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Efectos del uso de astaxantina, lúpulo o un aceite esencial sobre la calidad y vida útil de la carne y preparados cárnicos de cordero

por

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"¿Las aventuras nunca tienen un final?
Supongo que no. Alguien más siempre tiene
que continuar con la historia."

J. R. R. Tolkien

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Resumen

La carne y los preparados cárnicos son alimentos altamente perecederos. La pérdida de vida útil está ocasionada principalmente por deterioro microbiano y oxidación. Una estrategia actual contemplada por la industria cárnica es el uso de ingredientes naturales con propiedades antioxidantes o antimicrobianas. En el presente trabajo, en diferentes experimentos, se evaluaron los efectos de la astaxantina (carotenoide extraído de algas como *Haematococcus pluvialis*), de un extracto acuoso de lúpulo y del aceite esencial de *Zataria multiflora* Boiss, sobre la estabilidad y vida útil de la carne de cordero y diversos preparados cárnicos obtenidos con la misma.

En el artículo titulado "*Effects of dietary astaxanthin supplementation on the oxidative stability of meat from suckling lambs fed a commercial milk-replacer containing butylated hydroxytoluene*" se evalúan los efectos de la adición de astaxantina en un lactorremplazante comercial, que contiene butil hidroxi tolueno (BHT) en su formulación, sobre el color, producción de volátiles y oxidación lipídica de carne obtenida de los corderos.

El experimento se llevó a cabo con 20 corderos hembras Assaf colocados en jaulas individuales: 10 pertenecientes al grupo control y las restantes 10 alimentadas mediante la suplementación de 25 mg de astaxantina por kg de lactorremplazante. Durante los 22 días de lactación, los animales fueron periódicamente pesados y, después, sacrificados. Se evaluaron características relacionadas con el crecimiento de los corderos y la estabilidad oxidativa de la carne: estabilidad de color, formación de compuestos volátiles y producción de sustancias reactivas al ácido tiobarbiturico (TBARS). Se pudo observar una mayor acumulación de la grasa perirrenal en los corderos alimentados con la suplementación de astaxantina, un aumento del índice de rojos de carne y de la grasa perirrenal y un retraso en la oxidación lipídica en la carne almacenada en congelación (- 18 ^{o}C, 3 meses). Además, los corderos del grupo astaxantina mostraron una menor acumulación en el músculo del BHT presente en el lactoreemplazante.

En el artículo titulado "*Assessment of the antioxidant effect of astaxanthin in fresh, frozen and cooked lamb patties*" se evaluó el efecto de la adición de astaxantina sobre hamburguesas elaboradas con carne de cordero. Se prepararon por triplicado, hamburguesas con 4 concentraciones de astaxantina, desde 20 hasta 80 mg/kg y se prepararon 3 hamburguesas control, una sin aditivos y otras dos con aditivos antioxidantes: metabisulfito de sodio y ascorbato de sodio. Las características de calidad evaluadas fueron el color, la liberación de volátiles derivados de la cocción, la producción de TBARS durante el almacenamiento y de óxidos de colesterol generados por cocción, almacenamiento y recalentado en microondas. Los

resultados evidenciaron un efecto colorante y antioxidante, que fue directamente proporcional a la concentración de astaxantina añadida, en donde las mayores concentraciones lograban igualar o sobrepasar el efecto provocado por los antioxidantes de uso común. Por otro lado, la astaxantina produjo una marcada disminución en la liberación de volátiles durante la cocción, relacionados con procesos de oxidación y retrasó la oxidación lipídica, disminuyendo la cantidad de óxidos de colesterol como 7α-hidrocolesterol y 7-cetocolesterol.

Por otra parte, se publicaron dos artículos donde se evaluó el efecto antimicrobiano de un extracto acuoso de lúpulo y del aceite esencial de *Zataria multiflora* Boiss, solos o en combinación en salchichas frescas de cordero, elaboradas a partir de una receta de los Balcanes, almacenadas en condiciones anóxicas (80 % de N_2 y 20 % de CO_2) a refrigeración. A las salchichas, elaboradas por triplicado, se les agregó extracto de lúpulo (1,5 g de lúpulo/kg de masa cárnica), aceite esencial (1 mL/kg de carne) y/o una mezcla de ambos (0,75 g de lúpulo y 0,5 mL de aceite por kg de carne). El experimento fue dividido en dos partes, correspondientes a sendos artículos.

Por un lado, en el artículo titulado "*Microbial growth and biogenic amine production in a balkan-style fresh sausage during refrigerated storage under a CO_2-containing anaerobic atmosphere: Effect of the addition of Zataria multiflora essential oil and hops extract*" se evaluó el efecto individual y la combinación de los ingredientes previamente mencionados, sobre la población microbiana y la producción de aminas biógenas (AB).

Los resultados mostraron escaso efecto de ambos ingredientes tanto en la producción de AB como en la población total bacteriana. Sin embargo, los resultados evidenciaron con cierto detalle los cambios microbianos de los embutidos frescos durante su almacenamiento, destacando el protagonismo de *Lactobacillus sakei* y el crecimiento, hasta niveles considerados potencialmente peligrosos para la salud, de las aminas biógenas.

Por otro lado, en el artículo titulado "*The effects of storage and hop extract on aroma and flavour compounds in Balkan-style sausages packed under a CO_2-containing anaerobic atmosphere*" se evalúo el efecto individual de la adición de lúpulo sobre la producción de ácidos orgánicos y compuestos volátiles, que tienen impacto sobre características sensoriales, en la salchicha fresca mencionada anteriormente. Se observó el incremento con el tiempo de diversos compuestos volátiles responsables de la alteración de los embutidos, así como la aparición de olor a alteración a partir de los 14 días de almacenamiento. No se observó efecto del lúpulo sobre la producción de compuestos de origen microbiano por efecto del lúpulo. Sin embargo, se pudo observar que la adición de lúpulo generó una disminución en aquellos volátiles originados por oxidación lipídica como el hexanal, propanol y 1-octen-3-ol. La ausencia de efecto antimicrobiano tanto del lúpulo como del aceite esencial se atribuye a interacciones con la matriz de la salchicha o a la resistencia de los microorganismos que crecen en la salchicha en anaerobiosis, que disminuirían su eficacia.

1 Introducción

1.1. Generalidades

1.1.1. Carne

La definición de carne es compleja, siendo a veces más específica y otras más general en función del contexto en que se aplique. La Real Academia Española define a la carne (lat. *caro, carnis*) como la "parte muscular del cuerpo de un animal de tierra o aire, que es utilizada como alimento". Por otra parte, la definición de la Asociación Americana de Ciencia de la Carne (AMSA; Seman et al., 2018), en un sentido general, considera a la carne como el músculo esquelético y sus tejidos asociados (incluyendo el tejido conectivo, los nervios, los vasos sanguíneos, la piel, la grasa y los huesos), así como los despojos comestibles derivados de los mamíferos, las aves y las especies acuáticas consideradas como inocuas para el consumo humano. En el contexto del etiquetado de los derivados cárnicos, la Directiva 2000/13/CE (CE, 2000) incluye los músculos del esqueleto de la especies de mamíferos

y de aves reconocidas como aptas para el consumo humano, con los tejidos naturalmente incluidos o adheridos a ellos, en los que no se vean sobrepasados ciertos límites en los contenidos totales de materia grasa (aves y conejos 15 %, cerdos 30 %, mamíferos 25 %) y tejido conectivo (aves y conejos 10 %, cerdos 25 % y mamíferos 25 %).

1.1.2. Calidad de la carne

La carne, como la mayoría de los alimentos perecederos, presenta una estructura y composición compleja, en donde tienen lugar un conjunto de procesos bioquímicos, incluido el crecimiento microbiano (Gutiérrez, 2000; Schröder, 2003). Dicha estructura y composición y sus cambios *post-mortem* se verá traducida en cambios en su calidad (Rodriguez Caerio, 2005).

La calidad de la carne es un término que implica una amplia combinación de características concretas como la calidad de consumo o sensorial, su vida útil (estabilidad del color, oxidación lipídica y mantenimiento de la calidad sensorial inicial), su composición nutricional (contenido de grasa intramuscular, relación de carne magra/grasa, etc.), la aptitud para su procesado (capacidad de retención de agua, propiedad para ser trabajada como una emulsión, etc.), la facilidad para su preparación culinaria y consumo (almacenamiento) y su inocuidad (Biswas & Mandal, 2019). De forma más resumida, Hartung et al. (2009) agrupan los componentes de la calidad en cuatro aspectos fundamentales: i) componentes de la nutrición humana; ii) higiene y toxicología; iii) aptitud para el procesado o calidad tecnológica y

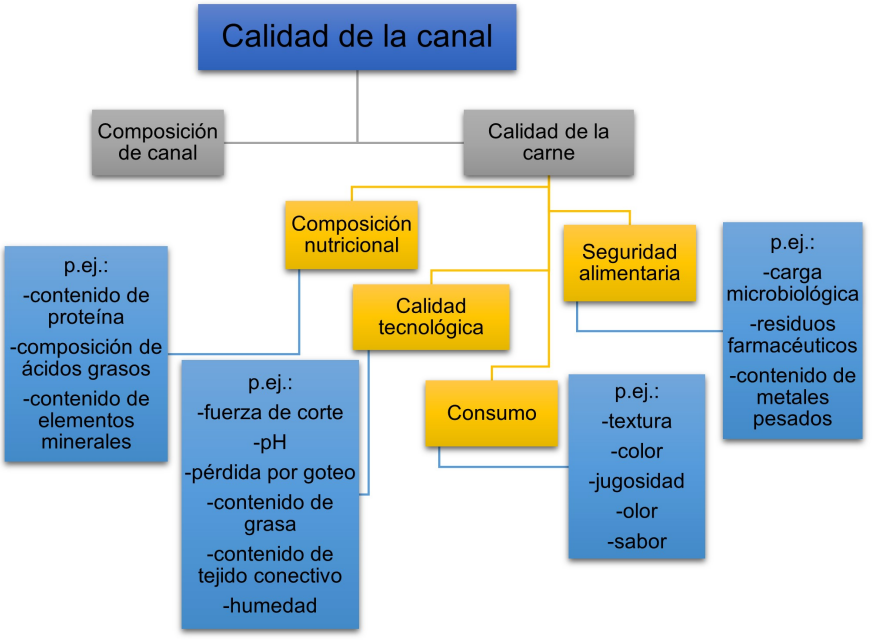

Figura 1: Parámetros constituyentes de la calidad cárnica y de la canal (Hartung et al., 2009)

finalmente, iv) parámetros sensoriales importantes que tendrán repercusión en la aceptabilidad por parte del consumidor (**Figura 1**).

La carne, como alimento, es considerada como una fuente idónea de nutrientes debido a su considerable contenido en proteínas con alto valor biológico, ácidos grasos esenciales n-*3*, vitaminas del grupo B o elementos minerales como hierro, zinc o fósforo (De Smet & Vossen, 2016; Martínez de Icaya & Koning Garlito, 2005).

Respecto a los atributos sensoriales, el color es el de mayor relevancia a la hora de la compra, asociándose al grado de frescura y la edad del animal. Este depende principalmente de la cantidad y estado químico de la

mioglobina, tornando de rojo a marrón con el tiempo de almacenamiento en presencia de oxígeno (Brugiapaglia & Destefanis, 2009; Calnan et al., 2016; Passetti et al., 2017). La textura afecta a la aceptación de la carne durante su consumo. Los principales componentes de la textura son la dureza y la jugosidad. Ambos están muy relacionados y vienen dados por aspectos estructurales y de composición como la longitud del sarcómero, la estructura y composición del colágeno y/o las proteínas miofibrilares, por el contenido en grasa intramuscular o el pH (Martínez-Cerezo et al., 2005; Pearce et al., 2011; Starkey et al., 2017). La aceptación de la carne durante el consumo también se ve afectada por el sabor, entendido como la sensación integrada a la hora de ingerir, masticar y deglutir. El sabor depende fundamentalmente de los compuestos volátiles generados durante la preparación culinaria de la carne a partir de compuestos precursores como ácidos grasos, tiamina, glucosa residual (Troy & Kerry, 2010; Webb & O'Neill, 2008).

La inocuidad de la carne es una de las principales preocupaciones a la hora de su consumo (Sofos, 2008). La principales problemas relacionados con la inocuidad son la presencia de contaminantes químicos (residuos de antibióticos, metales pesados, etc.) y microorganismos patógenos microbianos y/o sus toxinas (bacterias como *Salmonella* spp., *Campylobacter jejuni/coli*, *Yersinia enterocolitica*, *Escherichia coli* O157:H7, o parásitos como *Toxoplasma*, *Trichinella*, cestodos, etc.) (Lehotay et al., 2005; Nørrung et al., 2009; Saucier, 1999; Sofos, 2008).

La calidad tecnológica de la carne es de interés principal para la indus-

tria cárnica, donde se valora, por una parte, una carne con un pH adecuado y con unas buenas propiedades funcionales de sus proteínas, que garanticen una alta capacidad de retención de agua, de emulsión y gelificación y, por otra, una carne con buena estabilidad de la grasa y el color durante el procesado y almacenamiento, previniendo la pérdida de grasa, la oxidación y la decoloración (Cheng & Sun, 2008; Hugo & Roodt, 2007; Muchenje et al., 2009; Santhi et al., 2017).

Los aspectos sobre la calidad de la carne antes mencionados están relacionados con factores de la producción animal como la alimentación, la genética (Biswas & Mandal, 2019), así como en el manejo de los animales durante el sacrificio (Calnan et al., 2016; Sañudo et al., 1998). Por otro lado, la calidad de la carne cambia continuamente tras su obtención. En esos cambios de calidad influyen las condiciones de procesado, envasado y almacenamiento (Moschopoulou et al., 2019).

1.1.3. Preparados cárnicos

Es importante diferenciar la carne de los derivados cárnicos. La principal diferencia entre la carne y los derivados cárnicos frescos es que estos últimos pueden estar preparados total o parcialmente a base de carne, despojos o grasas y además en su elaboración incluyen ingredientes de origen vegetal o animal, condimentos, especias y aditivos autorizados (Rodriguez Caerio, 2005).

Los derivados cárnicos, a su vez, en función de su procesado y caracte-

rísticas del producto final, se clasifican en dos tipos: preparados cárnicos de carne y productos cárnicos (AECOSAN, 2019). Los preparados de carne, o preparados cárnicos, se definen según el Reglamento 853/2004/CE (CE, 2004) como "la carne fresca, incluida la carne que ha sido troceada, a la que se han añadido productos alimenticios, condimentos o aditivos, o que ha sido sometida a transformaciones que no bastan para alterar la estructura interna de la fibra muscular ni, por lo tanto, para eliminar las características de la carne fresca". Entre los preparados cárnicos se encuentran las hamburguesas, las salchichas frescas o los chorizos frescos. Por contra, los productos cárnicos son aquellos en los que la carne, adicionada con ingredientes autorizados, ha sido transformada por procesos como el secado o el tratamiento térmico, que alteran la estructura de la misma. Ejemplos de productos cárnicos son el jamón crudo curado, el chorizo crudo curado o el jamón cocido.

1.1.4. Producción y consumo de carne de ovino

La producción de ovino, especie caracterizada por su adaptabilidad y versatilidad – obteniendo de ellos carne, leche, lana, piel y abono orgánico – está presente en muchas regiones del mundo (Knapik et al., 2016). En 2018, los datos mundiales sobre producción de carne de ovino fueron de casi 10 millones de toneladas, de las cuales Europa produjo 1 162 308 toneladas (FAOSTAT, 2020). Hasta la última década, la Unión Europea se posicionaba como la mayor productora mundial de carne de ovino y la mayor importadora de este tipo de carne. Esta posición se ha visto dismi-

nuida hasta el tercer lugar por un incremento en la producción de carne en el continente africano y asiático y un descenso del consumo (Peña Ojeda, 2008). Dentro de la Unión Europea, España se situó como el segundo país productor de ovino, detrás del Reino Unido, con 119 642 toneladas. Entre las regiones de España, la comunidad autónoma de Castilla y León se posiciona en el primer lugar, con un 23,8 % de la producción total nacional (MAPA, 2020).

En los países europeos del Mediterráneo la carne de cordero es altamente apreciada por cierto sector de la población, que de forma característica la percibe como un producto tradicional (Sañudo et al., 1998). La carne de cordero de alta calidad (carne de pierna, costillar y espaldas de corderos jóvenes) es consumida principalmente en celebraciones y eventos sociales y llega a ser considerada en muchos casos como alimento de lujo (Linares et al., 2012).

A pesar de lo mencionado, la tendencia a la producción y consumo de carne de ovino y caprino no es halagüeña. El consumo ha disminuido tanto a nivel internacional como en España. En España, esta disminución ha sido de hasta un 55 % en la última década (con un consumo per cápita de 2,38 kg en 2009 y de 1,31 kg en 2019) (MAPA, 2020). Esta tendencia negativa ha provocado una movilización del sector hacia la búsqueda de estrategias para fomentar su consumo. Entre estas estrategias estaría la puesta en el mercado de derivados cárnicos de cordero con el propósito de aumentar la versatilidad de la oferta (Cruz, 2013; Garnier, 2010; Linares et al., 2012;

Paulos et al., 2015).

1.2. Vida útil de la carne y los preparados cárnicos

De acuerdo con el Reglamento 1169/2011 (UE, 2011b), la vida útil se define como la "fecha de durabilidad mínima de un alimento", lo cual se traduce como la fecha hasta la cual dicho alimento es capaz de conservar sus propiedades cuando éste se almacena de manera adecuada.

La alteración de la carne y preparados cárnicos produce grandes pérdidas económicas. Los tres factores principales responsables de su vida útil son el crecimiento microbiano, la pérdida de color y la oxidación (la oxidación de la mioglobina y los lípidos). El crecimiento microbiano y la pérdida de color afectan principalmente a la carne y preparados cárnicos crudos y la oxidación es especialmente importante a los cocinados. Estos fenómenos tienen gran impacto sobre las propiedades sensoriales, nutricionales y/o de inocuidad de carne y preparados de carne (Cunha et al., 2018; Estévez, 2017; Lambert et al., 1991; Love & Pearson, 1971; O'Grady & Kerry, 2009; Zagorec & Champomier-Vergès, 2017).

1.2.1. Crecimiento microbiano

1.2.1.1. Alteración de la carne y preparados cárnicos

El crecimiento microbiano es considerado como la causa principal del deterioro de la carne fresca y los preparados cárnicos frescos (Casaburi et al., 2015; Cocolin et al., 2004; Lonergan et al., 2019). Ambos son alimentos muy perecederos ya que proporcionan condiciones favorables para el rápido crecimiento de muchos microorganismos, incluso a temperaturas de refrigeración: riqueza en nutrientes como la glucosa residual, los compuestos nitrogenados no proteicos, los nucleótidos, un pH normalmente comprendido entre 5,5 y 6,5 y actividad de agua muy próxima a 1 (Labadie, 1999; Nychas et al., 2007, 2008). Su alteración se manifiesta por la aparición de sabores atípicos, formación de limosidad superficial y pérdida del color rojo (Martínez et al., 2005; Nychas et al., 2008). Además, la carne puede contener microorganismos patógenos sin mostrar signos de deterioro evidente (Guerrero-Legarreta, 2009) y el crecimiento de microorganismos puede llegar a afectar la salud del consumidor (Singh & Anderson, 2004).

A nivel muy general, los recuentos microbianos por encima de 10^{7-8} unidades formadoras de colonias (UFC)/cm^2 o g indican que el deterioro es evidente (Lonergan et al., 2019; Martínez et al., 2005). No obstante, el proceso de alteración es complejo y su comprensión requiere los enfoques químico, microbiano y sensorial. Los primeros permiten conocer los grupos microbianos alterantes y su dinámica, las vías metabólicas utilizadas por los microorganismos, así como los productos finales responsables de la

alteración de la carne (Doulgeraki et al., 2012).

La velocidad y el tipo de deterioro microbiano depende de la composición y pH de la carne, de la contaminación microbiana producida durante el sacrificio y manipulación posterior, así como de las condiciones del eventual procesado, almacenamiento y distribución (Doulgeraki et al., 2012; Huis In't Veld, 1996).

Para retrasar el crecimiento microbiano se emplea comúnmente la refrigeración, entre -1,5 a 5 ºC (Coombs et al., 2017; Doulgeraki et al., 2012; Lonergan et al., 2019; Nychas et al., 2008). También se puede modificar la atmósfera de almacenamiento aplicando vacío o atmósferas modificadas, con el fin de disminuir la disponibilidad de oxígeno y/o aumentar presencia de CO_2 en la superficie de la carne (Casaburi et al., 2015; Chiavaro et al., 2008). De esta forma, por ejemplo, con la presencia de 15-20 % de CO_2 en la atmósfera, la vida útil de filetes de carne de vacuno a 3 ºC, que en condiciones de aerobiosis suele ser inferior a 3 días, puede alargarse hasta 9 días (Lambert et al., 1991).

La vida útil de los preparados cárnicos es algo superior a la de la carne fresca debido a la presencia de sal, que baja la actividad de agua a valores próximos a 0,98, la eventual presencia de aditivos con efecto conservante, como los sulfitos o ácidos orgánicos, u otros ingredientes con efecto antimicrobiano como algunas especias (Björkroth, 2005; Gammariello et al., 2015; Hugo & Hugo, 2015). En condiciones de refrigeración aerobia a tempera-

turas de 3-4 °C la vida útil aproximada de los embutidos frescos convencionales sería inferior a los 10 días (Cocolin et al., 2004) y en anaerobiosis, con presencia de CO_2, podría alargarse hasta 20 días (Dias et al., 2013; Martínez et al., 2006; Ruiz-Capillas & Jiménez-Colmenero, 2010).

1.2.1.2. Microorganismos alterantes

Los microorganismos más vinculados al entorno de la carne son pseudomonas, enterobacterias, bacterias ácido-lácticas (BAL), *Brochothrix thermosphacta*, y algunos *Clostridia* (Borch et al., 1996; Doulgeraki et al., 2012; Pennacchia et al., 2011). Durante el almacenamiento de la carne y los preparados cárnicos, la refrigeración selecciona especies psicrotróficas. Los microorganismos responsables de la alteración en la carne y preparados cárnicos, en inglés *specif spoilage microorganisms*, forman parte de una asociación microbiana (Doulgeraki et al., 2012; Nychas et al., 2008) y la alteración solo depende de una pequeña fracción de esa asociación (Nychas et al., 2007, 2008). El desarrollo de los diferentes tipos de poblaciones microbianas de la asociación y la velocidad a la que se produce la alteración depende de la contaminación microbiana inicial de la carne, las condiciones de procesado, transporte y almacenamiento y la competición entre los grupos microbianos (Doulgeraki et al., 2012; Nychas et al., 2008).

De entre esos factores, posiblemente, el más notorio sea la composición de la atmósfera (Doulgeraki et al., 2012). De esta manera, cuando la carne se almacena en una atmósfera rica en oxígeno el consorcio de bacterias en su superficie suele estar habitualmente dominado por *Pseudomonas*

spp. (Koutsoumanis et al., 2006), como *Ps. Fragi, Ps. Fluorescens* y *Ps. Lundensis*. Las vías metabólicas usadas por estas Pseudomonas son: i) el catabolismo de D-glucosa, preferentemente, del L- y D-lactato, y de determinados aminoácidos libres, después de agotarse la glucosa (Nychas et al., 2008). También, *Pseudomonas* spp. es el grupo mayoritario en la superficie y en el interior embutidos frescos de calibre fino, cuando la presencia de oxígeno en la masa cárnica es suficientemente alta (Benson et al., 2014). No obstante, en el interior de la masa cárnica, debido al metabolismo microbiano, la concentración de O_2 disminuye con el tiempo, a la vez que la concentración de CO_2 aumenta (Koutsoumanis et al., 2008), lo que conlleva un cambio de la población microbiana, llegando a dominar otras especies como *Lactobacillus, Carnobacterium, Yersinia* y *Serratia* (Benson et al., 2014).

En la superficie de la carne, con condiciones de almacenamiento con baja concentración de oxígeno, menos al 6 %, como es el caso del envasado al vacío o las atmósferas modificadas, o el interior de una pieza de carne picada, genera una microbiota formada por microorganismos anaerobios facultativos, especialmente las BAL y *B. thermosphacta* (Doulgeraki et al., 2012; Pothakos et al., 2015). En relación con las BAL, cabe resaltar la presencia de *Lactobacillus* spp., *Carnobacterium* spp., y *Leuconostoc* spp. como responsables del deterioro (Casaburi et al., 2015). En preparados cárnicos frescos elaborados con carne picada y almacenados en anaerobiosis las bacterias predominantes son *B. thermosphacta* y *Lactobacillus sakei* (Cocolin et al., 2004; Dias et al., 2013; Raimondi et al., 2018).

Además de lo dicho, las enterobacterias, que también son tolerantes al frio, se pueden desarrollar tanto en carnes almacenadas refrigeradas en anaerobiosis como aeróbicamente, y su presencia es especialmente importante cuando el pH de la carne es alto. *Serratia liquefaciens* es el miembro más común de las *Enterobacteriaceae* de la carne (Casaburi et al., 2015). También se ha señalado en la bibliografía cómo algunos *Clostridia* se han asociado con el deterioro de la carne almacenada en estricta anaerobiosis siendo responsables de malos olores, decoloraciones e hinchamiento de los envases (Broda et al., 2003; Casaburi et al., 2015).

A modo de resumen, en la **Tabla 1** se recogen las principales especies de microorganismos capaces de causar deterioro en carne fresca o mínimamente procesada (preparados cárnicos frescos) según las condiciones de almacenamiento (Fernandes, 2010; Guerrero-Legarreta, 2009; Moschopoulou et al., 2019; Pellissery et al., 2019).

1.2.1.3. Técnicas utilizadas para la determinación de la alteración

Las técnicas más utilizadas para la determinación de la alteración es el recuento de microorganismos de deterioro. La técnica de recuento más general es el **recuento microbiano en placa** (Collins & Huey, 2014; Hameed et al., 2018). En general, se conoce que el deterioro de la carne comienza con recuentos de 6 Log UFC/g de colonias viables totales y que alcanzando recuentos de 8 Log UFC/g los signos de deterioro son muy evidentes

Tabla 1: Principales grupos de microorganismos alterantes de la carne fresca según sus condiciones de almacenamiento

Condiciones	Microorganismos
Almacenamiento aeróbico: desarrollo de malos olores y decoloración	**Pseudomonas:** *P. fragi, P. fluorescens, P. putida y P. lundensis* **Gram-negativos:** *Acinetobacter, Moraxella, Flavobacterium, Enterobacteriaceae (Kurthia, Enterobacter,* y *Hafnia)* **Gram-positivos:** bacterias ácido-lácticas, *Brochothrix thermosphacta, Micrococcus* (mayormente asociado a deterioro), *Achromobacter, y Staphylococcus*
Almacenamiento anaerobio: desarrollo de malos olores y producción de gas	**Clostridium:** *C. algidicarnis, C. algidixylanolyticum, C. estertheticum, C. frigidicarnis, C. gasigenes, C. laramiense, y C. putrefaciens* **Gram-positivos:** bacterias ácido-lácticas (especies *Lactobacillus, Carnobacterium, y Leuconostoc), Brochothrix thermosphacta* **Enterobacterias:** *Hafnia alvei, Serratia spp., y Enterobacter spp*

(Guerrero-Legarreta, 2009). También se puede utilizar la **microbiología predictiva** que considera diversos factores intrínsecos y extrínsecos, y utiliza modelos matemáticos que predicen el crecimiento microbiano. Los modelos usualmente utilizados, son las curvas de crecimiento y destrucción térmica (Blackburn, 2007).

Otro grupo de técnicas utilizadas son las que se basan el análisis químico instrumental para estudiar los metabolitos microbianos (metabolómica). Debido a que cada población microbiana produce metabolitos característicos, el poder de identificarlos y cuantificarlos puede ser de utilidad para

conocer la actividad microbiana y evaluar el impacto que tuvo cierto microorganismo sobre la carne.

Entre los metabolitos estudiados están las **aminas biógenas** (cadaverina, putrescina, espermidina, espermina, triptamina, tiramina e histamina; **Figura 2**), producidas a través de la descarboxilación de aminoácidos por parte de algunos microorganismos (Jairath et al., 2015). Las bacterias pertenecientes a los géneros *Enterobacteriaceae, Pseudomonadaceae, Micrococcaceae* y BAL, son los mayormente involucrados en producción de aminas biógenas en carne (Guerrero-Legarreta, 2009; Jairath et al., 2015; Ruiz-Capillas & Jiménez-Colmenero, 2004). Otro grupo de metabolitos estudiados son los **compuestos volátiles**, que pueden demostrar crecimiento y deterioro microbiano. Los volátiles comúnmente relacionados con el deterioro microbiano son la acetoina, el diacetil butanal y propanal (producidos en cantidades importantes por *B. thermosphacta* y BAL), ésteres (comúnmente producidos por *Pseudomonas* spp), ácido acético (producido en su mayoría por BAL) y ácido butírico (asociado con crecimiento de *Leuconostoc*) (Guerrero-Legarreta, 2009).

Por otra parte, las **técnicas moleculares**, aunque costosas, han probado ser altamente sensibles al momento de analizar las poblaciones microbianas, incluso a nivel de especie. Estás técnicas se dividen en los ensayos inmunológicos y los basados en ácidos nucleicos. Uno de los métodos inmunológicos es el ensayo por inmunoabsorción ligado a enzimas (ELISA), el cual está basado en la unión de anticuerpos a antígenos bacterianos es-

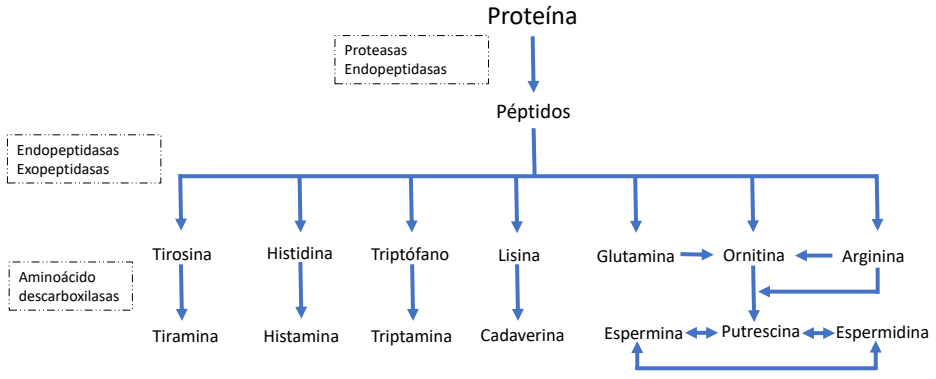

Figura 2: Producción de aminas biogenas a traves de descarboxilación de aminoácidos (Ruiz-Capillas & Jiménez-Colmenero, 2004)

pecíficos (Pellissery et al., 2019). La reacción en cadena de la polimerasa (PCR), es una de las técnicas, basadas en ácidos nucleicos, más empleadas. Esta técnica se centra en la amplificación de secuencias específicas de ADN, permitiendo comparar éstas con "huellas digitales" (*fingerprinting*) de microorganismos relacionados con el deterioro en la carne (Hill & Olsivk, 1995). Este método permite tanto la identificación a nivel de especie como a nivel intra-especie. Entre las técnicas más usadas para estudiar la microbiota mesófila y psicrófila responsable de la alteración de carne y preparados cárnicos encontramos el *randomly amplified polymorphic-PCR* (RAPD-PCR) y la *repetitive extragenic palindromic-PCR* (rep-PCR) (Casaburi et al., 2011; Ercolini et al., 2009; Vasilopoulos et al., 2010, 2008).

Existen otras **tecnologías emergentes** entre las que se encuentra la técnica especrométrica llamada "*matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry*" (MALDI-TOF). La espectrometría

de masas ha sido utilizada ampliamente en la química clínica, pero no fue hasta 1975 cuando fue utilizada para la caracterización de bacterias (Anhalt & Fenselau, 1975). En ese estudio se identificaron pequeñas moléculas de bacterias liofilizadas con lo cual fue posible hacer una diferenciación taxonómica de las mismas. Una década después el MALDI-TOF fue desarrollado para este propósito, permitiendo la caracterización de especies microbianas (Croxatto et al., 2012; Dingle & Butler-Wu, 2013).

En la **Figura 3** se pude observar un esquema del funcionamiento del MALDI-TOF. La muestra microbiana (colonia aislada) es mezclada con una matriz sobre un platillo metálico, donde después de cristalizarse, el platillo es introducido en el espectrómetro de masas (MS). Dentro del MS, la muestra es sometida a un bombardeo con pulsos de láser (usualmente de un láser de nitrógeno). La matriz absorbe la energía del láser y luego desorbe los analitos que serán ionizados en una fase gaseosa. Los iones son acelerados a través de un campo electrostático y propulsados a través del tubo de vuelo metálico, al vacío, hasta llegar al detector. Al final se obtiene un espectro, el cual podrá ser comparado con bases de datos pre-elaboradas, para la identificación del microorganismo.

La mencionada matriz, que tiene fuerte absorción óptica en la longitud del rayo láser, está compuesta de pequeñas moléculas acídicas, siendo los ácidos 2,5-dihidrobenzóico (DHB), α-ciano-4-hidroxicinámico, sinapínico, ferúlico, y 2,4-hidroxi-fenil benzóico los más utilizados. La elección para la utilización de cada uno de ellos dependerá de las biomoléculas a analizar (Croxatto et al., 2012).

Figura 3: Esquema de funcionamiento del MALDI-TOF (Croxatto et al., 2012; Dingle & Butler-Wu, 2013)

La identificación microbiana se centra en las proteínas ribosomales que tienen una relación m/z ente 3.000 y 12.000 Dalton. Posteriormente, el perfil obtenido de cada cepa es comparado con los perfiles que se encuentran en la librería (programa Biotyper). De esta manera, si obtiene una puntuación (score) que indica probabilidad de identificación a nivel de género y/o especie.

1.2.2. Estabilidad oxidativa

El deterioro oxidativo en la carne y derivados cárnicos es considerado como un proceso inevitable e irreversible que puede llegar a suceder durante el procesamiento, almacenamiento o distribución de los mismos (Estévez, 2017; Hwang, 2017). La resistencia de la carne a su oxidación (estabilidad oxidativa) depende del balance entre los agentes oxidantes de la carne y preparados cárnicos, p.ej. vitamina E, enzimas antoxidantes, sustancias reductoras, aditivos antioxidantes, y agentes que favorecen la oxidación como ácidos grasos poliinsaturados, hierro libre, etc. (Ladikos & Lougovois, 1990). Igualmente, la oxidación depende del procesado al que se somete la carne y las condiciones de almacenamiento. El ahumado, embutido en tripa, envasado anaerobio, oscuridad y bajas temperaturas de conservación retrasan la oxidación, mientras que el picado, salado, cocinado e irradiación la promueven (Ladikos & Lougovois, 1990; Soladoye et al., 2015).

El proceso oxidativo implica reacciones de oxidación tanto en fase acuosa (proteínas) como en fase lipídica (lípidos). Por un lado, la oxidación de

proteínas cárnicas resulta en una disminución en su funcionalidad (AMSA, 2012). Por otra parte, la oxidación de los lípidos en la carne, especialmente de los ácidos grasos insaturados, es una de las principales causas de pérdida de calidad o deterioro de la carne cocinada. La oxidación lipídica tiene implicaciones en el sabor (sabor a recalentado) y además implica la formación de compuestos no saludables como radicales libres y óxidos de colesterol (Faustman et al., 2010).

1.2.2.1. Oxidación del pigmento cárnico y de otras proteínas de la carne

La oxidación de la mioglobina de la carne o preparados cárnicos crudos tiene lugar por el efecto de sustancias reactivas presentes en la matriz cárnica, especialmente cuando el potencial redox, que aumenta a medida que avanza el tiempo de almacenamiento, es elevado (Santé-Lhoutellier et al., 2008). Esta oxidación produce un cambio en el color de la carne de rojo a marrón, que no es deseable para el consumidor, y genera consigo nuevas sustancias reactivas que pueden iniciar nuevos procesos de oxidación (Grunwald & Richards, 2006).

La oxidación de las proteínas cárnicas en general tiene como resultado la formación de enlaces cruzados entre proteínas y por tanto su agregación. Este fenómeno tiene consecuencias negativas sobre la textura y las propiedades funcionales de las proteínas cárnicas, como la capacidad de retención de agua o la emulsión, y también disminuye el valor biológico de la proteína (Estévez, 2011; Rodríguez-Carpena et al., 2011; Villaverde et al., 2014).

1.2.2.2. Oxidación lipídica

En la carne fresca y preparados cárnicos frescos las repercusiones de la oxidación durante su almacenamiento en crudo son limitadas. Dada la corta vida útil y la relativamente alta estabilidad oxidativa de la carne fresca, la alteración suele darse por crecimiento microbiano antes que por la oxidación de los lípidos. Sin embargo, durante el calentamiento y el almacenamiento de la carne y preparados cárnicos cocinados la oxidación tiene efectos notables. Por una parte, el calentamiento promueve la oxidación térmica de las grasas generando compuestos volátiles que influyen, dentro de los límites normales, de forma positiva sobre el aroma del producto cocinado (Domínguez et al., 2014). Por otra parte, la oxidación es la principal causa del deterioro de vida útil de la carne y preparados cárnicos durante su almacenamiento a refrigeración (Broncano et al., 2009). Durante el almacenamiento y el recalentado los lípidos de la carne experimentan una oxidación que conduce a la aparición de un sabor a carne recalentada, a veces descrito como sabor a cartón, y a la formación de compuestos tóxicos como los óxidos de colesterol (Domínguez et al., 2014). La carne cocinada es mucho más susceptible a la oxidación lipídica que la carne cruda puesto que las enzimas antioxidantes se desnaturalizan, la estructura celular se desorganiza y el hierro hémico se libera del grupo proteico (Brewer, 2007).

El proceso de oxidación de lípidos de la carne se puede dividir en tres etapas: iniciación, propagación y terminación (Gavahian et al., 2018; Ghaly et al., 2011; Hwang, 2017; Lorenzo et al., 2018; **Figura 4**). La primera eta-

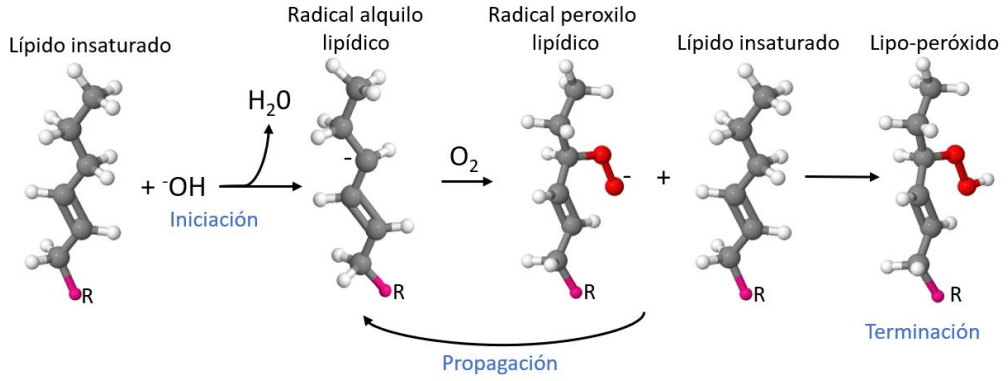

Figura 4: Fases de la oxidación lipídica. Radical carboxilo(-R), carbonos, oxígenos e hidrógenos representados por las esferas rosas, grises, rojas y blancas, respectivamente.

pa o de "iniciación" consiste en la formación de un radical libre (R•). Esta etapa comienza cuando un ácido graso insaturado (especies con alta susceptibilidad a la oxidación) pierde un hidrógeno (H•) y se forma un radical alquilo lipídico. En la segunda etapa o de "propagación" el radical reacciona con el oxígeno molecular (O_2), formando un radical peroxilo (ROO•). Durante esta etapa, el radical peroxilo reacciona con otras moléculas de ácidos grasos, normalmente insaturados, para formar un hidroperóxido (ROOH) y un nuevo radical (R•). El radical, reacciona con el oxígeno para producir otro radical peroxilo, resultando en un proceso cíclico. Finalmente, la tercera etapa o de "terminación", se caracteriza por la inactivación de los radicales libres. En esta etapa se pueden llevar a cabo dos reacciones para romper el proceso cíclico de la formación de radicales libres; la unión radical-radical para formar un producto no radical o por la unión radical libre con un donador de electrones en donde se produce el lipo-peróxido.

1.2.2.3. Metodología utilizada para determinación de la oxidación

Las pérdidas o cambios en **color** como resultado de oxidación en carne pueden ser determinadas mediante análisis instrumental, con el uso de colorímetro o análisis de visión computacional de imágenes digitales mediante la determinación espectrofotométrica/cuantitativa de formas oxidadas/reducidas de la mioglobina (ambos relacionados al sistema CIE L^* a^* b^* ;**Figura 5**), y también de manera visual por panelistas entrenados (Girolami et al., 2013; Mancini & Hunt, 2005; Tomasevic et al., 2019). Respecto al color instrumental, se utilizan variables como el cambio de color total (ΔE; que combina los cambios en coordenadas del sistema CIELab) durante el almacenamiento de carne, o los cambios en cociente a^*/b^* y los coeficientes de reflectancia de las diferentes formas oxidadas/reducidas de la mioglobina (R_{630nm}/R_{580nm} y R_{610nm}/R_{525nm} para la proporción de oximioglobina; y R_{572nm}/R_{525nm} para la proporción de metamioglobina) (AMSA, 2012; Girolami et al., 2013).

Por otra parte, la oxidación proteica causa pérdida en los grupos carbonilos de las proteínas. Con base en lo anterior, la cuantificación de carbonilos de las proteínas por el **método de la dinitrofenilhidracina** (DPNH) es el procedimiento más común para determinar la oxidación en las proteínas de la carne (Estévez, 2011). Este método se basa en la derivatización del grupo carbonilo, generando hidrazonas que pueden ser cuantificadas espectrofotométricamente a 370 nm (Armenteros et al., 2009).

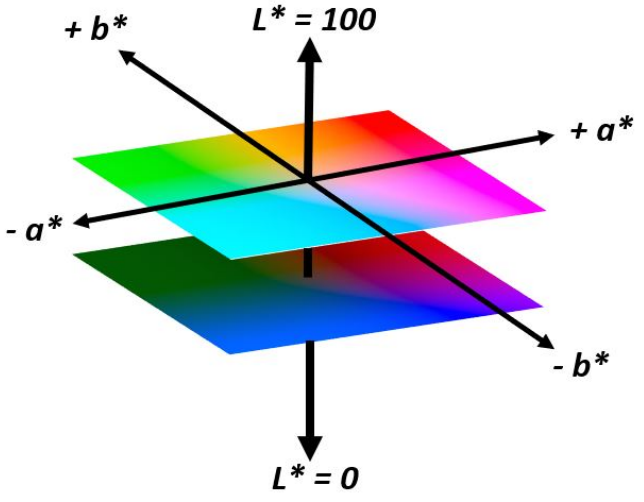

Figura 5: Coordenadas de color del sistema CIE L^* a^* b^* (CIE, 1976)

Finalmente, la oxidación lipídica de la carne se monitoriza por la determinación de diversos productos primarios o secundarios de la reacción oxidativa, que tiene como principales sustratos los triglicéridos, colesterol y fosfolípidos, producen varios metabolitos (Domínguez et al., 2019).

El método más utilizado como indicador de oxidación lipídica es la medida de las **sustancias reactivas al ácido tiobarbitúrico** (TBARS). El método determina fundamentalmente el malondialdehído (MDA), que es uno de los principales metabolitos secundarios. El método es colorimétrico y se basa en la formación un complejo cromóforo de coloración rosácea que absorbe de 532 a 535 nm al reaccionar el MDA en presencia de calor, con dos moléculas de ácido tiobarbitúrico (TBA; **Figura 6**). A la hora de utilizar este método hay que tener presente que puede plantear algunos inconvenientes. Uno de ellos radica en las posibles interferencias, ya que el TBA no

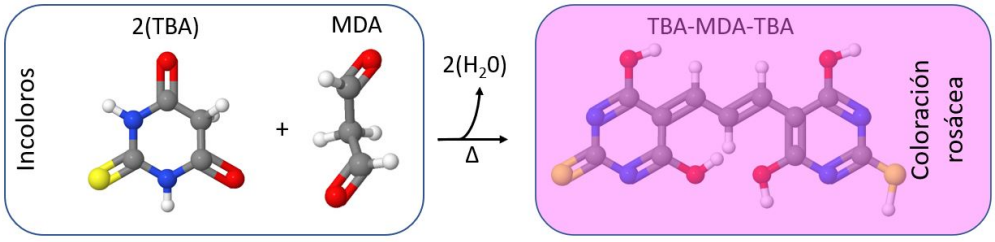

Figura 6: Reacción de dos moléculas de ácido tiobarbitúrico (TBA) con una molécula de malondialdehído (MDA). Carbonos, oxígenos, azufres, nitrógenos e hidrógenos representados por las esferas grises, rojas, amarillas, azules y blancas, respectivamente.

solo reacciona con el MDA, sino que puede interaccionar con aminoácidos, compuestos azufrados o nitritos (Estévez et al., 2009; Hwang, 2017; Irwin & Hedges, 2004; Jacobsen, 2010). Otro método frecuente para determinar la oxidación lipídica, especialmente útil en los inicios de la oxidación, es la medida de los hidroperóxidos, generalmente realizado mediante el **método yodométrico** (Estévez et al., 2009; Hwang, 2017). Aunque menos utilizado, también se describe en la bibliografía como indicador de oxidación lipídica la determinación **dienos conjugados**, que son creados a partir del re-arreglo de dobles enlaces en ácidos grasos poliinsaturados (Estévez et al., 2009; Hwang, 2017).

De forma más compleja y matizada es frecuente evaluar la oxidación lipídica mediante los metabolitos secundarios **volátiles** originados a partir de los ácidos grasos, especialmente aldehídos y cetonas, determinados por cromatografía gaseosa acoplada a espectroscopía de masas (CG:EM;

Estévez et al., 2009; Hwang, 2017). Estos compuestos han sido asociados a muchos de los olores y sabores de rancidez en la carne (Faustman et al., 2010). Los aldehídos como el propanal, 4-hidroxi-2-nonenal y hexanal han sido utilizados como índices del sabor a re-calentado de la carne cocinada (WOF; por las siglas en inglés de "*warmed-over flavor*"; Estévez et al., 2009).

Finalmente, otro método más específico de determinar la oxidación lipídica es la medida de los productos de la **oxidación del colesterol** (COPs). Los óxidos de colesterol o también conocidos como oxiesteroles, son metabolitos extensivamente estudiados debido su asociación con una amplia variedad de patologías tanto en animales como humanos (p. ej., aterogénesis, citotoxicidad, mutagénesis y carcinogénesis). La metodología comúnmente utilizada es la CG-EM (Estévez et al., 2009).

1.3. Ingredientes naturales para el alargamiento de la vida útil de la carne y los preparados cárnicos

Como se ha visto, la carne y los preparados cárnicos frescos son alimentos altamente perecederos. Por esta razón, el uso de ingredientes capaces de mantener las características de calidad durante más tiempo de almacenamiento, alargar la vida útil, es una de las estrategias empleadas por industria cárnica (Andrés et al., 2017; Jayasena & Jo, 2013).

Sin embargo, el uso de estos aditivos está regulado. En la carne no pueden utilizarse y en los preparados cárnicos su empleo está muy limitado. En la subcategoría de preparados cárnicos frescos del Reglamento (UE) No 1129/2011 (UE, 2011a) se enumeran los aditivos permitidos en este tipo de alimento, como ácido ascórbico, acético, láctico o sus sales, sulfitos y nitritos, además de sus condiciones de uso y una lista con los preparados cárnicos específicos donde se permite su utilización (SANCO, 2017). En la industria cárnica, los más utilizados han sido los sulfitos (Banks & Board, 1982; Farouk et al., 1997; Mathenjwa et al., 2012) , los ácidos orgánicos y sus sales, incluido ácido ascórbico y ascorbatos (Sahoo & Anjaneyulu, 1997; Stella et al., 2014) y los nitritos (Govari & Pexara, 2015).

Debido a estas limitaciones en el uso de aditivos y a la tendencia hacia la elaboración de productos sin aditivos, se está recurriendo al uso de ingredientes, no aditivos, ricos en antioxidantes o antimicrobianos, procedentes de plantas, tejidos animales, bacterias, hongos o algas (Falowo et al., 2014; Pisoschi et al., 2018).

Por una parte, se están utilizando ingredientes naturales ricos en antioxidantes (polifenoles, tocoferoles, etc.) subministrados en la alimentación de los animales de abasto de forma que puedan llegar a la carne y aumente así su estabilidad oxidativa (Jiang & Xiong, 2016). En esta estrategia se ha observado que la ingesta de determinados antioxidantes de la dieta de los animales puede retardar la oxidación de la carne y que, además, los antioxidantes pueden tener efecto positivo sobre la salud del animal. En el

caso de la alimentación de rumiantes el uso de subproductos ricos en antioxidantes combina su posible efecto beneficioso sobre la estabilidad de la carne con la disminución del impacto ambiental que supone la utilización de estos subproductos (Salami et al., 2019).

Por otra parte, se está recurriendo al empleo de ingredientes naturales, tanto con actividad antioxidante como antimicrobiana, en la formulación de los preparados cárnicos (Hugo & Hugo, 2015; Zhang et al., 2016). El uso de ingredientes con compuestos activos de origen natural en los preparados cárnicos se ha convertido en una de las estrategias de importancia en el desarrollo de preparados cárnicos (Davidson et al., 2015; Hygreeva et al., 2014; Islam et al., 2017; Munekata et al., 2020; Zhou et al., 2010). El uso de estos ingredientes se considera como alternativa potencial al uso de aditivos sintéticos (Martínez-Graciá et al., 2015).

Las investigaciones realizadas sobre el uso de antimicrobianos naturales en los derivados cárnicos se orientan a su efecto sobre la vida útil y sobre la inhibición del crecimiento de determinados microorganismos patógenos de interés (Calo et al., 2015; Hugo & Hugo, 2015; Pisoschi et al., 2018). Los ingredientes naturales con efecto antimicrobiano recogidos en diversos trabajos de investigación son de origen diverso: enzimas de origen animal (lisozima, lacto-peroxidasa), proteínas como la lactoferrina, péptidos antimicrobianos de diversos orígenes, plantas o sus extractos ricos en polifenoles y terpenoides, bacteriocinas o compuestos antimicrobianos procedentes de algas (Quinto et al., 2019).

Los materiales ricos en antioxidantes incluyen ingredientes comestibles, convencionales o no, como hierbas, especias, frutas (pulpa, piel, semillas), frutos secos, cereales, etc., o sus extractos. Ejemplos de antioxidantes que han demostrado efecto sobre la oxidación de los derivados cárnicos son las semillas de uva, la piel del grano de café, el tomillo, el salvado de arroz, la mejorana, entre otros (Ahmad et al., 2015; Embuscado, 2015; Regazzoni et al., 2016; Shahidi & Ambigaipalan, 2015). Los compuestos bioactivos que contienen son de diversa naturaleza, incluyendo polifenoles, terpenoides, sales, proteínas quelantes, productos de la reacción de Maillard, o hidrolizados de proteínas, entre otros.

Los estudios mencionados en los dos párrafos anteriores demuestran cómo el uso de determinados compuestos antimicrobianos o antioxidantes en los preparados cárnicos, solos o preferentemente en combinación con otros antimicrobianos o antioxidantes naturales, con aditivos, o con barreras como cambios en el pH, la actividad de agua o un envasado adecuado, constituye una herramienta eficaz para retardar el crecimiento de microorganismos alterantes o patógenos o los fenómenos oxidativos, respectivamente. No obstante, hay que considerar en cada caso las posibles interacciones del compuesto activo con la matriz del alimento, que pueden mermar su eficacia, su posible efecto sobre las propiedades sensoriales del preparado cárnico, la viabilidad de su uso respecto al incremento en el coste del alimento, los posibles efectos adversos sobre la salud de los consumidores o los impedimentos legislativos.

1.3.1. Astaxantina

La astaxantina (3,3'-dihidroxi-β,β-caroteno-4,4'-diona) es un ceto carotenoide de intensa coloración roja presente en los diversos tejidos de varias especies de crustáceos, salmónidos y aves, en levaduras, bacterias y microalgas como *Haematococcus pluvialis* (Ambati et al., 2014; Guerin et al., 2003; Kidd, 2011; Kobayashi et al., 1997). En la actualidad la astaxantina se utiliza como suplemento alimenticio para humanos (Turck et al., 2020).

La astaxantina presenta una estructura similar a los beta-carotenos (**Figura 7**). La diferencia principal radica en los dos anillos polares (hidrofílicos) localizados a los extremos de la molécula – la molécula presenta una naturaleza anfipática (Fakhri et al., 2018; Ng et al., 2020). La coloración rojiza de la astaxantina la obtiene debido a la absorción de luz que le otorga la parte no polar (hidrofóbica), es decir, los dobles enlaces de la región poliinsaturada en la cadena de carbonos.

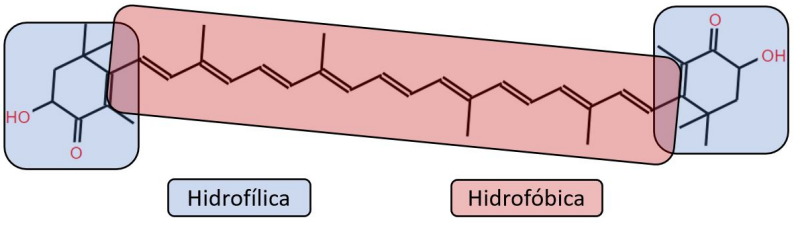

Figura 7: Estructura molecular de la astaxantina.

A consecuencia de dicha estructura anfipática, la molécula es capaz de ejercer acciones biológicas a través de las membranas celulares (**Figura 8**; Ambati et al., 2014; Ng et al., 2020). Se ha demostrado que entre dichas

acciones biológicas la astaxantina presenta una considerable actividad antioxidante – los grupos polares son capaces de atrapar radicales libres u otras especies reactivas al oxígeno –, además de actividades antinflamatoria, antidiabética o reparadora de DNA, entre otras (Ambati et al., 2014; Guerin et al., 2003; Higuera-Ciapara et al., 2006; Ng et al., 2020).

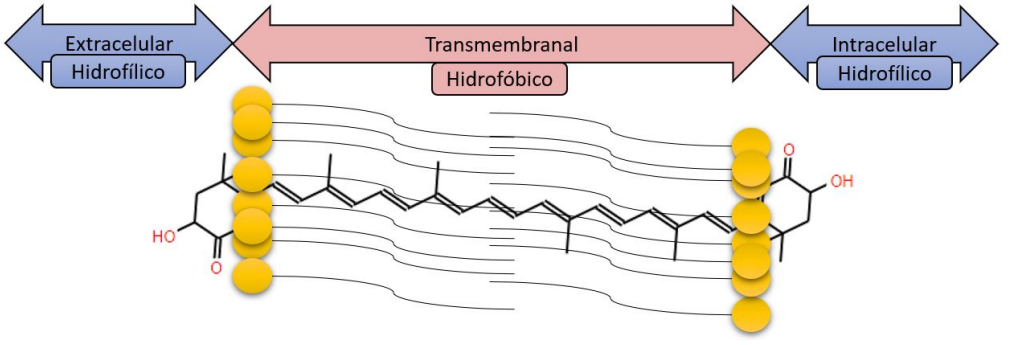

Figura 8: Posicionamiento transmembranal de la astaxantina (Ambati et al., 2014)

Aunque su uso actualmente como ingrediente en derivados cárnicos no está permitido, sí se permite en la cría de ciertos animales y, experimentalmente, se ha visto que puede ser de interés en la mejora del color y la estabilidad oxidativa de la carne y derivados. La astaxantina se usa corrientemente como suplemento de la dieta de trucha, salmón y crutáceos criados en cautividad (Turck et al., 2020). La adición de astaxantina en la dieta de salmones y truchas ha demostrado mejorar la estabilidad oxidativa y el color de su carne (Barbosa et al., 1999; Young et al., 2017). Hay estudios de investigación que prueban que el uso de astaxantina en dietas para animales de granja puede mejorar también la calidad de la carne. Se ha descrito que

cuando es añadida a la dieta de pollos puede aumentar la ganancia de peso y aumentar la coloración roja de su carne (Akiba et al., 2001; Jeong & Kim, 2014; Perenlei et al., 2014). Su suplementación en la dieta de cerdos puede tener efecto positivo sobre la estabilidad lipídica y la coloración roja de su carne (Carr et al., 2010; Lei & Kim, 2014). Por otra parte, hay un estudio que evalúa el efecto de la adición de astaxantina sobre la carne picada en la que se observa una mejora en el color y la estabilidad oxidativa durante su almacenamiento (Pogorzelska et al., 2018).

1.3.2. Lúpulo

El lúpulo (*Humulus lupulus*) es una planta perteneciente a la familia *Cannabaceae*, siendo su flor hembra sin fecundar comúnmente utilizada en la elaboración de cerveza. Se caracteriza por contener compuestos fenólicos prenilados (principalmente acil-floroglucinol y flavonoides), α- y β-ácidos, xanthohumol, humuleno, cariofileno, miriceno, β-cariofileno, y farneseno. Estos compuestos proporcionan a la cerveza su sabor/olor característico y algunos de ellos presentan cualidades antimicrobianas y antioxidantes (Attokaran, 2017; Bocquet et al., 2018; Bogdanova et al., 2018; Canbaş et al., 2001; Kramer et al., 2015; Steinhaus et al., 2007).

Los α-ácidos (humulonas) y β-ácidos (lupulonas) son los componentes principales de las llamadas resinas de la flor del lúpulo. Los α-ácidos, después de su isomerización por calor (**Figura 9**), son responsables del amargor de la cerveza y tienen cierta actividad antimicrobiana (Bocquet et al.,

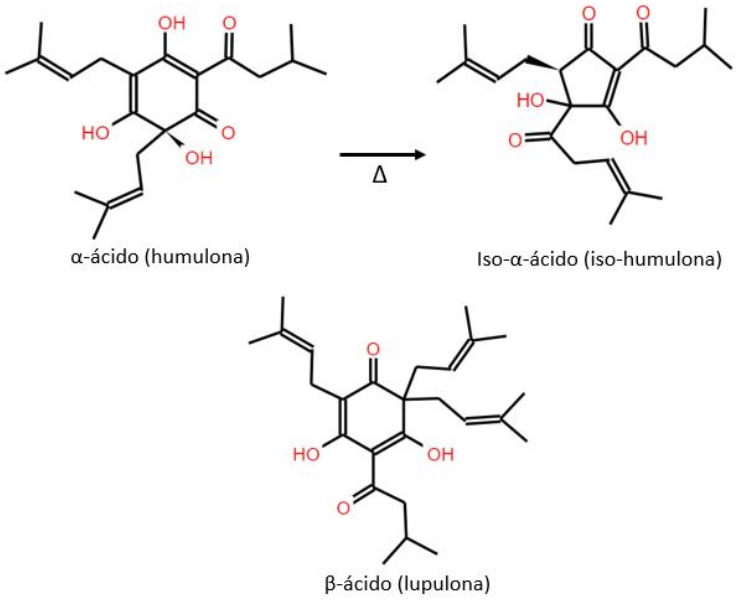

Figura 9: Componentes principales de las resinas de la flor de lúpulo.

2018). Por otro lado, los β-ácidos, debido no tener la capacidad de isomerizarse, presentan menor sabor, aunque una mayor actividad antimicrobiana (Attokaran, 2017; Bocquet et al., 2018; Haas & Barsoumian, 1994). Esta actividad antimicrobiana esta principalmente dirigida contra bacterias Gram-positivas y, en menor medida, contra bacterias Gram-negativas, hongos y levaduras (Bocquet et al., 2018; Bogdanova et al., 2018; Kramer et al., 2015). Adicionalmente el xantohumol, un flavonoide del lúpulo, ha mostrado efecto sobre el crecimiento de las bacterias Gram-positivas, en particular ante cepas de *Staphylococcus* y *Streptococcus* (Gerhäuser, 2005), además de ejercer actividad in-vitro quimiopreventiva contra el cáncer (Zanoli & Zavatti, 2008).

Además del efecto antimicrobiano, diversos compuestos del lúpulo pre-

sentan otras actividades biológicas como capacidad antioxidante, antiinflamatoria y anticancerígena (Hrnčič et al., 2019), siendo los compuestos fenólicos los principales responsables de la actividad antioxidante (Lermusieau et al., 2001; Villalobos-Delgado et al., 2015).

El lúpulo, con base en sus propiedades beneficiosas para la salud animal, ha sido aprobado como suplemento en la dieta de cerdos (Reglamento UE 2019/111; UE, 2019). Esta suplementación podría afectar a la estabilidad oxidativa de la carne. Se han desarrollado algunas investigaciones destinadas a la evaluación del efecto del lúpulo como suplemento en la dieta de los animales sobre la calidad carne de cerdo (Sbardella et al., 2018) y pollo (Zawadzki et al., 2018). En estos estudios se ha encontrado un aumento en la estabilidad oxidativa atribuible al lúpulo. No obstante, Blanco et al. (2018) evaluaron el efecto de la incorporación de pellets de lúpulo en alimento para cordero y sus resultados no mostraron efecto antioxidante en la carne de los corderos evaluados.

El uso de lúpulo como ingrediente funcional antimicrobiano y antioxidante en diversos alimentos, además de la cerveza, incluido los derivados cárnicos, es prometedor. Los β-ácidos de lúpulo como aditivo conservante en carne cocinada y tripas están permitido en EEUU (Singh et al., 2014). Los estudios de Shen et al. (2009), Kramer et al. (2015) y Nieto et al. (2020) muestran un efecto antimicrobiano del lúpulo o sus extractos frente a bacterias Gram-positivas cuando son agregados en las formulaciones de embutidos cocidos y las soluciones de marinado de la carne. Por otra par-

te, Villalobos-Delgado et al. (2015) demuestran que el uso de extractos de lúpulo propicia un aumento en la estabilidad lipídica en las hamburguesas.

1.3.3. Aceites esenciales: *Zataria multiflora*

Tradicionalmente, se han utilizado diversas plantas por su potencial terapéutico o medicinal, con la finalidad de tratar desordenes comunes y enfermedades degenerativas. La efectividad de estos tratamientos, en muchos casos, ha sido atribuida a diversos metabolitos secundarios presentes en los aceites esenciales (AEs) de dichas plantas (Duarte et al., 2017; Voon et al., 2012). Los AEs están formados por una mezcla compleja de compuestos volátiles y semi-volátiles, con potente aroma, que son solubles en disolventes orgánicos. Mayoritariamente, esta mezcla está compuesta por fenoles no polares, terpenoides, terpenos y fenilpropenos. Los compuestos de los AEs han sido asociados con actividades antioxidante y/o antimicrobiana (Hosseini et al., 2019).

Existen diversos métodos de obtención de AEs que comúnmente son divididos en dos grupos: a) **los convencionales**, como la hidro destilación, la destilación por vapor y la extracción con solventes, maceración, infusión y decocción; y b) los **métodos de extracción novedosos**, como la extracción asistida por ultrasonidos o microondas, la extracción por fluidos supercríticos, la extracción por campos eléctricos pulsados, la hidro destilación por microondas al vacío, o varias combinaciones de las anteriores (Roohinejad et al., 2017; Voon et al., 2012).

Debido a su poder antimicrobiano y antioxidante, el uso de AEs está presente en la industria alimentaria (Hosseini et al., 2019). No obstante, la cantidad de aceite esencial necesaria para producir efecto inhibidor sobre los microorganismos de los alimentos (0,5 y 2 g/100 g; Burt, 2004) puede afectar considerablemente al aroma de los alimentos a los que se añade. Para evitar usar cantidades tan elevadas se aconseja combinar los aceites esenciales con otras tecnologías que sumen efecto biológico o presenten sinergismo con los primeros (Matan et al., 2006). También se propone la incorporación de AEs en envases que actúen sobre los microorganismos de la superficie de los alimentos (Lorenzo et al., 2014; Ribeiro-Santos et al., 2017). Otros factores a considerar con respecto al uso de AEs en los derivados cárnicos son las interacciones con la matriz cárnica, la posible toxicidad o capacidad para desencadenar alergias o la estandarización del AEs de forma que su composición sea constante entre diferentes lotes (Ribeiro-Santos et al., 2017).

Uno de los aceites esenciales que ha mostrado potencial para su empleo en la industria cárnica es el de la planta *Zataria multiflora* Boiss (ZM), conocida también como *Zataria bracteata* Boiss y *Zataria multiflora* var. *elatior* Boiss. Es una planta que pertenece a la familia *Lamiaceae* y presenta propiedades similares al tomillo (*Thymus vulgaris*). Se usa frecuentemente en Irán, Pakistán y Afganistán para tratar dolores de cabeza, migrañas, el resfriado común, diarrea, vomito, fiebre, entre otros, y como agente saborizante en la cocina tradicional (Basti et al., 2016; Saei-Dehkordi et al., 2010; Sajed et al., 2013).

Sajed et al. (2013) mencionan que los mono terpenos oxigenados (principalmente timol y carvacrol) son los compuestos más abundantes (alrededor del 70 %) en el AE de ZM extraído por hidrodestilación, seguido por los hidrocarburos mono terpénicos, los hidrocarburos sesquiterpénicos y los sesquiterpenos oxigenados (Basti et al., 2016; Saleem et al., 2004). El timol y el carvacrol (**Figura 10**) tienen una probada actividad antimicrobiana (Saei-Dehkordi et al., 2010). Además de la actividad antimicrobiana, el AE de ZM ha demostrado presentar actividad antifúngica, antioxidante y ser repelente de insectos (Basti et al., 2016).

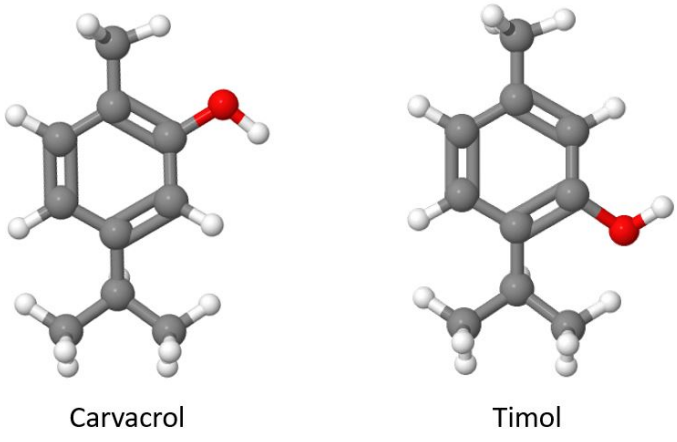

Figura 10: Principales monoterpenos presentes en el aceite esencial de *Zataria Multiflora*.

En la actualidad, existen diversos ensayos *in-vitro* que demuestran la actividad antioxidante y antimicrobiana, contra microorganismos patógenos y alterantes, del aceite esencial de ZM. Sin embargo, son pocos los estudios realizados que evalúen el efecto del aceite esencial dentro de una

matriz alimentaria. Tajik et al. (2015) evaluaron el efecto del aceite esencial de *Zataria multiflora* añadido al 0,1 % a carne picada de búfalo, donde observaron una disminución del crecimiento de todos los microorganismos evaluados (mesófilos totales viables, psicrófilos, *Pseudomonas* y BAL), además de un decremento en la oxidación lipídica al final del almacenamiento. Por otro lado, Torab et al. (2017) evaluaron el efecto de la incorporación de ZM (hasta 0,03 %) en carne para hamburguesa. Los resultados indicaron que la adición del AE lograba reducir significativamente el crecimiento de bacterias mesófilas, psicrófilos, hongos y levaduras y que además mejoraba la estabilidad oxidativa.

2

Justificación y Objetivos

Con el presente trabajo se ha buscado contribuir de forma amplia a la mejora del sector cárnico mediante el estudio de estrategias concretas que contemplan el uso de algunos ingredientes naturales con efecto antioxidante y/o antimicrobiano para la mejora de la calidad de la carne y los preparados cárnicos (principalmente que alarguen su vida útil).

Se ha trabajado con carne ovino dada la importancia de la carne de esta especie en la región y la tendencia al descenso en su consumo y como ingredientes naturales a evaluar, se optó por el uso de astaxantina, lúpulo y aceite esencial de *Zataria multiflora*.

La astaxantina se eligió por ser un potente antioxidante, potencialmente inocuo en dosis que no superen una cantidad determinada, que puede ser añadido en la dieta de animales. Su uso está permitido como suplemento alimentario y como ingrediente alimentario está despertando interés creciente.

El uso de lúpulo se consideró por ser altamente producido en la región y por haber hecho estudios previos por este grupo investigador. Además, el lúpulo ha sido tildado de producto inocuo y saludable, usado de forma tradicional en la elaboración de cerveza, y presenta efectos tanto antimicrobiano como antioxidante.

Igualmente se incluyó en el plan experimental un aceite esencial, también con efectos antimicrobianos y antioxidantes, cuyo uso puede ser de interés para la industria cárnica. El hecho de usar aceite esencial de *Zataria multiflora* es consecuencia de la colaboración con investigadores de Irán que han trabajado previamente con este aceite.

Los objetivos específicos planteados con la astaxantina fueron dos:

- a) evaluar el efecto de la incorporación de astaxantina en fórmula lacto-reemplazante para la alimentación de corderos lechales sobre la calidad de su carne
- b) conocer el efecto de la adición de astaxantina en un preparado cárnico fresco, hamburguesa de cordero, sobre el color, la estabilidad oxidativa de dichas hamburguesas.

Los objetivos específicos planteados con el lúpulo y el aceite esencial fueron los siguientes:

- a) estudiar el efecto de la adición de lúpulo y aceite esencial, por separado o de forma combinada, en la formulación de salchichas frescas de

cordero sobre el crecimiento de microorganismos alterantes y la formación de aminas biógenas durante su almacenamiento en atmósfera modificada anaerobia

- b) evaluar el efecto de la adición de lúpulo en las salchichas frescas sobre la formación de compuestos sápidos y volátiles del espacio de cabeza de los embutidos durante su almacenamiento en atmósfera modificada anaerobia.

3

Effects of dietary astaxanthin supplementation on the oxidative stability of meat from suckling lambs fed a commercial milk-replacer containing butylated hydroxytoluene

Diego E. Carballo, F. Javier Giráldez, Sonia Andrés, Irma Caro, Miguel Fernández-Gutiérrez, Javier Mateo

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Effects of dietary astaxanthin supplementation on the oxidative stability of meat from suckling lambs fed a commercial milk-replacer containing butylated hydroxytoluene

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ABSTRACT

Meat colour and lipid oxidative stability can be improved by adding antioxidants to animal diet. This study investigated the effects of the addition of astaxanthin to a butylated hydroxytoluene (BHT)-containing commercial milk-replacer, at a rate of 25 mg of astaxanthin/kg of milk-replacer powder, on suckling lamb meat quality. Twenty newborn (2 day old) lambs allocated to individual pens were artificially reared for 22 days. Ten lambs (Control) were fed a commercial milk-replacer and the other ten (Astaxanthin) received the same milk-replacer but included astaxanthin. After the feeding trial, meat and fat colour, astaxanthin and BHT levels in meat, oxidative stability in refrigerated and frozen raw meat and refrigerated cooked meat, and meat volatiles in cooked meat were determined. Astaxanthin in artificially reared suckling lambs at the levels used reduced the accumulation of BHT in the meat, slightly affected meat colour, by reducing meat lightness and increasing meat and fat redness, and increased the lipid stability of frozen meat.

1. Introduction

Meat colour and flavour can deteriorate during aerobic storage due to oxidation processes (Cunha et al., 2018). At present, there is a growing interest on the use of antioxidant-rich natural sources, or their extracts, in animal feeding in order to improve meat oxidative stability (Franz, Baser, & Windisch, 2010; Venskutonis, 2004). According to several studies, the combination of two or more dietary antioxidants can derive some advantage over the use of a single compound as oxidation processes follow several mechanisms; hence a mixture of bioactive molecules could affect different oxidizing agents and targets, which might result in additive or synergistic effects (Hamdo, Khayata, & Al-Assaf, 2014; Romano, Abadi, Repetto, Vojnov, & Moreno, 2009).

Dietary supplementation with astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) as a natural antioxidant has been the subject of recent research (Ambati, Phang, Ravi, & Aswathanarayana, 2014; Naguib, 2000). Astaxanthin is a red pigment belonging to the xanthophyll carotenoids and its special molecular structure compared with other carotenoids lets astaxanthin adopt a transmembrane orientation in animal cells that allows it to scavenge free radicals and inhibit the radical chain reaction both near the membrane surface and within the membrane (Goto et al., 2001; Kobayashi, 2000). Dietary supplementation of astaxanthin in the diet of orange-flesh fishes such as salmon or trout have been demonstrated to improve the oxidative stability of the flesh (Barbosa, Morais, & Choubert, 1999; Young, Pritchard, White, & Davies, 2017). Recent studies have extended the focus on the use of astaxanthin or astaxanthin-rich raw materials as a dietary supplement to meat animals such as broilers or pigs (Akiba et al., 2001; Carr, Johnson, Brendemuhl, & Gonzalez, 2010; Jeong & Kim, 2014; Lei & Kim, 2014; Perenlei et al., 2014; Yang et al., 2006). The amounts of astaxanthin tested in meat animal experimental diets have ranged from 1 to 66.7 mg of pure astaxanthin per kg of feed.

Butylated hydroxytoluene (BHT) is a synthetic antioxidant commonly used as an additive in animal feed to stabilize feed lipids and thus protect fat against oxidation. The BHT ingested by livestock can accumulate in animal tissues through absorption from the lumen and then distributed to body lipids (Nieva-Echevarría, Manzanos, Goicoechea, & Guillén, 2015). The presence of dietary BHT in meat, on one hand, can improve its oxidative stability, and, on the other hand, it can be a matter of concern. Consumption of food containing large

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amounts of BHT has been linked to undesired human health problems such as centrilobular necrosis and haemorrhage in the liver (Yehye et al., 2015). However, several studies have suggested that BHT intake at low dose may have beneficial effects reducing human cancer and protecting against toxic compounds originated by oxidation of fatty acids (Botterweck, Verhagen, Goldbohm, Kleinjans, & van den Brandt, 2000; Williams, Iatropoulos, & Whysner, 1999). Nevertheless, due to the widespread use of this synthetic antioxidant, the daily intake could be greater than the acceptable daily intake, i.e. 0.25 mg/kg of body weight/day (EFSA, 2012). Therefore, alternatives to reduce its content in feeds and meat should be evaluated.

In intensive dairy and/or sheep systems, lambs are usually artificially reared using milk-replacers that contain synthetic lipid soluble antioxidants such as butylated hydroxytoluene (BHT). In a previous study (Osorio, Zumalacárregui, Cabeza, Figueira, & Mateo, 2008), the presence of BHT in the suckling lamb meat from artificially reared lambs was suggested as being an important reason to explain the higher oxidative stability in suckling lamb meat from milk-replacer rearing systems compared to that from ewes' milk rearing systems.

It has been reported that the combination of astaxanthin with other antioxidants, i.e. tocotrienol, induces synergistic scavenging activity by intermolecular interactions between the two antioxidants (Kamezaki et al., 2016); however, as far as we know, there are no published studies evaluating the complementarity of astaxanthin with BHT either in in vitro or in vivo models. Taking into account that the combination of the natural antioxidant astaxanthin and the synthetic antioxidant BHT contained in suckling lamb milk-replacers could be a suitable approach to increase the oxidative stability of the meat from artificially reared suckling lambs and eventually to reduce the levels of BHT in milk-replacers, the aim of this study was to investigate the effects of astaxanthin supplementation, added at levels close to that recommended for humans as a dietary supplement, on the meat quality, i.e. colour and oxidative stability, of the meat of artificially reared suckling lamb.

2. Materials and methods

2.1. Animals and slaughtering

The animal practices carried out in this research followed the recommendations of the Directive 2010/63/EU of the European Parliament and of the European Council on the protection of animals used for scientific purposes and of the Animal Experimentation Committee from the Instituto Ganadería de Montaña - Consejo Superior de Investigaciones Científicas (IGM-CSIC; Grulleros, León, Spain; protocol number 100102/2017-1).

Twenty newborn female Assaf lambs were used in this experiment, which were reared between January and February 2017. After birth, all lambs were treated with an intramuscular injectable selenium + vitamin E complex at the prescribed dose (Vitasel: 5 g α-tocopherol acetate, 110 mg sodium selenite, excipient, per 100 mL; Laboratorios Ovejero, León, Spain) to prevent nutritional muscular dystrophy (white muscle disease) and they were kept with their dams for 2 days. The lambs were then randomly distributed in two experimental groups ($n = 10$), each animal was allocated to individual pens and artificially reared ad libitum using automatic feeders (Industrias JR, Leon, Spain). Lambs from the first group (Control) were fed a commercial milk-replacer (200 g/L of reconstituted milk, Cordevit50, Lemasa, Spain), whose ingredients and chemical composition are shown in Table 1. The lambs of the other group (Astaxanthin) received the same milk-replacer but included 1 g of astaxanthin-commercial powder (AstaReal®EL25, Nacka, Sweden; containing 2.5% natural astaxanthin) per kg of milk-replacer powder. This amount corresponds to 25 mg of pure astaxanthin/kg milk-replacer powder. The antioxidant potential showed by astaxanthin, as extracted from the astaxanthin commercial power with ethyl acetate and determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Serpen, Gökmen, & Fogliano, 2012), was 0.15 mmol Trolox equivalents per g of extract. The amount of astaxanthin added to the milk-replacer was such that the daily intake of pure astaxanthin by the lambs during the experiment was close to that recommended for humans as a dietary supplement, i.e. 6 mg/day (Ambati et al., 2014).

Table 1
Ingredients and chemical composition of the commercial milk-replacer.

Ingredients	Amount (g/kg)
Skim milk powder	500
Milk whey powder	160
Vegetable oil (cocoa)	140
Animal fat (pork lard)	120
Whey protein powder	40
Hydrolysed wheat protein	15
Yeast/sodium bicarbonate /dextrose	15
PreVIT mineral[a]	10
Chemical composition	
Dry matter	960
Crude protein (in dry matter)	235
Ether extract (in dry matter)	260
Ash (in dry matter)	70
Butylated hydroxytoluene[b]	0.078

[a] Concentration in milk replacer as provided the supplier: vitamin A, 80,000 IU; vitamin D_3, 4250 IU; vitamin E, 100 IU; iron, 40 mg; cobalt, 0.2 mg; copper, 5 mg; manganese, 25 mg; zinc, 30 mg; selenium, 0.2 mg.
[b] Concentration determined following the method described by Yang et al. (2002) with modifications.

The experimental feeding period lasted 22 days, during this period lambs were weighed twice weekly and at the end lambs were slaughtered after a 12 h fasting period. Animals were weighed, stunned, slaughtered by exsanguination from the jugular vein, eviscerated and skinned, and the corresponding carcasses were chilled at 4 °C. The mean growth rate and body weight at slaughtering of Control ($n = 10$) and Astaxanthin ($n = 10$) lambs were, respectively, 0.29 kg/day and 0.28 kg/day, and 11.6 kg and 11.9 kg ($P > .05$; Student-*t*-test).

2.2. Assessment of carcass and meat quality

At 24 h post-mortem the cold carcass weight (CCW) was recorded, the dressing percentage was calculated as percentage of body weight just before slaughtering, and the pH value was determined in the *M. longissimus thoracis* (LT) muscle using a puncture pH meter (Metrohm, Zofinger, Switzerland). The carcasses were then cut in two halves and the right halves were weighed and jointed. The joints were weighed and the percentage of each joint in total weight of joints were calculated (Colomer-Rocher, Morand-Fehr, Delfa, & Sierra Alfranca, 1988). Afterwards, LT and *M. longissimus lumborum* (LL) and *m. biceps femoris* (BF) and perirenal fat were dissected from the corresponding right-half carcass joint for meat quality analysis, and the perirenal fat was weighed.

Meat quality characteristics were determined in raw meat (fresh, refrigerated and frozen stored) and fat (fresh), and in cooked meat (Table 2). Fresh raw meat (LL) was analysed for chemical composition, i.e. proximate composition, and astaxanthin and BHT contents. Colour stability and oxidative stability of lipids (measured as thiobarbituric-acid reactive substances; TBARS) of raw meat (LT) during a 7 days aerobic refrigerated storage period were also investigated. Perirenal fat was used for fat colour analysis. The TBARS levels were also determined in frozen meat after a 3 month storage period (−18 °C) using BF samples. The analysis carried out on cooked meat consisted of TBARS determination in a cooked LT portion after a 1 day aerobic refrigerated storage period and the volatile profile of BF samples immediately after cooking, as an approach to evaluate the lipid degradation during meat heating.

Table 2
Quality characteristics evaluated in raw meat and fat and cooked meat, and anatomical region used for the analyses.

Analysis	Anatomical region
Refrigerated raw meat and fat	
Proximate composition, butylated hydroxytoluene, astaxanthin	*Longissimus lumborum*
Meat colour and colour an lipid oxidative stability after a 7-day refrigerated storage	*Longissimus thoracis*
Fat colour	Perirenal fat
Frozen raw meat	
Lipid oxidative stability after a 3-month frozen storage	*Biceps femoris*
Cooked meat	
Lipid oxidative stability after a 1-day refrigerated storage	*Longissimus thoracis*
Lipid degradation due to meat heating (volatiles)	*Biceps femoris*

2.3. Chemical composition, colour of meat and fat and meat colour stability

Before analyzing the chemical composition, LL were transversally divided in two similar portions – one was frozen at −80 °C, lyophilized and then used for proximate composition analysis; and the other was frozen at −80 °C before being used for BHT and astaxanthin analyses. Proximate composition analysis (moisture, protein, fat and ash) was carried out in duplicate following the methods described by the Association of Official Analytical Chemists (AOAC, 2003). The astaxanthin content was determined using the spectrophotometric method described by Tolasa, Cakli, and Ostermeyer (2005). A standard curve was generated using an astaxanthin standard (Sigma-Aldrich Química S.L, Madrid, Spain). Absorbance of both standard and sample solutions were measured at 472 nm using a VWR UV-3100PC spectrophotometer (VWR International, Auckland, New Zealand).

The BHT content (mg/kg of intramuscular fat) was determined in duplicate following the Bligh and Dyer (1959) method for lipid extraction and then using the method of Yang, Lin, and Choong (2002) with modifications for BHT chromatographic analysis. Briefly, the lipids from a 10 g muscle sample to which 8-quinolinol (8Q) was added (300 μL of a 0.01% *w/v* solution of 8Q in methyl-acetate) as an internal standard were first extracted and the solvents were evaporated. Then the lipids extracted were dissolved in 2 mL of toluene and analysed using the gas chromatography–mass spectrometry (GC–MS) technique. The injection volume was 2 μL and the injector operated at a temperature of 260 °C and in the splitless mode. The compounds were separated with a VF-5MS column (50 m × 0.25 mm ID × 0.25 μm film thickness; Varian, Palo Alto, CA, USA) using helium as carrier gas with a flow rate of 1.5 mL/min. The oven conditions were: initial temperature of 38 °C held for 1 min, increased to 50 °C at 10 °C/min, then to 146 °C at 4 °C/min, then to 200 °C at 20 °C/min and finally to 250 °C at 50 °C/min, maintaining this temperature for 11 min. The transfer-line temperature was 260 °C. The mass spectrometer operated in scan and selected ion monitoring (SIM) modes for identification and quantification of BTH and 8Q, respectively, with an electron source temperature of 240 °C, electron energy and emission currents of 70 eV and 35 μA, respectively, and a quadrupole temperature of 190 °C. The scanning range was from *m/z* 40 to *m/z* 250 and the observed ions within the SIM mode were (*m/z*) 117, 145, 205 and 220 – the first two are the major ions in the 8Q spectrum and the other two in the BHT spectrum.

Following dissection, the LT was transversally divided in two similar portions. One of them was cut into 2 cm width slices for meat colour analysis. One of the slices was allowed to bloom for 60 min at room temperature and the colour of the cut surface was immediately analysed (colour at day 0). Two other slices were placed after cutting in polypropylene rigid trays and covered with an 8.5 μm polyvinylchloride film, and the trays were then stored in darkness at 4 °C for 7 days in order to evaluate the colour stability. Meat display colour was determined in triplicate on the upper surface of each of the two slices on storage days 1, 3, 5 and 7. Additionally, fat colour was determined on the external surface of fresh recently dissected perirenal fat. For all meat and fat colour measurements, the instrumental colour was determined in triplicate directly on the meat slice or fat depot surface, using a Konica Minolta CM-700d colorimeter (Osaka, Japan) with D65 illuminant in SCI mode, a visual angle of 10°, an 11 mm aperture for illumination and 8 mm for measurement. The recorded parameters were the whole spectrum and lightness (L^*), redness (a^*) and yellowness (b^*). Moreover, the total colour change (ΔE) due to astaxanthin supplementation in the meat cut after the 60 min blooming period was calculated using the formula $[(L^*\ \text{Astaxanthin} - L^*\ \text{Control})^2 + (a^*\ \text{Astaxanthin} - a^*\ \text{Control})^2 + (b^*\ \text{Astaxanthin} - b^*\ \text{Control})^2)]^{1/2}$ (AMSA, 2012). Additionally, a*/b* ratio and the hue angle (degrees), as [arctangent (b*/a*) × (360/2π)], both related to meat discoloration (AMSA, 2012), were calculated for the stored slices on the different storage days.

2.4. Lipid oxidative stability due to storage and lipid degradation due to heating

Lipid oxidative stability was measured as 2-thiobarbituric acid reactive substances (TBARS) content of raw, frozen and cooked meat. TBARS levels in refrigerated raw meat were determined using the remaining slice of LT packaged in the polypropylene rigid tray (abovementioned) on storage day 7. Half of the BF, obtained by transversally cutting the muscle after dissection, was used for the analysis of TBARS in frozen raw meat (frozen in polyvinylchloride film at −18 °C for 3 months, then thawed at 4 °C for 24 h). Finally, TBARS of cooked meat was determined in a portion of LT, which after being obtained, was cooked using a pre-heated clam-shell grill at 180 °C to an internal temperature of 75 °C in the geometric center of the portion (measured by a Digi-Sense thermocouple probe, Cole-Parmer Instrument Company, Vernon Hills, IL, USA). Afterwards, the muscle portion was cooled to room temperature, wrapped with the polyvinylchloride film, stored at 4 °C for 24 h and finally reheated in a domestic microwave (400 W) for 2 min. The method used for all the TBARS analysis was that described by Nam and Ahn (2003).

The evaluation of lipid degradation during meat heating was carried out by analyzing in duplicate the volatile content in the head space of cooked meat samples using gas chromatography coupled with mass spectrometry (GC–MS) and following the method described by Carballo, Caro, Andrés, Giráldez, and Mateo (2018). Before analysis, the meat samples (the remaining half of the BF) were vacuum packaged, cooked in a water bath at 80 °C for 30 min and then cooled with tap water. The results were expressed in ng equivalents of hexanal/mL of headspace.

2.5. Statistical analysis

All the analysis were carried out by means of the general linear model analysis of variance (ANOVA) using the SPSS Statistics software (version 23; IBM, Somers, NY, USA). Carcass quality characteristics, proximate composition, BHT, TBARS analysis, colour of fat and meat, BHT and volatile contents were analysed by one-way ANOVA using the feeding treatment as a fixed factor. Colour changes during refrigerated storage were analysed by repeated measures ANOVA where the fixed factor was the feeding treatment and the repeated-measures factor was the storage time. A post-hoc analysis of the ANOVA results for the repeated-measures factor (storage day) was carried out using the Bonferroni test with a significance level of $P < .05$.

3. Results and discussion

3.1. Carcass characteristics and meat composition

Table 3 shows the results of carcass characteristics and meat pH and

Table 3
Effect of feeding suckling lambs with astaxanthin-supplemented[a] butylated hydroxytoluene-containing commercial milk-replacer on growth performance, lamb carcass characteristics and meat (*Longissimus thoracis*) composition.

	Control	Astaxanthin	RSD	*P*-value
	n = 10	*n* = 10		
Carcass characteristics				
pH (24 h)	5.42	5.50	0.170	NS
Cold carcass weight (CCW; kg)	5.74	5.81	0.748	NS
Dressing (%)	49.5	48.9	1.708	NS
Perirenal fat (% of CCW)	0.72	0.99	0.074	*
Meat composition (*L. thoracis*)				
Moisture (%)	76.64	76.62	0.444	NS
Protein (%)	19.32	19.30	0.730	NS
Fat (%)	1.72	1.95	0.789	NS
Ash (%)	1.20	1.20	0.044	NS
Astaxanthin (mg/kg of muscle)	–	< DL	–	–
BHT (mg/kg of intramuscular fat)	47.63	28.29	15.757	*

BHT: Butylated hydroxyltoluene.
RSD: Residual standard deviation; −: not determined.
DL: Detection limit: 1.2 mg/kg of meat.
P-value: NS = not significant; * = $P < .05$; − = non-calculated.
[a] 1 g of AstaREAL® EL25 (containing 2.5% of astaxanthin) was added to 1 kg of the commercial milk-replacer powder.

composition for Control- and Astaxanthin-fed lambs. Carcass weight and percentages of carcass dressing and joints were not affected by the feeding treatment. The percentages, with values being similar to those reported by Mateo et al. (2018), were not shown in the table for brevity. In agreement with the study by Carr et al. (2010) on pork, suckling lamb meat pH was not affected by astaxanthin supplementation. However, Lei and Kim (2014) found the inclusion of astaxanthin (at levels of approximately 20 and 40 mg/kg of feed) increased pork pH.

Perirenal fat weight percentage was significantly higher ($P = .022$) in carcasses from those lambs fed astaxanthin supplemented milk-replacer. This might indicate a higher fatness in these carcasses as perirenal fat appeared to be correlated with total carcass fat in suckling lamb carcasses (Miguélez, Zumalacárregui, Osorio, Beteta, & Mateo, 2006). These results would be in concordance with those of Jeong and Kim (2014) who observed that the inclusion of astaxanthin in broilers' feed (up to 4.6 mg of astaxanthin/kg of feed) tended to increase abdominal fat weight. However, in contrast, Yang et al. (2006) and Bergstrom et al. (2009) reported a decrease in backfat depth in pigs when their diet was supplemented with astaxanthin.

The proximate composition of meat was not affected by the feeding treatment. Furthermore, the astaxanthin level in the Astaxanthin-group meat could not be quantified, i.e. it resulted in levels lower than the detection limit, and the BHT content of intramuscular fat was significantly ($P < .05$) lower in Astaxanthin-meat. The concentration of BHT in muscle (mg/kg of muscle), as calculated from the BHT content in intramuscular fat (Table 3) and intramuscular fat content for Control and Astaxanthin-samples were, respectively, 0.72 and 0.48 ($P = .022$; data not shown in the tables for brevity). This difference in BHT levels could be at least partially explained by a higher fatness in astaxanthin-fed carcasses. BHT is a fat soluble molecule and assuming a similar intake of milk-replacer in lambs from both groups, the more the fat in the animal the lower the BHT concentration (Nieva-Echevarría et al., 2015). It could also be hypothesized that the presence of astaxanthin in the small intestine lumen could diminish the absorption of BHT by means of a competitive effect, or that astaxanthin could promote BHT catabolism in the animal tissues. Further investigation on these effects are warranted.

3.2. Colour of meat and fat and meat colour stability

The effect of using astaxanthin in the milk-replacer on instrumental colour of suckling lamb meat LT after a blooming period of 1 h (day 0) and of perirenal fat are shown in Table 4. The use of astaxanthin resulted in a decrease in meat lightness ($P = .005$) and an increase in redness ($P = .048$), the latter of which also increased in perirenal fat ($P = .016$). The mean transmittance spectra obtained from the Astaxanthin- and Control-perirenal fat are shown in Fig. 1. A near-significant lower ($P < .1$) transmittance at wavelengths between 470 and 500 nm was detected, with this wavelength range corresponding to the absorption peak of astaxanthin (Tolasa et al., 2005), thus suggesting the presence of astaxanthin in the fat of that group. With regard to meat spectra, the comparison did not result in significant nor near-significant differences ($P > .1$) at any wavelength and thus the transmittance spectra are not shown for brevity.

Table 4
Effect of feeding suckling lambs with astaxanthin-supplemented[a] butylated hydroxytoluene-containing commercial milk-replacer on colour of meat (day 0) and fat.

	Control	Astaxanthin	RSD	*P*-value
	n = 10	n = 10		
Longissimus thoracis				
Lightness (L^*)	42.1	40.1	1.418	**
Redness (a^*)	10.02	11.12	1.159	*
Yellowness (b^*)	8.68	8.54	0.730	NS
Perirenal fat				
Lightness (L^*)	65.38	65.12	1.800	NS
Redness (a^*)	4.34	6.26	1.609	*
Yellowness (b^*)	15.88	16.55	1.899	NS

RSD: Residual standard deviation.
P-value: NS = not significant; * = $P < .05$; ** = $P < .01$.
[a] 1 g of AstaREAL® EL25 (containing 2.5% of astaxanthin) was added to 1 kg of the commercial milk-replacer powder.

The ΔE in Astaxanthin-meat as compared with Control meat, calculated from the Table 4 data, was 2.3. Theoretically, the standard observer might appreciate small differences in colour of meat samples that are not side by side when $\Delta E > 1$ (AMSA, 2012), although colours are not easily distinguishable when $\Delta E < 3$ (Fleishman, McClintock, D'Eath, Brainard, & Endler, 1998). The change in suckling lamb meat colour due to the astaxanthin intake could negatively affect the consumers' preference because suckling lamb meat is characterized by a bright white to light pink colour (Erasmus, Muller, & Hoffman, 2017). In contrast, for the meat of weaned lambs a higher a^* would increase the consumer acceptability (Khliji, Van de Ven, Lamb, Lanza, & Hopkins, 2010).

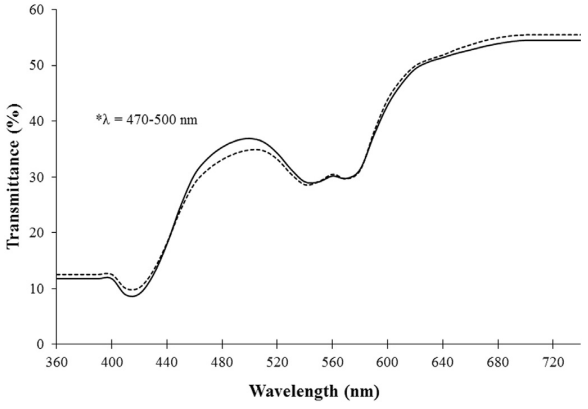

Fig. 1. Mean transmittance spectra obtained with the colorimeter from renal fat from control (—) and astaxanthin (—) fed suckling lambs. *λ = wavelengths where the astaxanthin treatment has a near to significant effect ($P < .1$).

Table 5
Effect of feeding suckling lambs with astaxanthin-supplemented[#] butylated hydroxytoluene-containing commercial milk replacer on meat (*Longissimus thoracis*) colour stability during aerobic storage.

	Treatment (Treat)[&]		Storage (Day)[&]				RSD		*P*-value		
	Control	Astaxanthin	1	3	5	7	Treat	Day	Treat	Day	Treat x Day
L^*	42.769	40.210	42.683^{a}	42.303^{a}	41.426^{b}	39.546^{c}	4.505	1.951	*	***	*
a^*	10.999	12.328	10.815^{b}	11.567^{b}	11.683^{b}	12.634^{a}	3.128	1.369	+	***	NS
a^*/b^*	1.050	1.246	1.079^{b}	1.120^{ab}	1.137^{ab}	1.258^{a}	0.397	0.228	*	***	NS
Hue angle (°)	44.107	39.456	43.318^{a}	42.214^{a}	41.992^{ab}	39.601^{b}	9.009	4.118	*	***	NS

RSD: Residual standard deviation.
a,b,c: different superscripts in the same row indicate statistical differences for storage day (Bonferroni test; $P < .05$).
P-value: NS = not significant; + = $P < .1$; * = $P < .05$; *** = $P < .001$.
[#] 1 g of AstaREAL® EL25 (containing 2.5% of astaxanthin) was added to 1 kg of the commercial milk replacer powder.
[&] Treatment values are averaged across the 7 days of display, and Storage values are averaged for Control and Astaxanthin treatments.

In agreement with the present study, Perenlei et al. (2014) also found an increase in the redness of breast meat in chickens fed a feed supplemented with 20 mg of astaxanthin per kg as compared with control meat (no supplementation), although a supplementation of 10 mg of astaxanthin per kg of feed showed no significant effect. Similarly, Carr et al. (2010) and Lei and Kim (2014) reported a positive effect of dietary astaxanthin in pork redness when used at levels of 67.2 and 20–40 mg/kg of feed, respectively. In contrast, neither Jeong and Kim (2014) in broilers nor Yang et al. (2006) in pigs found any effect of astaxanthin on meat colour. However, the level of astaxanthin in feed used in both studies seems to be lower than that tested in the other studies, i.e. < 5 mg/kg of feed, and it could explain the lack of effect. Taking into account this and the above mentioned studies, a supplementation of 20–25 mg astaxanthin/kg of feed for (functional) monogastric meat animals could be enough for increasing redness (a^*) in meat and at the same time would not be far from the recommended dose for humans (6 mg/day; Ambati et al., 2014).

The effect of feeding lambs with astaxanthin on the colour stability of lamb during aerobic refrigerated storage is shown in Table 5. This Table provides the lightness (L^*) and relevant instrumental colour characteristics indicating time-related meat discoloration (AMSA, 2012). For both treatments, storage resulted in a^* and a^*/b^* increases and hue angle decreases. These changes denote an increase in redness and, therefore, that meat discoloration due to storage would not have taken place. The increase in redness could be mainly attributed to meat surface desiccation occurring during meat storage in trays wrapped with PVC film (Callejas-Cárdenas et al., 2014). A significant interaction treatment x day was observed for L^* ($P = .018$; Fig. 2), consisting of a sharper decrease in L^* (lightness) for Astaxanthin-meat samples from day 5 of storage to day 7. Considering the mean colour values throughout the storage period, the Astaxanthin-meat samples showed lower L^* and higher a^*/b^* and hue angle ($P = .024$ for L^*, $P = .046$ for a^*/b^* and hue angle) and near-to-significantly higher a^* ($P = .082$) values than Control-meat samples. These differences are consistent with the differences explained above for fresh (day 0) meat. Longer aerobic storage periods (over 7 days) would result in meat discoloration, with the consequent decrease in a^* values (Khliji et al., 2010; Mateo et al., 2018). In this case, dietary astaxanthin might exert a positive effect in meat colour by keeping the a^* values higher than those of control meat and thus retarding the onset of discoloration.

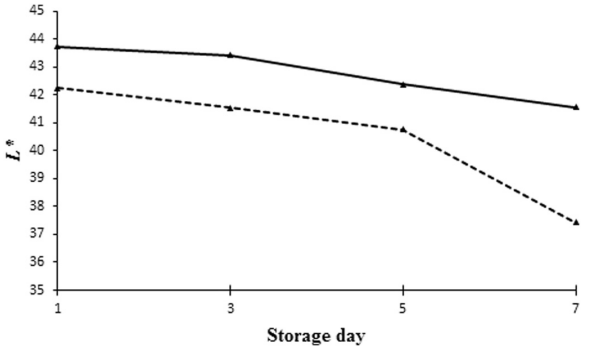

Fig. 2. Time-related changes in lightness (L^*) during storage of the meat (*Longissimus thoracis*) from control (—) and astaxanthin (—) fed suckling lambs.

3.3. Lipid stability due to storage and volatile compounds in cooked meat

In order to evaluate the effect of dietary astaxanthin supplementation on the lipid stability of suckling lamb meat during aerobic storage, TBARS values were determined in 7 day refrigerated and 3 month frozen raw meat, and 1 day refrigerated cooked meat (Table 6). Lipid oxidation was not evident (as per TBARS score < 0.1 mg MDA/kg meat) in 7 day refrigerated raw meat of either treatment, which is indicative of a high lipid oxidative stability in suckling lamb meat from both treatments. A high oxidative stability of lipids in suckling lamb meat from commercial milk-replacer-reared suckling lambs was also observed in a previous study (Osorio et al., 2008), which was attributed to the presence of BHT and high levels of vitamin E in meat due to the use of these antioxidants in the formulation of the commercial milk-

Table 6
Effect of feeding suckling lambs with astaxanthin-supplemented[a] butylated hydroxytoluene-containing commercial milk replacer on the levels of thiobarbituric acid reactive substances (TBARS; expressed as mg of malondialdehyde per kg; MDA/kg) in raw and cooked meat stored under aerobiosis at refrigeration and frozen temperatures.

Storage	Raw meat[b]		Cooked meat[c]
	7-Day refrigerated	3-Month frozen	1-Day refrigerated
Control	< DL	0.26	1.37
Astaxanthin	< DL	0.19	1.62
RSD	–	0.063	0.384
P-value	–	*	NS

DL: the detection limit of the method was 0.1 mg MDA/kg of meat.
RSD: Residual standard deviation.
P-value: NS = not significant; * = $P < .05$.
[a] 1 g of AstaREAL® EL25 (containing 2.5% of astaxanthin) was added to 1 kg of the commercial milk replacer powder.
[b] At 24 h postmortem 2-cm width *Longissimus thoracis* slices were individually packaged in trays wrapped with polyvinyl chloride and refrigerated stored (4 °C) for 7 days and *Biceps fermoris* halves were wrapped with polyvinyl chloride and frozen stored (−18 °C) for three months.
[c] At 24 h postmortem 2-cm width *Longissimus thoracis* slices were individually frozen under vacuum for 2 months, then thawed, cooked, packaged in trays wrapped with polyvinyl chloride, refrigerated stored (4 °C) for 1 day and then microwave reheated.

Table 7
Effect of feeding suckling lambs with astaxanthin-supplemented[a] commercial milk-replacer on the levels of volatile compounds of the headspace of cooked lamb expressed as ng of hexanal equivalents per mL (*Biceps femoris*).

	LRI	Treatment		RSD	*P*-value
		Control	Astaxanthin		
Aliphatic aldehydes					
Hexanal	806	26.981	36.299	11.35	+
Heptanal	904	0.930	1.260	0.521	NS
Octanal	1001	1.780	2.160	0.900	NS
Nonanal	1101	4.394	5.227	1.927	NS
Hexadecanal	1821	0.695	0.465	0.380	NS
Subtotal		34.957	45.627	0.318	+
Aliphatic alcohols					
1-Octen-3-ol	979	0.2835	0.4203	0.184	NS
Aliphatic ketones					
2,3-Octanedione	978	0.3239	0.5691	0.298	+
Furans					
2-Pentylfuran	988	0.287	0.393	0.298	NS
Terpenes					
Camphene	951	3.757	2.472	2.571	NS
Terpene $C_{10}H_{16}$	1086	2.646	1.549	2.204	NS
Subtotal		6.404	4.021	4.759	NS
Benzene compounds					
Benzaldehyde	966	0.009	0.020	0.044	NS
Butylated hydroxytoluene	1505	0.126	0.051	0.070	*
1-Phenyl-1,3,3-trimethylindane	1738	0.234	0.121	0.126	+
Subtotal		0.458	0.202	0.316	+
Total sum of volatiles		42.714	51.233	13.609	NS

LRI: Linear retention index.
RSD = residual standard deviation.
P-value: NS = not significant; + = $P < .1$; * = $P < .05$.
[a] 1 g of AstaREAL® EL25 (containing 2.5% of astaxanthin) was added to 1 kg of the commercial milk replacer powder.

replacers.

In frozen stored suckling lamb meat lipid oxidation could be detected after 3 months (TBARS > 0.1 mg MDA/kg meat) and the TBARS values were higher in Control-meat than in Astaxanthin-meat ($P = .029$). The difference could be attributed to the presence of astaxanthin in the Astaxanthin-treatment meat. The protective effect of astaxanthin against lipid oxidation during frozen storage would have been higher than that of the excess of BHT in Control-samples (as compared with Astaxanthin-samples, Table 3). In a previous study on fresh pork (Lei & Kim, 2014), decreased TBARS values with dietary astaxanthin addition were reported. In frozen lamb patties stored for 3 months at −18 °C, Carballo et al. (2018) found the presence of astaxanthin at a level of 20 mg per kg of patty (not animal diet) to exert a significant protection of meat lipids against oxidation. Studies concerning the antioxidant effect of dietary astaxanthin in fish products have suggested that the accumulation of astaxanthin in fish flesh could be capable of protecting lipids against oxidation in few-month frozen storage periods. However, this effect would be lost during long frozen periods due to a steady degradation of astaxanthin (Secci & Parisi, 2016).

No differences were found in the lipid oxidation status of cooked refrigerator stored meat. Since warmed-over-flavour (WOF) intensity in heated and then stored meat is positively correlated with TBARS levels (Tikk, Haugen, Andersen, & Aaslyng, 2008), the present results suggest that use of astaxanthin does not result in a reduction of WOF development in this type of meat. The samples in this study might not have developed the WOF or it would have been incipient as the TBARS levels found in this study were not above the range (0.6–2 mg/kg) suggested by Greene and Cumuze (1982) as a detection threshold of oxidized beef.

The volatile composition in the headspace of the heated suckling lamb meat samples from both experimental treatments are shown in Table 7 where, for brevity, only those volatiles with levels higher than 0.1 ng hexanal equivalents per mL of headspace gas were included. The volatiles detected belonged to the following chemical families: aliphatic aldehydes (six compounds), terpenes (two), aliphatic alcohols (one), aliphatic ketones (one), furans (one), terpenes (two) and benzene compounds (three). One of the terpenes could not be positively determined and was named $C_{10}H_{16}$. As expected, the majority of the compounds detected, i.e. aliphatic aldehydes, alcohols and ketones and furans, were presumably derived from lipid thermal degradation, and would have taken place during the meat cooking and headspace extraction processes (Resconi, Escudero, & Campo, 2013; Yang et al., 2015). The addition of astaxanthin to the milk-replacer did not significantly affect either the lipid derived volatiles or the total sum of volatiles in meat. The only compound significantly affected was BHT ($P < .05$), thus confirming the higher concentration of this compound in Control meat. Moreover, a tendency towards lower hexanal content ($P < .1$) and lower mean values on most of the lipid derived compounds were found in Control-samples. This suggests that the higher BHT content in these samples might have resulted in a higher protection of the lipids against thermal oxidation as compared to the Astaxanthin-meat samples.

4. Conclusions

Feeding suckling lambs a BHT-containing commercial milk-replacer supplemented with 25 mg of astaxanthin (1 g of AstaReal®EL25) per kg resulted in a slight but significant effect on meat and fat colour, which might slightly decrease consumer acceptability of suckling lamb meat colour. The contribution of astaxanthin to the lipid oxidative stability of meat from artificially reared suckling lambs, which is already high for the Control-meat, resulted to be useful only when frozen storage was used. The addition of astaxanthin to BHT-containing commercial milk-replacers, apart from the above-mentioned effects, reduced the BHT levels in meat from milk-replacer reared suckling lambs. The use of dietary astaxanthin in order to reduce BHT content in milk-replacers for lambs and suckling lamb meat warrants further studies, i.e. to gain knowledge on the physiological interaction between these two dietary antioxidants, and the effect of the reduction in the amount of BHT added to milk-replacers on the oxidative stability of lipids from both milk-replacer and meat from artificially reared lambs.

Declaration of interest

The authors guarantee that no competing interest exists.

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4

Assessment of the antioxidant effect of astaxanthin in fresh, frozen and cooked lamb patties

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Assessment of the antioxidant effect of astaxanthin in fresh, frozen and cooked lamb patties

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ABSTRACT

Astaxanthin is a natural red carotene exerting a strong antioxidant action. The effect of this carotene on the oxidative stability of raw and cooked lamb patties was evaluated. Seven experimental treatments were included in this study depending on the antioxidants added, which are: no antioxidant added (control), 450 mg/kg of sodium metabisulphite, 500 mg/kg of sodium ascorbate, and 20 mg/kg, 40 mg/kg, 60 mg/kg and 80 mg/kg of astaxanthin. The raw patties were either refrigerated for up to 11 days or frozen for 3 months under aerobic conditions. Changes in thiobarbituric reactive substances (TBARS), instrumental colour, pH and Eh were determined in the refrigerated patties and TBARS in the frozen patties. Volatile compounds were determined in cooked patties and cholesterol oxides in both cooked and after cooking microwave reheated patties. The changes in TBARS of cooked patties during a four-day refrigerated storage were also studied. Compared to the control patties, the use of astaxanthin reduced the TBARS generation in a manner depending on the dose for both raw and cooked patties during storage ($P < 0.05$). Astaxanthin added at levels of 60 and/or 80 mg/kg showed a greater antioxidant effect than ascorbate and metabisulphite. The presence of astaxanthin, like that of ascorbate, decreased the oxysterols levels of cooked patties with regard to controls. The amount of volatiles released from the cooked patties was also reduced by astaxanthin. This effect was not observed for ascorbate or metabisulphite. Astaxanthin in lamb patties at levels of 60–80 mg/kg could improve raw and cooked lamb patty oxidative stability during refrigerated aerobic storage, protect their lipids against thermal degradation more than ascorbate and metabisulphite, and reduce oxysterols formation during cooking in a similar way to ascorbate.

1. Introduction

Lipid oxidation is considered as an inevitable, irreversible and complex process that occurs in all food matrices during processing, storage or distribution, and remains a major concern in terms of loss of sensory quality besides loss of nutritional and economic value (Estévez, 2017). Furthermore, during oxidation, several reactive species (RS) are generated, which are the main agents responsible for health disorders in the consumer (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013). For example, in meat, as in other foods of animal origin, RS can react with cholesterol, originating the formation of at least 60 different oxidation products (Razzazi-Fazeli, Kleineisen, & Luf, 2000) which have been found to be mutagenic, cytotoxic and carcinogenic (Min et al., 2016). Furthermore, the presence of some of them in food has been associated with arteriosclerosis and neurodegenerative diseases (Poli, Biasi, & Leonarduzzi, 2013; Savage, Dutta, & Rodriguez-Estrada, 2002).

The use of antioxidant additives by the meat industry has proved to be a good strategy to delay or prevent oxidation processes (Liu, Xu, Dai, & Ni, 2015). Thus, ascorbic acid (or ascorbate) is a commonly used antioxidant additive in processed meat. This is a water-soluble molecule, used as an additive according to the principle of 'just enough', and is considered to have no toxic effect on consumers (Varvara et al., 2016). Moreover, sulphur dioxide, or its precursors such as metabisulphite, although less commonly used is allowed at maximum levels of 450 mg/kg in specific meat preparations such as burger meat (SANCO, 2017). The functions of this additive are to reduce microbial growth, protect against oxidation and enhance the red colour of meat (Mathenjwa, Hugo, Bothma, & Hugo, 2012; Ough & Were, 2005).

There is growing consumer demand for natural antioxidants over synthetic ones that have been associated with toxicological or antinutritional effects, such as sulphur dioxide, butylhydroxytoluene or butylhydroxyanisol (Shah, Bosco, & Mir, 2014). This has driven the use of natural materials or their extracts rich in antioxidant molecules as meat product ingredients, in order to delay oxidation processes, besides

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contributing to nutritional improvement (Kumar, Yadav, Ahmad, & Narsaiah, 2015; Shah et al., 2014; Villalobos et al., 2015).

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a red xanthophyll carotenoid which is naturally found in crustaceans, salmonids and some types of bird feathers, yeasts and algae, *Haematococcus pluvialis* being the main source of this antioxidant for human consumption (Ambati, Moi, Ravi, & Aswathanarayana, 2014; Guerin, Huntley, & Olaizola, 2003; Kidd, 2011; Kobayashi et al., 1997). Interest in astaxanthin extracts in the nutritional supplement and food industries is growing (Higuera-Ciapara, Félix-Valenzuela, & Goycoolea, 2006). Several functions have been attributed to this carotene regarding its use as food ingredient, i.e. natural colorant and antioxidant, or as a nutritional supplement, i.e. antioxidant, anti-inflammatory and anti-diabetic activity in the organism (Ambati et al., 2014; Guerin et al., 2003; Higuera-Ciapara et al., 2006). It is well known that its antioxidant capacity is greater than that shown by other carotenoid compounds (Ambati et al., 2014; Naguib, 2000).

In spite of the increasing interest within the food industry in extending the use of astaxanthin, there are few studies about the effect of the presence of this compound on meat quality. To the best of our knowledge, it has been reported that feeding of pigs (Carr, Johnson, Brendemuhl, & Gonzalez, 2010) or chickens (Perenlei et al., 2014) with appropriate doses of this carotene can lead to an improvement in meat colour and oxidative stability. Furthermore, Abdelmalek et al. (2016) demonstrated an antioxidant effect of astaxanthin in marinated chicken steaks during refrigerated storage. No studies have been carried out, however, on the effect of astaxanthin on the quality of red meat and specifically that of lamb, which is widely consumed in European Mediterranean countries where light lamb meat is considered a luxury meat (Linares, Bórnez, & Vergara, 2007).

Taking all this into account, the aims of the present study were to evaluate the effect of the addition of different levels of a commercial astaxanthin powder on the oxidative lipid stability of raw lamb patties during refrigerated and frozen storage and during their heat treatment and refrigerated storage of cooked patties. For a better assessment of this effect, patties without antioxidants and patties with ascorbate and metabisulphite were included in the study.

2. Materials and methods

2.1. Patty manufacturing and sampling

The meat used in this experiment was obtained from the legs of 12 lambs that were reared under a conventional system consisting of suckling until 13.5 kg body weight followed by weaning and fattening on a complete pelleted diet until a target body weight of 27 kg was achieved, as described by Santos, Giráldez, Mateo, Frutos, and Andrés (2018). Legs were obtained from the cold carcasses at 24 h post mortem, and deboned. The flesh was then cut into approximately 3-cm^3 pieces and trimmed of any visible fat. The meat pieces from the 12 lambs were mixed together, vacuum packaged in three different packaging bags and frozen-stored for a period of 8–9 months at −18 °C. Afterwards, the meat was thawed for 24 h at 5 °C in order to prepare three batches of patties, each one with the meat from one packaging bag. The patty batches were prepared on three different days at the Food Processing Hall of the Department of Food Hygiene and Technology (University of León, León, Spain).

Each batch (weighing 3.5 kg) consisted of seven 500-g sub-batches of patties according to the type or amount of antioxidant added, i.e. antioxidant treatment, which were labelled as CON (no addition of antioxidant), SULP (addition of 450 mg of sodium metabisulphite per kg of patty mixture: 450 ppm; Panreac, Barcelona, Spain), ASC (500 ppm of sodium ascorbate; Panreac), AST20 (20 ppm of astaxanthin), AST40 (40 ppm of astaxanthin), AST60 (60 ppm of astaxanthin) and AST80 (80 ppm of astaxanthin). The astaxanthin added to the patties was part of a commercial dietary supplement (Astaxantina-Lider, Naturlider, Ciudad Real, Spain) containing astaxanthin (1%) extracted from *H. pluvialis*, and excipients: maltodextrin, magnesium stearate and silicon dioxide. The amount of supplement added to the corresponding patties was adjusted to the above-mentioned astaxanthin levels. The amount of sodium ascorbate used was in the range generally applied in processed meat (400–600 ppm; Feiner, 2006), that of metabisulphite was the maximum allowed level for burger meat (SANCO, 2017), and those of astaxanthin were such that the amount contained in a 100-g patty was close to the dose recommended for astaxanthin as a dietary supplement for humans, i.e. approximately 6 mg/day (Ambati et al., 2014). The antioxidant potential of the antioxidants used in the experiment was determined in the lab using 2,2-diphenyl-1-picrylhydrazyl (DPPH; Serpen, Gökmen, & Fogliano, 2012) and the results obtained for sodium metabisulphite, sodium ascorbate, and astaxanthin, which was previously extracted from the dietary supplement with ethyl acetate, were respectively 0.34, 4.07 and 0.86 mmol Trolox equivalents per g of substance.

The formulation of patties was as follows: 79.5% of lean minced leg lamb (using a butcher's mincer equipped with a 5-mm diameter sieve), 4% of potato starch, 15% of water and 1.5% of common salt. Moreover, depending on the treatment, the corresponding antioxidant was added to the patties. All the components were manually mixed for 2 min. Finally, five 100-g patties were formed from each sub-batch; out of those, three patties were used to evaluate the oxidative status and stability when raw refrigerated, one when raw frozen, and the other when cooked and reheated (Table 1). The raw patties were analysed for thiobarbituric reactive substances (TBARS), colour, Eh and pH on the preparation day and after 5 and 11 days of refrigerated storage (4 °C) on polystyrene foam trays wrapped with polyvinyl chloride cling film, using one patty per storage day. The frozen patties, which had been previously wrapped with polyvinyl chloride cling film and then stored for 90 days at −18 °C, were analysed just after thawing (1 day at 4 °C) for TBARS. For the cooked patties, a patty from each antioxidant treatment was first stored raw under refrigeration (4 °C) for 5 days, then cooked in a convection oven at 150 °C for 15 min until reaching a core temperature of 70 °C (± 2 °C), and subsequently divided into four quarters. One quarter was vacuum packaged and frozen at −18 °C for up to 3 weeks before analysis of TBARS, cholesterol oxides and volatile compounds. The other three quarters were stored under refrigeration (4 °C) on polystyrene trays covered with polyvinylchloride cling film for 2 (two quarters) and 4 days (one quarter). One of the quarters stored for 2 days, and the quarter stored for 4 days were immediately frozen under vacuum (up to 3 weeks) before analysis of TBARS. The other quarter stored for 2 days was first reheated in a domestic microwave (400 W) for 2 min and then frozen under vacuum (up to 3 weeks) before analysis of cholesterol oxides. Moreover, a digital photograph of a raw patty per treatment and batch was taken just after patty preparation. A photo of a patty from each treatment has been included in Fig. 1 in order to show the reader the effect of the used levels of astaxanthin on the colour of

Table 1
Analysis carried out in three batches of raw refrigerated, raw frozen, cooked and reheated patties and the patty storage duration before analysis.

	Oxidative status analysis	Storage day
Raw refrigerated (three patties)	TBARS, colour, pH and redox potential	0, 5 and 11[a]
Raw frozen (one patty)	TBARS	90[a]
Cooked (after five days of refrigerated storage; three quarter patty)	TBARS	0, 2 and 4[b]
	Cholesterol oxides and volatile compounds	0[b]
Cooked and microwave reheated after two days of refrigerated storage (one quarter patty)	Cholesterol oxides	2[b]

[a] Day of sampling after the elaboration of patties during refrigerated storage.
[b] Day of sampling after the cooking of the patty.

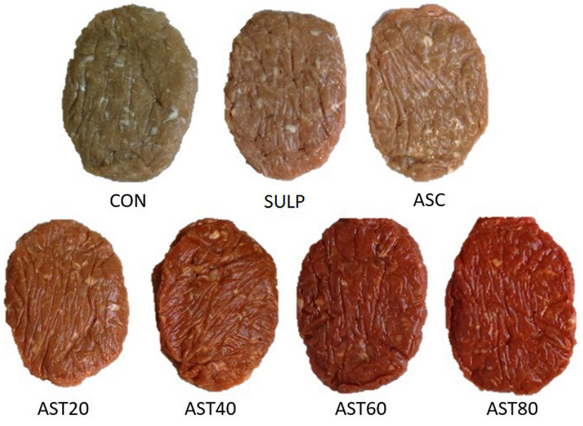

Fig. 1. Digital photographs of a recently-prepared lamb patty from each treatment: CON = control (no antioxidant additives); SULP = 450 mg sodium metabisulphite per kg of patty mixture; ASC = 500 mg sodium ascorbate per kg of patty mixture; AST20 = 20 mg of astaxanthin per kg of patty mixture; AST40 = 40 mg of astaxanthin per kg of patty mixture; AST60 = 60 mg of astaxanthin per kg of patty mixture; AST80 = 80 mg of astaxanthin per kg of patty mixture.

the patties. The photos were taken using a digital reflex camera (400D EOS camera; Canon, Ohta-ku, Tokyo) equipped with a 10.1-megapixel CMOS sensor and EF-S 18–55 mm lens. The operating conditions were as follows: camera in a horizontal position 40 cm above the patty, mode ISO 800, shutter speed 1/40, manual focus (MF), diaphragm aperture F11, exposure compensation −2/3, fluorescent white balance mode and flash on. Patties were placed on a table, and a matt white sheet was placed underneath the samples.

2.2. Instrumental and chemical analysis

The values of pH and Eh were determined in duplicate using a BasiC 20 pH meter (Crison Instruments, Barcelona, Spain) equipped with a 52–32 pH penetration electrode and a 32–61 redox platinum electrode, respectively, by inserting the electrodes into a ball-shaped 50-g raw patty sample. TBARS of raw and cooked patties was analysed following the procedure described by Nam and Ahn (2003). The instrumental colour parameters lightness (L^*), redness (a^*) and yellowness (b^*) were determined directly on the upper surface of the raw patty, in triplicate, using a Konica Minolta CM-700d colorimeter (Osaka, Japan) with D65 illuminant in SCI mode, an 11-mm aperture for illumination and 8 mm for measurement, and a visual angle of 10°.

The volatile compounds were analysed by gas chromatography coupled with mass spectrometry using the equipment and following the method described by Vieira et al. (2012) with modifications. Briefly, a 2-g aliquot of cooked patty was placed in 15-ml screw-cap vials (Agilent Technologies, Santa Clara, CA, USA). The vials were agitated intermittently (500 rpm, 5 s on, 2 s off) at 140 °C for 40 min in the chromatograph autosampler in order to extract the volatile compounds into the headspace. The syringe needle temperature was 120 °C, and the filling and injection speeds were 50 and 250 µl/s, respectively. The headspace injection volume was 1 ml, and the injector had a temperature of 260 °C and operated in the splitless mode. The volatile compounds were separated with a DB-5MS column (60 m × 0.25 mm ID × 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA) using helium as carrier gas with a flow rate of 1.5 ml/min. The oven conditions were as follows: initial temperature of 38 °C held for 1 min, increased to 50 °C at 10 °C/min, then to 146 °C at 4 °C/min, then to 200 °C at 20 °C/min and finally to 250 °C at 50 °C/min, maintaining this temperature for 11 min. The transfer-line temperature was 260 °C. The mass spectrometer operated with an electron source temperature of 240 °C, electron energy and emission currents of 70 eV and 35 µA, respectively, a quadrupole temperature of 190 °C, and a scanner range from m/z 40 to m/z 230. Identification of the volatile compounds detected was carried out by comparing their spectra with those contained in the NIST/EPA/NIH-98 Mass Spectral Database, together with personal interpretation, and their linear retention indices, calculated from the retention times of a series of n-alkanes (Hydrocarbons/C5–C20; Sigma-Aldrich, St. Louis, MO, USA), with those from the literature. The results were expressed in ng equivalents of hexanal/ml of headspace.

Cholesterol oxidation products (COPs) were determined in cooked and reheated patties using the method described by Grau, Codony, Grimpa, Baucells, and Guardiola (2001) with a few modifications. The patty sample was first lyophilized, and the lipids from 1 g of freeze-dried patty sample were extracted using a mixture of chloroform and methanol (1:1, v/v); then, 1 ml of 19-hydroxycholesterol (20 µg/ml) was added as an internal standard. After homogenization, the mixture was filtered using a vacuum, and then 9 ml of aqueous 0.88% KCl (w/v) and two drops of 6 M HCl were added to the sample. Afterwards, the sample was centrifuged, and the lower layer obtained was placed in a dark flask. Ten millilitres of 1.5 M methanolic KOH and 500 µl of BHT were added, and the mixture was kept in an orbital shaker (100 rpm) for 24 h to complete the saponification reaction, under N_2 atmosphere in darkness and at room temperature (20 °C). The unsaponifiable matter was then extracted and purified by solid-phase extraction following the procedure described by Guardiola, Codony, Rafecas, and Boatella (1995). Finally, oxysterols were derivatized to trimethylsilyl ethers, and the resulting solution was analysed using a gas chromatograph coupled with a mass spectrometer. A total of 1.5 µl was injected in splitless mode and at an injector temperature of 250 °C. Helium was used as carrier gas at a flow rate of 0.6 ml/min. The column used was a VF-5 ms (CP8947) capillary column (50 m × 250 µm × 0.25 µm; Varian, Palo Alto, CA, USA). The oven conditions were: initial oven temperature 75 °C, increased to 250 °C at 30 °C/min, then to 290 °C at 8 °C/min, and finally to 292 °C at 0.05 °C/min. The transfer-line temperature was 280 °C. The mass spectrometer operated with an electron source temperature of 260 °C, electron energy and emission currents of 70 eV and 35 µA, respectively, a quadrupole temperature of 200 °C, and a scanner range from m/z 40 to m/z 500. Identification was carried out by comparing the peak retention times of the sample with those of the standards analysed under the same conditions as the samples, and the peak spectra with those contained in the NIST/EPA/NIH-98 Mass Spectral Database, together with personal interpretation. The oxysterols used as external standards for identification and quantification were: 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), 5,6α-epoxycholesterol (α-CE), 5,6β-epoxycholesterol (β-CE), cholestanetriol (CT), 25-hydroxycholesterol (25-HC) and 7-ketocholesterol (7-KC) (Steraloids, Inc., Wilton, New Hampshire, USA). The results were expressed in µg/g of meat. Furthermore, an oxysterol different from the standard, cholesta-3,5-dien-7-one (CDO), was identified by its spectrum and was quantified as equivalents of α-CE (µg/g of meat).

2.3. Statistical analysis

The data from the chemical characteristics of the refrigerated raw patties and the TBARS values of the refrigerated cooked patties were analysed using the general linear model analysis of variance (ANOVA) with antioxidant treatment as fixed factor and storage time as repeated-measures variable. TBARS generation during frozen storage and the data on the volatile compounds were analysed by univariate ANOVA with antioxidant treatment being the fixed factor. Data for oxysterol contents in cooked samples and reheated samples were separately analysed by ANOVA (antioxidant treatment was the fixed factor). Furthermore, mean differences in oxysterols between cooked and reheated lamb within each antioxidant treatment were calculated using the Student's t-test. Post hoc analyses of the ANOVA results for

Table 2
Effect of different antioxidant treatments on selected quality traits of raw lamb patties stored at refrigeration temperature under aerobic packaging conditions.

	Antioxidant treatment (treat)[#]							Storage day (day)			RSD		Significance		
	CON	SULP	ASC	AST20	AST40	AST60	AST80	0	5	11	Treat	Day	Treat	Day	Treat × Day
pH	5.62^{ab}	5.64^{ab}	5.68^{a}	5.62^{ab}	5.60^{b}	5.59^{b}	5.58^{b}	5.62^{ab}	5.57^{b}	5.66^{a}	0.047	0.045	**	***	NS
Eh	92.83	70.50	29.28	71.89	72.72	71.72	71.11	49.21^{b}	88.62^{a}	67.9^{ab}	49.39	17.68	NS	**	NS
TBARS	8.35^{a}	7.98^{ab}	7.85^{ab}	6.55^{abc}	6.10^{abc}	5.43^{bc}	4.89^{c}	1.39^{c}	7.80^{b}	11.01^{a}	1.64	1.10	**	***	***
Colour															
L^*	49.92^{a}	47.53^{ab}	47.99^{ab}	44.61^{bc}	41.78^{cd}	40.76^{cd}	38.69^{d}	45.04	43.76	44.61	2.78	1.75	***	+	NS
a^*	3.85^{d}	5.19^{d}	6.24^{d}	14.02^{c}	17.99^{b}	22.17^{a}	24.12^{a}	14.21^{a}	13.7^{a}	12.2^{b}	1.80	0.69	***	***	NS
b^*	13.91^{c}	13.04^{c}	14.75^{bc}	18.46^{ab}	19.24^{a}	21.47^{a}	22.10^{a}	18.16^{a}	17.41^{ab}	17.13^{b}	2.47	1.27	***	*	NS

RSD = residual standard deviation.
Significance: NS = not significant; + = $P < 0.1$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.
TBARS = thiobarbituric acid-reactive substances (expressed as mg of malondialdehyde per kg of patty).
abcd = different superscripts in the same row indicate statistical differences by the Tukey-Kramer test ($P < 0.05$) for antioxidant treatment and by the Bonferroni test ($P < 0.05$) for storage day.
[#] CON = control (no antioxidant additives); SULP = 450 mg sodium metabisulphite per kg of patty mixture; ASC = 500 mg sodium ascorbate per kg of patty mixture; AST20 = 20 mg of astaxanthin per kg of patty mixture; AST40 = 40 mg of astaxanthin per kg of patty mixture; AST60 = 60 mg of astaxanthin per kg of patty mixture; AST80 = 80 mg of astaxanthin per kg of patty mixture.

antioxidant treatment factor were carried out with the Tukey–Kramer test, and for time in repeated-measurement factor (storage day) with the Bonferroni test. The significance level was always $P < 0.05$. Analyses were preformed using SPSS Statistics software (version 23; IBM, Somers, NY, USA).

3. Results and discussion

3.1. Refrigerated-stored fresh patties

The quality characteristics of the refrigerated patties stored under aerobic conditions are shown in Table 2. Among the traits evaluated, redox potential was the only characteristic not affected by antioxidant treatment ($P > 0.05$). The pH values of the patties with added antioxidant did not differ from those of CON; however, the pH of ASC patties, which showed the highest mean values, differed from those of AST patties with astaxanthin concentrations equal to or higher than 40 ppm ($P < 0.05$).

The TBARS levels at day 0 suggest the development of initial lipid oxidation in the meat during the previous deboning, cutting, vacuum frozen storage (8–9 months) and patty manufacture. The experimental refrigerated storage of patties led to a sharp increase in the TBARS concentration. These high rates of TBARS increase might be partially attributed to the initial oxidation of lamb lipids, i.e. high TBARS levels at day 0. Neither the presence of sulphites nor that of ascorbate resulted in a decrease in the increments of TBARS due to the storage as compared to CON patties. In agreement, Bañón, Díaz, Rodríguez, Garrido, and Price (2007) did not observe lipid antioxidant effect of metabisulphite (used at a level equivalent to 100 ppm of sulphur dioxide) in beef patties during a 9-day refrigerated aerobic display period. Also in agreement, Sánchez-Escalante, Djenane, Torrescano, Beltrán, and Roncalés (2001) observed that ascorbic acid at 500 ppm was ineffective in preventing lipid oxidation when added to ground beef during refrigerated storage under a high-oxygen modified atmosphere. To explain this, the authors hypothesised that the antioxidant effect of ascorbic acid, which would depend on its concentration, could be decreased by factors related to the meat preparation matrix such as salt presence, degree of mincing and oxygen concentration. Furthermore, taking into account our results, the initial oxidation of meat (at day 0) might be added to that listing of potential reasons.

The addition of astaxanthin at levels of 60 and 80 ppm, however, significantly reduced the TBARS formation ($P < 0.05$) during the patty storage with regard to CON patties. Moreover, the addition of 80 ppm of astaxanthin showed significantly ($P < 0.05$) higher effectiveness in preventing lipid oxidation than the addition of 450 ppm of sodium metabisulphite and 500 ppm of sodium ascorbate, which, as said before, did not show a significant protective effect with respect to CON values. Moreover, it is noteworthy that the time-related increase in TBARS showed a significant interaction with antioxidant treatment, which consisted of different rates of increase in TBARS values between treatments, i.e. higher rates for CON, SULP and ASC than for AST patties (data not shown). The antioxidant effect of astaxanthin on raw-meat lipid oxidation has been previously assessed by Abdelmalek et al. (2016) when adding 10 ppm of astaxanthin to marinated chicken steaks.

As regards instrumental colour, the addition of astaxanthin to the patties originated, as expected, a sharp increment in a^* and, to a lesser extent, in b^*, and a decrement in L^* (Table 2). On the other hand, instrumental colour characteristics of CON, SULP and ASC patties were apparently not different ($P < 0.05$). However, when the AST-added treatments are not considered, i.e. when repeated measures ANOVA was applied only to CON, SULP and ASC (data not shown in tables), the patties with sodium ascorbate showed a significantly higher degree of redness than CON ($P < 0.05$), while SULP patties were not significantly different either from CON or from ASC. This confirms the reported effectiveness of ascorbate in inhibiting metmyoglobin formation (Sánchez-Escalante et al., 2001). However, in contrast, the previously demonstrated protective effect of metabisulphite (at a level equivalent to 100 ppm of SO_2) on patty-surface discoloration (Bañón et al., 2007) was not statistically evidenced in this study. This lack of effect could be at least partially attributed to the initial oxidation of lipids at the moment of storage, which would have promoted the metmyoglobin formation (Faustman, Sun, Mancini, & Suman, 2010) and thus hindered the effect of of metabisulphite on improving the colour of the patties with respect to the colour of CON.

3.2. Frozen-stored patties

The increase in TBARS levels in raw lamb patties after 3 months of frozen storage (at −18 °C) is depicted in Fig. 2. The generation of TBARS did not exceed 1 mg/kg of meat except for the SULP treatment. Thus, the addition of metabisulphite to the patties significantly increased TBARS formation during frozen storage compared to the control and the rest of treatments. On the other hand, the addition of ascorbate practically stopped frozen storage-related TBARS formation. The greater amount of TBARS in the frozen SULP patties might be attributed to the formation of reactive sulphur species promoting lipid oxidation (Brannan, 2010), which would have somehow originated

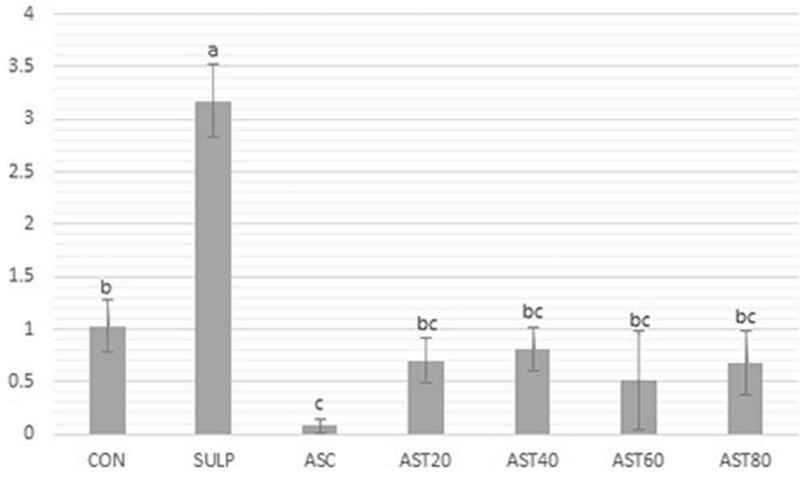

Fig. 2. Effect of different antioxidant treatments on the increment of the amount of thiobarbituric acid-reactive substances (TBARS; expressed as mg of malondialdehyde per kg of meat) in raw patties after a 3-month frozen storage at −18 °C under aerobic packaging conditions. CON = control (no antioxidant additives); SULP = 450 mg sodium metabisulphite per kg of patty mixture; ASC = 500 mg sodium ascorbate per kg of patty mixture; AST20 = 20 mg of astaxanthin per kg of patty mixture; AST40 = 40 mg of astaxanthin per kg of patty mixture; AST60 = 60 mg of astaxanthin per kg of patty mixture; AST80 = 80 mg of astaxanthin per kg of patty mixture. Error bars represent standard deviation from the mean ($n = 3$). ^{abc} = different superscripts in the same row indicate statistical differences by the Tukey-Kramer test ($P < 0.05$).

from the metabisulphite during the 3-month storage. In the frozen patties, the protective effect of astaxanthin against lipid oxidation, which in contrast was observed in refrigerated patties, was not evidenced in this study, i.e. the TBARS concentration increments due to frozen storage in AST patties were not different from those of CON patties ($P > 0.05$). To our knowledge, the effect of astaxanthin on lipid stability of frozen red meat has not been studied before and thus it was not possible to compare our results with literature. The lack of effect could be at least partially explained by the loss of astaxanthin content in patties during frozen storage. Astaxanthin significant losses during frozen storage of fish have been previously detected (Erickson, 1997; Jensen, Skibsted, Bertelsen, Birk, & Jokumsen, 1998), with losses depending on the storage conditions and period. Those authors explained that astaxanthin losses in frozen fish are enhanced by a reduced water activity and the eventual diffusion of oxygen through the oxygen-permeable packaging material, and suggested disruption of tissue as a contributing factor. The initial extend of lipid oxidation in meat (at the moment of frozen storage), which in this study was noticeable, might also be considered as another contributing factor.

3.3. Cooked patties

The lipid stability of the cooked patties during refrigerated storage as quantified by TBARS analysis is shown in Table 3. There were no significant differences between CON and SULP or between CON and ASC patties. Among these three treatments, the addition of ascorbate resulted in the lowest mean value and addition of metabisulphite the highest, so that the ASC levels differed statistically ($P < 0.05$) from the SULP levels. The use of astaxanthin as an antioxidant at levels of 40 ppm or higher decreased the lipid oxidation of cooked patties compared to CON patties, and there was a tendency for lower TBARS values with greater astaxanthin addition. The protective effect of astaxanthin against lipid oxidation in cooked patties was comparable to that observed in fresh patties; therefore, astaxanthin seems not to be affected by heat treatment. This is consistent with the high stability of astaxanthin when subjected to heat treatments of 70–90 °C (Ambati, Ravi, & Gokare Aswathanarayana, 2007). During storage, TBARS values increased ($P < 0.05$) but at different rates depending on the treatment, which explains the statistical effect ($P < 0.05$) found for the interaction day × treatment. During the entire storage period, the TBARS level increased by approximately 4 ppm in CON patties, 5 ppm in SULP patties, 3 ppm in ASC patties, 2 ppm in AST20 and AST40 patties, and by < 1 ppm in AST60 and AST80 patties (data not shown).

The COP levels (μg per g meat) in lamb patties for each of the treatments are shown in Table 4; CT and 25-HC were not detected (detection limit 0.1 μg/g) and thus were not included in the Table for brevity. Because heating is a main factor for COP formation in meat (Brzeska, Szymczyk, & Szterk, 2016; Khan et al., 2015; Min et al., 2016), and in order to know the effect of a second heat treatment on their formation, COP levels were determined in both recently cooked lamb patties and in patties reheated (2 days after cooking) using a microwave. As shown in the Table, reheating resulted in increased COP levels (approximately 30% higher), which were significantly different regarding total COPs in CON and SULP patties and 7-KC and β-CE in CON and AST20 patties, respectively.

The most abundant COPs in cooked lamb patties (with and without reheating) were 7-KC, β-CE, 7α-HC and 7β-HC, which have already been found as major COPs in cooked meat products (Orczewska-Dudek,

Table 3
Effect of different antioxidant treatments on thiobarbituric acid-reactive substances (TBARS; expressed as mg of malondialdehyde per kg of meat) of cooked lamb patties stored at refrigeration temperature under aerobic packaging conditions.

	Antioxidant treatment (treat)[#]							Storage days (day)			RSD		Significance		
	CON	SULP	ASC	AST20	AST40	AST60	AST80	0	2	4	Treat	Day	Treat	Day	Treat × Day
TBARS	11.84^{ab}	12.08^{a}	8.72^{bc}	8.67^{bc}	7.90^{c}	6.80^{c}	6.14^{c}	7.39^{a}	9.27^{b}	9.98^{b}	2.03	0.82	***	***	**

RSD = residual standard deviation.
Significance: ** = $P < 0.01$; *** = $P < 0.001$.
abc: different superscripts in the same row indicate statistical differences by the Tukey-Kramer test ($P < 0.05$) for antioxidant treatment and by the Bonferroni test ($P < 0.05$) for storage day.
[#] CON = control (no antioxidant additives); SULP = 450 mg sodium metabisulphite per kg of patty mixture; ASC = 500 mg sodium ascorbate per kg of patty mixture; AST20 = 20 mg of astaxanthin per kg of patty mixture; AST40 = 40 mg of astaxanthin per kg of patty mixture; AST60 = 60 mg of astaxanthin per kg of patty mixture; AST80 = 80 mg of astaxanthin per kg of patty mixture.

Table 4
Effect of different antioxidant treatments on oxysterols content (μg per g of meat) in cooked and microwave-reheated lamb patties.

	Antioxidant treatment[#]							RSD	Significance[1]
	CON	SULP	ASC	AST20	AST40	AST60	AST80		
7α-HC									
Cooked	3.64^{ab}	5.19^{a}	2.21^{b}	2.99^{ab}	2.40^{b}	2.32^{b}	1.92^{b}	0.589	**
Microwave-reheated	5.59^{ab}	8.94^{a}	3.11^{b}	3.29^{b}	2.79^{b}	2.81^{b}	2.25^{b}	1.110	**
SE	0.255	0.460	1.035	0.225	0.360	0.205	0.250		
Significance[2]	+	+	NS	NS	NS	NS	NS		
7β-HC									
Cooked	4.15	3.17	3.74	3.45	2.75	2.89	2.72	0.497	NS
Microwave-reheated	6.33^{a}	4.53^{ab}	4.52^{ab}	4.09^{ab}	3.70^{ab}	3.45^{ab}	2.89^{b}	0.814	*
SE	0.355	0.245	0.775	0.160	0.340	0.045	0.020		
Significance[2]	NS	NS	NS	NS	NS	+	+		
β-CE									
Cooked	4.36	4.01	3.34	2.77	2.71	2.85	3.16	1.069	NS
Microwave-reheated	7.15	5.56	5.69	3.98	3.52	3.36	3.85	1.516	NS
SE	0.310	0.505	0.980	0.755	0.575	0.800	1.290		
Significance[2]	+	NS	NS	*	NS	NS	NS		
α-CE									
Cooked	0.96^{a}	0.74^{ab}	0.71^{ab}	0.72^{ab}	0.60^{b}	0.59^{b}	0.54^{b}	0.084	*
Microwave-reheated	1.30	0.72	1.28	0.66	0.61	0.74	0.59	0.384	NS
SE	0.205	0.010	0.710	0.100	0.130	0.055	0.230		
Significance[2]	NS	NS	NS	NS	NS	NS	NS		
CDO									
Cooked	0.61	0.35	0.27	0.64	0.36	0.32	0.33	0.172	NS
Microwave-reheated	0.61	0.27	0.42	0.38	0.33	0.41	0.24	0.128	NS
SE	0.360	0.0350	0.265	0.070	0.0350	0.140	0.135		
Significance[2]	NS	NS	NS	NS	NS	NS	NS		
7-KC									
Cooked	9.94^{a}	3.47^{b}	5.96^{b}	6.88^{ab}	5.62^{b}	5.68^{b}	5.39^{b}	0.953	**
Microwave-reheated	15.89^{a}	4.63^{b}	8.44^{ab}	8.31^{ab}	7.33^{b}	7.29^{b}	5.99^{b}	1.961	*
SE	0.305	0.165	3.260	1.665	1.520	0.275	1.385		
Significance[2]	*	+	NS	NS	NS	NS	NS		
ΣCOPs									
Cooked	23.66	16.94	16.24	17.45	14.44	14.67	14.07	2.790	NS
Microwave-reheated	36.88	24.65	23.48	20.72	18.3	18.06	15.81	5.463	+
SE	0.065	0.070	7.030	2.970	2.965	1.430	3.305		
Significance[2]	**	**	NS	NS	NS	NS	NS		

7α-HC = 7a-hydroxycholesterol; 7β-HC = 7β-hydroxycholesterol; α-CE = 5,6-α-epoxycholesterol; β-CE = 5,6-β-epoxycholesterol; CDO = cholestan-3,5-dien-7-one (expressed as mg of α-CE equivalents per g of meat); 7-KC = 7-ketocholesterol; ΣCOPs = sum of cholesterol oxidation products.
RSD = residual standard deviation.
SE = standard error.
Significance[1] (Between antioxidant treatments); Significance[2] (Between heating treatment): NS = not significant; + = $P < 0.1$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.
abc: different superscripts in the same row indicate statistical differences in antioxidant treatment by the Tukey-Kramer test ($P < 0.05$).
[#] CON = control (no antioxidant additives); SULP = 450 mg sodium metabisulphite per kg of patty mixture; ASC = 500 mg sodium ascorbate per kg of patty mixture; AST20 = 20 mg of astaxanthin per kg of patty mixture; AST40 = 40 mg of astaxanthin per kg of patty mixture; AST60 = 60 mg of astaxanthin per kg of patty mixture; AST80 = 80 mg of astaxanthin per kg of patty mixture.

Bederska-Łojewska, Pieszka, & Pietras, 2012). The amounts found in this study for cooked (not reheated) CON patties were approximately four times higher than those detected in lamb steaks in a previous study (Andrés et al., 2014). The difference might be partially attributed to the mincing process and the presence of common salt in the patties, as well as variation in the cooking conditions.

The mean levels of total COPs decreased in all the antioxidant-treated patties compared to CON patties although only a near to significant difference due to antioxidant treatment was found in microwave reheated patties ($P < 0.1$). Moreover, the mean levels of total COPs for patties containing astaxanthin tended to decrease with the astaxanthin level, although significant differences between treatments with astaxanthin were not detected in ANOVA. In partial agreement, Osada, Hoshina, Nakamura, and Sugano (2000) found inhibition of cholesterol oxidation in meat due to the use of another radical scavenging antioxidant, i.e. apple polyphenols, and related fatty acid oxidation to cholesterol oxidation in meat products.

Among the major COPs detected, treatment significantly affected the amount of 7α-HC and 7-KC, which have been associated with neurodegenerative and cancerogenic diseases (Brzeska et al., 2016; Poli et al., 2013). With regard to 7-KC, metabisulphite, ascorbate and astaxanthin at levels ≥ 40 mg/kg showed a significant decreasing effect ($P < 0.05$) compared to CON cooked patties. On the other hand, the 7α-HC levels were significantly higher ($P < 0.05$) in SULP patties than in the other patties containing antioxidants, although no differences were detected between SULP and AST20 cooked (not reheated) patties and between SULP and CON patties.

Table 5 shows the volatiles identified in the headspace of cooked lamb patties. Only those compounds showing levels higher than 0.4 ng hexanal equivalents per ml of headspace are included for brevity. Volatiles were classified into eight chemical families: aliphatic hydrocarbons (five compounds), aliphatic aldehydes (21), aliphatic ketones (four), aliphatic alcohols (eight), furans (four), sulphur compounds (two) and benzene compounds (five). Most of them, i.e. the major part of the straight-chain hydrocarbons, aliphatic aldehydes, ketones and alcohols and alkylfurans, are presumably produced by lipid degradation

Table 5
Effect of different antioxidant treatments on volatile compounds (ng equivalent of hexanal per ml of headspace) of cooked lamb patties.

	LRI	Antioxidant treatment[#]							RSD	Significance
		CON	SULP	ASC	AST20	AST40	AST60	AST80		
Aliphatic hydrocarbons										
Octane	800	3.93^{a}	2.66^{abc}	3.09^{ab}	2.52^{abc}	1.57^{bc}	1.67^{bc}	1.18^{c}	0.659	**
Tridecane	1300	2.25^{a}	1.48^{abc}	2.01^{ab}	1.49^{abc}	1.29^{bc}	1.17^{bc}	0.81^{c}	0.339	**
Tetradecane	1400	1.37^{ab}	1.16^{ab}	1.47^{a}	1.07^{ab}	1.04^{ab}	0.95^{ab}	1.38^{b}	0.237	*
1-Pentadecene	1487	0.49	0.72	0.67	0.64	0.66	0.55	0.42	0.144	NS
Pentadecane	1492	2.17	1.89	2.16	1.86	1.73	1.55	1.28	0.330	+
Subtotal		12.08^{a}	9.21^{ab}	9.90^{ab}	8.83^{ab}	7.16^{ab}	6.64^{ab}	4.84^{b}	2.074	*
Aliphatic aldehydes										
Hexanal	806	165.67^{ab}	172.97^{a}	136.66^{abc}	123.66^{abc}	121.84^{abc}	94.63^{bc}	71.99^{c}	25.64	**
Heptanal	904	21.26^{a}	20.49^{a}	18.96^{ab}	13.99^{abc}	11.97^{bc}	10.33^{c}	7.30^{c}	2.987	***
Octanal	1001	16.77^{a}	15.06^{a}	14.92^{a}	10.14^{ab}	7.84^{b}	6.94^{b}	4.81^{b}	2.450	***
2-Octenal	1057	2.79^{a}	2.54^{ab}	2.24^{abc}	1.73^{abcd}	1.62^{bcd}	1.29^{cd}	0.93^{d}	0.386	***
Nonanal	1101	25.26^{a}	25.93^{a}	22.45^{ab}	17.77^{abc}	15.05^{bc}	13.36^{c}	10.20^{c}	3.067	***
2-Nonenal	1158	2.23^{a}	1.12^{ab}	1.58^{ab}	1.13^{ab}	0.83^{ab}	0.86^{ab}	0.62^{b}	0.547	*
2-Decenal	1260	3.27^{a}	2.30^{ab}	2.04^{ab}	1.03^{bc}	0.54^{bc}	0.54^{bc}	0.14^{c}	0.659	***
2,4-Decadienal	1323	2.53^{a}	2.12^{ab}	1.99^{abc}	1.27^{abc}	1.12^{bc}	1.03^{bc}	0.77^{c}	0.457	**
2-Undecenal	1370	4.11^{a}	3.06^{ab}	2.91^{ab}	1.55^{bc}	0.93^{bc}	0.89^{bc}	0.50^{c}	0.857	**
Dodecanal	1409	2.02^{ab}	2.24^{a}	1.88^{ab}	1.71^{ab}	1.80^{ab}	1.42^{ab}	1.25^{b}	0.346	*
Tridecanal	1514	2.12^{ab}	2.31^{a}	2.24^{a}	1.90^{ab}	1.54^{ab}	1.40^{ab}	1.09^{b}	0.403	**
Tetradecanal	1625	5.99^{ab}	8.03^{a}	5.73^{ab}	5.66^{ab}	4.28^{b}	3.86^{b}	2.94^{b}	1.234	**
Pentadecanal	1720	8.72^{ab}	12.01^{a}	10.18^{ab}	10.08^{ab}	8.11^{ab}	7.51^{ab}	6.23^{b}	1.994	*
14-Methylpentadecanal	1783	1.28	1.54	1.32	1.46	1.06	0.998	0.84	0.375	NS
Hexadecanal	1821	56.40	73.53	70.40	80.21	67.32	64.04	59.40	14.35	NS
14-Methylhexadecanal	1890	1.72	2.19	1.98	2.18	1.65	1.48	1.45	0.383	NS
15-Methylhexadecanal	1894	1.81	2.21	2.13	2.41	1.93	1.71	1.72	0.492	NS
Heptadecanal	1922	1.96	2.31	2.17	2.40	1.88	1.53	1.58	0.437	NS
(*E*)-9-Octadecenal	2005	2.60	3.14	3.44	3.72	2.87	2.36	2.57	0.781	NS
(*Z*)-9-Octadecenal	2010	2.18	2.53	2.91	3.15	2.32	1.84	2.04	0.783	NS
Octadecanal	2028	3.99	4.27	5.28	5.20	4.30	3.39	3.76	1.311	NS
Subtotal		338.63^{ab}	366.32^{a}	316.99^{ab}	295.36^{abc}	263.23^{abc}	223.99^{bc}	184.17^{c}	45.53	**
Aliphatic ketones										
2-Heptanone	892	8.24^{a}	5.93^{abc}	7.16^{ab}	6.71^{abc}	5.89^{abc}	4.38^{bc}	3.52^{c}	1.190	**
2,3-Octanodione	982	6.00^{a}	3.80^{abc}	4.26^{abc}	5.47^{abc}	5.67^{ab}	3.70^{bc}	3.31^{c}	0.806	**
2-Pentadecanone	1702	2.38^{a}	2.31^{ab}	2.17^{ab}	2.42^{a}	1.94^{ab}	1.88^{ab}	1.66^{b}	0.253	*
2-Heptadecanone	1903	2.16^{ab}	2.37^{a}	2.23^{ab}	2.33^{ab}	1.87^{ab}	1.62^{b}	1.67^{ab}	0.261	*
Subtotal		19.45^{a}	14.91^{abc}	16.71^{ab}	17.40^{ab}	16.04^{ab}	12.09^{bc}	10.32^{c}	2.025	**
Aliphatic alcohols										
Pentanol	773	12.72	8.34	10.95	7.44	7.62	6.44	4.10	3.189	+
Hexanol	872	4.38	2.53	3.78	3.10	2.31	2.70	2.10	1.182	NS
Heptanol	969	3.43^{a}	2.63^{ab}	2.80^{ab}	1.96^{abc}	1.35^{bc}	1.05^{c}	0.77^{c}	0.550	***
1-Octen-3-ol	978	14.88^{a}	12.40^{ab}	12.74^{ab}	12.05^{abc}	10.62^{abc}	8.30^{bc}	5.87^{c}	2.242	**
2-Ethyl-1-hexanol	1026	4.93^{c}	6.33^{bc}	9.57^{a}	6.53^{bc}	7.77^{abc}	6.83^{abc}	8.11^{ab}	1.091	**
2-Octen-1-ol	1065	2.12^{a}	1.72^{ab}	1.87^{a}	1.67^{ab}	1.58^{ab}	1.25^{ab}	0.84^{b}	0.362	*
Octanol	1068	4.52^{a}	3.85^{ab}	4.33^{a}	2.95^{abc}	2.58^{bc}	2.16^{c}	1.62^{c}	0.565	***
Dodecanol	1476	18.48	22.16	20.25	17.51	23.90	16.94	15.40	4.738	NS
Subtotal		68.23^{a}	62.44^{ab}	68.17^{a}	55.10^{abc}	58.61^{abc}	46.44^{bc}	39.36^{c}	7.484	**
Furans										
2-Pentylfuran	988	92.12^{a}	75.65^{ab}	72.24^{abc}	70.88^{abc}	64.72^{abc}	50.28^{bc}	41.60^{c}	11.88	**
(*E*)-2-Pentenylfuran	997	4.04^{a}	3.60^{ab}	3.71^{ab}	2.85^{abc}	2.47^{abc}	1.86^{bc}	1.40^{c}	0.689	**
2-Hexylfuran	1087	3.58^{a}	3.46^{a}	5.08^{b}	3.37^{a}	3.55^{a}	3.04^{a}	2.67^{a}	0.480	**
2-Octylfuran	1289	3.96^{a}	3.68^{ab}	3.26^{abc}	2.70^{abc}	2.17^{abc}	1.76^{bc}	1.38^{c}	0.786	**
Subtotal		105.45^{a}	87.51^{ab}	85.47^{ab}	80.75^{abc}	73.69^{abc}	57.52^{bc}	47.46^{c}	13.34	**
Sulphur compounds										
Dimethyl disulfide	755	5.71	5.92	4.05	4.69	5.57	6.43	6.24	2.650	NS
Dimethyl sulfide	973	2.04	2.43	2.04	2.65	2.64	2.33	2.22	0.686	NS
Subtotal		7.74	10.04	6.10	7.35	8.27	8.80	8.46	3.336	NS
Benzene compounds										
Benzaldehyde	966	7.64	7.38	8.65	8.18	9.77	9.75	8.59	1.682	NS
Benzeneacetaldehyde	1046	2.83	2.31	2.81	2.76	2.89	2.81	2.50	0.556	NS
1-Phenyl-1,3,3-trimethylindane	1738	0.90	1.41	1.64	1.50	1.81	1.42	1.35	0.560	NS
Diphenyl ethanedione	1856	1.57	0.78	0.65	1.01	1.32	1.14	0.67	1.033	NS
Benzil dimethyl ketal	1912	14.72	7.71	5.07	7.51	6.22	6.32	4.93	7.231	NS
Subtotal		30.01	23.04	21.52	23.67	25.12	25.05	21.56	9.293	NS

(*continued on next page*)

Table 5 (*continued*)

	LRI	Antioxidant treatment[#]							RSD	Significance
		CON	SULP	ASC	AST20	AST40	AST60	AST80		
Total sum of volatiles		583.22[a]	574.95[a]	527.53[a]	489.86[ab]	453.83[ab]	381.60[ab]	317.15[b]	73.34	**

LRI: Linear retention index.
RSD = residual standard deviation.
Significance: NS = not significant; + = $P < 0.1$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.
abc: different superscripts in the same row indicate statistical differences by the Tukey-Kramer test ($P < 0.05$).
[#] CON = control (no antioxidant additives); SULP = 450 mg sodium metabisulphite per kg of patty mixture; ASC = 500 mg sodium ascorbate per kg of patty mixture; AST20 = 20 mg of astaxanthin per kg of patty mixture; AST40 = 40 mg of astaxanthin per kg of patty mixture; AST60 = 60 mg of astaxanthin per kg of patty mixture; AST80 = 80 mg of astaxanthin per kg of patty mixture.

(Resconi, Escudero, & Campo, 2013; Yang et al., 2015), which would have originated due to thermal oxidation during both patty cooking and head-space extraction of volatiles.

The antioxidant treatment variable showed a significant effect on the total sum of volatiles and specifically on the sums of the above-mentioned chemical families (mainly composed of secondary products of lipid oxidation), as well as on most of the individual compounds within these families ($P < 0.05$; Table 5). Statistical differences ($P < 0.05$) were found between CON patties and AST40, AST60 and/or AST80 patties for octane, tridecane and tetradecane, the aliphatic aldehydes with less than sixteen carbons, the aliphatic ketones with seven and eight carbons and the four furans detected. Overall, the use of astaxanthin resulted in a dose-dependent decrease in those volatiles significantly affected. The results, thus, suggest that astaxanthin produced a protective effect against lipid thermal degradation and thus reduced the levels of lipid derived volatiles. This effect could be explained by the capacity of astaxanthin to scavenge lipid radicals, resulting in inhibition of the lipid oxidation free radical chain reaction (Skibsted, 2012). A decreasing effect of dietary natural antioxidants on the levels lipid derived volatiles in cooked lamb has been previously suggested by Resconi et al. (2010), who found a lower presence of ketones and unsaturated aldehydes in meat from lambs fed on pasture than in that from lambs fed on concentrate. They attributed this difference to a high lipid oxidative stability in the former due to the high concentration of antioxidants in fresh pastures. However, in partial disagreement with our study, they found that diet (levels of natural antioxidants in meat) did not affect the levels of volatile saturated aldehydes in meat. Notwithstanding, both studies are not totally comparable because, unlike the study by Resconi et al., in our study the fatty acid profile of meat would be similar between treatments. Moreover, the natural antioxidants involved in each study were different.

On the other hand, the use of or metabisulphite (SULP) or ascorbate (ASC) had no significant effect on the level of volatile compounds in the head space with regard to CON patties except for 2-ethyl-1-hexanol and 2-hexylfuran, the levels of which were higher in ASC patties ($P < 0.05$). Ascorbate, was thus not able to protect against lipid oxidation during lamb heating in spite of its demonstrated ability to reduce lipid oxidation during refrigerated storage (Jayathilakan, Sharma, Radhakrishna, & Bawa, 2007).

The changes in volatile profile due to astaxanthin could affect lamb patty flavour, probably towards a milder flavour. This is because among the volatiles detected and significantly affected, the aldehydes such as hexanal, 2-nonenal, 2-decenal, 2,4-decadienal or some of the ketones such as 2-heptanone, have been highly correlated with flavour modifications in lamb (Bueno et al., 2011; Descalzo & Sancho, 2008; Resconi et al., 2010), with some of them providing lamb with meaty flavours.

4. Conclusion

The findings of this study suggest that the inclusion of natural astaxanthin at levels from 20 to 80 mg/kg in meat preparations, particularly in raw and cooked lamb patties, can be used to delay lipid oxidation during their aerobic refrigerated storage, thus contributing to improve their shelf life. Furthermore, astaxanthin demonstrate a protective effect on lipids, including fatty acids and cholesterol, against their thermal degradation due to cooking. To some extent those effects appeared to be dose dependent and become greater than the effects shown by other conventional antioxidants, i.e. ascorbate and metabisulphite. In contrast, results suggested that astaxanthin as lipid antioxidant can be less effective in frozen storage as compared with refrigerated storage, since an antioxidant effect of astaxathin during a 3-month frozen storage of patties could not be evidenced.

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Microbial growth and biogenic amine production in a Balkan Style fresh sausage during refrigerated storage under a CO_2-containing anaerobic atmosphere: effect of the addition of *Zataria multiflora* essential oil and hops extract

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antibiotics

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Article

Microbial Growth and Biogenic Amine Production in a Balkan-Style Fresh Sausage during Refrigerated Storage under a CO_2-Containing Anaerobic Atmosphere: Effect of the Addition of *Zataria multiflora* Essential Oil and Hops Extract

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Abstract: Fresh sausages are highly perishable, and the preservatives allowed in these types of meat preparations are limited. Balkan-style fresh sausages were prepared in triplicate without antimicrobials (Control), with an aqueous hops extract (30 mL/kg), with *Zataria multiflora* Boiss essential oil (1 mL/kg), or a combination of both (15 and 0.5 mL/kg, respectively), and refrigerator-stored under a 20% CO_2 and 80% N_2 atmosphere. The spoilage microbial growth, i.e., lactic acid bacteria (LAB), *Brochothrix thermosphacta*, *Enterobacteriaceae*, *Micrococcaceae*, molds and yeasts, the pH value, and the production of biogenic amines in the sausages were monitored weekly and compared with a control sausage during a 35-day storage period. Furthermore, 349 colonies of presumptive LAB (isolated from the De Mann, Rogose-Sharpe agar plates) were identified using a MALDI-TOF-based method. Growth levels to ≈ 9 Log colony forming units (CFU) per g were reached by LAB, with a predominance of *Lactobacillus sakei*. *Enterobacteriaceae* and *B. thermosphacta* also showed significant growth (up to 6 Log CFU/g). Biogenic amine levels increased, and tyramine values overcame 250 mg/kg. The study could not demonstrate a significant effect of antimicrobial source treatments in any of the characteristics studied, and thus, the shelf-life of sausages.

Keywords: lamb sausage; lactic acid bacteria; shelf-life; natural antimicrobials; meat preparations; modified atmosphere packaging

1. Introduction

Fresh sausages are produced with comminuted meat, salt, species, and condiments and a limited number of allowed additives. Their formulation, preparation, and dimensions strongly depend on local preparation. Fresh sausages must be refrigerator-stored and cooked before consumption. They are considered to be highly perishable, with pH values >5.5 and water activity (a_w) ≥0.97 [1].

To retard microbial growth, fresh sausages are commonly stored at low temperatures under anaerobic CO_2-containing modified atmosphere packaging (MAP). The spoilage microbiota of fresh sausages on these conditions consists of facultative anaerobic microorganisms such as lactic acid bacteria (LAB), *Brochothrix thermosphacta*, and *Enterobacteriaceae*, with LAB being observed as the predominant group [2,3]. The shelf-life of fresh sausages refrigerator-stored under anaerobiosis depends on the hygienic quality of raw materials, pH, a_w, storage temperature, atmosphere, etc. [4]. Some authors, based on the appearance of off-odors and discoloration, have found a shelf-life for these sausages slightly longer than 10 days [3] and others of more than 20 days [5–7].

During refrigerated storage of fresh sausages under vacuum or anaerobic MAP, and most probably due to the growth of LAB and *Enterobacteriaceae*, a significant production of biogenic amines (BA) such as tyramine, putrescine, and cadaverine occurs [4,8]. In a previous study [2], levels of tyramine higher than 100 mg/kg were found in a Mexican fresh sausage stored in anaerobic MAP for more than two weeks, which represents a health risk and corroborates the need to control the production of BA in fresh sausages.

A current approach to extend the shelf-life of fresh sausages is the use of natural antimicrobials [9]. Hops, the strobiles (female flowers) of the *Humulus lupulus* L. plant, which are commonly used in brewery and have found application in other foods [10], appear to be a potentially suitable ingredient for this purpose. Hops contains antimicrobial compounds, such as prenylated acylphloroglucinols, bitter acids or xanthohumol, among others, which have been probed to inhibit Gram-positive bacteria [11,12]. The Food Safety and Inspection Service from the USA has approved the use of hops α-acids as antimicrobials for cooked meat and casings [13]. Moreover, Kramer et al. [11] found hops extract to inhibit total aerobic microbial growth in marinated pork. However, the effect of hops in fresh sausages packaged under anaerobic conditions seems to have been rarely studied. Hops could interfere in the growth of Gram-positive spoilage microorganisms such as LAB or *Brochothrix thermosphacta*, thus extending the sausage shelf-life.

Plant-derived essential oils (EO) obtained from aromatic and medical plant materials have proved wide antimicrobial spectra against bacteria, yeasts, and molds [14]. Nonetheless, among bacteria, the Gram-positive are more susceptible than the Gram-negative [15]. The effectiveness of EO at levels up to 2% in extending the lag phase or reducing the final population of spoilage microbiota in minced meat and meat products during refrigerated storage has been reported [16,17]. EO has been claimed to be one of the best alternatives to synthetic preservatives in meat and meat products [17]. However, the use of EO in meat as well as in other foods as natural preservatives present relevant limitations regarding deleterious effects in sensory quality due to their strong flavor, loss of antimicrobial activity due to interactions with food components, and regulatory or safety issues [18]. In this context, the use of EO combined with other synergistic or complementary natural antimicrobials has been suggested as a viable approach to using lower amounts of EO, thus not affecting the sensory acceptation, while achieving a significant antimicrobial effect.

Among the EO, that obtained from *Zataria multiflora* Boiss (ZM), which contains carvacrol and thymol as its main components, has shown a significant antimicrobial effect, this effect being greater on Gram-negative bacteria [19–21]. *Zataria multiflora* Boiss, belonging to the *Laminaceae* family, is cultivated in warm parts of the Middle East, where it is popularly used in traditional medicine and as food flavoring and preservative [22]. Regarding processed meat, it has been reported that the addition of ZM's EO at levels up to 0.1% reduced the growth of total viable microbiota, *Pseudomonas* spp., and LAB in buffalo burgers during aerobic storage [23,24]. Moreover, a chitosan film containing this EO (5–10 g/kg) also reduced the counts of total viable microbiota at the surface of mortadella-type slices packaged in oxygen permeable polyethylene bags during refrigerated storage [25]. The above-mentioned studies were carried out using aerobic storage; however, no study has been found in the literature addressing the antimicrobial effect of ZM's EO on meat or meat products stored under anaerobic MAP.

This study has aimed to evaluate the growth of spoilage microorganisms and BA production in a typical fresh sausage during refrigerated storage under CO_2 plus N_2 MAP, and to assess the effect

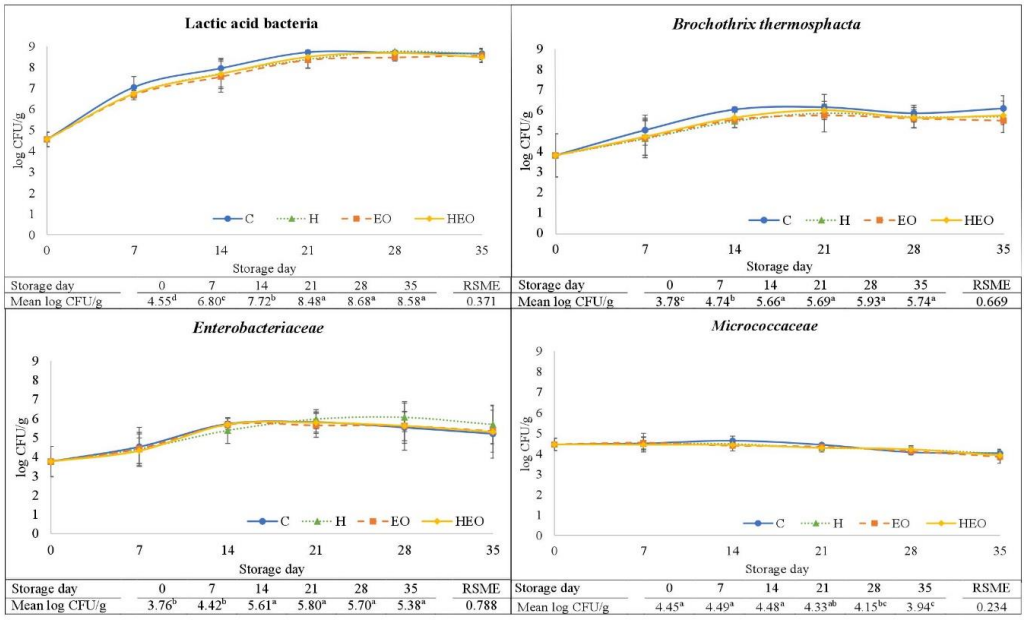
of two natural antimicrobials: hops and ZM's EO, alone or combined. The study focused on LAB population considering them as the predominant spoilage microorganisms in fresh sausages packaged under this type of atmosphere and responsible for the BA formation.

2. Results and Discussion

2.1. Water Activity, pH, Microbial Contents, and Biogenic Amine Production

The mean (standard deviation) a_w and pH values of the sausages from the three batches at day 0 were 0.987 (±0.004) and 6.01 (±0.02), respectively. During storage, the pH values decreased steadily ($P < 0.05$) from day 7 to day 28 for all the treatments, with the effect of either treatment or treatment x storage time interaction being non-significant. The mean values of pH in sausages (the four treatments) at days 7, 14, 28, and 35 were 6.05 (±0.01), 5.89 (±0.01), 5.66 (±0.02) and 5.61 (±0.03), respectively (data not shown in tables for brevity).

As is shown in Figure 1, the counts of LAB, *B. thermosphacta*, *Enterobacteriaceae*, and *Micrococcaceae* were not significantly affected by antimicrobial treatment. The mean counts of the Gram-positive LAB and *B. thermosphacta* tended to be higher in control (C) sausages, although the *P* values from the analysis of variance (ANOVA) were not significant, i.e., 0.263 and 0.397, respectively (not shown in figures). ZM's EO contains high amounts of antimicrobial molecules, i.e., thymol, carvacrol, *α*-terpinene, and a contrasted antimicrobial effect in in vitro experiments [19,21]. In this study, the lack of effect of ZM's EO on microbial growth in the fresh sausages could be explained by a loss of inhibitory efficacy due to interactions between the antimicrobials and sausage matrix compounds such as fat or specific proteins, which could be influenced by the sausage pH and a_w [15,18].



Lactic acid bacteria							
Storage day	0	7	14	21	28	35	RSME
Mean log CFU/g	4.55^{d}	6.80^{c}	7.72^{b}	8.48^{a}	8.68^{a}	8.58^{a}	0.371

Brochothrix thermosphacta							
Storage day	0	7	14	21	28	35	RSME
Mean log CFU/g	3.78^{c}	4.74^{b}	5.66^{a}	5.69^{a}	5.93^{a}	5.74^{a}	0.669

Enterobacteriaceae							
Storage day	0	7	14	21	28	35	RSME
Mean log CFU/g	3.76^{b}	4.42^{b}	5.61^{a}	5.80^{a}	5.70^{a}	5.38^{a}	0.788

Micrococcaceae							
Storage day	0	7	14	21	28	35	RSME
Mean log CFU/g	4.45^{a}	4.49^{a}	4.48^{a}	4.33^{ab}	4.15^{bc}	3.94^{c}	0.234

Figure 1. Effect of the addition of different natural antimicrobial sources on lactic acid bacteria: *Brochothrix thermosphacta*, *Enterobacteriaceae*, and *Micrococcaceae* counts (mean values, $n = 3$, and standard deviation, vertical bars) in fresh lamb sausages packaged under modified atmosphere (80% N_2, 20% CO_2) during refrigerated storage (4 °C). CFU: Colony forming units. RSME: Root square mean error. C: Control sausages; H: hops; EO essential oil (*Zataria multiflora* Boiss); HEO: hops and essential oil. ^{abcd}: Total means ($n = 12$) with different superscripts time-related indicate statistical differences (Tukey test, $P < 0.05$).

In contrast with our results, the addition of different EO, i.e., bay leaf, cassia, clove, holy basil, lemon, thyme, or sage, to fresh sausages at levels between 0.01% and 0.25% has shown significant reducing effects on the growth of spoilage microflora during refrigerated storage of fresh sausages packaged under aerobic atmosphere [26–29]. Moreover, the ZM's EO at levels up to 0.1% also significantly decreased the growth of total viable microbiota, *Pseudomonas* spp. and LAB in burgers during aerobic refrigerated storage [23,24]. Nevertheless, a clear difference between those studies and this one is that in the formers, the atmosphere was aerobic and in this study it was anaerobic. This suggests that the antimicrobial effect of EO in fresh comminuted meat products might be higher on the microbiota growing in fresh minced meat products with O_2 than in that growing when the presence of O_2 is restricted.

Hops extracts have been demonstrated to be useful as antimicrobials in casings, cooked ready-to-eat meat, and marinated meat products [11,13]. However, no study has been found investigating their antimicrobial effect in fresh comminuted meat products. In this study, hops extract given alone or combined with ZM's EO did not reduce the growth of spoilage bacteria. Again, chemical interactions between the hops antimicrobials and the food matrix would be the reasons for the lack of significant antimicrobial activity. In order to achieve a positive antimicrobial effect due to the use of hops extracts in fresh sausages stored under anaerobiosis, it is suggested to use a higher amount of hops antimicrobials or reduce the sausage pH, due to the reported higher effect of hops in food matrix with pH close to 5 [11].

Regarding the changes on microbial growth (Figure 1), LAB became the dominant microbial group from day 7 onwards, reaching final values slightly higher than 8 Log colony forming units (CFU) per g at day 21, which can be considered as the onset of the stationary growth phase. Psychrotrophic LAB have been found to become the major microorganisms in fresh sausages refrigerator-stored under anaerobic CO_2-containing MAP over the third week of storage [3,30], with the maximum LAB levels being comparable to those from this study. Lactic acid bacteria are considered as the principal spoilage-specific microorganisms in meat and fresh sausages stored under vacuum or CO_2-containing anaerobic MAP [31]. Thus, the appearance of off-flavors, i.e., sour or putrid, in fresh pork sausage has been related to LAB counts over 7–8 Log CFU/g [5,32].

Both *B. thermosphacta* and *Enterobacteriaceae* showed similar growth patterns between them, i.e., starting with counts near to 4 Log CFU/g at day 0 and reaching the stationary phase at day 14 with counts of 5–6 Log CFU/g (Figure 1). In both cases, the growth phase was slower and shorter than that for LAB, which suggests a competitive effect of LAB, probably due to a higher ability of LAB for the consumption of limiting nutrients under the anaerobic MAP fresh sausage conditions [33].

Other studies also described how *B. thermosphacta* steadily increases its levels in fresh meats during refrigerated storage under vacuum and anaerobic MAP, becoming one of the dominant spoilage species and originating cheesy, buttery, or sour odors [34]. According to Samelis [35], the levels of *B. thermosphacta* associated to fresh meat spoilage are around 7 Log CFU/g.

The control of *Enterobacteriaceae* in fresh sausages seems to be desirable since levels of 4–5 Log CFU/g [36] have been associated with meat spoilage—counts higher than this level were overcome in this study at day 14. The growth pattern of *Enterobacteriaceae* in fresh sausages stored under CO_2- and N_2-containing MAP has shown variability among studies. In agreement with our results, Benson et al. [32] reported an exponential growth of *Enterobacteriaceae* during the first two weeks of storage of a fresh pork sausage, reaching counts around 6 Log CFU/g; however, Ruíz-Capillas and Jiménez Colmenero [3] found the levels of *Enterobacteriaceae* in fresh pork sausages to decrease after 10 days of refrigerated storage. These differences might be explained by variations among studies in spice mixtures, sausage pH, or bacterial communities and their competence.

Micrococcaceae counts were stable up to day 21 of storage and then decreased slightly until day 35 (Figure 1). A decrease after some weeks of storage has been described in other studies on fresh sausages during anaerobic refrigerated storage [37,38], and attributed to both pH decrease and low O_2 and nutrients availability. No differences were found due either to treatment nor storage time in the molds and yeast counts (the mean values considering all the treatments and days were 2.99 ± 0.17 Log CFU/g; $n = 24$; data not shown in tables for brevity), which is probably due to their low growing ability under diminishing O_2 levels [38].

Changes in BA production in sausages are shown in Table 1. The levels of BA were not affected by antimicrobial treatment except for spermine ($P = 0.037$), with slightly higher amounts in the hops extract and essential oil (HEO) sausages than in the C sausages. Mono and diamines in fresh sausages are presumably produced from microbial enzymatic decarboxylation of free amino acids. In ripened sausages, this is mainly carried out by LAB and *Enterococci*, this ability being strain-dependent [39]. *Enterobacteriaceae* and *B. thermosphacta* can also contribute to the production of BA in LAB-fermented meats, with the first being more active in cadaverine and putrescine formation and the latter in histamine and tyramine [40–42]. The lack of effect of treatment on BA in the fresh sausage is thus coherent with the absence of a significant effect on microbial growth. However, the levels of tryptamine, putrescine, and histamine were significantly different between the experimental batches (data not shown in tables). Thus, the levels of putrescine and histamine in the second batch were respectively more than 5 times and 20 times higher than in the other two batches, which would corroborate the dependence of BA production on microbial strains.

In contrast with our results, Lu et al. [43] reported a reduction in biogenic mono and diamine production in Chinese smoked sausages as a result of the addition of a mixture of essential oils (from cinnamon, cloves, ginger, and anise; 0.12% in total) and tea polyphenols (0.19%) to the sausage mix. The discrepancy between both studies could be attributed to differences in the antimicrobial source used, the making process, and storage conditions (i.e., 50 °C smoking, 20–22 °C fermentation and 10–12 °C ripening-drying steps versus continuous refrigerated storage under CO_2-containing MAP).

Storage time affected the amounts of all mono and diamines in the fresh sausage, which increased steadily, indicating a continuous formation of those BA by the active microbiota during storage. However, time did not affect the content of polyamines originated from de novo synthesis in animal tissues [44]. Overall, the time-related changes in the content of BA in this study have been observed in other studies on fresh sausages stored under anaerobic MAP [2,8]. Biogenic monoamines can produce toxic effects on the consumers resulting in migraine, hypertensive crisis, or allergy [45]. Among them, histamine and tyramine present the highest health concern [46]. The maximum recommended levels for both amines in fermented sausages are over 100 mg/kg—although their toxicity depends not only on their levels in food but also on dietary factors and consumers' susceptibility [47]. Tyramine content in the fresh sausages from this study exceeded that limit (100 mg/kg) at day 14. On the other hand, although the diamines putrescine and cadaverine are not considered toxic per se, they can enhance the toxic effect of histamine and tyramine [48].

Table 1. Biogenic amine contents (mg/kg) in fresh sausages stored at 2 °C under anaerobic modified atmosphere storage as a function of antimicrobial treatment (Treat) and storage day (Time).

Biogenic Amine	Treat				Time						RMSE	*P*-Value		
	C	H	EO	HEO	0	7	14	21	28	35		Treat	Time	Treat × Time
Monoamines														
Tryptamine	17.66	20.48	17.52	19.08	5.58 ^{b}	6.28^{b}	16.85 ^{ab}	18.80 ^{ab}	29.61 ^{a}	34.99 ^{a}	16.33	0.942	0.000	1.000
Histamine	6.90	7.58	4.53	8.24	0.53 ^{b}	0.37 ^{b}	2.40 ^{b}	4.31 ^{b}	13.96 ^{ab}	19.29 ^{a}	17.48	0.926	0.046	1.000
Tyramine	143.04	152.28	150.89	150.40	19.88 ^{e}	99.04 ^{d}	138.37 ^{cd}	176.22 ^{bc}	206.08 ^{ab}	255.31 ^{a}	44.25	0.924	0.000	1.000
Diamines														
Putrescine	8.36	6.97	6.04	6.99	1.36 ^{b}	1.86 ^{b}	3.06 ^{ab}	7.03 ^{ab}	12.23 ^{ab}	17.00 ^{a}	12.35	0.955	0.015	1.000
Cadaverine	98.03	121.74	126.37	114.29	2.14 ^{c}	14.33 ^{c}	66.69 ^{c}	148.73 ^{b}	203.61 ^{ab}	255.14 ^{a}	54.56	0.432	0.000	0.996
Poliamines														
Spermine	25.52 ^{b}	28.88 ^{ab}	29.22 ^{ab}	29.89 ^{a}	30.12	30.17	26.57	26.88	27.16	29.34	4.72	0.037	0.200	0.506
Spermidine	5.83	5.80	6.06	6.22	5.56	6.02	5.97	6.06	5.87	6.37	1.24	0.715	0.738	0.587

C: Control sausages; H: hops; EO essential oil (*Zataria multiflora* Boiss); HEO: hops and essential oil. $^{a\text{-}e}$: Means in the same row within treatment or time showing different superscripts are significantly different ($P < 0.05$; Tukey test).

2.2. Identification of Lactic Acid Bacteria

Only eight out of the 346 isolates from the DeMan-Rogosa-Sharpe (MRS) agar plates were not positively identified. Among the identified isolates, 90% corresponded to LAB (70% of isolates were identified as LAB at day 0 and ≥90% at the other sampling days). Among the non-LAB bacteria, the genus identified in order of abundance were *Staphylococcus* spp., *Enterobacter* spp., *Serratia* spp., *Filifactor* spp., *Escherichia* spp., and *Macrococcus* spp. (not shown in tables). Table 2 shows the frequency (%) of the LAB species identified at different storage days considering the isolates in sausages from the four antimicrobial treatments. The genus *Lactobacillus* was the most abundant (84% of the LAB isolates) regardless of the storage time. *Lactobacillus sakei* was the predominant LAB, with its frequency overcoming 50% from day 7 onwards—when LAB counts showed significant growth (counts higher than 7 Log CFU/g; Figure 1). Among the isolates identified as *Lb. sakei*, 59% were identified as *Lb. sakei* subsp. *carnosus* (not shown in tables) and the others were identified as *Lb. sakei* (only species level). On the other hand, approximately 40% of the isolates ascribed to the *Lactobacillus* genus were not positively identified at species level (provided as *Lactobacillus* spp. in Table 2). Comparing between the first weeks (especially day 0) and the last weeks of storage, the diversity (number) of LAB species showed a tendency to decrease. On days 28 and 35, there was a clear dominance of *Lactobacillus*, and among them, *Lb. sakei* (<90% and ≤70% of total LAB, respectively).

Table 2. Lactic acid bacteria (LAB) species in the sausages [#] at the different storage days (expressed in % of total isolates identified as LAB).

Species	0 (n = 33)	7 (n = 50)	14 (n = 63)	21 (n = 52)	28 (n = 56)	35 (n = 53)
Aerococcus viridans	12	-	-	-	-	-
Carnobacterium maltraromaticum	-	-	5	2	-	4
Lactobacillus casei	3	-	-	-	-	-
Lactobacillus curvatus	-	2	2	-	-	2
Lactobacillus sakei	33	62	51	56	70	72
Lactobacillus spp.	12	14	38	37	30	23
Lactococcus lactis	24	2	-	-	-	-
Leuconostoc mesenteroides	12	14	3	6	-	-
Enterococcus faecalis	-	6	2	-	-	-
Streptococcus salivarius	3	-	-	-	-	-

n = number of isolates identified as lactic acid bacteria; [#] Results on each day include the isolates from the four antimicrobial treatments.

Figure 2 depicts the frequency of LAB species or genus, as identified by the matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis, obtained for each of the experimental treatments from day 7 to 35. *Lb. sakei* was predominant for each of the treatment-day combinations except for H-day 14 (ranging from 40% to 85%). The contingency chi-square test showed no significant effect of treatment either on the frequency of *Lb. sakei* or on *Lactobacillus* spp. (P = 0.419 and P = 0.729, respectively).

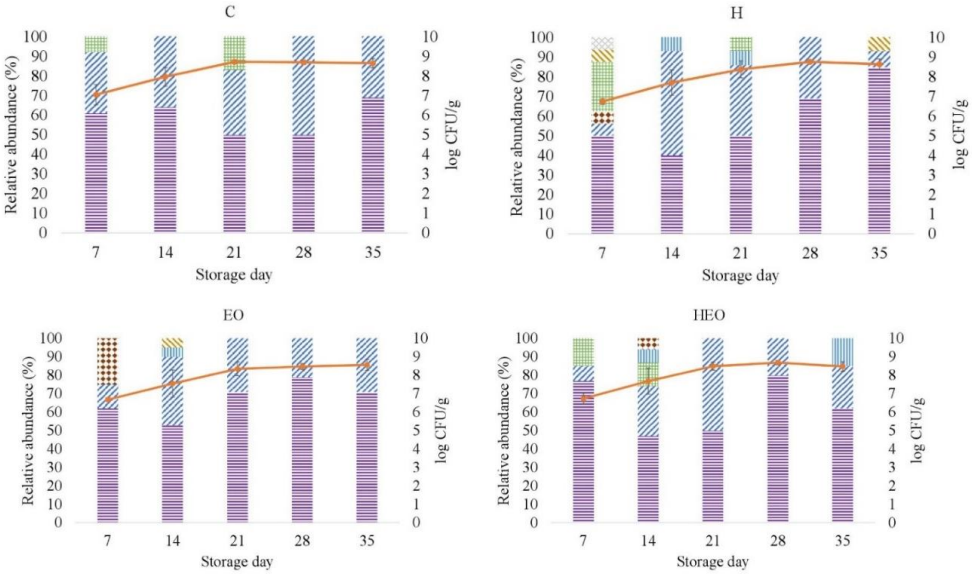


Figure 2. Relative abundance (%) and growth curve (mean and standard deviation, vertical bars; $n = 3$) of the isolates identified as lactic acid bacteria in fresh lamb sausages packaged under modified atmosphere (80% N_2, 20 %CO_2) during refrigerated storage (4 °C). CFU: Colony forming unit. C: Control sausages; H: hops; EO essential oil (*Zataria multiflora* Boiss); HEO: hops and essential oil. *Lactobacillus sakei* (), *Lactobacillus* spp. (), *Leuconostoc mesenteroides* (), *Enterococcus faecalis* (), *Carnobacterium maltromaticum* (), *Lactobacillus curvatus* (), *Lactococcus lactis* ().

Most of the studies on the succession of microbial population in fresh sausages during refrigerated storage have been carried out with the sausages stored under aerobic normal or modified atmosphere [1,4,6,32]. In these studies, LAB species have been one of the major microorganisms detected together with species belonging to *Enterobacteriaceae*, *Micrococcaceae*, *Pseudomonas* spp., and *B. thermosphacta*. However, LAB (*Lb. sakei*, *Lb. curvatus/graminis*) and *B. thermosphacta* tended to be the most predominant groups at the end of storage. The degree of abundance of LAB would directly depend not only on storage time but also on the reduction degree of redox potential during storage [49]. On the other hand, in the study by Fougy et al. [7], the sausages were packaged under vacuum and anaerobic 50% CO_2-containing atmosphere. The authors, in agreement with the results of the present study, found, using metagenetic 16S rRNA pyrosequencing, *Lb. sakei* to be the most abundant species in spoiled sausages (after 21 days of storage). Moreover, they also reported the presence of *Lactococcus piscium*, *Carnobacterium divergens*, *Carnobacterium maltaromaticum*, *Serratia proteamaculans*, and *B. thermosphacta*.

Lb. sakei together with *Lb. curvatus* appears to be the dominant LAB species in fermented sausages produced by spontaneous fermentation, and *Lb. sakei* is the most used LAB species as a starter culture for these sausages [50]. This species has been demonstrated to have an effective metabolic adaptation to the meat environment, and to cold temperature and high NaCl concentration. These abilities explain its growth in refrigerator-stored fresh sausages. This LAB have been demonstrated to be resistant to the presence of the antimicrobials tested in the present study at the levels used.

In contrast with traditional fermented sausages, fresh sausages became spoiled after fermentation, which would be due to differences in the LAB metabolism in a meat environment with higher a_w and the concomitant activity of other spoilage microorganisms, i.e., *B. thermosphacta* and *Enterobacteriaceae*.

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3. Materials and Methods

3.1. Experimental Plan

Three batches of ćevapi, a Bosnian-style fresh sausage, were prepared using lamb lean meat at the pilot plant at the Faculty of Veterinary Medicine, University of León (Spain). Each batch was composed of four treatments: control (C), with no antimicrobial additives in the formulation; hops extract (H), including an aqueous hops extract; essential oil (EO), with ZM's EO, and with both hops extract and essential oil (HEO). Sausages were packaged in bags under a modified atmosphere (20% of CO_2 and 80% N_2) and then stored under refrigeration at 2 °C for 35 days. Water activity (a_w) was determined at the day of packaging (day 0), and pH, the presence of relevant microbial groups, i.e., lactic acid bacteria (LAB), *Enterobacteriaceae, Brochothrix thermosphacta, Micrococcaceae* and yeast and mold, and the concentration BA were analyzed weekly (storage days 0, 7, 14, 21, 28, and 35). Moreover, 346 colonies (4–5 per batch, day, and treatment) were picked from De Man Rogosa agar plates used for LAB counts and identified by MALDI-TOF mass spectrometry.

3.2. Lamb Meat, Hops Extract, and Essential Oil

Lamb meat came from the legs of six male Assaf lambs reared at the Instituto de Ganadería de Montaña (CSIC; Grulleros, León, Spain). The lambs were weaned with 14 ± 2 kg of weight and fattened to 50 ± 4 kg of weight ad libitum on a pelleted complete diet based on straw (150 g/kg), cereals (barley, corn and soybean meal; 810 g/kg), molasses (10 g/kg), a mineral-vitamin premix (25 g/kg), and sodium bicarbonate (5 g/kg). The animals were then slaughtered in a local abattoir, and their legs were separated from the right-hand carcasses after 24 h post-mortem and then deboned. The meat was then cut into approximately 3 cm cubes, which were trimmed of visible fat. The lean meat from each leg was packaged under vacuum and frozen (−20 °C) until being used (up to 3 months).

The aqueous hops extract used in the study was obtained from recently cropped Nugget variety hop, with α-acid, β-acid, and co-humulone composition of 4.8–5.3%, 12–16%, and 22–28%, respectively. The hops was provided by a local producer (Orbigo Valley S.L., Madrid, Spain). An amount of 50 g of hops was boiled into 1 L of water for 30 min and the final volume was filled up to 1 L, which was filtered through a Whatman number 1 filter paper (GE Healthcare Europe, Barcelona, Spain) and frozen at −18 °C until further use. ZM's EO was obtained from the Faculty of Agriculture, University of Tehran, Iran. Crushed dried leaves of ZM plant were transferred to an all-glass Clevenger-type apparatus and steam distilled for 2.5 h. The essential oil was then dried over anhydrous Na_2SO_4 and stored in opaque glass bottles until further use.

3.3. Sausage Manufacture

Three batches of sausages were produced on different days using the meat from the legs of 2 among the 6 lambs for each of the batches. The sausage-making process was based on a Balkans-style ćevapi recipe. Lamb meat was thawed at 5 °C for 24 h and minced using a butcher's mincer equipped with a 5 mm diameter sieve. A total of 3.8 kg of minced meat was mixed with salt (80 g) for 10 min and placed into a bowl covered with cling film and stored at 4 °C until the next day (24 h). A mixture of finely cut fresh garlic and pepper was boiled in water for 2 min. The mixture (spices solution) was cooled, filtered, and then stored (4 °C) until the next day. The salted minced meat was divided into four parts of 950 g each, one for each of the four above-mentioned treatments (C, H, EO, and HEO) using the ingredients provided in Table 3. All the portions were mixed (for 5 min) with the spice solution (20 mL/kg) and 3 g/kg of sodium bicarbonate. C, EO, and HEO were also mixed with an amount of water, H and HEO treatment with hops extract, and EO and HEO with essential oil (see Table 3). The amount of hops extract added to the H sausage was equivalent to 1.5 g of hops per kg of sausage (i.e., 30 mL of the solution obtained from boiling 50 g of hops per L), which is that commonly used in brewery. The amount of ZM's EO used (1 mL/kg) was within the concentration

ranges reported for antimicrobial activity of EOs in food [16], i.e., around 0.5–20 mL of EO per kg. When both antimicrobial sources were added, their amounts were halved.

Table 3. Ingredients and amounts (expressed in g or mL, solid or liquids, respectively) used in the sausage preparation for the experimental treatments.

Ingredients	Treatments			
	C	H	EO	HEO
Lamb meat	980	980	980	980
Salt	20	20	20	20
Sodium bicarbonate	3	3	3	3
Spice infusion [a]	20	20	20	20
Water	30	-	30	15
Hops extract	-	30	-	15
Essential oil	-	-	1	0.5

C: Control sausages; H: hops; EO essential oil (*Zataria multiflora* Boiss); HEO: hops and essential oil. [a]: Filtered solution obtained by boiling garlic and pepper in water.

The sausage mixtures were stuffed into lamb casings (20/22 cm diameter) and drained for 3 h at 12 °C. The sausages were then cut into 100 g portions, which were individually packaged in bags (150 µm plastic film, oxygen permeability of 30 $cm^3/(m^2 \times bar \times 24\,h)$ at 23 °C and 0% relative humidity) under a 20% CO_2 and 80% N_2 atmosphere at 750 mbars, and refrigerator-stored (2 °C). One C portion was used for analysis at day 0 and one packaged portion for each of the treatments was sampled after 7, 14, 21, 28, and 35 days of storage for subsequent analysis.

3.4. Analysis of Water Activity, pH, Microbial Content, and Biogenic Amine Production

Water activity (a_w) was determined in duplicate at 25 °C using a CX-2 hygrometer (Decagon Devices Inc., Pullman, WA, USA) following the manufacturer's instructions, and pH using a pHmeter (Model 507; Crison, Barcelona, Spain) according to the International Organization for Standardization (ISO) guideline 2917 [51]. For microbiological analysis, samples of 25 ± 0.1 g of sausages were homogenized with 225 mL of peptone water (0.1% peptone) for 2 min in sterile bags using a Stomacher-400 circulator (Seward, West Sussex, UK). Serial decimal dilutions were prepared, and aliquots of the appropriate dilutions were cultured in duplicate on the corresponding media and incubated, according to the procedure described by the culture media manufacturer, as follows: 1 mL on the De Man-Rogosa-Sharpe agar (Oxoid) with double agar layer at 30 °C for 72 h for LAB; 1 mL in Mannitol Salt Agar (Oxoid) at 35 °C for 48 h for *Micrococcaceae*; 1 mL in Violet Red Bile Glucose Agar (VRBGA; Oxoid) with double agar layer at 35 °C for 48 h for *Enterobacteriaceae*; 1 mL in Oxytetracycline Glucose Yeast extract agar (Oxoid) at 22 °C for 5 days for molds and yeast, and 0.1 mL onto the surface of STAA Agar Base (CM 0881; Oxoid) plates containing STA Selective Supplement (0.4 mL/100 mL) and sterilized glycerol (1.5 g/100 mL), at 22 °C for 48 h (only the straw colored oxidase-negative colonies were considered).

Biogenic amine contents were analyzed following the Eerola, Hinkkanen, Lindfors, and Hirvi [52] procedure using a high performance liquid chromatograph (HPLC) Alliance (Waters 2695) equipped with a double wavelength detector (Waters 2996, Waters Corporation, Milford, MA, USA) and a Spherisorb ODS2 column (125 × 4 mm ID; 5 µm; Waters). The standards used for detection and quantification were tryptamine cadaverine dihydrochloride, histamine dihydrochloride, putrescine dihydrochloride, spermidine, spermine, tryptamine hydrochloride, and tyramine hydrochloride (Sigma-Aldrich Química, Madrid, Spain).

3.5. Identification of Lactic Acid Bacteria

From the growth in the MRS plates, 4–5 colonies were picked for each experimental treatment (4), sampling day (7) and batch (3), giving 346 colonies in total. These isolates were then grown in Tryptone

Soy Broth (TSB; Bacto, Mt Printchard, Australia) with 0.5% (*w*/*v*) of yeast extract (YE; Difco, Leeuwarden, The Netherlands) (TSB-YE) at 37 °C for 24 h. One mL aliquot was centrifuged (12,000 rpm, 3 min) in Eppendorf tubes (Eppendorf Ibérica, San Sebastián de los Reyes, Madrid). The supernatants were discarded, and the pellets were suspended in 1 mL of MRS broth with 50% (*v*/*v*) of glycerol. The isolates were maintained at −40 °C for storage purposes. Isolates were recovered for their identification as follows: they were grown at 30 °C on MRS broth (Oxoid) with 0.5% (*w*/*v*) of YE (Difco) at 37 °C for 24 h, and then a loopful of bacteria was sub-cultured in MRS agar (Oxoid).

The analysis was carried out at the Laboratory for Instrumental Analysis, University of Valladolid (Valladolid, Spain). For the analysis, one colony from the MRS plate was picked using a sterilized toothpick and smeared gently onto a MALDI-TOF target plate (Bruker Daltonik GmbH, Leipzig, Germany). After air-drying, 1 µL of formic acid was added. The dried sample was overlaid with 1 µL matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid (HCCA) in a mixture of acetonitrile, deionized water, and trifluoracetic acid (50/47.5/2.5, *v*/*v*/*v*). The target plate with samples were introduce in the MALDI-TOF equipment for analysis. Not all the bacteria were amenable to analysis: approximately 30% of the isolates were not accurately identified, i.e., identification score at genus level <1.7. For these isolates, the analysis was repeated including an ethanol extraction tube protocol before analysis to extract ribosomal proteins according to the manufacturer's instructions (Bruker Daltonik). Briefly, one colony from MRS plates was sub-cultured in TSB + 0.5% (*w*/*v*) of yeast extract at 35 °C overnight. One mL aliquot of the isolate was transferred into an Eppendorf tube and centrifuged at 12,000 rpm for 2 min. The supernatant was discarded, and the pellet was mixed thoroughly with 1 mL of deionized water and centrifuged at the same speed and time. This stage was performed twice. Afterward, 900 µL of absolute ethanol and 300 µL deionized water were added, mixed for 2 min, and the tube was centrifuged at 15,000 rpm for 5 min. The supernatant was discarded, and the pellet was air-dried for a minimum of 30 min until dryness. The pellet was re-suspended with 15 µL of formic acid (70%) and mixed thoroughly. Moreover, the mix was kept for 5 min at room temperature and then 15 µL of acetonitrile was added and mixed. The mixture was centrifuged at 15,000 rpm for 3 min and subsequently, 1 mL of the supernatant was spotted onto a MALDI-TOF target plate. After being air-dried, the sample was overlaid with 1 µL of matrix solution (HCCA).

For identification, each series of measurements was preceded by a calibration step with a bacterial test standard (BTS 155 255343; Bruker Daltonik) to validate the run. Mass spectra were generated by a Flex Analysis MALDI-TOF mass spectrometer (Bruker Daltonik) equipped with a nitrogen laser (l1⁄4337 nm) operating in linear positive ion detection mode under the Bruker Flex Control software (Bruker Daltonik). The Autoflex LT Speed was periodically calibrated by using the Bruker Daltonik *Escherichia coli* bacterial test standard DH5. Automated analysis of the raw spectral data was performed by the MALDI BioTyper automation (version 3.1) software (Bruker Daltonik) using a library of 5627 main spectra (MSPs; database update of 7/15/2015). Identifications at species or genus level were considered if scores were above 2.0 and 1.7 respectively, according to the report generated by Bruker Compass [53,54].

3.6. Statistical Analysis

Data on microbial counts and BA levels were analyzed by two-way analysis of variance (ANOVA) with treatment and storage day as fixed factors. When the fixed factors or their interaction showed significant differences ($P < 0.05$), the ANOVA was followed by the Tukey's post-hoc test. For the results of the LAB identification, a contingency table (4 by 2; treatment by positive or negative) chi-square analysis was used to test the eventual dependence between treatment and the frequency of the presence in the sausages of the main genus or species identified, considering the entire storage period. The statistical analysis was performed using the SPSS Statistics software (version 24; IBM, Somers, NY, USA).

4. Conclusions

The results from this study demonstrate that Balkan-style fresh sausages stored under anaerobic atmosphere are already fermented in the first week of storage and the predominant responsible species are *Lactobacillus* spp., specifically *Lb. sakei*. The fermentation was compatible with a controlled growth of *B. thermosphacta* and *Enterobacteriaceae* and resulted in BA production to a concerning level, thus suggesting that the contents of BA in anaerobic MAP fresh sausages should be controlled. The use of *Zataria multiflora* Boiss EO, hops extract, or the combination of both at the levels used did not significantly affect the microbial development in the sausages. More studies using higher amounts of these antimicrobial sources, different combinations with other antimicrobials, extracts with higher concentrations of active compounds, or previous encapsulation, would be needed to achieve their effectiveness in fresh sausage preservation.

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6

The effects of storage and hop extract on aroma and flavour compounds in Balkan-style sausages packed under a CO_2-containing anaerobic atmosphere

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ABSTRACT

Changes in the levels of pH, lactic acid, acetic acid, headspace volatile compounds, and spoilage odour intensity during a 35 day refrigerated storage period in two sets of a Balkan-style fresh sausage, a control sausage (CON) and a sausage containing an aqueous hop extract (HOP), packaged under a 20% CO_2 and 80% N_2 atmosphere were evaluated. Storage resulted in progressive sausage acidification and increased the levels of acetic acid, 1-methylbutanol, ethyl acetate, ethyl hexanoate, and 2-ethylhexanol; all of which are associated with anaerobic microbial metabolism under sugar restricted conditions. Storage decreased the levels of hexanal, heptanal, pentanol, and garlic-derived organosulfur compounds. Hop extract showed oxygen scavenging abilities, and decreased the levels of the volatile compounds derived from lipid auto-oxidation while contributing to the presence of specific terpene compounds. The use of hop extract did not improve the shelf life of sausages packed under anaerobic atmosphere. The spoilage odour appeared in both types of sausages on the 14th day, and it was considered strong from day 21 onwards.

1. Introduction

Fresh sausages are commonly made from minced meat to which salt and different spices and condiments are added. These sausages are highly perishable. In order to extend their shelf life, low storage temperatures combined with packaging under CO_2-containing anaerobic atmospheres are utilised (Cocolin et al., 2004; Hugo and Hugo, 2015). In fresh sausages stored under these conditions, lactic acid bacteria (LAB), mainly *Lactobacillus* species, together with *Brochothrix thermosphacta* and different *Enterobacteriaceae* species become the predominant microbiota and the principal spoilage organisms (Benson et al., 2014). The growth of these microorganisms has been monitored in different studies and associated with a negative effect on the sausage flavour, resulting in a shelf life of 10–20 days (Casaburi et al., 2015; Fougy et al., 2016; Martínez et al., 2005; Pothakos et al., 2015; Ruiz-Capillas and Jiménez-Colmenero, 2010; Salinas et al., 2014). The volatile compounds associated with the spoilage of meat packed under anaerobic atmosphere have been extensively described (Casaburi et al., 2015). However, to the best of our knowledge, no studies on storage-related changes in the flavour compounds of fresh sausages are available, with the exception of that by Rux et al. (2019). These authors reported volatile compounds originated from spices, meat, and microbial activity, and suggested that further investigation was needed to elucidate the role of microorganisms in the generation of volatile compounds.

Antimicrobial additives are frequently used to increase the shelf life of fresh sausage and, due to the trend of replacing synthetic antimicrobials, the use of natural bioactive compounds is increasingly being considered (Hugo and Hugo, 2015). Considering their high content of antimicrobials, which are especially effective against Gram-positive bacteria (Hrnčič et al., 2019), hop extracts, commonly used in brewing, seem to be an appropriate natural antimicrobial agent for use in the meat industry (Singh et al., 2014). No studies where hop extracts have been added to fresh sausages to improve their shelf life have been found in the literature.

Therefore, the aims of this study were to describe and gain knowledge of the changes in flavour compounds in the headspace of Balkan-style

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fresh lamb sausages (CON treatment) during refrigerated storage under a CO_2-containing anaerobic atmosphere and to assess the effect of using hop extract (HOP treatment) as a natural antimicrobial and antioxidant ingredient against those changes. This is a continuation of a previous study (Carballo et al., 2019) which evaluated the changes in microbial populations and biogenic amine formation due to storage time and the use of hops.

2. Materials and methods

2.1. Sausage preparation and treatments

The sausages used in the study were the same as those previously described by Carballo et al. (2019). The right-hand leg meat from six male Assaf lambs (49.5 ± 2 kg body weight) reared under a conventional intensive feeding system was used in this research. All handling practices were approved by the Spanish Research Council (CSIC) Animal Experimentation Committee (protocol number 100102/2017-4).

The legs were separated from the carcasses and deboned 24 h post-mortem. The leg meat was cut into approximately 3 cm cubes and trimmed of visible fat. Lean meat from each leg was vacuum-packaged and frozen (−20 °C) until further use (no more than three months). An aqueous hop extract was prepared using recently cropped Nugget-variety hops that were kindly provided by a local producer (Orbigo Valley S.L., Madrid, Spain). It contained α-acids, β-acids, and co-humulone at levels of 4.8–5.3%, 12–16%, and 22–28%, respectively. In brief, 50 g of hops were boiled in water for 30 min, and the mixture was filtered through a Whatman no. 1 filter paper (GE Healthcare Europe GmbH, Barcelona, Spain). The volume of the filtrate was made up to 1 l with water, and the hop extract was frozen (−18 °C) until further use. The antioxidant potential of the hop extract used in the experiment was 0.004 mmol Trolox per ml of extract as evaluated by 2,2-diphenyl-1-picrylhydrazyl analysis (Serpen et al., 2012).

Three batches of lamb sausages were produced on different days at the Faculty of Veterinary Medicine, University of León (Spain). The sausage preparation was based on a Balkan region recipe (ćevapi sausages): minced lean lamb, 950 g (two lamb legs were used per batch); common salt, 20 g; sodium bicarbonate, 3 g; spice infusion (filtered solution obtained by boiling garlic and black pepper in water), 20 ml; water, 30 ml. Two sets of sausages were prepared per batch: a control sausage (CON), following the recipe; and a hop sausage (HOP), where the same volume of hop extract replaced the water (30 ml). This amount of hop extract (30 ml of extract per kg of sausage mix) contained the water-extractable compounds from 1.5 g of hops, which is a comparable amount to that commonly used per litre of wort in brewing, i.e. 1-2 g hops/l. Furthermore, this amount did not negatively affect the Balkan-style sausage flavour, i.e. unpleasant bitterness, according to the results of preliminary taste studies carried out with consumers at the University of Sarajevo.

The day before the sausage preparation, the leg meat and hop extract were thawed at 5 °C. The sausage-making process consisted of an initial step of mincing the meat using a butcher's mincer (5 mm diameter sieve) and mixing the meat with common salt for 10 min. The mixture was then kept covered with cling film at 4 °C for 24 h. Next, the spice infusion, sodium bicarbonate, and water (CON) or hop extract (HOP) were added to the salted minced meat, which was mixed for 5 min. The sausage mixtures were then stuffed into lamb casings (20/22 cm diameter), and the sausages were air-dried for 3 h at 12 °C and then cut into 100 g portions. The portions were individually packed into plastic film bags [12 × 20 cm; 150 μm thickness; oxygen permeability of 30 $cm^3/(m^2$ bar 24 h) at 23 °C and 0% relative humidity] under a CO_2 and N_2 (20% and 80%) atmosphere at 7500 kPa. Finally, the packed sausage portions were stored in darkness at 2 ± 1 °C for up to 35 days.

2.2. Sausage sampling

One portion of each of CON and HOP raw sausage was sampled at days 0 (day of packaging), 7, 14, 21, 28, and 35 of storage for microbial and chemical analysis (carried out in duplicate), i.e. total aerobic mesophilic counts, pH, lactic and acetic acids contents, and volatile compound contents.

Total aerobic mesophilic counts and pH analysis were carried out just after sampling; the remaining sausage sample was frozen at −30 °C for up to 3 months and then thawed (24 h, 4 °C) before analysis. Another sausage portion of CON and HOP raw sausages were sampled on each storage day and frozen at −18 °C for evaluation of spoilage odour. Prior to analysis, the CO_2 and O_2 concentrations inside the packaging bags were analysed on days 7–28. Due to technical reasons, it was not possible to obtain the measurements at day 35, and we assumed that at day 0 the concentrations were that of the gas cylinder (20% of CO_2 and 80% of N_2).

2.3. Microbiological analysis

For the aerobic mesophilic counts (AMC), 25 g (±0.1 g) of raw sausage samples were homogenised for 2 min with 225 ml of 0.1% peptone water and 0.85% NaCl in a Stomacher-400 circulator (Seward, West Sussex, UK). Serial decimal dilutions were then prepared, and 1 ml aliquots of the appropriate dilutions were plated out onto Standard Plate Count Agar (PCA; Oxoid Ltd, Basingstoke, UK). Finally, the cultured plates were incubated at 30 °C for 48 h.

2.4. Instrumental chemical analysis

The CO_2 and O_2 composition of the modified atmosphere in the packaging bags was analysed using OXIBABY equipment (Cambridge Sensotec, St Ives, UK). The pH was determined using a BasiC 20 pH meter (Crison Instruments, Barcelona, Spain) equipped with a 52–32 pH penetration electrode.

Lactic and acetic acid contents in the sausage samples were extracted by homogenising 10 g of sausage in 40 ml of 4.5 mM H_2SO_4 using a T10 basic Ultraturrax (IKA-Werke, Staifen, Germany), with further filtration through Whatman no. 54 filter papers (GE HealthCare, Little Chalfont, United Kingdom). The concentrations were determined following the method described by Bruna et al. (2003). A high-pressure liquid chromatograph (Model 2690; Waters Corporation, Milford, MA, EEUU equipped with a 300 mm × 7.8 mm Aminex HP-87H ion-exchange column (Bio-Rad Laboratories, Hercules, CA, USA) and 3 mM H_2SO_4 as eluent were used for separation. Detection and quantification were carried out using a diode array detector (Model 996, Waters Corporation) and adequate solutions of lactic and acetic acid standards (Sigma-Aldrich Química, Madrid, Spain) in 4.5 mM H_2SO_4.

The extraction of volatile compounds from the sausages was carried out using solid-phase micro-extraction (SPME) fibres (75 μm Carboxen/polydimethylsiloxane-coated fused silica fibre, 1 cm coating length) from 4 g of homogenised sausage sample in 15-ml screw cap vials. Vials with the samples were incubated at 40 °C in a water bath with sonication (Bransonic 221; Branson, Danbury, CT, USA) for 15 min (equilibration) and then an additional 40 min (exposition to the fibre). Extraction was followed by injection (for 2 min at 260 °C in splitless mode) and gas chromatography separation, using a CG 7890A equipment (Agilent Technologies, Santa Clara, FL, USA) equipped with a 60 m × 0.25 mm ID, 0.25 μm film thickness, DB-5MS column (J&W Scientific, Folsom, CA, USA). The oven temperature was programmed at 35 °C (1 min), 35 °C–50 °C (10 °C/min), 50 °C–200 °C (4 °C/min), 200 °C–250 °C (50 °C/min), and 250 °C (11 min). The carrier gas (helium) flow was 1 ml/min. Detection was carried out using an MSD 5975C simple quadrupole mass spectrometry detector (Agilent Technologies) operating in the electron impact mode (70 eV, 50 lA) and scanning from 40 m/z to 350 m/z at 3.94 scans/s.

Identification of volatile compounds was carried out by spectra comparison using the MSD ChemStation software, searching the NIST/EPA/NIH-98 Mass Spectral Database, personal interpretation, and comparison of the linear retention indexes, experimentally calculated using a series of n-alkanes (Hydrocarbons/C5–C20; Sigma-Aldrich, St. Louis,

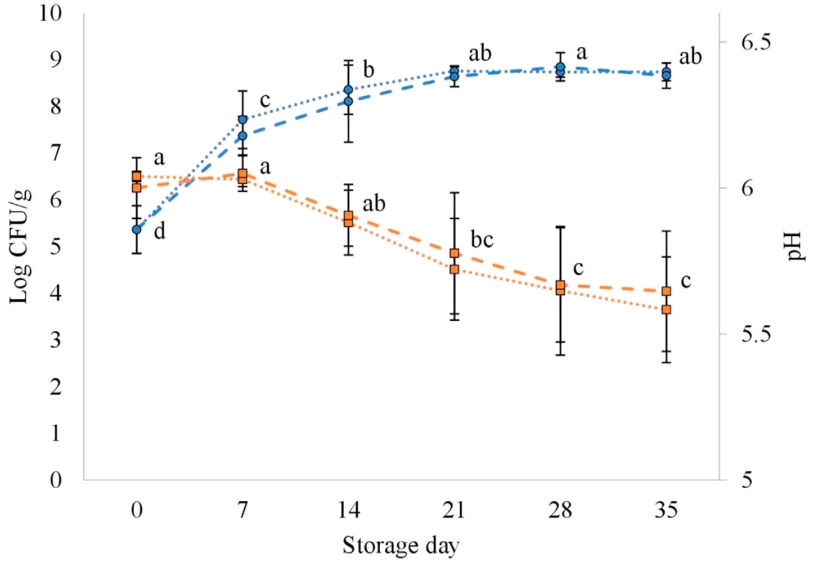

Figure 1. Changes in total viable bacterial counts (●) and pH (■) in fresh lamb sausages stored refrigerated (2 °C) under a 20% CO_2 and 80% N_2 atmosphere. Dotted lines, control sausages (CON); dashed lines, sausages containing 30 ml/kg of a hop extract (HOP). Vertical bars represent the standard deviation from the mean of each treatment (n = 3). a–d: different superscripts on the storage day values indicate statistical difference ($P < 0.05$; Duncan's multiple range test).

MO, USA), with those from the literature for a DB-5 capillary column or similar (Adams, 2007; Kondjoyan and Berdagué, 1996; Linstrom and Mallard, 2020; Vázquez-Araújo et al., 2013). The methods followed for extraction, identification, and detection were those described by Del Blanco et al. (2017) with a modification in the sample incubation phase, which was carried out in this study using sonication. The peak areas were transformed to ng of hexanal equivalents by comparing them with those of a hexanal external standard curve in hexane. Hexanal was used because it is one of the major volatile compounds in sausages and has an intermediate polarity and chromatography retention time.

2.5. Spoilage odour analysis

The odour analysis was carried out at the Instituto de Ciencia y Tecnología de Alimentos (University of León, Spain). It consisted of evaluating the odour intensity associated with spoilage of CON and HOP sausages at the different storage times. Six assessors were selected and trained before evaluation (four males and two females between 25 to 60 years old). The procedure followed for training and the test was based on that described by Martínez et al. (2016). Training consisted of three one-hour open discussion sessions to recognise and memorise the spoilage odour using reference sausages and to establish and practice the testing procedure and scale used. The reference sausages consisted of 100 g portions of the experimental sausages that were frozen on the day they were stuffed into casings (sausage with no perceivable spoilage odour) and after 42 days of refrigerated storage (sausage with extremely perceivable spoilage odour). Reference samples were prepared (for each panellist) by placing 10 g of reference sausage (thawed for 24 h at 4 °C) into tightly closed screw-cap vials (50 ml headspace, 22 mm diameter screw cap) covered with a layer of aluminium foil and tempered at room temperature (22 °C) for no less than an hour.

The analysis was carried out in individual booths under artificial green light to prevent panellists from associating the eventual colour differences in the sausage batter with the day it was stored. There were seven tasting sessions in total. In each session, up to six samples were tasted by at least five out of the six trained panellists; five is the lowest number of panellists recommended for product-oriented sensory analysis (Watts et al., 1989). All panellists smelled the whole sample set in random order. At the beginning of each session, panellists smelled vials containing the above-mentioned reference samples as a reminder. Samples were then analysed one by one as a blind testing. Panellists were given randomly ordered vials with the testing sausage samples prepared as explained for reference samples. They were asked to open the vial, take it with the fingers by the bottom, place the neck of the vial so it almost touched their nose, slowly inhale the air contained in the vial for a few seconds with both nostrils open, and then evaluate the spoilage odour. They were permitted to repeat the inhalation if needed. Spoilage odour was rated using a four-point scale (0 = not perceivable, 1 = slightly perceivable, 2 = considerably perceivable, and 3 = extremely perceivable, as in the reference spoiled sample). Between samples, panellists had to smell a vial with a mixture of odorant spices and herbs to avoid the adaptation of olfactory stimuli, as recommended by Doty (2017).

2.6. Statistical analysis

The microbial and chemical characteristics of the CON and HOP sausages were analysed in duplicate on each of the sampling days for each of the three batches produced, and each treatment. The mean value of the duplicates was calculated, and the means were analysed by two-way analysis of variance (ANOVA) with treatment and storage day as fixed factors. When the fixed factors or their interaction showed significant differences ($P < 0.05$), ANOVA was followed by Duncan's multiple range test.

For the spoilage odour analysis, panel reliability was assessed by testing 12 samples in duplicate (six CON and six HOP, one per sampling day and treatment, each duplicate was tested on the same day) and calculating the mean of the errors for each panellist. The errors were calculated as $\sqrt{[\sum (Sr_1 - Sr_2)^2/2N]}$, where Sr_1 and Sr_2 are the scores for replicates 1 and 2, respectively, and N is the number of samples tested in duplicate for each panellist. The mean error (±standard deviation) obtained was 0.6 (±0.3). To calculate the mean scores given to the sausages per day and treatment (as given in Table 3), the median value from the panellist's scores for each sample tested was calculated first, and then the mean value from the medians for each of the three sausage batches was obtained and rounded up to the nearest 0.5.

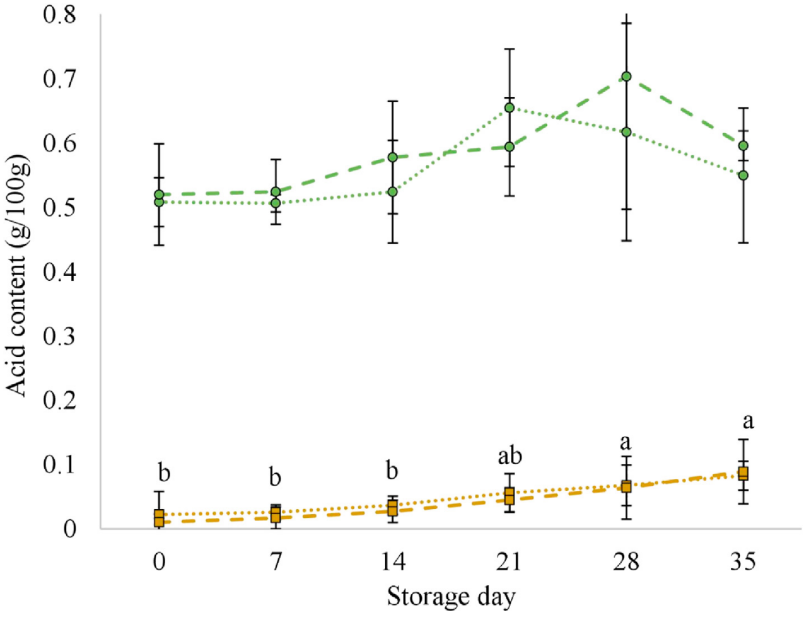

Figure 2. Changes in lactic acid (●) and acetic acid (■) content in fresh lamb sausages stored refrigerated (2 °C) under a 20% CO_2 and 80% N_2 atmosphere. Dotted lines, control sausages (CON); dashed lines, sausages containing 30 ml/kg of a hop extract (HOP). Vertical bars represent the standard deviation from the mean of each treatment (n = 3). abc: different superscripts for the storage day values indicate statistical difference ($P < 0.05$; Duncan's multiple range test).

3. Results and discussion

3.1. Changes in microbial counts, pH, lactic and acetic acids content, and atmosphere composition

The time-related changes in total aerobic mesophilic counts (AMC) and pH of CON and HOP sausages are depicted in Figure 1. Counts increased sharply during the first week of storage and reached final values of between 8 and 9 Log colony-forming units (CFU) per gram of sausage on day 21 when the stationary growth phase was reached. As seen in a previous study, LAB was the most predominant group by far in the CON and HOP sausages from day seven onwards (Carballo et al., 2019). This study also reported that among LAB species, *Lactobacillus sakei* was the principal one. *B. thermosphacta* and facultative anaerobic *Enterobacteriaceae* were also abundant among the sausage microbiota. Similar AMC growth patterns to those of the present study have been described for fresh sausages refrigerated under O_2 deprivation (Lerasle et al., 2014; Ruiz-Capillas and Jiménez-Colmenero, 2010).

The pH of the sausages decreased steadily ($P < 0.05$) from day 7 to day 28, by approximately 0.3 units. Significant differences from day 0 were found from day 21 onwards. This decrease was due to LAB fermentation of the sugars present in the sausage mix, originating from

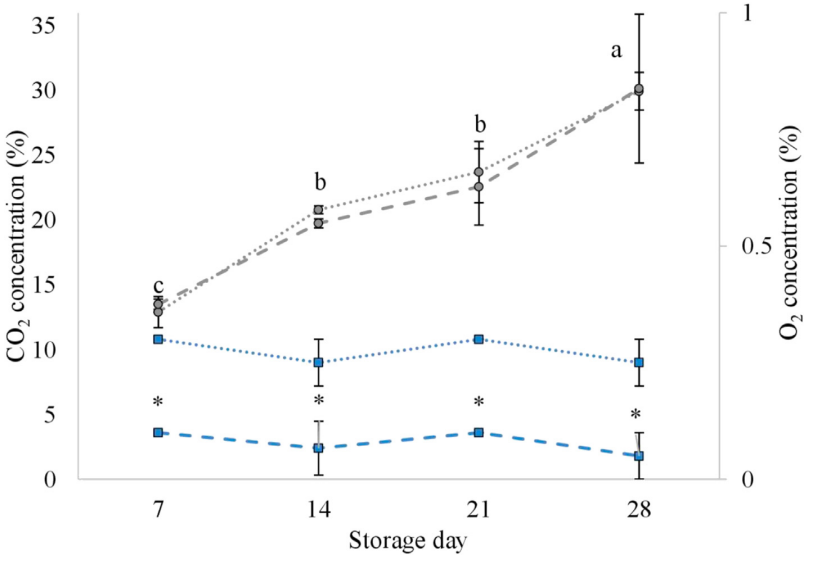

Figure 3. Changes in CO_2 (●) and O_2 (■) concentrations in the modified atmosphere of fresh lamb sausages stored refrigerated (2 °C) under a 20% CO_2 and 80% N_2 atmosphere (initial conditions). Dotted lines, control sausages (CON); dashed lines, sausages containing 30 ml/kg of a hop extract (HOP). Vertical bars represent the standard deviation from the mean of each treatment (n = 3). *: indicates the statistical difference in gas concentration between sausage type within a storage day ($P < 0.05$). abc: different superscripts for the storage day values indicate statistical difference ($P < 0.05$; Duncan's multiple range test).

Table 1. The total sum of volatile compounds (expressed as ng of hexanal equivalents) extracted from the headspace of fresh sausages stored at 2 °C under an anaerobic CO_2-containing modified atmosphere, and percentage of the different groups of volatiles extracted over the total sum, as a function of storage day (n = 6) and sausage treatment (control sausages, CON, or sausages containing 30 ml/kg of a hop extract, HOP; n = 18).

Volatile group†	Storage day (time)						Treatment (treat)		RMSE	*P*-value	
	0	7	14	21	28	35	CON	HOP		time	treat
Total sum of volatiles (46)	1410.6	813.2	938.7	816.3	1085.8	1174.4	1106.1	972.0	387.9	NS	NS
Abundance of groups (%)											
Acetoin and derivatives (3)	0.2^{b}	11.5^{ab}	10.7^{ab}	18.2^{a}	10.6^{ab}	11.2^{ab}	10.7	10.0	10.0	*	NS
Branched-chain and aromatic aldehydes (3)	0.1^{c}	3.8^{c}	6.0^{bc}	10.7^{ab}	10.5^{ab}	12.8^{a}	7.2	7.4	5.0	**	NS
Aliphatic esters (2)	n.d.	0.5^{c}	1.9^{bc}	3.9^{ab}	4.1^{ab}	4.6^{a}	2.8	2.2	1.8	***	NS
Aliphatic carbonyls (4)	13.5^{ab}	16.1^{a}	6.1^{bc}	6.0^{bc}	3.2^{c}	2.8^{c}	10.3	5.6	6.3	**	*
Aliphatic alcohols (4)	6.0	6.4	4.0	6.1	6.7	7.2	7.7	4.4	2.9	NS	**
Aliphatic hydrocarbons (11)	8.3	10.2	17.5	11.8	16.2	12.6	12.4	13.1	10.1	NS	NS
Sulphur compounds (7)	60.1^{a}	39.9^{b}	44.0^{b}	30.0^{b}	31.8^{b}	31.5^{b}	39.1	40.0	10.9	***	NS
Terpenes (12)	11.8^{ab}	11.7^{ab}	9.8^{b}	13.4^{ab}	17.0^{a}	17.3^{a}	9.7	17.3	4.3	*	***

n.d.: not detected; under the detection limit (0.1 ng of hexanal equivalents); ^{abc}: time-related means in the same row showing different superscripts are significantly different ($P < 0.05$; Duncan's multiple range test); RMSE: root mean square error; *P*-level: NS: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.
† Total number of volatiles or number of volatiles in each group in brackets.

the meat, garlic, and pepper, and also to dissolution of CO_2 gas from the packaging into the sausage (Lerasle et al., 2014). The maintenance of pH from day 0–7, despite great microbial growth (and thus fermentation) in this week, could be explained by the bicarbonate–carbonic acid buffer (pK = 6.1), since sodium bicarbonate was used as an ingredient.

The changes in lactic and acetic acids content are shown in Figure 2. No significant changes were observed for lactic acid levels due to storage. The mean concentrations of this acid were always in the range between 0.5 and 0.7 g/100 g. In contrast, acetic acid content increased significantly during storage until reaching amounts of approximately 0.1 g/100 g; significant differences from day 0 were found at days 28 and 35. The use of hop extract had no significant effect on the levels of both acids.

Lactic and acetic acids would have been produced in the sausages as end-products of sugar fermentation by the predominant bacteria, i.e. lactic acid by LAB, *B. thermosphacta*, and *Enterobacteriaceae*, and acetic acid by heterofermentative LAB and *Enterobacteriaceae* (Müller, 2008; Pothakos et al., 2015; Stanborough et al., 2017). The lack of a significant accumulation of lactic acid, despite it being produced via sugar fermentation, could be explained by its simultaneous degradation by lactate-utilising *lactobacilli*, which would result in additional acetic acid production (Gänzle, 2015). The generation of acetic acid could also be the result of the microbial use of pyruvate, which is highly generated from microbial catabolism of amino acids in the absence of sugars. The ability to use pyruvate by *L. sakei*, the most predominant LAB species in the sausages of this study (Carballo et al., 2019), for the production of energy has been reported (Montanari et al., 2018).

The percentages of CO_2 and O_2 in the packaging atmosphere during the storage of CON and HOP sausages are shown in Figure 3. Over time, the amount of CO_2 increased. It reached levels close to 30% on day 28 of storage, which was accompanied by pack distension. The increase in CO_2 levels could be directly attributed to bacterial metabolism, i.e. decarboxylation and oxidation reactions of amino acids, sugars, pyruvate, keto-acids (Carroll et al., 2016; Lerasle et al., 2014; Müller, 2008; Xiao and Lu, 2014). Furthermore, in this study, an important contribution to the CO_2 accumulated in the pack would have been the result of the gradual decomposition of sodium bicarbonate due to the also gradual pH decrease from 6.2 to 5.7 (Figure 1). The use of hop extract had no effect on the proportion of CO_2 in the atmosphere. The percentage of O_2 in the atmosphere was constant during storage. As can be seen in Figure 3, the hop extract reduced the residual O_2 content in a similar amount regardless of the storage time. This O_2-consumption effect might be attributed to the action of the polyphenols contained in the hop extract (Hrnčič et al., 2019) that, as part of their antioxidant mechanism, would scavenge O_2. The oxygen-scavenging ability of polyphenols has been reported previously (Gaikwad and Lee, 2016). The use of hop extract as an ingredient in sausages packed under anaerobic atmosphere would be recommendable to improve their lipid oxidative stability by reducing the presence of residual O_2.

3.2. Changes in volatile compounds

A total of 46 volatile compounds were detected in the headspace of both CON and HOP sausages during the whole storage period. The mean values of the total amount (sum of all) of volatile compounds (expressed as ng of hexanal equivalents) per day or sausage type, and the percentages of the volatile compounds grouped in different chemical families, over the total amount of volatile compounds, are shown in Table 1. The grouping of volatile compounds was carried out following both chemical (functional groups) and potential origin criteria. As explained below, a large part of the volatile compounds identified could be assigned to a single origin, i.e. microbial metabolism, lipid auto-oxidation, or spices and condiments. However, the other part would have had more than one main origin so that its presence and changes are more difficult to explain. The list of individual volatile compounds detected in the sausages and their levels are shown in Table 2. The *P*-levels for the interaction treatment × time were omitted from tables for brevity because they were always >0.1.

Time had a no significant effect on the total amount of volatile compounds ($P = 0.078$; Table 1), and this amount was not affected using hop extract ($P = 0.204$). Regarding the group percentages, time had a significant effect on the percentages of most of the chemical groups. Acetoin (3-hydroxy-2-butanone) and derivatives (Xiao and Lu, 2014), branched-chain and aromatic aldehydes, and aliphatic esters increased. In contrast, aliphatic carbonyls and sulphur compounds decreased. Significant differences were found between day 0 and days 21 or 28 (the last in the case of aliphatic carbonyls). The use of hop extract affected the aliphatic carbonyl and alcohol percentages negatively, and the terpene percentage positively (Table 1).

As for the individual volatile compounds, some of them were significantly affected by storage time and others by the use of hop extract, with the effect of time being strongest. In general, changes in the volatile compounds detected in the sausage headspace during storage could be explained by molecule degradation or formation reactions, and also to changes in the characteristics of the sausage matrix affecting the release of volatile compounds into the headspace, e.g. the pH changes (Figure 1). Changes in pH modify the capacity of meat proteins to adsorb volatile compounds. It has been reported that a pH change from 6 to 5 in a meat protein solution increases the surface hydrophobicity of myofibrillar proteins. This has been explained by the rebuilding of the protein conformation, and the subsequent changes in protein capacity to absorb

Table 2. Levels of individual volatile compounds (expressed as ng of hexanal equivalents) extracted from the headspace of fresh sausages stored at 2 °C under anaerobic CO_2-containing modified atmosphere as a function of storage day (n = 6) and sausage treatment (control sausages, CON, or sausages containing 30 ml/kg of a hop extract, HOP; n = 18).

Compound†	Storage day (time)						Treatment (treat)		RMSE	*P*-value	
	0	7	14	21	28	35	CON	HOP		Time	treat
Acetoin and derivatives											
Diacetyl (600)	3.1^{b}	71.1^{a}	92.3^{a}	96.3^{a}	103.7^{a}	84.6^{a}	77.1	73.2	67.4	*	NS
Acetoin (740)	n.d.	8.1	9.4	5.8	12.4	13.4	10.8	5.6	11.1	NS	NS
2,3-Butanediol (833)	n.d.	n.d.	n.d.	n.d.	12.0	35.6	4.8	11.1	28.3	NS	NS
Branched-chain and aromatic aldehydes											
Methyl-3-butanal (658)	n.d.	22.7	22.4	13.3	13.8	12.7	18.2	10.1	14.0	NS	NS
Methyl-3-butanol (767)	1.2^{c}	4.1^{c}	31.0^{bc}	52.1^{bc}	68.6^{ab}	108.7^{a}	39.9	48.7	45.1	**	NS
Phenylacetaldehyde (1077)	n.d.	n.d.	0.2^{a}	1.0^{a}	33.2^{b}	34.6^{b}	6.0	17.0	40.6	*	NS
Esters											
Ethylacetate (630)	n.d.	2.74^{b}	18.58^{ab}	30.86^{a}	34.14^{a}	39.64^{a}	21.97	20.01	19.7	**	NS
Ethylhexanoate (1015)	n.d.	n.d.	0.29^{b}	3.43^{b}	9.77^{a}	10.82^{a}	4.31	3.8	4.0	***	NS
Aliphatic carbonyls											
Hexanal (811)	176.0^{a}	125.7^{ab}	52.3^{ab}	62.2^{bc}	20.1^{c}	15.9^{c}	108.2	42.5	73.5	**	*
Heptanal (914)	15.6^{a}	4.4^{b}	5.0^{b}	5.5^{b}	1.8^{b}	2.6^{b}	7.4	4.3	7.0	*	NS
Nonanal (1118)	7.0	4.7	5.1	12.2	11.7	9.6	8.29	8.45	10.6	NS	NS
2-Heptanone (906)	2.6	1.6	1.8	1.3	1.4	1.8	2.11	1.42	1.7	NS	NS
Aliphatic alcohols											
Pentanol (798)	42.5^{a}	20.1^{b}	9.6^{bc}	11.2^{bc}	7.2^{bc}	5.3^{c}	21.9	10.1	11.2	***	**
Hexanol (899)	5.2	6.8	7.7	21.4	26.8	20.5	14.0	15.5	21.0	NS	NS
2-Ethyl-1-hexanol (1051)	2.6^{b}	6.5^{b}	7.3^{b}	9.5^{b}	17.6^{a}	19.2^{a}	14.6	6.3	5.9	***	***
1-Octen-3-ol (990)	34.7	21.4	14.5	17.4	20.1	33.0	33.0	14.0	23.7	NS	*
Aliphatic hydrocarbons											
Heptane (700)	0.8	0.6	2.3	3.5	2.8	2.5	2.1	2.1	2.1	NS	NS
Octane (800)	10.8	13.1	18.9	16.7	22.0	20.0	15.7	18.1	10.0	NS	NS
Decane (1000)	13.7	10.9	10.9	19.0	20.3	12.3	14.4	15.4	23.5	NS	NS
Undecane (1100)	1.0	0.5	1.5	3.4	3.4	3.1	2.1	2.0	2.9	NS	NS
Dodecane (1200)	1.0	0.5	0.5	1.1	1.5	1.0	0.8	1.1	1.8	NS	NS
Tridecane (1300)	0.6	0.2	0.4	0.7	1.2	1.3	0.8	0.7	1.2	NS	NS
Tetradecane (1400)	0.3	0.5	1.1	2.1	2.4	3.4	1.5	1.8	2.5	NS	NS
Octadiene (830)	3.6	6.9	5.7	3.7	6.1	2.0	5.8	3.5	5.2	NS	NS
2,2,4,6,6-Pentamethylheptane (991)	80.6	49.0	128.2	79.9	90.4	89.2	95.2	76.3	117.5	NS	NS
Branched alkane (1030)	8.3	4.4	15.6	7.8	9.3	9.1	10.7	7.5	16.1	NS	NS
Branched alkane (1115)	3.7	1.6	4.0	3.3	3.8	5.4	3.9	3.3	7.0	NS	NS
Sulphur compounds											
Allyl mercaptan (600)	94.7	35.8	46.7	12.0	36.2	56.8	47.0	47.0	44.2	NS	NS
Allyl methyl sulphide (706)	599.8^{a}	239.2^{b}	290.3^{b}	160.2^{b}	224.4^{b}	225.0^{b}	302.8	270.2	125.0	***	NS
Methyl 1-propenyl sulphide (740)	4.6	1.7	0.7	0.1	0.2	0.6	1.3	1.3	2.8	NS	NS
Dimethyl disulphide (751)	3.8^{a}	1.1^{ab}	2.4^{ab}	1.3^{ab}	0.2^{b}	n.d	0.6	2.3	2.5	NS	NS
Diallyl sulphide (861)	77.0	20.2	26.8	17.9	36.6	36.8	48.5	22.6	34.2	NS	*
Methyl allyl disulphide (925)	19.4	7.2	7.5	7.6	9.8	13.9	12.0	9.8	7.9	NS	NS
Diallyl disulphide (1090)	44.3	26.4	18.9	24.6	35.2	32.7	34.5	26.2	27.2	NS	NS
Terpenes											
α-Pinene (938)	11.6^{ab}	6.1^{c}	7.2^{bc}	5.0^{c}	11.7^{ab}	13.3^{a}	8.6	9.6	4.3	**	NS
Camphene (959)	0.9	3.0	1.4	1.2	1.3	1.6	1.4	1.8	2.2	NS	NS
β-Pinene (963)	3.3	2.3	3.8	1.4	2.2	1.7	2.9	2.1	2.7	NS	NS
Sabinene (980)	6.5^{ab}	4.8^{bc}	$2.4^{c\&}$	3.3^{bc}	6.3^{ab}	8.4^{a}	4.7	5.8	2.7	**	NS
Myrcene (998)	31.2	10.4	13.2	12.7	24.2	41.0	5.2	32.6	22.6	NS	*
Carene (1018)	38.8^{abc}	16.3^{c}	23.5^{bc}	22.2^{bc}	43.3^{ab}	51.0^{a}	31.7	33.3	19.4	*	NS
Cymene (1032)	3.0	3.0	2.9	2.8	4.6	4.7	3.3	3.7	2.6	NS	NS
Limonene (1038)	47.6	37.1	39.1	54.0	72.4	69.5	42.6	64.0	33.1	NS	NS
Dimethyl styrene (1108)	4.1	5.8	4.8	5.2	7.5	7.6	4.6	7.1	4.3	NS	NS
Copaene (1364)	1.0	0.5	0.2	0.8	2.0	2.2	0.6	1.6	1.3	NS	*
β-Caryophyllene (1410)	3.3	1.8	2.0	2.4	4.5	6.6	1.6	5.3	3.3	NS	**
Humulene (1465)	4.0	3.4	2.3	3.5	7.5	10.7	0.3	10.1	5.5	NS	***

n.d.: not detected, under the detection limit (0.1 ng of hexanal equivalents); ^{abc}: time-related means in the same row showing different superscripts are significantly different ($P < 0.05$; Duncan's multiple range test; RMSE: root mean square error; *P*-level: NS: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

† Experimental relative retention index in brackets.

volatile compounds (Yang et al., 2017). This capacity has appeared to be dependent not only on the protein conformation change but also on the chemical structure of volatile compounds, i.e. functional group and carbon chain length.

Considering both CON and HOP treatments, the levels of acetoin, diacetyl (2,3-butanedione), and 2,3-butanediol increased during storage (Table 2). Diacetyl, the most abundant, showed a significant increase from day 0–7 ($P < 0.05$). The levels of acetoin could not be quantified until day 7, and those of 2,3-butanediol until day 28, well after the onset of the microbial growth stationary phase (Figure 1). The presence of these compounds in meat has been attributed to microbial use of pyruvate or other intermediary metabolites formed from glucose and amino acid catabolism, the latter occurring under nutritional limiting conditions, such as glucose restriction (Casaburi et al., 2015; Montanari et al., 2018). Thus, in the fresh sausages studied, these volatiles would have been generated as metabolic end-products from sugars at the beginning of the storage and, more importantly, from amino acid catabolism later on, when the sausages supported significant microbial growth. According to Casaburi et al. (2015); Montanari et al. (2018), the presence of those volatile compounds can be related to flavour deterioration and spoilage of meat and fresh meat products during aerobic and anaerobic refrigerated storage.

Taking into account the changes in the microbial population in the sausages described in our previous study (Carballo et al., 2019), the production of acetoin and derivatives could be attributed, to a large degree, to the growth of *Lactobacillus* spp., specifically *L. sakei*. It could also be associated with the growth of *B. thermosphacta* and facultative anaerobic *Enterobacteriaceae*. All those microorganisms are able to generate acetoin from pyruvate (Montanari et al., 2018; Stanborough et al., 2017).

Storage also resulted in a net production of 3-methylbutanal, 3-methylbutanol, and phenylacetaldehyde (Table 2). These are intermediaries from the amino acid catabolism of leucine (the two former) and phenylalanine (the latter) and could have been produced by LAB and *B. thermosphacta* due to their aminotransferase activity (Ardö, 2006; Stanborough et al., 2017). These compounds have been commonly identified in stored meat, where they may play an important role in flavour due to their low odour thresholds, especially that of 3-methylbutanal (Casaburi et al., 2015; Smit et al., 2009). However, this role appears not to have been clearly stated so far.

The other two volatile compounds generated by microorganism metabolism that increased during storage were ethyl acetate and ethyl hexanoate (Table 2). Both have been found to be major esters among those detected in spoiled meat, with the latter having a potentially great impact on odour (Casaburi et al., 2015). It has been suggested that ethyl esters are formed in fermented food by the LAB species via esterification (e.g. from ethanol and acetate) or a transferase reaction, i.e. alcoholysis (from alcohols and acylglycerols), when ethanol is available (Liu et al., 2004). They can also be produced by *Enterobacteriaceae* species and *B. thermosphacta* (Casaburi et al., 2015).

The use of hop extract in the sausage formulation did not affect the levels of any of the above-mentioned volatile compounds related to microbial origin. The scarce effect of hops on the microbial metabolism, in spite of hops being rich in natural antimicrobials, is coherent with the lack of effect of hops on microbial growth in these sausages (Carballo et al., 2019), which was attributed to interactions between hop antimicrobials and the sausage matrix reducing their antimicrobial efficacy.

Most of the straight medium-chain carbonyls and alcohols detected in sausages has been reported to be secondary products from the oxidation of polyunsaturated fatty acids (Meynier et al., 1999). Among them, hexanal appears to be the most abundant in muscle food, and its levels have been proposed as indicators of lipid oxidative status in fresh and cooked meat (Shahidi and Zhong, 2005). Hexanal, heptanal, and pentanol levels showed a steady decrease in the sausages during storage, significantly different for hexanal from day 21, which suggests that lipid oxidation was not relevant during storage under anaerobic atmosphere and low temperatures. The decrease in the levels of those aldehydes can be explained mainly as a result of a negative balance between their formation by lipid oxidation and degradation by reactions with meat components (Shahidi and Zhong, 2005). The levels of hexanal, pentanol, and 1-octen-3-ol were significantly lower in HOP sausages (like the percentages of carbonyls and alcohols). The antioxidant effect of hop extracts (Villalobos-Delgado et al., 2015) was responsible for these reductions.

Apart from lipid auto-oxidation, several aliphatic carbonyls and alcohols, such as hexanal, nonanal, 2-heptanone, hexanol, and 1-octen-3-ol, could also be formed from microbial metabolism during fresh meat aerobic or anaerobic storage, i.e. amino acid catabolism, fatty acid oxidation, or de-hydrogenation of secondary alcohols, with the latter only in the case of 2-heptanone (Casaburi et al., 2015; Ercolini et al., 2009; Pothakos et al., 2015). Individual differences in their microbial and oxidation production rates, and in their reactivity with other sausage compounds, could explain the differences between the patterns followed during sausage storage by the carbonyls and alcohols detected.

The levels of 2-ethylhexanol in sausages showed a steady increase with storage time (significant from day 28). It is an alcohol commonly detected in stored meat that can negatively affect its flavour (Casaburi et al., 2015). To our knowledge, in the literature consulted, its presence in stored meat has not been related to lipid oxidation but to LAB and *B. thermosphacta* metabolism (Casaburi et al., 2015), and especially that of *Carnobacterium maltromaticum* (Ercolini et al., 2009). In agreement, the presence of this species has been detected in the sausages of this study (Carballo et al., 2019). This compound might also have originated from the molecular migration phenomena from the multilayer packaging material (Rivas-Cañedo et al., 2009), probably derived from the plasticiser di(2-ethylhexyl)phthalate (Horn et al., 2004). The levels of 2-ethylhexanol were lower in the HOP sausages. This might be explained as an inhibitory effect of the hop extract on the pathways involved in its production.

Eleven aliphatic hydrocarbons, representing close to 10% of the total volatile compounds, were detected in the sausages without being affected either by storage or hops. Their levels varied highly among samples, and the most abundant was 2,2,4,6,6-pentamethylheptane. Most of the aliphatic hydrocarbons found in the sausages were previously detected in the headspace of meat and fresh meat products (Del Blanco et al., 2017; Rivas-Cañedo et al., 2011). Their origin in meat has been generically associated with lipid oxidation (Madruga et al., 2009) and, more specifically, with migration from packaging (Rivas-Cañedo et al., 2009). The same authors reported the presence of some of the alkanes detected in the present study in the meat, i.e. undecane, tridecane, and 2,2,4,6,6-pentamethylheptane (the most abundant), as the result of migration from the multilayer plastic film used for packaging. The contribution of microorganisms to the formation of hydrocarbons and their impact on flavour seemed to be low (Casaburi et al., 2015).

All of the sulphur-containing volatile compounds detected in this study are major organo-sulphur compounds described in crushed and heated garlic, which are derived from allicin via thiosulphinate degradation (Kim et al., 2011). Five of them, namely allyl mercaptan, allyl methyl sulphide, diallyl sulphide, methyl propenyl disulphide and diallyl disulphide, were the garlic-derived sulphur compounds detected in the headspace of pork patties containing 1.4% or 2.8% fresh garlic as an ingredient (Park and Chin, 2014). As shown in Table 2, the percentage of sulphur compounds and the amount of allyl methyl sulphide (the most abundant) showed a significant decrease from day 0 to day 7. In line with the present results, Park and Chin (2014) also found that garlic-derived sulphur compounds in patty headspace decreased during storage. These experiments were carried out in trays covered by air-permeable film. They attributed part of the decrease to loss of volatile compounds from the patties to the environment. In this study, the packaging material had a reduced gas permeability; thus, the observed decrease might be associated with the loss of sulphur compounds from the sausages to the gas in the bags and to chemical interactions of the sulphur volatiles with

Table 3. Spoilage (off-odour) scores (median value obtained from 5-6 panellists per sample) given to fresh lamb sausages stored refrigerated (2 °C) under a 20% CO_2 and 80% N_2 atmosphere for up to 35 days as a function of sausage treatment (n = 3; control sausages, CON, or sausages containing 30 ml/kg of a hop extract, HOP).

Treatment	Storage day					
	0	7	14	21	28	35
CON	0	0	0.5	2	2	2
HOP	0	0	1	1.5	2.5	2.5

Spoilage off-odour scale: 0 = none, 1 = slight, 2 = considerable, 3 = extremely perceivable.

compounds in the packaging material or the sausage matrix. All these hypothesised reasons need further studies for validation. The effect of hop extract on sulphur compounds was limited to reduced amounts of diallyl sulphide, which might have interacted with hop components present in the sausages.

Two types of terpenes were found in the sausage headspace: monoterpenes and sesquiterpenes. Their origin could be attributed to the use of black pepper (Jagella and Grosch, 1999) and hop extract (Vázquez-Araújo et al., 2013), which are rich in the terpenes detected. The proportion of terpenes tended to be lower by the middle of storage and higher at the end. This pattern was accentuated in monoterpenes and statistically significant for three, α-pinene, sabinene, and carene. The change in terpene concentration could be attributed, at least partially, to a sausage matrix effect so that more terpenes were released into the headspace at the end of storage as compared with the middle. The lower pH of sausages at the end of storage might have an increased release of terpenes from the sausage to the headspace. Further studies are needed to confirm this hypothesis. Moreover, the hop extract, as expected, contributed significantly to the levels of myrcene, copaene, β-caryophyllene, and humulene, with all of them being reported as the major terpenes in hops (Vázquez-Araújo et al., 2013).

3.3. Odour analysis

The scores for spoilage odour intensity are summarised in Table 3. The spoilage score increased during storage for both sausage types. The off-flavour was detected on day 14 and became considerable from day 21 onwards. These patterns were consistent with those of acetic acid and several volatile compounds mainly produced by microbial metabolisms, such as 3-methylbutanol and the ethyl esters, supporting the microbial role in the formation of the spoilage off-odour. Moreover, these odour results suggest a shelf life for both CON and HOP sausages of no more than 14 days, when the spoilage odour was detected. This period is in general agreement with the shelf life of fresh sausages refrigerated under anaerobic atmospheres in other studies (Martínez et al., 2005; Ruiz-Capillas and Jiménez-Colmenero, 2010).

4. Conclusions

Flavour compounds in fresh sausages packaged under anaerobic CO_2-containing atmospheres experience complex changes during refrigerated storage as a result of different biochemical reactions that cause odour spoilage. The changes result in a progressive accumulation of a number of volatile compounds considered as end-products of meat spoilage specific microbiota growing under anaerobic and sugar restriction conditions. Among them, acetic acid, 1-methylbutanol, ethyl acetate, and ethyl hexanoate showed levels in sausage headspace directly correlated with storage time. In contrast, lipid oxidation could be not noticeable, i.e. secondary products of lipid oxidation would decrease or maintain their concentration in the sausage headspace during storage. Changes in pH during storage might induce a matrix effect resulting in changes in the release of volatile compounds such as organosulfur compounds or monoterpenes from sausage to the headspace. Further research is needed in this regard.

Hop extract, at the levels used, scarcely interfered with the metabolism of Gram-positive bacteria and appeared not to retard the appearance of sausage odour spoilage. Further research is needed to evaluate the possible effect of using higher amounts of hop extract. In contrast, hop extract at the used amount seemed to reduce the levels of secondary oxidation products in sausages and could exert an oxygen scavenging action. However, the advantage of the use of hop extracts in sausages stored under anaerobic atmospheres would be limited because the formation of lipid auto-oxidation derived volatiles seems not to be the main problem regarding the sausage flavour deterioration. The hop extract also contributed to the terpene content in the sausage headspace, thus potentially affecting sausage flavour.

Declarations

Author contribution statement

Diego E. Carballo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sonia Andrés, Francisco Javier Giráldez: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ali Khanjari, Sabina Operta: Conceived and designed the experiments; Performed the experiments.

Irma Caro: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Diego Llamazares: Performed the experiments; Analyzed and interpreted the data.

Javier Mateo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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7 Discusión General

En el presente trabajo se han realizado diversos experimentos para evaluar, en determinadas condiciones: el efecto antioxidante de la astaxantina en carne de cordero lechal y en hamburguesas de carne de cordero y el efecto antimicrobiano del extracto acuoso de lúpulo y del aceite esencial de ZM en una salchicha de cordero envasada en atmósfera modificada anóxica. A continuación, se discutirán los resultados obtenidos de los experimentos realizados, presentados en los artículos expuestos con anterioridad a manera de capítulos.

7.1. Efecto de la adición de astaxantina en un lacto-remplazante comercial sobre el color y la estabilidad oxidativa de la carne de lechazo

En este experimento se criaron 20 corderos lechales mediante la utilización de lactorremplazante comercial. El lactorreemplazante ofrecido a 10 de esos corderos fue suplementado con 1 g de astaxantina AstaREAL®EL25 (25 mg de astaxantina por kg de lactorremplazante); la cantidad de asta-

xantina utilizada no superó los límites considerados inocuos para la salud en humanos (<8 mg/día). Las características de calidad evaluadas en la carne fueron el grado de engrasamiento, el color y la estabilidad oxidativa de la carne de lechal.

Todos los lactorremplazantes comercializados en la región llevan BHT como antioxidante en su composición; el utilizado en este trabajo contenía 0,078 mg de BHT/kg de lactorremplazante. Por este motivo, el esperado efecto antioxidante de la astaxantina sobre la carne será en realidad un efecto combinado astaxantina + BHT, ya que parte del BHT ingerido por el animal queda depositado en su carne.

De acuerdo con estudios previos, el efecto de la suplementación de astaxantina en la dieta de los animales sobre la cantidad de grasa de la canal y de la carne es contradictorio. Ao y Kim (2019) y Jeong y Kim (2014) observaron que la adición de astaxantina en alimento para aves (7 y 5 mg de astaxantina por kg de alimento, respectivamente) producía un incremento significativo en la grasa abdominal de éstas. Por el contrario, Bergstrom et al. (2009) y Yang et al. (2006) encontraron que la adición de astaxantina en la ración de cerdos disminuía los depósitos de la grasa dorsal. En el presente estudio se observó un incremento significativo del uso de astaxantina en la grasa renal de los lechazos ($P = 0{,}022$), aunque no tuvo efecto significativo en la cantidad de grasa intramuscular. El efecto sobre la grasa renal podría considerarse positivo ya que la cantidad de grasa renal es una característica valorada en la clasificación de canales de ovino (Reglamento

de la IGP lechazo de Castilla y León; ES/PGI/0005/0069; DOOR, 2019).

En el presente trabajo, la cantidad de astaxantina en músculo no pudo ser cuantificada por estar por debajo del límite de detección del análisis utilizado (1,2 mg/kg de músculo; extracción con disolventes y espectrofotometría ultravioleta visible), pero sí se pudo cuantificar la cantidad de BHT en la carne. Ésta resultó ser menor en la carne de corderos suplementados con astaxantina que en la carne de corderos control (28 vs 48 mg/kg de grasa intramuscular; P <0,05). Este efecto sugiere la posible presencia de procesos competitivos en la absorción a nivel intestinal (Nieva-Echevarría et al., 2015), que habría tenido lugar entre ambos antioxidantes. No obstante, también se puede explicar, al menos parcialmente, por la mayor cantidad de grasa en los corderos alimentados con astaxantina, puesta de manifiesto por el peso más elevado de la grasa perirrenal en este grupo de corderos. El BHT está presente de forma habitual en aceites de fritura. La menor cantidad de BHT en la carne de los corderos con astaxantina se puede considerar beneficiosa para la salud humana cuando llega a ser consumida, ya que la recomendación de ingesta de BHT en la dieta se limita a 0,25 mg/kg de peso corporal por día (EFSA, 2012). El consumo de 100 g de la carne de los lechazos utilizados como control aportaría aproximadamente 0,025 mg de BHT y de la de los alimentados con astaxantina 0,015 mg.

Los resultados obtenidos en la presente investigación con respecto al **color** demostraron que la astaxantina tuvo un efecto moderado pero significativo sobre el mismo. La carne de los corderos con astaxantina presentó

una disminución en luminosidad (L^*) y tanto músculo como la grasa perirrenal presentaban aumento en el valor de índice de rojos (a^*). Estos resultados podrían suponer un efecto negativo de cara a los consumidores de carne de cordero lechal, ya que ésta tiene la característica especial de presentar un color pálido blanco-rosáceo (Erasmus et al., 2017). Sin embargo, para comprobar dicha afirmación se necesitaría hacer análisis del tipo sensorial.

Los resultados obtenidos en el color son consistentes con los obtenidos por Perenlei et al. (2014) y Ao y Kim (2019), quienes encontraron que una suplementación de astaxantina en el alimento de pollos y patos de 20 mg/kg y 7 mg/kg de alimento incrementaba significativamente el índice de rojos en las pechugas de dichos animales. Sin embargo, en otro experimento con cerdos, tal vez por ser la carne de cerdo más roja que la de pollo y lechazo, la adición de astaxantina en el pienso (en cantidades de entre 3 y 60 mg/kg) no afectó significativamente al color (Carr et al., 2010; Lei & Kim, 2014; Yang et al., 2006).

Con respecto a la estabilidad de color durante el almacenamiento aeróbico de la carne de cordero lechal, se pudo observar que la suplementación con astaxantina lograba disminuir ligeramente su decoloración, que comúnmente se produce después de 7 días de almacenamiento (Mateo et al., 2018). Este resultado indica que la acción de la astaxantina como antioxidante retarda la oxidación de la mioglobina, con el consiguiente mantenimiento del color rojo durante más tiempo.

Finalmente, se observó que la adición de astaxantina en la dieta de los corderos no ejerció efecto significativo sobre la **estabilidad lipídica** de la carne durante su cocinado (determinada a partir de los compuestos volátiles generados de la degradación de la grasa) o en la carne cocinada durante su almacenamiento a refrigeración (determinada por el análisis de TBARS). Sin embargo, el efecto antioxidante de la astaxantina pudo observarse en la estabilidad lipídica de la carne cruda congelada durante tres meses a –18ºC.

Los resultados sobre el efecto antioxidante de la astaxantina hay que interpretarlos teniendo en cuenta que la carne de cordero lechal alimentado artificialmente presenta ya alta estabilidad a la oxidación debido a la presencia de BHT y altas cantidades de vitamina E, procedentes ambos del lactorremplazante (Osorio et al., 2008). El experimento demuestra que la astaxantina puede contribuir de forma moderada a la estabilidad oxidativa de la carne de lechal alimentado con lactorremplazantes, ya de por sí alta. A partir de este trabajo se sugiere realizar otro estudio para comparar la estabilidad oxidativa de la carne aportada por la presencia de BHT en el lactorremplazante con la aportada por la presencia sola de astaxantina, con el fin de valorar la posible sustitución del primero por el segundo.

7.2. Efecto de la adición de astaxantina sobre el color y la estabilidad oxidativa en una hamburguesa de cordero

En este experimento se evaluó el efecto antioxidante de la astaxantina añadida a varias concentraciones (de 20 a 80 mg/kg de masa cárnica) en la

masa cárnica de hamburguesas de cordero, comparando los resultados con hamburguesas control sin antioxidantes y con hamburguesas conteniendo dos aditivos comúnmente usados en productos cárnicos (metabisulfito de sodio y ascorbato de sodio; 450 y 500 mg/kg de carne, respectivamente). Para evaluar dicho efecto en distintas condiciones de almacenamiento, las hamburguesas fueron almacenadas crudas a refrigeración y a congelación y también fueron cocinadas y posteriormente almacenadas a refrigeración.

Los resultados mostraron un **efecto colorante** de la astaxantina adicionada en las carnes para hamburguesas, disminuyendo la luminosidad y aumentando el valor de a^* de forma dependiente de la concentración adicionada. Estos resultados son consistentes con los obtenidos en carne de cerdo picada por Pogorzelska et al. (2018) y en filetes de pollo marinados por Abdelmalek et al. (2016).

Los resultados de la evolución del contenido en **TBARS** como índice de oxidación lipídica en las hamburguesas refrigeradas muestran en general un efecto antioxidante de la astaxantina, el cual es dependiente de la dosis. Las concentraciones de 40, 60 y 80 mg/kg de carne mostraron diferencias significativas respecto a las hamburguesas control y las concentraciones más altas superaron el efecto antioxidante del ascorbato. El incremento en la estabilidad lipídica de las hamburguesas cocinadas debido a la astaxantina corrobora la estabilidad térmica de la misma (Ambati et al., 2014). Sin embargo, el efecto antioxidante de la astaxantina no se evidenció en las hamburguesas crudas congeladas. En este sentido, varios autores han des-

crito que durante la congelación del pescado se puede perder la actividad antioxidante de la astaxantina y lo atribuyen a fenómenos de disrupción celular, disminución de la actividad de agua o difusión de oxígeno a través del tejido muscular (Erickson, 1997; Jensen et al., 1998).

Otro efecto importante de la astaxantina que se pudo observar en las hamburguesas cocinadas fue una disminución en la cantidad de **óxidos de colesterol**. Los óxidos de colesterol principalmente afectados por acción de la astaxantina fueron el 7α-hidrocolesterol y 7-cetocolesterol, los cuales han sido previamente asociados a enfermedades neurodegenerativas y cancerígenas (Brzeska et al., 2016; Poli et al., 2013).

Por último, la producción de **compuestos volátiles** por efecto del calor sobre los lípidos se vio disminuida por la adición de la astaxantina. Los alcanos, aldehídos y las cetonas alifáticas de cadena media fueron los compuestos mayormente afectados. Los resultados sugieren un efecto protector de la astaxantina sobre la degradación térmica de los lípidos que tiene lugar durante el cocinado de la carne (Skibsted, 2012). Dicho efecto fue observado principalmente para concentraciones de astaxantina mayores a 40 mg/kg de carne.

Los compuestos volátiles derivados de los lípidos en la carne cocinada pueden contribuir positivamente en su sabor. La disminución observada en los niveles de esos compuestos volátiles, en su mayoría responsables del sabor en la carne de cordero (Bueno et al., 2011; Resconi et al., 2010),

probablemente traen consigo cambios sensoriales. Sería interesante conocer el efecto de dichos cambios sobre la aceptación del sabor de las hamburguesas por los consumidores. Trabajo que queda pendiente para futuras investigaciones.

7.3. Efecto antimicrobiano del lúpulo y el aceite esencial de ZM en una salchicha fresca de cordero

Con este experimento se evaluó la, previamente documentada, actividad antimicrobiana del lúpulo (Hrnčič et al., 2019) y del aceite esencial de ZM (Saei-Dehkordi et al., 2010; Sharififar et al., 2007), en un embutido fresco de cordero envasado en atmósfera modificada anóxica almacenado a refrigeración durante 5 semanas. Se elaboraron varios embutidos: un embutido control sin antimicrobianos, uno con lúpulo, otro con aceite esencial y uno más conteniendo ambos compuestos. La cantidad de lúpulo empleada en la salchicha (1,5 g de lúpulo/kg de masa cárnica) fue equivalente a la que normalmente se usa en la elaboración de cerveza y la cantidad de aceite esencial (1 mL/kg de carne) estuvo dentro del rango de concentraciones consideradas como efectivas frente al crecimiento microbiano en alimentos, tal como lo indica Burt (2004). Además, en la salchicha que llevaba ambos compuestos antimicrobianos, la concentración de cada uno fue la mitad a la de los valores antes indicados. De acuerdo con estudios sensoriales preliminares, las cantidades utilizadas no ejercieron un efecto negativo sobre la aceptación sensorial de la salchicha.

7.3.1. Efecto del extracto de lúpulo y el aceite esencial de ZM sobre el crecimiento de microorganismos alterantes y la producción de aminas biógenas

Para evaluar la acción antimicrobiana de los compuestos ensayados se hicieron recuentos de los principales grupos microbianos responsables de la alteración microbiana de los embutidos frescos (aerobios totales, BAL, *Enterobacteriaceae, Brochothrix thermosphacta* o *Micrococcus*) y también se cuantificó la producción de aminas biógenas. Los datos de **crecimiento microbiano** mostraron que ninguno de los tratamientos con antimicrobianos naturales (lúpulo, aceite esencial o ambos) tuvo efecto significativo sobre el crecimiento de los microorganismos estudiados.

En estudios previos, sin embargo, se detectó actividad antimicrobiana para estos dos compuestos frente las bacterias alterantes de la carne o derivados cárnicos (Kramer et al., 2015; Nieto et al., 2020; Tajik et al., 2015; Torab et al., 2017). La discrepancia entre los resultados de esos estudios y el presente se puede atribuir a la diferencia en el tipo de derivado cárnico y las condiciones de almacenamiento. En este sentido, este estudio fue el único llevado a cabo con un embutido fresco almacenado en condiciones anaerobias (los otros se llevaron a cabo con carne marinada o hamburguesas con almacenamiento aerobio).

La ausencia de acción antimicrobiana podría atribuirse a un efecto bloqueante de la actividad por parte de compuestos de la matriz cárnica (masa de carne picada con un pH en torno a 5,5 - 6,0), unido a una escasa

sensibilidad de las bacterias del embutido a las condiciones de almacenamiento ensayadas (psicrotrofos anaerobios facultativos). Autores como Bor et al. (2016) relacionan la baja actividad antimicrobiana de aceites esenciales agregados directamente en los alimentos a dos hechos: 1) su baja solubilidad en agua, y 2) que los componentes como grasa y proteína en los alimentos posiblemente se unan a compuestos presentes en aceites esenciales, como el timol y eugenol, ocasionando la disminución o eficacia de su acción antimicrobiana.

En este contexto, sería conveniente hacer más estudios probando el posible efecto antimicrobiano de cantidades mayores de lúpulo o aceite esencial a las utilizadas en el experimento, siempre y cuando no ejercieran efecto negativo sobre el sabor de los embutidos, o la combinación de estos dos antimicrobianos con otros compuestos antimicrobianos.

En cualquier caso, el estudio tiene importancia pues recoge con cierto detalle la evolución microbiana de embutidos frescos durante su almacenamiento anaerobio, que ha sido escasamente estudiada. Las BAL fueron los microorganismos mayoritarios en los embutidos estudiados, siguiendo el patrón descrito en otros estudios realizados con carne y embutidos frescos almacenados en condiciones anóxicas (Pothakos et al., 2015; Sukumaran et al., 2018). Este crecimiento microbiano pudo correlacionarse con el descenso progresivo observado en el **pH** después de la primera semana de almacenamiento. La identificación de bacterias ácido-lácticas, que fue realizada por MALDI-TOF, demostró que el género y especie de mayor abundancia

en las salchichas frescas fueron *Lactobaccillus* (84 % del total de BAL) y *Lactobaccillus sakei* (54 % del total de *Lactobaccillus*). Estos resultados fueron consistentes con los encontrados por Fougy et al. (2016), en donde por pirosecuenciación del 16S rRNA demostraron que *Lb. sakei* era la especie de mayor abundancia en salchichas después de 21 días de almacenamiento.

Los resultados de la producción de **aminas biógenas** (AB) mostraron un escaso efecto de los tratamientos de lúpulo y aceite esencial. Solo se observó un efecto significativo en la cantidad de espermina. Ese escaso efecto de los tratamientos es coherente con la falta de efecto antimicrobiano anteriormente comentado. Lu et al. (2015), por el contrario, observaron una reducción en la producción de AB en salchichas ahumadas como resultado de la adición de una mezcla de aceites esenciales (canela, clavo, jengibre y anís, 0.12 % en total) junto con una mezcla de polifenoles de té (0.19 %).

En todos los tratamientos se pudo observar un aumento significativo de mono y diaminas analizadas durante el almacenamiento, en concordancia con los demostrados en otros estudios sobre embutidos frescos almacenados en anaerobiosis (González-Tenorio et al., 2013; Ruiz-Capillas et al., 2012). En la presente investigación, las cantidades de tiramina, amina capaz de producir migrañas o alergias (Ruiz-Capillas & Jiménez-Colmenero, 2004), superaron hacia el final del almacenamiento (a partir de la tercera semana) la cantidad de 100 mg/kg, cantidad propuesta como límite máximo recomendado en los alimentos (Halász et al., 1994). Este resultado sugiere la conveniencia de controlar la cantidad de tiramina en los embutidos frescos

almacenados a refrigeración con vida útil superior a las dos semanas.

7.3.2. Efecto del extracto de lúpulo sobre la producción de ácidos acético y láctico, y compuestos volátiles

En esta parte del estudio se determinó el efecto de la adición de lúpulo sobre la evolución de diversos metabolitos susceptibles de ser generados por los microorganismos en la salchicha durante su almacenamiento anaerobio a refrigeración: los ácidos acético y láctico, los gases de la atmósfera modificada, los compuestos volátiles extraídos de los embutidos mediante la técnica SPME de su espacio de cabeza y un análisis sensorial de detección de olor a alteración. Al estudiar el perfil volátil de los embutidos se pudo comprobar la presencia de compuestos presumiblemente derivados de orígenes no microbianos, como la oxidación lipídica o las especias, por lo que también se obtuvo información de la evolución de compuestos presuntivamente. No se incluyó el aceite esencial en este estudio por la gran cantidad de compuestos volátiles que aporta el aceite al embutido y la interferencia analítica que esto supone para el análisis de los compuestos volátiles de interés y el olor de la salchicha.

La formación de los **ácidos láctico y acético** durante el almacenamiento anaerobio de la carne y los productos cárnicos frescos ha sido relacionada con el metabolismo microbiano sobre los azúcares y otros compuestos y principalmente atribuido al crecimiento de bacterias ácido-lácticas (BAL), *Enterobacterias* y *Brochothrix thermosphacta* (Müller, 2008; Pothakos et al., 2015; Stanborough et al., 2017). No pudo observarse efecto

de la adición de extracto de lúpulo en las concentraciones de ambos ácidos orgánicos. Durante el periodo de almacenamiento de las salchichas, no se observaron cambios significativos en el contenido de ácido láctico ($P > 0.05$) pero sí para los del ácido acético, que fueron aumentando hasta alcanzar los 0,1 g/100 g de embutido. El comportamiento del ácido láctico podría ser explicado por su producción y simultanea degradación, mediada por lactobacilos, generando, a su vez, ácido acético (Gänzle, 2015).

Tampoco se observó efecto del extracto de lúpulo sobre las cantidades de los **compuestos volátiles** asociados al deterioro microbiano, como el diacetilo, el 1-metil-butanol, el etilacetato y el etilhexanoato. Estos compuestos, que han sido comúnmente relacionados con el deterioro de carne fresca y preparados cárnicos durante el almacenamiento a refrigeración (Casaburi et al., 2015; Montanari et al., 2018; Smit et al., 2009), aumentaron de forma similar en el embutido control y en el que contenía lúpulo. La ausencia de diferencias entre tipo de embutido es compatible con la similitud en el patrón de crecimiento de los grupos microbianos antes comentado (en el anterior apartado).

El análisis **sensorial de olor** con catadores, previamente entrenados, demostró que el olor a deterioro era perceptible en los embutidos a los 14 días de almacenamiento, y llegaba a ser elevado a los 21 días. Dichos datos se correlacionan con la concentración de ácido acético y con la de compuestos volátiles relacionados directamente con el crecimiento microbiano, como el 3-metilbutanol y los etil ésteres. Adicionalmente, los 14 días, en

los que se percibió el olor a deterioro, concuerda a grandes rasgos con la vida útil indicada en estudios previos para salchichas frescas almacenadas en condiciones anaerobias (Martínez et al., 2005; Ruiz-Capillas & Jiménez-Colmenero, 2010).

Aunque era un resultado no buscado de forma específica, mediante la evaluación de la producción de volátiles en las salchichas de cordero adicionadas con lúpulo (refrigeradas en condiciones anóxicas durante 35 días), se pudo evidenciar el poder antioxidante del extracto de lúpulo. Esta afirmación fue observada por la reducción en los contenidos de alcoholes y aldehídos, los cuales son compuestos relacionados con oxidación en la carne (específicamente hexanal, propanol y 1-octen-3-ol), en las salchichas adicionadas con el extracto de lúpulo durante el periodo de almacenamiento. Además, el lúpulo contribuyó a que los niveles de oxígeno residual en la atmósfera de los embutidos fuesen más bajos durante todo el almacenamiento. Por otro lado, también se observó que la adición de lúpulo tuvo efecto sobre los terpenoides, llegando a aumentar significativamente. Este resultado era esperable ya que el lúpulo es la fuente de los terpenoides afectados (Nuutinen, 2018; Vázquez-Araújo et al., 2013).

Después de todo lo visto sobre el uso de lúpulo y el aceite esencial de ZM como agentes antimicrobianos para su empleo en la formulación de embutidos frescos envasados en atmosferas modificadas, cabe decir que en las condiciones utilizadas en este trabajo no se constató efecto sobre el alargamiento de la vida útil de los embutidos. No obstante, el estudio no carece

de relevancia. Por una parte, pone nuevas condiciones para el punto de partida para futuros estudios en los que se usen estos antimicrobianos. Por otra parte, el estudio es pionero en describir los cambios en los compuestos volátiles en embutidos frescos durante su almacenamiento a refrigeración y en condiciones de anaerobiosis. Hay estudios previos que determinan los compuestos volátiles en carne fresca (en trozo o picada), pero no existen investigaciones detalladas, como esta, que estudien los compuestos volátiles en embutidos frescos que, a diferencia de la carne fresca, se caracterizan por contener sal y especias.

8

Conclusiones

- La alimentación artificial de los corderos lechales con el lacto reemplazante comercial, que contiene BHT, suplementado con 25 mg de astaxantina (1 g de AstaREAL®EL25) por kg, fue capaz de producir un efecto leve pero significativo sobre el color de la carne y la grasa, además de mejorar su estabilidad oxidativa durante el almacenamiento en congelación y reducir los niveles de deposición de BHT en su carne.

- La adición de astaxantina a niveles de 20 a 80 mg/kg en preparados cárnicos de carne de cordero tipo hamburguesa mejoró significativamente su estabilidad lipídica durante el almacenamiento refrigerado aeróbico, llegando a crear un efecto protector sobre la oxidación térmica lípidos, incluidos los ácidos grasos y el colesterol. Los resultados mostraron que los efectos dependían directamente de la dosis y que éstos eran iguales o mayores a los mostrados por otros antioxidantes convencionales ascorbato y metabisulfito.

- Las salchichas frescas de cordero al estilo de los Balcanes almacenadas en atmósfera anaeróbica a refrigeración presentaron una intensa fermentación por bacterias ácido lácticas (BAL) desde la primera semana de almacenamiento, siendo especies de *Lactobacillus spp.*, específicamente *Lb. sakei*, las de mayor predominancia. Además de las BAL, crecieron *B. thermosphacta* y *Enterobacteriaceae*, resultando en una producción de aminas biógenas a un nivel potencialmente peligroso. La adición de lúpulo y aceite esencial de *Zataria multiflora* Boiss en las condiciones ensayadas prácticamente no afectó ni al crecimiento microbiano, ni a la producción de aminas biógenas.

- Se observó una progresiva acumulación de compuestos volátiles considerados como metabolitos microbianos durante el almacenamiento salchichas frescas de cordero refrigeradas en condiciones anóxicas que llegó a producir mal olor a partir del día 14 de almacenamiento. Los volátiles ácido acético, 1 metil-butanol, etil acetato y etil hexanoato fueron los de mayor relevancia. A pesar de que los volátiles originados generalmente por procesos oxidativos no fueron altos, el extracto de lúpulo a las cantidades utilizadas resultó en una reducción significativa de éstos. Sin embargo, el extracto de lúpulo no modificó los metabolitos de origen microbiano detectados en la salchicha, ni el tiempo de aparición del mal olor.

Como resumen:

- La astaxantina, tanto administrada a los corderos lechales como adicionada a directamente a las hamburguesas tiene un potencial significativo sobre el retraso de las reacciones de oxidación de la mioglobina y de los lípidos de la carne, especialmente en carne cocinada. Son necesarios, no obstante, nuevos estudios que evalúen el efecto de la astaxantina sobre el color, por su contribución a los tonos rojos-naranjas, y sobre el sabor, por la disminución de compuestos volátiles típicamente formados en la carne durante su cocinado.

- El lúpulo y el aceite esencial de *Zataria multiflora* Boiss en salchichas frescas almacenada en anaerobiosis con CO_2 y a las dosis utilizadas, no contribuyen a retrasar el crecimiento microbiano, ni aumentar la vida útil. Con el trabajo se establece un nuevo punto de partida para plantear nuevos experimentos, que mediante modificación de condiciones puedan sacar partido de las propiedades antioxidantes de estos dos ingredientes naturales en su uso en preparados cárnicos.

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