacteria N / kg of OM truly digested for (i) a control diet without NPN supplementation (“control”), (ii) a corn silage-based diet with urea supplementation (“urea”) or (iii) a corn silage-based diet with supplementation with a urea-biuret mixture according to the present application (“UB”), after 24 hours of incubation is shown. These parameters define the capacity of rumen microbes to use N.

*Table 10: Effect of NPN supplementation on microbial synthesis and microbial efficiency production in a corn-based diet*

| Variable       | Control | Urea  | UB    |
|----------------|---------|-------|-------|
| Bacteria N, mg | 55.66   | 67.34 | 59.55 |
| EMPS           | 27.07   | 34.46 | 41.98 |

Out of Table 10, it can be concluded that the supplementation of the control diet with NPN increases the amount of N assimilated by bacteria. Supplementation with the urea/biuret mixture according to the present application particularly resulted in an increased bacteria efficiency/increased efficiency of microbial protein synthesis. These results are in line with the results of example 2.

### ***Methane production***

When feed is eaten by a ruminant, the nutrients are initially in the form of carbohydrates, proteins and fats (or lipids). During the fermentation of the carbohydrates, carbon dioxide and methane are produced.

The amount of methane produced was determined by measuring total gas production and the contribution of methane to it. The production of gas was recorded using an Ankom Gas Monitoring System from Ankom Technologies. This system was adapted to 500 ml bottles during the entire fermentation process. The kinetics of the production was fitted to a Gompertz model to determine the rate of the total maximal gas production (M), fractional rate of gas produced (kf) and lag phase (L). A gas subsample was analyzed to measure methane concentration and calculate the total methane production during the fermentation. The concentration of methane was determined using a gas chromatograph Agilent 7820 GC from Agilent Technologies with flame ionization and a capillary column (Plot Fused Silica 25 m x 0.32 mm, coating Molsieve 5A, Varian CP 7536 from Varian Inc.).

In Table 11, the effect of NPN supplementation on the production of methane in a corn silage-based diet is shown. More in particular, methane gas production expressed in mM/g organic matter incubated (OMi) and expressed in mM/g organic matter digested (OMd), in a control diet without NPN supplementation ("control"), a corn silage-based diet either urea supplementation ("urea") or with a urea/biuret mixture according to the present application ("UB"), are shown.

Table 11 shows that supplementation with a UB mixture according to the present disclosure resulted in a decreased production of methane compared to the control diet or the diet with urea as NPN. Methane production decreased by about 4-5% when expressed per g of OMi or per g of OMd, compared to urea as NPN.

*Table 11: Effect of NPN supplementation on methane production in a corn silage-based diet*

| Variable | Control | Urea | UB   |
|----------|---------|------|------|
| mM/g OMi | 1.24    | 1.23 | 1.18 |
| mM/g OMd | 6.93    | 6.86 | 6.58 |

**Example 5 – increasing doses of UB on fermentation and methane production *in vitro***

The main objective of example 5 was to assess the impact of increasing doses of a mixture of urea and biuret according to the present disclosure, hereinafter UB, with composition as in example 1 (41 % N, YARA International) on *in vitro* fermentation, nutrient digestibility, and methane production in a corn silage-based diet.

***Approach and Research Procedures***

Two ruminally cannulated steers were used as ruminal fluid donors for the *in vitro* batch culture incubations. The steers were fed corn silage, cotton gin trash, and a premix of vitamins and minerals (70, 28, and 2 % on a dry matter basis, respectively) *ad libitum* at least 35 d before collecting ruminal fluid to perform the *in vitro* incubations. To adapt steers to the presence of NPN, each steer received the N equivalent to 100 g of urea per day (46 g of N daily/steer) but divided into two NPN sources. Thus, each steer was fed daily with 33 g of urea or 37 g of a urea/biuret mixture according to the present application. These amounts were mixed in with the diet daily. *In vitro* incubations were conducted on three separate days (replicates) using the same silage fed to the steers as the incubation substrate. The sources of NPN to be tested were analyzed for total N content and incubated on an isonitrogenous basis.

***Treatments***

The incubation substrates (treatments) to test were control (CON, without NPN supplementation) and four increasing inclusions (from 1× to 4×) of the urea-biuret mixture according to the present application (UB). Treatments at 1× were designed to be iso-nitrogenous and equivalent to 1% of the inclusion in dry matter basis of urea (Yara International), in the diet, except for the CON treatment.

### ***Batch culture incubations***

*In vitro* incubations were conducted on three separate days (replicates) using the same silage fed to the steers as the incubation substrate. A representative sample of rumen  
5 digesta was collected from different places in the rumen from the ruminally-cannulated steers and strained through 4 layers of cheesecloth, placed in pre-warmed thermos containers, and transported to the laboratory within 30 min of collection. In the laboratory, ruminal fluid from the two steers was maintained under constant CO<sub>2</sub> flux and was combined in equal proportions. Combined ruminal fluid was mixed with  
10 McDougall buffer in relation 1:4 (i.e., inoculum).

Two bottles of 120 mL per treatment containing 0.7 g and 50 mL of inoculum were incubated for 24 h at 39°C with gentle agitation (60 rpm). Further, two bottles without substrate were incubated as blank bottles. Gas pressure was recorded at 24 h, and a sample of gas was stored for further analysis. At the end of the 24 h of incubation, the  
15 final pH of fermentation fluid was recorded, and two 10-mL samples were collected and acidified by adding 20 µL of a 20% (vol/vol) of H<sub>2</sub>SO<sub>4</sub> solution and were frozen at -20°C until further analysis.

Measurement of *in vitro* organic matter digestibility (IVOMD) was performed by incubating a separate set of duplicate 100-mL polypropylene tubes for 24 h at 39°C on  
20 each of the replicate days. These flasks contained 0.7 g of the substrate and were inoculated with 50 mL of a 4:1 McDougall's buffer:ruminal fluid mixture. After 24-h incubation with ruminal fluid, a pepsin-HCl solution was added, and tubes were incubated for another 48 h before filtering, drying at 105°C in a forced air oven for 24 h, and ashing at 550°C for 6 h to determine the undigested organic matter.

25

### ***Sample analysis***

Concentrations of NH<sub>3</sub>-N in the incubation fluid were measured after centrifuging at 10,000 x g for 15 min at 4°C (Avanti J-E, Beckman Coulter Inc., Palo Alto, CA) following the methodology described by Broderick and Kang (1980). Briefly, 1 mL of  
30 a phenol reagent was pipetted into 12 × 75 mm borosilicate disposable culture tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA). A 20-µL aliquot of the supernatant from the centrifuged sample was transferred to the phenol-containing culture tubes. After vortexing, 0.8 mL of a hypochlorite solution was added to the

mixture and vortexed again. The culture tubes were covered with glass marbles and placed in a water bath at 95°C for 5 min. Absorbance was read on 200 µL samples at OD<sub>620</sub> in flat-bottom 96-well plates (Corning Costar 3361, Thermo Fisher Scientific Inc., Waltham, MA) using a plate reader (Fisherbrand UV/VIS AccuSkan GO Spectrophotometer, Thermo Fisher Scientific Inc., Hampton, NH). All assays were conducted in duplicate determinations with subsequent analyses performed when CVs were above 5%.

Total gas production was measured using a manual transducer (Digital Test Gauge, Ashcroft Inc., Stratford, CT, USA), and a subsample was analyzed to measure CH<sub>4</sub> concentration by gas chromatography to calculate total CH<sub>4</sub> production during the course of the fermentation. To determine concentrations of CH<sub>4</sub>, a gas chromatograph (Agilent 7820A GC, Agilent Technologies, Palo Alto, CA) using flame ionization and a capillary column (Plot Fused Silica 25 m × 0.32 mm, Coating Molsieve 5A, Varian CP7536; Varian Inc., Lake Forest, CA) was used. Temperatures of the injector, column, and detector were 80, 160, and 200°C, respectively, and N<sub>2</sub> was the carrier gas flowing at 3.3 mL/min. The split ratio for the injected CH<sub>4</sub> sample was 100:1.

Additionally, total volatile fatty acids (VFA) concentrations were measured by gas chromatography in the incubation fluid at the end of the fermentation to assess potential changes in the profile of VFA being produced, following the methodology described by Ruiz-Moreno et al. (2015). Briefly, samples were centrifuged at 10,000 × g for 15 min at 4°C. Two milliliters of the supernatant were mixed with 0.4 mL (5:1 ratio) of a metaphosphoric:crotonic acid (internal standard) solution, and samples were frozen overnight. Samples were thawed and centrifuged again at 10,000 × g for 15 min at 4°C. The supernatant was transferred into 12 mm × 75 mm borosilicate disposable culture tubes (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA) and mixed with ethyl acetate to form a 2:1 ethyl acetate:supernatant mixture. Culture tubes were vigorously shaken and followed by a 5 min rest time to allow the separation of the ethyl acetate. A subsample of the ethyl acetate was transferred into small vials prior to analysis. Samples were analyzed with a gas chromatograph (Agilent 7820A GC, Agilent Technologies) using a flame ionization detector and a capillary column (CP-WAX 58 FFAP 25 m × 0.53 mm, Varian CP7767; Varian Inc.). The column temperature was maintained at 110°C, and the injector and detector temperatures were 200 and 220°C, respectively.

***Increasing dose of urea/biuret on in vitro fermentation and CH<sub>4</sub> emissions - results***

The results are represented in Table 12.

5 *Table 12. Effect of increasing the UB inclusion on fluid fermentation pH, the concentrations of volatile fatty acids and ammonia-N, in vitro organic matter digestibility, and gas and methane production in a corn silage-base diet.*

| Variable <sup>1</sup>          | UB inclusion |        |        |        |        |
|--------------------------------|--------------|--------|--------|--------|--------|
|                                | 0x           | 1x     | 2x     | 3x     | 4x     |
| pH                             | 6.50         | 6.49   | 6.54   | 6.59   | 6.63   |
| Total volatile fatty acids, mM | 88.72        | 105.78 | 101.83 | 99.92  | 96.03  |
| Acetate, mM                    | 55.57        | 65.41  | 52.45  | 61.03  | 59.15  |
| Propionate, mM                 | 18.19        | 22.12  | 21.14  | 21.09  | 19.68  |
| Butyrate, mM                   | 10.79        | 13.21  | 13.36  | 13.02  | 12.69  |
| Acetate, mol/100 mol           | 62.64        | 61.76  | 61.32  | 61.00  | 61.56  |
| Propionate, mol/100 mol        | 20.52        | 20.89  | 20.77  | 21.07  | 20.52  |
| Butyrate, mol/100 mol          | 12.15        | 12.57  | 13.11  | 13.13  | 13.25  |
| Acetate:Propionate             | 3.05         | 2.97   | 2.97   | 2.90   | 3.01   |
| Ammonia-N, mM                  | 3.25         | 6.96   | 9.96   | 13.88  | 15.39  |
| IVOMD, %                       | 54.40        | 54.96  | 55.13  | 58.77  | 57.48  |
| Gas production, mL/g OMd       | 300.26       | 301.34 | 297.04 | 263.94 | 262.43 |
| Methane production, mM/g OMd   | 33.73        | 32.74  | 32.43  | 28.84  | 23.02  |

<sup>1</sup> IVOMD: In vitro organic matter digestibility; OMd: Organic matter degraded.

10 Increasing doses of UB linearly increased the concentration of NH<sub>3</sub>-N, the rumen fluid pH, and the IVOMD. Also, the concentration of total VFA, acetate, propionate, and butyrate quadratically increased when increasing UB inclusion. Additionally, increasing the inclusion of UB linearly reduced the acetate:propionate ratio and gas and CH<sub>4</sub> production (Table 12).

15 In the present experiment, the inclusion of UB increased the NH<sub>3</sub>-N concentration resulting in greater IVOMD and concentration of total and individual VFA, indicative of a greater fermentation. Rumen microbes can utilize NH<sub>3</sub>-N to synthesise microbial protein promoting greater fermentation. Additionally, the greater concentration of NH<sub>3</sub>-N is in line with the linear increase in the rumen fluid pH.

Increasing the UB inclusion promoted other metabolic routes different than acetate formation, with a linear decrease in acetate proportion upon increasing the UB supplementation.

Furthermore, increasing the UB supplementation linearly decreased gas and CH<sub>4</sub> production but increased VFA and IVOMD, suggesting changes in rumen microbial communities, possibly Archaeas and fibrolytic bacteria, explaining the lower production of CH<sub>4</sub> and the lower proportion of acetate. Without wishing to be bound by theory, the greater concentration of NH<sub>3</sub>-N during the incubation results in a greater fermentation rate, thereby increasing the concentration of VFA and IVOMD. However, the faster accumulation of NH<sub>3</sub>-N may result in a toxic effect on some rumen microbial communities affecting the gas and CH<sub>4</sub> production.

Further particular statements (features) and embodiments and uses of a NPN composition according to the present disclosure are set herein below.

15

Statement 1. Use of a composition comprising biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed supplement composition to decrease the acetate to propionate ratio produced by rumen microbes in a ruminant.

20 Statement 2. Use of a composition comprising biuret, urea and N-containing by-product of the biuret production out of urea as a NPN-source in a ruminant feed supplement composition to improve the efficiency of microbial protein synthesis (EMPS).

Statement 3. Use of a composition comprising biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed supplement composition to increase the dry matter (DM) and organic matter (OM) digestibility.

25 Statement 4. Use of a ruminant feed supplement composition comprising urea, biuret and N-containing by-products of the biuret production out of urea, as a NPN-source to reduce methane emission produced by ruminant animals.

Statement 5. Use of a ruminant feed supplement composition comprising urea, biuret and N-containing by-products of the biuret production out of urea as a NPN-source to improve the dry matter intake (DMI) of ruminant animals.

30 Statement 6. Use according to any one of statements 1 to 5, wherein the composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret,

particularly between 35 and 55 wt.% of urea and between 35 and 50 wt.% of biuret, based on the total weight of the composition.

Statement 7. Use according to statement 6, wherein the composition comprises between 37 and 52 wt.% of urea and between 39 and 46 wt.% of biuret, based on the total weight of the composition.

Statement 8. Use according to any one of statements 1 to 7, wherein the composition further comprises between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.

Statement 9. Use according to statement 8, wherein the composition further comprises between 2.5 and 8 wt.% of triuret, and/or between 0.1 and 5 wt.% of ammelide, and/or between 2.5 and 15 wt.% of cyanuric acid, and between 0.1 and 2.0 wt.% of moisture, based on the total weight of the composition.

Statement 10. Use according to statement 8, wherein the composition further comprises: between 3 and 6 wt.% of triuret, and/or between 0.5 and 3 wt.% of ammelide, and/or between 3 and 10 wt.% of cyanuric acid, and between 0.1 and 1.5 wt.% of moisture.

Statement 11. Ruminant feed composition comprising one or more nutritional ingredients and an NPN composition, wherein the NPN composition comprises (a) between 35 and 55 wt.% of urea; (b) between 35 and 50 wt.% of biuret; and (c) between 5 wt.% and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the NPN composition, particularly wherein the N-containing by-products are triuret, ammelide and/or cyanuric acid.

Statement 12. Ruminant feed composition according to statement 11, wherein the NPN composition comprises (a) between 37 and 52 wt.% of urea; (b) between 39 and 46 wt.% of biuret; (c) between 3 wt.% and 6 wt.% of triuret; (d) between 0.5 and 3 wt.% of ammelide; and (e) between 3 and 10 wt.% of cyanuric acid, based on the total weight of the NPN composition.

Statement 13. Ruminant feed composition according to statement 11 or 12, wherein the one or more nutritional ingredients form a nutritionally balanced ration or a basal ration for a ruminant.

**CLAIMS**

1. Use of a composition comprising biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed or in a ruminant feed supplement composition for decreasing the enteric methane emissions of a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source, wherein the composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret.
2. Use according to claim 1, for further and simultaneously decreasing the acetate-to-propionate ratio produced by rumen microbes in a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
3. Use according to claim 1 or 2, for further and simultaneously improving the efficiency of microbial protein synthesis (EMPS) by rumen microbes in a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
4. Use according to any one of claims 1 to 3, for further and simultaneously increasing the dry matter (DM) and organic matter (OM) digestibility, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
5. Use according to any one of claims 1 to 4, for further and simultaneously increasing the dry matter intake (DMI) of a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
6. Use of a composition comprising biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed or in a ruminant feed supplement composition for:

- decreasing the acetate-to-propionate ratio produced by rumen microbes in a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source;
  - improving the efficiency of microbial protein synthesis (EMPS) by rumen microbes in a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source;
  - increasing the dry matter (DM) and organic matter (OM) digestibility particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source; and/or
  - increasing the dry matter intake (DMI) of a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source, wherein the composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret.
7. Use according to any one of claims 1 to 6, wherein the composition comprises between 35 and 55 wt.% of urea and between 35 and 50 wt.% of biuret, based on the total weight of the composition.
8. Use according to claim 7, wherein the composition comprises between 37 and 52 wt.% of urea and between 39 and 46 wt.% of biuret, based on the total weight of the composition.
9. Use according to any one of claims 1 to 8, wherein the composition further comprises between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.
10. Use according to claim 9, wherein the composition comprises between 2.5 and 8 wt.% of triuret, and/or between 0.1 and 5 wt.% of ammelide, and/or between 2.5 and 15 wt.% of cyanuric acid, and between 0.1 and 2.0 wt.% of moisture, based on the total weight of the composition.

11. Use according to claim 10, wherein the composition comprises between 3 and 6 wt.% of triuret, and/or between 0.5 and 3 wt.% of ammelide, and/or between 3 and 10 wt.% of cyanuric acid, and between 0.1 and 1.5 wt.% of moisture, based on the total weight of the composition.

5

12. A method for reducing the enteric methane emissions of a ruminant animal, comprising administering to the ruminant animal a NPN composition comprising biuret, urea and N-containing by-products of the biuret production, wherein the NPN composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret, particularly wherein the NPN composition comprises between 35 and 55 wt.% of urea, between 35 and 50 wt.% of biuret, and between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.

13. The method according to claim 12, wherein the enteric methane emissions of the ruminant animal, when expressed as g methane/kg dry matter intake (DMI) or as g methane/kg organic matter intake, are reduced by at least 5%, particularly by at least 10%, when the NPN composition is administered to a ruminant as NPN source, compared to a diet comprising urea as NPN source.

20

14. The method according to claim 12 or 13, wherein said method is further simultaneously a method for:

- decreasing the acetate-to-propionate ratio produced by rumen microbes in a ruminant animal, particularly wherein the acetate-to-propionate ratio produced by rumen microbes in a ruminant, particularly determined via an *in vitro* continuous flow fermentation with rumen fluid to simulate rumen fermentation, is decreased by at least 5%, in particular by at least 10%, when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source,

- improving the efficiency of microbial protein synthesis (EMPS) by rumen microbes in a ruminant animal, wherein the EMPS, particularly determined via an *in vitro* continuous or batch rumen fluid incubation to simulate rumen fermentation, was increased by at least 5%, in particular by at least 10%, when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source;

- increasing the dry matter (DM) and organic matter (OM) digestibility; and/or
- increasing the dry matter intake (DMI) of a ruminant animal, particularly wherein the DMI, expressed in kg/day is increased by at least 5% when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source.

15. The method according to any one of claims 12 to 14, wherein the NPN composition comprises between 35 and 55 wt.% of urea and between 35 and 50 wt.% of biuret, based on the total weight of the composition, more in particular comprises between 37 and 52 wt.% of urea and between 39 and 46 wt.% of biuret, based on the total weight of the composition.

16. The method according to any one of claims 12 to 15, wherein the NPN composition further comprises between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.

17. The method according to claim 16, wherein the NPN composition comprises between 2.5 and 8 wt.% of triuret, and/or between 0.1 and 5 wt.% of ammelide, and/or between 2.5 and 15 wt.% of cyanuric acid, and between 0.1 and 2.0 wt.% of moisture, based on the total weight of the composition, more in particular, wherein the composition comprises between 3 and 6 wt.% of triuret, and/or between 0.5 and 3 wt.% of ammelide, and/or between 3 and 10 wt.% of cyanuric acid, and between 0.1 and 1.5 wt.% of moisture, based on the total weight of the composition.

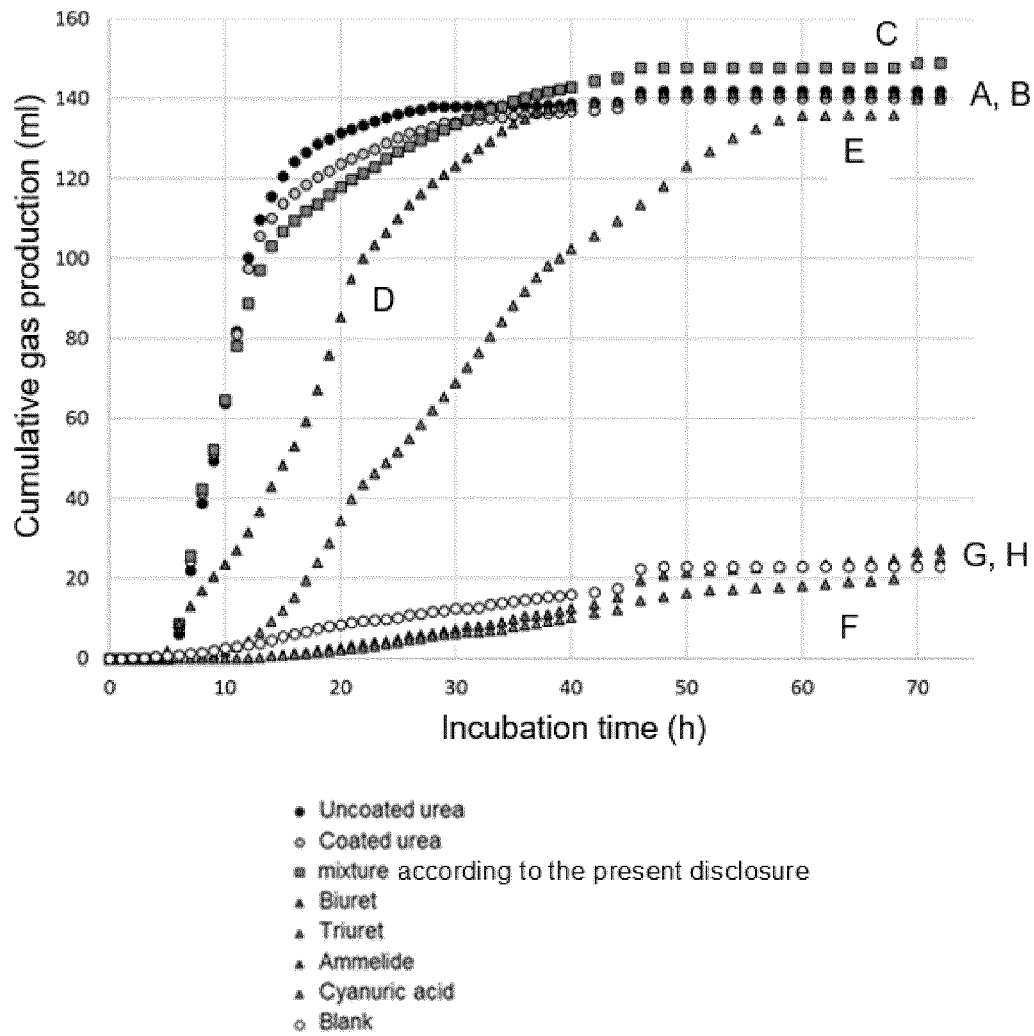
18. The method according to any one of claims 12 to 17, comprising the steps of preparing or providing a ruminant feed supplement composition comprising the NPN composition, or preparing or providing a ruminant feed composition comprising the NPN composition, and administering the ruminant feed supplement or the ruminant feed composition to the ruminant animal.

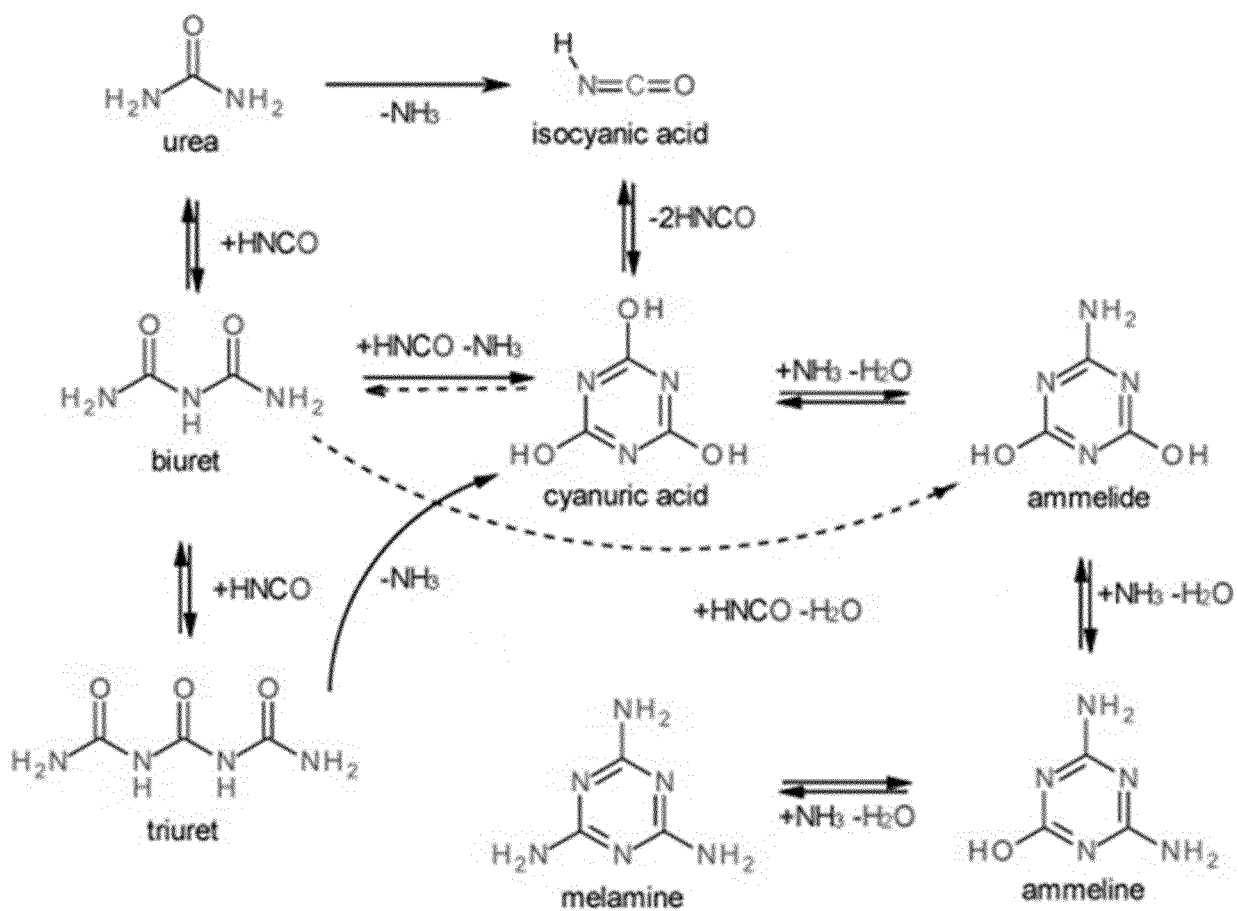
19. The method according to claim 18, wherein the NPN composition is present in the ruminant feed composition in a concentration between 0.5 and 4 wt.%, more in particular between 0.5 and 3.5 wt.% or between 0.5 and 3 wt.%, even more in particular between

1 and 2.5 wt.%, with wt% based on the total dry matter basis of the ruminant feed composition.

20. The method according to claim 18 or 19, wherein the ruminant feed composition  
5 further comprises one or more nutritional ingredients, wherein the one or more nutritional ingredients form a nutritionally balanced ration or a basal ration for a ruminant.

## FIGURES

**FIG. 1**

**FIG. 2**

# INTERNATIONAL SEARCH REPORT

International application No  
**PCT/EP2023/054080**

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| <b>A. CLASSIFICATION OF SUBJECT MATTER</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                       |                                                                             |
| <b>INV.</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | <b>A23K20/105</b>                                                                                                     | <b>A23K20/20</b>                                                            |
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| <b>ADD.</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                       | <b>A23K50/10</b>                                                            |
| According to International Patent Classification (IPC) or to both national classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                       |                                                                             |
| <b>B. FIELDS SEARCHED</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                                                                       |                                                                             |
| Minimum documentation searched (classification system followed by classification symbols)<br><b>A23K</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                       |                                                                             |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                       |                                                                             |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)<br><b>EPO-Internal, CHEM ABS Data, FSTA, WPI Data</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                       |                                                                             |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                       |                                                                             |
| Category*                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Citation of document, with indication, where appropriate, of the relevant passages                                    | Relevant to claim No.                                                       |
| <b>Y</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | <b>US 2 768 895 A (JONAS KAMLET)</b><br><b>30 October 1956 (1956-10-30)</b><br><b>claims; examples</b><br>-----       | <b>6-11</b>                                                                 |
| <b>Y</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | <b>CA 1 241 663 A (MOORMAN MFG CO)</b><br><b>6 September 1988 (1988-09-06)</b><br><b>examples 7-9</b><br>-----<br>-/- | <b>6-11</b>                                                                 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                       |                                                                             |
| * Special categories of cited documents :<br>"A" document defining the general state of the art which is not considered to be of particular relevance<br>"E" earlier application or patent but published on or after the international filing date<br>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"P" document published prior to the international filing date but later than the priority date claimed<br>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br>"&" document member of the same patent family |                                                                                                                       |                                                                             |
| Date of the actual completion of the international search<br><br><b>22 June 2023</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                       | Date of mailing of the international search report<br><br><b>03/07/2023</b> |
| Name and mailing address of the ISA/<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                       | Authorized officer<br><br><b>Saettel, Damien</b>                            |

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2023/054080

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                       |
|------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                                        | Relevant to claim No. |
| A                                                    | <p>XIUMIN ZHANG ET AL: "Effects of urea plus nitrate pretreated rice straw and corn oil supplementation on fiber digestibility, nitrogen balance, rumen fermentation, microbiota and methane emissions in goats",<br/>JOURNAL OF ANIMAL SCIENCE AND BIOTECHNOLOGY, BIOMED CENTRAL LTD, LONDON, UK,<br/>vol. 10, no. 1,<br/>23 January 2019 (2019-01-23), pages 1-10, XP021270875,<br/>DOI: 10.1186/S40104-019-0312-2<br/>the whole document<br/>-----</p> | 1-20                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

**PCT/EP2023/054080**

| Patent document<br>cited in search report |          | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|----------|---------------------|----------------------------|---------------------|
| <b>US 2768895</b>                         | <b>A</b> | <b>30-10-1956</b>   | <b>NONE</b>                |                     |
| -----                                     |          |                     |                            |                     |
| <b>CA 1241663</b>                         | <b>A</b> | <b>06-09-1988</b>   | <b>NONE</b>                |                     |
| -----                                     |          |                     |                            |                     |

# PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

# PCT

To:

Van Ballaer, Evi Maria August  
De Clercq & Partners  
Edgard Gevaertdreef 10a  
9830 Sint-Martens-Latem  
BELGIQUE

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL SEARCH REPORT AND  
THE WRITTEN OPINION OF THE INTERNATIONAL  
SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

|                                                       |  |                                                                                |
|-------------------------------------------------------|--|--------------------------------------------------------------------------------|
| Applicant's or agent's file reference<br>YARA-250-PCT |  | Date of mailing<br>(day/month/year)<br>3 July 2023 (03-07-2023)                |
| International application No.<br>PCT/EP2023/054080    |  | International filing date<br>(day/month/year)<br>17 February 2023 (17-02-2023) |
| Applicant<br>YARA INTERNATIONAL ASA                   |  |                                                                                |

- ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.  
**Filing of amendments and statement under Article 19:**  
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):  
**When?** The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.  
**How?** Directly to the International Bureau preferably through ePCT, or on paper to:  
The International Bureau of WIPO, 34 chemin des Colombettes, 1211 Geneva 20, Switzerland  
**For more detailed instructions**, see the *PCT Applicant's Guide*, International Phase, paragraphs 9.004 - 9.011.
- ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.
- ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
  - ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.
  - ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.
- Reminders**  
The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.  
Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90bis.1 and 90bis.3).  
Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see [www.wipo.int/pct/en/texts/time\\_limits.html](http://www.wipo.int/pct/en/texts/time_limits.html) and the *PCT Applicant's Guide*, National Chapters.  
Within **22 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45bis.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

|                                                                                                                                                                                                                                                                                    |                                                                          |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Name and mailing address of the International Searching Authority<br> European Patent Office, P.B. 5818 Patentlaan 2<br>NL-2280 HV Rijswijk<br>Tel. (+31-70) 340-2040<br>Fax: (+31-70) 340-3016 | Authorized officer<br>RADU, Gabriela-Cristina<br>Tel: +31 (0)70 340-2664 |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|

## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

|                                                       |                                                                                                  |                                                                                     |
|-------------------------------------------------------|--------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| Applicant's or agent's file reference<br>YARA-250-PCT | <b>FOR FURTHER ACTION</b><br>see Form PCT/ISA/220<br>as well as, where applicable, item 5 below. |                                                                                     |
| International application No.<br>PCT/EP2023/054080    | International filing date ( <i>day/month/year</i> )<br>17 February 2023 (17-02-2023)             | (Earliest) Priority Date ( <i>day/month/year</i> )<br>17 February 2022 (17-02-2022) |
| Applicant<br><br>YARA INTERNATIONAL ASA               |                                                                                                  |                                                                                     |

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed  
☐ a translation of the international application into \_\_\_\_\_, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6bis(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (See Box No. II)

3. ☐ **Unity of invention is lacking** (see Box No III)

4. With regard to the **title**,

- ☒ the text is approved as submitted by the applicant  
☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- ☒ the text is approved as submitted by the applicant  
☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings**,

- a. the figure of the **drawings** to be published with the abstract is Figure No. \_\_\_\_\_  
☐ as suggested by the applicant  
☐ as selected by this Authority, because the applicant failed to suggest a figure  
☐ as selected by this Authority, because this figure better characterizes the invention
- b. ☐ none of the figures is to be published with the abstract

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2023/054080

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                       |                                                                             |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| <b>A. CLASSIFICATION OF SUBJECT MATTER</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                                                                       |                                                                             |
| INV.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | A23K20/105                                                                                                            | A23K20/20                                                                   |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | A23K20/137                                                                                                            | A23K20/22                                                                   |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                       | A23K50/10                                                                   |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                       | A23K50/15                                                                   |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                       | A23K20/111                                                                  |
| <b>ADD.</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                       |                                                                             |
| According to International Patent Classification (IPC) or to both national classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                       |                                                                             |
| <b>B. FIELDS SEARCHED</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                                                                       |                                                                             |
| Minimum documentation searched (classification system followed by classification symbols)<br><b>A23K</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                       |                                                                             |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                       |                                                                             |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)<br><b>EPO-Internal, CHEM ABS Data, FSTA, WPI Data</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                       |                                                                             |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                                                                       |                                                                             |
| Category*                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Citation of document, with indication, where appropriate, of the relevant passages                                    | Relevant to claim No.                                                       |
| <b>Y</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | <b>US 2 768 895 A (JONAS KAMLET)</b><br><b>30 October 1956 (1956-10-30)</b><br><b>claims; examples</b><br>-----       | <b>6-11</b>                                                                 |
| <b>Y</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | <b>CA 1 241 663 A (MOORMAN MFG CO)</b><br><b>6 September 1988 (1988-09-06)</b><br><b>examples 7-9</b><br>-----<br>-/- | <b>6-11</b>                                                                 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                       |                                                                             |
| * Special categories of cited documents :<br>"A" document defining the general state of the art which is not considered to be of particular relevance<br>"E" earlier application or patent but published on or after the international filing date<br>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"P" document published prior to the international filing date but later than the priority date claimed<br>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br>"&" document member of the same patent family |                                                                                                                       |                                                                             |
| Date of the actual completion of the international search<br><br><b>22 June 2023</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                       | Date of mailing of the international search report<br><br><b>03/07/2023</b> |
| Name and mailing address of the ISA/<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                       | Authorized officer<br><br><b>Saettel, Damien</b>                            |

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2023/054080

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                       |
|------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                                        | Relevant to claim No. |
| A                                                    | <p>XIUMIN ZHANG ET AL: "Effects of urea plus nitrate pretreated rice straw and corn oil supplementation on fiber digestibility, nitrogen balance, rumen fermentation, microbiota and methane emissions in goats",<br/>JOURNAL OF ANIMAL SCIENCE AND BIOTECHNOLOGY, BIOMED CENTRAL LTD, LONDON, UK,<br/>vol. 10, no. 1,<br/>23 January 2019 (2019-01-23), pages 1-10, XP021270875,<br/>DOI: 10.1186/S40104-019-0312-2<br/>the whole document<br/>-----</p> | 1-20                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

**PCT/EP2023/054080**

| Patent document<br>cited in search report |          | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|----------|---------------------|----------------------------|---------------------|
| <b>US 2768895</b>                         | <b>A</b> | <b>30-10-1956</b>   | <b>NONE</b>                |                     |
| -----                                     |          |                     |                            |                     |
| <b>CA 1241663</b>                         | <b>A</b> | <b>06-09-1988</b>   | <b>NONE</b>                |                     |
| -----                                     |          |                     |                            |                     |

# PATENT COOPERATION TREATY

From the  
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

## PCT

### WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY (PCT Rule 43bis.1)

Date of mailing  
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference  
see form PCT/ISA/220

**FOR FURTHER ACTION**  
See paragraph 2 below

|                                                    |                                                          |                                              |
|----------------------------------------------------|----------------------------------------------------------|----------------------------------------------|
| International application No.<br>PCT/EP2023/054080 | International filing date (day/month/year)<br>17.02.2023 | Priority date (day/month/year)<br>17.02.2022 |
|----------------------------------------------------|----------------------------------------------------------|----------------------------------------------|

International Patent Classification (IPC) or both national classification and IPC  
INV. A23K20/105 A23K20/20 A23K20/24 A23K50/15 A23K20/111 A23K20/137 A23K20/22 A23K50/10

Applicant  
YARA INTERNATIONAL ASA

1. This opinion contains indications relating to the following items:
- ☒ Box No. I Basis of the opinion
  - ☐ Box No. II Priority
  - ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
  - ☐ Box No. IV Lack of unity of invention
  - ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
  - ☐ Box No. VI Certain documents cited
  - ☒ Box No. VII Certain defects in the international application
  - ☒ Box No. VIII Certain observations on the international application

#### 2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

|                                                                                                                                                                                                                                                                       |                                                                      |                                                                   |                                                                                       |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------|-------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| Name and mailing address of the ISA:<br><br>European Patent Office<br>P.B. 5818 Patentlaan 2<br>NL-2280 HV Rijswijk - Pays Bas<br>Tel. +31 70 340 - 2040<br>Fax: +31 70 340 - 3016 | Date of completion of<br>this opinion<br><br>see form<br>PCT/ISA/210 | Authorized Officer<br><br>Saettel, Damien<br><br>Telephone No. -0 |  |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------|-------------------------------------------------------------------|---------------------------------------------------------------------------------------|

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**Box No. I Basis of the opinion**

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1. With regard to the **language**, this opinion has been established on the basis of:
  - ☒ the international application in the language in which it was filed.
  - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).
2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))
3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:
  - a. ☐ forming part of the international application as filed.
  - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
    - ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
4. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this opinion has been established to the extent that a meaningful opinion could be formed without a WIPO Standard ST.26 compliant sequence listing.
5. Additional comments:

---

**Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

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1. Statement

|                               |             |                   |
|-------------------------------|-------------|-------------------|
| Novelty (N)                   | Yes: Claims | <u>1-20</u>       |
|                               | No: Claims  |                   |
| Inventive step (IS)           | Yes: Claims | <u>1-5, 12-20</u> |
|                               | No: Claims  | <u>6-11</u>       |
| Industrial applicability (IA) | Yes: Claims | <u>1-20</u>       |
|                               | No: Claims  |                   |

2. Citations and explanations

**see separate sheet**

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**Box No. VII    Certain defects in the international application**

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The following defects in the form or contents of the international application have been noted:

**see separate sheet**

---

**Box No. VIII    Certain observations on the international application**

---

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see separate sheet**

**Re Item V**

**Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1 Prior art:**

Reference is made to the following documents:

- D1 US 2 768 895 A (JONAS KAMLET) 30 October 1956 (1956-10-30)
- D2 CA 1 241 663 A (MOORMAN MFG CO) 6 September 1988 (1988-09-06)
- D3 XIUMIN ZHANG ET AL: "Effects of urea plus nitrate pretreated rice straw and corn oil supplementation on fiber digestibility, nitrogen balance, rumen fermentation, microbiota and methane emissions in goats", JOURNAL OF ANIMAL SCIENCE AND BIOTECHNOLOGY, BIOMED CENTRAL LTD, LONDON, UK, vol. 10, no. 1, 23 January 2019 (2019-01-23), pages 1-10, XP021270875, DOI: 10.1186/S40104-019-0312-2

**2 Inventive step**

The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 6-11 does not involve an inventive step in the sense of Article 33(3) PCT.

**2.1 Independent claim 6**

The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claim 6 does not involve an inventive step in the sense of Article 33(3) PCT.

The document D1 discloses (see ex. 2, 3) the use of a mixture of biuret, triuret, cyanuric acid, ammelide and urea (see cl. 1; ex. 1) to enhance the average daily gain (see ex. 2, tab. 1, first experiment with the composition) and to enhance daily milk yield (see ex. 3; tab. 2, first two experiments with the composition).

It is known to the skilled person that the increase of dry matter and organic matter digestibility is the underlying mechanism for the two effects shown by D1, such that D1 implicitly discloses the use of the composition for this purpose.

The subject-matter of claim 6 of the present application differs from the subject-matter of D1 by using a composition with 30-60% urea and 30-60% biuret.

No technical effect could be found due to this difference, as there is no comparison with the teaching of D1.

The objective technical problem to be solved can therefore be regarded as the provision of the use of an alternative composition containing biuret and urea as well as other N-containing by-products of the biuret production from urea for increasing the digestibility of organic and dry matter.

There is only one composition disclosed in D1 for the feeding of the ruminants, the one of example 1. However, a skilled person would understand that this composition is not the only one which would have the demonstrated effect and would look at the available prior art to know what other similar compositions could be used. By doing so, the person will find D2, which discloses (see ex. 7-9, tab. 1; p. 1, l. 5-16; p. 7, l. 18 - p. 8, l. 1) a ruminant feed with such an NPN composition, which contains:

|       | urea  | biuret | cyanuric acid | triuret |
|-------|-------|--------|---------------|---------|
| ex. 7 | 36%   | 40.6%  | 15.2%         | 8.2%    |
| ex. 8 | 36.2% | 42.5%  | 13%           | 8.3%    |
| ex. 9 | 37.4% | 41.8%  | 16.6%         | 4.2%    |

This person would then use one of the amounts disclosed in D2 in the composition of D1 in a simple routine work and without any inventive input, as both documents relate to a ruminant feed with the same components.

Based on the teaching of the prior art documents D1 and D2, claim 6 is therefore not considered as being inventive.

## 2.2 Dependent claims 7-11

The features of these claims are known from D2, see above. Claims 7-11 are therefore not considered as being inventive.

3 Independent claim 1

D3 is regarded as being the prior art closest to the subject-matter of claim 1, and discloses (see the abstract; tab. 3, 4) the use of a composition containing urea (containing traces of biuret, like for all commercial urea products) and nitrates for decreasing enteric methane emissions of a ruminant.

The subject-matter of claim 1 therefore differs from this known use in that the composition contains 30-60% urea, 30-60% biuret and N-containing by-products of the biuret production out of urea and is therefore new (Article 33(2) PCT).

In the application, there is a comparison in the example 3 between the use of commercial urea alone and a composition falling under the scope of claim 1. However, there is no comparison between the composition according to claim 1 and the one of D3 containing nitrates, such that no effect could be proven due to the differences.

The problem to be solved by the present invention may be regarded as the provision of the use of an alternative composition containing urea for decreasing enteric methane emissions of a ruminant.

The solution to this problem proposed in claim 1 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

There is no hint in D3 towards the use of biuret nor N-containing by-products of the biuret production out of urea. D3 is dealing with the use of urea+nitrates alone or with corn oil, such that the skilled person would not be directed to the solution provided in claim 1.

This person would know that a composition containing all the ingredients of claim 1 is used in ruminant feed (see D1 or D2), but the use of such a composition for reducing the enteric methane emission is not disclosed in these documents: D2 is silent on the effects while D1 uses the composition to improve daily weight gain or milk yield. The skilled person would therefore not combine the teachings of D1 or D2 with the one of D3, the documents dealing with different problems.

Inventivity can therefore be acknowledged.

Claims 2-5 are dependent on claim 1 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

4 Independent claim 12

D3 is regarded as being the prior art closest to the subject-matter of claim 12, and discloses (see the abstract; tab. 3, 4) a method for decreasing enteric methane emissions of a ruminant, comprising administering a composition containing urea (containing traces of biuret, like for all commercial urea) and nitrates.

The subject-matter of claim 12 therefore differs from this known use in that the composition contains 30-60% urea, 30-60% biuret and N-containing by-products of the biuret production out of urea and is therefore new (Article 33(2) PCT).

In the application, there is a comparison in the example 3 between the use of commercial urea alone or a composition falling under the scope of claim 12. However, there is no comparison between the composition according to claim 12 and the one of D3 containing nitrates, such that no effect could be proven due to the differences.

The problem to be solved by the present invention may be regarded as the provision of the use of an alternative composition containing urea for decreasing enteric methane emissions of a ruminant.

The solution to this problem proposed in claim 12 of the present application is considered as involving an inventive step (Article 33(3) PCT) with the same reasons as the one written for claim 1.

Claims 13-20 are dependent on claim 12 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

## **Re Item VII**

### **Certain defects in the international application**

To meet the requirements of Rule 5.1(a)(ii) PCT, D1-D3 should be identified in the description and the relevant background art disclosed therein should be briefly discussed.

**Re Item VIII**

**Certain observations on the international application**

The claims are not supported by the description as required by Article 6 PCT, as their scope is broader than justified by the description and drawings. There is indeed no limit to the amount of the composition containing urea and biuret introduced in the ruminant feed. It is however not believed that any amounts, and especially high amounts of urea, will have the claimed effects. In the description, only limited amounts of the composition are introduced (see the examples and p. 16, l. 31 - p. 17, l. 2).

**Information on Search Strategy - Pilot phase (see OJ 2015, A86)**

The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number

**PCT/EP2023/054080**

**TITLE: A MIXTURE OF UREA, BIURET AND N-CONTAINING BY-PRODUCTS CREATED DURING BIURET PRODUCTION AS A DIETARY NON-PROTEIN NITROGEN-SOURCE FOR RUMINANTS AND USES THEREOF**

**APPLICANT: YARA INTERNATIONAL ASA**

**IPC CLASSIFICATION: A23K20/105, A23K20/20, A23K20/24, A23K50/15, A23K20/111, A23K20/137, A23K20/22, A23K50/10**

**EXAMINER: Saettel, Damien**

**CONSULTED DATABASES: NPL, CAPLUS, DOSYS, EPODOC, FSTA, WPI**

**CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:**

**IPC:**

**CPC: A23K20/105, A23K20/20, A23K20/24, A23K50/15, A23V2200/32**

**FI/F-TERMS:**

**KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:**

**use of a mixture of urea, biuret and N-containing by-products of urea  
auto-condensation to reduce methane production in ruminants.**

Bitte beachten Sie, dass angeführte Nichtpatentliteratur (wie z. B. wissenschaftliche oder technische Dokumente) je nach geltendem Recht dem Urheberrechtsschutz und/oder anderen Schutzarten für schriftliche Werke unterliegen könnte. Die Vervielfältigung urheberrechtlich geschützter Texte, ihre Verwendung in anderen elektronischen oder gedruckten Publikationen und ihre Weitergabe an Dritte ist ohne ausdrückliche Zustimmung des Rechtsinhabers nicht gestattet.

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Please be aware that cited works of non-patent literature such as scientific or technical documents or the like may be subject to copyright protection and/or any other protection of written works as appropriate based on applicable laws. Copyrighted texts may not be copied or used in other electronic or printed publications or re-distributed without the express permission of the copyright holder.

## Possible steps after receipt of the international search report (ISR) and written opinion of the International Searching Authority (WO/ISA)

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### General information

For all international applications, the competent International Searching Authority (**ISA**) will establish an international search report (**ISR**) accompanied by a written opinion of the International Searching Authority (**WO/ISA**). The **WO/ISA** may be responded to by

- filing informal comments with the **International Bureau of WIPO (IB)** (where no demand for international preliminary examination (**demand**) is filed)
- filing amendments under Art. 19 PCT (this can be done whether or not a **demand** is filed)
- filing amendments under Art. 34 PCT and/or formal observations in response to objections raised in the **WO/ISA** (where a **demand** is actually filed)

This document explains these possibilities.

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### Filing informal comments

After receipt of the **ISR** and **WO/ISA**, the applicant may file informal comments on the **WO/ISA**, **directly with the IB** (see International Search and Preliminary Examination Guidelines 2.15). These will be communicated to the designated/elected Offices, together with the International Preliminary Report on Patentability (**IPRP**) at 30 months from the priority date.

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### Amending claims under Art. 19 PCT

The applicant may file **amended claims** under Art. 19 PCT, **directly with the IB** by the later of the following dates:

- 2 months from the date of mailing of the **ISR** and the **WO/ISA**
- 16 months from the priority date

**However**, any such amendment received by the **IB** after the expiration of the applicable time limit shall be **considered to have been received on time** by the **IB**, if it reaches it **before** the technical preparations for international publication have been completed (the 15th day prior to the date of publication, see PCT Applicant's Guide, International Phase, 9.013).

For further information, please see Rule 46 PCT as well as form PCT/ISA/220.

Please also note that, when filing amended claims under Art. 19 PCT, such amendments shall be **accompanied by a letter** identifying the amendments made and also the basis for the amendments in the application as originally filed (Rule 46.5(b) PCT). Where a **demand** is filed, failure to comply with this requirement may result in the amendments being ignored in the International Preliminary Examination Report (**IPER**), see Rule 70.2(c-bis) PCT.

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**Filing a demand  
for international  
preliminary  
examination**

In principle, the **WO/ISA** will be considered to be the written opinion of the International Preliminary Examining Authority (**IPEA**). Where the **WO/ISA** issued by the **EPO** as **ISA** gives a positive opinion on the international application and the invention to which it relates, filing a **demand** with the **EPO** as **IPEA** would normally be unnecessary, since a positive IPRP would anyway be established by the **IB** based on the **WO/ISA** (see also further below).

If the applicant wishes to file a **demand** (for example, to allow him to argue his case in international preliminary examination with regard to objections raised in a negative **WO/ISA** before the **IPEA** issues an **IPEP**), this must be done before expiration of **3 months after the date of mailing of the ISR and WO/ISA** or **22 months after priority date**, whichever expires later (Rule 54*bis* PCT). Amendments under Art. 34 PCT can be filed with the **IPEA**, normally at the same time as filing the demand (Rule 66.1(b) PCT) or within the time limit set for reply to any written opinion issued during international preliminary examination by the **IPEA**.

If a **demand** is filed at the **EPO** as **IPEA** and no comments/amendments have been received by the time the **EPO** starts drawing up the **IPEP** (Rule 66.4*bis* PCT), the **WO/ISA** will be transformed by the **IPEA** into an **IPEP** (also called the **IPRP (Chapter II)** which would merely reflect the content of the **WO/ISA** (OJ 10/2011, 532). The **demand** can still be withdrawn (Art. 37 PCT).

Please also note that, when filing amendments under Art. 34 PCT, such amendments shall be accompanied by a letter which identifies the amendments made and also the basis for the amendments in the application as originally filed (Rule 66.8(a) PCT). Failure to comply with this requirement may result in the amendments being ignored in the **IPEP (IPRP (Chapter II))**, see Rule 70.2(c-*bis*) PCT.

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**Filing a request  
for supplementary  
international  
search**

The applicant may, with the **IB**, file a request for **supplementary international search** under Rule 45*bis*.1 PCT. The present **ISR** and **WO/ISA** may also be taken into account in the execution of that supplementary international search, provided that these are available to the Authority charged with this task before it starts the supplementary search (Rule 45*bis*.5 PCT).

This kind of request **cannot be filed specifying the ISA** who did the **international search**.

More information on this topic can be found in the **PCT Applicant's Guide**, Chapter 8 (<http://www.wipo.int/pct/en/guide/ip08.html>).

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**End of the  
international  
phase**

Where no **demand** is filed, at the end of the international phase, the **IB** will transform the **WO/ISA** into the **IPRP (PCT Chapter I)** (Rule 44*bis* PCT), which will then be transmitted together with possible informal comments to the designated Offices. Where a demand is filed, the **WO/ISA** is not transformed into an **IPRP (Chapter I)** by the **IB**, but rather the **IPEA** will establish an **IPEP**, (the **IPEP** is the same as the **IPRP (PCT Chapter II)**, see Rule 70.15 PCT).

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# PATENT COOPERATION TREATY

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

|                                                                                        |
|----------------------------------------------------------------------------------------|
| To:                                                                                    |
| De Clercq & Partners<br>Edgard Gevaertdreef 10a<br>9830 Sint-Martens-Latem<br>BELGIQUE |

## PCT

### NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY (PCT Rule 71.1)

Date of mailing  
(day/month/year)

24.01.2024

Applicant's or agent's file reference  
YARA-250-PCT

#### IMPORTANT NOTIFICATION

International application No.  
PCT/EP2023/054080

International filing date (day/month/year)  
17.02.2023

Priority date (day/month/year)  
17.02.2022

Applicant  
Yara International ASA

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary report on patentability and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

#### 4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary report on patentability. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

The applicant's attention is drawn to Article 33(5), which provides that the criteria of novelty, inventive step and industrial applicability described in Article 33(2) to (4) merely serve the purposes of international preliminary examination and that "any Contracting State may apply additional or different criteria for the purposes of deciding whether, in that State, the claimed inventions is patentable or not" (see also Article 27(5)). Such additional criteria may relate, for example, to exemptions from patentability, requirements for enabling disclosure, clarity and support for the claims.

Name and mailing address of the international  
preliminary examining authority:



European Patent Office P.B. 5818 Patentlaan 2  
NL-2280 HV Rijswijk - Pays Bas  
Tel. +31 70 340 - 2040  
Fax: +31 70 340 - 3016

Authorized Officer

Muñoz Roda, Toñi

Tel. +31 70 340-2310



# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                   |                                                                                                                                                                         |                       |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Applicant's or agent's file reference<br>YARA-250-PCT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | <b>FOR FURTHER ACTION</b>                                         |                                                                                                                                                                         | See Form PCT/IPEA/416 |
| International application No.<br>PCT/EP2023/054080                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | International filing date ( <i>day/month/year</i> )<br>17.02.2023 | Priority date ( <i>day/month/year</i> )<br>17.02.2022                                                                                                                   |                       |
| International Patent Classification (IPC) or national classification and IPC<br>INV. A23K20/105                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                   |                                                                                                                                                                         |                       |
| Applicant<br>Yara International ASA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                   |                                                                                                                                                                         |                       |
| <p>1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of <u>8</u> sheets, including this cover sheet.</p> <p>3. This report is also accompanied by ANNEXES, comprising:</p> <p>a. <input checked="" type="checkbox"/> a total of <u>9</u> sheets, as follows:</p> <p style="margin-left: 20px;"><input checked="" type="checkbox"/> sheets of the description, claims and/or drawings which have been amended and/or sheets containing rectifications authorized by this Authority, unless those sheets were superseded or cancelled, and any accompanying letters (see Rules 46.5, 66.8, 70.16, 91.2, and Section 607 of the Administrative Instructions).</p> <p style="margin-left: 20px;"><input type="checkbox"/> sheets containing rectifications, where the decision was made by this Authority not to take them into account because they were not authorized by or notified to this Authority at the time when this Authority began to draw up this report, and any accompanying letters (Rules 66.4bis, 70.2(e), 70.16 and 91.2).</p> <p style="margin-left: 20px;"><input type="checkbox"/> superseded sheets and any accompanying letters, where this Authority either considers that the superseding sheets contain an amendment that goes beyond the disclosure in the international application as filed, or the superseding sheets were not accompanied by a letter indicating the basis for the amendments in the application as filed, as indicated in item 4 of Box No. I and the Supplemental Box (see Rule 70.16(b)).</p> <p>b. <input type="checkbox"/> a separate electronic file containing a sequence listing (<i>sent to the International Bureau only</i>)</p> |                                                                   |                                                                                                                                                                         |                       |
| <p>4. This report contains indications relating to the following items:</p> <p><input checked="" type="checkbox"/> Box No. I Basis of the report</p> <p><input type="checkbox"/> Box No. II Priority</p> <p><input type="checkbox"/> Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</p> <p><input type="checkbox"/> Box No. IV Lack of unity of invention</p> <p><input checked="" type="checkbox"/> Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p><input type="checkbox"/> Box No. VI Certain documents cited</p> <p><input checked="" type="checkbox"/> Box No. VII Certain defects in the international application</p> <p><input type="checkbox"/> Box No. VIII Certain observations on the international application</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                   |                                                                                                                                                                         |                       |
| Date of submission of the demand<br><br>15.12.2023                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                   | Date of completion of this report<br><br>24.01.2024                                                                                                                     |                       |
| Name and mailing address of the international preliminary examining authority:<br> European Patent Office P.B. 5818 Patentlaan 2<br>NL-2280 HV Rijswijk - Pays Bas<br>Tel. +31 70 340 - 2040<br>Fax: +31 70 340 - 3016                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                   | Authorized officer<br><br>Saettel, Damien<br><br>Telephone No. +31 70 340-9528<br> |                       |

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**Box No. I Basis of the report**

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1. With regard to the **language**, this report is based on
- ☒ the international application in the language in which it was filed
  - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of:
    - ☐ international search (under Rules 12.3(a) and 23.1(b))
    - ☐ publication of the international application (under Rule 12.4(a))
    - ☐ international preliminary examination (under Rules 55.2(a) and/or 55.3(a) and (b))
2. With regard to the **elements\*** of the international application, this report is based on (*replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report*):

**Description, Pages**

1-45 as originally filed

**Claims, Numbers**

1-22 filed in electronic form on 15-12-2023

**Drawings, Sheets**

1/2, 2/2 as originally filed

- ☐ a sequence listing - see Supplemental Box Relating to Sequence Listing.
3. ☐ The amendments have resulted in the cancellation of:
- ☐ the description, pages
  - ☐ the claims, Nos.
  - ☐ the drawings, sheets/figs
  - ☐ the sequence listing (*specify*):
4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since either they are considered to go beyond the disclosure as filed, or they were not accompanied by a letter indicating the basis for the amendments in the application as filed, as indicated in the Supplemental Box (Rules 70.2(c) and (c-bis)):
- ☐ the description, pages
  - ☐ the claims, Nos.
  - ☐ the drawings, sheets/figs
  - ☐ the sequence listing (*specify*):
5. ☐ This report has been established:
- ☐ taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rules 66.1(d-bis) and 70.2(e)).
  - ☐ without taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91(Rules 66.4bis and 70.2(e)).

**INTERNATIONAL PRELIMINARY REPORT  
ON PATENTABILITY**

International application No.  
PCT/EP2023/054080

6. ☒ With regard to top-up searches (Rules 66.1 *ter* and 70.2(f)):
- ☒ A top-up search was carried out by this Authority on 11.01.2024 (all discovered documents are listed in the Supplemental Box Relating to Top-up Search).
  - ☐ Additional relevant documents have been discovered during the top-up search.
  - ☐ No top-up search was carried out by this Authority because it would serve no useful purpose.
7. ☐ Supplementary international search report(s) from Authority(ies) has/have been received and taken into account in establishing this report (Rule 45bis.8(b) and (c)).

\* If item 4 applies, some or all of those sheets may be marked "superseded".

---

**Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

---

1. Statement

|                               |             |             |
|-------------------------------|-------------|-------------|
| Novelty (N)                   | Yes: Claims | <u>1-22</u> |
|                               | No: Claims  |             |
| Inventive step (IS)           | Yes: Claims | <u>1-22</u> |
|                               | No: Claims  |             |
| Industrial applicability (IA) | Yes: Claims | <u>1-22</u> |
|                               | No: Claims  |             |

2. Citations and explanations (Rule 70.7):

**see separate sheet**

---

**Box No. VII Certain defects in the international application**

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The following defects in the form or contents of the international application have been noted:

**see separate sheet**

**Re Item I**

**Basis of the report**

The amended claims filed with the letter dated 15/12/2023 seem to be allowable according to Article 34(2)(b) PCT. The amendments are the following:

- claims 1-5 are original claims 1-5,
- claim 6 is based on the first 2 options of original claim 6, with added feature "for improving rumen fermentation, wherein.." with a basis on pages 9 and 10 making the link between the acetate:propionate ratio and/or the EMPS and the rumen fermentation,
- claim 7 is based on the 2 other options of original claim 6,
- claims 8-12 are original claims 7-11,
- claim 13 has a basis on original claims 14+19 or on p. 8, l. 18-30,
- claims 14-22 are original claims 12-20.

**Re Item V**

**Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1 Prior art

Reference is made to the following documents:

- |    |                                                                                                                                                                                                                                                                                                                                                                                                   |
|----|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| D1 | US 2 768 895 A (JONAS KAMLET) 30 October 1956 (1956-10-30)                                                                                                                                                                                                                                                                                                                                        |
| D2 | CA 1 241 663 A (MOORMAN MFG CO) 6 September 1988 (1988-09-06)                                                                                                                                                                                                                                                                                                                                     |
| D3 | XIUMIN ZHANG ET AL: "Effects of urea plus nitrate pretreated rice straw and corn oil supplementation on fiber digestibility, nitrogen balance, rumen fermentation, microbiota and methane emissions in goats", JOURNAL OF ANIMAL SCIENCE AND BIOTECHNOLOGY, BIOMED CENTRAL LTD, LONDON, UK, vol. 10, no. 1, 23 January 2019 (2019-01-23), pages 1-10, XP021270875, DOI: 10.1186/S40104-019-0312-2 |

The document D1 discloses (see ex. 2, 3) the use of a mixture of biuret, triuret, cyanuric acid, ammeline and urea (see cl. 1; ex. 1) to enhance the average daily gain (see ex. 2, tab. 1, first experiment with the composition) and to enhance daily milk yield (see ex. 3; tab. 2, first two experiments with the composition).

It is known to the skilled person that the increase of dry matter and organic matter digestibility is the underlying mechanism for the two effects shown by D1, such that D1 implicitly discloses the use of the composition for this purpose.

The document D2 discloses (see ex. 7-9, tab. 1; p. 1, l. 5-16; p. 7, l. 18 - p. 8, l. 1) a ruminant feed with an NPN composition, which contains: 36-37.4% urea, 40-42.5% biuret, 13-16.6% cyanuric acid and 4.2-8.3% triuret.

The document D3 discloses (see the abstract; tab. 3-6; p. 7-8) the use of a composition containing urea (containing traces of biuret, like for all commercial urea products) and nitrates for decreasing enteric methane emissions of a ruminant and for shifting the rumen fermentation by reducing the acetate:propionate ratio.

## 2 Assessment of the independent claims

### 2.1 Independent claim 1

D3 is regarded as being the prior art closest to the subject-matter of claim 1, as it discloses (see the abstract; tab. 3; p. 7-8) the use of a composition containing urea (containing traces of biuret, like for all commercial urea products) and nitrates for decreasing enteric methane emissions of a ruminant.

The subject-matter of claim 1 therefore differs from this known use in that the composition contains 30-60% urea, 30-60% biuret and N-containing by-products of the biuret production out of urea and is therefore new (Article 33(2) PCT).

In the application, there is a comparison in the example 3 between the use of commercial urea alone and a composition falling under the scope of claim 1. However, there is no comparison between the composition according to claim 1 and the one of D3 containing nitrates, such that no effect could be proven due to the differences.

The problem to be solved by the present invention may be regarded as the provision of the use of an alternative composition containing urea for decreasing enteric methane emissions of a ruminant.

The solution to this problem proposed in claim 1 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

There is no hint in D3 towards the use of biuret nor N-containing by-products of the biuret production out of urea. D3 is dealing with the use of urea+nitrates alone or with corn oil, such that the skilled person would not be directed to the solution provided in claim 1.

This person would know that a composition containing all the ingredients of claim 1 is used in ruminant feed (see D1 or D2), but the use of such a composition for reducing the enteric methane emission is not disclosed in these documents: D2 is silent on the effects while D1 uses the composition to improve daily weight gain or milk yield. The skilled person would therefore not combine the teachings of D1 or D2 with the one of D3, the documents dealing with different problems.

Inventivity can therefore be acknowledged.

Claims 2-5 are dependent on claim 1 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

## 2.2 Independent claim 6

D3 is regarded as being the prior art closest to the subject-matter of claim 6, as it discloses (see the abstract; tab. 4-6; p. 7-8) the use of a composition containing urea (containing traces of biuret, like for all commercial urea products) and nitrates for shifting the rumen fermentation by reducing the acetate:propionate ratio.

The subject-matter of claim 6 therefore differs from this known use in that the composition contains 30-60% urea, 30-60% biuret and N-containing by-products of the biuret production out of urea and is therefore new (Article 33(2) PCT).

In the application, there is a comparison in the example 2 between the use of commercial urea alone and a composition falling under the scope of claim 1. However, there is no comparison between the composition according to claim 6 and the one of D3 containing nitrates, such that no effect could be proven due to the differences.

The problem to be solved by the present invention may be regarded as the provision of the use of an alternative composition containing urea for decreasing the acetate:propionate ratio in a rumen.

The solution to this problem proposed in claim 6 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

A reasoning similar to the one written for claim 1 can be applied for claim 6: there is no hint in D3 towards the use of biuret nor N-containing by-products of the biuret production out of urea. D3 is dealing with the use of urea+nitrates alone or with corn oil, such that the skilled person would not be directed to the solution provided in claim 6.

This person would know that a composition containing all the ingredients of claim 1 is used in ruminant feed (see D1 or D2), but the use of such a composition for improving the rumen fermentation by decreasing the acetate:propionate ratio is not disclosed in these documents: D2 is silent on the effects while D1 uses the composition to improve daily weight gain or milk yield. The skilled person would therefore not combine the teachings of D1 or D2 with the one of D3, the documents dealing with different problems.

Inventivity can therefore be acknowledged.

Claims 7-13 are dependent on claim 6 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

### 2.3 Independent claim 14

D3 is regarded as being the prior art closest to the subject-matter of claim 14, and discloses (see the abstract; tab. 3, 4) a method for decreasing enteric methane emissions of a ruminant, comprising administering a composition containing urea (containing traces of biuret, like for all commercial urea) and nitrates.

The subject-matter of claim 14 therefore differs from this known use in that the composition contains 30-60% urea, 30-60% biuret and N-containing by-products of the biuret production out of urea and is therefore new (Article 33(2) PCT).

In the application, there is a comparison in the example 3 between the use of commercial urea alone or a composition falling under the scope of claim 12. However, there is no comparison between the composition according to claim 14 and the one of D3 containing nitrates, such that no effect could be proven due to the differences.

The problem to be solved by the present invention may be regarded as the provision of the use of an alternative composition containing urea for decreasing enteric methane emissions of a ruminant.

The solution to this problem proposed in claim 14 of the present application is considered as involving an inventive step (Article 33(3) PCT) with the same reasons as the one written for claim 1.

Claims 15-22 are dependent on claim 14 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

### **Re Item VII**

#### **Certain defects in the international application**

To meet the requirements of Rule 5.1(a)(ii) PCT, D1-D3 should be identified in the description and the relevant background art disclosed therein should be briefly discussed.

European Patent Office  
P.B. 5818 Patentlaan 2  
2280 HV Rijswijk  
The Netherlands

**Online filing**

Sint-Martens-Latem, December 15, 2023

Our Ref.: YARA-250-PCT

Re.: International Patent Application No. PCT/EP2023/054080 filed on February 17, 2023  
"A MIXTURE OF UREA, BIURET and N-CONTAINING BY-PRODUCTS CREATED DURING  
BIURET PRODUCTION AS A DIETARY NON-PROTEIN NITROGEN SOURCE FOR  
RUMINANTS AND USES THEREOF"  
In name of Yara International ASA

Dear Madam, dear Sir,

In response to the International Search Report and Written Opinion in connection with the above-mentioned international application, issued by the International Searching Authority (ISA) on July 3, 2023, the applicant respectfully submits the following amendments and observations.

A request for Preliminary International Examination under Chapter II PCT is hereby filed.

The Written Opinion particularly relies upon the documents US2768895 (D1), CA1241663 (D2), Xiumin Zhang et al. (D3), and the same numbering is used herein.

**1. Amendments**

An amended set of claims 1-22 is submitted herewith to replace claims 1-20 currently on file.

Currently amended claim 6 is now limited to the use of the composition specified in the claims as a NPN source for improving rumen fermentation wherein the acetate-to-propionate ratio produced by rumen microbes is increased and/or wherein the efficiency of microbial protein synthesis (EMPS) by rumen microbes is improved, as supported by the sections between p.9, line 29 and p.10, line 9, and p.10, lines 16-24, and former claim 6.

Newly introduced claim 7, dependent on claim 6, is limited to the features of increasing the dry matter (DM) and organic matter (OM) digestibility and/or increasing the dry matter intake (DMI) of former claim 6.

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Newly introduced claim 13 specifies the concentration of the NPN composition in a ruminant feed composition, as supported by former claim 19.

Claim numbering and claim dependency have been adjusted accordingly.

It is respectfully submitted that these amendments do not extend beyond the content of the application as filed.

## **2. Observations**

### **2.1 Novelty and inventive step**

It is gratefully acknowledged that the examiner considers the subject-matter of former claims 1-5 and 12-20 novel and inventive over the cited prior art.

However, the examiner finds the subject-matter of former claim 6 not inventive over D1 in combination with D2. According to the examiner, an increase in dry matter and organic matter intake is the underlying mechanism for the effects shown in D1 for a mixture of biuret, urea, triuret, cyanuric acid, and ammelide, and that the skilled person would amend the composition of D1 by the amounts disclosed in D2, for the same purpose.

Without acquiescence to this objection, currently amended claim 6 now specifies the use of a composition comprising between 30 and 60 wt% of urea and between 30 and 60 wt% of biuret for improving rumen fermentation wherein the acetate-to-propionate ratio produced by rumen microbes is increased and/or wherein the efficiency of microbial protein synthesis (EMPS) by rumen microbes is improved.

These effects are demonstrated in the examples of the present application. In particular, Tables 4 and 10 demonstrated that supplementation of the diet with the urea/biuret mixture as specified in the claims as a NPN source resulted in a significant increase in EMPS, particularly compared to a diet comprising urea as NPN source. Table 4 also demonstrates that the acetate-to-propionate ratio is reduced compared to a diet comprising urea as NPN source. As explained on p.10, lines 3-9 of the PCT application as filed/published, the EMPS is a measure for how much microbial protein is produced due to the microbial activity per unit of fermentable substrate. As further explained on p.10, lines 16-24, using a urea/biuret mixture as NPN source results in the production of more propionate and less acetate, through which more energy is provided to the ruminant.

It is respectfully submitted that the use of a NPN composition comprising between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret for increasing the rumen fermentation, particularly for increasing the acetate-to-propionate ratio produced by rumen microbes or by increasing the efficiency of microbial protein synthesis (EMPS) by rumen microbes is not disclosed in D1-D3.

D1 discloses the use of a composition comprising urea and biuret for improving daily weight gain or milk yield. D2 relates to the production of feed-grade biuret, and is silent on the effects thereof. D3 relates to the pretreatment of rice straw with urea to improve fiber digestibility. D3 is completely silent on any effect of a urea/biuret mixture as feed additive.

The skilled person would thus not obtain the subject-matter claimed in currently amended claim 6, either alone or taken in combination, as the cited prior art documents deal with different problems.

Since D1-D3 does not disclose the subject-matter of currently amended claim 6, nor provide any incentive how to obtain the claimed effect, it is respectfully submitted that currently amended claim 6, as well as currently amended claims 7-13 dependent thereon, involve an inventive step in view of the cited prior art.

## 2.2 Clarity and support (Art 6 PCT)

The examiner is of the opinion that the claims are not supported by the description as their scope is broader than justified by the description and drawings. According to the examiner, there is no limit to the amount of the composition containing urea and biuret introduced in the ruminant feed.

The applicant respectfully does not agree with this opinion.

Currently amended claims 1 and 6 explicitly specify the use of a composition “as a NPN source” and currently amended claim 14 specify “administering (...) a NPN composition”.

It is respectfully submitted that the use of NPN sources/compositions, i.e. non-protein nitrogen sources/compositions, in the diet of ruminants is well known to the skilled person. As *inter alia* explained in the background of the present application, non-protein nitrogen is a source of supplemental nitrogen for ruminants. In the literature, it can be found that NPN is a term used in animal nutrition that refers to components that are not (true) proteins but can be converted to proteins by microbes in the ruminant stomach, or in other words such compounds that supply nitrogen other than in the form of protein. NPN sources can be used to supply some of the needed dietary nitrogen for the ruminants. While NPN serves as a cheaper alternate of protein for ruminants, feeding only NPN cannot replace the whole protein requirement. Furthermore, NPN should be administered to the ruminants according to strict feeding guidelines, since some NPN sources such as urea can be toxic for the ruminant animal when administered in too high amounts.

In view of these observations, the skilled person thus understands that the claimed subject-matter, by referring to the term “as a NPN source” implicitly but necessarily imposes a limit on the amount of the NPN composition as specified in the claims that may be introduced in the ruminant diet, particularly wherein the combination of the NPN composition, as such or as

present in a ruminant feed supplement, and the nutritional ingredients of the ruminant feed provide for a balanced diet.

### **3. Request**

In view of the arguments presented herein, it is submitted that the claimed subject matter complies with the requirements of the PCT with respect to clarity, novelty and inventive step, and a positive opinion is therefore requested. Should the examiner nevertheless still be of the opinion that the claims are not meeting the requirements of patentability, the applicant would appreciate a possibility to present further arguments, either orally or in writing.

Respectfully submitted,

A handwritten signature in black ink, appearing to read 'Kris Pappaert', with a stylized flourish above the name.

Mr. PAPPAERT, Kris  
Mr. GOESAERT, Hans  
Professional Representatives before the EPO

Encl: Amended claims (version showing amendments and retyped version)

**CLAIMS (clean copy)**

1. Use of a composition comprising biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed or in a ruminant feed supplement composition for decreasing the enteric methane emissions of a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source, wherein the composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret.
2. Use according to claim 1, for further and simultaneously decreasing the acetate-to-propionate ratio produced by rumen microbes in a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
3. Use according to claim 1 or 2, for further and simultaneously improving the efficiency of microbial protein synthesis (EMPS) by rumen microbes in a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
4. Use according to any one of claims 1 to 3, for further and simultaneously increasing the dry matter (DM) and organic matter (OM) digestibility, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
5. Use according to any one of claims 1 to 4, for further and simultaneously increasing the dry matter intake (DMI) of a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source.
6. Use of a composition comprising biuret, urea and N-containing by-products of the biuret production out of urea as a NPN-source in a ruminant feed or in a ruminant feed supplement composition for improving rumen fermentation, wherein the acetate-to-propionate ratio produced by rumen microbes in a ruminant animal is decreased,

- particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source; and/or wherein the efficiency of microbial protein synthesis (EMPS) by rumen microbes in a ruminant animal is improved, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source;
- 5 wherein the composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret.
7. Use according to claim 6, for further and simultaneously
- 10 - increasing the dry matter (DM) and organic matter (OM) digestibility particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising urea as a NPN-source; and/or
- increasing the dry matter intake (DMI) of a ruminant animal, particularly when compared to a ruminant feed or a ruminant feed supplement composition comprising
- 15 urea as a NPN-source,
8. Use according to any one of claims 1 to 7, wherein the composition comprises between 35 and 55 wt.% of urea and between 35 and 50 wt.% of biuret, based on the total weight of the composition.
- 20
9. Use according to claim 8, wherein the composition comprises between 37 and 52 wt.% of urea and between 39 and 46 wt.% of biuret, based on the total weight of the composition.
- 25 10. Use according to any one of claims 1 to 9, wherein the composition further comprises between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.
11. Use according to claim 10, wherein the composition comprises between 2.5 and 8
- 30 wt.% of triuret, and/or between 0.1 and 5 wt.% of ammeline, and/or between 2.5 and 15 wt.% of cyanuric acid, and between 0.1 and 2.0 wt.% of moisture, based on the total weight of the composition.

12. Use according to claim 11, wherein the composition comprises between 3 and 6 wt.% of triuret, and/or between 0.5 and 3 wt.% of ammelide, and/or between 3 and 10 wt.% of cyanuric acid, and between 0.1 and 1.5 wt.% of moisture, based on the total weight of the composition.

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13. Use according to any one of claims 1 to 12, wherein the NPN composition is present in the ruminant feed composition in a concentration between 0.5 and 4 wt.%, more in particular between 0.5 and 3.5 wt.% or between 0.5 and 3 wt.%, even more in particular between 1 and 2.5 wt.%, with wt% based on the total dry matter basis of the ruminant feed composition.

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14. A method for reducing the enteric methane emissions of a ruminant animal, comprising administering to the ruminant animal a NPN composition comprising biuret, urea and N-containing by-products of the biuret production, wherein the NPN composition comprises between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret, particularly wherein the NPN composition comprises between 35 and 55 wt.% of urea, between 35 and 50 wt.% of biuret, and between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.

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15. The method according to claim 14, wherein the enteric methane emissions of the ruminant animal, when expressed as g methane/kg dry matter intake (DMI) or as g methane/kg organic matter intake, are reduced by at least 5%, particularly by at least 10%, when the NPN composition is administered to a ruminant as NPN source, compared to a diet comprising urea as NPN source.

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16. The method according to claim 14 or 15, wherein said method is further simultaneously a method for:

- decreasing the acetate-to-propionate ratio produced by rumen microbes in a ruminant animal, particularly wherein the acetate-to-propionate ratio produced by rumen microbes in a ruminant, particularly determined via an *in vitro* continuous flow fermentation with rumen fluid to simulate rumen fermentation, is decreased by at least

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5%, in particular by at least 10%, when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source,

- improving the efficiency of microbial protein synthesis (EMPS) by rumen microbes in a ruminant animal, wherein the EMPS, particularly determined via an *in vitro* continuous or batch rumen fluid incubation to simulate rumen fermentation, was increased by at least 5%, in particular by at least 10%, when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source;
- increasing the dry matter (DM) and organic matter (OM) digestibility; and/or
- increasing the dry matter intake (DMI) of a ruminant animal, particularly wherein the DMI, expressed in kg/day is increased by at least 5% when the NPN composition is administered to a ruminant as NPN source compared to a diet comprising urea as NPN source.

17. The method according to any one of claims 14 to 16, wherein the NPN composition comprises between 35 and 55 wt.% of urea and between 35 and 50 wt.% of biuret, based on the total weight of the composition, more in particular comprises between 37 and 52 wt.% of urea and between 39 and 46 wt.% of biuret, based on the total weight of the composition.

18. The method according to any one of claims 14 to 17, wherein the NPN composition further comprises between 5 and 30 wt.% of N-containing by-products of the biuret production out of urea, based on the total weight of the composition.

19. The method according to claim 18, wherein the NPN composition comprises between 2.5 and 8 wt.% of triuret, and/or between 0.1 and 5 wt.% of ammelide, and/or between 2.5 and 15 wt.% of cyanuric acid, and between 0.1 and 2.0 wt.% of moisture, based on the total weight of the composition, more in particular, wherein the composition comprises between 3 and 6 wt.% of triuret, and/or between 0.5 and 3 wt.% of ammelide, and/or between 3 and 10 wt.% of cyanuric acid, and between 0.1 and 1.5 wt.% of moisture, based on the total weight of the composition.

20. The method according to any one of claims 14 to 19, comprising the steps of preparing or providing a ruminant feed supplement composition comprising the NPN

composition, or preparing or providing a ruminant feed composition comprising the NPN composition, and administering the ruminant feed supplement or the ruminant feed composition to the ruminant animal.

- 5      21. The method according to claim 20, wherein the NPN composition is present in the ruminant feed composition in a concentration between 0.5 and 4 wt.%, more in particular between 0.5 and 3.5 wt.% or between 0.5 and 3 wt.%, even more in particular between 1 and 2.5 wt.%, with wt% based on the total dry matter basis of the ruminant feed composition.
- 10      22. The method according to claim 20 or 21, wherein the ruminant feed composition further comprises one or more nutritional ingredients, wherein the one or more nutritional ingredients form a nutritionally balanced ration or a basal ration for a ruminant.

15

# PATENT COOPERATION TREATY

From the RECEIVING OFFICE

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## NOTIFICATION OF THE INTERNATIONAL APPLICATION NUMBER AND OF THE INTERNATIONAL FILING DATE

(PCT Rule 20.2(c))

Date of mailing (day/month/year) 03.03.2023

Applicant's or agent's file reference  
YARA-250-PCT

### IMPORTANT NOTIFICATION

International application No.

PCT/EP2023/054080

International filing date (day/month/year)

17 February 2023 (17-02-2023)

Priority date (day/month/year)

17 February 2022 (17-02-2022)

Applicant

YARA INTERNATIONAL ASA

Title of the invention

1. The applicant is hereby notified that the international application has been accorded the international application number and the international filing date indicated above.

2. The applicant is further notified that the record copy of the international application:



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| Date | 18.11.2022 |
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|                                                |                                                 |
|------------------------------------------------|-------------------------------------------------|
| Reference<br>P22834EP00                        | Application No./Patent No.<br>22382539.9 - 1105 |
| Applicant/Proprietor<br>Yara International ASA |                                                 |

**Communication**

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the European search report (R. 61 EPC) or the partial European search report/ declaration of no search (R. 63 EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☒ 0 additional set(s) of copies of such documents is (are) enclosed as well.

The following have been approved:

☒ Abstract ☒ Title

☐ The Abstract was modified and the definitive text is attached to this communication.

The following figure(s) will be published together with the abstract:

**Refund of search fee**

If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Should you wish to further prosecute this application in the examination phase, your attention is drawn to the provisions of Rule 70a EPC. An invitation to respond to the extended European search report will be issued once the date of publication of the European search report has been mentioned in the European Patent Bulletin (R. 69(1), R. 70(2) EPC).



# EUROPEAN SEARCH REPORT

Application Number  
**EP 22 38 2539**

| DOCUMENTS CONSIDERED TO BE RELEVANT                                                                                                                                                                                                                    |                                                                                                                                                                                     |                                                                                                                                                                                                                                                                                       |                                                                        |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------|
| Category                                                                                                                                                                                                                                               | Citation of document with indication, where appropriate, of relevant passages                                                                                                       | Relevant to claim                                                                                                                                                                                                                                                                     | CLASSIFICATION OF THE APPLICATION (IPC)                                |
| X                                                                                                                                                                                                                                                      | CA 1 241 663 A (MOORMAN MFG CO)<br>6 September 1988 (1988-09-06)<br>* page 7, line 18 - page 8, line 1;<br>claims; examples 7-9; table 1 *<br>* page 1, line 5 - line 16 *<br>----- | 7-11                                                                                                                                                                                                                                                                                  | INV.<br>A23K20/105<br>A23K50/10<br>A23K50/15<br>A23K10/30<br>A23K10/37 |
| X                                                                                                                                                                                                                                                      | US 2 768 895 A (JONAS KAMLET)<br>30 October 1956 (1956-10-30)<br>* claims; examples *<br>-----                                                                                      | 1-11                                                                                                                                                                                                                                                                                  | A23K20/111<br>A23K20/137<br>A23K20/22<br>A23K20/24                     |
| X                                                                                                                                                                                                                                                      | US 3 878 304 A (MOORE WILLIAM PERCY)<br>15 April 1975 (1975-04-15)<br>* example 3 *<br>-----                                                                                        | 7-11                                                                                                                                                                                                                                                                                  |                                                                        |
| X                                                                                                                                                                                                                                                      | CA 976 189 A (AFRICAN EXPLOSIVES & CHEM)<br>14 October 1975 (1975-10-14)<br>* claims; examples *<br>-----                                                                           | 7-11                                                                                                                                                                                                                                                                                  |                                                                        |
|                                                                                                                                                                                                                                                        |                                                                                                                                                                                     |                                                                                                                                                                                                                                                                                       | TECHNICAL FIELDS SEARCHED (IPC)                                        |
|                                                                                                                                                                                                                                                        |                                                                                                                                                                                     |                                                                                                                                                                                                                                                                                       | A23K                                                                   |
| The present search report has been drawn up for all claims                                                                                                                                                                                             |                                                                                                                                                                                     |                                                                                                                                                                                                                                                                                       |                                                                        |
| Place of search<br><b>The Hague</b>                                                                                                                                                                                                                    |                                                                                                                                                                                     | Date of completion of the search<br><b>1 November 2022</b>                                                                                                                                                                                                                            | Examiner<br><b>Saettel, Damien</b>                                     |
| CATEGORY OF CITED DOCUMENTS<br>X : particularly relevant if taken alone<br>Y : particularly relevant if combined with another document of the same category<br>A : technological background<br>O : non-written disclosure<br>P : intermediate document |                                                                                                                                                                                     | T : theory or principle underlying the invention<br>E : earlier patent document, but published on, or after the filing date<br>D : document cited in the application<br>L : document cited for other reasons<br>.....<br>& : member of the same patent family, corresponding document |                                                                        |

# ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO.

EP 22 38 2539

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on  
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01-11-2022

| Patent document<br>cited in search report |   | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---|---------------------|----------------------------|---------------------|
| CA 1241663                                | A | 06-09-1988          | NONE                       |                     |
| US 2768895                                | A | 30-10-1956          | NONE                       |                     |
| US 3878304                                | A | 15-04-1975          | NONE                       |                     |
| CA 976189                                 | A | 14-10-1975          | AU 451816 B2               | 22-08-1974          |
|                                           |   |                     | CA 976189 A                | 14-10-1975          |
|                                           |   |                     | DE 2152550 A1              | 27-04-1972          |
|                                           |   |                     | ES 396245 A1               | 16-04-1974          |
|                                           |   |                     | FR 2113063 A5              | 23-06-1972          |
|                                           |   |                     | GB 1324738 A               | 25-07-1973          |
|                                           |   |                     | JP S516052 B1              | 25-02-1976          |
|                                           |   |                     | NL 7114489 A               | 25-04-1972          |
|                                           |   |                     | ZA 707156 B                | 28-06-1972          |

**Information on Search Strategy - Pilot phase (see OJ 2015, A86)**

The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number

EP 22 38 2539

**TITLE:** USE OF A MIXTURE OF UREA, BIURET AND MINOR N-CONTAINING COMPOUNDS  
CREATED DURING BIURET PRODUCTION AS A DIETARY NPN-SOURCE IN A RUMINANT FEED  
SUPPLEMENT COMPOSITION

**APPLICANT:** Yara International ASA

**IPC CLASSIFICATION:** A23K20/105, A23K50/10, A23K50/15, A23K10/30, A23K10/37,  
A23K20/111, A23K20/137, A23K20/22, A23K20/24

**EXAMINER:** Saettel, Damien

**CONSULTED DATABASES:** FSTA, MEDLINE, NPL, WPI

**CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:**

**IPC:**

**CPC:** A23K20/105, A23K50/10, A23K50/15, A23K10/30, A23K10/37, A23K20/111,  
A23K20/137, A23K20/22, A23K20/24

**FI/F-TERMS:**

**KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:**

Use of biuret, containing urea and other compounds obtained during the  
condensation of urea to biuret, such as triuret, ammelide and cyarunic acid, in  
ruminant feed. Results are: decrease of acetate:propionate ratio, improvement  
of efficiency of microbial protein synthesis, and increase of dry matter and  
organic matter digestibility

The examination is being carried out on the **following application documents**

**Description, Pages**

1-18 as originally filed

**Claims, Numbers**

1-11 as originally filed

**Drawings, Sheets**

1/2, 2/2 as originally filed

1 Prior Art

Reference is made to the following documents; the numbering will be adhered to in the rest of the procedure.

- D1 CA 1 241 663 A (MOORMAN MFG CO) 6 September 1988 (1988-09-06)
- D2 US 2 768 895 A (JONAS KAMLET) 30 October 1956 (1956-10-30)
- D3 US 3 878 304 A (MOORE WILLIAM PERCY) 15 April 1975 (1975-04-15)
- D4 CA 976 189 A (AFRICAN EXPLOSIVES & CHEM) 14 October 1975 (1975-10-14)

2 Clarity

Claims 1-3 are not supported by the description as required by Article 84 EPC, as their scopes are broader than justified by the description and drawings. In these claims, there is no limit to the amount of the composition containing urea and biuret introduced in the ruminant feed. It is however not believed that any amounts, and especially high amounts of urea, will have the claimed effects: in the description, only limited amounts of the composition are indeed introduced.

### 3 Novelty

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 7, 10 and 11 is not new within the meaning of Article 54(1) and (2) EPC.

The document D1 discloses (see ex. 7-9, tab. 1; p. 1, l. 5-16; p. 7, l. 18 - p. 8, l. 1) a ruminant feed with an NPN composition and implicitly other nutritional ingredients for either a nutritionally balanced ration or a basal ration.

The NPN composition contains:

|       | urea  | biuret | cyanuric acid | triuret |
|-------|-------|--------|---------------|---------|
| ex. 7 | 36%   | 40.6%  | 15.2%         | 8.2%    |
| ex. 8 | 36.2% | 42.5%  | 13%           | 8.3%    |
| ex. 9 | 37.4% | 41.8%  | 16.6%         | 4.2%    |

D1 therefore removes novelty from the subject-matter of claims 7, 10 and 11.

### 4 Inventive step

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claims 1-6, 8 and 9 does not involve an inventive step within the meaning of Article 56 EPC.

#### 4.1 Independent claim 1

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claim 1 does not involve an inventive step within the meaning of Article 56 EPC.

The document D2 discloses (see cl. 1) a composition containing urea, biuret as well as the products of the condensation of urea such as triuret, cyanuric acid or ammelide. D2 discloses a ruminant feed containing this composition (see cl. 6-8) and the use of this feed to improve the daily weight gain of this ruminants (see ex. II, tab. I) when used as for example 16% in the feed, or to improve the milk yield (see ex. III, tab. II).

The subject-matter of claim 1 of the present application differs from the subject-matter of D2 by using this feed to decrease the acetate to propionate ratio produced by rumen microbes in a ruminant.

The objective technical problem to be solved can therefore be regarded as the provision of an alternative use of a ruminant feed containing biuret, urea and the by-products of the urea condensation.

In D2, the use of the claimed composition leads to an improvement of milk yield and average or daily weight gain, as shown in the examples, at least with a reasonable amount of the composition.

It is known to the skilled person that the use of claim 1, i.e. the decrease of the acetate : propionate ratio is one of the underlying mechanisms which will lead to the results shown by D2. By using the composition of D2 for the use of D2, the skilled person will automatically fall under the scope of claim 1, as the underlying mechanism will be present in the rumen of the treated ruminant.

Based on the teaching of the prior art document D2, claim 1 is therefore not considered as being inventive.

#### 4.2 Independent claim 2

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claim 2 does not involve an inventive step within the meaning of Article 56 EPC.

The document D2 discloses (see cl. 1) a composition containing urea, biuret as well as the products of the condensation of urea such as triuret, cyanuric acid or ammelide. D2 discloses a ruminant feed containing this composition (see cl. 6-8) and the use of this feed to improve the daily weight gain of this ruminants (see ex. II, tab. I) when used as for example 16% in the feed, or to improve the milk yield (see ex. III, tab. II).

The subject-matter of claim 2 of the present application differs from the subject-matter of D2 by using this feed to improve the efficiency of microbial protein synthesis.

The objective technical problem to be solved can therefore be regarded as the provision of an alternative use of a ruminant feed containing biuret, urea and the by-products of the urea condensation.

The same reasoning as the one written for claim 1 can be applied for claim 2: the improvement of the efficiency of microbial protein synthesis is indeed another one of the mechanism leading to the results shown by D2. Claim 2 is therefore not considered as being inventive.

#### 4.3 Independent claim 3

The present application does not meet the requirements of Article 52(1) EPC because the subject-matter of claim 3 does not involve an inventive step within the meaning of Article 56 EPC.

The document D2 discloses (see cl. 1) a composition containing urea, biuret as well as the products of the condensation of urea such as triuret, cyanuric acid or ammelide. D2 discloses a ruminant feed containing this composition (see cl. 6-8) and the use of this feed to improve the daily weight gain of this ruminants (see ex. II, tab. I) when used as for example 16% in the feed, or to improve the milk yield (see ex. III, tab. II).

The subject-matter of claim 3 of the present application differs from the subject-matter of D2 by using this feed to increase the dry matter and organic matter digestibility.

The objective technical problem to be solved can therefore be regarded as the provision of an alternative use of a ruminant feed containing biuret, urea and the by-products of the urea condensation.

The same reasoning as the one written for claim 1 can be applied for claim 3: the increase of the dry matter and organic matter digestibility is indeed another one of the mechanism leading to the results shown by D2. Claim 3 is therefore not considered as being inventive.

#### 4.4 Dependent claims

##### 4.4.1 Claims 4-6, dependent on independent claim 1-3

In D2, the "autocondensate A" has 30% urea and 58.1% of biuret (see ex. I). The feature of claim 4 is therefore known from D2, such that this claims is not considered as being inventive.

The subject-matter of claims 5 and 6 consists in the selection of a biuret, urea, triuret, ammeline, cyanuric acid and/or moisture content subranges from the ranges of possible values implied by D2.

Such a selection can only be regarded as inventive if the subranges present unexpected effects or properties in relation to the rest of the ranges.

However, no such effects or properties are indicated in the application, as there is only 1 example of the composition according to the claims without comparison with another composition having different amounts of one or more of these compounds. Hence no inventive step is present in the subject-matter of claims 5 and 6 (Articles 52(1) and 56 EPC).

#### 4.4.2 Claims 8 and 9, dependent on independent claim 7

The subject-matter of claim 8 consists in the selection of a a biuret, urea, triuret, ammeline and/or cyanuric acid content subranges from the ranges of possible values implied by D1 or D2. Such a selection can only be regarded as inventive if the subranges present unexpected effects or properties in relation to the rest of the range. However, no such effects or properties are indicated in the application. Hence no inventive step is present in the subject-matter of claim 8 (Articles 52(1) and 56 EPC).

Claim 9 relates to the presence of other compounds. These compounds do not seem to add any unexpected effects and are moreover known to be used in ruminants' feed compositions such that a skilled person would add them in a simple routine work, without any inventive input, with the basic knowledge of the field, depending on the desired properties of the final product. Claim 9 is therefore not considered as being inventive.

#### 4.5 Remark on D3 and D4

D3 and D4 disclose a composition obtained after the condensation of urea and containing urea, biuret and the other by-products (see the relevant passages cited in the search report).

The subject-matter of independent claim 7 consists in the selection of a a biuret, urea, triuret, ammeline and/or cyanuric acid content subranges from the ranges of possible values implied by D3 or D4. Such a selection can only be regarded as inventive if the subranges present unexpected effects or properties in relation to the rest of the range.

However, no such effects or properties are indicated in the application. D3 and D4 are therefore considered as being highly relevant for the assessment of the inventive step of independent claim 7 at least.

## 5 Amendments

The applicant is invited to file new claims which takes account of the above comments.

In order to comply with the requirements of Rule 137(4) EPC, the applicant should clearly identify the amendments made, irrespective of whether they concern amendments by addition, replacement or deletion, and indicate the passages of the application as filed on which these amendments are based (see Guidelines H III, 2.1).

When filing amended claims the applicant should at the same time bring the description into conformity with the amended claims. Care should be taken during revision, especially of the introductory portion and of any statements of problem or advantage, not to add subject-matter which extends beyond the content of the application as originally filed (Article 123(2) EPC).

The description should be amended where appropriate to fulfil the following requirements:

- The technical field of the invention (Rule 42(1)(a) EPC; Guidelines F-II.4.2) must correspond to the amended set of claims.
- The summary of the invention (Rule 42(1)(c) EPC; Guidelines F-II.4.5) must correspond to the amended set of claims. Where appropriate, statements such as “The invention is set out in the appended set of claims” or “The invention is as defined in claim X” may be used instead of repeating the claims verbatim.
- Embodiments which are no longer covered by the claims must be deleted, unless they can reasonably be considered to be useful for highlighting specific aspects of the amended subject-matter. If this is the case, they must be clearly marked as not being part of the present invention (T 1808/06 and Guidelines F-IV, 4.3):
  - Any combinations of features which do not fall under the scope of the amended independent claims must be clearly marked as not being part of the present invention.
  - Statements such as “The following examples/aspects/embodiments X, Y, Z are not according to the invention and are present for illustration purposes only” may be used at the start of the description or in the list of figures.

- The technical features of the independent claims may not be presented as optional in the description. Where they precede a feature of an independent claim, terms such as “for example”, “may”, “can”, “exemplary”, “optionally”, “preferably” and the like must be removed.
- To meet the requirements of Rule 42(1)(b) EPC, D1-D4 should be identified in the description and the relevant background art disclosed therein should be briefly discussed.

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17/02/2023

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☒ This declaration is continued on the following sheet, "Continuation of Box No. VIII (iv)".

**Continuation of Box No. VIII (i) to (v) DECLARATION**

*If the space is insufficient in any of Boxes Nos. VIII (i) to (v) to furnish all the information, including in the case where **more than three inventors are to be named** in Box No. VIII (iv), in such case, write "Continuation of Box No. VIII ..." (indicate the item number of the Box) and furnish the information in the same manner as required for the purposes of the Box in which the space was insufficient. If additional space is needed in respect of two or more declarations, a separate continuation box must be used for each such declaration. If this Box is not used, this sheet should not be included in the request.*

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**PCT DEMAND FOR INTERNATIONAL  
PRELIMINARY EXAMINATION (CHAPTER II)**

|              |                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                        |
|--------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>0</b>     | <b>For International Preliminary Examining Authority use only</b>                                                                                                                                                                                                                                                            |                                                                                                                                                                        |
| <b>0-1</b>   | Identification of IPEA                                                                                                                                                                                                                                                                                                       |                                                                                                                                                                        |
| <b>0-2</b>   | Date of receipt of DEMAND                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                        |
| <b>0-3</b>   | <b>Form - PCT/IPEA/401 PCT Demand</b>                                                                                                                                                                                                                                                                                        |                                                                                                                                                                        |
| <b>0-3-1</b> | Prepared Using                                                                                                                                                                                                                                                                                                               | <b>eOLF</b><br><b>Version: FMMNGR5183, IBR4015182, FM_FOP0205</b>                                                                                                      |
| <b>0-4</b>   | <b>Demand under Article 31 of the Patent Cooperation Treaty:</b><br>The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated). |                                                                                                                                                                        |
| <b>0-5</b>   | <b>International Preliminary Examining Authority (specified by the applicant)</b>                                                                                                                                                                                                                                            | <b>IPEA/EP</b>                                                                                                                                                         |
| <b>I</b>     | <b>Identification of the International application</b>                                                                                                                                                                                                                                                                       |                                                                                                                                                                        |
| <b>I-1</b>   | International Application No.                                                                                                                                                                                                                                                                                                | <b>PCT/EP2023/054080</b>                                                                                                                                               |
| <b>I-2</b>   | International Filing Date                                                                                                                                                                                                                                                                                                    | <b>17 February 2023 (17.02.2023)</b>                                                                                                                                   |
| <b>I-3</b>   | (Earliest) Priority Date                                                                                                                                                                                                                                                                                                     | <b>17 February 2022 (17.02.2022)</b>                                                                                                                                   |
| <b>I-4</b>   | Applicant's or agent's file reference                                                                                                                                                                                                                                                                                        | <b>YARA-250-PCT</b>                                                                                                                                                    |
| <b>I-5</b>   | Title of Invention                                                                                                                                                                                                                                                                                                           | <b>A MIXTURE OF UREA, BIURET AND N-CONTAINING BY-PRODUCTS CREATED DURING BIURET PRODUCTION AS A DIETARY NON-PROTEIN NITROGEN-SOURCE FOR RUMINANTS AND USES THEREOF</b> |

**PCT DEMAND FOR INTERNATIONAL  
PRELIMINARY EXAMINATION (CHAPTER II)**

|              |                                                                                                                                                                                                                |                                                                             |
|--------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| <b>II-1</b>  | <b>Applicant</b>                                                                                                                                                                                               |                                                                             |
| II-1-1       | Name                                                                                                                                                                                                           | <b>YARA INTERNATIONAL ASA</b>                                               |
| II-1-2       | Address                                                                                                                                                                                                        | <b>Drammensveien 131<br/>0277 Oslo<br/>Norway</b>                           |
| II-1-3       | State of nationality                                                                                                                                                                                           | <b>NO</b>                                                                   |
| II-1-4       | State of residence                                                                                                                                                                                             | <b>NO</b>                                                                   |
| II-1-5       | Telephone No.                                                                                                                                                                                                  |                                                                             |
| II-1-6       | e-mail                                                                                                                                                                                                         |                                                                             |
| II-1-7       | Applicant's registration No. with the Office                                                                                                                                                                   |                                                                             |
| <b>III-1</b> | <b>Agent or common representative; or address for correspondence</b>                                                                                                                                           |                                                                             |
|              | The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:                                                              | <b>Attorney<br/>(appointed earlier)</b>                                     |
| III-1-1      | Name                                                                                                                                                                                                           | <b>De Clercq &amp; Partners</b>                                             |
| III-1-2      | Address                                                                                                                                                                                                        | <b>Edgard Gevaertdreef 10a<br/>9830 Sint-Martens-Latem<br/>Belgium</b>      |
| III-1-3      | Telephone No.                                                                                                                                                                                                  | <b>+3292802340</b>                                                          |
| III-1-4      | e-mail                                                                                                                                                                                                         | <b>info@dcp-ip.com</b>                                                      |
| III-1-4a     | The International Preliminary Examining Authority is authorised to use this e-mail address, if the Authority so wishes, to send notifications issued in respect of this international preliminary examination: | <b>exclusively in electronic form (no paper notifications will be sent)</b> |
| III-1-5      | Agent's registration No.                                                                                                                                                                                       | <b>458</b>                                                                  |

**PCT DEMAND FOR INTERNATIONAL  
PRELIMINARY EXAMINATION (CHAPTER II)**

|                |                                                                                                                                                                    |                                                                                                                                           |                             |                                         |
|----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|-----------------------------------------|
| <b>IV</b>      | <b>Basis for International Preliminary Examination</b>                                                                                                             |                                                                                                                                           |                             |                                         |
| IV-1-1         | Statement concerning amendments:<br>The applicant wishes the International Preliminary Examination to start on the basis of:                                       | <b>-the description as originally filed</b><br><b>-the claims as amended under Article 34</b><br><b>-the drawings as originally filed</b> |                             |                                         |
| IV-2           | Language for the purposes of international preliminary examination:                                                                                                | <b>English</b><br><b>-(which is the language in which the international application was filed )</b>                                       |                             |                                         |
| <b>V</b>       | <b>Election of States</b>                                                                                                                                          | The filing of this demand constitutes the election of all Contracting States which are designated and are bound by Chapter II of the PCT. |                             |                                         |
| <b>VI</b>      | <b>Check list</b>                                                                                                                                                  | Number of sheets                                                                                                                          | Electronic file(s) attached | <b>Received Y/N (For IPEA use only)</b> |
|                | <b>The demand is accompanied by the following elements, in the language referred to under item IV-2 for the purposes of international preliminary examination:</b> |                                                                                                                                           |                             |                                         |
| VI-2 (1)       | Amendments under Art. 34                                                                                                                                           | <b>5</b>                                                                                                                                  | <b>AMDA34-1.pdf</b>         |                                         |
| VI-6           | Letter accompanying the amendments under Article 34 (Rule 66.8)                                                                                                    | <b>4</b>                                                                                                                                  | <b>LETT.pdf</b>             |                                         |
| VI-7           | Other: Other document                                                                                                                                              | <b>5</b>                                                                                                                                  | <b>OTHERDOC-1</b>           |                                         |
|                | <b>Accompanying Items</b>                                                                                                                                          | Details                                                                                                                                   | Electronic file(s) attached | <b>Received Y/N (For IPEA use only)</b> |
|                | <b>The demand is also accompanied by the following item(s):</b>                                                                                                    |                                                                                                                                           |                             |                                         |
| VI-8           | Fee calculation sheet                                                                                                                                              | <b>1</b>                                                                                                                                  | <b>fees.pdf</b>             |                                         |
| <b>VII-1</b>   | <b>Signature of applicant, agent or common representative</b>                                                                                                      | <b>/Hans GOESAERT/</b>                                                                                                                    |                             |                                         |
| <b>VII-1-1</b> | <b>Name</b>                                                                                                                                                        | <b>De Clercq &amp; Partners</b>                                                                                                           |                             |                                         |
| <b>VII-1-2</b> | <b>Capacity</b>                                                                                                                                                    | <b>Representative</b>                                                                                                                     |                             |                                         |
| <b>VIII</b>    | <b>Note(s) to IPEA/EP</b>                                                                                                                                          |                                                                                                                                           |                             |                                         |

**PCT DEMAND FOR INTERNATIONAL  
PRELIMINARY EXAMINATION (CHAPTER II)**

**FOR INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY USE ONLY**

|       |                                                                                                                                                                  |  |
|-------|------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|
| 8-1   | Date of actual receipt of DEMAND                                                                                                                                 |  |
| 8-2   | Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b)                                                                                         |  |
| 8-3   | The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and items 8-4 and 8-5 below do not apply.                          |  |
| 8-3-1 | The applicant has been informed accordingly                                                                                                                      |  |
| 8-4   | The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.                                   |  |
| 8-5   | Although the date of receipt of the demand is after the expiration of the 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82. |  |
| 8-6   | The date of receipt of the demand is AFTER the expiration of the time limit under Rule 54bis, 1(a) and item 7 or 8, below, does not apply.                       |  |
| 8-7   | The date of receipt of the demand is WITHIN the time limit under Rule 54bis.1(a) as extended by virtue of Rule 80.5.                                             |  |
| 8-8   | Although the date of receipt of the demand is after the expiration of the time limit under Rule 54bis.1(a), the delay in arrival is EXCUSED pursuant to Rule 82. |  |

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|-----|------------------------------|--|
| 9-1 | Demand received from IPEA on |  |
|-----|------------------------------|--|

RESEARCH

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# Effects of urea plus nitrate pretreated rice straw and corn oil supplementation on fiber digestibility, nitrogen balance, rumen fermentation, microbiota and methane emissions in goats

Xiumin Zhang<sup>1,2</sup>, Rodolfo F. Medrano<sup>3,4</sup>, Min Wang<sup>1\*</sup>, Karen A. Beauchemin<sup>5</sup>, Zhiyuan Ma<sup>1,2</sup>, Rong Wang<sup>1,4</sup>, Jiangnan Wen<sup>1,4</sup>, Lukuyu A. Bernard<sup>6</sup> and Zhiliang Tan<sup>1</sup>

## Abstract

**Background:** Urea pretreatment is an efficient strategy to improve fiber digestibility of low quality roughages for ruminants. Nitrate and oil are usually used to inhibit enteric methane (CH<sub>4</sub>) emissions from ruminants. The objective of this study was to examine the combined effects of urea plus nitrate pretreated rice straw and corn oil supplementation to the diet on nutrient digestibility, nitrogen (N) balance, CH<sub>4</sub> emissions, ruminal fermentation characteristics and microbiota in goats. Nine female goats were used in a triple 3 × 3 Latin Square design (27 d periods). The treatments were: control (untreated rice straw, no added corn oil), rice straw pretreated with urea and nitrate (34 and 4.7 g/kg of rice straw on a dry matter [DM] basis, respectively, UN), and UN diet supplemented with corn oil (15 g/kg soybean and 15 g/kg corn were replaced by 30 g/kg corn oil, DM basis, UNCO).

**Results:** Compared with control, UN increased neutral detergent fiber (NDF) digestibility ( $P < 0.001$ ) and copies of protozoa ( $P < 0.001$ ) and *R. albus* ( $P < 0.05$ ) in the rumen, but decreased N retention ( $-21.2\%$ ,  $P < 0.001$ ), dissolved hydrogen concentration ( $-22.8\%$ ,  $P < 0.001$ ), molar proportion of butyrate ( $-18.2\%$ ,  $P < 0.05$ ), (acetate + butyrate) to propionate ratio ( $P < 0.05$ ) and enteric CH<sub>4</sub> emissions ( $-10.2\%$ ,  $P < 0.05$ ). In comparison with UN, UNCO increased N retention ( $+34.9\%$ ,  $P < 0.001$ ) and decreased copies of protozoa ( $P < 0.001$ ) and methanogens ( $P < 0.001$ ). Compared with control, UNCO increased NDF digestibility ( $+8.3\%$ ,  $P < 0.001$ ), reduced ruminal dissolved CH<sub>4</sub> concentration ( $-24.4\%$ ,  $P < 0.001$ ) and enteric CH<sub>4</sub> emissions ( $-12.6\%$ ,  $P < 0.05$ ).

**Conclusions:** A combination of rice straw pretreated with urea plus nitrate and corn oil supplementation of the diet improved fiber digestibility and lowered enteric CH<sub>4</sub> emissions without negative effects on N retention. These strategies improved the utilization of rice straw by goats.

**Keywords:** Dissolved hydrogen, Methane, Nitrate, Oil, Rumen fermentation, Urea

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## Background

Rice straw is an abundant crop residue in rice producing areas, such as China and Southeast Asia. However, rice straw has low nutritive value, which limits its wide and efficient use in ruminant diets, because of the high content of indigestible structural polysaccharides and low crude protein (CP) content [1]. Enteric methane ( $\text{CH}_4$ ) resulting from ruminants is an important source of greenhouse gas and represents a loss of 2–14% of dietary energy [2]. Feeding straw results in greater  $\text{CH}_4$  emissions per unit of feed digested than high quality forage, because it has a slow passage rate and prolonged residency time in the rumen [3]. Before promoting the use of rice straw in ruminant diets, strategies are needed to improve its digestibility and decrease its  $\text{CH}_4$  emissions.

Urea can be used to pretreat rice straw to enhance its nutritional quality by destroying fiber structure and increasing non-protein nitrogen (N) content [1], leading to increased ruminal fiber digestibility [4, 5]. Nitrate supplementation has been identified as a possible strategy to reduce  $\text{CH}_4$  emissions [6, 7], because nitrate reduction to ammonia incorporates metabolic hydrogen ( $[\text{H}]$ ) away from methanogenesis [8]. Urea plus nitrate pretreatment has been reported to increase degradation and reduce  $\text{CH}_4$  production of rice straw *in vitro* [9], and further verification of these combined effects *in vivo* is needed.

Oil supplementation is another option to decrease enteric  $\text{CH}_4$  emissions from ruminants and improve final quality of product (i.e., milk and meat). Oil supplementation increases energy density of the diet, provides additional unsaturated fatty acids and decreases numbers of protozoa and associated methanogens, but may also decrease ruminal organic matter (OM) fermentation [3]. Additionally, inhibition of ruminal protozoa can decrease microbial N cycling in the rumen [10] and increase the flow of microbial protein from the rumen to the small intestine [11], leading to an improvement of N utilization. Thus, oil supplementation of diets containing urea plus nitrate pretreated rice straw may further help to improve efficiency of dietary N use in ruminants.

In the present study, we hypothesized that pretreatment of rice straw with urea plus nitrate would enhance fiber digestibility and inhibit  $\text{CH}_4$  production, and supplementation of corn oil to a diet containing the pretreated straw would further exert additional beneficial effects on dietary N use efficiency and  $\text{CH}_4$  mitigation. To test these hypotheses, we used goats as the experimental animal, and measured diet digestibility, N balance,  $\text{CH}_4$  emissions, rumen fermentation, and selected microorganisms.

## Methods

### Urea plus nitrate pretreated rice straw

The rice straw originated from Jiangxi province of China. Whole plant material was obtained and thrashed to remove

the grain and the straw was sun-cured and chopped into pieces of approximately 3 cm in length. Straw was pre-treated with urea (34 g/kg straw dry matter [DM]) and 6 g/kg DM of ammonium nitrate (to supply 4.7 nitrate g/kg straw DM) for the urea plus nitrate treatment (UN). The pretreatment procedure was fully described by Zhang et al. [9]. Briefly, urea and ammonium nitrate were dissolved in water (40%, w/v) and sprayed onto the straw to achieve the appropriate concentrations. The pretreated straw was then placed into sealed 200 L plastic vessels and incubated at  $15 \pm 3.0^\circ\text{C}$  (means  $\pm$  SD) for 4 weeks. After incubation, the straw was sun-cured for feeding.

### Experimental design, goats, and diets

Nine female Liuyang Black goats (a local breed in southern China, 1 year of age) with body weight of  $19.0 \pm 1.22$  kg (mean  $\pm$  SD) at the start of the experiment were used. The experiment was a triple  $3 \times 3$  Latin Square design with 3 dietary treatments (Table 1). The control diet was formulated to meet appropriately 1.2 to 1.3 times the digestible energy (DE) and CP requirements of non-pregnant female goats according to Zhang and Zhang [12], and consisted of 50% rice straw and 50% concentrate (Table 1). The UN diet was formulated by replacing untreated rice straw with UN pretreated rice straw. The combination of UN and corn oil supplementation (UNCO) diet was formulated by replacing soybean meal (15 g/kg of dietary DM) and corn grain (15 g/kg of dietary DM) with corn oil (30 g/kg of dietary DM). Goats were fed individually at 07:30 and 17:30 h with equal portions of feed offered at each meal.

The experiment consisted of 3 periods, each with 27 d including 14 d for diet adaptation, 5 d for collecting feces and urine, 6 d for measuring  $\text{CH}_4$  emissions and 2 d for rumen sampling. All goats were housed in individual metabolism cages and had free access to drinking water.

### Apparent total-tract digestibility and N balance

Total feces and urine were collected and weighed twice daily from d 15 to 19. A subsample (~1%) of feces and urine from each animal was obtained at each collection time and frozen immediately at  $-20^\circ\text{C}$ , and another subsample (~1%) was acidified using 10% (w/w)  $\text{H}_2\text{SO}_4$  to prevent N loss and then frozen immediately at  $-20^\circ\text{C}$ . The subsamples were then individually combined by day and goat within period. The acidified samples were used for total N analysis, whereas non-acidified samples were used for other chemical analysis.

The N balance, including N excretion and retention, was calculated using the following equations:

$$\text{N excretion (g/d)} = \text{fecal N (g/d)} + \text{urinary N (g/d)}.$$

$$\text{N excretion (\%)} = [\text{N excretion (g/d)} / \text{N intake (g/d)}] \times 100\%.$$

$$\text{N retention (g/d)} = \text{N intake (g/d)} - \text{N excretion (g/d)}.$$

**Table 1** Ingredients and chemical composition of the diets (DM basis)

| Items                                                | Treatment <sup>1</sup> |      |      |
|------------------------------------------------------|------------------------|------|------|
|                                                      | Control                | UN   | UNCO |
| Ingredients, g/kg                                    |                        |      |      |
| Untreated rice straw <sup>2</sup>                    | 500                    | –    | –    |
| Urea plus nitrate pretreated rice straw <sup>3</sup> | –                      | 500  | 500  |
| Soybean meal <sup>4</sup>                            | 80.0                   | 80.0 | 65.0 |
| Corn grain                                           | 292                    | 292  | 277  |
| Wheat bran                                           | 98.0                   | 98.0 | 98.0 |
| CaCO <sub>3</sub>                                    | 1.00                   | 1.00 | 1.00 |
| CaH <sub>2</sub> PO <sub>4</sub>                     | 4.00                   | 4.00 | 4.00 |
| NaCl                                                 | 5.00                   | 5.00 | 5.00 |
| Premix                                               | 20.0                   | 20.0 | 20.0 |
| Corn oil <sup>5</sup>                                | –                      | –    | 30.0 |
| Chemical composition, g/kg                           |                        |      |      |
| OM                                                   | 910                    | 910  | 921  |
| CP                                                   | 105                    | 121  | 119  |
| NDF                                                  | 468                    | 458  | 440  |
| ADF                                                  | 271                    | 266  | 257  |
| GE, MJ/kg                                            | 16.2                   | 16.3 | 16.3 |
| EE                                                   | 10.2                   | 10.2 | 40.2 |
| Nitrate                                              | –                      | 2.33 | 2.33 |

<sup>1</sup>UN urea plus nitrate pretreated rice straw, UNCO combination of UN and corn oil

<sup>2</sup>Rice straw contained 898 g OM, 34.4 g CP, 766 g NDF and 470 g ADF per kg DM

<sup>3</sup>Urea plus nitrate pretreated rice straw contained 898 g OM, 68.1 g CP, 744 g NDF and 458 g ADF per kg DM

<sup>4</sup>Contained 442 g CP per kg DM

<sup>5</sup>The corn oil was supplied by Wilmar International Ltd., Wuhan, China.

Composed of 15% saturated fatty acids, 32% monounsaturated fatty acids and 53% polyunsaturated fatty acids

N retention (%) = [N retention (g/d) / N intake (g/d)] × 100%.

### Enteric methane emission

After total collection, CH<sub>4</sub> emissions were measured for 2 d per goat by moving each goat into a respiration chamber as described by Wang et al. [13]. As only three chambers were available, it took 6 d (from d 20 to 25) to complete the measurements for all goats. Nine goats were assigned to 3 blocks, with each block containing 3 goats (1 goat from each treatment). The goats in each block were assigned to the same chamber and 1 goat from each block was used for each of the 2-day sequential measurements. The goats were restrained but had free access to a feed bin and drinking water within the chamber. On average, the flow rate of air was maintained at 40 m<sup>3</sup>/h. The CH<sub>4</sub> concentration in outlet gas from each chamber and ambient gas were measured using an Ultraportable Greenhouse Gas Analyzer (MIU-374-8, Los Gatos Research, San Jose, CA

95134, USA). The cycling time to separately measure CH<sub>4</sub> concentration from the 3 chambers was 30 min, with 8 min for each chamber gas and 6 min for ambient gas. Calibrations were done by discharging purified CH<sub>4</sub> into each chamber at 25 cm<sup>3</sup>/min controlled by a gas flow meter (C100L-CRWE-DD, SIYA Detection Instrument Co., Ltd., Shanghai, China). The gas flow data from both the gas flow meter and chamber were calculated and compared, and then the data for the chamber were adjusted to 100% recovery according to the data of the gas flow meter. The chambers were opened twice a day at 07:30 and 17:30 h to deliver the diets, and the chambers were cleaned before the morning feeding.

### Rumen sampling

Rumen contents were collected at 0, 2.5 and 6 h after morning feeding on 2 consecutive days (d 26 and d 27) using an oral stomach tube according to Wang et al. [13]. About 150 mL of rumen contents were rapidly collected after discarding the initial 100 mL of rumen contents. Two subsamples (15 mL each) were immediately frozen at –80 °C in liquid nitrogen for DNA extraction and subsequent microbial analysis. Two other subsamples (35 mL each) were immediately transferred into 50-mL plastic syringes for measuring dissolved hydrogen (dH<sub>2</sub>) and dissolved CH<sub>4</sub> (dCH<sub>4</sub>) concentrations according to the procedures outlined by Wang et al. [14]. About 10 mL of rumen contents were used for the measurement of ruminal pH using a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd., Shanghai, China). An aliquot of 2.5 mL of rumen contents was centrifuged at 15,000×g for 10 min at 4 °C, and supernatant (1.5 mL) was transferred into tubes containing 0.15 mL of 25% (w/v) metaphosphoric acid, vigorously hand-shaken and stored at –20 °C for subsequent determination of volatile fatty acid (VFA) and ammonia concentration.

### Sample analyses

The samples of feed, orts and feces were dried in a forced-air oven at 65 °C for 48 h, ground through a 0.25-mm screen (WJX-100, Shanghai, Yuanwo Company) and analyzed in triplicate for DM, OM, ether extract (EE), neutral detergent fiber (NDF), acid detergent fiber (ADF), N content and gross energy (GE). The DM (method 930.15), OM (method 942.05), EE (method 963.15) and N (method 970.22) were analyzed according to published methodologies [15]. Neutral detergent fiber and ADF were assayed according to the method of Van Soest et al. [16], and expressed inclusive of residual ash. Heat stable α-amylase was added during the NDF analysis. Gross energy was determined using an isothermal automatic calorimeter (5E-AC8018, Changsha Kaiyuan Instruments Co., Ltd., China). Individual VFA concentrations were measured using a gas chromatograph (Agilent 7890, Palo Alto, CA,

USA) according to the procedure described by Wang et al. [14]. Ammonia-N concentration was measured by UV-Vis spectrophotometer (Shimadzu UV-2450, Kyoto, Japan) according to the protocol described by Chaney and Marbach [17].

### Microbial analyses

Rumen samples taken 2.5 h after the morning feeding were freeze-dried (CHRIST RVC2–25 CDPIUS, Marin Christ CO., Ltd., Osterode, Germany) for microbial analysis. The DNA was extracted with repeated bead beating plus column purification as described by Yu and Morrison [18], and eluted by 300 µL TE buffer (Tris 10 mmol/L, EDTA 1 mmol/L, pH = 8.0). The quality and quantity of DNA were measured based on absorbance at 260 and 280 nm using a NanoDrop ND-2000 (NanoDrop Technologies Inc., Wilmington, USA).

The 16S rRNA gene V3-V4 hypervariable regions of bacteria genomic DNA were used for PCR amplification with the primers 5'-ACTCCTACGGGAGGCAGCAG-3' (338F) and 5'-GGACTACHVGGGTWTCTAAT-3' (806R). The PCR reactions were performed in triplicate using a 20-µL mixture containing 0.8 µL of each primer, 10 ng of template DNA, 2 µL 2.5 mmol/L dNTPs, 0.4 µL of FastPfu polymerase (Transgen, Beijing, China) and 4 µL 5 × FastPfu Buffer (Transgen, Beijing, China). The thermal cycling programming was performed as follows: initial denaturation step, 95 °C, 3 min; denaturation, 27 cycles, 95 °C, 30 s; annealing, 55 °C, 30 s; elongation, 72 °C, 45 s; and final extension, 72 °C, 10 min. The PCR products were excised from 2% agarose gels and purified using a QIAquick Gel extraction kit (Qiagen, Hilden, Germany). Amplicons from each reaction mixture were quantified fluorometrically, normalized and pooled at equimolar

ratios based on the concentration of each amplicon. Amplicons were sequenced with the Illumina MiSeq platform (PE300, Majorbio Bio-Pharm Technology, Shanghai, China). Quality control of the sequence reads was performed using MOTHR v.1.39.5 [19] following the protocol described by Kozich et al. [20]. The high-quality reads were clustered into operational taxonomic units (OTU) at 97% similarity using Usearch v.7.0 [21]. Representative sequences defined by abundance from each OTU were identified using PyNAST [22] against the SILVA database for bacteria [23]. Taxonomy analysis was performed using the Ribosomal Database Project classifier v.11.1 [24] with a minimum support threshold of 80%. Alpha diversity analyses were generated, including observed species (Sobs), Chao, Ace, Shannon-Weiner, and Simpson's indices. All 16S rRNA gene sequences were deposited into the NCBI Sequence Read Archive under accession number SRP155601.

Real-time quantitative PCR (qPCR) was performed according to the procedures described by Jiao et al. [25]. Briefly, the plasmid DNA containing exact 16S or 18S rRNA gene inserts were used to make a standard curve for selected microbial groups. All standard curves met the following requirements ( $R^2 > 0.99$ ,  $90\% < E < 120\%$ ). The protozoa, fungi, total bacteria, total methanogens, and four selected bacteria were studied. *Fibrobacter succinogenes* (*F. succinogenes*), *Ruminococcus albus* (*R. albus*) and *Ruminococcus flavefaciens* (*R. flavefaciens*) were selected because of their predominant role in fiber digestion [26]; and *Selenomonas ruminantium* (*S. ruminantium*) was selected because it plays a role in nitrate and nitrite reduction [27]. Forward and reverse primers of the selected microbial groups are shown in Table 2.

**Table 2** Primers for qPCR assay

| Microbial Species                | Primer Sets (5'→3')                                                | Product size, bp | References |
|----------------------------------|--------------------------------------------------------------------|------------------|------------|
| Protozoa                         | F: GCTTTCGWTGGTAGTGTATT;<br>R: CTGGCCCTCYAATCGTWCT                 | 223              | [47]       |
| Fungi                            | F: GAGGAAGTAAAAGTCGTAACAAGGTTTC;<br>R: CAAATTCACAAAGGGTAGGATGATT   | 121              | [48]       |
| Bacteria                         | F: CGGCAACGAGCGCAACCC;<br>R: CCATTGTAGCACGTGTGTAGCC                | 146              | [48]       |
| Methanogens                      | F: GGATTAGATACCCSGGTAGT;<br>R: GTTGARTCCAATTAACCGCA                | 192              | [49]       |
| Selected groups of bacteria      |                                                                    |                  |            |
| <i>Fibrobacter succinogenes</i>  | F: GTTCGGAATTACTGGGCGTAAA;<br>R: CGCCTGCCCTGAACCTATC               | 121              | [48]       |
| <i>Ruminococcus albus</i>        | F: CCCTAAAAGCAGTCTTAGTTCG;<br>R: CCTCCTTGC GGTTAGAACA              | 176              | [50]       |
| <i>Ruminococcus flavefaciens</i> | F: GAACGGAGATAATTTGAGTTTACTAGG;<br>R: CGGTCTCTGTATGTTATGAGGTATTACC | 132              | [48]       |
| <i>Selenomonas ruminantium</i>   | F: CAATAAGCATTCCGCCTGGG;<br>R: TTCACCTCAATGTCAAGCCCTGG             | 138              | [51]       |

**Table 3** Effect of urea plus nitrate pretreated rice straw (UN) and combination of UN and corn oil supplementation (UNCO) on apparent total-tract digestibility, N balance and methane emissions in goats

| Items                                    | Treatment         |                    |                   | SEM   | P - value |
|------------------------------------------|-------------------|--------------------|-------------------|-------|-----------|
|                                          | Control           | UN                 | UNCO              |       |           |
| DMI, g/d                                 | 484 <sup>b</sup>  | 501 <sup>a</sup>   | 496 <sup>ab</sup> | 4.2   | 0.03      |
| Apparent total-tract digestibility, g/kg |                   |                    |                   |       |           |
| DM                                       | 656 <sup>b</sup>  | 666 <sup>ab</sup>  | 674 <sup>a</sup>  | 7.0   | 0.05      |
| OM                                       | 688 <sup>b</sup>  | 711 <sup>a</sup>   | 720 <sup>a</sup>  | 10.5  | < 0.001   |
| NDF                                      | 542 <sup>b</sup>  | 577 <sup>a</sup>   | 587 <sup>a</sup>  | 11.1  | < 0.001   |
| ADF                                      | 530               | 539                | 538               | 10.9  | 0.62      |
| CP                                       | 631               | 634                | 638               | 14.0  | 0.76      |
| GE                                       | 629 <sup>b</sup>  | 641 <sup>b</sup>   | 668 <sup>a</sup>  | 12.1  | < 0.001   |
| Nitrogen balance                         |                   |                    |                   |       |           |
| N intake, g/d                            | 8.41 <sup>c</sup> | 9.81 <sup>a</sup>  | 9.13 <sup>b</sup> | 0.051 | < 0.001   |
| Urinary N, g/d                           | 2.94 <sup>b</sup> | 4.35 <sup>a</sup>  | 3.32 <sup>b</sup> | 0.198 | < 0.001   |
| Urinary N to N intake ratio, %           | 35.0 <sup>b</sup> | 44.3 <sup>a</sup>  | 34.4 <sup>b</sup> | 2.19  | < 0.001   |
| Fecal N, g/d                             | 3.10 <sup>b</sup> | 3.60 <sup>a</sup>  | 3.30 <sup>b</sup> | 0.141 | < 0.001   |
| Fecal N to N intake ratio, %             | 36.9              | 36.6               | 36.2              | 1.39  | 0.77      |
| N excretion, g/d                         | 6.05 <sup>b</sup> | 7.95 <sup>a</sup>  | 6.62 <sup>b</sup> | 0.238 | < 0.001   |
| N excretion to N intake ratio, %         | 71.9 <sup>b</sup> | 81.0 <sup>a</sup>  | 72.5 <sup>b</sup> | 2.19  | < 0.001   |
| N retention, g/d                         | 2.36 <sup>a</sup> | 1.86 <sup>b</sup>  | 2.51 <sup>a</sup> | 0.208 | < 0.001   |
| N retention to N intake ratio, %         | 28.1 <sup>a</sup> | 19.1 <sup>b</sup>  | 27.5 <sup>a</sup> | 2.40  | < 0.001   |
| Methane emissions                        |                   |                    |                   |       |           |
| g/d                                      | 9.91 <sup>a</sup> | 9.24 <sup>ab</sup> | 8.92 <sup>b</sup> | 0.779 | 0.04      |
| g/kg DMI                                 | 20.6 <sup>a</sup> | 18.5 <sup>b</sup>  | 18.0 <sup>b</sup> | 1.59  | 0.03      |
| g/kg OM digested                         | 32.8 <sup>a</sup> | 28.5 <sup>b</sup>  | 27.3 <sup>b</sup> | 2.32  | < 0.001   |
| g/kg NDF digested                        | 76.3 <sup>a</sup> | 61.2 <sup>b</sup>  | 63.7 <sup>b</sup> | 4.44  | < 0.001   |
| % of GE intake                           | 6.41 <sup>a</sup> | 5.69 <sup>b</sup>  | 5.32 <sup>b</sup> | 0.481 | 0.004     |

<sup>a-c</sup>Means with different superscripts within a row are significantly different ( $P \leq 0.05$ )

### Statistical analyses

The statistical analysis was performed using the mixed linear model procedure of SPSS 19.0 (Chicago, IL, USA). Fermentation and CH<sub>4</sub> emissions data were averaged to obtain a mean value for the 2 consecutive days before further analysis. For the data of CH<sub>4</sub> emissions, digestibility, qPCR, relative abundance and alpha diversity indexes estimated from 16S rRNA gene library sequences, the model used included treatment ( $n = 3$ ) as a fixed effect, and period ( $n = 3$ ) and animal ( $n = 9$ ) as random effects. When sampling time was included, the model included dietary treatment ( $n = 3$ ) and the interaction of treatment and sampling time as a fixed effect, sampling time ( $n = 3$ ) as a repeated measurement, and animal ( $n = 9$ ) and period ( $n = 3$ ) as random effects. When significant differences were found, a multiple comparison was conducted to elucidate differences between two particular treatments, and  $P$ -values were adjusted using the Bonferroni method. Statistical significance was declared at  $P \leq 0.05$ , with a tendency towards significance declared at  $0.05 < P \leq 0.10$ .

### Results

Goats fed UN diet had a greater dry matter intake (DMI) than control goats ( $P = 0.03$ , Table 3), but DMI of goats fed UNCO was similar to those fed UN and control diets. Total-tract digestibility of OM ( $P < 0.001$ ) and NDF ( $P < 0.001$ ) was greater for goats fed UN or UNCO compared with those fed control diet. However, goats fed UNCO had greater total-tract digestibility of GE than those fed UN ( $P < 0.001$ ) or control diets ( $P < 0.001$ ).

Goats fed UN had greater N intake (+16.6%,  $P < 0.001$ ) and total N excretion (+31.4%,  $P < 0.001$ ), and less N retention (−21.2%,  $P < 0.001$ ) than those fed control (Table 3). In comparison with goats fed UN, those fed UNCO consumed less N (−6.9%,  $P < 0.001$ ) and excreted less N (−16.7%,  $P < 0.001$ ), and retained a greater amount of N (+34.9%,  $P < 0.001$ ). Compared with control, goats fed UNCO had greater N intake (+8.5%,  $P < 0.001$ ), but did not differ in N excretion and retention.

Compared with control, goats fed UN diet had less ( $P < 0.05$ ) daily CH<sub>4</sub> emission expressed as g/kg DMI, g/kg

OM digested, g/kg NDF digested and % of GE intake (Table 3). Goats fed UNCO also had less ( $P < 0.05$ )  $\text{CH}_4$  emissions expressed as g/d than those fed the control diet.

Goats fed UN had greater mean ruminal pH ( $P < 0.001$ ) and ammonia-N concentration (+30.8%,  $P < 0.05$ ), lower  $\text{dH}_2$  concentration (−26.8%,  $P < 0.001$ ), molar proportions of butyrate (−18.2%,  $P < 0.001$ ), isobutyrate ( $P < 0.001$ ) and valerate ( $P < 0.05$ ), and (acetate + butyrate) to propionate ratio ( $P < 0.05$ ) in comparison with those fed control (Table 4). Corn oil supplementation to the UN diet decreased mean pH ( $P < 0.001$ ), and increased molar proportions of butyrate (+31.7%,  $P < 0.001$ ) and valerate ( $P < 0.05$ ). Goats fed UNCO had lower ( $P < 0.001$ )  $\text{dCH}_4$  concentrations than those fed control or UN. No significant interactions between treatment and sampling time were observed for any of the fermentation variables reported.

Goats fed UN had greater copies of protozoa ( $P < 0.001$ ), methanogens ( $P < 0.001$ ), *R. albus* ( $P < 0.05$ ) and *S. ruminantium* ( $P < 0.001$ ) in comparison with those fed control (Table 5). Corn oil supplementation to the UN diet decreased copies of protozoa ( $P < 0.001$ ), methanogens ( $P < 0.001$ ) and *S. ruminantium* ( $P < 0.001$ ). Goats on all treatments had similar bacterial richness when expressed as Chao and Ace values and as Shannon-Weiner and Simpson indexes (Table 6). However, goats fed UN had greater abundance of Ruminococcaceae ( $P < 0.001$ ) than those fed control, but supplementation of corn oil to the UN diet decreased the abundance of Ruminococcaceae ( $P < 0.001$ ).

## Discussion

### Feed digestibility

It has been reported that UN pretreatment improved ruminal degradation of rice straw measured in in vitro batch incubation [9]. The present in vivo study also indicates that goats fed UN had greater total-tract digestibility of NDF than those fed control. Urea in the UN pretreatment can be converted to ammonia during incubation with rice straw, which removes the polymerized silica-waxy compounds from the leaf fractions and destroys the covalent association between lignocellulose and exposes the inner tissues to bacterial colonization [28]. Increased in vivo fiber digestibility indicates that UN pretreatment allows greater access of ruminal fibrolytic bacteria to the fiber substrate. Griffith et al. [29] reported that ammoniation of fiber facilitates its degradation by rumen microbes. The rumen has a wide-range of fibrolytic microbial groups that utilize the fibrous substrates in feed. Protozoa and fungi are active fiber degraders, while other predominant fibrolytic bacteria include *R. albus*, *R. flavefaciens* and *F. succinogenes* [26]. In our study, UN pretreatment increased protozoa and *R. albus* populations, which may have contributed to increased total-tract digestibility of NDF in goats.

Although supplementation of corn oil to the UN diet did not further improve nutrient digestibility, fiber digestibility remained greater than that of the control treatment. It has been reported that high concentrations (> 40 g fat/kg DM) of fat supplementation exert negative effects on feed digestibility [30]. Thus,

**Table 4** Effect of urea plus nitrate pretreated rice straw (UN) and combination of UN and corn oil supplementation (UNCO) on dissolved gases and fermentation end products in the rumen of goats

| Items                                           | Treatment         |                   |                    | Time after morning feeding |                   |                    | SEM   | P - value |         |                  |
|-------------------------------------------------|-------------------|-------------------|--------------------|----------------------------|-------------------|--------------------|-------|-----------|---------|------------------|
|                                                 | Control           | UN                | UNCO               | 0 h                        | 2.5 h             | 6 h                |       | Treatment | Time    | Treatment × Time |
| pH                                              | 6.34 <sup>b</sup> | 6.47 <sup>a</sup> | 6.33 <sup>b</sup>  | 6.51 <sup>a</sup>          | 6.30 <sup>b</sup> | 6.34 <sup>b</sup>  | 0.072 | < 0.001   | < 0.001 | 0.42             |
| $\text{NH}_4^+$ , mmol/L                        | 8.26 <sup>b</sup> | 10.8 <sup>a</sup> | 11.2 <sup>a</sup>  | 10.9                       | 10.8              | 8.6                | 1.31  | 0.02      | 0.06    | 0.58             |
| Dissolved gases                                 |                   |                   |                    |                            |                   |                    |       |           |         |                  |
| Dissolved hydrogen, $\mu\text{mol/L}$           | 0.57 <sup>a</sup> | 0.44 <sup>b</sup> | 0.46 <sup>b</sup>  | 0.30 <sup>c</sup>          | 0.71 <sup>a</sup> | 0.46 <sup>b</sup>  | 0.038 | < 0.001   | < 0.001 | 0.61             |
| Dissolved methane, mmol/L                       | 1.31 <sup>a</sup> | 1.23 <sup>a</sup> | 0.99 <sup>b</sup>  | 1.55 <sup>a</sup>          | 1.01 <sup>b</sup> | 0.96 <sup>b</sup>  | 0.067 | < 0.001   | < 0.001 | 0.18             |
| Total VFA, mmol/L                               | 82.3              | 82.1              | 77.5               | 77.4 <sup>b</sup>          | 85.6 <sup>a</sup> | 79.0 <sup>ab</sup> | 3.73  | 0.15      | < 0.001 | 0.99             |
| Molar proportion of individual VFA, mol/100 mol |                   |                   |                    |                            |                   |                    |       |           |         |                  |
| Acetate                                         | 70.1              | 70.5              | 69.2               | 69.7                       | 70.4              | 69.7               | 0.89  | 0.19      | 0.57    | 0.97             |
| Propionate                                      | 19.3              | 20.6              | 19.9               | 19.4                       | 20.1              | 20.4               | 0.92  | 0.12      | 0.21    | 0.99             |
| Butyrate                                        | 7.52 <sup>a</sup> | 6.15 <sup>b</sup> | 8.10 <sup>a</sup>  | 7.50                       | 6.91              | 7.36               | 0.472 | < 0.001   | 0.28    | 0.72             |
| Iso-butyrate                                    | 1.05 <sup>a</sup> | 0.90 <sup>b</sup> | 0.91 <sup>b</sup>  | 1.16 <sup>a</sup>          | 0.85 <sup>b</sup> | 0.84 <sup>b</sup>  | 0.058 | < 0.001   | < 0.001 | 0.77             |
| Valerate                                        | 0.65 <sup>a</sup> | 0.57 <sup>b</sup> | 0.65 <sup>a</sup>  | 0.62 <sup>ab</sup>         | 0.67 <sup>a</sup> | 0.59 <sup>b</sup>  | 0.029 | 0.02      | 0.03    | 0.85             |
| Iso-valerate                                    | 1.40              | 1.21              | 1.31               | 1.66 <sup>a</sup>          | 1.13 <sup>b</sup> | 1.14 <sup>b</sup>  | 0.102 | 0.09      | < 0.001 | 0.96             |
| Acetate/Propionate ratio                        | 3.83              | 3.50              | 3.66               | 3.73                       | 3.67              | 3.59               | 0.20  | 0.06      | 0.58    | 0.99             |
| (Acetate + butyrate)/Propionate ratio           | 4.23 <sup>a</sup> | 3.82 <sup>b</sup> | 4.09 <sup>ab</sup> | 4.13                       | 4.03              | 3.97               | 0.141 | 0.01      | 0.52    | 0.99             |

<sup>a-c</sup>Means with different superscripts within a row and heading are significantly different ( $P \leq 0.05$ )

**Table 5** Effect of urea plus nitrate pretreated rice straw (UN) and combination of UN and corn oil supplementation (UNCO) on selected groups of microorganisms ( $\log_{10}$  copies/g DM rumen contents) in the rumen of goats

| Items                            | Treatment         |                   |                    | SEM  | P - value |
|----------------------------------|-------------------|-------------------|--------------------|------|-----------|
|                                  | Control           | UN                | UNCO               |      |           |
| Protozoa                         | 10.6 <sup>b</sup> | 11.0 <sup>a</sup> | 10.4 <sup>b</sup>  | 0.42 | < 0.001   |
| Fungi                            | 10.4              | 10.5              | 10.7               | 0.14 | 0.13      |
| Bacteria                         | 13.7              | 13.8              | 13.6               | 0.10 | 0.36      |
| Methanogens                      | 12.0 <sup>b</sup> | 12.4 <sup>a</sup> | 12.1 <sup>b</sup>  | 0.08 | < 0.001   |
| Selected groups of bacteria      |                   |                   |                    |      |           |
| <i>Fibrobacter succinogenes</i>  | 10.9              | 11.1              | 10.9               | 0.13 | 0.13      |
| <i>Ruminococcus albus</i>        | 9.98 <sup>b</sup> | 10.3 <sup>a</sup> | 10.1 <sup>ab</sup> | 0.13 | 0.047     |
| <i>Ruminococcus flavefaciens</i> | 10.2              | 10.1              | 10.1               | 0.10 | 0.34      |
| <i>Selenomonas ruminantium</i>   | 12.1 <sup>b</sup> | 12.5 <sup>a</sup> | 12.1 <sup>b</sup>  | 0.09 | < 0.001   |

<sup>a,b</sup>Means with different superscripts within a row are significantly different ( $P \leq 0.05$ )

the relatively low dose of corn oil (30 g/kg DM) supplementation was not expected to decrease fiber digestibility. Similarly, Machmüller and Kreuzer [31] reported that 35 g oil /kg DM supplementation did not alter fiber digestibility in sheep. Although protozoa are major fiber degraders in the rumen [26], the decreased protozoa population observed for UNCO did not negatively affect fiber digestibility.

**Table 6** Effect of urea plus nitrate pretreated rice straw (UN) and combination of UN and corn oil supplementation (UNCO) on alpha diversity of bacterial community, and abundance of major phyla (relative abundance > 1%) and select families in the rumen of goats

| Items                  | Treatment         |                   |                   | SEM   | P - value |
|------------------------|-------------------|-------------------|-------------------|-------|-----------|
|                        | Control           | UN                | UNCO              |       |           |
| Alpha diversity index  |                   |                   |                   |       |           |
| Number of OTU          | 36,693            | 33,587            | 35,882            | 4527  | 0.88      |
| Shannon-Weiner index   | 4.02              | 4.04              | 3.59              | 0.339 | 0.38      |
| Simpson index          | 0.07              | 0.07              | 0.12              | 0.018 | 0.08      |
| Chao                   | 786               | 716               | 734               | 79.2  | 0.74      |
| Ace                    | 777               | 703               | 729               | 77.4  | 0.71      |
| Sobs                   | 642               | 581               | 567               | 74.9  | 0.66      |
| Relative abundances, % |                   |                   |                   |       |           |
| Bacteroidetes          | 64.8              | 54.2              | 61.2              | 3.52  | 0.12      |
| Firmicutes             | 27.5              | 34.8              | 27.4              | 3.65  | 0.27      |
| Ruminococcaceae        | 10.2 <sup>b</sup> | 14.7 <sup>a</sup> | 10.9 <sup>b</sup> | 1.28  | < 0.001   |
| Spirochaetae           | 1.25              | 2.47              | 1.40              | 0.357 | 0.06      |
| Unclassified           | 2.14              | 1.72              | 1.24              | 0.711 | 0.69      |
| Others                 | 4.31              | 6.81              | 8.76              | 0.618 | 0.39      |

<sup>a,b</sup>Means with different superscripts within a row are significantly different ( $P \leq 0.05$ )

### Nitrogen balance

The greater N intake of goats fed UN did not alter total-tract digestibility of CP, but it increased rumen ammonia-N concentration and urinary N excretion, leading to a decrease in N retention. Djajanegara and Doyle [32] reported that urea pretreatment of rice straw increased fecal N excretion in sheep. Gunun et al. [5] reported that urea pretreated rice straw increased fecal and urinary N excretion in dairy cows. The urea and nitrate in the UN pretreatment provided additional non-protein N for rumen microbes. The lack of treatment effects on 16S rRNA gene copies of bacteria would suggest that the additional N supplied did not promote bacterial growth. It is likely that the soluble protein in the control diet was adequate for microbial growth, and additional N supplementation did not exert further benefits to the host animals. Furthermore, the greater observed protozoa population for the goats fed UN may have contributed to the decreased capture of ruminal N, resulting in increased ruminal ammonia concentration and urinary N excretion, because protozoa can prey on bacteria and reduce the amount of N used for bacterial protein synthesis [10].

Corn oil supplementation to the UN diet decreased N excretion and increased N retention. Benchaar et al. [33] reported that 40 g oil/kg DM reduced N excretion in dairy cows. Doranalli and Mutsavangwa [34] found that 60 g oil/kg DM decreased total N excretion and increased N retention in growing lambs. Improved N retention might be due to decreased intra-ruminal N recycling and increased microbial N flow to the duodenum [10]. In our study, corn oil supplementation to the UN diet decreased ruminal protozoa numbers, which has been reported to decrease predation of bacteria, and thus facilitate N capture by bacteria [10]. Therefore, supplementing corn oil to the UN diet offset the negative impact of UN pretreatment on N utilization, leading to an improvement of N retention in goats.

### Methane emission and rumen environment

Enteric CH<sub>4</sub> emission was on average  $9.9 \pm 1.35$  g/d, which is less than in other studies using goats or sheep. This discrepancy is likely due to the lower body weight ( $19.0 \pm 1.22$  kg) of goats used in the present study. Machmüller and Kreuzer [31] reported 29.4 g/d CH<sub>4</sub> emissions in sheep with body weight of  $78 \pm 7.0$  kg, while Abecia et al. [35] observed 15.9 g/d CH<sub>4</sub> emissions in goats with average body weight of  $43 \pm 1.7$  kg.

The UN treatment greatly decreased CH<sub>4</sub> emissions in terms of g/kg DMI and g/kg OM digested, but did not alter daily CH<sub>4</sub> emission in terms of g/d. This effect is due to the slight increase in DMI and OM digestibility in goats fed the UN diet. Nitrate acts as an alternative hydrogen sink and inhibits CH<sub>4</sub> production in the rumen. When compared with control, the observed increase in protozoa

and *S. ruminantium* populations for UN is consistent with the roles of these microorganisms in nitrate and nitrite reduction [27, 36]. Stoichiometrically, 4 mol of 2[H] can be redirected to ammonia away from methanogenesis during 1 mol of nitrate reduction to ammonia ( $\text{NO}_3^- + 4 [2\text{H}] + 2\text{H}^+ \rightarrow \text{NH}_4^+ + 3\text{H}_2\text{O}$ ), equivalent to 258.1 g  $\text{CH}_4$ /kg nitrate. If 2.33 g nitrate/kg dietary DM in UN was completely reduced to ammonia,  $\text{CH}_4$  reduction would have been 0.6 g/kg DMI. However, the observed  $\text{CH}_4$  reduction of 2.1 g/kg DMI is far more than that theoretical reduction. Toxic effects of nitrate and nitrite have been reported for methanogens, but the dietary nitrate content (2.33 g/kg DM) in the present study is far less than that in published studies (6.7 and 5.3 g/kg DM, respectively) [7, 27], indicating that toxic effects may not have been the main cause of  $\text{CH}_4$  reduction for the UN treatment.

The shift of rumen fermentation may contribute to the  $\text{CH}_4$  reduction for the UN treatment. The decreased butyrate molar proportion and (acetate + butyrate) to propionate ratio of the goats fed UN is consistent with the decrease in  $\text{CH}_4$  emission of UN compared with control. Acetate and butyrate formation results in net [H] production, while propionate formation incorporates [H] [37]. A shortage of [H] would result in reduced methanogenesis. Our previous in vitro study found that urea plus nitrate pretreatment for rice straw decreased  $\text{CH}_4$  production with a reduction of acetate to propionate ratio [9]. van Zijderfeld et al. [38] observed that nitrate supplementation caused  $\text{CH}_4$  inhibition, which was accompanied by increased propionate molar proportion at the expense of acetate and butyrate.

Oil supplementation inhibits  $\text{CH}_4$  production [39], due to a reduction in OM intake, rumen fermentation and protozoa associated methanogens. Guyader et al. [40] reported that a combination of 40 g linseed oil/kg dietary DM and 30 g calcium nitrate/kg dietary DM shows additive effects on  $\text{CH}_4$  mitigation. However, we did not find additive effects on  $\text{CH}_4$  mitigation when corn oil was supplemented to the UN diet. Therefore, the combination of UN and corn oil supplementation effectively decreases  $\text{CH}_4$  emissions, but not to a greater extent than UN alone.

As nitrate acts as an electron acceptor in anaerobic environments, reduction of nitrate to ammonia is associated with decreased  $\text{H}_2$  availability for methanogens [41]. However, nitrate or nitrite may exert toxic effect on methanogens, leading to an increase in ruminal  $\text{H}_2$  accumulation or  $\text{dH}_2$  concentration [39]. The balance between nitrate reduction and nitrite toxicity determines the available  $\text{H}_2$  in the rumen. In our study, UN treatment decreased ruminal  $\text{dH}_2$  concentration, indicating that nitrate in UN treatment incorporates [H] for ammonia synthesis during nitrate reduction. The low nitrate content (2.33 g/kg dietary DM) of the UN treatment was unlikely to have been toxic to the methanogens. Urea plus nitrate treatment did

not alter  $\text{dCH}_4$  concentration, possibly because increased DMI and NDF digestibility can cause greater  $\text{CH}_4$  synthesis. When compared with control, goats fed UNCO had lower  $\text{dCH}_4$  concentration in rumen fluid, which is consistent with decreased  $\text{CH}_4$  emissions. However, in comparison with control, UNCO increased the fiber digestibility and ruminal ammonia concentration, but did not alter major individual VFA profile. These results are surprising and the underlying mechanism needs further investigation.

### Bacterial community

Urea plus nitrate treatment did not alter diversity of bacterial community, as shown by the similar Shannon-Weiner and Simpson indexes between UN and control groups. Bacteroidetes and Firmicutes are the two predominant phyla in the rumen bacterial community [42]. Although UN did not alter abundances of these phyla in comparison with control, the large increase in abundance of Ruminococcaceae family, which plays a very important role in fiber degradation [43], is consistent with the observed increase in fiber digestibility [44]. The greater 16S rRNA gene copies of *R. albus* of goats fed UN compared with control goats is consistent with the literature. Nguyen et al. [4] fed swamp buffaloes rice straw pretreated with urea plus limestone (20 + 20 g/kg DM of rice straw) and reported an increase in 16S rRNA gene copies of *R. albus*. It seems that the urea component in the UN treatment destroys the indigestible structural polysaccharide matrix, which facilitates colonization of fibrolytic bacteria by providing attachment sites for adherence of the bacteria. When corn oil was supplemented to the UN diet, abundance of Ruminococcaceae family was reduced, indicating that the unsaturated fatty acids in the oil had negative effects on microbiota of the rumen [45]. High-fat diets can decrease the abundance of Ruminococcaceae family [46]. When compared with control, UNCO did not alter bacterial diversity and abundance of major dominant bacteria and 16S rRNA gene copies of selected bacteria. Thus, it appears that corn oil supplementation can reverse the negative effects of UN on bacteria, resulting in a similar bacterial community as the control group.

### Conclusions

Urea plus nitrate pretreated rice straw increased total-tract fiber digestibility and decreased  $\text{CH}_4$  emissions but with decreased N retention. Greater fiber digestibility was associated with increased protozoa and *R. albus* populations and greater abundance of Ruminococcaceae. The urea in the UN pretreatment destroyed the covalent association between lignocellulose and exposed the inner tissues for attachment of fibrolytic microorganisms. Nitrate in the UN pretreatment appeared to incorporate [H] away from methanogenesis, leading to a reduction in ruminal  $\text{dH}_2$

concentration and methanogenesis. Supplementation of corn oil to the UN diet increased N retention, which may have been due to the decreased protozoa population. Compared with the control group, UNCO treatment increased fiber digestibility and decreased CH<sub>4</sub> emissions, although most of the selected ruminal microorganisms were not changed. A combination of UN pretreatment and corn oil supplementation improved fiber digestibility and decreased CH<sub>4</sub> emissions without affecting N balance, and thus may provide a potential strategy to increase nutritive value of rice straw for ruminant production.

#### Abbreviations

[H]: Metabolic hydrogen; ADF: Acid detergent fiber expressed inclusive of residual ash; CH<sub>4</sub>: Methane; CP: Crude protein; dCH<sub>4</sub>: Dissolved methane; DE: Digestible energy; dH<sub>2</sub>: Dissolved hydrogen; DM: Dry matter; DMI: Dry matter intake; EE: Ether extract; *F. succinogenes*: *Fibrobacter succinogenes*; GE: Gross energy; N: Nitrogen; NDF: Neutral detergent fiber expressed inclusive of residual ash; OM: Organic matter; qPCR: Real-time quantitative polymerase chain reaction; *R. albus*: *Ruminococcus albus*; *R. flavefaciens*: *Ruminococcus flavefaciens*; *S. ruminantium*: *Selenomonas ruminantium*; UN: Urea plus nitrate treatment; UNCO: UN diet supplemented with corn oil; VFA: Volatile fatty acid

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#### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

#### Authors' contributions

XZ, MW, ZT and LB designed research; XZ, RM, ZM, RW and JW conducted research; XZ and MW analyzed data; XZ, KB and MW wrote the paper. All authors read and approved the final manuscript and take full responsibility for the final content.

#### Ethics approval and consent to participate

All animal procedures used in this study were reviewed and approved by the Animal Care Committee (Approval number: ISA-Wang-201705), Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare no competing interests.

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Sint-Martens-Latem, December 15, 2023

Our Ref.: YARA-250-PCT

Re.: International Patent Application No. PCT/EP2023/054080 filed on February 17, 2023  
"A MIXTURE OF UREA, BIURET and N-CONTAINING BY-PRODUCTS CREATED DURING  
BIURET PRODUCTION AS A DIETARY NON-PROTEIN NITROGEN SOURCE FOR  
RUMINANTS AND USES THEREOF"  
In name of Yara International ASA

---

Dear Madam, dear Sir,

In response to the International Search Report and Written Opinion in connection with the above-mentioned international application, issued by the International Searching Authority (ISA) on July 3, 2023, the applicant respectfully submits the following amendments and observations.

A request for Preliminary International Examination under Chapter II PCT is hereby filed.

The Written Opinion particularly relies upon the documents US2768895 (D1), CA1241663 (D2), Xiumin Zhang et al. (D3), and the same numbering is used herein.

**1. Amendments**

An amended set of claims 1-22 is submitted herewith to replace claims 1-20 currently on file.

Currently amended claim 6 is now limited to the use of the composition specified in the claims as a NPN source for improving rumen fermentation wherein the acetate-to-propionate ratio produced by rumen microbes is increased and/or wherein the efficiency of microbial protein synthesis (EMPS) by rumen microbes is improved, as supported by the sections between p.9, line 29 and p.10, line 9, and p.10, lines 16-24, and former claim 6.

Newly introduced claim 7, dependent on claim 6, is limited to the features of increasing the dry matter (DM) and organic matter (OM) digestibility and/or increasing the dry matter intake (DMI) of former claim 6.

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Newly introduced claim 13 specifies the concentration of the NPN composition in a ruminant feed composition, as supported by former claim 19.

Claim numbering and claim dependency have been adjusted accordingly.

It is respectfully submitted that these amendments do not extend beyond the content of the application as filed.

## **2. Observations**

### **2.1 Novelty and inventive step**

It is gratefully acknowledged that the examiner considers the subject-matter of former claims 1-5 and 12-20 novel and inventive over the cited prior art.

However, the examiner finds the subject-matter of former claim 6 not inventive over D1 in combination with D2. According to the examiner, an increase in dry matter and organic matter intake is the underlying mechanism for the effects shown in D1 for a mixture of biuret, urea, triuret, cyanuric acid, and ammelide, and that the skilled person would amend the composition of D1 by the amounts disclosed in D2, for the same purpose.

Without acquiescence to this objection, currently amended claim 6 now specifies the use of a composition comprising between 30 and 60 wt% of urea and between 30 and 60 wt% of biuret for improving rumen fermentation wherein the acetate-to-propionate ratio produced by rumen microbes is increased and/or wherein the efficiency of microbial protein synthesis (EMPS) by rumen microbes is improved.

These effects are demonstrated in the examples of the present application. In particular, Tables 4 and 10 demonstrated that supplementation of the diet with the urea/biuret mixture as specified in the claims as a NPN source resulted in a significant increase in EMPS, particularly compared to a diet comprising urea as NPN source. Table 4 also demonstrates that the acetate-to-propionate ratio is reduced compared to a diet comprising urea as NPN source. As explained on p.10, lines 3-9 of the PCT application as filed/published, the EMPS is a measure for how much microbial protein is produced due to the microbial activity per unit of fermentable substrate. As further explained on p.10, lines 16-24, using a urea/biuret mixture as NPN source results in the production of more propionate and less acetate, through which more energy is provided to the ruminant.

It is respectfully submitted that the use of a NPN composition comprising between 30 and 60 wt.% of urea and between 30 and 60 wt.% biuret for increasing the rumen fermentation, particularly for increasing the acetate-to-propionate ratio produced by rumen microbes or by increasing the efficiency of microbial protein synthesis (EMPS) by rumen microbes is not disclosed in D1-D3.

D1 discloses the use of a composition comprising urea and biuret for improving daily weight gain or milk yield. D2 relates to the production of feed-grade biuret, and is silent on the effects thereof. D3 relates to the pretreatment of rice straw with urea to improve fiber digestibility. D3 is completely silent on any effect of a urea/biuret mixture as feed additive.

The skilled person would thus not obtain the subject-matter claimed in currently amended claim 6, either alone or taken in combination, as the cited prior art documents deal with different problems.

Since D1-D3 does not disclose the subject-matter of currently amended claim 6, nor provide any incentive how to obtain the claimed effect, it is respectfully submitted that currently amended claim 6, as well as currently amended claims 7-13 dependent thereon, involve an inventive step in view of the cited prior art.

## 2.2 Clarity and support (Art 6 PCT)

The examiner is of the opinion that the claims are not supported by the description as their scope is broader than justified by the description and drawings. According to the examiner, there is no limit to the amount of the composition containing urea and biuret introduced in the ruminant feed.

The applicant respectfully does not agree with this opinion.

Currently amended claims 1 and 6 explicitly specify the use of a composition “as a NPN source” and currently amended claim 14 specify “administering (...) a NPN composition”.

It is respectfully submitted that the use of NPN sources/compositions, i.e. non-protein nitrogen sources/compositions, in the diet of ruminants is well known to the skilled person. As *inter alia* explained in the background of the present application, non-protein nitrogen is a source of supplemental nitrogen for ruminants. In the literature, it can be found that NPN is a term used in animal nutrition that refers to components that are not (true) proteins but can be converted to proteins by microbes in the ruminant stomach, or in other words such compounds that supply nitrogen other than in the form of protein. NPN sources can be used to supply some of the needed dietary nitrogen for the ruminants. While NPN serves as a cheaper alternate of protein for ruminants, feeding only NPN cannot replace the whole protein requirement. Furthermore, NPN should be administered to the ruminants according to strict feeding guidelines, since some NPN sources such as urea can be toxic for the ruminant animal when administered in too high amounts.

In view of these observations, the skilled person thus understands that the claimed subject-matter, by referring to the term “as a NPN source” implicitly but necessarily imposes a limit on the amount of the NPN composition as specified in the claims that may be introduced in the ruminant diet, particularly wherein the combination of the NPN composition, as such or as

present in a ruminant feed supplement, and the nutritional ingredients of the ruminant feed provide for a balanced diet.

### **3. Request**

In view of the arguments presented herein, it is submitted that the claimed subject matter complies with the requirements of the PCT with respect to clarity, novelty and inventive step, and a positive opinion is therefore requested. Should the examiner nevertheless still be of the opinion that the claims are not meeting the requirements of patentability, the applicant would appreciate a possibility to present further arguments, either orally or in writing.

Respectfully submitted,

A handwritten signature in black ink, appearing to read 'K. Pappaert', with a stylized flourish above it.

Mr. PAPPAERT, Kris  
Mr. GOESAERT, Hans  
Professional Representatives before the EPO

Encl: Amended claims (version showing amendments and retyped version)