TESIS DOCTORAL

# DESARROLLO DE EMBUTIDOS CON MENORES NIVELES DE NITRIFICANTES INOCULADOS CON LEVADURAS SELECCIONADAS PARA MANTENER LAS CARACTERÍSTICAS SENSORIALES PROPIAS, SU SEGURIDAD E INCREMENTAR EL AROMA A CURADO

PROGRAMA DE DOCTORADO:

**CIENCIAS DE LA ALIMENTACIÓN**


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Junio de 2020

Las Doctoras Mónica Flores Llovera y Carmela Belloch Trinidad, investigadora científica y científica titular de los departamentos "Ciencia de los Alimentos" y "Biotecnología de los Alimentos" respectivamente, del Instituto de Agroquímica y Tecnología de los Alimentos" (IATA-CSIC)

**INFORMAN QUE:**

La graduada en Ciencia y Tecnología de los Alimentos, Dña. Laura Perea Sanz, ha realizado bajo su dirección el trabajo que lleva por título "**Desarrollo de embutidos con menores niveles de nitrificantes inoculados con levaduras seleccionadas para mantener las características sensoriales propias, su seguridad e incrementar el aroma a curado**". El trabajo ha dado lugar a cuatro artículos publicados en los que ha participado en el 100% y firma como primera autora. Dichas publicaciones no han sido utilizadas en otras tesis y están publicadas con el factor de impacto que se indica a continuación:

1. Nitrate reduction in the fermentation process of salt reduced dry sausages: Impact on microbial and physicochemical parameters and aroma profile. International Journal of Food Microbiology (2018), 282, 84-91. Índice de impacto JCR (2018): 4.006 Q1. Food Science & Technology.
2. Microbial changes and aroma profile of nitrate reduced dry sausages during vacuum storage. Meat Science (2019), 147, 100-107. Índice de impacto JCR (2018): 3,483 Q1. Food Science & Technology.
3. *Debaryomyces hansenii* metabolism of sulfur amino acids as precursors of volatile sulfur compounds of interest in meat products. Journal of Agricultural and Food Chemistry (2019), 67, 9335-9343. Índice de impacto JCR (2018): 3,571 Q1. Food Science & Technology.
4. Counteracting the effect of reducing nitrate/nitrite levels on dry fermented sausage aroma by *Debaryomyces hansenii* inoculation. Meat Science (2020), 108103. Índice de impacto JCR (2018): 3,483 Q1. Food Science & Technology.

Autorizan la presentación de la Tesis Doctoral para optar al Grado de Doctor en Ciencias de la Alimentación. Y para que conste a los efectos oportunos,

En Paterna, 5 de junio de 2020

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**INFORMAN QUE:**

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La doctoranda Laura Perea Sanz ha disfrutado de una ayuda para la contratación de personal investigador en formación de carácter predoctoral dentro del Programa VALI+D (ACIF/2016/107) de la Conselleria d'Educació de la Generalitat Valenciana, en el periodo entre julio de 2016 y julio de 2019 (Resolución de 16 de junio 2016).

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## RESUMEN

Los agentes de curado (nitratos y nitritos) desempeñan funciones importantes en la elaboración de embutidos curado-madurados, entre las que se encuentra el desarrollo del aroma característico de estos productos. El uso exclusivo de nitrato, de nitrito o de una mezcla de los dos, puede tener diferente repercusión en el perfil aromático. Sin embargo, existe una tendencia a la reducción del uso de estos aditivos por su relación con la generación de N-nitrosaminas que ha hecho que en los últimos años exista una gran preocupación a nivel de los consumidores. Por ello, en la presente tesis doctoral se ha estudiado el efecto que produce la reducción de la cantidad añadida de nitrato o de la mezcla de nitrato y nitrito en los embutidos curado-madurados, centrándose en los cambios fisicoquímicos, microbiológicos y de los compuestos volátiles, y en especial en aquellos compuestos que participan en el perfil aromático. También se ha determinado la estabilidad de los embutidos reducidos en nitrificantes durante el almacenamiento a vacío y 18-20 °C con el fin de determinar el efecto durante la vida útil del producto. Además, se han estudiado varias cepas de la levadura *Debaryomyces hansenii* procedentes de diferentes alimentos, de entre las cuales se ha seleccionado una de ellas por su potencial de generación de aromas que proceden de la degradación de aminoácidos azufrados. Estos aminoácidos azufrados, principalmente la metionina, se degradan a aromas de interés mediante la ruta de "Ehrlich", la cual ha sido evaluada mediante el estudio de varios genes que intervienen en ella. La levadura seleccionada ha sido utilizada en la elaboración de embutidos curado-madurados reducidos en nitrificantes como posible alternativa a los efectos producidos por la reducción de nitrificantes en el perfil aromático.

La reducción de la cantidad añadida de agentes de curado en la fabricación de embutidos curado-madurados, así como el almacenamiento a vacío y 18-20 °C, modifica el perfil aromático del producto. El uso de nitrito como agente de curado en las mezclas, ejerce un mayor control del crecimiento y metabolismo de los microorganismos responsables de la producción de aromas, minimizando los cambios en el perfil aromático causados por la reducción de la cantidad añadida. La inoculación en embutidos curado-madurados de la cepa seleccionada de *Debaryomyces hansenii*, demuestra su capacidad para contrarrestar los cambios en el aroma por la reducción de la cantidad añadida de nitrato y nitrito debido al

aumento de ésteres con notas a “fruta” y a “dulce”, y por la disminución en la producción de ácido acético.

## ABSTRACT

Curing agents (nitrates and nitrites) play an important role on the quality of dry fermented sausages, participating in the development of the characteristic cured aroma. Moreover, the exclusive use of nitrate, nitrite or a mixture of both, may have different impact on the aroma profile. In recent years, a relationship of these additives with the generation of N-nitrosamines has been found, and the consumer's concern have propiciated a trend to reduce their use in food. The present doctoral thesis has focused on the effect of reducing the ingoing amount of nitrate or the mixture of nitrate and nitrite added to the quality of dry fermented sausages. The physicochemical, microbiological and aroma changes, especially in those volatile compounds that participate in the aroma profile, have been our main point of interest. Besides, in order to determine the effect of the curing agents' reduction during the shelf-life of the product, the stability of the sausages reduced in curing agents during storage under vacuum and at 18-20 °C has been studied. Yeast of the species *Debaryomyes hansenii* strains isolated from different food sources have been evaluated for their potential to produce aroma compounds of interest. Among them, strain *D. hansenii* L1 has been selected due to its capacity to produce aromas from sulfur amino acid degradation. Yeast metabolism of sulfur amino acids generates aromas of interest using the "Ehrlich" pathway. The genes in this pathway have been evaluated to test their involvement in the generation of specific sulfur volatile compounds. The selected strain has been used in the formulation of dry fermented sausages reduced in curing agents to counteract the effects in the aroma profile produced by the reduction of nitrifying agents.

The reduction of the ingoing amounts of curing agents added in dry fermented sausages production, in addition to the storage under vacuum at 18-20 °C, modifies the aroma profile of the product. The use of nitrite as curing agent in the mixtures exercises greater control over the growth and metabolism of microorganisms responsible for the aroma compounds production. The inoculation of *Debaryomyces hansenii* strain L1 counteracta the changes in the aroma caused by the reduction of the ingoing amounts of nitrate and nitrite due to the increase in ester compounds with "fruit" and "sweet" notes and the decrease in acetic acid production.

**RESUM**

Els agents de curat (nitrats i nitrits) exercixen diverses funcions importants en l'elaboració d'embotits curat-madurats, entre les que es troba el desenvolupament de l'aroma característic d'estos productes. L'ús exclusiu de nitrat, de nitrit o d'una mescla dels dos, pareix tindre diferent repercussió en el perfil aromàtic. No obstant això, hi ha una tendència a la reducció de l'ús d'estos additius per la seua relació amb la generació de N-nitrosamines que ha fet que en els últims anys existisca una gran preocupació a nivell dels consumidors. Per això, la present tesi doctoral ha estudiat l'efecte que produïx la reducció de la quantitat afegida de nitrat o de la mescla de nitrat i nitrit en els embotits curat-madurats, centrant-se en els canvis fisicoquímics, microbiològics i dels compostos volàtils, i en especial en aquells compostos que participen en el perfil aromàtic. També s'ha determinat l'estabilitat dels embotits reduïts en nitrificants durant l'emmagatzemament al buit i 18-20 °C a fi de determinar l'efecte durant la vida útil del producte. A més, s'han estudiat diversos ceps del rent *Debaryomyces hansenii* procedents de diferents aliments, d'entre les quals s'ha seleccionat un d'ells pel seu potencial de generació d'aromes que procedixen de la degradació d'aminoàcids ensofrats. Estos aminoàcids ensofrats, principalment la metionina, es degraden a aromes d'interés per mitjà de la ruta de "Ehrlich", la qual ha sigut avaluada per mitjà de l'estudi de diversos gens que intervenen en ella. El rent seleccionat ha sigut utilitzat en l'elaboració d'embotits curat-madurats reduïts en nitrificants com a possible alternativa als efectes negatius produïts en el perfil aromàtic per la reducció de nitrificants.

La reducció de la quantitat afegida d'agents de curat en la fabricació d'embotits curat-madurats, així com l'emmagatzemament al buit i 18-20 °C, modifica el perfil aromàtic del producte. L'ús de nitrit com a agent de curat en les mescles, exercix un major control del creixement i metabolisme dels microorganismes responsables de la producció d'aromes, minimitzant els canvis en el perfil aromàtic causats per la reducció de la quantitat afegida. La inoculació en embotits curat-madurats del cep seleccionat de *Debaryomyces hansenii*, demostra la seua capacitat per a contrarestar els canvis en l'aroma per la reducció de la quantitat afegida de nitrat i nitrit a causa de l'augment d'esters amb notes a "fruita" i "dolç" i per la disminució en la producció d'àcid acètic.

## ABREVIATURAS

**AESAN**: Agencia Española de Seguridad Alimentaria y Nutrición

**AU**: Unidades de abundancia

**BHT**: Butilhidroxitolueno

**CAR/PDMS**: Carboxeno/Polidimetilsiloxano

**CG**: Cromatografía de gases

**MS**: Espectrometría de masas

**CN-BP**: Cocos Gram positivos coagulasa negativos cultivados en el medio Agar Baird Parker

**CN-M**: Cocos Gram positivos coagulasa negativos cultivados en el medio Agar Manitol Sal

**DF**: Frecuencia de detección

**EB**: Enterobacterias

**EFSA**: Autoridad Europea de Seguridad Alimentaria

**EIC**: Corriente de iones extraída

**FCEC**: Consorcio para la Evaluación de la Cadena Alimentaria

**FID**: Detector de ionización de llama

**GCN**: Cocos Gram positivos coagulasa negativos

**GC-O**: Cromatógrafo de gases-olfatómetro

**GLM**: Modelo lineal generalizado

**GPY**: Medio Glucosa Pepetona Extracto de Levadura Agar

**HS**: Espacio de cabeza

**IARC**: Centro Internacional de Investigaciones sobre el Cáncer

**KMBA**: Ácido α-cetometiltiobutanoico

**LAB**: Bacterias ácido-lácticas

**LCA**: Medio Agar Cromogenico para Listeria

**LRI**: Índice de retención lineal

**LSM**: Mínimo cuadrado medio

**MDA**: Malonaldehído

**MKTTN**: Medio Caldo Muller-Kauffman con tetrationato y novobiocina

**MRS**: Medio Agar Man Rogosa Sharpe

**MSA**: Medio Agar Manitol Sal

**MTL**: Metanotiol

**DMS**: Dimetil sulfuro

**DMDS**: Dimetil disulfuro

**DMTS**: Dimetil trisulfuro

**MTA**: Metil tioacetato

**NDMA**: N-nitrosodimetilamina

**NPYR**: N-nitrosopirrolidina

**NDEA**: N-nitrosodietilamina

**NPIP**: N-nitrosopiperidina

**NHPRO**: N-nitrosohidroxiprolina

**NTCA**: Ácido N-nitrosotiazolidina-4-carboxilico

**HMNTCA**: Ácido 2-(hidroximetil)-3-nitrosotiazolidina-4-carboxilico

**NPRO**: N-nitrosoprolina

**NDBA**: N-nitrosodi-n-butilamina

**NSAR**: N-nitrososarcosina

**NMEA**: N-nitrosometiletilamina

**NDPA**: N-nitrosodipropilamina

**NMTCA**: Ácido N-nitroso-2-metiltiazolidina-4-carboxilico

**NMA**: N-nitrosometilanilina

**NOC**: Nitrosocompuestos

**OD**: Densidad óptica

**SPBS**: Medio Caldo Peptona Sorbitol y Sales Biliares

**PCA**: Análisis de componentes principales

**PCR**: Reacción en Cadena de la Polimerasa

**RH**: Humedad relativa

**RI**: Fiabilidad de la identificación

**RMSE**: Raíz del error cuadrático medio

**RT-PCR**: Transcriptasa Reversa-PCR

**RT-qPCR**: Transcriptasa Reversa-PCR cuantitativa

**SIM**: Monitoreo de iones selectivos

**SPME**: Microextracción en fase sólida

**TBARS**: Prueba de sustancias reactivas del ácido tiobarbitúrico

**TIC**: Corriente total de iones

**TMB**: Bacterias aerobias mesófilas totales

**UPLC**: Cromatografía líquida de ultra alto rendimiento

**VRBG**: Medio Agar Glucosa Bilis Violeta cristal y Rojo neutro

**XLD**: Medio Agar Xilosa Lisina Desoxicolato

**YM**: Levaduras y hongos

**YSA**: Medio Agar Selectivo para Yersinia

# INTRODUCCIÓN

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La industria cárnica ocupa la primera posición en producción económica dentro del sector de alimentos y bebidas en España. En cuanto a la producción cárnica en Europa en 2018, España se encuentra en segundo lugar con un 19%, siguiendo de cerca a Alemania con un 22%. El porcino es el principal sector ganadero en España por su aportación económica. En el año 2018, el valor alcanzado por este sector fue de 7.417,2 millones de euros. Dentro del sector cárnico, la producción de los productos cárnicos elaborados en España llega a 1.413.000 toneladas, de las cuales 209.000 pertenecen a embutidos curado-madurados, situados en tercer lugar por detrás de los elaborados cocidos (429.800) y siguiendo de cerca a los jamones y paletas curadas (299.000) (Mercasa, 2019).

La importancia del subsector de los embutidos curado-madurados en la alimentación de los españoles, indica el gran valor de estos productos para el consumidor. Esto es debido a las cualidades organolépticas especialmente positivas de estos productos, tales como el chorizo y salchichón. Además, a pesar de que poseen un contenido de grasa y sal elevado y se recomienda un consumo ocasional (Muguerza, Gimeno, Ansorena & Astiasarán, 2004), los embutidos curado-madurados pueden aportar beneficios para la salud del consumidor. Por un lado, debido a que son productos elaborados con carne de cerdo, son una importante fuente de vitaminas del complejo B (especialmente B12), vitamina D y minerales como el hierro, zinc y selenio (Arihara & Ohata, 2008). Por otro lado, durante la fermentación y la maduración del producto, se producen compuestos como el ácido láctico, péptidos y aminoácidos libres que pueden aportar beneficios para la salud del consumidor por su efecto antiinflamatorio, antioxidante, antimicrobiano, hipocolesterolemiante, antihipertensivo, inmunomodulatorio, etc… (Zhang, Xiao, Samaraweera, Lee & Ahn, 2010; Gallego, Mora, Escudero & Toldrá, 2018). Además de generar compuestos bioactivos, la presencia de microorganismos viables en el producto final ($10^6$-$10^9$ células/g o ml), convierte a los embutidos curado-madurados en un potencial vehículo para microorganismos probióticos (Zhang et al., 2010; Leroy et al., 2018). Por último, las reformulaciones de los embutidos curado-madurados en cuanto a sal (NaCl) y grasa han sido estudiadas para mejorar la salubridad del producto (Muguerza et al., 2004; Olivares, Navarro & Flores, 2011; Corral, Salvador & Flores, 2013).

La adición de nitratos y nitritos en embutidos curado-madurados está regulada (DOUE, 2008) y su empleo se basa en sus propiedades tecnológicas y su contribución a la seguridad microbiológica. Sin embargo, hoy día se tiende a disminuir el uso de aditivos en alimentos demandado principalmente por los consumidores. Además, en los productos cárnicos podrían darse las condiciones necesarias que den lugar a la formación de N-nitrosaminas, las cuales son cancerígenas (Honikel, 2008) especialmente durante los periodos largos de maduración y durante el almacenamiento y preparación para el consumo en los hogares. Debido a ello, existe un interés a nivel internacional de reducir al máximo posible la cantidad que se adiciona de nitratos y nitritos en productos cárnicos curados (EFSA, 2017a, 2017b; CODEX, 2019). Sin embargo, tal reducción debe ser minuciosamente estudiada por sus implicaciones en la seguridad y calidad del producto final. Por ello, debido a que los embutidos curado-madurados se caracterizan por sus propiedades organolépticas únicas, es necesario conocer el efecto de la reducción de la cantidad añadida de nitratos y nitritos en su aroma y diseñar nuevas estrategias que contrarresten los posibles efectos de dicha reducción.

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# ANTECEDENTES BIBLIOGRÁFICOS

# ANTECEDENTES BIBLIOGRÁFICOS

## 1. PRODUCTOS CÁRNICOS CURADO-MADURADOS

Los derivados cárnicos se describen como los *productos alimenticios preparados total o parcialmente con carnes o menudencias y sometidos a operaciones específicas antes de su puesta al consumo* (BOE, 2014). Se clasifican en derivados cárnicos tratados por el calor y no tratados por el calor (Figura 1).


**Figura 1. Clasificación de los derivados cárnicos (BOE, 2014).**

Los productos cárnicos curado-madurados se encuentran dentro de los derivados no tratados por el calor, y se definen como *productos sometidos a un proceso de salazón y de curado-maduración, suficiente para conferirles las características organolépticas propias y de estabilidad a temperatura ambiente, pudiéndose someterse opcionalmente a ahumado* (BOE, 2014). Se entiende por curado la aplicación de una solución que contiene nitratos y/o nitritos, sal y otros componentes (Flores, 1997). A su vez, los derivados cárnicos curado-madurados se clasifican en productos que provienen de piezas enteras o de carnes troceadas y picadas, y dentro de estas últimas, se encuentran los embutidos curado-madurados. Los embutidos curado-madurados más comunes en España son el salchichón y el chorizo, que se definen como *embutidos elaborados con carnes y grasa generalmente de cerdo, picados y sometidos a un proceso de salazón. Se les añade pimentón (chorizo) o pimienta (salchichón) como ingrediente caracterizante, y son sometidos a un proceso de curado-madurado, acompañado o no de fermentación, y opcionalmente ahumados* (BOE, 2014).

## 1.1. Embutidos curado-madurados

Existe una gran variedad de embutidos curado-madurados debido a las distintas formas de producirlos (BOE, 2014). A continuación, se presentan los ingredientes y los pasos comúnmente utilizados en la elaboración de estos productos.

### 1.1.1. Ingredientes

Los ingredientes principales en la elaboración de embutidos curado-madurados son la carne y la grasa. Éstas deben ser de gran calidad para proporcionar las características óptimas para la elaboración de estos productos (Solignat, 2002). El origen de estos ingredientes suele ser porcino, aunque la carne puede proceder de otros animales como ternera o una mezcla de ellos (Vignolo et al., 2010).

Además de la carne y la grasa, se utilizan varios ingredientes y aditivos con funciones importantes. Los más relevantes son la sal común, sales de nitrito y nitrato, azúcar, agentes reductores, especias y cultivos iniciadores.

La adición de sal tiene como papel principal la reducción de la actividad de agua, que evitará el crecimiento de microorganismos indeseables que puedan estar presentes. Además, la sal solubiliza las proteínas miofibrilares ayudando a estabilizar la emulsión de carne y contribuye a la liberación de compuestos responsables del aroma (Ruusunen & Puolanne, 2005).

Las sales de nitrito, comúnmente añadido como nitrito sódico, ejercen un papel crucial en la elaboración de embutidos curado-madurados, ya que intervienen en el desarrollo del color y aroma característicos. Su utilización también evita el enranciamiento del producto por su actividad antioxidante, e inhiben el crecimiento de microorganismos patógenos como *Clostridium botulinum* (Rahman, 2007). Las funciones y características del nitrito se explican en el apartado 3.

La adición de azúcar es necesaria para estimular el proceso de fermentación llevado a cabo por parte de los microorganismos presentes en el produto. El azúcar es utilizado como fuente de energía, produciendo a su vez ácidos orgánicos importantes para la seguridad del producto cárnico (Kumar et al., 2017).

El papel reductor de algunos ingredientes como el ascorbato, facilita la reducción del nitrito a óxido nítrico acelerando el proceso de curado y disminuyendo la formación de N-nitrosaminas. Además, también interviene en la generación del color evitando la oxidación de la mioglobina, el pigmento responsable (Shimokomaki, Youssef Youssef & Terra, 1993).

Las especias se añaden por su aporte sensorial, caracterizando el sabor, el color y el aroma del producto cárnico.

Los cultivos iniciadores son microorganismos inoculados a los ingredientes y cuya función es llevar a cabo la fermentación del embutido curado-madurado, utilizando los azúcares anteriormente mencionados (Leroy, Verluyten & De Vuyst, 2006).

### 1.1.2. Proceso de elaboración

Antes de empezar con la elaboración de los embutidos curado-madurados, se debe asegurar que todos los ingredientes que se van a utilizar estén en condiciones higiénicas adecuadas. La carne y la grasa deben ser refrigeradas antes de su uso, incluso a veces congeladas sobre todo en el caso de la grasa, para evitar la aparición de alteraciones microbiológicas que puedan producir contaminación o enranciamiento, además de evitar el "embarrado" de la masa cárnica y así facilitar su corte durante su posterior picado o triturado (Solignat, 2002).

Una vez se tienen los ingredientes preparados, se procede en primer lugar con el picado de la carne y la grasa. Se realiza en picadoras o en trituradoras de tipo "cutter", dando como resultado una masa de carne y grasa fragmentada con un tamaño variable en función del tipo de producto que se quiere fabricar.

La masa de carne y grasa picada es posteriormente mezclada y amasada junto con el resto de ingredientes y aditivos (Apartado 1.1.1). Esta mezcla se lleva a cabo en máquinas mezcladoras-amasadoras provistas de paletas giratorias a fin de conseguir una masa uniforme. Este proceso suele realizarse a vacío y a temperatura de refrigeración, como se ha comentado anteriormente.

Tras la obtención de la masa cárnica con los ingredientes y aditivos incorporados, ésta se deja en reposo durante 24 horas para favorecer la adaptación de la flora microbiana al medio y la ligazón de la masa (Solignat, 2002). Una vez transcurrido este tiempo, la masa se embute

en tripas, las cuales pueden ser de origen natural o artificial. Las de origen natural proceden de los intestinos delgado y grueso de distintas especies animales o de esófagos y vejigas de bovino y porcino. Las de origen artificial suelen ser de colágeno o celulosa y tienen diversos calibres. El proceso de embutido es un punto importante en la elaboración de embutidos curado-madurados, durante el cual se debe evitar la presencia de aire que pueda dar lugar a la formación de cavidades en el producto, que a su vez puedan provocar contaminaciones o alteración del color (Juillard, 2002).

#### *1.1.2.1. Fermentación*

En esta etapa, la masa cárnica embutida se mantiene en condiciones ambientales óptimas para que ocurra la fermentación de los azúcares por parte de los cultivos iniciadores. La fermentación es un proceso metabólico llevado a cabo por las bacterias y/o levaduras, (Apartado 1.1.3) mediante el cual estas obtienen energía de los azúcares produciendo ácido láctico que ocasionará una bajada del pH del producto y dificultará el crecimiento de microorganismos contaminantes no deseables (Hugas & Monfort, 1997). Dicha acidificación alcanza un pH cercano al punto isoeléctrico de las proteínas, lo que disminuye su capacidad de retención de agua, ayudando al posterior paso de secado, además de provocar su coagulación mejorando la cohesividad y posterior loncheado (Hugas & Monfort, 1997). Por lo tanto, la incorporación de una etapa de fermentación en la elaboración de embutidos curado-madurados proporciona un aumento de la seguridad y estabilidad del producto, además de una mayor aceptabilidad por el desarrollo de atributos sensoriales como el aroma, color y la terneza (Kumar et al., 2017).

#### *1.1.2.2. Maduración y desecación*

Tras la etapa de fermentación, se llevan a cabo de forma simultanea la maduración y desecación del embutido bajo condiciones de temperatura y humedad relativa controladas. Durante esta etapa, los embutidos experimentan varias transformaciones químicas, microbiológicas y bioquímicas que darán lugar a la pérdida de agua, la degradación de proteínas y lípidos y la formación de compuestos volátiles por distintas vías bioquímicas en las que participan los microorganismos presentes y las enzimas musculares (Toldrá, 2017). Finalmente, se obtiene un producto estable con características organolépticas distintivas. La

duración de este periodo suele ser de 1 a 3 meses en función de las características del producto.

### 1.1.3. Cultivos iniciadores

En productos cárnicos tradicionales se evita el uso de los cultivos iniciadores mencionados en el apartado 1.1.1, y la fermentación se lleva a cabo por la microbiota autóctona (Toldrá & Flores, 2014). En este caso, el procesado es más lento, dando lugar a embutidos curado-madurados con un pH final superior a 5, lo que obliga a la utilización de temperaturas de fermentación y maduración más bajas (inferiores a 15 °C) para controlar el crecimiento de microorganismos alterantes y patógenos. Debido a la industrialización implantada a mitad del siglo XIX, existe una mayor demanda de alimentos, lo que crea una nueva necesidad de elaboración de un mayor número de embutidos curado-madurados de manera uniforme y manteniendo la calidad y seguridad. Por ello, aparece el uso de cultivos iniciadores que favorecen una elaboración más rápida, uniforme y segura (Kumar et al., 2017). Los cultivos iniciadores utilizados no deben ser patógenos ni producir metabolitos nocivos para la salud. Además, deben poseer la habilidad de generar productos de similar calidad de manera repetida y deben tener una alta tasa de multiplicación y crecimiento, para controlar el crecimiento de patógenos o alterantes. Los microorganismos más utilizados como iniciadores son los que se encuentran naturalmente presentes en la carne, de esta manera se asegura su adaptabilidad al medio y la producción de productos cárnicos con características sensoriales cercanas a las de los productos tradicionales (Hugas & Monfort, 1997). Comúnmente, se emplean bacterias ácido-lácticas por su actividad metabólica y su capacidad de producir ácidos orgánicos (ácido láctico) de manera eficiente. Además, estas bacterias producen bacteriocinas útiles para evitar el crecimiento de microorganismos indeseables. Los géneros más utilizados son *Lactobacillus* y *Pediococcus*. Sin embargo, el género *Lactobacillus* suele ser el de más común elección debido a su alta prevalencia en estos productos. Dentro de este género, las especies más utilizadas suelen ser *L. sakei*, *L. curvatus* y *L. plantarum*. Además de estas bacterias, también se emplean otras bacterias Gram positivas capaces de reducir el nitrato en nitrito y con capacidad de generar aromas. Entre ellas se encuentran las bacterias coagulasa negativas del género *Staphylococcus*, siendo *S. xylosus* y *S. carnosus* las especies más comúnmente utilizadas. Estas bacterias

poseen actividades nitrato-reductasa, catalasa, proteolítica, lipolítica y esterasa, favoreciendo la modulación de la generación de compuestos volátiles e interviniendo positivamente en el perfil aromático del producto final (Talon, Chastagnac, Vergnais, Montel & Berdagué, 1998; Talon, Walter, Chartier, Barrière & Montel, 1999). Además de las bacterias mencionadas, también pueden utilizarse mohos y levaduras como cultivos iniciadores cuyo papel principal es su aportación a las características sensoriales del producto final (Flores, Corral, Cano-García, Salvador & Belloch, 2015). El crecimiento de estas levaduras y mohos en embutidos curado-madurados suele ser en la superficie ya que son microorganismos aerobios, creando una película superficial que ayuda a controlar el secado del embutido. En el caso de productos fermentados, las levaduras pueden crecer en el interior del producto contribuyendo positivamente al desarrollo de características sensoriales por su actividad proteolítica y lipolítica. Además, se ha descrito que consumen ácidos orgánicos que, junto con la producción de amonio a partir de aminoácidos, incrementan ligeramente el pH del producto disminuyendo las notas ácidas finales sin afectar a su seguridad (Durá, Flores & Toldrá, 2004).

## 2. EL AROMA DE LOS EMBUTIDOS CURADO-MADURADOS

La aplicación de los procesos de fermentación y curado-maduración para la elaboración de productos cárnicos, como los embutidos curado-madurados, se lleva realizando desde antes de la época de los Romanos y se utilizaba como técnica de conservación (Shimokomaki et al., 1993). Sin embargo, hoy en día existen otras técnicas de conservación de alimentos, tales como la refrigeración o los tratamientos térmicos, por lo que la fermentación y el curado se siguen utilizando para obtener productos con una gran variedad de características sensoriales (Flores, 1997).

El aroma es una de las características sensoriales importantes en embutidos curado-madurados, generando aceptabilidad o rechazo por parte del consumidor en su primer contacto con el producto. Se han detectado cientos de compuestos volátiles en estos embutidos (Olivares, Navarro & Flores, 2013). Sin embargo, solo un pequeño porcentaje de ellos interviene en el aroma final, siendo éstos los volátiles cuya cantidad en el producto es superior a su umbral de detección (Flores, 2018). A continuación, se presentan las

principales reacciones relacionadas con la formación de precurosres y de compuestos volátiles de interés en embutidos curado-madurados.

## 2.1. Generación de precursores

Los ácidos grasos y los aminoácidos libres son moléculas susceptibles de ser degradadas a compuestos volátiles que intervienen en el perfil aromático final. Su generación es debida a procesos de lipólisis y proteólisis llevados a cabo durante la fermentación y maduración de embutidos curado-madurados (Toldrá & Flores, 2007).

### 2.1.1. Lipólisis

La hidrólisis de los lípidos proporciona sustratos (ácidos grasos libres) susceptibles de ser metabolizados a compuestos volátiles involucrados en el aroma. En productos cárnicos, las lipasas y fosfolipasas musculares son responsables de esta hidrólisis (Toldrá & Flores, 2007). En embutidos curado-madurados, la actividad microbiana también ejerce un papel importante. Los cocos Gram positivos coagulasa negativos del cultivo iniciador bacteriano, junto con algunas levaduras como *Debaryomyces hansenii* son los principales proveedores de las enzimas lipasas que degradan triglicéridos en ácidos grasos libres (Flores & Olivares, 2015).

### 2.1.2. Proteólisis

Los aminoácidos son precursores importantes en la generación de compuestos volátiles con alto potencial aromático. La formación de estos ocurre por la hidrólisis de las proteínas presentes en el producto cárnico (Toldrá, 2008). Las proteasas involucradas en este proceso pueden tener origen en los tejidos de la carne (enzimas endógenas) o en los microorganismos. La mayor contribución a la actividad proteolítica es debida a las proteasas endógenas (alrededor de un 60%). Sin embargo, se ha comprobado que determinadas especies bacterianas contienen actividades enzimáticas capaces de hidrolizar proteínas cárnicas generando precursores de compuestos aromáticos, así como levaduras entre las que destaca *Debaryomyces hansenii* (Flores & Olivares, 2015).

## 2.2. Origen químico

Las reacciones químicas son importantes en la generación de aromas en embutidos curado-madurados.

### 2.2.1. Oxidación de lípidos

La oxidación de los ácidos grasos presentes en la carne y de los generados por la lipólisis pueden sufrir reacciones de oxidación. Esta oxidación puede provocar una pérdida de calidad del producto cárnico. Sin embargo, productos de esta reacción intervienen de manera esencial en el aroma del producto final por su bajo umbral de detección: aldehídos, cetonas, alcoholes, hidrocarburos alifáticos, ácidos carboxílicos, etc (Flores, 2018). Los aldehídos lineales son los compuestos más característicos de esta reacción, considerándose el hexanal como un marcador indicativo de oxidación lipídica (Olivares, Navarro & Flores, 2011).

### 2.2.2. Reacción de Maillard y degradación de Strecker

Estos procesos se llevan a cabo principalmente durante el cocinado de la carne y en productos cárnicos cocidos, ya que se necesitan altas temperaturas para que se produzcan (Mottram, 1998; Flores, 2017). Sin embargo, en embutidos curado-madurados, los cuales no se someten a tratamiento térmico, también se han descrito estas reacciones favorecidas por largos periodos de secado y baja actividad de agua. La reacción de Maillard se produce entre azúcares reductores y aminoácidos, dando lugar a pirroles, pirazinas, piridinas, tiofenos y tiazoles. Los compuestos formados por la reacción de Maillard pueden reaccionar con aminoácidos por la degradación de Strecker mediante desaminación oxidativa seguida de descarboxilación, dando lugar al correspondiente aldehído y α-aminocetona (Mottram, 1998). Todos estos compuestos volátiles, una vez formados, tienen un gran impacto aromático en los productos cárnicos debido a que poseen un umbral de detección bajo (Flores, 2017, 2018).

### 2.2.3. Degradación de la tiamina

Al igual que la reacción de Maillard y degradación de Strecker, la temperatura es un factor esencial para la degradación de la tiamina (Flores, 2017). Debido a los largos periodos de secado en embutidos curado-madurados, esta degradación podría tener lugar; sin embargo,

existen pocos trabajos que demuestren la degradación de la tiamina durante la maduración (Flores, 2018). Como resultado de esta degradación, en productos cárnicos cocidos se generan furanos, tiofenos, tiazoles y compuestos azufrados alifáticos que además poseen un bajo umbral de detección que hace que su aportación al perfil aromático sea significativa (Vermeulen, Gijs & Collin, 2005).

## 2.3. Origen microbiano

Los microorganismos presentes en los embutidos curado-madurados son responsables de la generación de diferentes compuestos volátiles por diferentes mecanismos que se describen a continuación.

### 2.3.1. β-Oxidación de lípidos

Los ácidos grasos presentes en la carne y los formados por lipólisis durante la elaboración de embutidos curados-madurados pueden ser metabolizados por microorganismos activos mediante β-oxidación, dando lugar a ácidos grasos de cadena corta y β-cetoácidos. Los últimos se descarboxilan y dan lugar a metil cetonas con potencial aromático, las cuales pueden seguir metabolizándose a alcoholes secundarios (Montel, Masson & Talon, 1998; Ravyts, Vuyst & Leroy, 2012). A diferencia de las metil cetonas, los alcoholes secundarios formados no poseen gran potencial aromático. Los microorganismos que llevan a cabo esta β-oxidación de lípidos son principalmente las bacterias del género *Staphylococcus* y levaduras (Toldrá & Flores, 2014).

### 2.3.2. Degradación de aminoácidos

Los aminoácidos presentes en la carne pueden ser degradados por microorganismos como cocos Gram positivos coagulasa negativos y algunas levaduras mediante desaminación y descarboxilación, dando lugar a aldehídos que pueden reducirse u oxidarse a sus respectivos alcoholes y ácidos (Montel et al., 1998). Los aldehídos (2-metilpropanal, 2-metilbutanal y 3-metilbutanal) formados por degradación de los aminoácidos ramificados (valina, isoleucina y leucina, respectivamente), así como sus alcoholes y ácidos derivados, representan una fracción aromática importante en el aroma de embutidos curado-madurados (Leroy et al., 2006). De la misma manera, la degradación del aminoácido metionina da lugar a compuestos volátiles azufrados con un particular carácter aromático con gran impacto

debido a su bajo umbral de detección (Landaud, Helinck & Bonnarme, 2008). Los derivados de la metionina son el metanotiol, dimetil sulfuro/disulfuro/trisulfuro, metional, metionol y metil tioacetato. Éstos proporcionan notas cárnicas, azufradas, a cebolla y vegetales cocidos que intervienen en el aroma a curado en este tipo de productos (Flores & Olivares, 2015).

### 2.3.3. Fermentación de carbohidratos

La fermentación de los hidratos de carbono presentes en el producto llevado a cabo por las bacterias ácido-lácticas genera ácidos orgánicos, principalmente lactato y acetato. Además de bajar el pH contribuyendo a la textura y seguridad del embutido (Apartado 1.1.3), la fermentacion de éstos compuestos también afecta al aroma aportando notas ácidas (Toldrá, 2008). Además de ácidos orgánicos, estas bacterias también forman otros compuestos como el etanol, 2,3-butanediona (diacetilo), 3-hidroxi-2-butanona (acetoina) y 2,3-butanediol, que también intervienen en el perfil aromático final. Sin embargo, también se forman otros compuestos volátiles (aldehídos de cadena corta) que no intervienen de manera significativa, tales como el acetaldehído y el formaldehído (Flores & Olivares, 2015).

### 2.3.4. Formación de ésteres

Los microorganismos con actividad esterasa, principalmente las bacterias del género *Staphylococcus* y levaduras como *Debaryomyces hansenii*, generan compuestos esterificados por la reacción entre un ácido y un alcohol (Flores et al., 2015). Los ésteres formados en embutidos curado-madurados aportan un gran impacto aromático con notas frutales y dulces. Principalmente se forman los esteres de etilo, por lo que el alcohol que interviene en la reacción de formación es el etanol. Sin embargo, los alcoholes secundarios también son precursores de esteres de gran interés (Talon et al., 1998; Flores, Durá, Marco & Toldrá, 2004).

## 2.4. Otros orígenes

Además de las reacciones químicas y el metabolismo microbiano, el uso de determinados ingredientes en la elaboración también interviene en el desarrollo del aroma de embutidos curado-madurados.

El uso de especias en su elaboración aporta compuestos químicos (terpenos) con notas aromáticas típicas de las especias utilizadas: pimienta negra, pimentón, ajo, cebolla, mostaza, nuez moscada, orégano y anís (Silvis, Luning, Klose, Jansen & van Ruth, 2019). Además, las especias presentan actividad antioxidante (Martínez, Bastida, Castillo, Ros & Nieto, 2019), por lo que pueden disminuir los procesos de oxidación lipídica existentes durante el proceso de elaboración.

## 2.5. Compuestos aromáticos en embutidos curado-madurados

Como se ha mencionado anteriormente, los compuestos aromáticos son aquellos compuestos volátiles que producen un impacto en el aroma, siendo para ello necesario que estén presentes a una concentración superior a su umbral de detección (Belitz, Grosch & Schieberle, 2009). Así, los compuestos que posean un umbral muy bajo tendrán un impacto importante en el aroma, aunque se encuentren en baja concentración (Flores, 2017). La técnica más utilizada para determinar aquellos volátiles con impacto aromático es la olfatometría tras separación cromatográfica (cromatografía de gases) (Zellner, Dugo, Dugo & Mondello, 2008). Se han descrito más de 100 compuestos volátiles que participan en el aroma de los embutidos curado-madurados (Olivares, 2011). En la tabla 1 se presenta una recopilación de los compuestos detectados más frecuentemente, clasificados según su estructura química junto con su umbral de detección y su descripción olfativa. Los compuestos que han sido más frecuentemente descritos como responsables de aromas en embutidos curado-madurados son: los aldehídos hexanal, heptanal y 3-metilbutanal, seguido de nonanal, octanal y benzenoacetaldehído con aromas verdes y a grasa; los ácidos acético y butanoico, seguidos de los ácidos 3-metilbutanoico, propanoico y 2-metilbutanoico con aromas a ácido, queso y rancio; las cetonas 2,3-butanediona y 2,3-pentanediona con aromas a mantequilla y dulce; los ésteres butanoato de etilo y 2-metilbutanoato de etilo seguidos de 3-metilbutanoato de etilo, 2-metilpropanoato de etilo y penanoato de etilo con aromas a fruta y dulce; el 1-octen-3-ol con aroma a setas; y los azufrados metional y metanotiol con aromas a cebolla y carne (Tabla 1). El umbral de detección de los compuestos mencionados da una idea del potencial aromático de cada uno; sin embargo, su participación en el perfil aromático del embutido dependerá de la concentración en que se encuentren (Olivares, 2011). De entre los compuestos aromáticos con un umbral de detección más bajo, se encuentran el metional

y el metanotiol (Tabla 1), por lo que su contribución al perfil aromático es de esperar que sea importante. Además, los compuestos azufrados son de gran interés debido a que aportan notas cárnicas (Mottram & Madruga, 1994). En embutidos curados madurados, además del metanotiol y metional (Tabla 1), también se han detectado los compuestos azufrados dimetil disulfuro (Schmidt & Berger, 1998) y dimetil trisulfuro (Corral, Salvador & Flores, 2013) como compuestos aromáticos, aunque de manera menos frecuente. Sin embargo, su análisis en los productos cárnicos es una tarea complicada debido a que no solo se encuentran en muy bajas concentraciones, a nivel de µg/kg, sino que además son poco estables, lo que dificulta su análisis (Corral, Leitner, Siegmund & Flores, 2016).

**Tabla 1. Umbral de detección y descripción olfativa de compuestos aromáticos detectados en embutidos curado-madurados.**

| Compuesto aromático | Descripción olfativa[a,b] | Umbral de detección[c] (µg/kg) |
|---|---|---|
| *Aldehídos* | | |
| Hexanal[1,2,3,4,5,6,7,8] | Grasa, verde, hierba | 330 |
| Heptanal[1,2,3,5,6] | Grasa, verde | 140 |
| 3-Metilbutanal[2,3,5,6,8] | Pungente, fruta, grasa | 1,6 |
| Nonanal[2,4,5,8] | Grasa, fresco, cáscara de naranja | 20 |
| Octanal[1,2,3,5] | Grasa, cítrico, miel | 21 |
| Benzenoacetaldehído[1,2,3,8] | Ceniza, verde, dulce, floral | 0,72 |
| 2,4-Nonadienal[3,4,8] | Grasa, floral | 0,4 |
| Pentanal[2,4,5] | Acre, pungente, afrutado | 17500 |
| 2-Metilbutanal[5,6,8] | Dulce, fruta, chocolate, café | - |
| 2-Heptenal (Z)[1,2] | Pungente, verde, grasa | 56 |
| 2-Nonenal (E)[2,8] | Grasa, verde, penetrante | 0,39 |
| 2-Nonenal (Z)[3,8] | | 0,022 |
| 3-Metilhexanal[5,6] | Dulce, verde | - |
| 2-Octenal (E)[2,6] | Hoja verde, grasa | 250 |
| 2,4-Decadienal (E,E)[5](E,Z)[7] | Pollo, grasa, dulce, naranja | - |
| *Ácidos* | | |
| Ácido acético[1,2,3,4,5,6,7,8] | Vinagre | 25 |
| Ácido butanoico[1,2,3,4,5,6,7,8] | Queso, mantequilla, fruta, ácido | 13 |
| Ácido 3-metilbutanoico[2,5,6,7,8] | Rancio, queso, dulce | 14 |
| Ácido propanoico[2,6,7,8] | Queso, pungente, rancio | 440-580 |

**Tabla 1. Continuación.**

| Compuesto aromático | Descripción olfativa[a,b] | Umbral de detección[c] (µg/kg) |
|---|---|---|
| Ácido 2-metilbutanoico[2,6,7,8] | Ácido, queso, pungente | 20 |
| Ácido heptanoico[1,2,4] | Rancio, ácido, sudor, grasa | 22 |
| Ácido 2-metilpropanoico[2,5,6,7,8] | Rancio, mantequilla, queso, ácido | 240-330 |
| Ácido octanoico[2,3] | Desagradable, queso, rancio, vegetal | 0,065 |
| Ácido pentanoico[5,7] | Desagradable, rancio, ácido, mantequilla | 30-120 |
| Alcohol feniletílico[1,2] | Rosas, floral | 50-100 |
| *Cetonas* | | |
| 2,3-Butanodiona[1,2,3,4,5,6,7] | Mantequilla, dulce | 5 |
| 2,3-Pentanodiona[1,2,5,6] | Dulce, mantequilla | 63 |
| 2-Nonanona[2,4,7] | Fresco, dulce, verde, tierra | 5500 |
| 2-Octanona[3,4] | Floral, madera, verde, fruta | 230 |
| 2-Heptanona[2,6] | Fruta, dulce, picante, canela, plátano | 45 |
| 1-Octen-3-ona[6,8] | Setas, tierra, herbal, rancio | 0,03-0,12 |
| *Alcoholes* | | |
| 1-Octen-3-ol[1,2,3,4,5,6] | Setas, tierra, verde | 48 |
| 1-Pentanol[2,4] | Dulce | 20 |
| *Ésteres* | | |
| Butanoato de etilo[1,2,3,4,5,6,7,8] | Fruta, piña | 0,017-0,023 |
| 2-Metilbutanoato de etilo[1,2,5,6,8] | Verde, fruta, dulce | - |
| 3-Metilbutanoato de etilo[1,6,7,8] | Fruta, vino, dulce | 4600 |
| 2-Metilpropanoato de etilo[1,2,6,8] | Fruta, aromático, dulce | - |
| Pentanoato de etilo[1,2,5,6] | Fruta, dulce, verde | 330-390 |
| Acetato de etilo[1,2,7] | Fruta, dulce, verde | 4600 |
| Hexanoato de etilo[1,2,6] | Fruta, piña, plátano | 10-20 |
| Butanoato de metilo[1,7] | Fruta, piña, manzana | 77-100 |
| 3-Metilbutanoato de metilo[1,3] | Fruta, piña, manzana | - |
| *Furanos* | | |
| 2-Etilfurano[2,4] | Ahumado, quemado, dulce, tierra | - |
| 2-Pentilfurano[2,3] | Fruta, verde, vegetal, tierra | 270 |

**Tabla 1. Continuación.**

| Compuesto aromático | Descripción olfativa[a,b] | Umbral de detección[c] (µg/kg) |
|---|---|---|
| *Terpenos* | | |
| Terpineno (α)[1,7] (γ)[7] | Madera, limón, hierba, medicinal | 7900 (α) – 55000 (γ) |
| Linalol[7,8] | Floral, cítrico, dulce, verde | 6000 |
| Limoneno[2,7] | Limón | 1700 |
| α-Pineno[7,8] | Pino, fresco, dulce, tierra | 105000 |
| *Hidrocarburos aromáticos* | | |
| 4-Metilfenol[1,3,4,7,6,8] | Fenol, narciso, animal | 0,18 |
| 2-Metoxifenol (guaiacol)[6,8] | Dulce, vainilla, carne, picante, madera | 2.6 |
| Eugenol[7,8] | Dulce, picante, madrea | 5-5,5 |
| 2-Feniletanol[7,8] | Rosa, dulce, floral, fresco | 50-100 |
| *Azufrados* | | |
| Metional (3-(metiltiopropanal)[1,2,3,4,8] | Cebolla, carne, patata | 0,063 |
| Metanotiol[1,2,3,5] | Col, ajo podrido | 0,24 |
| Metilsulfuro de alilo[7,8] | Cebolla, ajo | - |

Descripción olfativa: [a]Fenaroli's Handbook of Flavor Ingredients (Burdock, 2002) o [b]The Good Scents Company (http://www.thegoodscentscompany.com/). [c]Umbral de detección en aire: van Gemert (2004). Referencias: **1** (Olivares et al., 2013), **2** (Marco, Navarro & Flores, 2007), **3** (Olivares et al., 2011), **4** (Corral et al., 2013), **5** (Stahnke, 1994), **6** (Stahnke, 1995), **7** (Schmidt & Berger, 1998), **8** (Söllner & Schieberle, 2009).

## 3. NITRATOS Y NITRITOS EN PRODUCTOS CÁRNICOS

Para llevar a cabo la elaboración de embutidos curado-madurados, se emplean los agentes de curado (sales nitrificantes) empleados principalmente para garantizar la seguridad del producto (Rahman, 2007). Normalmente, la adición de nitrificantes se realiza en forma de nitrito sódico y/o nitrato sódico o nitrato potásico. El nitrito es el que ejerce el papel principal para llevar a cabo el proceso de curado, mientras que el nitrato sirve de fuente de nitrito tras su reducción, la cual esta llevada a cabo por microorganismos presentes en el producto o procedentes del cultivo iniciador, principalmente estafilococos con actividad nitrato reductasa (Sánchez Mainar & Leroy, 2015). La química del nitrito en la matriz cárnica es complicada debido a la variedad de reacciones que pueden participar en ella (Honikel, 2010). Al pH de la carne fresca (≈5-6) el nitrito ($NO_2$) se encuentra en equilibrio con el ácido nitroso ($HNO_2$), mientras que, al bajar el pH por la conversión del músculo en carne y por el efecto

de la fermentación, aumenta su transformación. A su vez, el $HNO_2$ se encuentra en equilibrio con el ácido nitroso anhidro ($N_2O_3$), que vuelve a formar $NO_2$ además de óxido nítrico (NO) (Figura 2).

$$KNO_2/NaNO_2 + H^+ \rightleftharpoons HNO_2 + K^+/Na^+$$

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$$

$$N_2O_3 \rightleftharpoons NO + NO_2$$

**Figura 2. Reacciones del nitrito en carne. Adaptado de Honikel (2008).**

El NO puede reaccionar con varios componentes y compuestos presentes en la carne, como la mioglobina y los aminoácidos, y es el responsable de las funciones específicas de curado que se describen a continuación.

Es importante destacar que el $NO_2$ o $HNO_2$ puede oxidarse y dar lugar a nitrato ($NO_3$) u óxido nítrico ($HNO_3$), razón por la que puede haber nitrato residual en productos curados exclusivamente con nitrito (Marco, Navarro & Flores, 2006) lo que se contempla en el reglamento 1333/2008 (DOUE, 2008). Hoy en día, se suele utilizar ascorbato para acelerar la reducción del $NO_2$ a NO. Además, el ascorbato reacciona con el oxígeno dando lugar a dehidroascorbato, disminuyendo la oxidación del $NO_2$ a $NO_3$ (Majou & Christieans, 2018).

## 3.1. Funciones del nitrito en productos cárnicos

### 3.1.1. Actividad antimicrobiana

El óxido nítrico procedente del nitrito añadido al embutido curado-madurado ejerce actividad bactericida y/o bacteriostática, evitando el crecimiento de microorganismos patógenos o indeseables que puedan estar presentes en el producto cárnico debido a contaminación o procedentes de la materia prima (Sindelar & Milkowski, 2011; Lee et al., 2018). El control del crecimiento microbiano por parte del nitrito ha sido ampliamente estudiado. Se ha visto que actúa inhibiendo el crecimiento de bacterias patógenas Gram negativas tales como enterobacterias (Sanz, Vila, Toldra, Niet & Flores, 1997; Gonzalez & Diez, 2002; Wójciak, Stasiak & Keska, 2019), *Salmonella thyphimurium* (Hospital, Hierro & Fernández, 2014; Christieans, Picgirard, Parafita, Lebert & Gregori, 2018) entre otras, así

como de bacterias Gram positivas tales como *Listeria monocytogenes* (Christieans et al., 2018) y *Clostridium botulinum* (Keto-Timonen, Lindström, Puolanne, Niemistö & Korkeala, 2012). La razón principal por la que se adiciona nitrito en embutidos curado-madurados es por su acción inhibitoria del crecimiento de *Clostridium botulinum* y la formación de su toxina (Lee et al., 2018). El mecanismo de acción bactericida y/o bacteriostática llevada a cabo por el nitrito no se conoce en profundidad. Uno de los mecanismos por los que el nitrito puede inhibir el crecimiento del *C. botulinum* es la unión del óxido nítrico con sus enzimas de hierro y azufre, produciendo una desactivación de las mismas necesarias para su metabolismo (Reddy, Lancaster & Cornforth, 2016). Además, se ha visto cómo el peroxinitrito ($ONOO^-$) también puede tener un papel importante en su acción antimicrobiana. Este compuesto se forma a partir de la reacción del óxido nítrico con el anión superóxido o con el peróxido de hidrógeno presente en los tejidos de la carne (Figura 3) (Brannan, Connolly & Decker, 2002; Majou & Christieans, 2018), y daña a las células patógenas mediante diversos mecanismos: (i) oxidación de componentes estructurales y moleculas esenciales; (ii) promoviendo modificaciones y roturas de la cadena de ADN; (iii) inhibiendo enzimas importantes para su metabolismo y defensa; y (iv) aumentando la producción de ácido acético en bacterias fermentativas (Rahman, 2007; Majou & Christieans, 2018). *C. botulinum* no posee la enzima catalasa, lo que daría lugar a que el óxido nítrico pudiese reaccionar con el peróxido de hidrogeno y formar peroxinitrito en su interior (Majou & Christieans, 2018).

Existen varios factores que intervienen en la actividad bactericida y/o bacteriostática del nitrito en embutidos curado-madurados, tales como el pH, la presencia de oxígeno, la temperatura utilizada y la presencia de otros compuestos como el hierro (Rahman, 2007). En valores de pH ácidos, especialmente por debajo de 6, aumenta el efecto del nitrito. En condiciones anaeróbicas se incrementa el efecto inhibitorio del nitrito. Las altas temperaturas favorecen la inhibición del crecimiento de *Clostridium botulinum*. Sin embargo, la presencia de hierro inhibe la acción antibacteriana del nitrito.

El uso de nitrato o nitrito podría producir un efecto diferente sobre el crecimiento y metabolismo de los microorganismos presentes en el producto. En este sentido, Fettahoğlu, Çinar, Kaya & Kaban (2019) observaron que el crecimiento de bacterias ácido-lácticas,

*Micrococcus/Staphylococcus* y levaduras y hongos en "Pastirma" (producto cárnico típico de Turquía) era menor con la adición de nitrito que cuando se añadía nitrato.

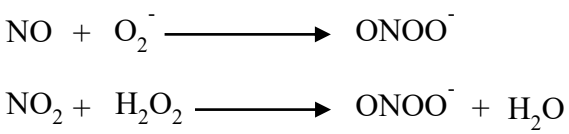



**Figura 3. Formación de peroxinitrito en carne curada.**

### 3.1.2. Actividad antioxidante

La presencia de nitrito en embutidos curado-madurados también ejerce un papel antioxidante (Berardo et al., 2016). Se ha comprobado que la disminución o eliminación de nitrificantes en embutidos curado-madurados aumenta el estado de oxidación del producto (Zanardi, Ghidini, Battaglia & Chizzolini, 2004; Hospital et al., 2015; Aksu, Erdemir & Çakici, 2016; Berardo et al., 2016; Braghieri, Piazzolla, Carlucci, Bragaglio & Napolitano, 2016; Wójciak et al., 2019). Se han propuesto varios mecanismos de acción a partir de los cuales se lleva a cabo esta actividad. La que parece ser la principal vía antioxidante del óxido nítrico es su unión y estabilización del hierro hemo presente en los pigmentos de la carne (Sebranek, 2009; Skibsted, 2011; Stoica, 2019). De esta manera se evita que el hierro se oxide, bloqueando su actividad catalítica en reacciones de oxidación lipídica, de forma que también disminuiye la cantidad de hierro liberado en el caso de que el producto fuese cocinado (Parthasarathy & Bryan, 2012). De la misma forma, el óxido nítrico también se une al hierro libre/no hemo presente en el embutido, evitando de nuevo su participación en reacciones de oxidación de lípidos (Bergamaschi & Pizza, 2011). Además de su acción sobre el hierro, se han propuesto otros medios mediante los cuales el óxido nítrico ejerce esta acción antioxidante: su capacidad quelante de iones metálicos, ser aceptor de radicales libres y por su capacidad para secuestrar moléculas de oxígeno y otras especies reactivas del oxígeno, todas ellas potentes oxidantes (Honikel, 2008; Sebranek, 2009; Stoica, 2019). Además, el óxido nítrico es capaz de reaccionar con diferentes constituyentes de la carne formando otros compuestos con capacidad antioxidante (Sebranek, 2009). El uso de nitrito o nitrato también parece influir en los procesos antioxidantes ocurridos en el producto, ya que se ha visto que el uso de nitrato disminuye el grado de oxidación lipídica con respecto al uso de nitrito en

embutidos curado-madurados de maduración lenta (Marco et al., 2006), lo contrario que en embutidos curado-madurados de maduración rápida (Navarro, Nadal, Nieto & Flores, 1998) y en "Pastirma" (Hazar, Kaban & Kaya, 2017).

Por otra parte, tal como se ha descrito en el apartado 3.1.1, el óxido nítrico procedente del nitrito puede producir peroxinitrito (Figura 3), el cual podría provocar la oxidación de constituyentes de la carne como lípidos y proteínas.

### 3.1.3. Desarrollo del color

El efecto más obvio producido por el nitrito en productos cárnicos es el desarrollo del color característico de los mismos, uno de los atributos más importantes en la aceptación por parte del consumidor. El papel crucial del nitrito en el desarrollo del color rojo en productos cárnicos se ha comprobado en embutidos curado-madurados (Aksu et al., 2016; Braghieri et al., 2016; Ben-Slima et al., 2017; Hwang et al., 2018), en productos cárnicos cocinados (Hustad et al., 1973; Dubose, Cardello & Maller, 1981; Jonas et al., 2017) y salchichas cocidas ("hot dogs") (Ruiz-Capillas et al., 2015), así como en sistemas modelo (Posthuma, Rasmussen & Sullivan, 2018). En estos estudios se ha visto cómo la adición de nitrito es el componente principal que interviene en el desarrollo del color. El color de la carne depende del estado de oxidación del hierro hemo presente en el pigmento mioglobina (Stoica, 2019). Si el hierro hemo de la mioglobina se oxida (de $Fe^{+2}$ a $Fe^{+3}$) se denomina metamioglobina produciendo un color marrón y si se oxigena se denomina oximioglobina produciendo un color rojo brillante. El nitrito, actúa en primer lugar como un agente oxidante produciendo la mencionada metamioglobina. En segundo lugar, a partir del nitrito se produce el óxido nítrico el cual se une a la metamioglobina y produce nitrosilmioglobina, que posee un color rojo brillante más estable que el color producido por la oximioglobina (Figura 4).

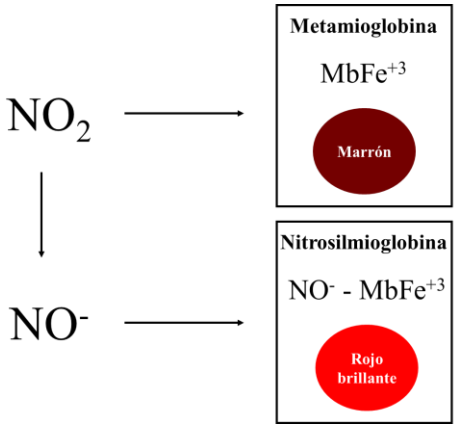



**Figura 4. Efecto de la adición de nitrito en el estado de la mioglobina de la carne y sus efectos en el color.**

Si al producto cárnico se le aplica calor, como en los productos cocidos, la nitrosilmioglobina sufre una desnaturalización de su porción proteica produciendo el complejo nitrosohemocromo con un color rosa también estable (Honikel, 2008). Para que ocurra la produccion de nitrosilmioglobina, no son necesarias grandes cantidades de nitrito (Rahman, 2007; Sindelar & Milkowski, 2011). Entre 5 y 20 ppm de nitrito sódico son suficientes para el desarrollo del color, pero para que dicho color sea estable con el tiempo y uniforme en el producto, son necesarias cantidades más altas. Sin embargo, cuando las cantidades son demasiado altas (> 600 ppm), se produce una decoloración del producto conocida como la "quemadura por nitrito". Aparece un color verde-marrón debido a la formación de nitrihemina (Stoica, 2019). Recientemente se ha visto cómo el nitrito puede actuar de manera sinérgica con otros componentes como los aminoácidos L-lisina, L-arginina y L-cisteina, aumentando el desarrollo del color rojo característico en embutidos curados cocidos (Ning et al., 2019).

### 3.1.4. Desarrollo del aroma

La adición de nitrificantes en productos cárnicos curados también contribuye a la generación del aroma característico de este tipo de productos. Es bien conocido que el uso del nitrito en productos cárnicos mejora sensorialmente el aroma general de los mismos (Hustad et al., 1973; Noel, Briand & Dumont, 1990). Sin embargo, este es el aspecto menos conocido de la química llevada a cabo por los nitrificantes en carnes curadas (Sebranek, 2009; Sindelar

& Milkowski, 2011). Hay varias teorías que apoyan que el nitrito interviene en el desarrollo del aroma a curado. Una de ellas, que parece ser la más relevante, es la capacidad del nitrito de disminuir el grado de oxidación lipídica del producto (Thomas, Mercier, Tournaire, Martin & Berdagué, 2013) y de esta manera disminuir la producción de compuestos aromáticos responsables de las notas rancias propias de un producto oxidado. Sin embargo, se ha visto cómo el uso de otros antioxidantes disminuye esta oxidación lipídica pero no produce el aroma que sí genera el uso de nitrificantes (Sebranek & Bacus, 2007), por lo que se cree que puede existir otro mecanismo para el desarrollo del aroma a curado. El efecto de la reducción o eliminación del uso de las sales de curado en el aroma característico de productos cárnicos curados ha sido escasamente estudiado, lo que serviría de ayuda para entender los mecanismos implicados. En este sentido, se han determinado cambios en la producción de compuestos volátiles en embutidos curado-madurados tras la reducción o eliminación de la adición de nitratos y nitritos (descritos en el apartado 3.5.1.3).

El uso de nitrato podría producir un perfil aromático distinto al producido por el uso de nitrito en productos cárnicos curados. Por un lado, se ha observado que el uso de nitrato en embutidos curado-madurados potencia la producción de compuestos volátiles derivados de la degradación de aminoácidos (Olesen, Meyer & Stahnke, 2004; Marco et al., 2006) y de la fermentación de carbohidratos (Marco et al., 2006), además de producir un aroma sensorialmente más aceptable (Marco et al., 2006, 2008). Por otro lado, el uso de nitrito potencia la producción de compuestos volátiles derivados de la β-oxidación (Marco et al., 2006). Sin embargo, estas diferencias no siempre han sido percibidas (Sanz et al., 1997; Marco et al., 2007). Una posible justificación sobre el diferente efecto en el aroma llevado a cabo por el uso de nitrato frente al nitrito, puede residir en el diferente efecto sobre los microorganismos implicados en la generación de compuestos aromáticos (Olesen et al., 2004) y en las diferencias en los procesos de fabricación. Olesen et al. (2004) han observado que el nitrito disminuye el crecimiento de los estafilococos por su mayor potencial antimicrobiano, y que el nitrato parece potenciar su crecimiento ya que puede actuar como aceptor de electrones en su respiración en ausencia de oxígeno. De esta manera, la implicación de este microorganismo en el aroma es diferente con el uso de cada nitrificante, modificando el perfil aromático. Además, es importante tener en cuenta que el efecto de estas sales difiere en función de la especie de *Staphylococcus* (Olesen et al., 2004).

## 3.2. Análisis del contenido en nitrito/nitrato en productos cárnicos

El nitrato y nitrito sufren cambios durante el procesado y vida útil del producto cárnico al que se añaden (Apartado 3, Figura 2). En consecuencia, la cantidad residual de nitrato y nitrito no se puede relacionar directamente con la cantidad añadida. Sin embargo, es importante su determinación porque la presencia de estos nitrificantes en el producto final podría implicar la formación de N-nitrosaminas, como se explica en el apartado 3.4, además de por la creciente tendencia por parte de los consumidores para apreciar la disminución de la presencia de aditivos en estos productos. En el caso del nitrito, al tratarse de un compuesto muy reactivo, en el análisis de su concentración residual se debe evitar al máximo posible su pérdida durante la aplicación de los métodos de determinación empleados.

Recientemente, se ha realizado una revisión bibliográfica sobre los métodos empleados en el análisis de nitrito y nitrato (Wang et al., 2017). Esta revisión recoge un gran número de artículos donde se analiza el nitrito y/o nitrato principalmente en agua, muestras biológicas y en menor medida en alimentos. En la tabla 2 se recogen diferentes estudios sobre métodos de análisis empleados en productos cárnicos. En todos los casos, la metodología consiste en una primera fase de extracción, una fase opcional de precipitación y una posterior determinación del nitrito extraido mediante diferentes métodos de detección.

### 3.2.1. Extracción de nitrito y nitrato en productos cárnicos

La fase de extracción se suele realizar en fase acuosa. La mayoría de autores utilizan agua caliente, entre 50 - 100 °C durante 5 min - 2 h (Tabla 2). Sin embargo, Hamano et al. (1998), Yu, Chen, Nie & Yao (2001), Badea et al. (2004), He, Zhang, Huang & Hu (2007) y Wu et al. (2019), optan por una extracción acuosa en frío. Otros autores, como Yu et al. (2001) utilizan ultrasonidos, mientras que Liu et al. (2019) han combinado la extracción acuosa de nitrito con ultrasonidos y un baño de agua caliente.

Tras este primer paso, algunos autores optan por realizar una precipitación del extracto obtenido para eliminar las posibles interferencias, como las proteínas que puedan afectar la sensibilidad y selectividad del análisis. Los productos de precipitación más utilizados son el $ZnSO_4$ (Hassan, Marzouk & Sayour, 2003; He et al., 2007; Kumar, Gonçalves, Sukeri, Araki & Bertotti, 2018; Kong et al., 2019; Yang et al., 2019), el acetato de Zn (Promsuwan,

Thavarungkul, Kanatharana & Limbut, 2017; Pourreza & Abdollahzadeh, 2019; Li et al., 2019; Han & Chen, 2019) y los reactivos Carrez I y II (Andrade, Viana, Guadagnin, Reyes & Rath, 2003; Merino, 2009; Dinçkaya, Akyilmaz, Sezgintürk & Ertaş, 2010; Hu et al., 2019). Sin embargo, muchos de los autores no realizan este paso de precipitación con agentes desnaturalizantes, sino que solo emplean el calentamiento previo para desnaturalizar las proteínas (Tabla 2).

### 3.2.2. Detección de nitrito y nitrato en productos cárnicos

El siguiente paso en el análisis de nitrificantes en productos cárnicos es la determinación del nitrito a partir del extracto obtenido previamente. En la tabla 2 se muestran las técnicas espectrofotométricas, electroquímicas, de fluorescencia, de quimioluminiscencia, de cromatografía iónica, por UPLC-MS, CG-MS y electroforesis capilar más utilizadas. La técnica más empleada es la basada en la espetrofotometría, seguida de las técnicas electroquímicas y de fluorescencia. En cuanto al límite de detección mostrado por estas técnicas, no todos han sido determinados de la misma manera. Algunos han sido determinados en la matriz cárnica (con unidades en mg/kg) y otros en la solución de ensayo (con unidades en μg/L). Con respecto a los límites de detección de nitrito determinados en la matriz cárnica, la técnica electroquímica llevada a cabo por Gill et al. (2019) muestra el valor más bajo (0.0038 mg/kg). Las técnicas cromatográficas con detector de espectrometría de masas (Siddiqui et al., 2018; Luckovitch & Pagliano, 2019) también muestran límites de detección bajos (0.05 y 0.114 mg/kg, por CG y UPLC respectivamente), seguidas de las técnicas espectrofotométricas (Zatar, Abu-Eid & Eid, 1999; Casoni, Badiu & Frentiu, 2019) con limites de 0.2-0.4 mg/kg. Con respecto a los límites de detección de nitrito determinados en la solución de ensayo, las técnicas con valores más bajos fueron las llevadas a cabo por Ensafi & Amini (2010), Kumar et al. (2018) y Li et al. (2019) mediante espectrofotometría (0.08 μg/L), electroquímica (0.046 μg/L) y fluorescencia (0.031 μg/L) respectivamente.

Para la determinación de nitrato es necesario su reducción a nitrito, para posteriormente realizar la detección de éste utilizando las técnicas arriba mencionadas. Algunos autores utilizan metales de transición para esta reducción, tales como Zn (Merino, 2009) y columnas de Cd con recubrimiento de Cu (Andrade et al., 2003; Ensafi, Rezaei & Nouroozi, 2004). Además, Martínez Calatayud, García Mateo & David (1998) llevaron a cabo una

fotoreducción. Cabe destacar que Dinçkaya et al. (2010) han diseñado un método electroquímico para la determinación directa de nitrato mediante un biosensor, basado en la actividad nitrato-reductasa. En cuanto a los límites de detección del nitrato mediante las técnicas de determinación de nitrito mencionadas anteriormente, los valores más bajos (Tabla 2) se obtienen mediante la técnica de cromatografía iónica llevada a cabo por D'Amore, Di Taranto, Vita, Berardi & Iammarino (2019) en la matriz cárnica (0.68 mg/kg) y mediante la técnica electroquímica llevada a cabo por Dinçkaya et al. (2010) en la solución de ensayo (0.1 μg/L). Sin embargo, también se obtienen valores bajos mediante espectrofotometría (1-5 mg/kg y 2.04-3 μg/L).

Como se ha mencionado anteriormente, la técnica espectrofotométrica es la más usada para la determinación de nitrato y nitrito en productos cárnicos. Esta técnica se basa en la generación de un compuesto que posee su máxima absorción a una determinada longitud de onda, donde el nitrito actúa formando parte de la reacción de formación de este compuesto, o como catalizador o inhibidor de la misma. De esta manera, la absorbancia del compuesto final será directamente o inversamente proporcional a la concentración de nitrito. Se trata de una técnica sencilla y de bajo coste y con límites de detección razonablemente bajos. En cuanto a la reducción del nitrato a nitrito para su posterior determinación, en embutidos curado-madurados se suelen utilizar con más asiduidad enzimas con actividad nitrato reductasa proporcionadas por kits enzimáticos comerciales con esta finalidad (Marco et al., 2006, 2008; Hospital et al., 2015).

**Tabla 2. Métodos de análisis de nitrito y nitrato en productos cárnicos.**

| Muestra | Método de extracción | Precipitación | Detección | LOD[1] | *Ref.*[2] |
|---|---|---|---|---|---|
| ***Espectrofotometría*** | | | | | |
| Salami<br>Embutido<br>Jamón cocido | Recomendaciones AOAC | - | Reacción de Griess | 0,4 mg/kg $NO_2$ | *1* |
| Embutido | Agua caliente + baño de agua 90 °C 30 min | Acetato de Zn.<br>Filtrar (papel de filtro) | Azul metileno + hidrazina → ↓azul<br>Abs 662 nm<br>Catalizador: nanopartículas de paladio<br>Nitrito: inhibe reacción colorimétrica | 9,66 µg/L $NO_2$ | *2* |
| Embutido | Agua 80 °C + NaOH, 15 min<br>Filtrar (Whatman no 45) | - | Azul cresyl brillante + bromato → ↓azul<br>Abs 570 nm<br>Nitrito: catalizador de la reacción | 0,08 µg/L $NO_2$ | *3* |
| Carne picada | Agua 50-60 °C | Carrez I y II<br>Filtrar (Whatman) | Reacción de Griess<br>Reducción $NO_3$ a $NO_2$ con Zn | 3 mg/kg $NO_2$<br>5 mg/kg $NO_3$ | *4* |
| Carne | Agua, calentar a 80 °C 15 min +<br>Baño vapor 2 h<br>Filtrar | - | Azul de fosfomolibdeno + $NO_2$ → azul de fosfomolibdeno oxidado<br>Monitoreo de la reacción 600-900 nm | 34 µg/L $NO_2$ | *5* |
| Embutido de ternera | Agua, calentar 2 h<br>Filtrar (Whatman no 2) | - | Reacción redox entre pirogalol sulfoneftaleína y bromato de potasio, Abs 465 nm<br>Nitrito: catalizador<br>Reducción $NO_3$ a $NO_2$ con columna de reducción de Cd con recubrimiento de Cu | 1,6 µg/L $NO_2$<br>3 µg/L $NO_3$ | *6* |
| Embutido de cerdo<br>"Hot-dogs" | Agua 70 °C, 15 min | Carrez I y II<br>Filtrar (papel de filtro) | $NO + Fe^{+2}$ + tiocianato → $FeSCNNO^{+}$<br>Abs 460 nm<br>Nitrito: fuente de NO (reducción por ácido sulfhídrico)<br>Reducción $NO_3$ a $NO_2$ con columna de reducción de Cd con recubrimiento de Cu | 13 mg/kg $NO_2$<br>20mg/kg $NO_3$ | *7* |
| Ternera enlatada | Agua, calentar a 80 °C 15 min +<br>baño de agua 2 h<br>Filtrar | - | Ácido fosfomolíbdico + $Na_2S$ → complejo azul de fosfomolibdeno Abs 814 nm<br>$NO_2$ oxida complejo azul | 0,2 mg/kg $NO_2$ | *8* |

**Tabla 2. Continuación.**

| Muestra | Método de extracción | Precipitación | Detección | LOD[1] | *Ref.*[2] |
|---|---|---|---|---|---|
| "Frankfurt" | Agua 100 °C, 2 h | Carbón<br>Filtrar (filtro 2 µm) | $NO_2$ + sulfato de proflavina → Abs 328 nm | 8,74 µg/L $NO_2$<br>2,04 µg/L $NO_3$ | *9* |
| Embutido tipo "Viena" | Tampón fosfato pH 7<br>Filtrar (conos de membrana Centriflo) | - | $NO_2^-$ + $NADH^+$ + $4H^+$ → $3NAD^+$ + $NH_4OH$ + $H_2O$<br>↓NADH = ↓Abs 340 nm | 1 mg/kg $NO_2$ | *10* |
| ***Electroquímica*** | | | | | |
| Jamón cocido<br>"Hot-dogs"<br>Salami.<br>"Bologna" | Tampón fosfato pH 7.4 + baño de agua a 80 °C 30 min<br>Filtrar (papel de filtro de textura media) | - | Reducción:<br>$NO_2$ + KI + $H_2SO_4$ → NO gas<br>Detector fase gaseosa:<br>• Amperímetro Pt-Nafion (laboratorio)<br>• Sensor "Alphasense" (comercial) | Pt-Nafion:<br>0,0038 mg/kg $NO_2$<br>Alphasense:<br>0,0098 mg/kg $NO_2$ | *11* |
| "Hot-dogs"<br>Embutido de cerdo | Solución saturada de Borax + agua caliente + baño de agua a 70 °C 20 min | $ZnSO_4$<br>Filtrar | Oxidación.<br>Electrodo: NPG (película de oro nanoporoso) en $H_2SO_4$<br>Detector: Amperímetro | 0,046 µg/L $NO_2$ | *12* |
| Jamón cocido<br>Bacon<br>Emb. curado-madurado | Solución saturada de Borax + agua 70 °C, calentar 100 °C 15 min | Acetato de Zn<br>Filtrar | Oxidación.<br>Electrodo: AgCMs-PAA/PVA/SPCE (microtubos de plata de ácido poliacrílico/alcohol polivinilico/electrodo de carbono impreso en pantalla)<br>Detector: Amperímetro | 207 µg/L $NO_2$ | *13* |
| Pastrami<br>Embutido<br>Salami | Agua + baño de agua a 90 °C 1h | Carrez I y II<br>Filtrar (membrana 0.45 µm) | Reducción.<br>Electrodo: carbono vítreo con biosensor nitrato reductasa<br>Detector: Voltímetro | 0,1 µg/L $NO_3$ | *14*[3] |
| Salami<br>Jamón cocido<br>Jamón de Parma<br>Bologna | Tampón acetato 4 °C, 30 min | EDTA y ácido metafosfórico<br>Filtrar (papel filtro textura media) | Oxidación.<br>Electrodo: pasta de carbono<br>Detector: Amperímetro | 2,8 mg/kg $NO_2$ | *15* |

**Tabla 2. Continuación.**

| Muestra | Método de extracción | Precipitación | Detección | LOD[1] | *Ref.*[2] |
|---|---|---|---|---|---|
| Carne de ternera enlatada Hamburguesa de ternera | Agua, pH 8 + calentar a 50 °C, 5 min | $ZnSO_4$ + baño de agua a 80 °C 2 h Filtrar (Whatman no 1) | Reacción de Griess + potenciometría Electrodo: tris(bathofenantrolina)Ni(II)/SPAN | 23 µg/L $NO_2$ | *16* |
| ***Fluorescencia*** | | | | | |
| Bacon Embutido | Agua + ultrasonidos 15 min + baño de agua a 75 °C 5 min Filtrar (membrana 0.22 µm) | - | Sensor: Nanosondas fluorescentes RYDE CDs λexcitación 520 nm λemisión 566/621 nm | 1,45 µg/L $NO_2$ | *17* |
| Jamón cocido | Solución de Borax + agua 70 °C + baño de agua 100 °C 15 min | Carrez I y II Filtrar (filtro 0.45 µm) | Sensor: C-dots-NR λexcitación 517 nm λemisión 575 nm | 13,5 µg/L $NO_2$ | *18* |
| Embutido | Tampón fosfato pH 4.5 + calentar a 80 °C 30 min | $ZnSO_4$ Filtrar (filtro 0.22 µm) | Sensor: nanosonda fluorimétrica y colorimétrica g-CNQDs- BPS-Fe2+ λexcitación 400 nm λemisión 515 nm | 2,3 µg/L $NO_2$ | *19* |
| Embutido | Solución saturada de Borax + agua 70 °C, 15 min | Ferrocianuro de potasio Acetato de Zn Filtrar (papel de filtro) | Sensor: MoS2 QDs-AuNCs λexcitación 365 nm λemisión 430/615 nm | 0,031 µg/L $NO_2$ | *20* |
| Carne enlatada Jamón cocido Embutido | Agua | - | Sensor: AC-$NO_2$ (derivado de carboximida de antraceno) λexcitación 440 nm λemisión 522 nm | 3,86 µg/L $NO_2$ | *21* |
| Jamón cocido Embutido | Solución saturada de Borax + agua 70 °C + baño agua 100 °C 15 min | $ZnSO_4$ Filtrar | Sensor: ZnCdS QDs ternarios λexcitación 365 nm λemisión 505 nm | 35,88 µg/L $NO_2$ | *22* |

**Tabla 2. Continuación.**

| Muestra | Método de extracción | Precipitación | Detección | LOD[1] | *Ref.*[2] |
|---|---|---|---|---|---|
| ***Quimioluminiscencia*** | | | | | |
| Embutido | Agua caliente + solucion de Borax 5% + baño agua a 100 °C 15 min | Ferrocianuro de potasio<br>Acetato de Zn<br>Filtrar (membrana 0.45 µm) | DPA (diperiodatoargentado (II)) + Ácido fólico → Reacción Quimioluminiscente<br>$NO_2$ inhibe la reacción | 3,17 µg/L $NO_2$ | *23* |
| Jamón cocido<br>Cerdo curado<br>Embutido | Agua | $ZnSO_4$ + calentar a 60 °C 10 min<br>Filtrar | $NO_2$ + Ferrocianuro + HCl → Ferricianuro<br>Ferricianuro + Luminol → Reacción Quimioluminiscente | 4 µg/L $NO_2$ | *24* |
| ***Cromatografía iónica*** | | | | | |
| Carne fresca de ternera, cerdo, pollo/pavo y equina<br>Jamón cocido<br>Embutido especiado | Agua + baño agua a 80 °C 5 min<br>Filtrar (filtro 0.22 µm) | - | Detector de conductividad<br>Fase móvil: flujo isocrático KOH<br>Flujo: 0.015 ml/min<br>Elución (38 min): | 1,2 mg/kg $NO_2$<br>0,68 mg/kg $NO_3$ | *25* |
| Carne fresca de ternera, cerdo, pollo y equina | Agua + calentar a 70 °C 5 min<br>Filtrar (Whatman no 40) | - | Detector electroquímico<br>Fase móvil: $Na_2CO_3$ 9 mM<br>Flujo: 1 ml/min<br>Elución: 20 min | 1,5 mg/kg $NO_2$<br>3,2 mg/kg $NO_3$ | *26* |
| Carne fresca<br>Carne curada | Ultrasonidos<br>Filtrar (membrana 0.45 µm + cartucho $C_{18}$) | Dowex 50W-X8 resina de intercambio catiónico ($Ag^+$) (eliminar exceso de cloro) | Detector: sensor de ondas acústicas<br>Fase móvil: KHP (hidrogenoftalato de potasio) 1.5 mM<br>Flujo: 1.2 ml/min | 200 µg/L $NO_2$<br>300 µg/L $NO_3$ | *27* |
| Salami<br>Jamón cocido | Agua + calentar a 70-80 °C 15 min<br>Filtrar (Whatman no 2 + GF/A + Acrodisc. 1.2 y 0.2 µm) | - | Detector: UV 225 nm<br>Fase móvil: NaOH 5 mM<br>Flujo: isocrático 1 ml/min<br>Elución: 10 min | 300 mg/kg $NO_2$<br>500 mg/kg $NO_3$ | *28* |

**Tabla 2. Continuación.**

| Muestra | Método de extracción | Precipitación | Detección | LOD[1] | *Ref.*[2] |
|---|---|---|---|---|---|
| ***Espectrometría de masas*** | | | | | |
| Jamón cocido ahumado<br>Jamón horneado<br>"Bologna"<br>Embutido ahumado | Agua 70 °C, 20 min<br>Espacio de cabeza estático 60 °C 2 min | - | CG<br>Derivatización: $NaHCO_3$ + $Et_3OBF_4$<br>Detección MS:<br>$NO_3$ ($EtONO_2$) m/z: 92, 93<br>$NO_2$ ($EtNO_2$) m/z: 76, 77 | 0,05 mg/kg $NO_2$<br>1 mg/kg $NO_3$ | *29* |
| Carne de ternera, pollo y cordero | Agua + calentar a 80 °C 15 min<br>Filtrar (papel de filtro) | - | UPLC<br>Fase móvil Dodecilsulfato sódico:Acetonitrilo 30:70<br>Detector: MS triple cuadrupolo<br>SCAN Polaridad $ESI^-$ | 0,114 mg/kg<br>$NO_2$ | *30* |
| ***Electroforésis capilar*** | | | | | |
| Salami<br>Jamón cocido<br>Embutido de pavo<br>Embutido | Agua, 50 °C 15 min<br>Flitrar (Whatman + acetato de celulosa 0.45 μm) | - | Capilar PEI (polietilenimina)<br>Flujo electroosmótico reverso (hacia ánodo)<br>Detección por UV 210 nm | 105 μg/L $NO_2$<br>99 μg/L $NO_3$ | *31* |

[1]LOD: límite de detección. [2]Referencias: **1** (Casoni et al., 2019), **2** (Pourreza & Abdollahzadeh, 2019), **3** (Ensafi & Amini, 2010), **4** (Merino, 2009), **5** (Niazi, Ghasemi & Yazdanipour, 2005), **6** (Ensafi et al., 2004), **7** (Andrade et al., 2003), **8** (Zatar et al., 1999), **9** (Martínez Calatayud et al., 1998), **10** (Hamano et al., 1998), **11** (Gill et al., 2019), **12** (Kumar et al., 2018), **13** (Promsuwan et al., 2017), **14** (Dinçkaya et al., 2010), **15** (Badea et al., 2004), **16** (Hassan et al., 2003), **17** (Liu et al., 2019), **18** (Hu et al., 2019), **19** (Kong et al., 2019), **20** (Li et al., 2019), **21** (Wu et al., 2019), **22** (Yang et al., 2019), **23** (Han et al., 2019), **24** (He et al., 2007), **25** (D'Amore et al., 2019), **26** (Iammarino & Taranto, 2012), **27** (Yu et al., 2001), **28** (Siu & Hensall, 1998), **29** (Luckovitch & Pagliano, 2019), **30** (Siddiqui et al., 2018), **31** (Öztekin, Nutku & Erim, 2002). [3]Método de análisis de nitrato.

## 3.3. Estado actual del uso de nitrito/nitrato en España y en la Unión Europea

Las funciones esenciales llevadas a cabo por los nitrificantes en la elaboración de productos cárnicos curados (Apartado 3.1) implican que se debe controlar su uso en este tipo de productos. En los últimos 25 años, la legislación en Europa y España sobre el uso de nitrificantes en productos cárnicos ha sufrido muchos cambios. En 1995, la Directiva Europea 95/2/CE *relativa a aditivos alimentarios distintos de los colorantes y edulcorantes* (DOCE, 1995) se transpuso a la reglamentación española mediante el Real Decreto 145/1997 (BOE, 1997), *por el que se aprueba la lista positiva de aditivos distintos de colorantes y edulcorantes para su uso en la elaboración de productos alimenticios, así como sus condiciones de utilización*, posteriormente actualizada (BOE, 2002). En esta reglamentación se establecieron "cantidades añadidas indicativas" de 150 y 300 mg/kg, además de "niveles residuales" de 50 y 250 mg/kg, de nitrito y nitrato sódico respectivamente. Debido a los comentarios de varios estados miembros sobre la necesidad de reevaluar las dosis utilizadas de dichos aditivos, la Comisión Europea se planteó la posibilidad de la reducción de nitrificantes en productos cárnicos y, para ello, acudió a la EFSA para que emitiera una opinión sobre la seguridad de estos aditivos. A raíz de ello, en 2003 la EFSA publicó la opinión de su Panel Científico sobre el efecto del nitrito/nitrato en la seguridad microbiológica de los productos cárnicos (EFSA, 2003), donde se presentaron las siguientes conclusiones:

- Los productos cárnicos curados son aquellos que por definición contienen sales de curado generalmente sal (cloruro de sodio) y nitrito o nitrato.

- El nitrito contribuye a la seguridad microbiológica y también al sabor, color y estabilidad oxidativa de los productos cárnicos curados. El efecto inhibitorio del nitrito añadido sobre el crecimiento microbiano depende de su concentración y del pH, siendo mayor a valores ácidos de pH. No hay evidencia convincente de que la cantidad residual de nitrito contribuya a la seguridad microbiológica de los productos cárnicos.

- No existe relación entre las cantidades inicialmente añadidas y las residuales de nitrito, ya que está influenciado por varios factores como el pH, la temperatura de

almacenamiento, tratamiento térmico aplicado y la presencia de sustancias reductoras como el ascorbato/isoascorbato.

- Tampoco existe relación entre las cantidades inicialmente añadidas y las residuales de nitrato.
- En la mayoría de los productos cárnicos, el nitrato no proporciona una protección directa contra el crecimiento de *C. botulinum*. Sin embargo, el uso de nitrato como reservorio de nitrito parece necesario en productos cárnicos curado-madurados tradicionales.
- El nivel mínimo de nitrito para alcanzar el efecto protector ante un riesgo microbiológico, como *C. botulinum*, dependerá de factores como el tratamiento térmico aplicado, el pH, la actividad de agua y la concentración de sal.
- El nitrito aumenta la protección contra *C. botulinum* y no se ha encontrado ninguna alternativa a su uso en productos cárnicos.
- El control de nitritos y nitratos en productos cárnicos debe ser mediante los niveles añadidos inicialmente y no mediante los niveles residuales, ya que estos últimos no contribuyen a la actividad inhibitoria frente a *C. botulinum*.
- La adición de 50-100 mg/kg de nitrito sódico debe ser suficiente para la mayoría de productos cárnicos, excepto para los que posean un contenido de sal bajo y una vida útil prolongada, para los que serán necesario 50-150 mg/kg para inhibir el crecimiento de *C. botulinum*.
- El uso de nitrito solo se permite mediante su mezcla con cloruro sódico para evitar la adición excesiva del mismo, así como por la prevención de accidentes.
- La "cantidad añadida indicativa" actual de nitrito y nitrato debe ser modificada por "cantidad máxima añadida".

Tras este dictamen, en 2006 se publica una nueva directiva, la Directiva 2006/52/CE (DOUE, 2006) por la que se modifica la Directiva 95/2/CE *relativa a aditivos alimentarios distintos de los colorantes y edulcorantes*. Finalmente, en 2008 el Reglamento 1333/2008 *sobre aditivos alimentarios* (DOUE, 2008) establece las normas sobre los aditivos alimentarios usados en los alimentos incluyendo un apartado “8.3 Productos cárnicos” el cual incluye a su vez, un subapartado “8.3.4 Productos cárnicos tradicionales con

disposiciones específicas para nitritos y nitratos". En esta nueva reglamentación, se sustituyó el término "cantidad añadida indicativa" por el de "cantidad máxima añadida", además de eliminar las "cantidades residuales" excepto en jamón curado y productos similares. De esta manera, tras las correspondientes modificaciones, actualmente se permiten "cantidades máximas añadidas" de 150 mg/kg de nitrito y nitrato sódicos en productos cárnicos no tratados por el calor, entre los que se encuentran los embutidos curado-madurados. En el caso de productos tipo salchichón y chorizo de larga curación (período de maduración de 30 días como mínimo), la "cantidad máxima añadida" permitida de nitrato sódico es de 250 mg/kg siempre y cuando no se adicione nitrito (DOUE, 2008). Cabe destacar que los productos cárnicos curados tradicionales con disposiciones específicas para nitritos y nitratos sí tienen especificada una "cantidad residual máxima", que oscilan entre 50 y 175 mg/kg de nitrito sódico y 250 mg/kg para nitrato sódico (DOUE, 2008).

### 3.4. Problemática del uso de nitrito y nitrato

La carne es considerada una importante fuente de nutrientes en la dieta debido a su contenido en proteínas de alto valor biológico, además de vitaminas principalmente del grupo B y minerales, tales como zinc y hierro. Sin embargo, en 2015 la agencia "International Agency for Research on Cancer" (IARC, OMS) publicó los resultados de un estudio monográfico (finalmente publicado en V114, 2018; IARC, 2018) donde concluyó que la carne roja se clasifica como probablemente cancerígena (grupo 2A) y la carne procesada como cancerígena (grupo 1) en humanos. Esta publicación ha creado muchas dudas en cuanto al sistema de identificación de riesgo empleado (Boobis et al., 2016), puesto que la carne procesada se clasificaba en la misma categoría de carcinógenos como el gas mostaza, lo que demostraba que el sistema no tenia en cuenta el grado de riesgo. Por ello, Boobis et al., 2016 indican la necesidad de llevar a cabo evaluaciones que determinen el peligro y el riesgo, para evitar el lanzamiento de mensajes confusos, afectando no solo a las políticas de salud sino a la economía. Además, otros autores indican que las categorías y descripciones de los alimentos de origen cárnico evaluados en los estudios epidemológicos son substancialmente diferentes entre los estudios contemplados, incluso dentro del mismo estudio, o no coinciden con las definiciones empleadas en la regulación (O'Connor et al., 2020). Por ello, la falta de una clara clasificación para los alimentos de origen animal impide llevar a cabo una

adecuada interpretación de la evidencia sobre el consumo de alimentos de origen animal y las enfermedades crónicas. Por otra parte, Breda et al., 2019 demuestran en un estudio de intervención que el nitrato del agua potable puede tener una contribución significativa a la formación endógena de nitrosocompuestos (NOC), independientemente del tipo de carne consumida, aunque evidencian que el efecto es más pronunciado en sujetos que consumen carne roja procesada. Por ello, indican que se deben mejorar los estudios de evaluación de riesgo realizados para evaluar la carcinogeneicidad de los productos cárnicos ya que se deben tener en cuenta los niveles de nitrato del agua potable (Breda et al., 2019).

Sin embargo, la relación entre el consumo de carnes procesadas y cáncer viene dada, entre varios factores, por la presencia de determinados compuestos tales como N-nitrosaminas e hidrocarburos aromáticos policíclicos, procedentes del proceso de curado y ahumado (Bouvard et al., 2015). Existe suficiente evidencia de que muchas N-nitrosaminas producen cáncer en animales, así que algunas de ellas han sido clasificadas como probablemente cancerígenas para humanos (IARC, 1978). En los productos cárnicos procesados, las N-nitrosaminas se forman a partir de la reacción de un agente nitrosante con una amina secundaria (Honikel, 2008), siendo el nitrito el principal agente nitrosante en productos cárnicos curados. La presencia de N-nitrosaminas en productos cárnicos curados empezó a visualizarse en los años 70, cuando se demostró que el bacon frito contenía cantidades considerables de N-nitrosopirrolidina (Honikel, 2008). En la tabla 3 se presenta una recopilación de estudios que analizan el contenido de N-nitrosaminas en productos cárnicos curados. Sin embargo, la nomenclatura empleada para definir los productos cárnicos es ambigua, lo que en ocasiones imposibilita la apropiada identificación del proceso tecnológico empleado en la fabricación. Esto pone de manifiesto, que el empleo de una terminología adecuada es esencial para interpretar adecuadamente los estudios realizados, como se ha indicado anteriormente. Debido a ello, en la tabla 3 solo se recogen aquellos estudios en los que el producto analizado se ha podido identificar y clasificar correctamente en: bacon (sin tratar, frito, ahumado o al microondas), embutidos curado-madurados (tipo salami y “sucuk”), embutidos curados cocidos (“Frankfurt”, bologna y mortadela) y jamón (curado, ahumado o cocido).

**Tabla 3. Contenido (µg/kg) de N-nitrosaminas en productos cárnicos curados.**

| Producto cárnico curado | NDMA | NPYR | NDEA | NPIP | NHPRO | NTCA | HMNTCA | NPRO | NDBA | NSAR | NMEA | NDPA | NMTCA | NMA | [1]Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Bacon** | | | | | | | | | | | | | | | |
| **Bacon** | 0 | 0 | 0 | 0 | - | - | - | - | 0 | - | - | 0 | - | - | *1* |
| | 2-5 | 2-9 | [2]- | - | 5-186 | 7-310 | 5-130 | 74-437 | - | 10-74 | - | - | - | - | *2* |
| | 1.8 | 0.4 | 0 | 0 | 0 | 41 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | *3* |
| | 1,2 | 7,5 | 0,4 | 1,2 | - | - | - | - | 0,72 | - | - | - | - | - | *4* |
| **Bacon frito** | 1-5 | 1-200 | 1-20 | 1-20 | - | - | - | - | - | - | - | - | - | - | *1* |
| | - | 1-65 | - | - | - | - | - | - | - | - | - | - | - | - | *9* |
| | 1, 2-1,4 | 3,09-9,4 | - | - | - | - | - | - | - | - | - | - | - | - | *10* |
| | 0-2,9 | 0,4-21 | - | 0-0,9 | - | - | - | - | - | - | - | - | - | - | *11* |
| **Bacon ahumado** | 2-8 | 2-19 | - | - | 5-340 | 8-1900 | 26-670 | 58-840 | - | 90-210 | - | - | - | - | *2* |
| | 0-1,3 | - | 0,1-3,8 | 0,1-0,3 | - | - | - | - | 0-0,8 | - | - | - | - | - | *5* |
| **Bacon microondas** | 0 | 4.2 | - | - | - | - | - | - | - | - | - | - | - | - | *10* |
| | 0-1,2 | 0-0,8 | - | 0-0,3 | - | - | - | - | - | - | - | - | - | - | *11* |
| **Embutidos curado-madurados (Emb cur-mad)** | | | | | | | | | | | | | | | |
| **Salami** | 1-5 | - | | | - | - | - | - | - | - | - | - | - | - | *1* |
| | 0-2,1 | - | 1,5-12 | 0-0,06 | - | - | - | - | 0,3-2 | - | - | - | - | - | *5* |
| | 0 | 0-1,4 | 0 | 0 | 0-3,9 | 76-217 | - | 0-7,5 | 0 | 0 | 0 | 0 | 7,5-39 | 0-0,6 | *3* |
| | 0,8 | 0,9 | 0,7 | 0,6 | - | - | - | - | 0,8 | - | - | - | - | - | *4* |
| | 0-0,3 | 0-0,5 | 0-0,3 | 0,2-1,4 | - | - | - | - | 0-0,6 | - | - | 0-0,5 | - | - | *6* |
| **Salami con pimienta** | - | 1,5 | - | 12,3 | - | - | - | - | - | - | - | - | - | - | *12* |
| **"Sucuk"** | 0-0,9 | 0,1-1,4 | 0-1 | 0,2-2,7 | - | - | - | - | 0-1,7 | - | - | 0-0,1 | - | - | *6* |
| **Emb cur-mad** | 0-5 | 1,3-2 | 0 | 3,4-4,1 | - | - | - | - | 0,2-1,5 | - | 1,2-6,5 | 4,1-34,8 | - | - | *7* |
| | 2,2-4,1 | 0-2,6 | 0-3,6 | 0-2,2 | - | - | - | - | 0-3,3 | - | 0 | 0-1 | - | - | *8* |
| | >0,8 | 0,3 | - | - | - | - | - | - | - | - | - | - | - | - | *12* |
| **Chorizo** | - | 0,8 | 0 | - | - | - | - | - | - | - | - | - | - | - | *12* |

**Tabla 3. Continuación.**

| Producto cárnico curado | NDMA | NPYR | NDEA | NPIP | NHPRO | NTCA | HMNTCA | NPRO | NDBA | NSAR | NMEA | NDPA | NMTCA | NMA | [1]Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Embutidos curados cocidos** | | | | | | | | | | | | | | | |
| **"Frankfurt"** | 0,2 | 0,5 | 0 | 0 | - | - | - | - | 0 | - | - | - | - | - | *4* |
| | 2-2,2 | 0-2,2 | 0 | 1,4-2,3 | - | - | - | - | 0 | - | 0 | 0-1,9 | - | - | *8* |
| **Bologna** | 0-0,7 | 0,7-0,8 | 0 | 2,4-1,5 | - | - | - | - | 0,3-0,6 | - | 0 | 5,5-5,6 | - | - | *7* |
| **Mortadela** | 1,5-3,4 | 1,4-1,6 | 0 | 0,7-0,8 | - | - | - | - | 0-2,2 | - | 0 | 0-1,2 | - | - | *8* |
| **Jamón** | | | | | | | | | | | | | | | |
| **Jamón curado** | 4-6,6 | 2,7-3,1 | 0 | 1,8-2 | - | - | - | - | 0 | - | 0,7-1,4 | 5,8-6,7 | - | - | *7* |
| | 2 | 2,9 | 0 | 1,8 | - | - | - | - | 0 | - | 2,5 | 0 | - | - | *8* |
| **Jamón curado ahumado** | 0-4,8 | - | 0-12 | 0-1,3 | - | - | - | - | 0-1,1 | - | - | - | - | - | *5* |
| | 0 | 1 | 0 | 0 | 9,4 | 171 | - | 5,5 | 0 | 0 | 0 | 0 | 22 | 0 | *3* |
| **Jamón cocido** | 2,6 | 3,4 | 2,4 | 1,9 | - | - | - | - | 0 | - | 0 | 0 | - | - | *8* |

NDMA: N-nitrosodimetilamina; NPYR: N-nitrosopirrolidina; NDEA: N-nitrosodietilamina; NPIP: N-nitrosopiperidina; NHPRO: N-nitrosohidroxiprolina; NTCA: Ácido N-nitrosothiazolidina-4-carboxilico; HMNTCA: Ácido Ácido 2-(hidroximetil)-3-nitrosothiazolidina-4-carboxilico; NPRO: N-nitrosoprolina; NDBA: N-nitrosodi-n-butilamina; NSAR: N-nitrososarcosina; NMEA: N-nitrosometiletilamina; NDPA: N-nitrosodipropilamina; NMTCA: Ácido N-nitroso-2-metilthiazolidina-4-carboxilico; NMA: N-nitrosometilanilina. [1]Referencias: **1** (Bara et al., 2011), **2** (Massey et al., 1991), **3** (Herrmann et al., 2014), **4** (Yurchenko & Mölder, 2007), **5** (Mavelle et al., 1991), **6** (Ozel et al., 2010), **7** (Scheeren et al., 2015), **8** (Campillo et al., 2011), **9** (Canas et al., 1986), **10** (Miller et al., 1989), **11** (Österdahl & Alriksson, 1990), **12** (de Mey et al., 2014). [2]-: no analizado.

Los contenidos recogidos en cada producto son muy variables debido a que los productos analizados son muy heterogéneos, y a que la producción de N-nitrosaminas en ellos depende de diversos factores. Las N-nitrosaminas más frecuentemente detectadas y cuantificadas en productos cárnicos curados son: N-nitrosodimetilamina (NDMA), N-nitrosopirrolidina (NPYR), N-nitrosodietilamina (NDEA), N-nitrosopiperidina (NPIP), N-nitrosodi-n-butilamina (NDBA) y N-nitrosodipropilamina (NDPA) (Tabla 3).

Las N-nitrosaminas volátiles son las más importantes desde el punto de vista tóxico y por ello son las más controladas (Tabla 3, Herrmann, 2014). Dentro de este grupo, la NDMA, NPYR y NPIP son las encontradas y analizadas más frecuentemente. Por ello, la figura 5 representa las cantidades máximas de NDMA, NPYR y NPIP detectadas en los diferentes productos cárnicos recogidos en la tabla 3 y clasificados en función de su procesado, con el fin de comparar su efecto en la generación de N-nitrosaminas.

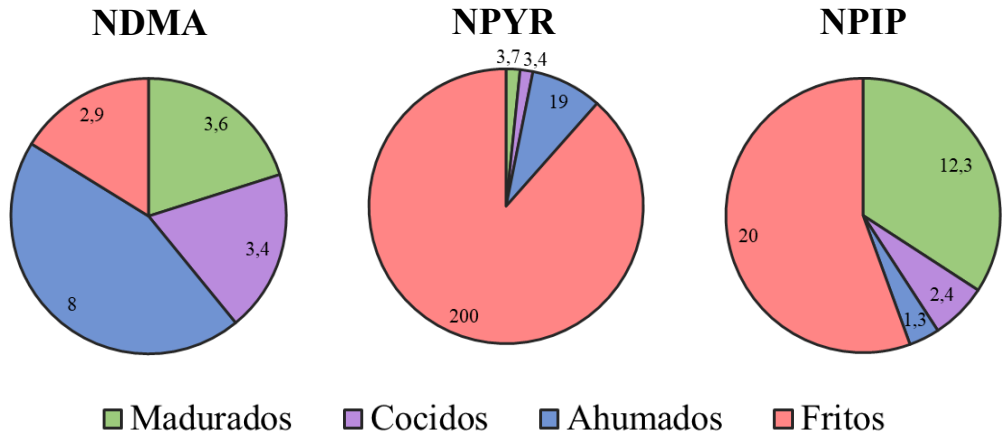



**Figura 5. Contenido máximo de NDMA, NPYR y NPIP (µg/kg) detectados en productos cárnicos curados.**

En la figura 5 se observa que en los productos cárnicos curados ahumados se ha detectado un mayor contenido de NDMA. En este grupo se han incluido los resultados de estudios obtenidos en jamón curado ahumado y en bacon ahumado (Tabla 3), siendo el bacon ahumado en el que se ha encontrado mayor contenido (hasta 8 µg/kg, Massey et al., 1991). En cuanto a NPYR y NPIP, los productos en los que se han detectado mayor contenido son los tratados mediante fritura. En este grupo se incluyen los resultados de estudios obtenidos

en bacon frito (Tabla 3), siendo Bara et al. (2011) los que han encontrado mayor contenido de estas N-nitrosaminas (hasta 200 y 20 μg/kg de NPYR y NPIP, respectivamente). En los productos madurados y cocidos se han detectado menores cantidades de NDMA, NPYR y NPIP, sin llegar a los 4 μg/kg en ninguno de los productos incluidos en cada grupo (Figura 5).

Como se ha mencionado anteriormente, son varios los factores que intervienen en el desarrollo de N-nitrosaminas durante el procesado de productos cárnicos, siendo el más importante el tratamiento térmico aplicado (De Mey, De Maere, Paelinck & Fraeye, 2017). Sin embargo, no todos los tratamientos térmicos actúan de la misma manera, siendo la fritura el que mayor producción de N-nitrosaminas genera (Figura 5, Yurchenko & Mölder, 2007). Los productos crudos que se someten a procesos de secado (sin tratamiento térmico), como los embutidos curado-madurados, no presentan una producción significativa de N-nitrosaminas, aunque el tratamiento que posteriormente se le realice podría desencadenar en un aumento de su producción. La cocción con agua o microondas no parece modificar el contenido de N-nitrosaminas en embutidos curado-madurados con respecto a la carne fresca, lo contrario que la fritura que aumenta su generación (Li, Wang, Xu & Zhou, 2012).

Además del tratamiento térmico, se ha comprobado la intervención de otros factores en la formación de N-nitrosaminas (De Mey et al., 2017). La composición del producto es importante en cuanto a la disponibilidad de los precursores necesarios. En este sentido, la presencia de aminoácidos (aminas secundarias), aminas biógenas y nitrificantes está relacionada con la formación de N-nitrosaminas. El uso de especias en estos productos, comúnmente utilizados en embutidos curado-madurados como el chorizo o salchichón, puede proporcionar aminas secundarias acelerando la formación de N-nitrosaminas. El ejemplo más ampliamente conocido es el contenido en pirrolidina y piperidina en la pimienta negra y su relación con la formación de NPYR y NPIP (Nakamura, Katoh & Kawabata, 1981; De Mey et al., 2014).

### 3.5. Tendencias actuales y futuras

Debido a la problemática expuesta en el apartado 3.4 en el uso de nitrificantes por la posible formación de N-nitrosaminas, la Unión Europea ha establecido la necesidad de reevaluar el uso de nitratos y nitritos en productos cárnicos curados. En este sentido, en 2010 se inicia

un programa para la reevaluación de algunos aditivos alimentarios (DOUE, 2010) que aparecen en el Reglamento de la Unión Europea 1333/2008 sobre aditivos alimentarios (DOUE, 2008), entre los que figuran los nitratos y nitritos.

### 3.5.1. Reducción de los límites máximos de adición de nitrificantes

En la reevaluación de los nitrificantes como aditivos de productos cárnicos curados, uno de los puntos clave es el conocimiento de las consecuencias producidas por la disminución de la cantidad inicial añadida de nitrificantes en las propiedades fisicoquímicas, seguridad microbiológica y características sensoriales. En concreto, en el caso de los embutidos curado-madurados, se han realizado pocos estudios con este objetivo. Las consecuencias microbiológicas y fisicoquímicas de la reducción de la cantidad añadida, hasta un 50% de nitrato y nitrito, han sido estudiadas por Hospital et al. (2012, 2014, 2015, 2016). Christieans et al. (2018) han analizado los efectos de la reducción de hasta un 47% de la cantidad añadida de nitrificantes (nitrato o mezclas de nitrato y nitrito) en las propiedades fisicoquímicas, además de su estabilidad microbiológica como resultado de esta reducción. Por otra parte, en otro producto cárnico de gran consumo, como el jamón curado, también se han estudiado los cambios fisicoquímicos producidos por la eliminación de la adición de estos aditivos (Gratacós-Cubarśi et al., 2013).

#### *3.5.1.1. Consecuencias en las propiedades fisicoquímicas*

En cuanto a las consecuencias fisicoquímicas, la reducción de hasta un 50% de nitrificantes (mezcla de nitrito y nitrato) no afecta a la bajada de la actividad de agua y del pH, como factores esenciales para la seguridad microbiológica en embutidos curado-madurados (Hospital et al., 2015, 2014, 2016; Christieans et al., 2018), lo que se observa incluso en ausencia de nitrificantes (Hospital et al., 2014, 2015, 2016). El pH tampoco se ve afectado por la eliminación total de la adición de nitratos y nitritos en jamón curado (Gratacós-Cubarśi et al., 2013). Sin embargo, la ausencia de nitrificantes produce un aumento en la oxidación lipídica del jamón curado (Gratacós-Cubarśi et al., 2013), que se explica por la acción antioxidante del nitrito mencionada anteriormente (Apartado 3.1.2).

#### *3.5.1.2. Consecuencias en la seguridad microbiológica*

En cuanto a la seguridad microbiológica, los estudios realizados en embutidos curado-madurados demuestran que la reducción de hasta un 50% de nitrificantes no modifica el crecimiento de las bacterias ácido-lácticas (Hospital et al., 2015, 2016; Christieans et al., 2018). Ésta sería la razón por la que el pH tampoco se ve afectado. Por un lado, Christieans et al. (2018) no observan efecto en el crecimiento de cocos Gram positivos catalasa positivos. Sin embargo, Hospital et al. (2012, 2014, 2015) si detectan un aumento en el crecimiento de estos microorganismos como consecuencia de la reducción de nitrificantes (50 y 100%). Estos resultados se explican por la actividad antimicrobiana del nitrito (Apartado 3.1.1). En este sentido, la estabilidad microbiológica de estos productos puede verse comprometida por la reducción de la cantidad añadida en un 50% de nitrato y nitrito, considerando al nitrito como una barrera esencial en el control de *Salmonella typhimurium*, *Listeria innocua* y Enterobacteriaceae, y en el caso de las dos últimas especies con una reducción del 25% (Hospital et al., 2014, 2015). Sin embargo, Christieans et al. (2018) observan que dicha estabilidad no se ve afectada por la reducción de hasta un 47% de la adición de nitrato y nitrito, en lo que respecta a *Listeria monocytogenes* y *Salmonella typhimurium*, aunque sí considera que la presencia de nitrito en la mezcla de sales nitrificantes es esencial para la estabilidad microbiológica de los embutidos curado-madurados. En cuanto a *Clostridium botulinum*, como principal bacteria patógena objetivo del nitrito en productos cárnicos curados (Apartado 3.1.1), no se ha podido detectar la presencia de clostridios sulfito-reductores en estos productos con reducción de nitratos y nitritos, e incluso en ausencia de los mismos (Hospital et al., 2015). Tampoco se ha detectado la presencia de la toxina botulínica como producto de las esporas de *Clostridium botulinum* inoculadas en embutidos curado-madurados sin adición de nitrificantes (Hospital, Hierro, Stringer & Fernández, 2016). Por lo tanto, parece que la combinación de pH, actividad de agua, temperatura y la presencia de bacterias competidoras como factores de inhibición, son suficientes para impedir el crecimiento de *Clostridium botulinum* y la formación de su toxina (Leistner, 2000).

#### *3.5.1.3. Consecuencias en las características sensoriales*

En cuanto al efecto sensorial que produce la reducción o eliminación de la adición de nitrito/nitrato en los productos cárnicos, éste ha sido escasamente evaluado. Previamente, se ha indicado que la reducción de nitrificantes puede alterar la producción de compuestos volátiles en productos cárnicos curados y, como consecuencia, su aroma final. En este sentido, Hospital et al. (2012, 2015) han observado que, con un 25% de reducción de nitrato y nitrito añadido inicialmente a embutidos curado-madurados, se incrementa la producción de compuestos volátiles derivados de la degradación de aminoácidos y de la fermentación de carbohidratos llevados a cabo por microorganismos. Tal incremento se relaciona con el aumento en el crecimiento también observado de los cocos Gram positivos catalasa positivos, responsables de la producción de parte de dichos compuestos volátiles. La variación en el estado de oxidación lipídica también puede modificar los compuestos volátiles producidos en los productos curado-madurados. Así, se ha observado un aumento en la producción de compuestos volátiles derivados de la oxidación lipídica, como consecuencia de la eliminación de nitrificantes en embutidos curado-madurados (Hospital, Hierro & Fernández, 2012) y en jamón curado (Gratacós-Cubarśi et al., 2013). Estos cambios en los compuestos volátiles producidos por la reducción de nitrificantes en embutidos curado-madurados (Hospital et al., 2012, 2015) tienen repercusión en el aroma de estos productos. Teniendo en cuenta los compuestos volátiles con propiedades aromáticas encontrados en los embutidos (Tabla 1), Hospital et al. (2012, 2015) han observarado un cambio en los siguientes compuestos volátiles como consecuencia de tal reducción: disminución del alcohol 2-pentenol y del acetato de etilo, y aumento en la producción de ácidos acético, propanoico, 2- y 3-metilbutanoico y 2-metilpropanoico; de las cetonas 2-heptanona, 2-nonanona y 2,3-butanediona; de los alcoholes 2-feniletanol, 1-octen-3-ol y 1-pentanol; de los furanos 2-etilfurano y 2-pentilfurano; y de los aldehídos hexanal, 2-hexenal, heptanal, 2-heptenal, octanal, 2-octenal, nonanal 3-metilbutanal y benzenoacetaldehído. A pesar de los cambios encontrados en compuestos volátiles estos autores no demuestran el impacto sensorial producido en el aroma.

Como conclusión, la reducción o eliminación de nitrificantes en la elaboración de productos curado-madurados puede afectar principalmente a su estabilidad microbiológica y al nivel

de oxidación del producto y, como consecuencia, provocar variaciones en su aceptabilidad por afectar a la producción de compuestos volátiles que intervienen en el perfil aromático. Sin embargo, debido a resultados contradictorios entre los estudios presentados y a la escasez de los mismos, es necesario profundizar en estudios centrados en el aspecto microbiológico, haciendo hincapié en el crecimiento de *Clostridium botulinum* y la producción de su toxina (Lee et al., 2018), así como en el aspecto sensorial, ya que el papel de los nitrificantes es crucial en el desarrollo del color y aroma en productos cárnicos curados (Apartados 3.1.3 y 3.1.4).

### 3.5.2. Sustitutos alternativos

Otro de los puntos clave en el estudio de los nitrificantes como aditivos de productos cárnicos curados, es la búsqueda de alternativas que sustituyan parcial o totalmente su uso (Gassara, Kouassi, Brar & Belkacemi, 2016). Alahakoon et al. (2015) hicieron una revisión bibliográfica hasta el año 2015 sobre los posibles sustitutos del uso de nitrificantes en productos cárnicos curados.

#### *3.5.2.1. Extractos vegetales*

En la revisión nombrada anteriormente (Alahakoon et al., 2015), las alternativas basadas en plantas han sido las más ampliamente estudiadas. Se han estudiado extractos de plantas o vegetales en general, tales como extractos de apio, acelgas, espinacas, tomate, arándanos, ajo, romero o tomillo entre otros, en forma de extractos en polvo, en zumos o sus aceites esenciales. Es importante recalcar que en algunos casos su uso implica la adición de nitratos de manera indirecta, ya que algunos extractos poseen concentraciones de nitrato incluso superiores a 2500 mg/kg. En esta revisión se recogen una gran variedad de estudios, en los que el uso de estos extractos naturales aporta funciones en la elaboración de productos cárnicos curados con una reducción total o parcial de nitrificantes. Una de estas funciones es su alta actividad antioxidante debido a su contenido en compuestos fenólicos y ácidos orgánicos principalmente, como se ha reportado en estudios sobre extractos de tomate, ajo, romero y albedo de limón, entre otros. Otra de las funciones llevadas a cabo por estos extractos naturales es su actividad antimicrobiana. Se ha reportado esta actividad frente a *Listeria monocytogenes* y *Clostridium botulinum* por parte de extractos de arándano, clavo, nuez moscada y orégano. Además, algunos extractos naturales como el de tomate pueden

aportar una mejoría en el desarrollo del color por presentar pigmentos en su composición, además de mejorar el aspecto aromático. Sin embargo, su uso también presenta efectos negativos en la elaboración de productos con reducción o eliminación de nitrificantes. El uso de extractos vegetales a altas concentraciones puede deteriorar la calidad organoléptica a nivel de sabor y desarrollo de olores anómalos.

Los estudios recogidos por Alahakoon et al. (2015) utilizan estas alternativas al nitrito en productos cárnicos curados elaborados mediante tratamiento térmico. Desde el 2015 hasta la actualidad se ha seguido estudiando el uso de extractos naturales como sustitutos de nitrificantes en productos cárnicos curados (Riazi, Zeynali, Hoseini, Behmadi & Savadkoohi, 2016; Wójciak & Dolatowski, 2016; Riel, Boulaaba, Popp & Klein, 2017; Aquilani et al., 2018; Riyad, Ismail & Abdel-Aziz, 2018; Sucu & Turp, 2018; Jin, Choi, Yang, Park & Yim, 2018). Al igual que en Alahakoon et al. (2015), casi la totalidad de estos estudios se han realizado en embutidos curados con tratamiento térmico, principalmente por cocción, a excepción de Aquilani et al. (2018) y Sucu & Turp (2018). Aquilani et al. (2018) estudiaron el uso de extractos de semilla de uva y de castaña como sustitutos de nitratos y nitritos en embutidos curado-madurados, sin resultados totalmente satisfactorios por una disminución en la aceptación general del producto. Por su parte, Sucu & Turp (2018) estudiaron el uso del perejil en polvo como sustituto parcial y total de nitratos y nitritos en "sucuk", un embutido curado-madurado de ternera típico de Turquía. El mayor interés por utilizar estas alternativas en productos cárnicos curados cocidos es porque existe un mayor consumo de éstos a nivel internacional que de embutidos curado-madurados, que se consumen en mayor proporción en Europa.

#### *3.5.2.2. Ácidos orgánicos y bacteriocinas*

Alahakoon et al. (2015) recoge estudios donde el lactato, benzoato y sorbato son posibles alternativas al empleo de nitrificantes como conservantes por su actividad antioxidante y antibacteriana en productos cárnicos curados como el bacon, mortadela o salchichas "Frankfurt".

Alahakoon et al. (2015) también tiene en cuenta el uso de bacteriocinas, como la nisina o enterocinas, para ejercer una amplia protección contra microorganismos patógenos. En esta revisión, se recogen estudios donde las enterocinas disminuyen el crecimiento de *Listeria*

*monocytogenes* en embutidos curado-madurados tipo fuet y salami. Sin embargo, solo se contempla el efecto del uso de nisina en carne fresca y salchichas "Frankfurt" como sustitución de parte de los nitrificantes utilizados, mejorando la inhibición de la producción de esporas de *Clostridium perfringens* y *Clostridium esporogenes*, siendo necesario el empleo de grandes cantidades de nisina.

#### *3.5.2.3. Microorganismos*

Otra alternativa poco estudiada al uso de nitrificantes en productos cárnicos, es el uso de microorganismos. Los microorganismos más utilizados en este sentido en embutidos curado-madurados han sido bacterias ácido-lácticas (Kawahara, Nakamura, Sakagami & Suzuki, 2006; Zhang, Kong & Xiong, 2007; Cenci-Goga et al., 2012, 2016, 2018; Yu, Wu & Zhang, 2015; Nikodinoska et al., 2019; Zhu et al., 2019). Muchas de estas bacterias han demostrado ejercer efecto antimicrobiano frente a patógenos en este tipo de productos. Sin embargo, se han reportado casos en los que no se ha encontrado este efecto. Por ejemplo, Nikodinoska et al. (2019) han observado que la inoculación de *L. plantarum* en embutidos curado-madurados tipo chorizo no es efectiva frente a *Salmonella* spp, y el uso de *L. delbrueckii* tampoco lo es frente a este patógeno ni frente a *L. monocytogenes*. Por lo tanto, se necesitan estudios más profundos para demostrar la estabilidad microbiológica que estos microorganismos pueden aportar a los embutidos curado-madurados con reducción o sustitución de nitrificantes, especialmente frente a *Clostridium botulinum*. En el aspecto sensorial, se ha estudiado la aportación de estas bacterias ácido-lácicas a la coloración de embutidos curado-madurados (Zhang et al., 2007; Zhu et al., 2019). Sin embargo, es necesario determinar si la formación del color por parte de estas bacterias es estable en el tiempo, además de profundizar en otros aspectos sensoriales importantes como es el aroma.

El uso de hongos o levaduras puede ser una alternativa interesante para reemplazar el empleo de nitratos y nitritos en productos cárnicos curados, entre ellos en embutidos curado-madurados. Sin embargo, esta alternativa ha sido escasamente estudiada. Yu et al. (2015) proponen la inoculación del hongo *Monascus* como sustituto de nitrificantes en carne fermentada, por su capacidad de formación de color y de reducción de la oxidación lipídica. Este hongo ha demostrado tener otras propiedades beneficiosas, como efecto antimicrobiano y anticancerígeno, entre otras. Sin embargo, su uso conlleva riesgos asociados debido a la

formación de la micotoxina citrina (Agboyibor, Kong, Chen, Zhang & Niu, 2018). Por lo tanto, se necesitarían más estudios sobre el uso de este hongo en productos cárnicos curados, sobre su control microbiológico, su aportación al aspecto sensorial y en el riesgo de su uso por la formación de micotoxinas. A pesar de ello, existen otros hongos y levaduras que ejercen efectos beneficiosos en productos cárnicos (Flores et al., 2015), lo que abriría una ventana nueva en la reducción de la adición de nitrificantes en productos cárnicos curados.

## 4. *DEBARYOMYCES HANSENII* COMO CULTIVO INICIADOR EN PRODUCTOS CÁRNICOS CURADO-MADURADOS

Los productos cárnicos curados poseen un microbiota compleja formada por diferentes microorganismos. De entre estos microorganismos, destacan por su funcionabilidad las bacterias ácido-lácticas y los cocos Gram positivos coagulasa negativos. Éstas bacterias han sido ampliamente estudiadas, tanto los géneros encontrados en estos productos como su aportación tecnológica a la elaboración de los mismos. Sin embargo, en este ecosistema complejo también se encuentran otros tipos de microorganismos como las levaduras, las cuales han sido menos estudiadas que las bacterias anteriormente mencionadas, debido a las dificultades asociadas a su aislamiento e identificación además de las continuas modificaciones en su taxonomía (Selgas & García, 2015). Mediante métodos tradicionales (cultivo en placa) y métodos moleculares (independientes de cultivo), se han identificado diferentes géneros de levaduras en productos cárnicos, entre las que se encuentran *Debaryomyces*, *Candida*, *Yarrowia*, *Pichia*, *Rhodotorula*, *Cryptococcus* y *Trichosporon* (Mendoza, Padilla, Belloch & Vignolo, 2004; Cocolin, Urso, Rantsiou, Cantoni & Comi, 2006; Asefa et al., 2009). La población de levaduras en estos productos varía en función de la etapa de su procesado. Sin embargo, se ha observado que la levadura más frecuente y dominante durante todo el proceso de fermentación y maduración es *Debaryomyces hansenii*, tanto en jamón curado (Andrade, Rodríguez, Casado, Bermúdez & Córdoba, 2009), como en embutidos curado-madurados procedentes de España e Italia (Encinas, López-Díaz, García-López Otero, & Moreno, 2000; Cocolin et al., 2006; Aquilanti et al., 2007), embutidos tradicionales "Sucuk" de Turquía (Ozturk & Sagdic, 2014), y en productos cárnicos curados a los que se les ha aplicado una etapa de ahumado (Asefa et al., 2009). Esta levadura se lleva utilizando como cultivo iniciador en embutidos curado-madurados desde

los años 70 (Flores et al., 2015), debido a su dominancia en estos productos junto a las posibles aportaciones funcionales que se describen en los siguientes apartados.

### 4.1. *Debaryomyces hansenii*

El género *Debaryomyces* pertenece a la familia *Saccharomycetaceae.* La especie *Debaryomyces hansenii* es un hemiascomiceto normalmente haploide, su reproducción es principalmente asexual por gemación multilateral y su reproducción sexual es por conjugación entre la célula madre y la gema. Esta levadura presenta un metabolismo aerobio con una capacidad de fermentación débil. Puede metabolizar diferentes azúcares (como la glucosa, sacarosa, galactosa, lactosa, almidón soluble, además de etanol, glicerol, succinato, D-L-lactato, y citrato) y asimilar diferentes fuentes de nitrógeno (como el sulfato amónico, urea, nitrito, bases púricas y aminoácidos) (Breuer & Harms, 2006; Al-Qaysi, Al-Haideri, Thabit, Al-Kubaisy & Ibrahim, 2017). En su taxonomía, se diferencian dos variedades: *hansenii* y *fabryii*, diferenciadas principalmente por su temperatura óptima de crecimiento, siendo 31-35 °C y 36-39 °C respectivamente (Breuer & Harms, 2006). *Debaryomyces hansenii* es una especie muy heterogénea, debido a que se encuentran bastantes diferencias entre sus cepas en cuanto a la capacidad de asimilar y fermentar varias fuentes de carbono, en la expresión de diferentes actividades lipasa y proteasa, y en las condiciones óptimas de crecimiento, entre otras. Por lo tanto, las aportaciones funcionales llevadas a cabo por esta levadura variarán en función de la o las cepas presentes en el producto.

#### 4.1.1. Características específicas de *D. hansenii*

De manera general, las cepas de *D. hansenii* poseen características que contribuyen a su alto interés tecnológico en la elaboración de embutidos curado-madurados.

*D. hansenii* es capaz de crecer en condiciones ambientales extremas, tanto de pH (Masoud & Jakobsen, 2005; Al-Qaysi et al., 2017; Ramos, Melero, Ramos-Moreno, Michán & Cabezas, 2017) como de NaCl (Gori, Mortensen, Ameborg & Jespersen, 2005; Prista, Michan, Miranda & Ramos, 2016; Ramos et al., 2017), lo que le permite crecer en alimentos con alto contenido en sal como es el caso de los embutidos curado-madurados.

*D. hansenii* también posee cierta actividad antimicrobiana frente a otros hongos. Se ha observado que es capaz de disminuir el crecimiento de otras levaduras y hongos patógenos

además de la formación de sus toxinas (Andrade, Thorsen, Rodríguez, Córdoba & Jespersen, 2014; Núñez et al., 2015; Peromingo, Núñez, Rodríguez, Alía & Andrade, 2018; Peromingo, Andrade, Delgado, Sánchez-Montero & Núñez, 2019).

Por último, su metabolismo es capaz de modificar el perfil aromático de los productos cárnicos, como se muestra en el siguiente apartado.

#### 4.1.2. Efecto de *D. hansenii* en las características sensoriales en productos cárnicos curados

La dominancia de *D. hansenii* en productos curados, como los embutidos curado-madurados, así como sus características específicas, han favorecido el estudio del efecto de su inoculación en los cambios sensoriales en estos productos. En la tabla 4 se resumen los resultados obtenidos de los estudios sobre las aportaciones sensoriales y la producción de compuestos volátiles de interés por parte de *D. hansenii* en embutidos curado-madurados. Sensorialmente se ha observado que, de manera general, la inoculación de *D. hansenii* mejora el aroma del producto por un aumento en las notas frutales y dulces y una disminución de las notas rancias, además de mejorar su color. Estos efectos acrecientan la calidad global del embutido mejorando su apariencia y aumentando su aceptabilidad. Sin embargo, hay autores que muestran que la inoculación de *D. hansenii* en embutidos curado-madurados no produce cambios sensoriales generales (Olesen & Stahnke, 2000; Selgas, Ros & García, 2003), ni en rasgos individuales importantes tales como en aroma, gusto, terneza o jugosidad del embutido (Iucci et al., 2007; Cano-García, Belloch & Flores, 2014a), aunque sí mejora la aceptabilidad general del producto.

**Tabla 4. Efecto de la inoculación de *D. hansenii* en las características sensoriales y en los compuestos volátiles producidos de embutidos curado-madurados.**

| Efecto sensorial | Cambios en compuestos volátiles | Referencia |
|---|---|---|
| Aumenta:<br>Aroma frutal, dulce y queso.<br>Disminuye:<br>Aroma rancio y ácido.<br>Metional y 2-acetil-1-pirrolina. | - | Corral et al. (2018) |
| Aumenta:<br>Aroma ácido, frutal y dulce.<br>Disminuye:<br>Aroma rancio.<br>Enmascara el olor sexual ("boar traint"). | Aumenta:<br>Ésteres, alcoholes, azufrados, aldehídos ramificados.<br>Disminuye:<br>Cetonas, aldehídos lineales, furanos, 2-acetyl-1-pyrroline. | Corral et al. (2017) |
| - | Aumenta:<br>Ésteres y volátiles de degradación de aminoácidos.<br>Disminuye:<br>Volátiles de oxidación lipídica. | Corral et al. (2015) |
| Mejora:<br>Apariencia general.<br>No cambia:<br>Rasgos individuales: aroma, gusto, terneza, jugosidad y calidad general. | Aumenta:<br>*Cepa 1:* Ácidos, cetonas y volátiles de fermentación de carbohidratos.<br>*Cepa 2:* Volátiles de fermentación de carbohidratos, ésteres y volátiles de oxidación lipídica (aldehídos, alcanos)<br>Disminuye:<br>*Cepa 1:* Azufrados, alcoholes y volátiles de oxidación lipídica (aldehídos y alcanos). | Cano-García et al. (2014a) |
| Mejora:<br>Aceptabilidad general.<br>Disminuye:<br>Dureza.<br>No cambia:<br>Elasticidad y cohesividad. | - | Cano-García et al. (2014b) |
| Aumenta:<br>Aceptabilidad general.<br>No cambia:<br>Rasgos individuales: gusto, color y rancidez. | Aumenta:<br>Azufrados y alcoholes.<br>Disminuyen:<br>Aldehídos. | Iucci et al. (2007) |
| Mejora:<br>Color, aroma, gusto, calidad global. | Aumenta:<br>Volátiles de oxidación lipídica, degradación de aminoácidos, fermentación de carbohidratos, ésteres y ácidos. | Bolumar et al. (2006) |

**Tabla 4. Continuación**.

| Efecto sensorial | Cambios en compuestos volátiles | Referencia |
|---|---|---|
| Aumenta:<br>Preferencia general. | Aumenta:<br>Ácidos y ésteres.<br>Disminuye:<br>Aldehídos y volátiles de oxidación lipídica. | Flores et al. (2004) |
| No hay cambios | - | Selgas et al. (2003) |
| No hay cambios | No hay cambios | Olesen & Stahnke (2000) |

Los cambios sensoriales en el aroma vienen dados por cambios en los compuestos volátiles producidos en el embutido curado-madurado tras la inoculación de *D. hansenii*. Alguno de los cambios importantes (Tabla 4) son el aumento de la producción de ésteres y la disminución de los aldehídos lineales, estos últimos procedentes de la oxidación lipídica. Al igual que en los rasgos sensoriales, las cepas estudiadas por los diferentes autores muestran variabilidad en la producción de estos compuestos. En este sentido, la inoculación de las cepas estudiadas por Andrade, Córdoba, Casado, Córdoba & Rodríguez (2010) y Bolumar et al. (2006), además de una de las cepas estudiadas por Cano-García et al. (2014a), muestran un aumento en la producción de volátiles procedentes de la oxidación lipídica. Además de estos cambios, la inoculación de esta levadura también causa un aumento en los volátiles procedentes de la degradación de aminoácidos y de la fermentación de carbohidratos. Sin embargo, hay pocos estudios centrados en los cambios producidos por *D. hansenii* en los compuestos volátiles azufrados de productos cárnicos curados, procedentes principalmente de la degradación de aminoácidos azufrados. Esto es debido a que éstos volátiles son difíciles de analizar por su baja concentración y alta volatilidad y reactividad (Corral et al., 2016), aunque su impacto aromático en los productos cárnicos es muy elevado. Corral, Belloch, López-Díez, Salvador & Flores (2017) e Iucci et al. (2007) han observado que la inoculación de *D. hansenii* aumenta la producción de estos compuestos, lo contrario que una de las dos cepas de *D. hansenii* estudiadas por Cano-García et al. (2014a).

La gran producción de ésteres por parte de *D. hansenii* demuestra su alta capacidad de esterificación que, junto a su capacidad antioxidante y de degradación de aminoácidos, constituyen los principales mecanismos mediante los cuales la inoculación de *D. hansenii*

mejora el aroma en embutidos curado-madurados. Además de la capacidad de producción de compuestos aromáticos de interés, se ha demostrado que *D. hansenii* tiene la capacidad de producir precursores (aminoácidos y ácidos grasos libres) de estos volátiles a partir de proteínas y lípidos gracias a su actividad proteolítica y lipolítica (Sørensen, 1997; Durá et al., 2004; Patrignani et al., 2007), contribuyendo a la mejora sensorial del producto.

### 4.1.3. Rutas metabólicas implicadas en la generación de aromas

Los compuestos volátiles con mayor relevancia aromática generados por *D. hansenii* en embutidos curado-madurados son los ésteres y los procedentes de la degradación de aminoácidos. Las rutas metabólicas de estos compuestos se representan en la figura 6 y se explican a continuación.

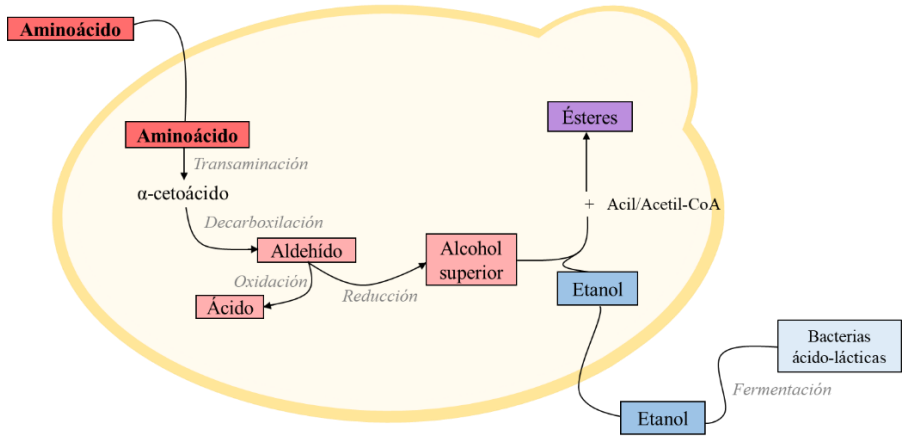
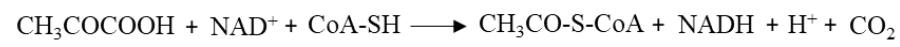



**Figura 6. Metabolismo de formación de volátiles procedentes de la degradación de aminoácidos y ésteres.**

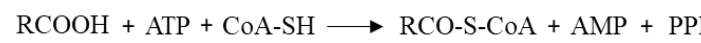
Los ésteres se pueden clasificar en dos grupos: los ésteres de acetato y los ésteres procedentes de ácidos grasos de cadena media (Mason & Dufour, 2000). La inoculación de *D. hansenii* en embutidos curado-madurados aumenta la producción de éstos dos tipos de compuestos, entre los que se encuentran, el acetato de etilo y el acetato de 3-metilbutilo pertenecientes al primer grupo, y el hexanoato de etilo perteneciente al segundo (Flores et al., 2004; Corral, Salvador, Belloch & Flores, 2015). Su síntesis ocurre por condensación de un ácido carboxílico y un alcohol (Figura 7.2). En levaduras, dicha reacción se lleva a cabo mayoritariamente de manera enzimática, siendo catalizada por las enzimas acetil/acil

transferasas. Para que se lleve a cabo tal condensación se requiere energía, en este caso aportada por acetil/acil-CoA procedente de la deshidrogenación del piruvato o del metabolismo de ácidos grasos (Figura 7.1a y b). Una vez formados en el interior celular, éstos salen al exterior debido a su naturaleza lipídica.

**1a Formación de Acetil-CoA. Reacción de deshidrogenación del piruvato.**



**1b Formación de Acil-CoA a partir de ácidos grasos.**



**2 Formación ésteres. Catalizada por alcohol aciltransferasas.**

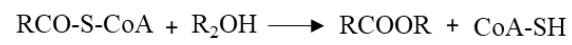


**Figura 7. Formación de ésteres. Adaptación de Saerens, Delvaux, Verstrepen & Thevelein (2010).**

El alcohol utilizado en la formación de ésteres puede ser el etanol o un alcohol superior, éste último solo en el caso de ésteres de acetato (Pires, Teixeira, Brányik & Vicente, 2014). En embutidos curado-madurados, el etanol procede de la fermentación de carbohidratos llevada a cabo principalmente por las bacterias ácido-lácticas. Y los alcoholes superiores que acabarán siendo sustrato en la producción de ésteres, proceden de la degradación de aminoácidos.

La degradación de aminoácidos, por la que se obtienen aromas de la actividad de los microorganismos entre los que se encuentran las levaduras, viene dada por la conocida ruta de "Ehrlich" (Pires et al., 2014; Cordente, Schmidt, Beltran, Torija & Curtin, 2019). Esta ruta se puede clasificar en tres pasos (Figura 6). El primer paso es una transaminación o desaminación del aminoácido objetivo catalizada por transaminasas. En este paso el glutamato y el α-cetoglutarato actúan como dador y aceptor de electrones y se obtiene como producto un α-cetoácido. El segundo paso se lleva a cabo mediante una descarboxilación del α-cetoácido, catalizada por descarboxilasas, obteniendo como producto el correspondiente aldehído. Por último, el tercer paso puede ocurrir por varios caminos, ya que el aldehído producido en el paso anterior puede reducirse a su correspondiente alcohol u oxidarse a su

correspondiente ácido, mediante la acción de alcohol o aldehído deshidrogenasas respectivamente (Hazelwood, Daran, Van Maris, Pronk & Dickinson, 2008).

La ruta de degradación de la metionina representa un caso especial. Además de la ruta mencionada, este aminoácido y su α-cetoácido pueden sufrir una desmetiolización (catalizada por desmetiolasas) y formar el compuesto también aromático metanotiol (Hazelwood et al., 2008). Éste último puede servir de precursor para la generación de otros compuestos aromáticos azufrados de interés (Landaud et al., 2008) (Figura 8).

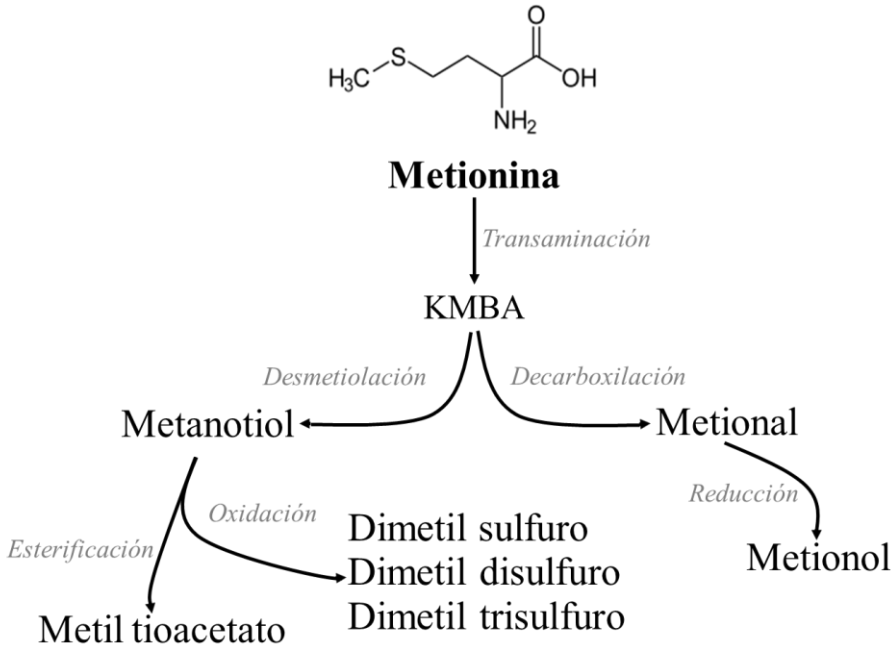



**Figura 8. Ruta de "Ehrlich" de la metionina. KMBA: ácido α-cetometiltiobutanoico.**

### 4.1.4. Genes implicados en la formación de aromas

El estudio de la producción de ésteres se ha realizado principalmente por su interés en la industria cervecera, por lo que los genes involucrados se han estudiado mayoritariamente en *Saccharomyces cerevisiae*. Éstos genes se corresponden con los que codifican las mencionadas alcohol acetil/aciltransferasas. En el caso de los ésteres de acetato, estos genes son ATF1 y ATF2 (Mason & Dufour, 2000; Lilly et al., 2006; Saerens et al., 2010; Pires et

al., 2014) siendo el primero el más importante. Los ésteres procedentes de ácidos grasos de cadena media han sido menos estudiados, debido a que los anteriores son los ésteres con mayor relevancia aromática en la elaboración de cerveza. Sin embargo, se ha confirmado que ATF1 y ATF2 también intervienen en su formación, además de otros genes como EHT1 y EEB1 que codifican dos acil-CoA:etanol O-aciltransferasas (Saerens et al., 2010; Pires et al., 2014).

En el caso de los aromas procedentes de la degradación de aminoácidos, la mayor parte de los trabajos enfocados en la ruta de "Ehrlich" también se han llevado a cabo en *Saccharomyces cerevisiae*. Así, se han observado los siguientes genes involucrados (Hazelwood et al., 2008; Cordente et al., 2019): ARO8, ARO9, BAT1 y BAT2 que codifican aminotransferasas; ARO10, PDC1, PDC5 y PDC6 que codifican descarboxilasas; ADH1 – ADH6 que codifican alcohol deshidrogenasas y ALD1 – ALD6 que codifican aldehído deshidrogenasas.

#### *4.1.4.1. Aromas azufrados*

De entre los compuestos volátiles generados por degradación de aminoácidos, los compuestos azufrados tienen un papel importante en el perfil aromático de embutidos curado-madurados (Corral et al., 2016). El metional es el más comúnmente detectado, el cual aporta aroma a "patata cocida" (Burdok, 2002). Varios autores han observado que diferentes cepas de *D. hansenii* aisladas de quesos madurados son capaces de producir metanotiol, dimetil sulfuro, dimetil disulfuro, dimetil trisulfuro, metionol, metional y metil tioacetato a partir de la metionina como principal aminoácido precursor (Tabla 5).

**Tabla 5. Compuestos volátiles azufrados producidos a partir de metionina por *D. hansenii* aislada de quesos.**

| Compuestos volátiles azufrados | Referencia |
|---|---|
| DMDS, MTA, metionol, metional | López Del Castillo-Lozano et al. (2007) |
| MTL, MTA, DMDS, DMTS, metional | Arfi et al. (2002) |
| MTL, DMDS, DMTS, MTA | Spinnler et al. (2001) |
| MTL, DMS, DMDS, DMTS | Bonnarme et al. (2001) |

MTL: metanotiol; DMS: dimetil sulfuro; DMDS: dimetil disulfuro; DMTS: dimetil trisulfuro; MTA: metil tioacetato.

Sin embargo, no se ha estudiado la capacidad de producción de aromas azufrados por parte de cepas de *D. hansenii* aisladas de embutidos curado-madurados, aunque sería útil debido al interés de estos aromas en estos productos y debido a la heterogeneidad metabólica de esta levadura.

El estudio de los genes implicados en la formación de estos aromas azufrados se ha realizado por su interés en la elaboración de quesos y vino, por lo que se han llevado a cabo principalmente en levaduras presentes en estas matrices (Tabla 6). Estos genes, en los que se ha comprobado su intervención en la producción de aromas azufrados mediante degradación de metionina como principal precursor, son: ARO8, BAT1 y BAT2 que codifican transaminasas y PDC1, PDC5 y PDC6 que codifican descarboxilasas. Los autores indicados en la tabla 6 han encontrado estos genes sobreexpresados tras la adición de metionina en el medio, excepto Perpète et al. (2006) que no observa producción de metionol en cepas mutantes de *Saccharomyces cerevisiae* en las que se han eliminado los genes PDC1, PDC5 y PDC6.

Sin embargo, los genes implicados en la formación de aromas azufrados por parte de *D. hansenii* no han sido estudiados. Aunque sería de gran interés debido a que estos compuestos tienen un papel importante en el perfil aromático de embutidos curado-madurados.

**Tabla 6. Genes que intervienen en la producción de aromas azufrados a partir de metionina.**

| Genes | Levadura | Referencia |
|---|---|---|
| *BAT1* | *Yarrowia lipolytica* | Hébert et al. (2013) |
| *BAT2, ARO8-03* | *Geotrichum candidum* | Pracharova et al. (2019) |
| *ARO8* | *Kluyveromyces lactis* | Hébert et al. (2011) |
| *ARO8, PDC1, PDC6* | *Yarrowia lipolytica* | Cholet (2008) |
| *PDC1, PDC5, PDC6* | *Saccharomyces cerevisiae* | Perpète et al. (2006) |

### 4.1.5. Técnicas de análisis transcriptómico

Para el estudio de los genes responsables de la producción de volátiles azufrados por *D. hansenii*, una de las maneras más utilizadas es el estudio de su transcriptoma. El

transcriptoma es el resultado de la transcripción del genoma, es decir, el conjunto de moléculas de ARN mensajero (ARNm) y de ARN no codificante presente en una célula. De esta manera, obtendremos información de los genes que el organismo expresa y que son responsables de su fenotipo. Existen diversas técnicas para el análisis del transcriptoma, que pueden clasificarse en función de si el análisis se realiza de todo o de gran parte del ARNm (análisis no dirigido) o si por el contrario se focaliza a uno o unos pocos genes concretos (secuencias de ARNm) (análisis dirigido).

#### *4.1.5.1. Análisis no dirigido*

Dentro de los análisis no dirigidos podemos encontrar el EST (Expressed Sequence Tag) collection, SAGE (Serial Analysis of Gene Expresion), CAGE (Cap Analysis of Gene Expression), microarrays o DNA chips y RNA-seq, entre otros. Los más utilizados son los microarrays y el RNA-seq (Ness, 2006; Wolf, 2013). Los microarrays o DNA chips se basan en un soporte sólido con moléculas de ADN concretas a las que se une el ARN extraído de la muestra. La intensidad de hibridación se corresponde con el nivel de expresión de los genes. Por su parte, el RNA-seq se basa en la secuenciación completa del ARN extraído del organismo objetivo.

#### *4.1.5.2. Análisis dirigido*

Sin embargo, cuando se tiene conocimiento o sospecha de qué genes pueden ser los responsables del fenotipo a estudiar, el uso de análisis del transcriptoma dirigido puede ser una buena opción. Dentro de este tipo de análisis, los más conocidos son el ensayo de Northern-blot y la RT-PCR. La técnica de Northern-blot, requiere de gran cantidad de ARN y su elaboración lleva mucho tiempo (Josefsen & Nielsen, 2011). Sin embargo, la RT-PCR puede realizarse para una gran cantidad de muestras y/o genes diferentes en el mismo experimento, requiere menor cantidad de ARN y los resultados son fácilmente interpretables.

##### *4.1.5.2.1. RT-PCR*

A continuación, se expone el fundamento general del análisis transcriptómico por RT-PCR.

El paso inicial en ésta técnica es la producción de ADNc (ADN complementario) a partir del ARN de la muestra por la acción de la enzima transcriptasa inversa.

El segundo paso es la amplificación por PCR de los genes de interés presentes en el ADNc. El número de ciclos llevados a cabo en la PCR depende de la cantidad presente del gen de interés y de la eficiencia de reacción. Suelen utilizarse uno o varios genes de expresión constitutiva (housekeeping) para normalizar la expresión de los genes de interés (Rebouças et al., 2013).

El último paso es la detección y cuantificación de los productos de amplificación. Existen dos técnicas de cuantificación: análisis del punto final ("end point") y el control a tiempo real de la formación de los productos:

- Análisis del punto final ("end point"): analiza la reacción después de que se haya completado y se lleva a cabo normalmente mediante el uso de colorantes fluorescentes intercalados, que se detectan por HPLC o por electroforesis capilar.
- Control a tiempo real: controla la reacción de PCR en el termociclador a medida que avanza, lo que permite una mejora de la cuantificación. Se minimizan los errores por la manipulación de los productos de amplificación en la cuantificación del punto final y se obtiene mucha más información sobre cada ciclo de PCR (Freeman, Walker & Vrana, 1999). Esta cuantificación puede realizarse mediante el uso de detectores específicos o detectores no específicos.
    - Detectores específicos: son sondas que hibridan con el gen de interés, cuya degradación por la acción de la polimerasa de ADN hace que se emita fluorescencia. El detector especifico más comúnmente utilizado es el "TaqMan". Sin embargo, estos detectores son caros y con gran probabilidad de obtener falsos resultados.
    - Detectores no específicos: su uso es más barato, fácil de producir y manejar y son sensibles y efectivos. El uso de primers específicos solucionan el problema de especificidad de estos detectores. El más utilizado es el SYBR Green. Es un tinte fluorescente, que al unirse a secuencias de ADN de doble hebra (las amplificadas por los primers específicos) emite fluorescencia (absorbe a 497 nm y emite a 520 nm).

Los dos métodos de cuantificación pueden dar lugar a una cuantificación absoluta o relativa. Por un lado, una cuantificación absoluta se utiliza para cuantificar muestras desconocidas

mediante la interpolación de su cantidad a partir de una curva de patrones. Por otro lado, la cuantificación relativa se utiliza para analizar los cambios en la expresión génica en una muestra determinada en relación con otra muestra de referencia que suele ser la muestra sin tratar.

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# OBJETIVOS

## OBJETIVOS

Debido a la importancia del nitrito tanto en materia de seguridad como tecnológica en la elaboración de embutidos curado-madurados, en la presente tesis se plantean los siguientes objetivos generales:

1. Evaluación del efecto de la reducción de nitratos y nitritos en embutidos curado madurados y su repercusión en el perfil aromático.
2. Estudio del empleo de levaduras como estrategia para mantener la calidad e incrementar el aroma a curado.

Para ello, se ha llevado a cabo el siguiente plan de trabajo:

1. Evaluación del efecto de la reducción de nitrificantes en la calidad y seguridad de embutidos curado-madurados y determinar la estabilidad del embutido durante el almacenamiento a vacío.
2. Evaluación del potencial de las levaduras para producir volátiles a partir de aminoácidos azufrados. Evaluar mediante análisis transcriptómico, las rutas metabólicas y los genes implicados en la formación de compuestos aromáticos azufrados.
3. Desarrollar embutidos curado-madurados reducidos en nitrificantes y de alta calidad aromática mediante el empleo de levaduras generadoras de compuestos aromáticos.

# RESULTADOS

# Capítulo 1

## Reducción de nitrato en el proceso de fermentación de embutidos curado-madurados reducidos en sal: impacto en los parámetros microbiológicos y fisicoquímicos y en el perfil aromático.

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# Nitrate reduction in the fermentation process of salt reduced dry sausages: Impact on microbial and physicochemical parameters and aroma profile

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## Abstract

Slow fermented sausages with reduced ingoing amounts of sodium nitrate were manufactured: control (250ppm), 15% (212.5ppm) and 25% (187.5ppm) reduction. The effect of nitrate reduction on microbiology and chemical parameters, volatile compounds and aroma production was studied. Parameters like, pH, $a_w$ and colour decreased during ripening, without being affected by nitrate reduction. Lipid oxidation increased during ripening and it was higher in control sausages due to fat content. Residual nitrite was below the detection limit during the whole process and residual nitrate decreased during ripening, with higher reduction in RN25 sausages. Lactic acid bacteria, total mesophilic bacteria and yeasts and moulds increased during ripening but Gram positive cocci decreased. Microbial counts from nitrate reduced sausages at the end of the manufacturing process were not statistically different from the control sausages with nitrate. Regarding volatile compounds formation, compounds derived from amino acid degradation were increased by nitrate reduction. Aroma compounds derived from amino acid degradation and responsible for strong odours, dimethyl disulphide (toasted, garlic) and methional (cooked potato) and, to a lesser extent, compounds derived from esterase activity producing fruity odours (ethyl acetate, ethyl butanoate, ethyl-2-hydroxypropanoate, ethyl-2-methylbutanoate and ethyl-3-methylbutanoate) and several compounds from carbohydrate fermentation acetic acid (vinegar odour) and 2-butanone (fruity) were related to the high nitrate reduction (25%). Despite nitrate reduction up to 25% produced minor effect on microbial growth, their

metabolism is regulated by nitrate content and therefore by nitrite generation affecting the production of key aroma compounds that alter the sausage aroma profile.

Keywords: Nitrate reduction; Fermented sausage; Flavour; Aroma; Microbial safety.

## 1. Introduction

Nitrite has several important functions in the manufacturing of meat products. Among them, colour improvement through its reduction to nitric oxide and development of cured flavour, are the most important for consumer acceptability (Sindelar and Milkowski, 2011). Moreover, nitrite is able to control the oxidative stability by preventing lipid oxidation (Berardo et al., 2016) and plays an important role inhibiting the growth and toxin production of *Clostridium botulinum* (Rahman, 2007). On the other hand, generation of nitrosamines in meat products by reactions between nitrosating agents, derived from nitrite, and secondary amines, derived from protein and lipid degradation, has been of safety concern due to their carcinogenic potential (De Mey et al., 2015). Generally, intensive heat treatments are necessary to obtain carcinogenic nitrosamines and in case of fermented sausages, the mild acidic conditions and low water activities prevent their formation (De Mey et al., 2015). The search for alternatives to nitrite have focused on the use of spices and fruits (Gassara et al., 2016), organic acids, antimicrobial peptides, etc. (Alahakoon et al., 2015). However, none of them fulfils all technological needs (colour, flavour, microbiological safety and antioxidant activity) and the actual trend is directed to reduce the ingoing amount of nitrite and nitrate in meat products (EFSA, 2010; FCEC, 2016). Previous studies have demonstrated that the reduction of nitrate and nitrite in dry fermented sausages produces changes in quality (Hospital et al., 2015) and microbial safety (Hospital et al., 2012, 2014, 2016). Dry sausages with reduced amounts of nitrate and nitrite contained the highest amounts of volatile compounds derived from carbohydrate fermentation and amino acid degradation (Hospital et al., 2015) which could be attributed to increased counts of Gram-positive catalase-positive cocci and Enterobacteriaceae at the end of the ripening. Furthermore, the presence of nitrite is essential to control *Salmonella typhimurium* growth in fermented sausages; although, reduced concentrations of the compound are sufficient to provide equal protection (Hospital et al., 2014). Regarding *Listeria*, nitrite reduction increased the growth of inoculated *L. innocua* in the final sausage.

Production of *C. botulinum* toxin has not been detected in nitrate and nitrite reduced fermented sausages, although this could depend on existing hurdles and specific ripening conditions (Hospital et al., 2016). The effect of reduced ingoing amounts of nitrate and nitrite in fermented meat products seems to depend on the presence of both agents (nitrite and

nitrate) and specific ripening conditions. Nevertheless, the specific provision concerning nitrites and nitrates (Regulation (EC) no 1333/2008) indicates the possibility of the exclusively use of nitrates (250 ppm) without added nitrite in traditional slow ripened sausages such as "salchichón" and "chorizo" with maturation period of at least 30 days. Presently, nothing is known about the effect of nitrate reduction on these traditionally cured meat products where nitrate is used exclusively as curing agent. Slow-fermented sausages are typical Mediterranean products subjected to a first ripening step at low temperatures and a second step at higher temperatures in order to develop special sensory characteristics (Toldrá & Flores, 2014). These sausages have a mild pH decline, although starters are added in the process to improve the microbial safety of the product. The long process allows the exclusively use of nitrate as a nitrite reservoir by the activity of acid sensitive bacteria from the Staphylococcaceae family (Sánchez Mainar and Leroy, 2015). Nitrate slow fermented sausages have a preferred flavour than those made with nitrite and provide better organoleptic characteristics (Marco et al., 2008). In fact, the use of only nitrate instead of nitrite in dry fermented sausages produces the inhibition of lipid oxidation and affects the generation of volatile compounds derived from microbial metabolism, but as Marco et al. (2006) indicated it depends greatly on the fermentation process applied. The longer ripening times employed in traditional dry sausages and the use exclusively of nitrates without added nitrite can produce different effects on the quality and safety of reduced nitrate sausages. Factors such as final pH, temperature and $a_w$ are closely interrelated with microbial growth. Moreover, the actual trends to reduce sodium in meat products may also affect microbial growth and sausage safety (Aaslayng et al., 2014; Laranjo et al., 2016). In view of these actual trends, the aim of this study was to evaluate the reduction of nitrate used exclusively without added nitrite on the overall quality of traditional dry sausages with reduced sodium content.

## 2. Materials and methods

### *2.1. Dry fermented sausage manufacture*

Three different formulations (6 kg/formulation) of dry fermented sausages were manufactured: sodium nitrate at 250 ppm (C), 15% reduction (RN15) and 25% reduction (RN25). Three replicates of each formulation were carried out. Pork's lean and belly fat were purchased from a local producer (Cárnicas La Cope, Torrente, Spain). For each manufacture

batch, lean (50% lean pork meat) and fat (50% pork belly) were ground through a 10 mm diameter mincing plate and mixed with the following ingredients: 20 g/kg lactose, 20 g/kg dextrin, 7 g/kg glucose, 20.25 g/kg sodium chloride (NaCl), 6.75 g/kg potassium chloride (KCl), 0.5 g/kg sodium ascorbate, and sodium nitrate at 250 ppm (C), 212.5 ppm (RN15) or 187.5 ppm (RN25) depending on the batch. Also, a commercial starter culture (0.125 g/kg) TRADI-302 (Danisco, Cultor, Madrid, Spain) containing *Lactobacillus sakei*, *Staphylococcus xylosus* and *Staphylococcus carnosus* was added. Each formulation was kept at 3–5°C for 24h and then stuffed into 95 mm diameter collagen casings (Fibran, S.A., Girona, Spain), being the final weight of each sausage approximately 500 g. Sausages were dried for 62 days at 10 °C and 75–90% relative humidity (RH). In order to control the ripening process, temperature and RH of the ripening chambers were continuously recorded. Two sausages from each batch were weighed almost every day to control weight losses. Also, one sausage from each batch was used to control the pH by introducing a pH meter HI99163 (Hanna Instruments Inc., Hoonsocket, USA) into the centre of the sausage as described by Olivares et al. (2010). A total of 9 batches were produced and approximately 10 sausages were obtained from each batch. Before stuffing, approximately 200 g of the minced meat mixture were collected for further analysis. At the end of the process, two sausages from each batch were randomly chosen for analysis. A slice of approximately 25 g was taken for microbial analyses. Several slices were taken, wrapped in aluminium foil, vacuum packed and storage at −80 °C for volatile analysis. Sausage colour was measured and approximately 100 g of sausage were minced and used for moisture, water activity ($a_w$) and pH analysis. The remaining minced sausage was vacuum packed and frozen at −20 °C for subsequent physicochemical analyses (TBARS, lipid, protein, residual nitrite and nitrate).

### *2.2. Physicochemical analysis*

#### *2.2.1. pH, aw, colour and moisture*

pH was measured with a pH meter HI 99163 (Hanna Instruments Inc.) using minced sausage and distilled water (1:1, p/v) (ISO 2917:, 1999). Water activity was measured with a Fast-lab water activity meter (Gbx, Romans sur Isère Cédex, France) and colour was analysed with a colorimeter (CR-400/410, Konica Minolta Sensing Inc., Japan) with D65 illuminant

(Olivares et al., 2010). Moisture was determined by drying at 100 °C until constant weight (BOE, 1979).

### *2.2.2. Lipid and protein content*

Lipid content was determined by organic extraction with $Cl_2CH_2$:$CH_3OH$ (2:1) (Folch et al., 1957) as described Olivares et al. (2010). Nitrogen content was determined by the Kjeldahl method and protein estimated by multiplying the nitrogen content by 6.25.

### *2.2.3. Oxidation and nitrifying agents*

Lipid oxidation was evaluated using the thiobarbituric acid reactive substances test (TBARS) as described Corral et al. (2013). Values were expressed as μg of malonaldehyde per gram of dry matter (μg MDA/ g dm). Nitrate and nitrite contents were extracted with hot water (Mohamed et al., 2008) and determined using an enzymatic kit (Boehringer) (Arneth and Herold, 1988).

## *2.3. Microbiological analysis*

Microbial counts were done on 25 g of dry fermented sausage. Samples were finely sliced, blended with 225 ml of buffered peptone water (Pronadisa, Spain) and homogenized in a Pulsifier (Microgen Biotech, Spain). Homogenates were used to prepare decimal dilutions which were spread on appropriate media plates. Microbial counts were determined on the following media: bacterial starter containing lactic acid bacteria (LAB) on MRS Agar (Scharlau, Spain) at 30 °C for 3 days and Gram positive cocci on Mannitol Salt Agar (MSA) (CN-M) (Scharlau, Spain), at 30 °C for 3 to 5 days and Baird Parker Agar (BP) (CN-BP) (Pronadisa, Spain) at 37 °C for 48 h. Coagulase test was carried out in Gram positive cocci isolates from BP (EN ISO 6888–1) using lyophilised rabbit plasma (Scharlau, Spain). Mesophilic bacteria (TMB) were determined on Plate Count Agar (Pronadisa, Spain) at 30 °C for 3 days, yeasts and moulds on Rose Bengal Agar with chloranphenicol (Scharlau, Spain) at 30 °C for 5 to 7 days. Enterobacteriaceae were counted on Violet Red Bile Agar with Glucose (VRBG) (Pronadisa, Spain) at 37 °C for 24 h in anaerobiosis. Sulphite reducing clostridia were determined from 1 ml homogenate sample inoculated in freshly prepared Lactose Sulphite Broth supplemented with sodium metabisulfite and ferric ammonium citrate (Pronadisa, Spain) dispensed into tubes with Durham gas collecting tubes and incubated in anaerobiosis (bioMerieux, Spain) at 46 °C for 48 h. Twenty-five ml of

homogenated sample were used for enrichment of *Yersinia enterolytica* in Sorbitol Peptone Broth and Bile Salts (SPBS) (Pronadisa, Spain) at 25 °C for 5 days and subsequently plated on Yersinia Selective Agar (YSA) (Pronadisa, Spain) at 30 °C for 48 h. Twenty-five ml of homogenated sample were used for enrichment of *Listeria* spp. in 1/2 Fraser and Fraser Broth supplemented with ferric ammonium citrate (Pronadisa, Spain) at 30 °C for 24 and 48 h, respectively. Dilutions of the *Listeria* enriched Fraser medium were inoculated onto Listeria Chromogenic Agar (Pronadisa, Spain) and incubated at 37 °C for 24 h.

The remaining homogenate was incubated at 37 °C during 16–20 h for *Salmonella* pre-enrichment. One millilitre of the incubated homogenate was used for enrichment of *Salmonella* spp. in Rappaport Soy Broth (Pronadisa, Spain) at 41.5 °C for 24 h and Muller Kauffmann Broth Base w/Brilliant Green and Novobiocin supplemented with iodine and potassium iodide solution (MKTTN) (Pronadisa, Spain) at 37 °C for 24 h. Enriched cultures were plated on Xylose Lysine Desoxycholate Agar (XLD) (Pronadisa, Spain) and incubated at 37 °C for 24 h.

### *2.4. Volatile compound analysis*

#### *2.4.1. Gas chromatography-mass spectrometry*

The analysis of headspace (HS) volatile compounds was carried out by solid phase micro extraction (SPME) with an 85 μm Carboxen/ Polydimethylsiloxane (CAR/PDMS) fibre (Supelco, Bellefonte, PA) using a gas chromatograph (Agilent HP7890 series II (Hewlett-Packard, Palo Alto, CA) with a mass detector (HP 5975C (Hewlett-Packard) equipped with an autosampler (Gerstel MPS2 multipurpose sampler (Gerstel, Germany), as described Corral et al. (2015) with minor modifications. Briefly, 5 g of sausage with 0.75 mg BHT to avoid oxidation, was weighed into a headspace vial. The vial was incubated at 37 °C for 30 min. Then, the fibre was exposed into the head space vial for 120 min while maintaining the sample at 37 °C. The compounds adsorbed by the fibre were desorbed in the injection port of the GC–MS for 5 min at 240 °C in splitless mode. The compounds were identified by comparison with mass spectra from the library database (Nist'05), with linear retention indices (Van Den Dool and Kratz, 1963) and with authentic standards. The results were expressed as abundance units (AU) $10^5$.

#### *2.4.2. Gas chromatography-olfactometry/FID*

The analysis of aroma compounds was carried out by a gas chromatograph (Agilent 6890, USA) equipped with a FID detector and sniffing port (ODP3, Gerstel, Mülheim an der Ruhr, Germany) as described Corral et al. (2015). Each assessment was carried out with 5 g of sample using the detection frequency method (Pollien et al., 1997). Four trained panellists evaluated the odours from the GC-effluent of the sausages at the end of the maturation (62 days). A total of 12 assessments were carried out. The aroma compounds were identified by comparison with mass spectra, with linear retention indices of authentic standards injected in GC–MS and GC-O and by the coincidence of the assessor's descriptors with those in the Fenaroli's handbook of flavour ingredients (Burdok, 2002).

### *2.5. Statistical analysis*

Data were analysed using the Generalized Linear Model (GLM) procedure of statistical software (XLSTAT 2011, v5.01, Addinsoft, Barcelona, Spain). Data was analysed using the linear mixed model and included nitrate reduction and ripening time as fixed effects, and replicates as random effect. When significant effect of the treatment group was detected ($P < 0.05$), least squares means (LSM) were compared using Tukey test. Principal component analysis (PCA) was done to evaluate the relationships among sausage formulation and different parameters (pH, water activity, TBARS, protein and fat content, nitrate residual, colour, microbiota and aroma compounds).

## 3. Results and discussion

### *3.1. Physicochemical analyses*

Physicochemical parameters are shown in Table 1. Observed differences between batches in fat and protein content were due to random formulation variations which resulted in a higher fat content in control sausages and small differences in weight losses (C 39.78%, RN15 41.97%, RN25 42.58%). Final dry fermented sausages had an average pH value of 4.9, as expected for this type of product (Marco et al., 2008), and the $a_w$ decreased from 0.977 to 0.893 (average values). These results guarantee the stability and safety of the sausages and, therefore, nitrate reduction did not seem to affect them, as observed in nitrite and nitrate reductions by Hospital et al. (2015). The colour parameters evaluated (L*, a* and b* values) decreased during ripening due to drying with values within the expected range (Olivares et

al., 2010). Nitrate reduction did not affect the colour of sausages neither at the beginning nor at the end of process, although a higher L* value was detected in the control raw sausage than in nitrate reduced ones (RN15 and RN25). Regarding lipid oxidation, it increased with ripening in all sausages, although at the end of process C batch had a significant higher TBARS value than RN15 and RN25. The antioxidant activity of nitrite is well known (Zanardi et al., 2004), therefore, the higher lipid oxidation in C batch could be due to its higher fat content (Olivares et al., 2011).

At the beginning of the process, nitrite was not detected because only nitrate was added and there was not enough time for its reduction. At the same time, residual nitrate content confirmed the different ingoing amounts added to each batch. At the end of the process, the nitrate decline was 33, 39 and 52% respect to the initial amount (C, RN15 and RN25). Nitrite was never detected; this compound was always below the detection limits. Marco et al. (2006, 2008) also observed a decrease of nitrate concentration during ripening of slow fermented sausages with only nitrate added. These authors found a nitrate decline of approximately 30% in sausages with a final pH value of 4.8 (Marco et al., 2006), however, higher nitrate drops were observed in sausages with pH slightly above 5 (Marco et al., 2008). In both cases, residual nitrate amounts were close to 100 ppm dm. In contrast, the residual amounts of the manufactured fermented sausages were above 100 ppm dm but the differences may be due to the fermentation process and its effect on the nitrate reductase enzyme (Cachaldora et al., 2013).

### *3.2. Microbiological analyses*

Microbial counts are shown in Table 2. At the beginning of the process, total mesophilic bacteria (TMB) and lactic acid bacteria (LAB) had mean counts of 6.8 and 5.8 log cfu/g respectively, in accordance with the amount of starter inoculated. Moreover, the increase by 2 log in TMB and 1 log in LAB during ripening is in the range expected for this type of sausage (Fontana et al., 2005). The population of Gram positive cocci that include staphylococcal starters numerated either on MSA (CN-M) or on BP (CN-BP) decreased during the process. This could be due to the pH decrease produced by LAB which is affecting coagulase negative staphylococci (Leroy et al., 2006) as well as NaCl reduction and replacement by KCl as observed by Corral et al. (2014). Coagulase test on CN-BP cocci

isolates (about 400 studied) classified all of them as coagulase negative and probably representative of *Staphylococcus* species also included in the bacterial starter. Enterobacteria counts decreased by 2 log from the beginning to the end of the process. Yeasts and moulds increased until $10^3$ cfu/g in final sausages. Sulphite reducing clostridia, *Salmonella* spp. and *Yersinia enterolitica* were not present during the whole process. *Listeria* counts were also negative in all samples except for two positive colonies grown on LCA media in a replicate of RN25 reduced nitrate sausages at the end of the fermentative process.

In summary, microbial counts in control samples were in the range expected and followed the dynamics observed in slow fermented sausages (Rantsiou et al., 2005; Samelis et al., 1998; Di Luccia et al., 2016; Tabanelli et al., 2016). Hospital et al. (2015) studied the effect of nitrate and nitrite reduction on the microbial growth of dry fermented sausages. In agreement with our results, these authors did not find differences in LAB counts at the beginning of the process, but neither in Gram positive catalase positive cocci. The differences between results in the present study and those of Hospital et al. (2015) can also be attributed to the initial addition of nitrite together with nitrate and the kind of fermentation (Marco et al., 2008; Sanz et al., 1997). Nitrate reduction appeared to affect mostly growth of yeasts and moulds which counts were significantly higher in nitrate reduced samples.

**Table 1. Effect of nitrate reduction on physicochemical parameters of dry fermented sausages at initial and end of process.**

| | 0 days | | | 62 days | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **C[a]** | **RN15** | **RN25** | **C** | **RN15** | **RN25** | **RMSE[c]** | $P_t$[d] | $P_n$ | $P_{txn}$ |
| **pH** | 5.85 a[b] | 5.83 a | 5.88 a | 4.99 b | 4.95 b | 4.93 b | 0.038 | *** | ns | ns |
| **Aw** | 0.981 a | 0.974 a | 0.975 a | 0.890 b | 0.894 b | 0.895 b | 0.004 | *** | ns | * |
| **Moisture (%)** | 63.12 a | 64.18 a | 64.88 a | 40.33 b | 41.03 b | 42.61 b | 2.091 | *** | ns | ns |
| **Proteins (% dm)** | 49.11 d | 49.37 cd | 50.05 cd | 54.58 b | 56.15 b | 61.23 a | 2.409 | *** | ** | ns |
| **Fat (% dm)** | 35.97 ab | 34.07 abc | 31.68 bc | 36.30 a | 33.15 abc | 30.57 c | 2.052 | ns | *** | ns |
| **L*** | 64.92 a | 61.15 a | 61.04 a | 50.50 b | 49.84 b | 49.27 b | 1.716 | *** | * | ns |
| **a*** | 26.05 a | 27.14 a | 26.47 a | 17.66 b | 18.21 b | 17.88 b | 0.994 | *** | ns | ns |
| **b*** | 15.73 a | 15.69 a | 15.32 a | 6.59 b | 6.76 b | 6.26 b | 0.538 | *** | ns | ns |
| **TBARS[e]** | 0.56 b | 0.46 b | 0.41 b | 0.93 a | 0.69 ab | 0.61 b | 0.139 | *** | ** | ns |
| **$NO_3$ (ppm dm)** | 445.21 a | 378.58 ab | 338.50 bc | 299.79 c | 231.81 d | 163.52 e | 27.214 | *** | *** | ns |

[a] C: control batch. RN15: 15% sodium nitrate reduction batch. RN25: 25% sodium nitrate reduction batch.
[b] Different letters in the same row indicate significant differences at $p < 0.05$ among batches.
[c] RMSE: Roost mean square error.
[d] *P*: *P* value of time (t), nitrate content (n) and time and nitrate content (txn) effect, ns: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
[e] TBARS expressed as µg malonaldehyde/g dm.

**Table 2. Effect of nitrate reduction on microbial counts (log cfu/g) of dry fermented sausages at initial and end of process.**

| | Culture Medium[e] | 0 days | | | 62 days | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C[a] | RN15 | RN25 | C | RN15 | RN25 | RMSE[c] | $P_t$[d] | $P_n$ | $P_{txn}$ |
| **Total mesophilic bacteria (TMB)** | PCA | 6.84 b[b] | 6.88 b | 6.87 b | 8.89 a | 8.83 a | 8.96 a | 0.12 | *** | ns | ns |
| ***Lactobacillus* (LAB)** | MRS | 5.85 b | 5.83 b | 5.75 b | 6.98 a | 6.94 a | 6.96 a | 0.07 | *** | ns | ns |
| **Yeasts and molds (YM)** | RB | - | - | - | 2.32 b | 3.24 a | 3.33 a | 0.20 | ** | ** | ns |
| **Enterobacteriaceae (EB)** | VRBGA | 4.84 a | 4.83 a | 4.81 a | 2.39 b | 2.34 b | 2.33 b | 0.03 | *** | ns | ns |
| **Gram positive cocci CN (CN-M)** | MSA | 5.47 a | 5.41 a | 5.28 a | 4.49 b | 4.33 b | 4.39 b | 0.13 | *** | ns | ns |
| **Gram positive cocci CN (CN-BP)** | BP | 4.86 b | 4.84 ab | 4.78 ab | 4.45 c | 4.56 bc | 4.64 abc | 0.10 | *** | ns | ns |

[a] C: control batch. RN15: 15% sodium nitrate reduction batch. RN25: 25% sodium nitrate reduction batch.
[b] Different letters in the same row indicate significant differences at $p < 0.05$ among batches.
[c] RMSE: Roost mean square error.
[d] *P*: *P* value of time (t), nitrate content (n) and time and nitrate content (txn) effect, ns: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
[e] PCA: Plate Count Agar, RB: Rose Bengal Agar, VRBGA: Violet Red Bile Agar with Glucose, MSA: Mannitol Salt Agar, BP: Baird Parker Agar.

### *3.3. Volatile compound analysis*

The effect of nitrate reduction on the volatile compounds of dry fermented sausages was studied through the analysis of the volatile compounds present in the headspace (HS) of the different sausages analysed by SPME-GC–MS (Table 3). Fifty-three volatile compounds were identified and quantified in the HS of dry fermented sausages using a CAR/PDMS fibre. The volatile compounds identified and quantified in the HS can be compared with previous studies using the same extraction technique and fibre. The volatile compounds present in the HS of sausages were derived from chemical and metabolic reactions during ripening. Table 3 shows the volatile compounds classified by their possible origin: microbial activity: amino acid degradation (14), carbohydrate fermentation (9), esterase activity (6) and β-oxidation (3), and from lipid oxidation reaction (20) and unknown origin (1). All of these compounds have been previously identified in slow fermented sausages (Corral et al., 2013; Marco et al., 2006; Olivares et al., 2011). Volatile compounds derived from amino acid degradation were significantly affected by nitrate reduction. In RN25 sausages a significant increase was observed for 2-methylpropanal, 2-methylpropanol, 3-methylbutanol, 2-methylbutanol, and 2,6-dimethylpyrazine, which come mainly from branched chain amino acids (Søndergaard and Stanhke, 2002). However, only benzene was significantly reduced in nitrate reduced sausages ($p < 0.01$). Thus, amino acid degradation took place in more abundance when nitrate was reduced. This fact can be related to the growth of LAB, Staphylococcaceae, yeasts and moulds as responsible for the degradation of amino acids (Toldrá, 2008) although only yeasts and moulds were significantly high in RN25 sausages (Table 2). Hospital et al. (2012, 2015) also found an increase in volatile compounds derived from amino acid degradation in reduced nitrite and nitrate sausages. These authors also observed an increase on 3-methylbutanol in reduced nitrite and nitrate sausages. However, it is important to notice that differences among studies are due to the use of different curing agents, since different effect is produced by the use of nitrate versus nitrite in the generation of volatile compounds as already observed by Marco et al. (2006), specially volatiles from high branched chain amino acid degradation (Olesen et al., 2004). Carbohydrate fermentation was the group with the highest proportion of volatile compounds in all formulations (75–80%) (Corral et al., 2013) being acetic acid, ethanol and 3-hydroxy-2-butanone the most abundant compounds in this group. Nitrate reduction did not affect the total abundance of compounds derived from

carbohydrate fermentation. As observed in Table 2, starter culture (LAB and Gram positive cocci) were not affected by nitrate reduction, being these microorganisms responsible for the generation of these volatile compounds (Toldrá, 2008). Nevertheless, the effect of nitrate reduction affected the abundance of several compounds especially at the highest reduction (RN25) producing an increase in acetone, 2-butanone and, acetic and butanoic acids. Similar increases in acid compounds were also reported in nitrite and nitrate reduced dry fermented sausages (Hospital et al., 2012, 2015).

Among volatile compounds derived from esterase activity, the most abundant was ethyl acetate as showed by other authors in similar sausages (Corral et al., 2013). The total abundance of compounds derived from esterase activity was higher in RN25 batch, although the increase was not significant. The abundance of compounds ethyl acetate, ethyl butanoate, ethyl-2-hydroxypropanoate, ethyl-2-methylbutanoate and ethyl-3-methylbutanoate was significantly higher in nitrate reduced than in C sausages. However, Gram positive cocci (CN-M and CN-BP) growth was not significantly different among sausages (Table 2). In contrast, Hospital et al. (2012, 2015) reported a reduction of ethyl acetate in dry fermented sausages containing reduced nitrite and nitrate levels. These differences among studies can be due to the exclusive use of nitrate (Marco et al., 2008) and for the differences in ethanol abundance, which is a substrate in esterase activity (Talon et al., 1998). Compounds 2,3-pentanedione, 2-heptanone and 1-octen-3-ol were identified as volatiles derived from β-oxidation (Corral et al., 2013). These compounds, found in the smallest proportion (<1%), seemed not to be affected by nitrate reduction. Although high fat content has been related to high abundance of these compounds (Olivares et al., 2010), no differences were observed among nitrate reduced sausages nor due to different fat content. Hospital et al. (2012) found significantly higher amounts of 2-heptanone and 1-octen-3-ol in absence of nitrite and nitrate, demonstrating the antioxidant activity of curing agents. Compounds derived from lipid oxidation reactions have an important role in dry fermented sausages aroma. The highest abundance of volatiles from lipid oxidation was detected in the control batch, although the differences with nitrate reduced sausages were not significant. This is in accordance with the high TBARS values detected in control sausages (Table 1). Among the lipid oxidation compounds, 2-methylfuran, butanal, 1-pentanol, hexanal, heptanal, 2-heptenal and hexanoic acid were produced in significantly increased amounts in control

sausages. On the other hand, 1-propanol, 2-hexenal and decane appeared in significantly higher amounts in nitrate reduced sausages. The antioxidant activity of nitrite (Berardo et al., 2016) was difficult to appreciate in the nitrate reduced fermented sausages because the higher fat content in control sausages produced a higher susceptibility to oxidation. In addition, the nitrate reduced sausages had the lowest residual nitrate content, indicating a higher conversion of nitrate to nitrite and probably a higher antioxidant effect.

**Table 3. Effect of nitrate reduction on volatile compounds generated (expressed as AU×10$^5$) in dry fermented sausages at the end of process.**

| | LRI[a] | RI[b] | C[c] | RN15 | RN25 | RMSE[d] | $P_n$[e] |
|---|---|---|---|---|---|---|---|
| **Microbial origin** | | | | | | | |
| **Amino acid degradation** | | | 400.589 b | 392.157 b | 534.289 a | 72.31 | ** |
| 2-Methylpropanal | 594 | a | 4.852 ab | 4.428 b | 7.655 a | 1.75 | * |
| Benzene | 676 | a | 2.011 a | 1.982 a | 1.034 b | 0.47 | ** |
| 2-Methyl-1-propanol | 683 | a | 12.449 ab | 9.707 b | 17.083 a | 4.22 | * |
| 3-Methylbutanal | 691 | a | 34.320 | 27.215 | 39.208 | 8.98 | ns |
| Dimethyl disulfide | 773 | a | 2.439 | 3.904 | 3.148 | 1.38 | ns |
| Toluene | 788 | a | 27.531 | 101.371 | 70.830 | 58.49 | ns |
| 3-Methyl-1-butanol | 795 | a | 227.773 ab | 176.059 b | 286.989 a | 57.99 | * |
| 2-Methyl-1-butanol | 797 | a | 49.137 b | 40.051 b | 68.571 a | 12.58 | ** |
| 2,6-Dimethylpyrazine | 945 | a | 3.230 b | 3.509 b | 4.586 a | 0.61 | ** |
| Methional | 968 | a | 2.186 | 3.145 | 5.137 | 1.24 | ns |
| Benzaldehyde | 1020 | a | 8.538 | 11.533 | 11.050 | 3.45 | ns |
| Benzene acetaldehyde | 1110 | a | 3.253 | 2.295 | 3.686 | 1.14 | ns |
| Phenol | 1114 | a | 17.529 | 17.635 | 16.779 | 1.55 | ns |
| Methanethiol | 473 | a | 12.395 | 10.746 | 8.2407 | 2.62 | ns |
| **Carbohydrate fermentation** | | | 6845.361 | 8221.824 | 7426.739 | 1448.965 | ns |
| Acetaldehyde | 466 | a | 75.874 | 72.257 | 61.998 | 17.915 | ns |

**Table 3. Continued.**

| | LRI[b] | RI[b] | C[c] | | RN15 | | RN25 | | RMSE[d] | $P_n$[e] |
|---|---|---|---|---|---|---|---|---|---|---|
| Ethanol | 507 | a | 1858.751 | | 2126.592 | | 2326.289 | | 565.348 | ns |
| Acetone | 529 | a | 31.026 | b | 51.857 | a | 38.867 | ab | 8.163 | ** |
| 2,3-Butanedione | 627 | a | 78.890 | a | 47.539 | b | 45.482 | b | 18.699 | * |
| 2-Butanone | 631 | a | 41.494 | b | 47.258 | b | 101.245 | a | 20.633 | ** |
| Acetic acid | 718 | a | 2685.579 | b | 2601.611 | b | 3031.631 | a | 233.298 | * |
| 3-hydroxy-2-butanone | 781 | a | 1333.706 | | 1021.151 | | 1025.607 | | 448.975 | ns |
| 2,3-Butanediol | 888 | a | 702.097 | | 2259.924 | | 1449.120 | | 1172.704 | ns |
| Butanoic acid | 896 | a | 55.761 | b | 50.373 | b | 95.959 | a | 15.088 | ** |
| **Esterase activity** | | | 718.76 | | 713.97 | | 1093.10 | | 304.53 | ns |
| Ethyl acetate | 635 | a | 571.12 | b | 704.31 | b | 1049.16 | a | 136.75 | *** |
| Ethyl butanoate | 832 | a | 47.01 | | 46.30 | | 68.99 | | 14.16 | ns |
| Ethyl 2-hydroxypropanoate | 867 | a | 45.84 | b | 55.66 | ab | 67.80 | a | 13.87 | * |
| Ethyl 2-methylbutanoate | 878 | a | 22.84 | b | 28.77 | ab | 40.31 | a | 9.44 | * |
| Ethyl 3-methylbutanoate | 882 | a | 29.34 | b | 27.45 | b | 62.31 | a | 14.84 | * |
| Ethyl octanoate | 123 | a | 7.51 | | 10.75 | | 9.60 | | 2.60 | ns |
| **β-Oxidation** | | | 43.95 | | 38.70 | | 38.14 | | 10.46 | ns |
| 2,3-Pentanedione | 745 | a | 16.64 | | 11.84 | | 19.44 | | 4.51 | ns |
| 2-Heptanone | 935 | a | 11.41 | | 11.35 | | 10.40 | | 1.97 | ns |
| 1-Octen-3-ol | 1033 | a | 17.49 | | 16.58 | | 12.18 | | 4.20 | ns |

**Table 3. Continued.**

| | LRI[a] | RI[b] | C[c] | RN15 | RN25 | RMSE[d] | $P_n$[e] |
|---|---|---|---|---|---|---|---|
| **Chemical origin** | | | | | | | |
| **Lipid oxidation** | | | 831.43 | 805.59 | 560.18 | 186.39 | ns |
| Pentane | 500 | a | 51.79 | 55.47 | 37.52 | 17.295 | ns |
| Propanal | 524 | a | 8.74 | 6.40 | 7.65 | 2.043 | ns |
| Hexane | 600 | a | 19.80 | 20.40 | 15.01 | 4.808 | ns |
| 1-Propanol | 612 | a | 11.88 ab | 10.71 b | 16.55 a | 3.038 | * |
| 2-Metylfuran | 616 | a | 1.80 a | 1.25 b | 1.36 ab | 0.317 | * |
| Butanal | 622 | a | 2.11 a | 0.48 b | 0.93 b | 0.703 | ** |
| Heptane | 700 | a | 94.23 | 94.22 | 78.82 | 24.149 | ns |
| Pentanal | 739 | a | 55.17 | 48.45 | 36.12 | 12.601 | ns |
| Octane | 800 | a | 126.91 | 121.26 | 93.47 | 22.281 | ns |
| 1-Pentanol | 827 | a | 34.12 | 30.49 | 21.56 | 7.228 | ns |
| Hexanal | 842 | a | 355.29 a | 322.76 ab | 183.14 b | 93.305 | * |
| 2-Hexenal | 907 | a | 1.55 b | 1.78 b | 2.96 a | 0.520 | ** |
| 1-Hexanol | 924 | a | 57.22 | 53.23 | 46.12 | 13.174 | ns |
| Heptanal | 941 | a | 34.88 a | 31.88 ab | 22.97 b | 6.441 | * |
| Decane | 1000 | a | 2.59 b | 3.19 ab | 3.63 a | 0.603 | * |
| 2-Pentylfuran | 1010 | a | 7.16 | 8.50 | 7.08 | 1.516 | ns |
| (E)- 2-Heptenal | 1013 | a | 3.41 a | 2.88 ab | 1.93 b | 0.835 | * |

**Table 3. Continued.**

| | LRI[a] | RI[b] | C[c] | RN15 | RN25 | RMSE[d] | $P_n$[e] |
|---|---|---|---|---|---|---|---|
| Octanal | 1049 | a | 21.69 | 22.12 | 16.26 | 4.872 | ns |
| Hexanoic acid | 1079 | a | 14.34 a | 12.27 ab | 9.53 b | 2.164 | * |
| Nonanal | 1151 | a | 24.45 | 26.76 | 21.95 | 5.178 | ns |
| **Unknown compounds** | | | | | | | |
| Carbon disulfide | 537 | a | 57.80 b | 73.28 ab | 104.65 a | 20.871 | * |

[a] LRI: Linear retention index of the compounds eluted from the GC–MS.
[b] RI: Reliability of identification: a, identification by mass spectrum, coincidence with the LRI of an authentic standard; b, tentatively identification by mass spectrum.
[c] C: control batch, RN15: 15% sodium nitrate reduction batch, RN25: 25% sodium nitrate reduction batch.
[d] RMSE: Roost mean square error.
[e] *P*n: *P* value of nitrate content effect, ns: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, different letters in the same row indicate significant differences at $p < 0.05$ among batches.

*3.4. Aroma analysis*

**Table 4. Odour active compounds identified in dry fermented sausages at the end of process.**

| | LRI GC-O[a] | LRI std GC-O[b] | RI[c] | Descriptor | DF[d] |
|---|---|---|---|---|---|
| **Methanethiol** | 472 | 471 | a | Rotten, unpleasant | 8 |
| **2-Methylfuran** | 615 | 619 | a | Green, garlic, toasted, yeast, malt | 4 |
| **2,3-Butanedione** | 629 | 632 | a | Cheese, butter, floral, fresh | 4 |
| **2-Butanone** | 636 | 638 | a | Fruity, sweet, cheese, butter, dairy | 8 |
| **Acetic acid** | 699 | 700 | a | Vinegar, acid, unpleasant | 8 |
| **2,3-Pentanedione** | 740 | 739 | a | Sweet, candy, fruit, glue | 4 |
| **Dimethyl disulfide** | 769 | 774 | a | Toasted, garlic | 3 |
| **3-Hydroxy-2-butanone** | 782 | 777 | a | Strawberry, sweet, fruity, green | 9 |
| **Ethyl butanoate** | 824 | 825 | a | Sweet, fruity, floral | 10 |
| **Hexanal** | 834 | 836 | a | Fresh cut grass, vegetable, fresh | 10 |
| **Ethyl 2-hydroxypropanoate** | 865 | 859 | a | Cheese, fruit, strawberry, sweet | 11 |
| **Ethyl 3-methylbutanoate** | 874 | 876 | a | Strawberry, fruit, glue, sweet | 9 |
| **1-Hexanol** | 920 | 919 | a | Cheese, oxidized fat, humidity | 11 |
| **2-Heptanone** | 931 | 931 | a | Rancid, burnt, strawberry, fruit | 4 |
| **Heptanal** | 938 | 937 | a | Green, unpleasant, toasted | 5 |
| **2-Acetyl-1-pyrroline** | 960 | 960 | a | Toasted, fried corn, bread | 12 |
| **Methional** | 965 | 969 | a | Cooked potato, roast meat | 10 |

**Table 4. Continued.**

| | LRI GC-O[a] | LRI std GC-O[b] | RI[c] | Descriptor | DF[d] |
|---|---|---|---|---|---|
| **2-Pentylfuran** | 1008 | 1011 | a | Garlic, onion, unpleasant, grass | 8 |
| **1-Octen-3-ol** | 1023 | 1028 | a | Mushrooms, humidity, spicy | 11 |
| **Unknown** | 1031 | - | c | Burnt, mushrooms, humidity, herbs | 8 |
| **Unknown** | 1037 | - | c | Green, grass, earth, burnt, herbs | 6 |
| **Octanal** | 1045 | 1047 | a | Orange, sweet | 4 |
| **Unknown** | 1162 | - | c | Spices, fried corn, unpleasant | 5 |
| **Unknown** | 1178 | - | c | Fried corn, toasted | 10 |
| **Ethyl octanoate** | 1223 | 1226 | a | Cooked vegetable, onion, fruity | 7 |

[a] Linear retention index of the compounds eluted from the GC-FID-O.
[b] Linear retention index of standard compounds in the GC-FID-O.
[c] Reliability of identification: a, identification by mass spectrum, coincidence with LRI of an authentic standard and by coincidence of the assesors's descriptors with those described in the Fenaroli's handbook of flavour ingredients (Burdok, 2002); b, tentatively identification by mass spectrum; c, unknown compounds.
[d] Detection frequency value.

Aroma compounds were analysed by gas chromatography-olfactometry (Table 4). Twenty-five aroma active zones were detected. From these, 21 compounds were identified by mass spectra, liner retention indices and by comparison with the odour description in literature. Four odour zones were not identified and were labelled as unknown compounds. The most important aroma compounds with highest DF values were ethyl butanoate (sweet and fruity odour), hexanal (fresh cut grass), ethyl 2-hydroxypropanoate (cheese and fruity odour), 1-hexanol (cheese and rancid odour), 2-acetyl-1-pyrroline (popcorn odour), methional (cooked potato odour), 1-octen-3-ol (mushrooms odour) and 1 unknown aromatic compound (cooked potato, fried corn, toasted and dried fruit odour) as already observed by Corral et al. (2013) and Olivares et al. (2011). To examine the relationship of the instrumental variables with the ingoing amounts of nitrate, a principal component analysis was done using the following parameters; chemical composition, TBARS values, pH, colour parameters, $a_w$, residual nitrate concentration, aroma active volatile compounds and microorganism's groups (Fig. 1). Two principal components were able to explain the 52.87% of the total variance. PC2 accounted for 18.19% of the variance and showed the variability among replicates of the same formulation. PC1 accounted for 34.68% of the variance and distinguished sausages by nitrate ingoing amounts placing C sausages on the left quadrant and nitrate reduced sausages, RN15 and RN25, on the centre and right quadrant, respectively. C batch was related to lipid oxidation and aroma compounds derived from oxidation reactions (hexanal, octanal, heptanal and 2-methylfuran) and carbohydrate fermentation (2,3-butanedione and 3-hydroxy-2-butanone). Other aroma impact compounds, methanethiol and 1-octen-3-ol, were also related to the control sausages. In contrast, nitrate reduced sausages (RN25) were related to aroma compounds derived from amino acid degradation (dimethyl disulphide and methional), esterase activity (ester compounds) and several compounds from carbohydrate fermentation (acetic acid and 2-butanone). Moreover, nitrate reduced sausages (RN25) were related to Enterobacteriaceae, Gram positive cocci (CN-BP) and yeast and moulds growth indicating the presence of other microorganisms which can be responsible for the high volatile compounds derived from microorganism's metabolism. In summary, nitrate reduction affected the aroma profile of fermented sausages due to their effect on microbial metabolism.

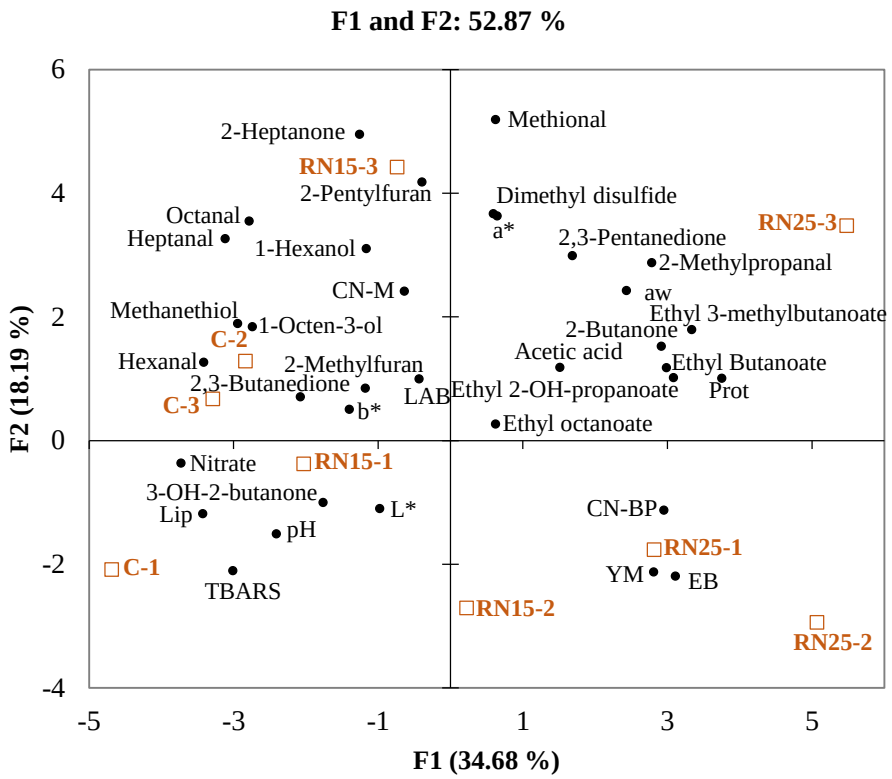


**Figure 1.** Loadings of the first two principal components (PC1-PC2) of dry fermented sausages with different nitrate ingoing amounts: C (control batch, 250 ppm), RN15 (15% sodium nitrate reduction, 212.5 ppm), RN25 (25% sodium nitrate reduction, 187.5 ppm) and instrumental variables (physicochemical, microbiological parameters and aroma compounds). Abbreviations are indicated in Tables 1 and 2.

## 4. Conclusion

The reduction of in going amounts of nitrate when used exclusively without nitrite addition in slow fermented sausages produced changes in the production of volatile compounds affecting the aroma profile. Microbial counts dynamics were mostly influenced by fermentation time and only slightly by nitrate reduction in case of yeasts and moulds. Manufacturing with reduced amount of nitrate up to 15% did not have a significant impact on microbial counts. Nitrate reduction resulted in an increase of volatile compounds derived from aminoacid degradation and, to a lesser extent, from esterase activity, affecting the production of key aroma compounds that alter the sausage aroma profile. Our results indicate

that nitrate reduction does not seem to affect directly microbial growth but affects microbial metabolism. Moreover, microbial metabolism can affect nitrate reduction into nitrite and hence its effect on lipid oxidation. More studies are necessary to know the extent and significance of nitrate reduction into the manufacturing process of traditional dry sausages.

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# Capítulo 2

## Cambios microbiológicos y del perfil aromático de embutidos curado-madurados reducidos en nitrato durante el almacenamiento a vacío.

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# Microbial changes and aroma profile of nitrate reduced dry sausages during vacuum storage

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## Abstract

Slow fermented sausages with reduced ingoing amounts of sodium nitrate (control, 15% and 25% reduction) were stored under vacuum up to three months. Changes in microbiology, chemical parameters and volatile compounds were studied. Residual nitrate was not affected by vacuum storage and its reduction resulted in a reduction of sausage redness. General microbial counts decreased during vacuum storage, though nitrate reduction increased the growth of total mesophilic bacteria and Gram positive cocci. Long storage time and 25% nitrate reduction affected microbial activity and sausage aroma profile. Short vacuum storage times and moderate nitrate reduction (15%) were related to compounds producing pleasant odours (3-hydroxy-2-butanone, ethyl octanoate, ethyl-3-methylbutanoate and 2,3-pentanedione) and cheesy/buttery odour (2,3-butanedione and ethyl-2-hydroxypropanoate). In contrast, 25% nitrate reduction increased compounds like heptanal (green, unpleasant odour) and those related to unpleasant odours, methanethiol (rotten odour) and methional (cooked potato).

Keywords: Nitrate; Fermented sausage; Storage; Vacuum; Flavour; Health safety.

## 1. Introduction

Consumers demands healthier meat products reduced in additives such as nitrite due to the generation of nitrosamines with carcinogenic potential (De Mey, De Maere, Paelinck, & Fraeye, 2015). However, nitrate and nitrite are used in fermented sausage manufacture as curing salts due to the nitrite effect on the control of *Clostridium botulinum* and its toxin production (Sindelar & Milkowski, 2011). Moreover, nitrite influences several technological parameters like colour development, typical cured flavour and antioxidant effect (Honikel, 2008). In this term, the interest of producers is directed to the knowledge of the reasonable nitrite/nitrate reduction to operate with safety warrant and maintain the high organoleptic properties of traditional meat products. Recently, Christieans, Picgirard, Parafita, Lebert, and Gregori (2018) have demonstrated the impact of reducing the ingoing amount of nitrate/nitrite in dry fermented sausages manufacture and its effect on the growth of pathogens like *Salmonella* and *Listeria*. However, scientific studies should provide information not only regarding microbial risks but also on organoleptic properties like aroma and the changes that may be produced during the long shelf life of this type of products. Different storage conditions are used depending on product type to extent their shelf-life while maintaining quality and safety. Dry fermented sausages can be kept unpackaged or packaged as whole or slices pieces under modified atmospheres or under vacuum conditions. Among these, vacuum packed is widely used to extend the shelf-life of dry sausages. Therefore, many studies have reported the changes observed during storage under vacuum conditions (Ansorena & Astiasarán, 2004; Dos Santos, Campagnol, Fagundes, Wagner, & Pollonio, 2015, 2017; Kim et al., 2012; Rubio et al., 2007; Rubio, Martinez, Garcia-Cachan, Rovira, & Jaime, 2008; Ščetar, Kovacic, Kurek, & Galic, 2013; Summo, Caponio, Paradiso, Pasqualone, & Gomes, 2010; Summo, Caponio, & Pasqualone, 2006; Summo, Caponio, Pasqualone, & Gomes, 2011; Zanardi, Dorigoni, Badiani, & Chizzolini, 2002), modified atmospheres (Rubio et al., 2007, 2008; Ščetar et al., 2013; Tabanelli, Montanari, Grazia, Lanciotti, & Gardini, 2013; Viallon et al., 1996; Zanardi et al., 2002;) and perforated packages (Bañon, Serrano, & Bedia, 2014; Lorenzo, Bedia, & Bañon, 2013). Changes in pH, water activity ($a_w$), red colour (a*) and oxidation parameters (TBARS) during shelf-life have been reported. Moreover, microbiology counts show a general decrease, except for LAB (Kim et al., 2012; Rubio et al., 2007). Overall, sausage acceptability decreases during storage

due to colour, aroma, and taste deterioration. The most common changes are the decrease in red intensity, ripened flavour and firmness and the increase in rancid aroma and hardness (Kim et al., 2012; Rubio et al., 2007; Summo et al., 2010; Zanardi et al., 2002) which is apparently accentuated by vacuum storage versus modified atmosphere (Rubio et al., 2008) and unpackaged storage (Summo et al., 2006). In addition to the physicochemical, microbiological and sensory changes attributed to storage, several studies have dealt with the effect on volatile compounds responsible for ripened aroma (Summo et al., 2011; Tabanelli et al., 2013; Viallon et al., 1996). Viallon et al. (1996) described the variation in sausage volatile profile with packaging under modified atmosphere as an increase of compounds derived from carbohydrate and amino acid degradations. Recent studies revealed that microbial and endogenous enzyme activities during modified atmosphere packaging depended on the initial sausage water activity and, therefore, changes in the latter affected the aroma profile (Tabanelli et al., 2013). Regarding the effect of vacuum storage on aroma profile, differences in lipid oxidation compounds like aldehydes (Ansorena & Astiasarán, 2004) and a significant increase of volatile compounds derived from carbohydrate and amino acid degradation reactions have been described in dry fermented sausages (Marco, Navarro, & Flores, 2006). Moreover, recent studies have shown a general increase in volatile compounds derived from lipid oxidation reactions (Dos Santos et al., 2015; Summo et al., 2011) and a decrease of those derived from spices under vacuum storage (Dos Santos et al., 2015). In summary, reported volatile changes during storage are highly dependent on sausage properties like $a_w$ (Tabanelli et al., 2013), lipid profile (Ansorena & Astiasarán, 2004), curing agents (Marco et al., 2006) and salt substitutes (Dos Santos et al., 2015) in addition to packaging conditions like temperature and time (Ščetar et al., 2013).

The latest trends in fermented sausage composition are directed to the reduction of additives such as nitrifying agents (EFSA, 2010; FCEC, 2016). Until now, only Hospital, Hierro, and Fernández (2014) have studied the effect of nitrate and nitrite reduction in rapid fermented sausages on microbial evolution during 1 month of vacuum storage. These authors reported changes in microbial counts but the impact of nitrifying agents and storage conditions on aroma was not investigated. Furthermore, the possibility of the exclusive use of nitrates (250 ppm) without added nitrite in traditional slow ripened sausages such as “salchichón” and “chorizo” with maturation period of at least 30 days is indicated in a specific provision

concerning nitrites and nitrates (EC Regulation no 1129/2011). Therefore, the aim of the present study is to determine the effect of vacuum storage and nitrate reduction on the aroma quality and microbial counts of slow fermented sausages manufactured with reduced sodium content.

## 2. Materials and methods

### *2.1. Dry fermented sausages manufacture*

Three replicates of the experiment were performed. In each replicate, three different formulations of dry fermented sausages were manufactured: Control with 250 ppm sodium nitrate (C) and two formulations with a reduction of 15% (RN15) and 25% (RN25) of ingoing amounts of sodium nitrate. Lean pork meat (50%) and pork fat (bellies boneless and skinless) (50%) were minced with the following ingredients (g/kg): lactose (20), dextrin (20), glucose (7), sodium chloride (NaCl) (20.25), potassium chloride (KCl) (6.75), sodium ascorbate (0.5), sodium nitrate at 250 ppm (C), 212.5 ppm (RN15) or 187.5 ppm (RN25) depending on the batch. A commercial starter culture (0.125) TRADI-302 containing *Lactobacillus sakei*, *Staphylococcus xylosus* and *Staphylococcus carnosus* (Danisco, Cultor, Madrid, Spain) was added. The mixture was stuffed into 95 mm diameter collagen casings (Fibran, S.A., Girona, Spain). After ripening for 62 d, dry sausages were vacuum packaged and stored at 18–20°C. For each of three replications, two sausages per batch were randomly taken after 1, 2 and 3 months of storage. In each sausage, colour was measured and a portion of 100 g was minced and used for moisture, water activity ($a_w$) and pH analyses. The remaining minced sausage was vacuum packed and frozen at −20 °C for physicochemical analyses (TBARS, lipid, protein and residual nitrite and nitrate). A slice of approximately 25 g was taken for microbial analyses. Several slices were wrapped in aluminium foil, vacuum packed and stored at −80 °C for volatile analysis.

### *2.2. Physicochemical analysis*

pH was measured with a pH meter HI 99163 (Hanna Instruments Inc.) with an electrode including built-in temperature sensor and calibration was performed automatically at two points (4 and 7) using standard buffers. Water activity was measured with a Fast-lab water activity meter (Gbx, Romans sur Isère Cédex, France). Colour (CIE L*a*b* system) was analysed with a portable colorimeter (CR-400/410, Konica Minolta Sensing Inc., Japan) with

a fixed aperture (8 mm diameter diaphragm with optical glass) and measurements were made with a D65 iluminant and 0° viewing angle. Three colour measurements were made on each sausage. Moisture was determined by the dehydration method until constant weight (BOE, 1979). Lipid content was determined by organic extraction (Folch, Lees, & Stanley, 1957), lipid oxidation was evaluated using the thiobarbituric acid reactive substances test (TBARS) and protein content was determined by the Kjeldahl method as described in Olivares, Navarro, Salvador and Flores (2010). Residual nitrate and nitrite contents were extracted with hot water (Mohamed, Mubarak, Fawy & El-Shahat, 2008) and determined using an enzymatic kit (Boehringer) (Arneth & Herold, 1988).

### *2.3. Microbiological analysis*

Microbial counts were done on 25 g of dry fermented sausage. Samples were finely sliced, blended with 225 ml of buffered peptone water (Pronadisa, Spain) and homogenized in a Pulsifier (Microgen Biotech, Spain). Homogenates were used to prepare decimal dilutions which were spread on appropriate media plates. Microbial counts were determined on the following media: bacterial starter containing lactic acid bacteria (LAB) on MRS Agar (Scharlau, Spain) at 30 °C for 3 days and Gram positive cocci on Mannitol Salt Agar (MSA) (CN-M) (Scharlau, Spain), at 30 °C for 3 to 5 days and Baird Parker Agar (BP) (CN-BP) (Pronadisa, Spain) at 37 °C for 48 h. Gram positive cocci isolates from BP were tested for coagulase activity (EN ISO 6888-1) using lyophilised rabbit plasma (Scharlau, Spain). Mesophilic bacteria (TMB) were determined on Plate Count Agar (Pronadisa, Spain) at 30 °C for 3 days, yeasts and moulds on Rose Bengal Agar with chloramphenicol (Scharlau, Spain) at 30 °C for 5 to 7 days. Enterobacteriaceae were counted on Violet Red Bile Agar with Glucose (VRBG) (Pronadisa, Spain) at 37 °C for 24 h in anaerobiosis. Sulphite reducing clostridia were determined from 1 ml homogenate sample inoculated in freshly prepared Lactose Sulphite Broth supplemented with sodium metabisulfite and ferric ammonium citrate (Pronadisa, Spain) dispensed into tubes with Durham gas collecting tubes and incubated in anaerobiosis (bioMerieux, Spain) at 46 °C for 48 h. Twenty-five ml of homogenated sample were used for enrichment of *Yersinia enterocolytica* in Sorbitol Peptone Broth and Bile Salts (SPBS) (Pronadisa, Spain) at 25 °C for 5 days and subsequently plated on Yersinia Selective Agar (YSA) (Pronadisa, Spain) at 30 °C for 48 h. Twenty-five ml of homogenated sample were used for enrichment of *Listeria* spp. in ½ Fraser and Fraser

Broth supplemented with ferric ammonium citrate (Pronadisa, Spain) at 30 °C for 24 and 48 h, respectively. Dilutions of the *Listeria* enriched Fraser medium were inoculated onto Listeria Chromogenic Agar (Pronadisa, Spain) and incubated at 37 °C for 24 h. The remaining homogenate was incubated at 37 °C during 16–20 h for *Salmonella* pre-enrichment. One millilitre of the incubated homogenate was used for enrichment of *Salmonella* in Rappaport Soy Broth (Pronadisa, Spain) at 41.5 °C for 24 h and Muller Kauffmann Broth Base w/Brilliant Green and Novobiocin supplemented with iodine and potassium iodide solution (MKTTN) (Pronadisa, Spain) at 37 °C for 24 h. Enriched cultures were plated on Xylose Lysine Desoxycholate Agar (XLD) (Pronadisa, Spain) and incubated at 37 °C for 24 h.

### *2.4. Volatile compound analysis*

The analysis of headspace (HS) volatile compounds was carried out by solid phase micro extraction (SPME) with an 85 μm Carboxen/ Polydimethylsiloxane (CAR/PDMS) fibre (Supelco, Bellefonte, PA) using a gas chromatograph Agilent 7890 series II with a mass spectrometer detector MS 5975C, (Agilent, Palo Alto, CA) equipped with an autosampler (Gerstel MPS2 multipurpose sampler, Gerstel, Mülheim an der Ruhr, Germany), as described Corral, Salvador, Belloch and Flores (2015) with minor modifications. Sausage sample (5 g) was weighed into a 20 ml headspace vial and 0.75 mg BHT was added. The vial was incubated at 37 °C for 30 min and volatile compounds were extracted by exposing the fibre to the headspace for 120 min at 37 °C. The fibre was desorbed in the injection port of the GC–MS for 5 min at 240 °C in splitless mode. The compounds were identified by comparison with mass spectra from the library database (Nist'05), by comparison to linear retention indices (Van Den Dool & Kratz, 1963) and using authentic standards. Quantification was based on the total extracted area (TIC). The results were expressed as abundance units ($AU \times 10^6$).

### *2.5. Statistical analysis*

Data were analysed using the Generalized Linear Model (GLM) procedure of statistical software (XLSTAT 2011, v5.01, Addinsoft, Barcelona, Spain). The data was analysed using the linear mixed model and included nitrate reduction and storage time as fixed effects, and replicates as random effect. The interaction between fixed effects was tested, and it was not

significant and was excluded from the model. The replication was not significant ($P > .10$) for any of the traits. When significant effect of the treatment group was detected ($P < .05$), least squares means (LSM) were compared using Tukey test. Principal component analysis (PCA) was done to evaluate the relationships among sausage formulation (nitrate reduction), storage time and different parameters (pH, water activity, TBARS, protein and fat content, nitrate residual, colour, microbiota and volatile compounds).

## 3. Results

The results of the statistical analysis on physicochemical, microbiology and volatile compounds are shown in Tables 1 to 3 and supplementary tables have been included reporting the results of all nitrate groups at each storage time.

### *3.1. Physicochemical analyses*

Physicochemical parameters were analysed taking into account the two factors vacuum storage time and nitrate reduction (Table 1). During vacuum storage, pH and water activity values suffered a significant decrease, as well as the redness parameter (a*) which decreased significantly after 3 months of storage. In addition, lipid oxidation values (TBARS) showed a significant decrease after the second month of vacuum storage that was maintained up to the third month of storage. About residual nitrite and nitrate levels, residual nitrite was below the detection limits while residual nitrate was not affected by vacuum storage. Variation in sausage composition (protein and fat content) among nitrate batches was attributed to variations in the trimming of the pork meat. Control sausages (C) had the highest fat content. Sausages with the smallest ingoing amount of nitrate (RN25) presented a slightly low pH value. Similarly, the redness parameter (a*) was lower in reduced sausages (RN25) than in C batch. Regarding lipid oxidation, TBARS values were lower in reduced nitrate sausages than in C sausage. Residual nitrate was lower in nitrate reduced sausages and confirmed the reduced ingoing amount used in formulation.

### *3.2. Microbiology analyses*

The changes in microbiota are shown in Table 2. Total mesophilic bacteria (TMB) and lactic acid bacteria (LAB) decreased a logarithm cycle ($p < .001$) after three months, whereas Gram positive cocci (CN-M and CN-BP) decreased between 1.5 and 2 logarithm cycles. Coagulase test on CN-BP cocci isolates (about 200 isolates) classified all of them as coagulase negative

suggesting that they are probably *Staphylococcus* from the bacterial starter. In the case of Enterobacteriaceae, yeast and moulds, sulphite reducing clostridia, *Salmonella* spp. and *Yersinia enterocolytica* no counts were detected in the whole vacuum storage period. *Listeria* counts were also negative in all samples except for one positive (blue-green) colony found in a LCA replicate of a RN25 sample at one month of vacuum storage. No positive colonies were found in the equivalent sample in successive months of vacuum storage. On the other hand, nitrate reduction (Table 2) produced a general increase in microbial counts, especially in case of Gram positive cocci, and for TMB only when nitrate was 15% reduced.

**Table 1. Effect of vacuum storage time and nitrate reduction on physicochemical parameters of dry fermented sausages. Values are presented as least squares means.**

| | Vacuum storage time | | | Nitrate reduction | | | $P_t$[2] | $P_n$ | RMSE[3] |
|---|---|---|---|---|---|---|---|---|---|
| | 1m | 2m | 3m | C[1] | RN15 | RN25 | | | |
| **pH** | 5.09 a | 5.03 b | 4.96 c | 5.05 a | 5.04 a | 5.00 b | *** | ** | 0.05 |
| **Aw** | 0.887 a | 0.883 b | 0.876 c | 0.883 | 0.881 | 0.881 | *** | ns | 0.01 |
| **Moisture (%)** | 40.3 b | 42.4 a | 42.3 a | 40.6 b | 41.9 a | 42.5 a | *** | ** | 1.38 |
| **Protein (% dm)** | 55.4 ab | 56.4 a | 54.0 b | 51.7 c | 55.5 b | 58.5 a | ** | *** | 2.00 |
| **Fat (% dm)** | 30.7 b | 33.8 a | 33.8 a | 36.4 a | 32.3 b | 29.6 c | *** | *** | 2.01 |
| **L*** | 48.1 | 48.1 | 47.8 | 48.4 | 48.2 | 47.5 | ns | ns | 1.27 |
| **a*** | 18.3 ab | 18.6 a | 17.9 b | 18.5 a | 18.4 ab | 17.9 b | * | * | 0.68 |
| **b*** | 6.7 b | 7.1 a | 7.3 a | 7.2 a | 7.2 a | 6.7 b | *** | *** | 0.36 |
| **TBARS[4]** | 0.99 a | 0.75 b | 0.74 b | 1.02 a | 0.86 b | 0.62 c | *** | *** | 0.18 |
| **$NO_3$ (ppm dm)** | 197.8 | 190.2 | 196.1 | 235.7 a | 185.7 b | 162.6 b | ns | *** | 35.0 |

[1] C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm).
[2] *Pt*: P value of storage time effect and *Pn*: P value of nitrate reduction effect. Different letters in the same row of each group indicate significant differences at *** $P < .001$, ** $P < .01$, * $P < .05$. ns: $P > .05$.
[3] RMSE: root mean square error.
[4] TBARS expressed as μg malonaldehyde/g dm.

**Table 2. Effect of vacuum storage time and nitrate reduction on microbial counts (log cfu/g) of dry fermented sausages. Values are presented as least squares means.**

| | Culture medium | Vacuum storage time | | | Nitrate | | | $P_t$[2] | $P_n$ | RMSE[3] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1m | 2m | 3m | C[1] | RN15 | RN25 | | | |
| **Total mesophilic bacteria (TMB)** | PCA[4] | 7.6 a | 7.5 a | 6.5 b | 7.1 b | 7.3 a | 7.2 ab | *** | * | 0.2 |
| ***Lactobacillus* (LAB)** | MRS | 6.6 a | 6.5 a | 5.6 b | 6.2 | 6.3 | 6.2 | *** | ns | 0.2 |
| **Gram positive cocci (CN-M)** | MSA | 3.7 a | 2.0 b | 1.6 b | 1.4 b | 2.9 a | 2.9 a | *** | *** | 0.7 |
| **Gram positive cocci (CN-BP)** | BP | 4.1 a | 3.4 b | 2.6 c | 3.2 b | 3.5 a | 3.4 a | *** | *** | 0.2 |

[1] C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm).
[2] *Pt: P* value of storage time effect and *Pn: P* value of nitrate reduction effect. Different letters in the same row of each group indicate significant differences at *** $P < .001$, ** $P < .01$, * $P < .05$. ns: $P > .05$.
[3] RMSE: root mean square error.
[4] PCA: Plate Count Agar, MRS: Man Rogosa Sharpe agar, MSA: Mannitol Salt Agar, BP: Baird Parker Agar.

### *3.3. Volatile compound analysis*

Volatile compounds were analysed in the headspace of sausages by SPME-GC–MS. Fifty-three volatile compounds were identified and quantified (Table 3) using the CAR/PDMS fibre. These volatile compounds were classified by their possible origin: microbiota activity (amino acid degradation (14), carbohydrate fermentation (9), lipid β-oxidation (3) and esterase activity reactions (6)), lipid oxidation reaction (20) and unknown origin (1). Fig. 1 shows the abundance of volatile compounds groups according to storage time and nitrate reduction. Volatile compounds derived from amino acid degradation were affected by storage time producing a decrease after 3 months of storage (Fig. 1a). This might be due to the significant decrease of benzene, 2-methyl-1-propanol, toluene, 3-methyl-1-butanol, and 2-methyl-1-butanol (Table 3). However, other compounds (2,6-dimethyl-pyrazine, methional and 3-methylbutanal) increased with vacuum storage time. In contrast, nitrate reduction did not affect the total abundance of volatiles derived from amino acid degradation, except for two compounds. An increase of 2,6-dimethyl-pyrazine and a decrease of benzene could be observed as nitrate concentration diminished. Carbohydrate fermentation was the group who represented the highest proportion of volatile compounds throughout vacuum storage (70–75%). Among them, acetic acid and ethanol were the most abundant compounds. Volatile compounds from carbohydrate fermentation decreased significantly after 3 months of storage (Fig. 1b). Ethanol, acetic acid and 2,3-butanediol were less abundant after 3 months, while a reduction in butanoic acid was observed since the second month (Table 3). On the contrary, acetone and 2-butanone increased with vacuum storage. Regarding nitrate reduction, an increase in the compounds generated by carbohydrate fermentation was observed (Fig. 1b). Acetone, acetic acid and 2,3-butanediol were more abundant in RN25 sausages. In contrast, 2,3-butanedione and butanoic acid were more abundant in C batch. Volatile compounds derived from esterase activity decreased after 3 months of storage (Fig. 1c). Ethyl acetate, ethyl butanoate, ethyl 2-hydroxypropanoate, ethyl-3-methylbutanoate and ethyl-2-methylbutanoate decreased at the third month of storage. However, nitrate reduction had not impact on production of these volatile compounds, except for ethyl octanoate which was less abundant in RN25 sausages (Table 3). Regarding volatile compounds derived from lipid β-oxidation, only nitrate reduction produced a significant effect on the total abundance (Fig. 1d). The effect of vacuum storage

time was only seen in few compounds such as 2-heptanone and 1-octen-3-ol which concentration increased and 2,3-pentanedione which showed the opposite effect (Table 3). Moreover, the highest reduction in nitrate (RN25) produced the decrease of 2-heptanone and 1-octen-3-ol. In the same way, lipid oxidation volatile compounds increased with storage time (Fig. 1e). This is the case of pentane, butanal, pentanal, 1-pentanol, hexanal, 2-pentylfuran, and (E)-2-heptenal (Table 3). However, several compounds decrease after 3 months of vacuum storage (propanal, 1-propanol, 2-hexenal and nonanal). Regarding the effect of nitrate content, only the highest nitrate reduction (RN25) produced a significant reduction of the total abundance (Fig. 1e). The strongest decrease in concentration was observed in pentane, heptane, octane, hexanal, hexanoic acid and octanal. Carbon disulphide was identified as an unknown compound which increased with storage time and nitrate reduction (Table 3). Among the 53 volatile compounds present in the sausages, 20 of them were identified as potential aroma contributors by gas chromatography-olfactometry (Perea-Sanz, Montero, Belloch & Flores, 2018). These compounds contribute to specific aroma notes as indicated in Table 3. In order to examine the relationship of the chemical and microbiological parameters with the aroma compounds, a principal component analysis (PCA) was performed (Fig. 2). Two principal components were able to explain the 55.34% of the total variability. PC1 accounts for 32.87% of the variability and distinguishes samples by vacuum storage time as seen by the time progression from right to left quadrant. First months of storage (1 and 2 months, right upper quadrant) were related to aromatic volatile compounds derived from microorganism metabolism (LAB and CN-BP, CN-M): carbohydrate fermentation (2,3-butanedione, 3-hydroxy-2-butanone and acetic acid), esterase activity (ethyl octanoate, ethyl butanoate, ethyl-2-hydroxypropanoate and ethyl-3-methylbutanoate), one compound from lipid β-oxidation (2,3-pentanedione) and amino acid degradation (dimethyl disulphide) as well as to aroma compounds derived from lipid oxidation (1-hexanol and heptanal). In contrast, longer vacuum storage times (3 months) were related to lipid oxidation volatile compounds (2-pentylfuran, 2-methylfuran, octanal, and hexanal), lipid β-oxidation (1-octen-3-ol and 2-heptanone) and compounds from sulphur amino acid degradation (methanethiol and methional) and one from carbohydrate fermentation (2-butanone). PC2 accounts for 22.46% of the variability and distinguishes samples by nitrate content. As can be observed, C and RN15 sausages are placed on the

upper quadrant, and RN25 sausages on the bottom quadrant. In addition, C and RN15 sausages appeared related to most of the aromatic volatile compounds analysed derived from microbial metabolism and lipid oxidation reactions. However, volatile compounds derived from sulphur amino acid degradation (methanethiol and methional) and 2-butanone were related to sausages with 25% nitrate reduction (RN25).

**Table 3. Effect of vacuum storage time and nitrate reduction on volatile compounds generated (expressed as AU×10$^6$) in dry fermented sausages. Values are presented as least squares means.**

| | | | | Vacuum Storage time | | | Nitrate | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LRI[1] | RI[2] | Aroma[6] | 1m | 2m | 3m | C[3] | RN15 | RN25 | $P_t$[4] | *Pn* | RMSE[5] |
| **Amino acid degradation** | | | | | | | | | | | | |
| 2-Methylpropanal | 594 | a | | 0.49 | 0.48 | 0.61 | 0.51 | 0.59 | 0.50 | ns | ns | 0.17 |
| Benzene | 676 | a | | 0.19 a | 0.17 a | 0.13 b | 0.19 ab | 0.18 a | 0.11 b | ** | *** | 0.04 |
| 2-Methyl-1-propanol | 683 | a | | 1.44 a | 0.68 b | 0.99 b | 0.87 | 1.25 | 0.99 | *** | ns | 0.48 |
| 3-Methylbutanal | 691 | a | | 2.23 b | 3.98 a | 4.20 a | 3.44 | 3.36 | 3.77 | *** | ns | 0.87 |
| Dimethyl disulfide | 773 | a | Toasted, garlic | 0.45 | 0.55 | 0.39 | 0.41 | 0.47 | 0.49 | ns | ns | 0.15 |
| Toluene | 788 | a | | 3.44 a | 3.78 a | 2.64 b | 3.55 | 3.09 | 3.22 | *** | ns | 0.73 |
| 3-Methyl-1-butanol | 795 | a | | 10.87 a | 9.89 a | 5.81 b | 9.12 | 8.64 | 8.96 | *** | ns | 2.41 |
| 2-Methyl-1-butanol | 797 | a | | 2.11 a | 1.86 a | 1.11 b | 1.68 | 1.67 | 1.72 | *** | ns | 0.51 |
| 2,6-Dimethylpyrazine | 945 | a | | 1.97 b | 2.65 a | 2.29 b | 1.79 c | 2.23 b | 2.88 a | *** | *** | 0.41 |
| Methional | 968 | a | Cooked potato | 0.65 b | 1.31 a | 1.17 a | 0.88 | 0.86 | 1.31 | * | ns | 0.39 |
| Benzaldehyde | 1020 | a | | 1.23 | 1.39 | 1.40 | 1.24 | 1.34 | 1.43 | ns | ns | 0.37 |
| Benzeneacetaldehyde | 1110 | a | | 0.81 | 0.86 | 0.80 | 0.77 | 0.87 | 0.84 | ns | ns | 0.17 |
| Phenol | 1114 | a | | 2.56 | 2.45 | 2.38 | 2.41 | 2.45 | 2.53 | ns | ns | 0.29 |
| Methanethiol | 473 | a | Rotten | 0.99 | 1.14 | 1.11 | 1.01 | 1.10 | 1.13 | ns | ns | 0.3 |

**Table 3. Continued.**

| | LRI[1] | RI[2] | Aroma[6] | Vacuum Storage time | | | Nitrate | | | $P_t$[4] | *Pn* | RMSE[5] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1m | 2m | 3m | C[3] | RN15 | RN25 | | | |
| **Carbohydrate fermentation** | | | | | | | | | | | | |
| Acetaldehyde | 466 | a | | 5.10 | 5.56 | 4.64 | 5.36 | 5.03 | 4.91 | ns | ns | 1.1 |
| Ethanol | 507 | a | | 291.12 a | 309.72 a | 228.76 b | 263.73 | 300.37 | 265.50 | ** | ns | 66.11 |
| Acetone | 529 | a | | 5.40 b | 7.37 a | 7.40 a | 5.41 b | 6.28 b | 8.47 a | ** | *** | 1.84 |
| 2,3-Butanedione | 627 | a | Cheese, butter | 1.25 | 1.13 | 1.01 | 1.33 a | 0.90 b | 1.16 ab | ns | * | 0.46 |
| 2-Butanone | 631 | a | Fruity, butter | 1.99 b | 3.13 a | 3.43 a | 2.93 | 2.44 | 3.18 | *** | ns | 0.9 |
| Acetic acid | 718 | a | Vinegar | 290.55 a | 300.94 a | 247.70 b | 260.05 b | 278.82 ab | 300.31 a | *** | * | 38.54 |
| 3-Hydroxy-2-butanone | 781 | a | Sweet, fruity | 21.67 | 18.23 | 15.52 | 21.74 | 15.36 | 18.31 | ns | ns | 8.36 |
| 2,3-Butanediol | 888 | a | | 72.17 a | 70.39 a | 45.03 b | 53.98 b | 62.97 ab | 70.64 a | *** | ** | 12.61 |
| Butanoic acid | 896 | a | | 8.51 a | 5.97 b | 3.23 c | 7.09 a | 5.18 b | 5.44 b | *** | *** | 0.68 |
| **Esterase activity** | | | | | | | | | | | | |
| Ethyl acetate | 635 | a | | 112.65 a | 122.35 a | 69.68 b | 94.63 | 109.45 | 100.62 | *** | ns | 28.73 |
| Ethyl butanoate | 832 | a | Fruity | 9.95 a | 9.58 a | 6.57 b | 8.84 | 9.54 | 7.71 | *** | ns | 2.41 |
| Ethyl-2-hydroxypropanoate | 867 | a | Fruity, sweet | 9.54 a | 9.70 a | 6.09 b | 7.45 | 9.31 | 8.57 | *** | ns | 2.2 |
| Ethyl-2-methylbutanoate | 878 | a | | 4.31 a | 4.46 a | 2.73 b | 3.54 | 4.09 | 3.87 | *** | ns | 1.16 |
| Ethyl-3-methylbutanoate | 882 | a | Fruity, sweet | 9.17 a | 10.67 a | 5.67 b | 7.76 | 9.13 | 8.63 | *** | ns | 3.17 |

**Table 3. Continued.**

| | | | | Vacuum Storage time | | | Nitrate | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **LRI[1]** | **RI[2]** | **Aroma[6]** | **1m** | **2m** | **3m** | **C[3]** | **RN15** | **RN25** | ***P*$_t$[4]** | ***Pn*** | **RMSE[5]** |
| Ethyl octanoate | 123 a | | Vegetable, fruity | 5.07 | 5.10 | 4.14 | 4.60 b | 5.93 a | 3.78 b | ns | *** | 1.48 |
| **Lipid β-oxidation** | | | | | | | | | | | | |
| 2,3-Pentanedione | 745 a | | Sweet, candy | 2.17 ab | 2.47 a | 1.66 b | 2.19 | 2.19 | 1.92 | ** | ns | 0.65 |
| 2-Heptanone | 935 a | | Rancid, fruity | 1.59 b | 1.99 a | 2.09 a | 1.97 a | 2.01 a | 1.70 b | *** | ** | 0.31 |
| 1-Octen-3-ol | 1033 a | | Mushroom | 2.31 b | 2.77 a | 2.75 ab | 2.75 a | 2.92 a | 2.16 b | * | *** | 0.55 |
| **Lipid oxidation** | | | | | | | | | | | | |
| Pentane | 500 a | | | 3.40 b | 5.28 a | 4.49 ab | 4.63 a | 5.40 a | 3.14 b | ** | *** | 1.43 |
| Propanal | 524 a | | | 0.73 ab | 0.91 a | 0.66 b | 0.76 ab | 0.93 a | 0.61 b | * | ** | 0.22 |
| Hexane | 600 a | | | 1.77 | 1.85 | 1.61 | 1.86 b | 2.30 a | 1.07 c | ns | *** | 0.5 |
| 1-Propanol | 612 a | | | 2.32 a | 1.37 b | 1.02 b | 1.09 b | 2.36 a | 1.26 b | * | ** | 0.8 |
| 2-Methylfuran | 616 a | | Green, garlic | 0.14 | 0.20 | 0.17 | 0.20 | 0.17 | 0.15 | ns | ns | 0.06 |
| Butanal | 622 a | | | 0.06 b | 0.13 a | 0.15 a | 0.14 a | 0.11 ab | 0.08 b | *** | *** | 0.04 |
| Heptane | 700 a | | | 15.36 | 16.81 | 17.45 | 19.65 a | 20.18 a | 9.77 b | ns | *** | 4.67 |
| Pentanal | 739 a | | | 2.62 c | 4.43 b | 5.68 a | 4.74 a | 4.91 a | 3.07 b | *** | *** | 0.92 |
| Octane | 800 a | | | 22.36 | 21.94 | 24.11 | 26.15 a | 28.54 a | 13.71 b | ns | *** | 6.95 |
| 1-Pentanol | 827 a | | | 2.20 b | 3.20 a | 2.97 a | 3.07 a | 3.38 a | 1.92 b | ** | *** | 0.87 |
| Hexanal | 842 a | | Fresh cut grass | 25.90 b | 33.44 ab | 40.19 a | 35.03 a | 40.30 a | 24.20 b | *** | *** | 9.41 |

**Table 3. Continued.**

| | | | | Vacuum Storage time | | | Nitrate | | | $P_t$[4] | *Pn* | RMSE[5] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LRI[1] | RI[2] | Aroma[6] | 1m | 2m | 3m | C[3] | RN15 | RN25 | | | |
| 2-Hexenal | 907 | a | | 0.24 a | 0.21 a | 0.14 b | 0.18 | 0.22 | 0.20 | *** | ns | 0.05 |
| 1-Hexanol | 924 | a | Oxidized fat | 6.70 | 7.11 | 5.35 | 6.17 | 7.34 | 5.65 | ns | ns | 2.08 |
| Heptanal | 941 | a | Green | 7.61 | 7.92 | 7.28 | 5.69 b | 8.21 a | 8.92 a | ns | *** | 2.15 |
| Decane | 1000 | a | | 0.40 | 0.43 | 0.42 | 0.39 | 0.45 | 0.42 | ns | ns | 0.09 |
| 2-Pentylfuran | 1010 | a | Garlic, onion | 1.42 b | 1.65 ab | 1.75 a | 1.60 ab | 1.82 a | 1.40 b | * | *** | 0.32 |
| (E)-2-Heptenal | 1013 | a | | 0.15 b | 0.24 a | 0.20 ab | 0.19 | 0.20 | 0.19 | ** | ns | 0.07 |
| Octanal | 1049 | a | Orange, sweet | 3.30 | 3.25 | 3.42 | 3.23 ab | 3.77 a | 2.98 b | ns | * | 0.81 |
| Hexanoic acid | 1079 | a | | 3.46 | 3.59 | 3.67 | 3.76 a | 4.10 a | 2.85 b | ns | *** | 0.83 |
| Nonanal | 1151 | a | | 5.70 a | 5.34 ab | 4.83 b | 4.59 b | 5.70 a | 5.58 b | * | *** | 0.92 |
| **Unknown compound** | | | | | | | | | | | | |
| Carbon disulfide | 537 | a | | 3.65 b | 6.58 a | 4.12 b | 4.16 b | 4.56 b | 5.65 a | *** | ** | 1.18 |

[1] LRI: Linear retention index of the compounds eluted from the GC–MS.
[2] RI: Reliability of identification: a, identification by mass spectrum, coincidence with the LRI of an authentic standard; b, tentatively identification by mass spectrum.
[3] C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm).
[4] *Pt*: *P* value of storage time effect and *Pn*: *P* value of nitrate reduction effect. Different letters in the same row of each group indicate significant differences at *** $P < .001$, ** $P < .01$, * $P < .05$. ns: $P > .05$.
[5] RMSE: root mean square error.
[6] Compounds detected as aroma active compound by GC-olfactometry (Perea-Sanz et al., 2018).

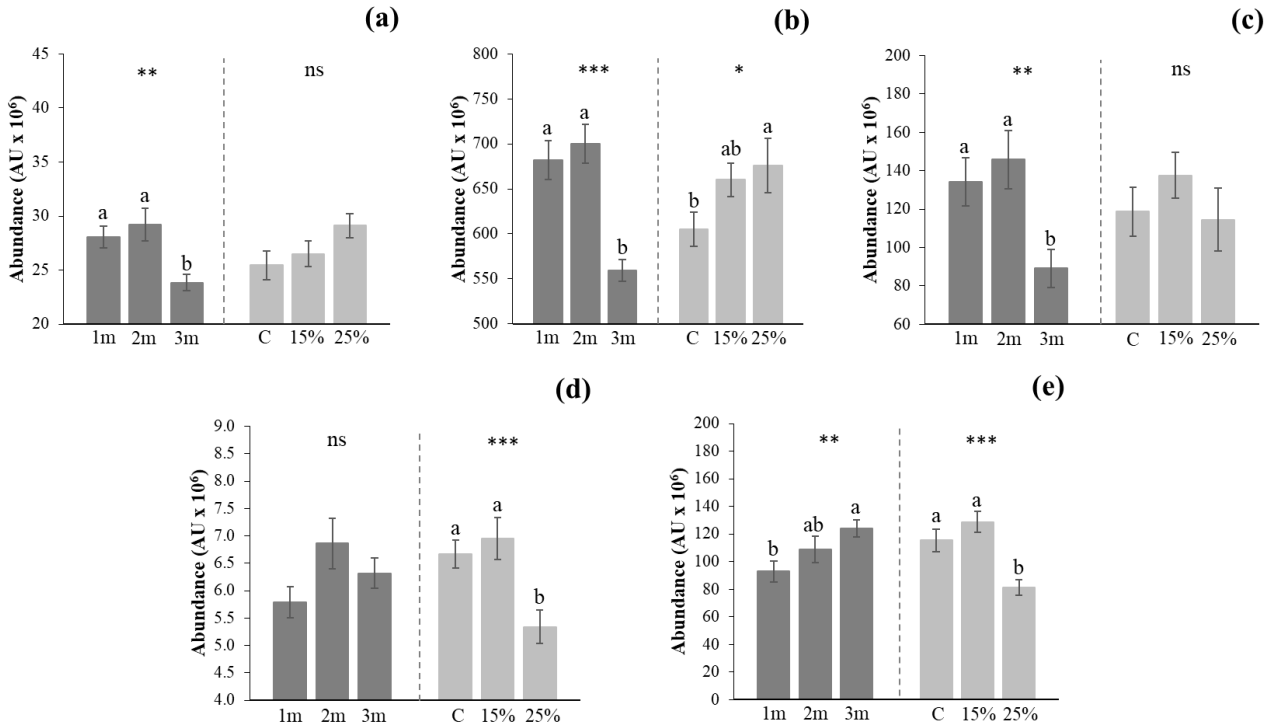


**Figure 1**. Abundance of volatile compounds (Au × $10^6$) according to storage time (1, 2 or 3 m of vacuum storage) and nitrate reduction (C: control batch 250 ppm sodium nitrate; 15% (RN15): 15% reduction 212.5 ppm; 25% (RN25): 25% reduction 187.5 ppm). Different letters in each group indicate significant differences: *** $P < .001$, ** $P < .01$, * $P < .05$. ns: $P > .05$. Volatile compounds grouped according to origin: derived from bacterial metabolism (a: amino acid degradation; b: carbohydrate fermentation; c: esterase activity, d: lipid β-oxidation reactions) and chemical reactions (e: lipid oxidation).

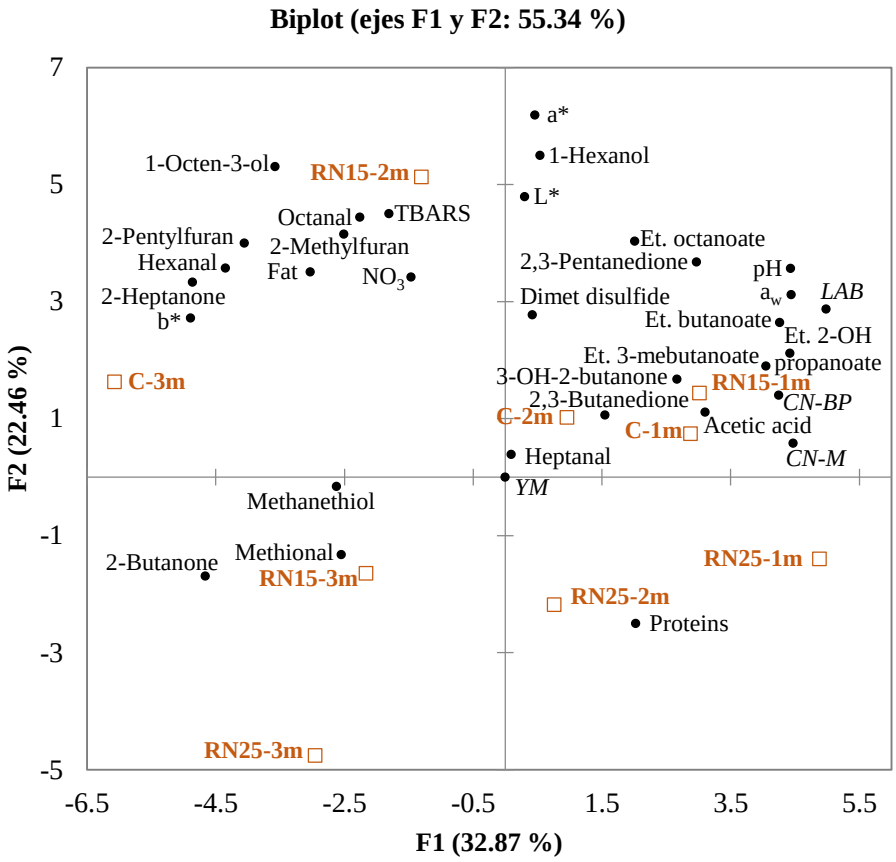


**Figure 2.** Loadings of the first two principal components (PC1-PC2) of the analysed parameters (physicochemical and microbiological parameters and aroma volatile compounds) in dry fermented sausages based on nitrate content: C: control batch 250 ppm sodium nitrate; RN15: 15% reduction 212.5 ppm; RN25: 25% reduction 187.5 ppm, and vacuum storage (1, 2 and 3 m). Abbreviations are indicated in Tables 1 and 2.

## 4. Discussion

During vacuum storage, dry fermented sausages underwent changes on physicochemical and microbiological characteristics as reported by Kim et al. (2012) and Rubio et al. (2007). The general decrease of microbial counts during vacuum storage (Table 2) might have an impact on organoleptic sausage quality. This general decline in microbial counts during vacuum storage appears to be the main consequence of low pH and $a_w$, which act as hurdles for microbial growth (Christieans et al., 2018; Leistner, 2000). The slight but continuous pH decrease observed at successive months of storage (Table 1) may be due to the metabolic activity of LAB, which are still active although to a lesser extent. Similar results were reported by Rubio et al. (2007) in sliced sausages under vacuum storage and modified atmospheres, despite no changes in $a_w$ were seen. In agreement with our results, Bañon et al. (2014) and Tabanelli et al. (2013) reported a general microbial growth inhibition possibly due to the $a_w$ decrease. Other authors have described few changes in microbial counts and no effect on pH values (Hospital et al., 2014; Kim et al., 2012). On the contrary, an increase in sausage pH under vacuum conditions (Ščetar et al., 2013), modified atmospheres (Ščetar et al., 2013; Tabanelli et al., 2013) and perforated packages (Bañon et al., 2014) has been demonstrated in other studies. Regarding sausage colour, a redness decrease (Table 1) was reported in sausages storage under vacuum (Summo et al., 2006; Summo et al., 2010) and in entire sausages stored in perforated packages (Bañon et al., 2014). In fact, several authors indicated that vacuum packaged produce less redness intensity than modified atmosphere packaging (Rubio et al., 2008; Zanardi et al., 2002) or perforated packaging (Summo et al., 2006). On the contrary, other authors indicated an increase in redness (a*) under vacuum packed storage (Kim et al., 2012; Rubio et al., 2008).

Concerning lipid oxidation, different results have been reported during vacuum storage of dry fermented sausages. Rubio et al. (2008) observed a decrease on lipid oxidation value in agreement with our results (Table 1), while others did not observe changes (Summo et al., 2010). However, many studies have reported an increase in lipid oxidation values during vacuum storage (Dos Santos et al., 2017; Kim et al., 2012; Summo et al., 2006, 2010; Ščetar et al., 2013; Zanardi et al., 2002) and under modified atmosphere (Ščetar et al., 2013; Zanardi et al., 2002;). Different patterns of lipid oxidation can be explained by different ingredients, such as spices with antioxidant activity (Yashin, Yashin, Xia, & Nemzer, 2017), in addition

to the manufacture process. Moreover, the low specificity of the TBARS test contributes to the observed differences since malonaldehyde is an unstable molecule and could react with other compounds present in the meat matrix (Janero, 1990).

The general decrease observed in microbial counts is in agreement with previous studies (Bañon et al., 2014; Rubio et al., 2007). Despite the decrease in LAB and Gram positive cocci inoculated with the bacterial starter, pH decreased slightly during storage suggesting the existence of bacteria metabolic activity. The low pH and $a_w$ effectively prevented growth of pathogenic bacteria as *Salmonella* spp., *Listeria* spp., Gram positive coagulase positive cocci and *Clostridium* spp. even in RN25 sausages (Bañon et al., 2014). Therefore, our results suggest that no apparent risk regarding microbial safety can be attributed to sausages stored in the conditions utilised in our study.

Microbial growth is related to volatile compounds production through their metabolism. LAB generate volatile compounds from amino acid degradation and carbohydrate fermentation reactions together with staphylococci, which also generate ethyl esters with fruity notes through their esterase activity (Flores & Olivares, 2015). During vacuum storage a general decrease of volatile compounds derived from microbial activity was observed (Fig. 1). Similar results under vacuum storage were reported by Summo et al. (2011). These authors found a decrease of volatile compounds derived from carbohydrate fermentation during its shelf-life under vacuum storage, in addition to an increase of volatile compounds derived from lipid oxidation (Fig. 1). On the contrary, Dos Santos et al. (2015) observed an increase of volatile compounds derived from amino acid degradation and carbohydrate fermentation in addition to those from lipid oxidation. Differences between studies can be due to different sausage manufacture process, use of spices and smoking process (Summo et al., 2011).

Nitrate residual content was not affected by the storage time under vacuum although the residual concentration detected declined between 44 and 51% respect to the initial amount measured in the minced meat in C, RN15 and RN25 sausages (Perea-Sanz etal., 2018). The absence of nitrate reduction during vacuum storage could be due to a low nitrate reductase activity available during storage due to the low Gram positive cocci counts (Table 2) and the pH value close to 5.0 that inhibit this activity (Sánchez Mainar & Leroy, 2015). Nevertheless, the reduction of nitrate ingoing amounts in fermented sausages produced

changes in the production of volatile compounds although nitrate reduction did not affect directly microbial growth but affected microbial metabolism (Perea-Sanz et al., 2018). Nitrate reduced sausages had less nitrite available and therefore, lowest antioxidant activity, but the highest oxidation reactions were detected in control sausages due to its high fat content (Olivares et al., 2010). This fact is in accordance with volatile compounds derived from lipid oxidation and lipid β-oxidation, which were in high abundance in control sausages. Moreover, reduced nitrite antimicrobial activity in nitrate reduced sausages may be the reason for high Gram positive cocci counts (CN-M and CN-BP) as observed by Hospital et al. (2014) after thirty days of vacuum storage. The higher counts of Gram positive cocci detected in nitrate reduced sausages (RN15 and RN25) would be responsible for the high amount of volatile compounds derived from carbohydrate fermentation. Similarly, the increment in the generation of volatile compounds from amino acid degradation and ester compounds observed in nitrate reduced sausages would be the result of high counts of *Staphylococci* (Flores & Olivares, 2015), as LAB were insignificantly affected by nitrate reduction.

Changes in volatile compounds produced by vacuum storage of slow fermented sausages (Table 3) affected the aroma profile of the product (Fig. 2). Under vacuum storage, several authors observed a decline of the characteristic sausage aroma and quality as reported by Kim et al. (2012), Rubio et al. (2007) and Summo et al. (2006). Packaging under modified atmosphere altered the sausage volatile profile and produced a more intense "raw meat" aroma and a less distinct "dry sausage" aroma (Viallon et al., 1996). The effect on the volatile profile was related to the increase in ethanol, diacetyl, acetoin and restriction of acetic acid, 1,3-butanediol and 2,3-butanediol (Viallon et al., 1996). Moreover, packaging under vacuum storage produced a limited number of lipid oxidation compounds as reported by Viallon et al. (1996), in opposition to the results observed in Fig. 2. The present results demonstrate the relationship of microbiological and physicochemical characteristics and the effect of factors, vacuum storage and nitrate reduction, on sausage aroma. The compounds with pleasant and sweet aroma (3-hydroxy-2-butanone, ethyl octanoate, ethyl-3-methylbutanoate and 2,3-pentanedione) and with cheesy/buttery odour (2,3-butanedione and ethyl-2-hydroxypropanoate) were related to short vacuum storage times and to control and 15% reduced nitrate sausages. In contrast, the characteristic "dry sausage" aroma loss might be

the result of the increase of volatile compounds such as heptanal (green, unpleasant odour) and compounds related to unpleasant odours, methanethiol (rotten odour) and methional (cooked potato) (Perea-Sanz et al., 2018). In summary, small nitrate reductions of 15% did not produce a significant effect on aroma profile in slow fermented sausages in contrast to the more negative effect produced by a reduction of 25% nitrate.

## 5. Conclusion

Vacuum storage and reduced amounts of ingoing nitrate influenced the shelf-life of slow fermented sausages in terms of microbial and organoleptic characteristics. Microbial growth was affected mainly by vacuum storage and to a lesser extent by nitrate content, leading to changes in the profile of volatile compounds. On the one hand, vacuum storage time produced a decrease in volatile compounds derived from amino acid degradation, carbohydrate fermentation and esterase activity after three months under vacuum. On the other hand, the reduction of ingoing nitrate amounts caused a decrease of volatile compounds derived from lipid oxidation and β-oxidation reactions. These changes affected the production of key aroma compounds and sausage aroma. More studies are necessary to elucidate the mechanism involved in the effect of nitrate reduction during vacuum storage in slow fermented sausages to determine the appropriate sausage shelf-life.

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# SUPPLEMENTARY MATERIAL

**Table 1 supplementary material. Effect of vacuum storage time and nitrate reduction on physicochemical parameters of dry fermented sausages. Values are presented as least squares means.**

| | 1m | | | | | | 2m | | | | | | 3m | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C[1] | | RN15 | | RN25 | | C | | RN15 | | RN25 | | C | | RN15 | | RN25 | |
| **pH** | 5.10 | (0.01) | 5.12 | (0.03) | 5.06 | (0.03) | 5.06 | (0.01) | 5.02 | (0.02) | 5.01 | (0.02) | 4.99 | (0.01) | 4.98 | (0.02) | 4.92 | (0.01) |
| **Aw** | 0.890 | (0.003) | 0.890 | (0.001) | 0.890 | (0.002) | 0.880 | (0.001) | 0.890 | (0.001) | 0.880 | (0.001) | 0.880 | (0.001) | 0.870 | (0.004) | 0.880 | (0.003) |
| **Moisture (%)** | 39.44 | (0.45) | 40.33 | (0.65) | 41.11 | (0.48) | 40.78 | (0.71) | 43.21 | (0.51) | 43.27 | (0.40) | 41.52 | (0.33) | 42.20 | (0.51) | 43.06 | (0.89) |
| **Protein (% dm)** | 52.63 | (0.70) | 54.95 | (0.27) | 58.50 | (1.10) | 52.32 | (0.45) | 57.43 | (1.07) | 59.37 | (0.66) | 50.18 | (0.81) | 54.09 | (0.92) | 57.75 | (0.85) |
| **Fat (% dm)** | 33.49 | (0.62) | 29.54 | (0.60) | 29.08 | (1.37) | 37.32 | (0.70) | 33.69 | (1.26) | 30.26 | (0.54) | 38.27 | (0.42) | 33.76 | (0.50) | 29.37 | (0.67) |
| **L*** | 47.77 | (0.75) | 48.57 | (0.48) | 47.97 | (0.67) | 48.86 | (0.41) | 48.12 | (0.51) | 47.36 | (0.66) | 48.54 | (0.59) | 47.89 | (0.64) | 47.05 | (0.28) |
| **a*** | 18.56 | (0.39) | 18.44 | (0.31) | 17.90 | (0.29) | 18.64 | (0.22) | 19.01 | (0.22) | 18.10 | (0.31) | 18.32 | (0.23) | 17.75 | (0.24) | 17.84 | (0.23) |
| **b*** | 6.70 | (0.08) | 6.94 | (0.13) | 6.39 | (0.10) | 7.19 | (0.14) | 7.37 | (0.19) | 6.71 | (0.15) | 7.60 | (0.11) | 7.39 | (0.16) | 7.03 | (0.20) |
| **TBARS[2]** | 1.14 | (0.11) | 1.08 | (0.03) | 0.78 | (0.07) | 1.07 | (0.05) | 0.66 | (0.08) | 0.53 | (0.05) | 0.84 | (0.07) | 0.82 | (0.06) | 0.55 | (0.09) |
| **$NO_3$ (ppm dm)** | 241.81 | (15.66) | 199.53 | (7.32) | 152.06 | (15.46) | 216.41 | (22.09) | 184.23 | (8.96) | 169.79 | (23.24) | 248.88 | (10.51) | 173.42 | (14.03) | 166.03 | (7.83) |

The numbers in parentheses represent standard errors. [1]C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm). [2]TBARS expressed as µg malonaldehyde/g dm.

**Table 2 supplementary material. Effect of vacuum storage time and nitrate reduction on microbial counts (log cfu/g) of dry fermented sausages. Values are presented as least squares means.**

| | Culture medium | 1m | | | | | | 2m | | | | | | 3m | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C[1] | | RN15 | | RN25 | | C | | RN15 | | RN25 | | C | | RN15 | | RN25 | |
| **Total mesophilic bacteria (TMB)** | PCA[2] | 7.64 | (0.03) | 7.68 | (0.07) | 7.59 | (0.07) | 7.39 | (0.07) | 7.57 | (0.05) | 7.44 | (0.01) | 6.33 | (0.11) | 6.74 | (0.02) | 6.47 | (0.19) |
| ***Lactobacillus* (LAB)** | MRS | 6.63 | (0.03) | 6.64 | (0.08) | 6.60 | (0.03) | 6.39 | (0.05) | 6.54 | (0.09) | 6.49 | (0.03) | 5.45 | (0.17) | 5.72 | (0.07) | 5.49 | (0.16) |
| **Gram positive cocci (CN-M)** | MSA | 3.63 | (0.06) | 3.76 | (0.17) | 3.66 | (0.13) | 0.70 | (0.70) | 2.53 | (0.21) | 2.76 | (0.10) | 0.00 | (0.00) | 2.38 | (0.06) | 2.44 | (0.05) |
| **Gram positive cocci (CN-BP)** | BP | 3.96 | (0.01) | 4.26 | (0.09) | 3.98 | (0.04) | 3.20 | (0.14) | 3.48 | (0.07) | 3.47 | (0.07) | 2.29 | (0.15) | 2.82 | (0.08) | 2.67 | (0.03) |

The numbers in parentheses represent standard errors. [1]C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm). [2]PCA: Plate Count Agar, MRS: Man, Rogosa and Sharpe agar, MSA: Mannitol Salt Agar, BP: Baird Parker Agar.

**Table 3 supplementary material. Effect of vacuum storage time and nitrate reduction on volatile compounds generated (expressed as AU x $10^6$) in dry fermented sausages. Values are presented as least squares means.**

| | LRI[1] | RI[2] | 1m | | | | | | 2m | | | | | | 3m | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C[3] | | RN15 | | RN25 | | C | | RN15 | | RN25 | | C | | RN15 | | RN25 | |
| **Amino acid degradation** | | | | | | | | | | | | | | | | | | | | |
| 2-Methyl propanal | 594 | a | 0.39 | (0.05) | 0.78 | (0.21) | 0.33 | (0.03) | 0.42 | (0.05) | 0.50 | (0.06) | 0.53 | (0.06) | 0.68 | (0.07) | 0.50 | (0.05) | 0.65 | (0.04) |
| Benzene | 676 | a | 0.19 | (0.01) | 0.18 | (0.03) | 0.19 | (0.02) | 0.22 | (0.02) | 0.19 | (0.03) | 0.08 | (0.01) | 0.16 | (0.02) | 0.17 | (0.01) | 0.05 | (0.01) |
| 2-Methyl 1-propanol | 683 | a | 1.63 | (0.18) | 0.95 | (0.11) | 1.70 | (0.41) | 0.47 | (0.03) | 0.84 | (0.15) | 0.73 | (0.10) | 0.37 | (0.07) | 1.83 | (0.35) | 0.60 | (0.12) |
| 3-Methyl butanal | 691 | a | 2.43 | (0.59) | 2.09 | (0.16) | 2.16 | (0.22) | 3.03 | (0.32) | 4.29 | (0.44) | 4.45 | (0.51) | 4.61 | (0.40) | 3.28 | (0.37) | 4.70 | (0.35) |
| Dimethyl disulfide | 773 | a | 0.46 | (0.11) | 0.40 | (0.03) | 0.46 | (0.06) | 0.38 | (0.04) | 0.69 | (0.08) | 0.52 | (0.07) | 0.39 | (0.03) | 0.30 | (0.06) | 0.50 | (0.06) |
| Toluene | 788 | a | 3.46 | (0.23) | 3.36 | (0.31) | 3.51 | (0.48) | 4.61 | (0.32) | 3.09 | (0.39) | 3.65 | (0.28) | 2.58 | (0.30) | 2.83 | (0.19) | 2.51 | (0.17) |
| 3-methyl 1-butanol | 795 | a | 10.71 | (1.31) | 10.18 | (0.80) | 11.72 | (1.05) | 11.29 | (1.41) | 9.52 | (1.42) | 9.09 | (1.13) | 5.04 | (0.45) | 6.20 | (0.34) | 6.07 | (0.47) |
| 2-methyl 1-butanol | 797 | a | 2.04 | (0.31) | 2.00 | (0.21) | 2.33 | (0.26) | 1.95 | (0.26) | 1.94 | (0.27) | 1.70 | (0.25) | 0.94 | (0.10) | 1.11 | (0.13) | 1.24 | (0.13) |
| 2.6-Dimethyl pyrazine | 945 | a | 1.53 | (0.13) | 1.75 | (0.04) | 2.62 | (0.19) | 1.97 | (0.12) | 2.54 | (0.17) | 3.45 | (0.27) | 1.88 | (0.20) | 2.43 | (0.15) | 2.58 | (0.14) |
| 3-(methylthio)propanal | 968 | a | 0.94 | (0.27) | 0.45 | (0.13) | 0.51 | (0.23) | 0.98 | (0.10) | 1.43 | (0.00) | 1.39 | (0.24) | 0.75 | (0.04) | 1.02 | (0.11) | 1.54 | (0.15) |
| Benzaldehyde | 1020 | a | 1.24 | (0.22) | 1.50 | (0.26) | 0.99 | (0.10) | 1.15 | (0.18) | 1.32 | (0.21) | 1.74 | (0.12) | 1.35 | (0.10) | 1.19 | (0.16) | 1.62 | (0.11) |
| Benzeneacetaldehyde | 1110 | a | 0.82 | (0.09) | 0.96 | (0.15) | 0.64 | (0.03) | 0.65 | (0.07) | 0.90 | (0.06) | 1.03 | (0.06) | 0.85 | (0.05) | 0.73 | (0.07) | 0.83 | (0.05) |
| Phenol | 1114 | a | 2.67 | (0.24) | 2.49 | (0.14) | 2.52 | (0.03) | 2.11 | (0.04) | 2.61 | (0.11) | 2.63 | (0.10) | 2.44 | (0.11) | 2.26 | (0.03) | 2.44 | (0.06) |
| Methanethiol | 473 | a | 0.97 | (0.09) | 1.13 | (0.13) | 0.87 | (0.08) | 0.88 | (0.09) | 1.18 | (0.16) | 1.35 | (0.18) | 1.19 | (0.14) | 0.98 | (0.08) | 1.17 | (0.07) |

**Table 3 supplementary material. Continued.**

| | LRI[1] | RI[2] | 1m | | | | | | 2m | | | | | | 3m | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C[3] | | RN15 | | RN25 | | C | | RN15 | | RN25 | | C | | RN15 | | RN25 | |
| **Carbohydrate fermentation** | | | | | | | | | | | | | | | | | | | | |
| Acetaldehyde | 466 | a | 5.65 | (0.52) | 5.40 | (0.46) | 4.23 | (0.22) | 5.67 | (0.63) | 4.98 | (0.39) | 6.02 | (0.64) | 4.62 | (0.29) | 4.70 | (0.44) | 4.48 | (0.54) |
| Ethyl alcohol | 507 | a | 287.42 | (30.36) | 298.02 | (19.12) | 287.92 | (26.68) | 294.14 | (11.93) | 340.53 | (49.25) | 294.48 | (33.27) | 209.62 | (27.07) | 262.56 | (11.50) | 214.10 | (21.03) |
| Acetone | 529 | a | 4.09 | (0.59) | 6.14 | (0.87) | 5.99 | (0.81) | 4.67 | (0.34) | 7.49 | (0.60) | 9.95 | (1.06) | 7.50 | (0.61) | 5.22 | (0.52) | 9.47 | (0.94) |
| 2.3-Butanedione | 627 | a | 1.36 | (0.24) | 0.93 | (0.11) | 1.46 | (0.18) | 1.40 | (0.29) | 0.95 | (0.29) | 1.01 | (0.16) | 1.21 | (0.22) | 0.80 | (0.07) | 1.02 | (0.11) |
| 2-Butanone | 631 | a | 2.32 | (0.45) | 1.62 | (0.18) | 2.04 | (0.20) | 2.78 | (0.44) | 3.09 | (0.51) | 3.48 | (0.40) | 3.73 | (0.48) | 2.58 | (0.34) | 3.99 | (0.30) |
| Acetic acid | 718 | a | 261.31 | (20.42) | 280.34 | (8.26) | 329.49 | (19.60) | 271.85 | (12.50) | 306.46 | (22.20) | 324.55 | (21.26) | 246.50 | (7.92) | 249.69 | (10.30) | 246.90 | (14.16) |
| 3-hydroxy-2-butanone | 781 | a | 20.98 | (2.70) | 17.77 | (1.62) | 26.21 | (5.61) | 24.93 | (4.88) | 15.72 | (5.39) | 13.55 | (2.54) | 18.28 | (3.65) | 13.14 | (1.22) | 15.66 | (2.08) |
| 2.3-Butanediol | 888 | a | 62.84 | (6.48) | 68.48 | (3.15) | 85.17 | (7.25) | 56.00 | (2.98) | 76.33 | (7.17) | 78.84 | (6.92) | 43.11 | (2.39) | 44.10 | (2.73) | 47.90 | (3.41) |
| Butanoic acid | 896 | a | 8.20 | (0.42) | 8.60 | (0.29) | 8.76 | (0.42) | 7.45 | (0.25) | 1.18 | (0.14) | 1.01 | (0.13) | 5.56 | (0.22) | 5.77 | (0.42) | 6.64 | (0.36) |
| **Esterase activity** | | | | | | | | | | | | | | | | | | | | |
| Ethyl acetate | 635 | a | 109.20 | (12.46) | 104.29 | (9.59) | 124.78 | (15.41) | 118.63 | (5.74) | 127.65 | (18.71) | 122.99 | (22.95) | 51.63 | (6.17) | 96.08 | (11.50) | 56.92 | (6.90) |
| Ethyl butanoate | 832 | a | 10.90 | (1.32) | 10.12 | (0.93) | 8.61 | (0.90) | 10.52 | (0.66) | 9.67 | (1.63) | 8.53 | (1.42) | 4.68 | (0.49) | 8.82 | (0.66) | 5.78 | (0.73) |
| Ethyl 2-hydroxy propanoate | 867 | a | 8.93 | (1.10) | 9.58 | (1.03) | 9.97 | (1.09) | 9.02 | (0.52) | 10.55 | (1.29) | 9.47 | (1.48) | 4.10 | (0.43) | 7.75 | (0.50) | 6.11 | (0.56) |
| Ethyl 2-methyl butanoate | 878 | a | 4.30 | (0.37) | 3.68 | (0.40) | 4.85 | (0.79) | 4.47 | (0.42) | 4.89 | (0.53) | 3.98 | (0.98) | 1.69 | (0.20) | 3.60 | (0.39) | 2.77 | (0.31) |
| Ethyl 3-methyl butanoate | 882 | a | 9.65 | (1.01) | 7.99 | (0.79) | 9.59 | (1.56) | 9.67 | (0.92) | 10.98 | (1.19) | 10.99 | (2.87) | 3.60 | (0.46) | 8.14 | (0.98) | 5.04 | (0.54) |
| Ethyl octanoate | 123 | a | 5.19 | (0.65) | 5.89 | (0.86) | 4.05 | (0.59) | 5.27 | (0.35) | 5.91 | (0.89) | 4.02 | (0.67) | 3.27 | (0.44) | 5.87 | (0.62) | 3.08 | (0.45) |

**Table 3 supplementary material. Continued.**

| | LRI[1] | RI[2] | 1m | | | | | | 2m | | | | | | 3m | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C[3] | | RN15 | | RN25 | | C | | RN15 | | RN25 | | C | | RN15 | | RN25 | |
| **β Oxidation** | | | | | | | | | | | | | | | | | | | | |
| 2.3-Pentanedione | 745 | a | 2.34 | (0.35) | 1.77 | (0.22) | 2.36 | (0.53) | 2.68 | (0.32) | 2.62 | (0.38) | 1.97 | (0.36) | 1.50 | (0.07) | 2.09 | (0.18) | 1.28 | (0.16) |
| 2-Heptanone | 935 | a | 1.63 | (0.16) | 1.72 | (0.13) | 1.41 | (0.09) | 1.76 | (0.05) | 2.32 | (0.16) | 1.89 | (0.15) | 2.50 | (0.16) | 1.98 | (0.11) | 1.78 | (0.06) |
| 1-Octen-3-ol | 1033 | a | 2.39 | (0.25) | 2.51 | (0.22) | 2.02 | (0.14) | 2.64 | (0.25) | 3.55 | (0.32) | 2.19 | (0.28) | 3.22 | (0.16) | 2.69 | (0.10) | 2.33 | (0.28) |
| **Lipid oxidation** | | | | | | | | | | | | | | | | | | | | |
| Pentane | 500 | a | 3.09 | (0.42) | 4.30 | (0.44) | 2.86 | (0.28) | 4.82 | (0.48) | 7.74 | (1.13) | 3.20 | (0.67) | 5.90 | (0.67) | 4.17 | (0.47) | 3.40 | (0.51) |
| Propanal | 524 | a | 0.80 | (0.03) | 0.73 | (0.07) | 0.68 | (0.04) | 0.71 | (0.08) | 1.30 | (0.25) | 0.72 | (0.07) | 0.76 | (0.07) | 0.78 | (0.07) | 0.44 | (0.06) |
| Hexane | 600 | a | 1.46 | (0.16) | 2.77 | (0.39) | 1.08 | (0.11) | 2.02 | (0.17) | 2.60 | (0.36) | 0.94 | (0.12) | 2.10 | (0.19) | 1.52 | (0.15) | 1.20 | (0.08) |
| 1-Propanol | 612 | a | 1.10 | (0.11) | 4.67 | (1.95) | 1.21 | (0.14) | 1.14 | (0.07) | 1.50 | (0.17) | 1.48 | (0.16) | 1.03 | (0.11) | 0.93 | (0.11) | 1.07 | (0.13) |
| 2-Metylfuran | 616 | a | 0.13 | (0.02) | 0.11 | (0.01) | 0.18 | (0.02) | 0.19 | (0.04) | 0.29 | (0.01) | 0.12 | (0.03) | 0.27 | (0.03) | 0.11 | (0.01) | 0.14 | (0.03) |
| Butanal | 622 | a | 0.08 | (0.01) | 0.05 | (0.01) | 0.05 | (0.01) | 0.14 | (0.01) | 0.17 | (0.04) | 0.07 | (0.01) | 0.22 | (0.02) | 0.11 | (0.01) | 0.12 | (0.02) |
| Heptane | 700 | a | 16.24 | (2.86) | 21.10 | (2.22) | 8.73 | (1.28) | 19.45 | (2.27) | 21.42 | (3.10) | 8.99 | (1.49) | 23.03 | (1.50) | 17.96 | (1.96) | 11.35 | (1.33) |
| Pentanal | 739 | a | 2.53 | (0.36) | 3.16 | (0.33) | 2.16 | (0.23) | 4.89 | (0.14) | 6.17 | (0.77) | 2.22 | (0.38) | 6.76 | (0.19) | 5.44 | (0.34) | 4.81 | (0.45) |
| Octane | 800 | a | 24.35 | (4.64) | 30.73 | (4.50) | 12.38 | (1.39) | 22.95 | (2.72) | 28.64 | (4.45) | 13.53 | (2.67) | 30.84 | (1.57) | 26.2 | (2.60) | 15.29 | (1.65) |
| 1-Pentanol | 827 | a | 2.74 | (0.36) | 2.44 | (0.46) | 1.36 | (0.17) | 3.23 | (0.23) | 4.56 | (0.51) | 1.82 | (0.53) | 3.23 | (0.15) | 3.13 | (0.36) | 2.55 | (0.28) |
| Hexanal | 842 | a | 25.21 | (3.90) | 34.27 | (3.72) | 18.23 | (1.50) | 33.47 | (3.02) | 47.17 | (7.62) | 18.81 | (3.24) | 46.55 | (4.32) | 38.58 | (2.08) | 35.43 | (3.82) |
| 2-Hexenal | 907 | a | 0.22 | (0.03) | 0.24 | (0.02) | 0.26 | (0.02) | 0.19 | (0.02) | 0.23 | (0.03) | 0.20 | (0.03) | 0.12 | (0.01) | 0.18 | (0.02) | 0.14 | (0.01) |

**Table 3 supplementary material. Continued.**

| | LRI[1] | RI[2] | 1m | | | | | | 2m | | | | | | 3m | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C[3] | | RN15 | | RN25 | | C | | RN15 | | RN25 | | C | | RN15 | | RN25 | |
| 1-Hexanol | 924 | a | 6.90 | (0.86) | 7.07 | (0.98) | 5.99 | (0.68) | 4.82 | (0.45) | 10.14 | (1.22) | 6.44 | (1.40) | 6.84 | (0.97) | 4.80 | (0.36) | 4.52 | (0.52) |
| Heptanal | 941 | a | 5.87 | (1.30) | 7.12 | (0.84) | 9.84 | (1.19) | 3.71 | (0.26) | 10.45 | (0.77) | 9.59 | (0.80) | 7.48 | (0.73) | 7.04 | (0.80) | 7.33 | (0.55) |
| Decane | 1000 | a | 0.41 | (0.02) | 0.45 | (0.03) | 0.35 | (0.02) | 0.35 | (0.03) | 0.48 | (0.07) | 0.44 | (0.04) | 0.39 | (0.04) | 0.42 | (0.05) | 0.46 | (0.03) |
| 2-Pentylfuran | 1010 | a | 1.43 | (0.22) | 1.56 | (0.16) | 1.26 | (0.11) | 1.37 | (0.13) | 2.08 | (0.16) | 1.50 | (0.12) | 2.00 | (0.10) | 1.81 | (0.08) | 1.43 | (0.10) |
| (E)- 2-Heptenal | 1013 | a | 0.16 | (0.02) | 0.17 | (0.01) | 0.13 | (0.01) | 0.19 | (0.04) | 0.25 | (0.05) | 0.26 | (0.03) | 0.24 | (0.03) | 0.17 | (0.02) | 0.18 | (0.01) |
| Octanal | 1049 | a | 3.17 | (0.49) | 3.84 | (0.49) | 2.88 | (0.36) | 2.76 | (0.25) | 4.08 | (0.26) | 2.94 | (0.30) | 3.76 | (0.16) | 3.38 | (0.24) | 3.13 | (0.20) |
| Hexanoic acid | 1079 | a | 3.91 | (0.49) | 3.64 | (0.37) | 2.80 | (0.21) | 2.87 | (0.31) | 5.22 | (0.44) | 2.68 | (0.26) | 4.50 | (0.39) | 3.44 | (0.17) | 3.07 | (0.23) |
| Nonanal | 1151 | a | 5.30 | (0.36) | 6.54 | (0.45) | 5.29 | (0.54) | 3.56 | (0.39) | 6.28 | (0.30) | 6.18 | (0.57) | 4.90 | (0.10) | 4.30 | (0.16) | 5.28 | (0.35) |
| **Unknown compound** | | | | | | | | | | | | | | | | | | | | |
| Carbon disulfide | 537 | a | 3.15 | (0.25) | 3.70 | (0.38) | 4.21 | (0.26) | 4.27 | (0.44) | 6.54 | (0.54) | 8.55 | (0.77) | 5.26 | (0.59) | 3.20 | (0.41) | 3.95 | (0.66) |

The numbers in parentheses represent standard errors. [1]LRI: Linear retention index of the compounds eluted from the GC-MS. [2]RI: Reliability of identification: a. identification by mass spectrum. coincidence with the LRI of an authentic standard; b. tentatively identification by mass spectrum.[3]C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm).

# Capítulo 3

## Metabolismo de los aminoácidos azufrados en *Debaryomyces hansenii* que actúan como precursores de compuestos volátiles azufrados de interés en productos cárnicos.

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# *Debaryomyces hansenii* metabolism of sulfur amino acids as precursors of volatile sulfur compounds of interest in meat products

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## Abstract

The ability of *Debaryomyces hansenii* to produce volatile sulfur compounds from sulfur amino acids and the metabolic pathway involved have been studied in seven strains from different food origins. Our results proved that L-methionine is the main precursor for sulfur compound generation. Crucial differences in the sulfur compound profile and amino acid consumption among *D. hansenii* strains isolated from different food sources were observed. Strains isolated from dry pork sausages displayed the most complex sulfur compound profiles. Sulfur compound production, such as that of methional, could result from chemical reactions or yeast metabolism, while according to this study, thioester methyl thioacetate appeared to be generated by yeast metabolism. No relationship between sulfur compounds production by *D. hansenii* strains and the expression of genes involved in sulfur amino acid metabolism was found, except for the *ATF2* gene in the L1 strain for production of methyl thioacetate. Our results suggest a complex scenario during sulfur compound production by *D. hansenii*.

Keywords: *Debaryomyces hansenii*; L-Methionine; Volatile sulfur compounds; Sulfur amino acids.

## 1. Introduction

A wide range of volatile compounds is responsible for meat product aromas, among which volatile sulfur compounds contribute to the characteristic meaty notes.[1] The main source of sulfur compounds in fermented meat is the transformation from sulfur amino acids. During meat processing for manufacturing dry fermented sausages, sulfur amino acids transformation is mainly conducted by microorganisms and, to a lesser extent, chemical reactions like Maillard and Strecker degradation.[2] L-Methionine, L-cysteine, and L-cystine are the main sulfur amino acids present in dry fermented sausages, with L-methionine being the most abundant followed by L-cysteine.[3] Microbial transformation of sulfur amino acids into volatile sulfur compounds is conducted by bacteria (lactic acid bacteria and staphylococci) and yeasts present in the meat product.[4]

The generation of volatile sulfur compounds from sulfur amino acids by yeasts metabolism has been thoroughly investigated in cheese.[5–7] The main sulfur compound precursor in yeasts seems to be L-methionine.[8–10] Methanethiol is believed to result from direct L-methionine demethiolation by β/γ-lyases activity (demethiolation pathway) or by a two-step transformation carried out by an aminotransferase, giving α-keto-γ-methylthio-oxobutyric acid (KMBA), followed by a demethiolase activity in the Ehrlich pathway. Further transformation of methanethiol leads to generation of other sulfur compounds such as dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide generated by an oxidation reaction, while thioester methyl thioacetate is generated by a chemical or enzymatic reaction. KMBA can be transformed directly to methional through decarboxylation, and methional can be subsequently reduced to methionol.

Among the yeasts isolated from dry fermented sausages, *Debaryomyces hansenii* is the dominant yeast species.[11] Addition of a *D. hansenii* starter has potential functionalities in the manufacture of dry fermented sausages, contributing to the proteolytic activity[12] and producing free amino acids which act as precursors of volatile compounds.[3,13] The ability of *D. hansenii* to generate ester and sulfur compounds among other volatiles and to reduce the production of oxidation derived compounds in dry fermented sausages has been demonstrated.[14,15] Moreover, addition of *D. hansenii* improves the sensory quality of the final dry fermented sausages product.[16,17] However, the metabolic pathways involved in

sulfur compounds generation in dry fermented sausages have not yet been elucidated. The aim of this study was to compare the ability of several *D. hansenii* strains to produce volatile sulfur compounds from sulfur amino acids and to obtain insight into the metabolic pathway involved in the generation of L-methionine-derived volatile compounds.

## 2. Materials and methods

### *2.1. Chemicals*

The following compounds were commercially purchased from Sigma-Aldrich (Missouri, USA): dimethyl sulfide, methyl thioacetate, dimethyl disulfide, ethyl thioacetate, methional, dimethyl trisulfide, methionol, L-methionine, L-cysteine, and L-cystine.

### *2.2. Yeast strains*

*D. hansenii* strains used in this study (Table 1) were isolated from different dry fermented pork and llama sausages, cheese, and vegetables.

**Table 1. *D. hansenii* strains used in this study.**

| Yeast strain | Source | Reference |
|---|---|---|
| L1 | Pork dry fermented sausage | Bolumar et al.[18] |
| L5 | Pork dry fermented sausage | Cano-García et al.[14] |
| L12 | Llama dry fermented sausage | Mendoza et al.[19] |
| L21 | Llama dry fermented sausage | Mendoza et al.[19] |
| L25 | Llama dry fermented sausage | Mendoza et al.[19] |
| L66 | Ewe's cheese | Padilla et al.[20] |
| L74 | Lupine | Flores et al.[13] |

### *2.3. Growth of D. hansenii on sulfur amino acids supplemented media*

*D. hansenii* strains were precultured during 48 h on a GPY liquid medium (2% glucose, 0.5% peptone, and 0.5% yeast extract) at 25 °C. After growth, each culture was centrifuged and washed three times with saline solution (0.9% NaCl). Cell suspensions were adjusted for inoculation in culture media at a final concentration of $10^6$ cells/mL. A control medium (C) composition was 0.5% yeast extract (amino acids content in Table S1), 10 g/L glucose, 30 g/L NaCl, 127.5 mg/L $NaNO_2$ and 127.5 mg/L $NaNO_3$. Composition of the sulfur amino-acids-supplemented media were the same as the control medium, except for the addition of

100 mg/L L-methionine (M), 250 mg/L L-cysteine (Cy), and 50 mg/L L-cystine (Ci), respectively. The media were adjusted to pH 6.5 and sterilized using a vacuum-driven filtration system of 0.22 μm. Experiments were conducted in 100 mL flasks containing 50 mL of C, M, Cy, and Ci media. Seven flasks were inoculated with each *D. hansenii* strain while a noninoculated (NI) flask of each medium was the control. Each experiment was conducted in triplicate (96 experiments in total). Flasks were incubated at 25 °C for 15 days for sulfur compounds analysis. After incubation, optical density (OD) was measured at 600 nm in a BioPhotometer (Eppendorf, Germany). The supernatant was recovered by centrifugation at 4000 rpm for 5 min at 20 °C, cell-free filtered (0.22 μm), and frozen at −20 °C until the volatiles and amino acid analyses. Additional experiments in media C and M inoculated with yeasts L1 and L74 were carried out in triplicate (12 in total) for studying gene expression. After 2 days of incubation, cells were collected by centrifugation at 4000 rpm for 5 min at 20 °C. The resulting yeast pellet was resuspended in sterile Milli-Q water and frozen at −80 °C until RNA isolation.

### *2.4. Volatile sulfur compounds analysis*

The sulfur compounds analyzed (Figure 1) were methanethiol (1), dimethyl sulfide (2), dimethyl disulfide (3), dimethyl trisulfide (4), methional (5), methionol (6), methyl thioacetate (7), and ethyl thioacetate (8). Analysis was carried out by headspace (HS) solid-phase microextraction (SPME) with an 85 μm carboxen/polydimethylsiloxane (CAR/PDMS) fiber (Supelco, Bellefonte, PA) using a gas chromatograph (Agilent HP 7890 series II (Hewlett-Packard, Palo Alto, CA)) with a quadrupole mass detector (HP 5975C (Hewlett-Packard)) and equipped with an autosampler (MPS2 multipurpose sampler (Gerstel, Germany)). Supernatants (7 mL) were placed into 20 mL headspace vials containing 2.37 g of NaCl to produce a salting out effect. To prevent oxidation, the vials were purged with nitrogen gas for 5 s before sealing. The internal standard 2-methyl-3-heptanone (256 ng) was added, and the vials were incubated at 37 °C for 30 min at 250 rpm for equilibration. Then, the fiber was exposed to the headspace for 30 min at 250 rpm while maintaining the sample at 37 °C, after it was desorbed in the injection port of the GC-MS for 5 min at 240 °C in splitless mode. The volatile compounds were separated using a DB-624 capillary column (30 m, 0.25 mm i.d., film thickness 1.4 μm, (J&W Scientific, Agilent Technologies, USA)). Helium was used as a carrier gas with a linear velocity of 34.3 cm/s.

The GC oven temperature was held at 38 °C for 13 min, ramped to 100 °C at 3°C/ min, held at 100 °C for 5 min, ramped to 150 °C at 4°C/min and to 210 °C at 10°C/min, and held at 210 °C for 5 min. The MS interface temperature was set to 240 °C. Sulfur compounds were identified in full scan mode and by their retention time according to authentic standards. The identified volatile compounds were quantified in SIM mode using specific m/z ions: 48 for (1), 62 for (2), 94 for (3), 126 for (4), 104 for (5), 106 for (6), 90 for (7), and 104 for (8). Calibration curves for each sulfur compound (except for methanethiol (1)), relative to the internal standard, were obtained using the same SPME conditions in water. The peak areas of the compounds were compared to their respective standard and expressed as nanograms per milliliters of supernatant. Sulfur compounds were counted based on the normalized area and using the response factors shown in Table 2. The calibration curve for methanethiol (1) was not obtained, and it was expressed as nanogram equivalents of dimethyl disulfide (3) per milliliters of supernatant. Experimental triplicates were analyzed in duplicate.

### *2.5. Amino acid analysis*

The analysis of free amino acids was done using the EZ-Faast kit bought from Phenomenex (Torrance, CA, USA). Media supernatants were diluted 1:5 (v/v) with distilled water and analyzed using the kit. The derived amino acids were analyzed using GC-FID. A gas chromatograph (Agilent Technologies 7890B) with a flame ionization detector (FID) equipped with an autosampler G4513A and a ZB-AAA 10 m × 0.25 mm GC column (Phenomenex) was used. The injection volume was 2.5 μL at 250 °C in split mode (15:1). Helium was used as a carrier gas at a constant flow of 27 mL/ min during the run, and the column head pressure was 8.78 psi. The GC oven temperature was initially held at 110 °C and then raised to 320 °C at 32°C/min; the inlet temperature was 250 °C, and the detector was set at 320 °C. Identification and quantitation was based on retention times and peak area integration of the reference amino acids (Phenomenex). Norleucine was the internal standard. Calibration curves for each amino acid were obtained with the standard amino acids solutions (Phenomenex). Results were expressed in milligrams per 100 mL of supernatant. Consumption of amino acids was analyzed for each yeast strain in each medium, subtracting the concentration measured in the respective NI medium, at the end of incubation time. The method used did not allow for detection of L-cysteine in liquid media.

### *2.6. RNA extraction and cDNA copy*

About $10^8$ yeast cells were used for RNA isolation. Total RNA was extracted as described by Sanvisens et al.[21] with modifications. Cell pellets were suspended in 400 μL of LETS buffer (2 M LiCl, 1 M EDTA, 1 M Tris-HCl pH 7.4, SDS 10%) and 400 μL of phenol, chloroform, and isoamyl alcohol (120:24:1); glass beads were added, and cells were lysed using a Millmix 20 Bead Beater (Tehtnica, Slovenia). The supernatant was collected by centrifugation, and RNA was precipitated in two steps with 5 M LiCl and 96% ethanol at −20 °C overnight and with 3 M sodium acetate and 96% ethanol at −80 °C for 2−3 h. RNA was isolated by centrifugation and washed with 70% ethanol. Isolated RNA was dried and suspended in 200 μL of RNase-free Milli-Q water (VWR, USA). RNA concentration and purity were determined using a NanoDrop (Thermo Scientific, USA) instrument, and DNA was removed using the DNA-free Kit (DNase Treatment and Removal, Invitrogen, USA). Reverse transcription of cDNA was made with Reverse Transcriptase SuperScript III (Invitrogen) with the $Oligo(dT)_{12–18}$ primer, using a Protector RNase inhibitor kit (Roche, Switzerland) according to the manufacturer's recommendations. DNA-free RNA and cDNA concentration and purity were determined using a NanoDrop (Thermo Scientific) instrument.

### *2.7. Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) experiments*

Expression of *S. cerevisiae* orthologous genes in L1 and L74 *D. hansenii* strains *ARO8, CYS3, AAT2, BNA3, ADH1, ILV6, ATF1, ATF2, PDC1, PDC6, STR3, PDB1,* and *BAT2* related to sulfur compounds generation[7,22] was studied. Primers, listed in Table 3, were designed using the OligoAnalyzer tool on the IDT webpage (https://eu.idtdna.com/pages/tools/oligoanalyzer). We searched close to the 3′ end of the orthologous gene sequences for conserved regions using available *D. hansenii* genome sequences, i.e., NRRLY-7426T (syn. CBS767), MTCC234, and J6. Our PCR and Sanger-sequencing of genes *PDB1* and *STR3* helped redesign the pairs of primers which failed in our first approach. The RT-qPCR was performed using a LightCycler (Roche) and LightCycler 480 SYBR Green I Master Kit (Roche), according to the manufacturer's recommendations. The *ACT1* gene was used as the reference gene. The quantity of cDNA for each gene was normalized to the quantity of the *ACT1* cDNA in each sample. The E-method (LightCycler 480, Roche) was used for accurate relative quantitation data analysis. The amplification efficiency of the

reference and target genes was calculated using LinRegPCR 2017.[23] The relative change in the expression of each gene was described as the ratio of normalized quantity of cDNA for each gene studied under different conditions, low L-methionine content in control medium (C) and high L-methionine content in (M) medium. A gene was considered overexpressed when the ratio of its transcriptomic response in treatment M and C conditions (M/C) was >2.

**Table 2. Response factors for the analysis of sulfur compounds in Selected-Ion-Monitoring (SIM) mode and using 2-methyl-3-heptanone as the internal standard.**

| Compound | Monitored ion (*m/z*) used for quantitation | Response factor |
|---|---|---|
| **Dimethyl sulfide** | 62 | 0.0941 |
| **Methyl thioacetate** | 90 | 0.0589 |
| **Dimethyl disulfide** | 94 | 1.2547 |
| **Ethyl thioacetate** | 104 | 0.0723 |
| **Methional** | 104 | 0.0370 |
| **Dimethyl trisulfide** | 126 | 1.0625 |
| **Methionol** | 106 | 0.0603 |

**Table 3. Primers Used in RT-qPCR.**

| ***D. hansenii* open reading frame** | ***D. hansenii* gene name** | **Enzyme** | **Direction** | **Sequence** |
|---|---|---|---|---|
| DEHA2D05412g | *DhACT1* | Actine | F<br>R | GGTAACATTGTTATGTCTGGTG<br>TACTTTCTTTCTGGAGGAGC |
| DEHA2C05236g | *DhAAT2* | Cytosolic aspartate aminotransferase | F<br>R | AACACCGTCAGAACCAAG<br>CAATTCAATAACTTGTTCAGC |
| DEHA2A06886g | *DhARO8* | Methionine aminotransferase | F<br>R | CAA GGT TGT TTG ATG ATC YCC<br>AACGGCAGCATATGTACCTC |
| DEHA2C09724g | *DhATF1* | Alcohol acetyltransferase | F<br>R | CTGGTGCAGCATTAGGAC<br>AAA TGG CTT YAA TCT GTC TC |
| DEHA2D14762g | *DhATF2* | Alcohol acetyltransferase | F<br>R | GCCTCAACTTGCTGRC<br>GTTCCAAGAGTTTTGTAGTAAAC |
| DEHA2D06952g | *DhBAT2* | Cytosolic branched-chain amino acid transferase | F<br>R | TTTAGAAGGTGTCACCAGAG<br>CYCTTTCTTCAATTTCGTGG |
| DEHA2A04818g | *DhBNA3* | Arylformamidase | F<br>R | CCYTATACATCTGCTCAAGG<br>TCCGATTTCYTTTATCAACCAG |
| DEHA2C15686g | *DhCYS3* | Cystathionine gamma-lyase | F<br>R | CACGGTGGTATTCCAAAAG<br>AAGCTTGTCTGACATCTTCG |
| DEHA2E21604g | *DhILV6* | Acetolactate synthase | F<br>R | GTT GAY ATT GCT GAT AGA AAC G<br>CTTGGTAATGCCATCATACC |
| DEHA2C09152g | *DhPDB1* | E1beta subunit of pyruvate dehydrogenase complex | F<br>R | ACGGTGTTAAGGCTGAAGTTAT<br>ATGATTTGGGCACAGATTTC |
| DEHA2B03872g | *DhPDC1* | Pyruvate decarboxylase | F<br>R | GGTACATCAGCATTTGGRATTG<br>CCA TKA CTG CTC CTA ATG TAG |
| DEHA2G18348g | *DhPDC6* | Pyruvate decarboxylase | F<br>R | GATTAATTCATGGTGAAAATGCC<br>AGC ATC GTA ATC CTC AGC AC |
| DEHA2A06798g | *DhSTR3* | Cystathionine beta-lyase | F<br>R | TATCTTTGGAATTGCCGTTTC<br>TTCTTCTCTGGTCTTGGCATC |
| DEHA2G21032g | *DhADH1* | Alcohol dehydrogenase | F<br>R | GGGCACATGGAGTWATTAATG<br>CACKACAGTACCACGAGATC |

### *2.8. Statistical analysis*

Data were analyzed using the generalized linear model (GLM) procedure in XLSTAT 2018.4 (Addinsoft, Paris, France). Sulfur compounds and amino acids consumption data were analyzed using the linear mixed model including the yeast strain as the fixed effect and replicates as the random effect. Differences in gene expression between L1 and L74 strains growing on C and M media were analyzed using the same model including replicates as the random effect. In case an effect because of yeast inoculation or media was detected ($P < 0.05$), least-squares mean (LSM) were compared using the Tukey test. Heatmaps of amino acid consumption in each medium were calculated, relative to the concentration in the control medium without inoculation (XLSTAT).

## 3. Results

### *3.1. Volatile sulfur compounds analysis.*

The sulfur compounds methanethiol (1), dimethyl sulfide (2), dimethyl disulfide (3), dimethyl trisulfide (4), methional (5), methionol (6), methyl thioacetate (7), and ethyl thioacetate (8) were identified and quantified in all experiments. The highest generation of sulfur compounds was seen on the M medium (Figure 2b). Comparisons between culture media show that the concentration of volatile sulfur compounds in the sulfur amino-acids-supplemented media inoculated with yeasts (Figure 2b−d) was higher than in their respective C flasks (Figure 2a). The amount of sulfur compounds produced in sulfur amino acids rich media (Ci, Cy, and M) inoculated with yeasts were similar except for medium M. Regarding the sulfur compounds profile, dimethyl trisulfide (4), methionol (6), and methyl thioacetate (7) were not produced in media Ci or Cy (Tables 4 and S2−S4). Generation of sulfur compounds occurred in NI flasks of all media. Methional (5) was exceptionally abundant in all NI flasks while methyl thioacetate (7) was absent in NI flasks of sulfur amino-acids-supplemented media. The compound dimethyl trisulfide (4) appeared only in NI flasks from Ci and Cy media. Significant differences were observed between *D. hansenii* strains regarding sulfur compounds generation on all media (Tables 4 and S2−S4). However, differences between replicates were not significant ($P > 0.10$). In medium M (Table 4), strain L74 was the highest producer of sulfur compounds in contrast to strain L25, which was the lowest. The most abundant sulfur compounds in medium M were methyl thioacetate (7) by strain L1, methionol (6) by strain L12, dimethyl disulfide (3) by strain L74, and methional

(5) by strain L21. It is worth noting that methyl thioacetate (7) was only produced by L1, L5, and L74. The volatile profile between strains was also different. Strains isolated from pork sausages, L1 and L5, produced the same sulfur compounds but in different amounts. Between strains isolated from llama sausages we found larger differences in the production of methionol (6) and methional (5) in strains L12 and L21. Strain L25 differed from the other strains from llama sausage, regarding its sulfur compound profile. Cheese strain L66 showed a sulfur compound profile like the strains isolated from meat. The most different strain regarding sulfur compounds profile was L74, isolated from lupine. For media Cy and Ci, inoculation of strain L66 had a remarkable effect on the generation of methionol (6) which was not produced in C (Tables S3 and S4).

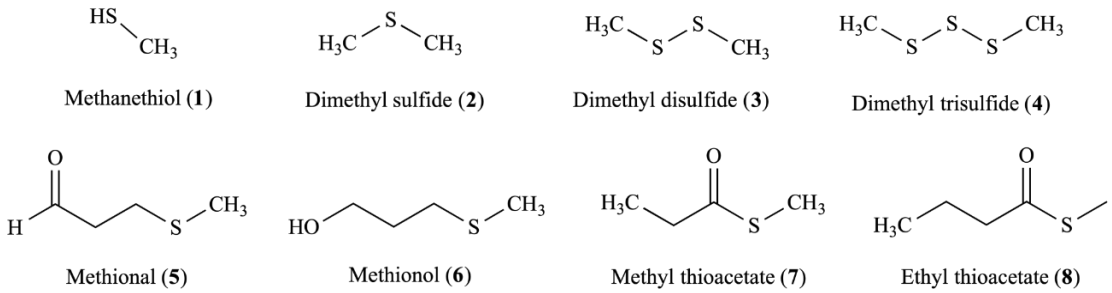


**Figure 1.** Chemical structures of the volatile sulfur compounds studied.

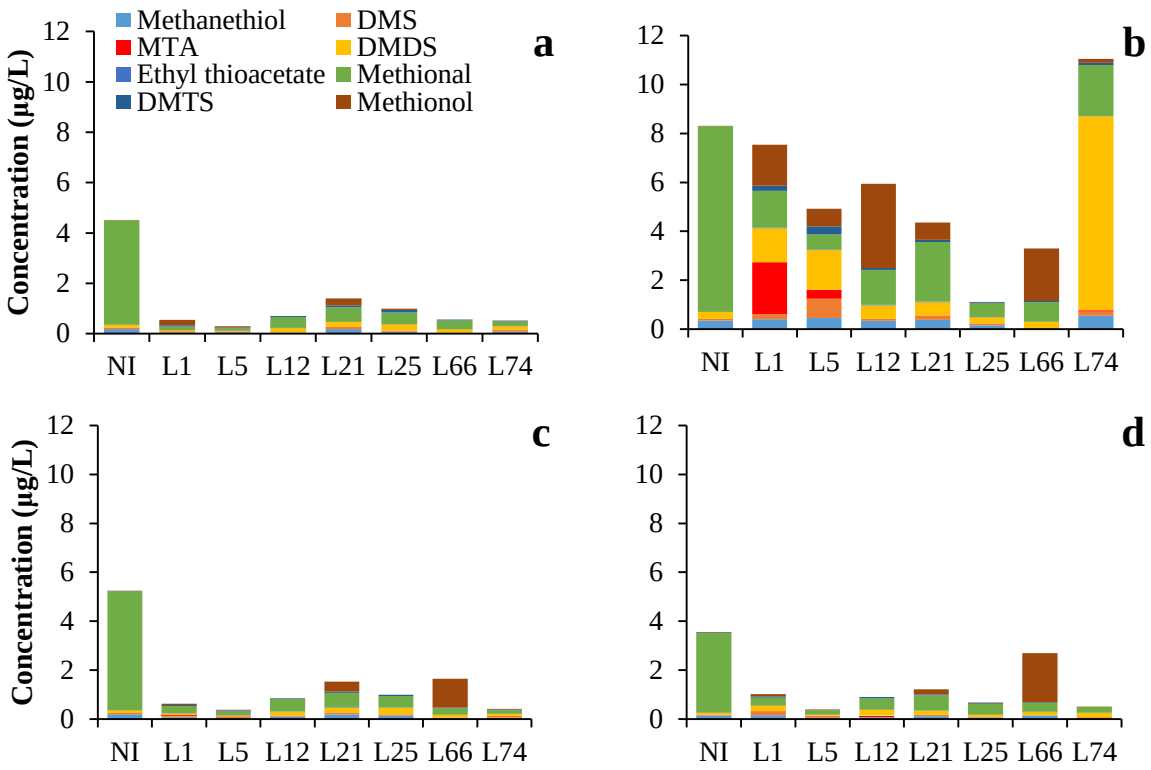


**Figure 2**. Volatile sulfur compounds generated in sulfur amino-acids-supplemented media noninoculated (NI) and inoculated with *D. hansenii* (Table 1) in different media: (a) control (C); (b) L-methionine-supplemented medium (M); (c) L-cysteine-supplemented medium (Cy); and (d) L-cystine-supplemented medium (Ci).

**Table 4. Concentration of Sulfur Compounds (µg/L) in L-Methionine Medium (M) after 15 d Incubation with *D. hansenii* Strains.**

| | NI[a] | L1 | L5 | L12 | L21 | L25 | L66 | L74 | RMSE[b] | *P*[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| **Methanethiol** | 0.349 ab | 0.402 ab | 0.453 a | 0.346 ab | 0.382 ab | 0.161 bc | 0.005 c | 0.546 a | 0.078 | *** |
| **Dimethyl sulfide** | 0.069 b | 0.216 b | 0.792 a | 0.072 b | 0.180 b | 0.074 bc | 0.034 b | 0.175 b | 0.066 | *** |
| **Methyl thioacetate** | n.d.[5] | 2.116 a | 0.366 b | n.d. | n.d. | n.d. | n.d. | 0.051 b | 0.309 | * |
| **Dimethyl disulfide** | 0.284 bc | 1.391 b | 1.621 b | 0.556 bc | 0.539 bc | 0.253 c | 0.268 c | 7.935 a | 0.370 | *** |
| **Ethyl thioacetate** | 0.009 b | 0.029 a | 0.026 a | 0.027 a | 0.025 ab | 0.007 b | 0.006 b | 0.012 b | 0.002 | *** |
| **Methional** | 7.585 a | 1.505 bc | 0.620 c | 1.428 bc | 2.438 b | 0.550 c | 0.781 bc | 2.079 bc | 0.617 | *** |
| **Dimethyl trisulfide** | n.d. | 0.204 ab | 0.330 a | 0.072 c | 0.090 c | 0.059 c | 0.051 c | 0.094 bc | 0.025 | ** |
| **Methionol** | 0.004 b | 1.680 ab | 0.711 ab | 3.447 a | 0.707 ab | 0.004 b | 2.149 ab | 0.153 b | 0.849 | * |
| **Total** | 8.300 | 7.543 | 4.919 | 5.948 | 4.361 | 1.108 | 3.294 | 11.045 | | |

[a]NI: medium noninoculated. [b]RMSE: root-mean-square error. [c]*P*: *P* value of inoculation effect of different yeasts (Table 1). Different letters in the same row indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. ns: $p > 0.05$. dn.d.: not detected.

### *3.2. Amino acid analysis.*

Amino acids asparagine (ASN), glutamine (GLN), and ornithine (ORN) were chemically produced after 15 days of incubation in NI media (Tables S5− S8). Data on consumption of amino acids by each yeast strain was represented in a heatmap with hierarchical clustering (Figure 3) and in Table 5 for sulfur amino acids. Concentration of the remaining amino acids in C, M, Cy, and Ci media are shown in Tables S5−S8. In the heatmap, consumption of each amino acid is relative to the total consumption of amino acids. The highest consumption is given a red color, while the lowest consumption is given a yellow color. Hierarchical clustering is done on the rows of the data matrix and is represented in the left part of the figure. Clustering in Figure 3 divided *D. hansenii* strains into two groups of yeasts, the low amino acid consumption group (top) and the high amino acid consumption group (bottom). The principal differences between both groups was consumption of tyrosine. Strains L66 and L12 showed the lowest amino acid consumption, while strains L21 and L25 displayed an intermediate amino acid consumption. The main differences between those two groups was the consumption of alanine, glycine, proline, hydroxyproline, glutamic acid, phenylalanine, and histidine. Strains L1, L5, and L74 were in the highest amino acid consumers group, where the consumption of ornithine was the main difference between L1 and the other strains.

Yeast consumption of individual amino acids was different depending on the culture media (Tables S5−S8). The most consumed amino acids in all media were glutamic acid and threonine, and the least were valine and tyrosine. Biogenic amine production was not observed in any of the strains assayed in the amino acid medium following the method of Aslankoohi et al.[24] In individual sulfur amino acids, L1, L5, and L74 consumed the highest amount of L-methionine in all media (Table 5). L-Cystine was only detected in media supplemented with L-cystine or L-cysteine (Ci and Cy), and L1, L5, L21 and, L74 were the highest consumers of this sulfur amino acid.

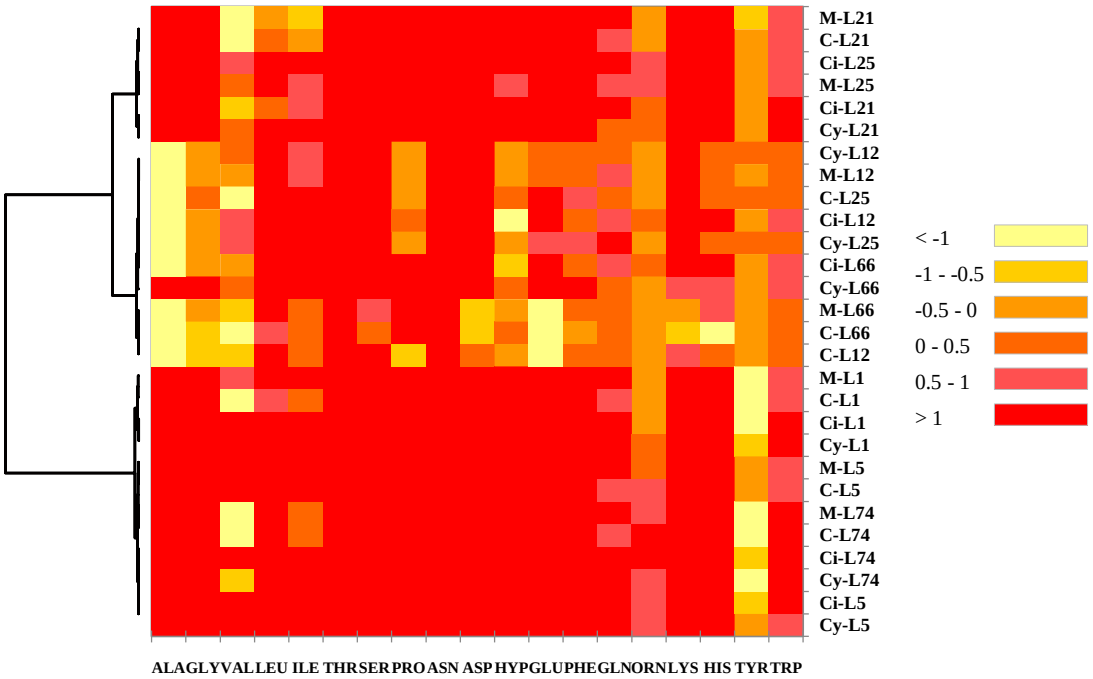


**Figure 3.** Heatmap showing the consumption of non sulfur amino acids by the metabolic activity of D. hansenii strains (Table 1) in all media. C (control medium), M (L-methionine-supplemented medium), Cy (L-cysteine-supplemented medium), and Ci (L-cystine-supplemented medium). Red color indicates relatively high amino acid consumption (>1), and the yellow color indicates the low amino acid consumption (>−1); the orange color indicates no difference.

### *3.3. Gene expression*

Expression of genes related to generation of sulfur compounds derived from L-methionine in media containing low L-methionine concentration (C) and high L-methionine concentration (M) was analyzed in RNA extracted from yeasts cultivated for 2 days (Table 6). Differential expression of few genes between M and C culture media (M/C ratio) was significant in strains L1 and L74. Expression of gene *DhATF2* (alcohol acetyltransferase) was overexpressed in L1 on media supplemented with L-methionine ($p < 0.01$). Expression of genes *DhAAT2* (cytosolic aspartate aminotransferase) and *DhPDC1* (pyruvate decarboxylase) was significantly different ($p < 0.05$) in C and M media in L74. However, none of these genes could be considered overexpressed or repressed.

## 4. Discussion

*D. hansenii* is added as a starter in the production of many Mediterranean meat products contributing to the generation of volatile compounds and overall quality of the products.[17] During the dry curing process many amino acids are released by different proteolytic activities.[25] The amounts of amino acids in the media used in this study are like the quantities found in dry fermented meat products.[3] The free amino acids are potential sulfur compound precursors, and especially, L-methionine catabolism has been investigated as the source of methanethiol (1). Methanethiol (1) is the first-step degradation product of L-methionine, is the main precursor for many sulfur compounds,[9] and appeared in medium M in very small amounts. The highest production of sulfur compounds by yeast was observed in the medium supplemented with L-methionine (M) confirming that methionine is the main precursor of sulfur compounds in *D. hansenii*, as observed by other authors in cheese yeasts.[5,26] *D. hansenii* strain L1 consumed the highest amount of L-methionine and produced the most complex sulfur compound profile. Overexpression of genes involved in sulfur compounds generation from L-methionine has been revealed in several cheese yeast species including *Geotrichum candidum*, *Yarrowia lipolytica*, *Kluyveromyces lactis*, and *Saccharomyces cerevisiae*.[27–29] Among the genes involved in conversion of L-methionine to methanethiol (1), we tested the expression of *DhAAT2* (cytosolic aspartate aminotransferase), *DhARO8* (aromatic amino acid transferase), *DhBAT2* (cytosolic branched-chain amino acid transferase), *DhBNA3* (arylformamidase), *DhCYS3* (cystathionine gamma-lyase), *DhSTR3* (cystathionine beta-lyase), and *DhILV6* (acetolactate synthase). However, we did not find any of these genes overexpressed in the strains and media evaluated, although *DhARO8* M/C ratios in L1 indicate high levels of expression of this gene in medium M respective to medium C.

Alternatively, chemical oxidation plays a key role in the generation of sulfur compounds. Dimethyl sulfide (2), dimethyl disulfide (3), and dimethyl trisulfide (4) are considered the result of the chemical oxidation of methanethiol (1).[9] These sulfur compounds appear in lesser amounts in media inoculated with *D. hansenii*, except for dimethyl disulfide (3) by L74. Strong chemical transformations of methional (5) into methanethiol (1) do not occur in NI M medium, therefore enzymatic production of methanethiol (1) is the most probable explanation for their presence in inoculated M media. Comparison between inoculated M

media revealed that L25 M medium was like the NI M medium, except for the number of compounds, indicating that strain L25 is preventing oxidation of L-methionine. The large amount of methionol (6) and methyl thioacetate (7) in L1 and L5 M media indicates that the L-methionine catabolic pathway plays an important role in their generation. The large amount of dimethyl disulfide (3), produced by oxidation, indicates that strain L74 is not preventing oxidation of L-methionine or any other sulfur compound. Taking into account that the precursor of dimethyl disulfide (3) is methanethiol (1), the presence of methyl thioacetate (7) indicates that its precursor is also methanethiol (1). The most probable explanation is that methanethiol (1) is being produced by catabolism of L-methionine and rapidly oxidized to dimethyl disulfide (3).

Chemical oxidation of amino acids and nonbiological generation of sulfur compounds take place in NI medium.[2] In addition, conversion from L-cysteine to L-methionine in the medium could have happened.[30] Methional (5) was the only sulfur compound whose generation in NI was always higher than in media inoculated with yeasts. The most probable explanation could be that methional (5) is the consequence of an oxidative chemical reaction from L-methionine (Strecker reaction).[31,32] Escudero et al.[31] established the relationship between oxidized wines and the generation of methional (5), but this compound was not found in not oxidized wines. Similarly, our results show that in NI, methional (5) generation is the consequence of chemical oxidation, while in the inoculated media, chemical oxidation was not prevalent. This would agree with earlier studies reporting the role of *D. hansenii* in prevention of lipid oxidation in meat products.[14] However, our study did not reveal changes in the expression of pyruvate decarboxylase genes (*PDC*) responsible for the conversion of KMBA into methional (5). On the contrary, Cholet et al.[22] found *PDC* expression of *DhPDC1* and *DhPDC6* genes in *Yarrowia lipolytica*, although the authors did not detect generation of methional (5). This might indicate that methional (5) is not the result of pyruvate decarboxylase activity from KMBA. Another explanation could be the rapid conversion of methional (5) into methionol (6), which would prevent accumulation of the former in inoculated media. This conversion occurred in some of our *D. hansenii* yeasts in M medium. Conversion of methionol (6) from methional (5) is conducted by alcohol dehydrogenases (*ADH*); however, these genes (*DhADH1*) were also not overexpressed in our *D. hansenii* strains. Moreover, the lower amounts of methional (5) found in the

inoculated media could result from methional oxidation and generation of methyl thiopropionic acid.[32] Nevertheless, this compound was not analyzed in our study. Thioesters generated from alcohol acetyltransferases activity (*ATF*), methyl thioacetate (7), and ethyl thioacetate (8) were detected in media inoculated with *D. hansenii* strains, although in very low amounts among the sulfur compounds analyzed. Generation of ethyl thioacetate (8) is absent in sulfur compounds produced by cheese yeasts.[6,8,27–29] Although, in most studies, including ours, methyl thioacetate (7) is the most produced thioester from L-methionine. *D. hansenii* strain L1 produced the highest methyl thioacetate (7) amount in medium M, and the *DhATF2* gene was overexpressed in this yeast strain.

Gene expression was similar in *D. hansenii* strains growing on M medium respective to C medium at 48 h except for *DhATF2*. Comparable experiments of gene expression were also conducted at 4 and 15 days (data not shown); however, the expression of all genes evaluated did not change between media M and C. Moreover, sulfur compounds were analyzed at 15 days because previous studies show a measurable concentration at this time.[15] However, after 15 days incubation yeasts are in stationary phase of growth and none of the selected genes must be overexpressed at this late time in the growth curve. Most authors used 48 and 96 h incubation to test overexpression of genes and its relationship with sulfur compounds production in other yeast species.[8,27–29] *D. hansenii* strains L1 and L74 were selected for gene expression analysis because of their great ability to generate sulfur compounds from amino acids, plus their different volatile production pattern. Medium M was selected for gene expression analysis because L-methionine is the precursor of most sulfur compounds. Other genes tested in previous studies dealing with sulfur compounds production by Ehrlich or demethylation pathways such as *ARO9*, *ARO10*, and *BAT1* were overexpressed in *S. cerevisiae* and *Y. lipolytica*[22,28,33] and assayed in high L-methionine medium. Liu et al.[28] found overexpression of the *STR3* gene (demethiolase activity) and *ADH4* and *ADH5* genes (alcohol dehydrogenase activity) in *S. cerevisiae*, although other studies did not find these genes overexpressed in *K. lactis* and *Y. lipolytica*.[27,34]

Our results showed that C, Cy, and Ci media contained all of the necessary amino acids but generated low amounts of sulfur compounds, in agreement with previous studies.[6,8,26,27] One explanation could be that L-cysteine is preferably consumed by yeasts producing $H_2S$.

However, $H_2S$ is highly volatile and reactive, preventing the production of its derived sulfur compounds, and is difficult to detect by GC-MS.[26]

Regarding the relationship between amino acid consumption and sulfur compounds generation in the *D. hansenii* strains tested, a clear link between these variables was found in strains L1 and L5, isolated from pork dry fermented sausages, and L74 isolated from lupine. Moreover, the high consumption of amino acids, besides L-methionine, by these yeast strains indicates the probable production of other volatile compounds plus other sulfur compounds, which can provide an interesting flavor profile to the product.[13] Nevertheless, the performance of *D. hansenii* strains in amino-acid-supplemented media for production of sulfur compounds could change with the composition of the media and environmental conditions in production of dry cured meat products.[14]

In summary, L-methionine-supplemented media (M) was the most efficient for production of sulfur compounds by yeasts. Methional (5) was preferably generated by chemical oxidation of L-methionine, while methyl thioacetate (7) was solely produced by yeasts. Profiles of sulfur compounds generated by yeast were different, and strains from pork meat origins presented the most complex sulfur compound profiles. Expression of genes in the metabolic pathway of L-methionine, for generation of sulfur compounds, could not be directly related to sulfur compound production in *D. hansenii*, except in case of methyl thioacetate (7), strain L1, and overexpression of gene *DhATF2*. Sulfur compounds produced by yeast were detected long after genes involved in the metabolic pathway of sulfur compounds generation were expressed. This could explain the absence of overexpression of most genes in M media. Nevertheless, this study has deeply investigated the metabolic pathway involved in the generation of L-methionine-derived volatile compounds in *D. hansenii* isolated from meat products. It has subsequently shown their relevance as a producer of volatile sulfur compounds with aroma impacts in meat products. The results have shown that different metabolic pathways are expressed in *D. hansenii* from fermented pork and llama sausages, and their impact on the final meat product aroma is different. However, the expression of the metabolic pathways depends on many processing conditions that affect the production of sulfur compounds from their precursor sulfur amino acids. Therefore, it is necessary to continue to elucidate in real conditions (sausage manufacture)

that the expression of genes impacts the produced sulfur compounds and, subsequently, the meat product aroma.

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## SUPPLEMENTARY MATERIAL

**Table 1 supplementary material. Initial amino acids concentration in control medium (C).**

| Amino acid | Concentration (mg/100ml) |
|---|---|
| Alanine | 43.5 |
| Arginine | 25 |
| Aspartic acid | 48.5 |
| Cystine | 4 |
| Glutamic acid | 80.5 |
| Glycine | 24.5 |
| Histidine | 10 |
| Isoleucine | 28 |
| Leucine | 38 |
| Lysine | 40 |
| Methionine | 6.5 |
| Phenylalanine | 19 |
| Proline | 20 |
| Serine | 23.5 |
| Threonine | 22 |
| Triptofano | 6 |
| Tyrosine | 11.5 |
| Valine | 29 |

**Table 2 supplementary material. Concentration of sulfur compounds (µg/L) in the control medium (C) inoculated with different *D. hansenii* strains.**

| | NI[1] | | L1 | | L5 | | L12 | | L21 | | L25 | | L66 | | L74 | | RMSE[2] | *P*[3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Methanethiol** | 0.176 | a | 0.041 | b | 0.036 | b | 0.035 | b | 0.185 | a | 0.077 | b | 0.024 | b | 0.077 | b | 0.027 | *** |
| **Dimethyl sulfide** | 0.047 | ab | 0.038 | bc | 0.051 | ab | 0.021 | c | 0.065 | a | 0.034 | bc | 0.018 | c | 0.067 | a | 0.008 | *** |
| **Methyl thioacetate** | 0.010 | | 0.014 | | nd | | nd | | 0.024 | | nd | | nd | | 0.006 | | 0.002 | ns |
| **Dimethyl disulfie** | 0.125 | bcd | 0.048 | cd | 0.022 | d | 0.166 | ab | 0.193 | ab | 0.258 | a | 0.136 | bcd | 0.147 | bc | 0.035 | *** |
| **Ethyl thioacetate** | 0.005 | b | 0.005 | b | 0.005 | b | 0.005 | b | 0.008 | a | 0.010 | a | 0.004 | b | 0.004 | b | 0.001 | *** |
| **Methional** | 4.136 | a | 0.141 | b | 0.106 | b | 0.434 | b | 0.571 | b | 0.464 | b | 0.341 | b | 0.184 | b | 0.174 | *** |
| **Dimethyl trisulfide** | nd | | 0.046 | | 0.024 | | 0.039 | | 0.076 | | 0.096 | | 0.034 | | 0.030 | | 0.029 | ns |
| **Methionol** | 0.002 | d | 0.213 | b | 0.055 | c | nd | | 0.277 | a | 0.049 | cd | nd | | 0.004 | d | 0.011 | *** |
| **Total** | 4.501 | | 0.546 | | 0.299 | | 0.7 | | 1.399 | | 0.988 | | 0.557 | | 0.519 | | | |

[1]NI: medium non inoculated. [2]RMSE: root mean square error. [3]*P*: *P* value of inoculation effect of different yeasts. Different letters in the same row indicate significant differences at *** p<0.001. ** p<0.01. * p<0.05. ns: p>0.05. [4] n.d.: not detected.

**Table 3 supplementary material. Concentration of sulfur compounds (µg/L) in the L-cysteine medium (Cy) inoculated with different *D. hansenii* strains.**

| | NI[1] | L1 | L5 | L12 | L21 | L25 | L66 | L74 | RMSE[2] | *P*[3] |
|---|---|---|---|---|---|---|---|---|---|---|
| **Methanethiol** | 0.185 a | 0.074 ab | 0.032 b | 0.093 ab | 0.181 ab | 0.135 ab | 0.038 ab | 0.044 ab | 0.049 | * |
| **Dimethyl sulfide** | 0.060 ab | 0.044 ab | 0.079 a | 0.043 ab | 0.094 a | 0.054 ab | 0.018 b | 0.076 ab | 0.021 | * |
| **Methyl thioacetate** | n.d. | 0.034 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | | |
| **Dimethyl disulfide** | 0.114 cd | 0.081 cd | 0.030 d | 0.157 bc | 0.196 ab | 0.258 a | 0.123 bcd | 0.099 cd | 0.027 | *** |
| **Ethyl thioacetate** | 0.005 c | 0.007 bc | 0.007 bc | 0.010 b | 0.016 a | 0.017 a | 0.006 c | 0.003 c | 0.002 | *** |
| **Methional** | 4.855 a | 0.289 bc | 0.159 b | 0.514 b | 0.579 b | 0.477 b | 0.260 b | 0.139 b | 0.435 | *** |
| **Dimethyl trisulfide** | 0.014 | 0.040 | 0.041 | 0.025 | 0.056 | 0.051 | 0.022 | 0.028 | 0.007 | ns |
| **Methionol** | 0.008 b | 0.060 b | 0.033 b | n.d. | 0.400 b | n.d. | 1.178 a | 0.029 b | 0.165 | *** |
| **Total** | 5.241 | 0.629 | 0.381 | 0.842 | 1.522 | 0.992 | 1.645 | 0.418 | | |

[1]NI: medium non inoculated. [2]RMSE: root mean square error. [3]*P*: P value of inoculation effect of different yeasts. Different letters in the same row indicate significant differences at *** $p<0.001$. ** $p<0.01$. * $p<0.05$. ns: $p>0.05$. [4] n.d.: not detected.

**Table 4 supplementary material. Concentration of sulfur compounds (µg/L) in the L-cystine medium (Ci) inoculated with different *D. hansenii* strains.**

| | NI[1] | | L1 | | L5 | | L12 | | L21 | | L25 | | L66 | | L74 | | RMSE[2] | *P*[3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Methanethiol** | 0.133 | a | 0.158 | a | 0.050 | b | 0.077 | ab | 0.113 | ab | 0.065 | ab | 0.132 | ab | 0.102 | ab | 0.020 | * |
| **Dimethyl sulfide** | 0.045 | cd | 0.159 | a | 0.090 | b | 0.026 | cd | 0.070 | bc | 0.021 | d | 0.023 | d | 0.070 | bc | 0.015 | *** |
| **Methyl thioacetate** | n.d. | | n.d. | | n.d. | | n.d. | | n.d. | | n.d. | | n.d. | | n.d. | | | |
| **Dimethyl disulfide** | 0.088 | cd | 0.240 | ab | 0.047 | d | 0.248 | a | 0.152 | abcd | 0.106 | bcd | 0.132 | abcd | 0.176 | abc | 0.043 | *** |
| **Ethyl thioacetate** | 0.004 | | 0.006 | | 0.005 | | 0.004 | | 0.007 | | 0.003 | | 0.005 | | 0.004 | | 0.001 | ns |
| **Methional** | 3.241 | a | 0.311 | b | 0.171 | b | 0.459 | b | 0.622 | b | 0.423 | b | 0.367 | b | 0.240 | b | 0.191 | *** |
| **Dimethyl trisulfide** | 0.027 | | 0.043 | | n.d. | | 0.042 | | 0.038 | | 0.043 | | 0.027 | | n.d. | | 0.009 | ns |
| **Methionol** | 0.002 | c | 0.099 | bc | 0.022 | c | | | 0.210 | b | 0.008 | c | 2.000 | a | 0.006 | c | 0.025 | *** |
| **Total** | 3.540 | | 1.016 | | 0.385 | | 0.856 | | 1.247 | | 0.669 | | 2.686 | | 0.598 | | | |

[1]C: medium non inoculated. [2]RMSE: root mean square error. [3]*P*: P value of inoculation effect of different yeasts. Different letters in the same row of each group indicate significant differences at *** $p<0.001$. ** $p<0.01$. * $p<0.05$. ns: $p>0.05$. [4] n.d.: not detected.

**Table 5 supplementary material. Concentration of amino acids (mg/100ml) in the control media (C) inoculated with different *D. hansenii* strains.**

| Amino acid | C[1] | L1 | L5 | L12 | L21 | L25 | L66 | L74 | RMSE[2] | *P*[3] |
|---|---|---|---|---|---|---|---|---|---|---|
| **Ala** | 16.01 ab | 4.28 c | 1.16 c | 19.17 a | 11.22 b | 17.12 a | 19.71 a | 0.35 c | 1.749 | *** |
| **Gly** | 7.06 a | 2.85 bc | 0.00 c | 7.69 a | 4.90 ab | 6.85 a | 7.80 a | 0.13 c | 1.112 | *** |
| **Val** | 12.32 | 16.38 | 11.24 | 13.22 | 13.59 | 15.25 | 13.36 | 14.65 | 2.302 | ns |
| **Leu** | 17.25 a | 16.61 a | 11.48 b | 16.10 a | 17.08 a | 14.75 ab | 16.59 a | 11.52 b | 1.177 | *** |
| **Ile** | 10.05 | 9.72 | 8.07 | 9.80 | 10.06 | 8.70 | 9.79 | 9.58 | 0.690 | * |
| **Thr** | 11.41 a | 0.00 d | 0.00 d | 7.07 b | 0.00 d | 4.67 c | 7.45 b | 0.00 d | 0.725 | *** |
| **Ser** | 8.68 a | 5.89 b | 0.10 c | 6.76 ab | 1.02 c | 5.25 b | 8.25 a | 0.00 c | 0.713 | *** |
| **Pro** | 3.26 ab | 0.80 cd | 0.06 d | 4.11 a | 1.82 c | 3.28 ab | 1.97 bc | 2.22 bc | 0.494 | *** |
| **Asn** | 7.69 a | 0.00 d | 0.00 d | 6.22 b | 0.00 d | 4.74 c | 6.21 b | 0.00 d | 0.327 | *** |
| **Asp** | 11.60 a | 0.00 c | 0.00 c | 11.11 a | 3.57 b | 9.59 a | 12.10 a | 0.00 c | 0.998 | *** |
| **Met** | 3.27 a | 0.00 d | 0.15 d | 2.13 bc | 1.70 c | 2.13 bc | 2.43 b | 0.00 d | 0.229 | *** |
| **Hpro** | 2.26 a | 0.00 b | 0.00 b | 2.29 a | 0.61 b | 1.84 a | 2.18 a | 0.00 b | 0.361 | *** |
| **Glu** | 27.71 a | 1.74 c | 0.00 c | 30.25 a | 16.43 b | 26.51 a | 30.09 a | 1.77 c | 2.507 | *** |
| **Phe** | 9.29 ab | 7.56 bcd | 5.94 de | 9.22 ab | 7.23 cde | 8.43 abc | 9.43 a | 5.65 e | 0.623 | *** |
| **Gln** | 0.65 | 0.00 | 0.00 | 0.30 | 0.00 | 0.18 | 0.62 | 0.00 | 0.292 | ns |
| **Orn** | 1.86 ab | 2.00 a | 0.97 b | 1.89 ab | 1.98 a | 1.89 ab | 1.92 a | 0.00 c | 0.320 | *** |
| **Lys** | 8.51 ab | 3.71 d | 0.49 e | 7.53 abc | 6.15 c | 6.79 bc | 9.41 a | 0.38 e | 0.681 | *** |
| **His** | 2.03 ab | 0.79 bc | 0.00 c | 1.97 ab | 0.17 c | 2.01 ab | 3.07 a | 0.40 bc | 0.611 | *** |
| **Tyr** | 3.32 c | 4.85 a | 3.78 abc | 3.45 bc | 3.70 bc | 3.23 c | 3.60 bc | 4.47 ab | 0.377 | ** |
| **Trp** | 4.33 a | 3.73 bcd | 3.57 cd | 4.17 ab | 3.63 cd | 4.04 abc | 4.25 ab | 3.21 d | 0.186 | *** |

[1]C: medium without yeast inoculation. [2]RMSE: root mean square error. [3]*P*: P value of inoculation effect of different yeasts. Different letters in the same row of each group indicate significant differences at *** p<0.001. ** p<0.01. * p<0.05. ns: p>0.05.

**Table 6 supplementary material. Concentration of amino acids (mg/100ml) in the L-methionine media (M) inoculated with different *D. hansenii* strains.**

| Amino acids | C[1] | | L1 | | L5 | | L12 | | L21 | | L25 | | L66 | | L74 | | RMSE[2] | *P*[3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Ala** | 16.40 | a | 2.60 | b | 1.88 | b | 19.05 | a | 10.18 | ab | 12.33 | ab | 19.64 | a | 0.83 | b | 4.142 | *** |
| **Gly** | 7.37 | a | 1.71 | b | 0.15 | b | 7.75 | a | 4.34 | ab | 5.11 | ab | 7.85 | a | 0.20 | b | 1.806 | *** |
| **Val** | 11.94 | ab | 11.03 | b | 10.48 | b | 12.31 | ab | 13.71 | a | 11.88 | ab | 12.74 | ab | 13.86 | a | 0.866 | ** |
| **Leu** | 17.87 | a | 15.88 | abc | 12.95 | bc | 16.38 | ab | 18.12 | a | 14.78 | abc | 16.86 | a | 12.27 | c | 1.309 | *** |
| **Ile** | 9.75 | ab | 8.46 | b | 8.23 | b | 9.11 | ab | 10.39 | a | 9.23 | ab | 9.43 | ab | 9.25 | ab | 0.645 | * |
| **Thr** | 11.58 | a | 0.00 | d | 0.00 | d | 6.54 | bc | 0.00 | d | 3.12 | cd | 6.96 | b | 0.00 | d | 1.262 | *** |
| **Ser** | 8.96 | a | 5.98 | abc | 0.00 | d | 6.56 | ab | 1.75 | cd | 3.60 | bcd | 8.13 | a | 0.00 | d | 1.560 | *** |
| **Pro** | 3.61 | a | 0.48 | b | 0.40 | b | 3.77 | a | 2.30 | ab | 2.33 | ab | 2.07 | ab | 2.35 | ab | 0.885 | ** |
| **Asn** | 8.12 | a | 0.04 | c | 0.00 | c | 6.20 | ab | 0.00 | c | 3.44 | b | 6.32 | ab | 0.00 | c | 1.148 | *** |
| **Asp** | 10.28 | a | 0.00 | b | 0.00 | b | 8.92 | a | 3.93 | ab | 5.55 | ab | 10.87 | a | 0.00 | b | 2.607 | *** |
| **Met** | 11.98 | a | 2.75 | d | 5.95 | cd | 10.86 | abc | 8.43 | abc | 8.75 | abc | 11.16 | ab | 6.48 | bcd | 1.705 | *** |
| **Hpro** | 2.48 | a | 0.00 | b | 0.00 | b | 2.65 | a | 0.42 | b | 1.62 | ab | 2.79 | a | 0.00 | b | 0.637 | *** |
| **Glu** | 25.28 | a | 0.93 | b | 0.00 | b | 24.98 | a | 16.99 | ab | 15.66 | ab | 27.27 | a | 0.71 | b | 6.596 | *** |
| **Phe** | 9.78 | a | 7.44 | abc | 6.60 | bc | 9.42 | ab | 7.46 | abc | 8.04 | abc | 9.61 | a | 6.08 | c | 1.027 | ** |
| **Gln** | 1.26 | a | 0.00 | b | 0.00 | b | 0.74 | ab | 0.00 | b | 0.28 | ab | 0.88 | ab | 0.00 | b | 0.356 | ** |
| **Orn** | 1.84 | | 1.91 | | 1.54 | | 1.88 | | 2.04 | | 1.29 | | 1.89 | | 1.11 | | 0.577 | ns |
| **Lys** | 9.39 | ab | 3.35 | cd | 1.62 | d | 7.79 | abc | 6.76 | abc | 4.71 | bcd | 9.63 | a | 0.78 | d | 1.675 | *** |
| **His** | 2.57 | a | 0.31 | b | 0.00 | b | 2.08 | ab | 0.13 | b | 1.01 | ab | 2.05 | ab | 0.28 | b | 0.779 | * |
| **Tyr** | 3.51 | b | 4.97 | a | 3.95 | b | 3.51 | b | 4.04 | b | 3.68 | b | 3.67 | b | 4.82 | a | 0.190 | *** |
| **Trp** | 4.61 | a | 3.72 | bc | 3.77 | bc | 4.26 | ab | 3.74 | bc | 3.88 | bc | 4.18 | ab | 3.27 | c | 0.212 | *** |

[1]C: medium without yeast inoculation. [2]RMSE: root mean square error. [3]*P*: P value of inoculation effect of different yeasts. Different letters in the same row of each group indicate significant differences at *** p<0.001. ** p<0.01. * p<0.05. ns: p>0.05.

**Table 7 supplementary material. Concentration of amino acids (mg/100ml) in the L-cysteine media (Cy) inoculated with different *D. hansenii* strains.**

| Amino acid | C[1] | L1 | L5 | L12 | L21 | L25 | L66 | L74 | RMSE[2] | *P*[3] |
|---|---|---|---|---|---|---|---|---|---|---|
| **Ala** | 17.45 a | 2.82 bc | 1.43 c | 19.20 a | 9.06 b | 18.74 a | 15.78 a | 0.78 c | 2.178 | *** |
| **Gly** | 7.66 a | 1.26 c | 0.00 c | 7.89 a | 4.13 b | 7.75 a | 6.23 ab | 0.12 c | 0.969 | *** |
| **Val** | 12.65 ab | 10.26 b | 10.38 b | 12.40 ab | 12.31 ab | 11.92 ab | 12.55 ab | 13.19 a | 0.887 | ** |
| **Leu** | 18.52 a | 14.89 b | 11.87 c | 16.69 ab | 16.63 ab | 16.30 ab | 15.58 ab | 11.53 c | 1.027 | *** |
| **Ile** | 10.65 a | 7.85 c | 8.19 bc | 9.73 ab | 9.45 abc | 9.45 abc | 9.14 abc | 9.53 abc | 0.637 | ** |
| **Thr** | 13.26 a | 0.00 c | 0.00 c | 8.45 ab | 0.00 c | 6.52 b | 5.35 bc | 0.00 c | 1.929 | *** |
| **Ser** | 10.02 a | 5.94 ab | 0.00 c | 7.82 a | 1.72 bc | 6.77 a | 6.33 ab | 0.08 c | 1.674 | *** |
| **Pro** | 3.48 a | 0.25 c | 0.00 c | 3.84 a | 2.17 b | 3.54 a | 1.97 b | 2.48 b | 0.297 | *** |
| **Asn** | 8.33 a | 0.03 c | 0.00 c | 6.48 ab | 0.00 c | 5.36 ab | 4.09 b | 0.00 c | 1.376 | *** |
| **Asp** | 12.58 a | 0.00 b | 0.00 b | 11.48 a | 3.15 b | 11.01 a | 9.44 a | 0.00 b | 1.713 | *** |
| **Met** | 3.09 a | 0.10 c | 0.23 c | 2.17 ab | 1.69 b | 2.17 ab | 1.97 b | 0.43 c | 0.359 | *** |
| **Hpro** | 2.16 a | 0.00 b | 0.00 b | 2.31 a | 0.12 b | 2.40 a | 1.98 a | 0.00 b | 0.212 | *** |
| **Glu** | 29.75 a | 2.39 c | 1.23 c | 29.62 a | 13.35 b | 28.96 a | 24.80 a | 2.29 c | 2.077 | *** |
| **Phe** | 9.79 a | 6.85 b | 6.15 b | 9.39 a | 6.84 b | 9.25 a | 8.64 a | 5.75 b | 0.591 | *** |
| **Gln** | 1.15 a | 0.00 b | 0.00 b | 0.94 a | 0.00 b | 0.91 a | 0.74 ab | 0.00 b | 0.257 | *** |
| **Orn** | 2.27 a | 2.13 ab | 1.76 bc | 2.29 a | 2.23 a | 2.30 a | 2.32 a | 1.57 c | 0.129 | *** |
| **Lys** | 9.51 a | 2.81 d | 0.80 e | 8.02 b | 4.56 c | 7.58 b | 8.53 ab | 0.47 e | 0.444 | *** |
| **His** | 3.73 a | 0.22 b | 0.02 b | 3.59 a | 0.17 b | 3.29 a | 2.76 a | 0.62 b | 0.486 | *** |
| **Tyr** | 3.51 c | 4.30 ab | 3.90 bc | 3.48 c | 3.64 c | 3.48 c | 3.64 c | 4.67 a | 0.226 | *** |
| **Trp** | 4.63 a | 3.56 d | 3.75 cd | 4.31 ab | 3.62 d | 4.22 ab | 4.08 bc | 3.34 d | 0.146 | *** |
| **C-C[4]** | 19.00 ab | 16.18 cd | 16.95 bc | 19.28 ab | 15.11 cd | 19.70 a | 20.40 a | 14.16 d | 0.810 | *** |

[1]C: medium without yeast inoculation. [2]RMSE: root mean square error. [3]*P*: P value of inoculation effect of different yeasts. Different letters in the same row of each group indicate significant differences at *** p<0.001. ** p<0.01. * p<0.05. ns: p>0.05. [4] C-C: cystine.

**Table 8 supplementary material. Concentration of amino acids (mg/100ml) in the L-cystine media (Ci) inoculated with different *D. hansenii* strains.**

| Amino acid | C[1] | L1 | L5 | L12 | L21 | L25 | L66 | L74 | RMSE[2] | *P*[3] |
|---|---|---|---|---|---|---|---|---|---|---|
| **Ala** | 17.38 a | 2.53 bc | 1.25 bc | 18.63 a | 10.64 abc | 12.32 ab | 19.34 a | 0.53 c | 3.924 | *** |
| **Gly** | 7.56 a | 1.28 bcd | 0.00 d | 7.63 a | 4.92 abc | 4.97 ab | 7.72 a | 0.14 cd | 1.671 | *** |
| **Val** | 13.54 | 11.71 | 12.20 | 12.97 | 14.42 | 12.68 | 13.83 | 12.52 | 1.546 | ns |
| **Leu** | 18.41 a | 15.10 abc | 12.43 bc | 16.49 ab | 17.98 a | 14.69 abc | 16.42 ab | 11.26 c | 1.453 | *** |
| **Ile** | 10.58 | 8.04 | 8.08 | 9.26 | 9.95 | 9.39 | 9.15 | 8.49 | 0.943 | ns |
| **Thr** | 13.04 a | 0.00 c | 0.00 c | 6.97 b | 0.00 c | 3.45 bc | 6.67 b | 0.00 c | 1.316 | *** |
| **Ser** | 9.95 a | 5.05 bc | 0.00 d | 6.44 ab | 1.49 cd | 3.75 bcd | 7.41 ab | 0.00 d | 1.422 | *** |
| **Pro** | 3.62 a | 0.56 bc | 0.01 c | 3.60 a | 2.53 ab | 2.32 ab | 1.93 abc | 2.06 abc | 0.767 | *** |
| **Asn** | 8.50 a | 0.00 c | 0.00 c | 6.15 ab | 0.00 c | 3.57 b | 6.33 ab | 0.00 c | 1.206 | *** |
| **Asp** | 12.69 a | 0.00 c | 0.00 c | 9.81 ab | 3.42 bc | 6.84 ab | 10.32 a | 0.00 c | 2.304 | *** |
| **Met** | 3.40 a | 0.00 c | 0.18 c | 2.32 ab | 1.91 ab | 1.40 bc | 2.45 ab | 0.22 c | 0.524 | *** |
| **Hpro** | 2.41 ab | 0.00 b | 0.00 b | 3.72 a | 1.13 ab | 1.27 ab | 3.13 ab | 0.00 b | 1.152 | ** |
| **Glu** | 30.11 a | 1.58 b | 0.00 b | 28.60 a | 15.23 ab | 19.63 a | 26.16 a | 1.59 b | 5.886 | *** |
| **Phe** | 9.75 a | 6.80 bc | 6.42 c | 9.48 ab | 7.53 abc | 8.00 abc | 9.40 ab | 5.57 c | 0.980 | ** |
| **Gln** | 1.21 a | 0.00 b | 0.00 b | 0.29 b | 0.00 b | 0.00 b | 0.69 ab | 0.00 b | 0.276 | ** |
| **Orn** | 1.99 | 2.02 | 1.03 | 1.97 | 1.98 | 1.30 | 1.84 | 0.48 | 0.620 | ns |
| **Lys** | 9.72 a | 3.65 bcd | 0.56 cd | 7.72 ab | 5.66 ab | 5.29 abc | 8.38 ab | 0.00 d | 1.766 | *** |
| **His** | 3.80 a | 0.21 b | 0.00 b | 1.12 b | 0.00 b | 1.06 b | 1.22 b | 0.00 b | 0.600 | *** |
| **Tyr** | 3.49 b | 4.58 a | 4.00 ab | 3.56 b | 3.95 ab | 3.71 ab | 3.66 ab | 4.24 ab | 0.344 | * |
| **Trp** | 4.75 a | 3.45 cd | 3.61 bcd | 4.14 b | 3.54 cd | 3.91 bc | 4.00 bc | 3.02 d | 0.209 | *** |
| **C-C[4]** | 6.53 a | 1.60 bc | 3.13 abc | 5.31 ab | 0.00 c | 3.09 abc | 5.60 ab | 0.00 c | 1.606 | ** |

[1]C: medium without yeast inoculation. [2]RMSE: root mean square error. [3]*P*: P value of inoculation effect of different yeasts. Different letters in the same row of each group indicate significant differences at *** p<0.001. ** p<0.01. * p<0.05. ns: p>0.05. [4]C-C: cystine.

# Capítulo 4

## Inoculación de *Debaryomyces hansenii* en embutidos curado-madurados para contrarrestar el efecto de la reducción de los niveles de nitrato/nitrito en el aroma del producto.

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# Counteracting the effect of reducing nitrate/nitrite levels on dry fermented sausage aroma by *Debaryomyces hansenii* inoculation

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## Abstract

The reduction of ingoing amounts of nitrate and nitrite in dry fermented sausages was studied together with the impact of *Debaryomyces hansenii* inoculation on aroma generation. Three different formulations of sausages were manufactured: control (C), reduced in nitrate and nitrite ingoing amounts (R) and reduced R inoculated with *D. hansenii* (RY). Changes in physicochemical and microbiological parameters, volatile compounds and aroma were investigated at different drying times. Nitrite/nitrate reduction did not seem to affect microbial growth but affected their metabolic activity. Moreover, nitrite/nitrate reduction decreased lipid oxidation and generation of derived volatile compounds. Yeast inoculation limited lipid oxidation and prevented nitrite oxidation. Sausage aroma profile was positively affected by *D. hansenii* inoculation which contributed to the generation of potent aroma compounds like ethyl ester compounds and 3-methylbutanal. Long drying time impacted sausage aroma profile as well as yeast metabolism. Yeast inoculation counteracted the negative influence of nitrite/nitrate reduction due to its antioxidant capacity, aroma generation and hindered nitrite oxidation.

Keywords: Nitrate; Nitrite; *D. hansenii*; Fermented sausage; Volatile compounds; Aroma

## 1. Introduction

The use of nitrite and nitrate as curing agents in dry fermented sausages have important technological functions. Nitrite has antioxidant and antimicrobial activities being the most important its activity against the pathogen *Clostridium botulinum* and control of toxin production. Furthermore, nitrite facilitates the generation and stabilization of the typical colour and enhances cured flavour (Honikel, 2008; Sindelar & Milkowski, 2011). Nitrate acts as nitrite reservoir in dry cured products with a long ripening time (Sanchez Mainar & Leroy, 2015). However, the controversy about the adequate amounts of nitrite used as additive in meat products processing is under concern (CODEX, 2019). Nitric oxide, from nitrite, can react with biogenic amines present in meat under heating conditions to form N-nitrosamines, with carcinogenic potential (De Mey, De Maere, Paelinck & Fraeye, 2015). Therefore, the actual trend is to reduce the use of nitrite and nitrate in cured meat products (EFSA, 2010), although nitrite is considered beneficial for improving the microbial safety of the products. Up to now, several alternatives to nitrite addition have been studied. Among them, plant derivatives are one of the most used, followed by organic acids, bacteria and bacteriocins (Alahakoon, Jayasena, Ramachandra & Jo, 2015). However, these alternatives do not provide all nitrite functions in dry cured fermented sausages. Nitrate and nitrite reduction below their allowed maximum levels in European legislation (150 mg/kg of sodium nitrate and 150 mg/kg of sodium nitrite, (Regulation (EC) no 1333/2008) have safety and sensory consequences. The reduction up to a 50 % of the allowed limits affects growth of pathogenic microorganisms (Hospital, Hierro & Fernandez, 2014), volatile compounds production (Hospital, Hierro, Stringer & Fernandez, 2016) and aroma profile (Perea-Sanz, Montero, Belloch & Flores, 2018). A recent study (Christieans, Picgirard, Parafita, Lebert & Gregori, 2018) has shown that the use of a mixture of sodium nitrate and sodium nitrite at a concentration of 80 mg/kg, was able to control the growth of *Listeria monocytogenes* and *Salmonella typhimurium*. Nevertheless, the importance of nitrite presence at the beginning of the process to control these pathogens was remarked due to the slow conversion of nitrate into nitrite (Christieans et al., 2018).

The consequences of the use of reduced nitrate and nitrite mixtures on aroma profile of dry cured meat products have been scarcely studied. The inoculation of yeasts such as *Debaryomyces (D.) hansenii* with potential to generate desirable aromas in dry fermented

sausages is a suitable alternative. This yeast has been shown to produce esters and sulfur compounds as cured aroma contributors to the aroma profile of dry fermented sausages (Cano-Garcia, Belloch & Flores, 2014; Perea-Sanz, Peris, Belloch & Flores, 2019a). The production of aroma compounds by *D. hansenii* strains is influenced by the concentration of curing agents (Flores, Moncunill, Montero, López-Díez & Belloch, 2017), as well as by the availability of specific amino acids used as volatile precursors (Perea-Sanz et al., 2019a). A recent study regarding the production of volatile sulphur compounds that contribute to meat aroma notes by *D. hansenii* strains revealed that one yeast strain may have an impact on dry fermented sausage aroma due to its ability to express a gene directly related to sulfur compound production (Perea-Sanz et al., 2019a). Consequently, the aim of the present study is to evaluate the effect of the inoculation of a *D. hansenii* strain on dry fermented sausages manufactured with reduced ingoing amounts of nitrite and nitrate and its effect on aroma production during the ripening time.

## 2. Materials and methods

### *2.1. Dry fermented sausages manufacture*

Three different formulations of dry fermented sausages were manufactured. Control (C), containing sodium nitrite at 150 ppm and potassium nitrate at 178 ppm, and two formulations with a 47 % reduction of both nitrite and nitrate (80 and 95 mg/kg, respectively) ingoing amounts (R) as proposed by Christieans et al. (2018), but one of them inoculated with *D. hansenii* strain L1 (RY). Three replicates of the experiment were performed. Pork's lean meat and belly fat (boneless and skinless) were purchased from a local producer (Cárnicas La Cope, Torrente, Spain). Lean (50% lean pork meat) and fat (50% pork belly) were ground through a 10 mm diameter minced plate and mixed with the following ingredients: 20 g/kg lactose, 20 g/kg dextrin, 6 g/kg glucose, 20.25 g/kg sodium chloride (NaCl), 6.75 g/kg potassium chloride (KCl), 0.5 g/kg sodium ascorbate and 1.5 g/kg black pepper. Commercial starter culture TRADI-302 (Danisco, Cultor, Madrid, Spain) containing *Lactobacillus sakei*, *Staphylococcus xylosus* and *Staphylococcus carnosus* was added to the meat (0.125 g/kg). In the three replications, each formulation was supplemented with the different ingoing amounts of nitrate/nitrite mixtures. Then, *D. hansenii* L1 was inoculated using 10 ml saline suspension containing approximately 6 x $10^9$ cells/g. This yeast suspension was added to 6 kg of meat batter to reach a final concentration of $10^6$ cells/g. To homogenize all ingredients

into the meat batter, a mixer-kneading machine provided with rotating blades was used. Formulations were kept at 3–5°C for 24 h to rest after yeast inoculation and then, stuffed into 95 mm diameter collagen casings (Fibran, S.A., Girona, Spain), being the final weight of each sausage approximately 500 g. All sausages were submitted to a slow ripening process and dried at 10 °C and 75–90 % relative humidity (RH). In order to control the ripening process, temperature and RH of the ripening chambers were continuously recorded. A total of 9 batches (3 x 3) were produced. One sausage from each formulation was weighed regularly to control weight losses. Also, one sausage per formulation was used to control the pH by introducing a pH meter HI 99163 (Hanna Instruments Inc., Hoonsocket, USA) into the sausage centre as described by Olivares, Navarro, Salvador & Flores (2010). Sausages from the different formulation and replicates were sampled for analyses at 0, 60 and 83 d of process. At 0 d, approximately 200 g of the minced meat mixture were collected for analysis. After 60 d of ripening (44-45 % weight loss), three sausages from each formulation and replicate (3 x 3 sausages) were randomly chosen for analyses. The remaining sausages were ripened for 23 additional days (50% weight loss), and afterwards they were collected as above. Slices were taken for microbial analyses (25 g) and other slices for volatile analysis were vacuum packed and stored at −80 °C. Sausage colour and physicochemical analysis (moisture, water activity $a_w$, pH, TBARS, protein and residual nitrite and nitrate content) were measured on minced sausage.

### *2.2. Physicochemical parameters measurement*

Measurements of pH, $a_w$, weight loss, colour (CIE L*, a*, b*), moisture, and protein was done as described in Perea-Sanz et al. (2018). Lipid autoxidation was measured by the thiobarbituric acid reactive substances (TBARS) method and results expressed as µg malonaldehyde (MDA)/g in dry matter (Perea-Sanz et al., 2018). Residual nitrate and nitrite contents were extracted by Carrez precipitation and determined after zinc reduction and Griess reaction as reported in Merino (2009). Measurements were expressed as mg/kg of sodium nitrite and potassium nitrate in dry matter.

### *2.3. Microbiological analysis*

Microbial counts of lactic acid bacteria (LAB), gram positive coagulase negative cocci (GCN), yeasts and moulds and enterobacteria were carried out as described in Perea-Sanz et al. (2018).

### *2.4. D. hansenii L1 monitoring by M13 RAPD PCR*

Fifteen yeast colonies were isolated (Perea-Sanz et al., 2018) from each sausage batch (C, R and RY) at the three ripening times (0, 60 and 83 d). Colonies were tested for purity and freshly isolated colonies selected and cultured overnight at 25 ºC in 5 ml of GPY (glucose 2%, peptone 0.5% and yeast extract 0.5%). DNA extraction and M13 minisatelite PCR amplification were carried out as described in Cano-García, Flores & Belloch (2013). PCR products were separated by electrophoresis on 2 % agarose gel in 1X TAE buffer at 90 V for 3 h, stained with Red safe nucleic acid staining solution 20000x (Intron biotechnology, Korea) and visualized under UV light. DNA fragment sizes were determined using 100 bp DNA ladder (Invitrogen, USA).

### *2.5. Volatile compound analysis*

Analysis of volatile compounds was carried out by headspace (HS) solid-phase microextraction (SPME) with an 85 μm carboxen/polydimethylsiloxane (CAR/PDMS) fibre (Supelco, Bellefonte, USA) using a gas chromatograph (Agilent HP 7890 series II, Hewlett-Packard, Palo Alto, USA) with a quadrupole mass detector (HP 5975C, Hewlett-Packard, Palo Alto, USA) and equipped with an autosampler (MPS2 multipurpose sampler (Gerstel, Germany). In summary, 5 g of sausage supplemented with 0.75 mg BHT to avoid oxidation, were placed into a headspace vial and incubated at 37 °C for 30 min. The extracted volatile compounds were adsorbed in the fibre for 90 min at 37 °C and desorbed in the injection port of the GC–MS for 5 min at 240 °C in splitless mode. The volatile compounds were separated using a DB-624 capillary column (30 m, 0.25 mm i.d., film thickness 1.4 μm, (J&W Scientific, Agilent Technologies, USA)) using the conditions described by Corral, Salvador, Belloch & Flores (2015). The MS interface temperature was set to 240 °C. The compounds were identified in full scan mode and by comparison with mass spectra from the library database (Nist'05), with linear retention indices (Van Den Dool & Kratz, 1963) and with

authentic standards. The quantitation was done in SCAN mode using either total or extracted ion current (TIC or EIC) on an arbitrary scale and expressed as abundance units (AU) x $10^5$.

### *2.6. Olfactometric analysis*

The analysis of aroma compounds was carried out in a gas chromatograph (Agilent 6890, USA) equipped with a FID detector and a sniffing port (ODP3, Gerstel, Mülheim an der Ruhr, Germany) as described in Corral et al. (2015). Each assessment was carried out on 5 g of sausage using the detection frequency method (Pollien et al., 1997). The olfactometry analysis was done in sausages ripened for 60 d. Five trained panellists evaluated the odours from the GC-effluent. A total of 12 assessments were carried out. Aroma compounds were identified by comparison with mass spectra, using linear retention indices of authentic standards injected in GC–MS and GC-O and by the coincidence of the assessor's descriptors with those in the Fenaroli's handbook of flavour ingredients (Burdok, 2002).

### *2.7. Statistical analysis*

Data were analysed using the Generalized Linear Model (GLM) procedure of statistical software (XLSTAT 2011, v5.01, Addinsoft, Barcelona, Spain). Data were analysed using the linear mixed model including sausage formulation and ripening time as fixed effects, and replicates as random effect. The interaction between fixed effects was tested and evaluated as not significant, therefore it was excluded from the model. When a significant effect of the treatment group was detected ($P < 0.05$), least squares means (LSM) were compared using Tukey test. Principal component analysis (PCA) was plotted to evaluate the relationships among sausage formulations and parameters (physicochemical, microbiological and aroma compounds) measured at each sampling time.

## 3. Results

### *3.1. Physicochemical characteristics of the sausages*

Measurements of physicochemical parameters from samples of the three formulations at the three sampling times are shown in Table 1. At the initial time (0 d), the three formulations were very similar regarding all parameters except nitrite and nitrate concentration. Residual nitrite differences between C and reduced R and RY formulations was 45-52%, whereas residual nitrate differences were about 31-33%. After 60 d of ripening, no residual nitrite levels were detected in any formulation, while residual nitrate levels in R and RY respect to

C were 40 % and 46 %, respectively. These differences in residual nitrate concentrations were maintained after 83 d of ripening. During the ripening time, residual nitrate decreased around 17 %, 36 % and 48 % within C, R and RY sausages, respectively. Colour parameter a* increased after 60 d of ripening. Lipid oxidation (TBARS) evolution was different among the formulations. C and R showed the same trend, an initial increase at 60 d and a slow decline after 83 d, while RY sausages did not show lipid oxidation maintaining low TBARS values during the ripening time. In addition, the highest pH value was reached at 60 d in RY sausages. These differences between RY and the other two formulations could be observed also in water activity and moisture at 83 d of ripening time.

### *3.2. Microbiological counts*

Results on microbial counts are shown in Fig. 1. Enterobacteriaceae were not found in any sausage batch at any sampling time (data not shown). LAB counts (Fig. 1A) increased from 0 d to 60 d and decreased slightly at 83 d. Significant differences between LAB counts were found at 0 d in RY respect to C and R batches, but at 60 and 83 d LAB counts were indistinguishable between batches. GCN counts (Fig. 1B) decreased constantly from 0 d and no significant differences were found among sausages at any ripening time. Yeast counts (Fig. 1C) were lower than 10 cfu/g in C (2 colonies) and R (5 colonies) sausages at 0 d, whereas the RY showed a yeast count in accordance with *D. hansenii* L1 inoculation. Yeast counts increased from 0 d to 60 d in all sausages and decreased slightly from 60 to 83 d, however counts in RY were higher than C and R.

### *3.3. D. hansenii monitoring by M13 RAPD-PCR along sausage ripening*

The implantation of the inoculated *D. hansenii* strain on RY sausages, as well as its presence in C and R sausages, was followed by comparison of M13 minisatellite PCR patterns of yeast isolates with the inoculated *D. hansenii* L1 strain (Fig. 2). M13 patterns of yeast isolates from RY sausages at any ripening time were identical to the pattern of the inoculated *D. hansenii* L1 strain. On the other hand, several yeast isolates from C sausages (Figure 2A) and R sausages (Figure 2B) showed M13 patterns different from the pattern of *D. hansenii* L1 strain. In C sausages, only one isolate at 60 d and in R batch 5 isolates at 60 d and 6 isolates at 83 d were found with identical pattern of the inoculated *D. hansenii* L1 strain.

**Table 1. Effect of nitrite/nitrate reduction and *D. hansenii* inoculation on physicochemical parameters of dry fermented sausages.**

| | C[1] | | | R | | | RY | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0d | 60d | 83d | 0d | 60d | 83d | 0d | 60d | 83d | RMSE[2] | $P_t$[3] | $P_f$ |
| **pH** | 5.79 a | 4.85 d | 4.94 bc | 5.79 a | 4.86 d | 4.94 bc | 5.80 a | 4.99 b | 4.92 c | 0.05 | *** | ** |
| **aw** | 0.973 a | 0.894 b | 0.809 d | 0.973 a | 0.897 b | 0.807 d | 0.969 a | 0.899 b | 0.837 c | 0.006 | *** | *** |
| **Moisture %** | 64.15 a | 42.44 b | 29.69 d | 64.19 a | 42.28 b | 29.77 d | 63.48 a | 41.92 b | 32.58 c | 1.18 | *** | ns |
| **Protein (% dm)** | 49.42 d | 52.89 cd | 54.14 bc | 50.71 cd | 58.08 a | 53.23 bcd | 49.97 d | 56.22 ab | 53.25 bcd | 2.81 | *** | ns |
| **L*** | 59.81 a | 52.85 b | 44.76 cd | 59.91 a | 52.21 b | 44.34 d | 60.26 a | 53.45 b | 46.64 c | 1.71 | *** | ns |
| **a*** | 13.79 c | 16.81 a | 14.40 bc | 14.49 bc | 16.76 a | 14.24 bc | 12.64 d | 17.04 a | 14.78 b | 0.51 | *** | ns |
| **b*** | 11.00 a | 6.07 b | 5.11 c | 11.28 a | 5.67 b | 5.07 c | 11.72 a | 6.03 b | 5.59 bc | 0.47 | *** | * |
| **TBARS[4]** | 0.59 d | 3.62 a | 2.21 b | 0.48 d | 2.23 b | 1.27 c | 0.47 d | 0.63 d | 0.47 d | 0.32 | *** | *** |
| **$NaNO_2$ mg/kg dm** | 217.40 a | 0.00 c | 0.00 c | 102.32 b | 0.00 c | 0.00 c | 119.10 b | 0.00 c | 0.00 c | 11.20 | *** | *** |
| **$KNO_3$ mg/kg dm** | 533.05 a | 396.77 bc | 442.44 b | 363.81 c | 235.32 d | 233.20 d | 364.66 c | 212.67 d | 188.39 d | 42.37 | *** | *** |

[1]C: control batch; R: 47% reduction in nitrite and nitrate; RY: 47% reduction in nitrite and nitrate and *D. hansenii* inoculated. [2]RMSE: root mean square error. [3]$P_t$: *P* value of time effect, $P_f$: *P* value of formulation effect. Different letters in the same row of each group indicate significant differences at *** P < 0.001, ** P < 0.01, * P < 0.05. ns: P > 0.05. [4]TBARS expressed as µg malonaldehyde/g dm.

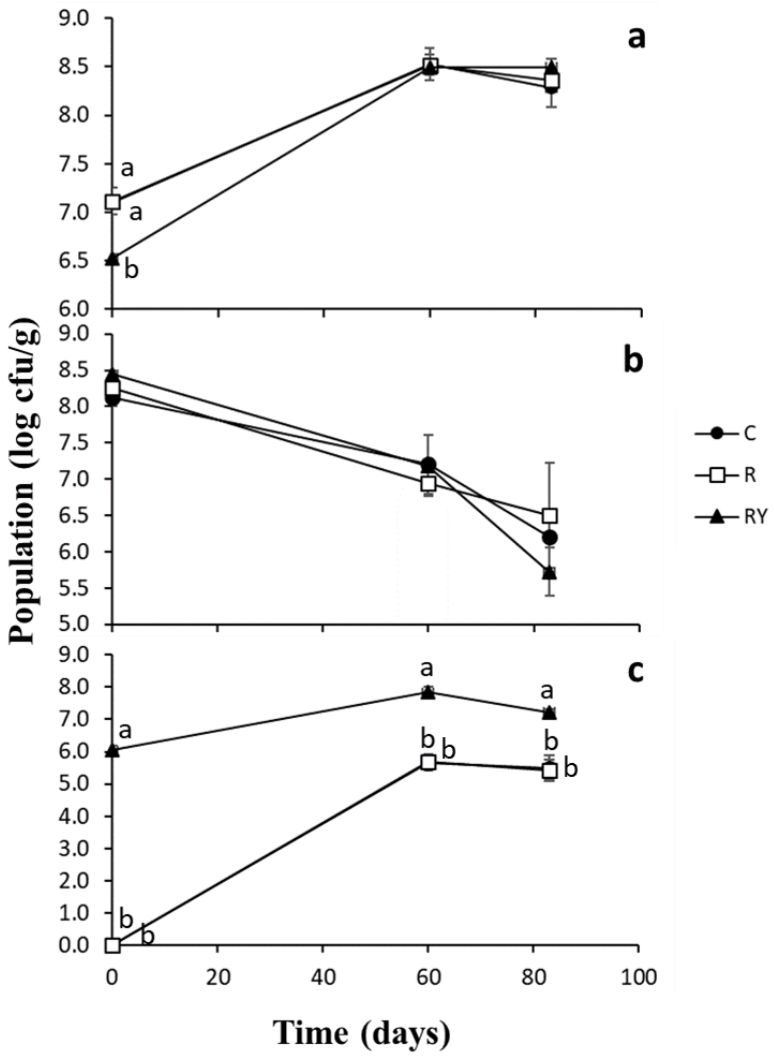


**Figure 1.** Changes in microbial populations during the ripening of dry fermented sausages in Control (●), R (□) and RY (▲) formulations. Lactic acid bacteria (LAB) (a), Gram positive coagulase negative cocci (GCN) (b) and yeasts and moulds (c). Different letters in the same ripening time indicate mean differences at *** $P < 0.001$ among sausages.

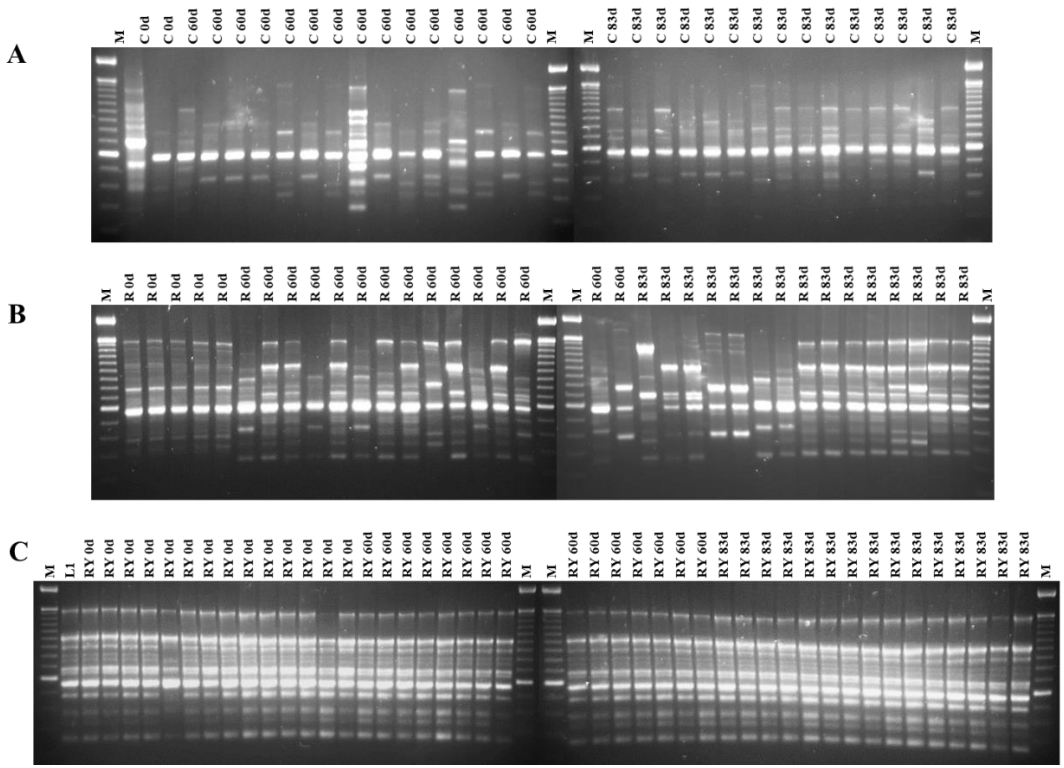


**Figure 2.** Electrophoretic patterns of minisatellite M13 PCR amplification of yeast isolates at 0, 60 and 83 d of ripening in C (A), R (B) and RY (C) formulations. The arrow indicates bands of isolated yeast in C and R sausages matching the pattern of the inoculated strain L1. Lane M is the 100 bp ladder. Lane L1 in panel C represents the pattern of the inoculated *D. hansenii* strain.

### *3.4. Volatile compounds generation*

Eighty volatile compounds were identified and quantified in the HS of dry fermented sausages using a CAR/PDMS fibre (Table 2). The volatile compounds were classified by their most probable origin. Within the bacterial metabolism origin, four subgroups were done: amino acid degradation (17), carbohydrate fermentation (8), esterase activity (10) and β-oxidation (1)), from the chemical reaction origin two subgroups: lipid oxidation reaction (22) and thiamine degradation (2)); and finally, from spices (17) and from unknown origin (3). Among the 80 volatiles identified, 61 compounds were confirmed while 11 were tentatively identified.

The RY sausages showed the highest concentration of volatile compounds from amino acid degradation at both drying times. The most abundant compounds were 3-methyl-1-butanol, 2-methyl propanoic acid and 2-methyl and 3-methyl butanoic acids, whereas dimethyl disulphide, toluene, 3-(methylthio)propanal, benzeneacetaldehyde, 2,6-dimethylpyrazine and phenol were the less abundant. On the other hand, methanethiol and 3-methylbutanal were the most abundant compounds found in C and R sausages. Comparison between C and R sausages revealed that nitrite and nitrate reduction produce significant differences only in two compounds; 3-(methylthio)propanal and benzeneacetaldehyde which were more abundant in C sausages. During the ripening period between 60 and 83 d, the total volatile compounds derived from amino acid degradation diminished in RY sausages. This decrease was significant in methanethiol, 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, and 3-methyl-1-butanol concentrations. Only benzeneacetaldehyde increased after the 60 d of ripening in RY sausages. Three volatile compounds derived from amino acid degradation were identified and only traces were detected of 2-acetyl-2-thiazoline, 2-acetyl-1-pyrroline and dimethyl trisulfide.

The sum of carbohydrate fermentation volatiles was significantly higher in C and R sausages than in RY sausages at both drying times. The most abundant compounds in C and R sausages respect to RY sausages were 2,3-butanedione, 2-butanone, acetic acid and 3-hydroxy-2-butanone. On the contrary, acetaldehyde and ethyl alcohol were present in significant high proportions in RY sausages. During the ripening time between 60 and 83 d,

the total abundance of volatile compounds derived from carbohydrate fermentation increased exclusively in C sausages due to the increase in acetic acid.

Ethyl acetate was the most abundant volatile compound derived from esterase activity in all sausage types, followed by ethyl-3-methylbutanoate in RY sausages. The total abundance of volatile compounds derived from esterase activity was significantly higher in RY sausages than in C and R sausages at both drying times. This difference was observed in all ester compounds except for ethyl pentanoate and ethyl hexanoate. In addition, 3-methylbutyl acetate and ethyl-2-methylpropanoate were exclusively present in RY sausages. Nitrate and nitrite reduction in R produced a decrease in two ester compounds, ethyl hexanoate and ethyl 2-hydroxypropanoate at 60 d although the additional drying time increased its abundance.

Among volatile compounds derived from chemical origin only those derived from lipid oxidation were analysed being the most abundant hexanal in all sausages. These compounds were the lowest in RY sausages at both ripening times. This was due to a significant decrease of most volatiles except for 1-propanol. The last step of ripening from 60 to 83 d, caused a decrease in the total abundance of volatile compounds derived from lipid oxidation although pentanoic and octanoic acids increased during this period.

Only two volatile compounds from thiamine degradation (2-methyl-3-furanthiol and methyl-2-methyl-3-furyldisulfide) were identified as traces. Finally, volatile compounds derived from spices were abundant in all sausage formulations but most of them were not affected by either formulation or drying time.

**Table 2. Effect of nitrite/nitrate reduction and *D. hansenii* inoculation on volatile compounds generated in dry fermented sausages (expressed as abundance (AU) of total ion current (TIC) or area of the target ion shown in parenthesis as AU×10$^5$).**

| | | | C[3] | | R | | RY | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | LRI[1] | RI[2] | 60 d | 83 d | 60 d | 83 d | 60 d | 83 d | RMSE[4] | $P_t$[5] | $P_f$ |
| **Microbial origin** | | | | | | | | | | | |
| **Amino acid degradation** | | | **155.29 c** | **202.53 c** | **135.14 c** | **210.42 c** | **457.22 a** | **355.54 b** | **51.70** | **ns** | ******* |
| Methanethiol | 473 | a | 28.43 c | 60.35 a | 30.51 c | 64.79 a | 43.02 b | 16.27 d | 5.59 | *** | *** |
| 2-Methylpropanal | 593 | a | 7.36 b | 5.25 b | 5.76 b | 4.46 b | 26.54 a | 5.78 b | 8.17 | ** | ** |
| 3-Methylbutanal | 690 | a | 35.96 ab | 32.74 b | 32.29 b | 26.29 b | 55.26 a | 24.05 b | 12.61 | ** | ns |
| 2-Methylbutanal (58) | 698 | a | 1.94 b | 1.73 b | 1.93 b | 1.61 b | 6.26 a | 1.62 b | 1.72 | ** | ** |
| Dimethyl disulfide | 773 | a | 18.21 a | 5.73 b | 9.61 ab | 5.77 b | 4.66 b | 7.60 ab | 6.14 | * | ns |
| Toluene (91) | 788 | a | 10.62 a | 8.98 ab | 8.14 ab | 7.04 b | 7.80 b | 6.79 b | 1.47 | * | *** |
| 3-Methyl-1-butanol (55) | 795 | a | 2.66 c | 2.14 c | 2.85 c | 2.52 c | 33.68 a | 16.31 b | 1.78 | *** | *** |
| 2-Methyl propanoic acid | 864 | a | 14.80 b | 32.68 b | 11.84 b | 48.07 b | 142.41 a | 161.01 a | 30.95 | * | *** |
| 3-Methyl butanoic acid (60) | 941 | a | ND b | ND b | ND b | ND b | 93.81 a | 127.11 a | 26.67 | ns | *** |
| 2-Acetyl-1-pyrroline | 961 | a | tr[6] | tr | tr | tr | tr | tr | | | |
| 2,6-Dimethylpyrazine (108) | 944 | a | 2.88 b | 21.26 a | 3.63 b | 18.85 a | 0.54 b | 2.54 b | 4.11 | *** | *** |
| 2-Methyl butanoic acid (74) | 946 | a | 6.54 b | 8.63 b | 8.59 b | 11.38 b | 29.36 a | 29.98 a | 5.46 | ns | *** |
| 3-(Methylthio)propanal (104) | 968 | a | 1.45 a | 1.06 ab | 0.79 bc | 0.68 bc | 0.33 c | 0.49 c | 0.27 | ns | *** |

**Table 2. Continued.**

| | | | C[3] | | | | R | | | | RY | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LRI[1] | RI[2] | 60 d | | 83 d | | 60 d | | 83 d | | 60 d | | 83 d | | RMSE[4] | $P_t$[5] | $P_f$ |
| Dimethyl trisulfide | 1003 | a | tr | | tr | | tr | | tr | | tr | | tr | | | | |
| Benzeneacetaldehyde | 1110 | a | 10.93 | a | 9.99 | ab | 7.11 | bc | 7.89 | abc | 5.11 | c | 8.73 | ab | 1.84 | ns | *** |
| Phenol | 1114 | a | 13.53 | a | 13.42 | a | 12.09 | a | 12.97 | a | 8.44 | b | 10.91 | ab | 1.90 | ns | *** |
| 2-Acetyl-2-thiazoline | 1175 | a | tr | | tr | | tr | | tr | | tr | | tr | | | | |
| **Carbohydrate fermentation** | | | **5631.62** | **b** | **7405.09** | **a** | **6280.08** | **ab** | **6877.28** | **ab** | **3473.90** | **c** | **3777.01** | **c** | **983.47** | ****** | ******* |
| Acetaldehyde | 467 | a | 25.14 | b | 20.89 | b | 18.13 | b | 16.98 | b | 73.09 | a | 28.18 | b | 13.21 | *** | *** |
| Ethyl alcohol | 507 | a | 887.72 | b | 739.85 | b | 670.60 | b | 854.35 | b | 2294.16 | a | 2192.11 | a | 151.08 | ns | *** |
| Acetone | 529 | a | 69.34 | a | 47.78 | ab | 61.17 | ab | 42.92 | b | 49.51 | ab | 54.99 | ab | 13.49 | ** | ns |
| 2,3-Butanedione | 627 | a | 129.85 | a | 129.93 | a | 160.14 | a | 119.13 | a | 19.36 | b | 9.69 | b | 27.37 | ns | *** |
| 2-Butanone | 631 | a | 72.68 | a | 48.17 | b | 79.28 | a | 43.59 | b | 28.79 | b | 26.80 | b | 13.22 | *** | *** |
| Acetic acid | 717 | a | 2805.24 | b | 4435.95 | a | 3122.49 | b | 3822.32 | ab | 813.68 | c | 1343.73 | c | 651.28 | *** | *** |
| 3-Hydroxy-2-butanone | 781 | a | 1586.48 | a | 1914.66 | a | 2109.15 | a | 1890.61 | a | 150.70 | b | 46.42 | b | 400.29 | ns | *** |
| Butanoic acid | 891 | a | 55.16 | de | 67.85 | bc | 59.12 | cd | 87.39 | a | 44.60 | e | 75.09 | b | 6.81 | *** | *** |
| **Esterase activity** | | | **404.73** | **b** | **364.80** | **b** | **415.44** | **b** | **423.13** | **b** | **1077.97** | **a** | **1134.48** | **a** | **112.28** | **ns** | ******* |
| Ethyl acetate | 635 | a | 229.96 | bc | 178.56 | c | 283.17 | b | 199.47 | bc | 430.45 | a | 462.49 | a | 58.33 | ns | *** |
| Ethyl 2-methylpropanoate (71) | 788 | a | ND | b | ND | b | ND | b | ND | b | 30.70 | a | 31.56 | a | 4.13 | ns | *** |
| Ethyl butanoate | 831 | a | 29.65 | b | 29.57 | b | 27.13 | b | 32.82 | b | 69.91 | a | 77.54 | a | 7.21 | ns | *** |
| Ethyl 2-hydroxypropanoate | 867 | a | 40.96 | bc | 46.99 | c | 26.86 | d | 51.52 | c | 81.60 | b | 143.68 | a | 10.39 | *** | *** |

**Table 2. Continued.**

| | | | C[3] | | | | R | | | | RY | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LRI[1] | RI[2] | 60 d | | 83 d | | 60 d | | 83 d | | 60 d | | 83 d | | RMSE[4] | $P_t$[5] | $P_f$ |
| Ethyl 2-methylbutanoate (57) | 878 | a | 2.41 | b | 3.26 | b | 2.60 | b | 5.20 | b | 31.95 | a | 32.78 | a | 5.84 | ns | *** |
| Ethyl 3-methylbutanoate | 882 | a | 33.96 | b | 55.91 | b | 32.89 | b | 94.84 | b | 387.96 | a | 386.12 | a | 84.17 | ns | *** |
| 3-Methylbutyl acetate (70) | 907 | a | ND | b | ND | b | ND | b | ND | b | 1.00 | a | 1.03 | a | 0.10 | ns | *** |
| Ethyl pentanoate | 928 | a | 21.53 | a | 12.56 | bc | 13.65 | b | 10.93 | bcd | 8.44 | cd | 6.76 | d | 2.82 | *** | *** |
| Ethyl hexanoate | 1030 | a | 45.43 | a | 37.45 | ab | 28.48 | b | 27.88 | b | 32.58 | b | 36.30 | ab | 6.71 | ns | *** |
| Ethyl octanoate (88) | 1230 | a | 0.83 | c | 0.50 | c | 0.66 | c | 0.46 | c | 3.37 | b | 4.10 | a | 0.28 | ns | *** |
| **β-Oxidation** | | | | | | | | | | | | | | | | | |
| 1-Octen-3-ol | 1032 | a | 30.49 | a | 29.09 | a | 25.23 | a | 24.13 | a | 10.04 | b | 12.43 | b | 5.00 | ns | *** |
| **Chemical origin** | | | | | | | | | | | | | | | | | |
| **Lipid oxidation** | | | **3441.38** | **a** | **2502.57** | **b** | **1768.25** | **bc** | **1192.48** | **cd** | **539.38** | **d** | **603.53** | **d** | **499.39** | ****** | ******* |
| Pentane | 500 | a | 31.95 | | 17.41 | | 28.03 | | 28.22 | | 32.05 | | 35.80 | | 11.09 | ns | ns |
| Hexane | 600 | a | 8.28 | | 5.01 | | 8.38 | | 7.40 | | 9.35 | | 7.43 | | 2.62 | * | ns |
| 1-Propanol | 312 | a | 2.02 | c | 1.99 | c | 1.95 | c | 1.74 | c | 21.69 | a | 13.16 | b | 1.66 | *** | *** |
| Butanal | 622 | a | 3.68 | a | 2.41 | b | 1.91 | bc | 1.51 | bcd | 0.77 | d | 1.22 | cd | 0.62 | ns | *** |
| Heptane | 700 | a | 9.49 | | 6.94 | | 9.76 | | 8.63 | | 6.92 | | 7.06 | | 3.20 | ns | ns |
| Pentanal | 739 | a | 136.60 | a | 88.75 | b | 69.27 | bc | 49.72 | bcd | 19.23 | d | 31.16 | cd | 24.34 | * | *** |
| Octane | 800 | a | 135.27 | a | 128.26 | ab | 120.72 | abc | 102.54 | abc | 74.20 | c | 82.53 | bc | 29.58 | ns | *** |
| Propanoic acid | 811 | a | 14.80 | bc | 21.55 | a | 12.39 | c | 18.80 | ab | 12.72 | c | 15.09 | bc | 2.77 | *** | ** |

**Table 2. Continued.**

| | | | C[3] | | | | R | | | | RY | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LRI[1] | RI[2] | 60 d | | 83 d | | 60 d | | 83 d | | 60 d | | 83 d | | RMSE[4] | $P_t$[5] | $P_f$ |
| 1-Pentanol (70) | 827 | a | 4.45 | a | 2.57 | b | 3.83 | a | 2.02 | bc | 1.82 | bc | 1.15 | c | 0.65 | *** | *** |
| Hexanal | 842 | a | 2774.14 | a | 1969.97 | b | 1223.18 | c | 768.59 | cd | 201.53 | d | 270.65 | d | 410.63 | ** | *** |
| 1-Hexanol | 924 | a | 81.87 | a | 39.79 | ab | 80.92 | ab | 37.78 | bc | 30.23 | c | 11.20 | c | 24.98 | *** | *** |
| Heptanal (70) | 941 | a | 17.36 | a | 17.18 | ab | 13.11 | ab | 8.69 | b | ND | b | ND | b | 5.62 | ns | ** |
| Pentanoic acid (60) | 980 | a | 4.00 | ab | 4.96 | a | 3.29 | b | 4.23 | ab | 1.08 | c | 1.30 | c | 0.73 | ** | *** |
| 2-Pentylfuran | 1010 | a | 25.20 | a | 25.22 | a | 15.42 | b | 17.78 | b | 3.79 | c | 4.28 | c | 3.51 | ns | *** |
| (Z)- 2-Heptenal | 1012 | a | 10.45 | a | 8.20 | ab | 5.90 | bc | 5.65 | c | 1.89 | d | ND | | 1.47 | * | *** |
| Octanal (56) | 1049 | a | 7.14 | a | 6.27 | a | 5.71 | ab | 3.71 | bc | 3.02 | c | 3.12 | c | 1.33 | * | *** |
| Hexanoic acid (60) | 1077 | a | 22.26 | a | 25.95 | a | 19.09 | a | 22.42 | a | 5.27 | b | 6.88 | b | 3.98 | 0.038 | *** |
| (E)-2-Octenal | 1117 | a | 13.03 | a | 11.01 | ab | 7.45 | bc | 7.76 | bc | 1.37 | d | 5.17 | cd | 2.25 | ns | *** |
| Nonanal | 1151 | a | 122.97 | | 95.75 | | 122.58 | | 75.31 | | 97.86 | | 87.20 | | 30.58 | ** | ns |
| (E)-2-Nonenal | 1224 | a | 3.69 | a | 3.59 | a | 2.36 | b | 2.15 | b | 1.11 | c | 1.92 | bc | 0.49 | ns | *** |
| Octanoic acid | 1267 | a | 11.02 | d | 18.10 | a | 11.42 | cd | 16.30 | ab | 12.29 | bcd | 16.16 | abc | 2.76 | *** | ns |
| Nonanoic acid (60) | 1359 | a | 1.70 | | 1.70 | | 1.56 | | 1.53 | | 1.51 | | 1.03 | | 0.96 | ns | ns |
| **Thiamine degradation** | | | | | | | | | | | | | | | | | |
| 2-Methyl-3-furanthiol | 896 | a | tr | | tr | | tr | | tr | | tr | | tr | | | | |
| Methyl-2-methyl-3-furyldisulfide | 1221 | a | tr | | tr | | tr | | tr | | tr | | tr | | | | |

**Table 2. Continued.**

| | | | C[3] | | R | | RY | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | LRI[1] | RI[2] | 60 d | 83 d | 60 d | 83 d | 60 d | 83 d | RMSE[4] | $P_t$[5] | $P_f$ |
| **Spices** | | | **3105.15** | **3210.12** | **3087.19** | **2804.85** | **3299.25** | **2941.91** | **376.00** | **ns** | **ns** |
| O-Xylene (91) | 917 | a | 0.84 | 0.72 | 0.70 | 0.67 | 0.69 | 0.59 | 0.14 | ns | ns |
| Styrene (104) | 919 | a | 0.57 c | 0.78 c | 0.51 c | 0.64 c | 3.17 a | 2.53 b | 0.19 | ns | *** |
| Unidentified terpene | 934 | c | 165.77 | 141.31 | 168.37 | 138.55 | 160.73 | 141.74 | 20.13 | *** | ns |
| β-Phellandrene | 988 | b | 460.31 | 419.40 | 486.90 | 407.33 | 490.25 | 440.91 | 66.65 | * | ns |
| β-Myrcene (93) | 1003 | a | 29.80 | 33.77 | 29.68 | 28.42 | 33.01 | 29.16 | 4.26 | ns | ns |
| Unidentified terpene | 1019 | c | 113.30 | 121.94 | 120.30 | 105.53 | 126.23 | 110.98 | 19.27 | ns | ns |
| 3-Carene | 1022 | a | 571.43 | 630.26 | 555.16 | 515.62 | 638.08 | 540.43 | 100.34 | ns | ns |
| Unidentified terpene | 1034 | c | 51.50 ab | 48.03 b | 55.76 ab | 48.49 b | 61.38 a | 55.69 ab | 6.19 | * | ** |
| D-limonene | 1046 | a | 1081.58 | 1170.91 | 1063.91 | 1004.14 | 1133.01 | 1022.63 | 129.31 | ns | ns |
| Unidentified terpene | 1075 | c | 68.52 | 68.29 | 68.15 | 61.41 | 83.39 | 66.98 | 17.45 | ns | ns |
| Unidentified terpene (93) | 1099 | c | 0.99 | 0.98 | 0.80 | 0.81 | 0.90 | 0.78 | 0.14 | ns | ns |
| Terpinolene (93) | 1101 | a | 3.70 | 3.86 | 3.75 | 3.31 | 4.08 | 3.48 | 0.50 | ns | ns |
| Unidentified terpene | 1121 | c | 15.05 ab | 19.32 a | 14.83 ab | 17.49 ab | 14.48 b | 19.14 a | 2.57 | *** | ns |
| Unidentified terpene | 1366 | c | 26.89 a | 26.61 a | 22.58 ab | 20.12 b | 23.74 ab | 21.74 ab | 3.62 | ns | ** |
| Unidentified terpene | 1377 | c | 8.24 | 9.49 | 6.77 | 7.60 | 7.66 | 8.29 | 2.50 | ns | ns |
| Unidentified terpene | 1407 | c | 71.50 ab | 77.51 a | 64.64 ab | 59.95 b | 66.96 ab | 64.48 ab | 9.45 | ns | ** |
| Caryophyllene | 1470 | a | 435.18 | 436.96 | 424.39 | 384.77 | 451.48 | 412.34 | 49.79 | ns | ns |

**Table 2. Continued.**

| | | | C[3] | | | | R | | | | RY | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LRI[1] | RI[2] | 60 d | | 83 d | | 60 d | | 83 d | | 60 d | | 83 d | | RMSE[4] | $P_t$[5] | $P_f$ |
| **Unknown origin** | | | **27.74** | **b** | **29.19** | **c** | **34.36** | **ab** | **39.25** | **a** | **63.34** | **ab** | **64.12** | **ab** | **13.80** | **ns** | ******* |
| 2-Methyl-1-propene (41) | 459 | b | 4.87 | a | 4.08 | ab | 2.80 | bc | 2.13 | c | 1.42 | c | 1.61 | c | 0.88 | ns | *** |
| Carbon disulfide | 537 | a | 21.97 | b | 23.95 | b | 30.58 | b | 36.01 | b | 66.95 | a | 61.37 | a | 13.87 | ns | *** |
| 4-Methylphenol (107) | 1197 | a | 0.90 | b | 1.16 | a | 0.98 | b | 1.11 | a | 0.97 | b | 1.14 | a | 0.07 | *** | ns |

[1]LRI: Linear retention index of the compounds eluted from the GC–MS using a DB-624 capillary column. [2]RI: Reliability of identification: a, identification by mass spectrum and by coincidence with LRI of authentic standard; b, tentative identification by mass spectrum; c: unknown. [3]C: control batch; R: 47% reduction in nitrite and nitrate; RY: 47% reduction in nitrite and nitrate and *D. hansenii* inoculated. [4]RMSE: Roost mean square error. [5]$P_t$: *P* value of ripening time effect, $P_f$: *P* value of formulation effect. Different letters in the same row indicate significant differences at *** P < 0.001, ** P < 0.01, * P < 0.05. ns: P > 0.05.[6]tr: traces, not quantified.

### *3.5. Aroma characteristics of the sausages*

Gas chromatography-olfactometry (Table 3) revealed the presence of 29 aroma active zones which were identified except for 1 odour zone attributed to an identified terpene. The most important aroma compounds with the highest DF (detection frequency) values (DF > 10) were 2,3-butanedione, acetic acid, hexanal, ethyl 2-methylbutanoate, 1-hexanol, 2-acetyl-1-pyrroline, methional, dimethyl trisulfide and 3-carene. The assessors were able to detect five odour zones that corresponded to compounds 2-methyl-3-furanthiol, 2-acetyl-1-pyrroline, dimethyl trisulfide, 2-acetyl-2-thiazoline and methyl-2-methyl-3-furyldisulfide which were not analysed by mass spectra due to their presence as trace levels.

The relationship between the instrumental variables and the ingoing amounts of nitrate and nitrite and *D. hansenii* inoculation at each ripening time, were examined by a principal component analysis (Fig. 3). Comparisons were done using the parameters (physicochemical, microbiological parameters and aroma compounds) measurements at 60 (Fig. 3 A and B) and 83 d (Fig. 3 C and D) of ripening. Regarding results at 60 d (Fig. 3A), the first two principal components were able to explain the 72.09 % of the total variance. PC1 accounted for 56.34 % of the variance and distinguished sausages by *D. hansenii* inoculation, placing C and R sausages on the left quadrant and RY sausages on the right quadrant. Sausages inoculated with *D. hansenii* RY, were associated with higher water activity, pH, yeast and moulds and colour parameters. Regarding aroma compounds, RY sausages were related to those derived from esterase activity, as well as 3-methylbutanal and methanethiol resulting from amino acid degradation. On the other hand, C and R sausages were related to an increase in TBARS and aroma compounds derived from lipid oxidation, β-oxidation and carbohydrate fermentation, as well as methional resulting from amino acid degradation. PC2 represented 15.76 % of the variance corresponding to the variability among replicates.

Regarding results at 83 d of drying (Fig. 3 C and D), two principal components were able to explain the 70.40 % of the total variance. PC1 accounted for 56.21 % of the variance and distinguished sausages by *D. hansenii* inoculation and nitrate and nitrite reduction, placing C sausages on the left quadrant, R sausages on the centre and RY sausages on the right quadrant. Sausages inoculated with *D. hansenii* RY, were again related to higher water

activity, yeast and moulds, colour parameters and aroma compounds derived from esterase activity. Moreover, C sausages were again related to TBARS and aroma compounds derived from lipid oxidation, while the R sausages were related to GCN counts and aroma compounds derived from amino acid degradation and carbohydrate fermentation. PC2 represented 14.19 % of the variance corresponding to the variability among replicates.

**Table 3. Odour active compounds identified in dry fermented sausages.**

| Compounds | IRL GC-O[1] | IRL std GC-O[2] | RI[3] | GC-O description | DF[4] |
|---|---|---|---|---|---|
| Methanethiol | 474 | 471 | a | Rotten, unpleasant | 9 |
| 1-Propanol | 611 | 614 | a | Acid, fermented | 4 |
| 2-Butanone | 628 | 632 | a | Wet, fresh, sweet | 3 |
| 2,3-Butanedione | 633 | 638 | a | Cheese, butter, dairy, fruit | 11 |
| 3-Methylbutanal | 690 | 691 | a | Sweet, green, spicy | 5 |
| Acetic acid | 701 | 700 | a | Acid, fermented, vinegar | 11 |
| 3-Hydroxy-2-butanone | 779 | 777 | a | Cardboard, green | 3 |
| Ethyl 2-methylpropanoate | 783 | 789 | a | Sweet, fruity | 4 |
| Ethyl butanoate | 824 | 825 | a | Sweet, fruity, fresh | 5 |
| Hexanal | 835 | 836 | a | Green, fresh cut grass, fatty | 12 |
| Ethyl 2-hydroxypropanoate | 861 | 859 | a | Fresh, floral, acid | 3 |
| Ethyl 2-methylbutanoate | 872 | 872 | a | Pineapple, sweet, acid, unpleasant, | 10 |
| Ethyl 3-methylbutanoate | 875 | 876 | a | Sweet, fruity, unpleasant, acid, | 7 |
| 2-Methyl-3-furanthiol | 899 | 897 | a | Fatty, medicinal, unpleasant, sulfur | 3 |
| 1-Hexanol | 925 | 919 | a | Fatty, rancid, rotten fruit, unpleasant | 12 |
| Heptanal | 942 | 939 | a | Cured, rancid, unpleasant | 6 |
| 2-Acetyl-1-pyrroline | 962 | 963 | a | Roasted, fried corn, roasted nuts, | 11 |
| Methional | 969 | 969 | a | Cooked potatoes, vegetable, meaty, sulfur | 11 |

**Table 3. Continued.**

| Compounds | IRL GC-O[1] | IRL std GC-O[2] | RI[3] | GC-O description | DF[4] |
|---|---|---|---|---|---|
| β-Myrcene | 1002 | 1003 | a | Green | 3 |
| Dimethyl trisulfide | 1011 | 1009 | a | Spicy, pungent, sulfur, rotten, vegetable | 11 |
| 3-Carene | 1021 | 1026 | a | Mushrooms, earthy, green, fresh | 12 |
| 1-Octen-3-ol | 1032 | 1028 | a | Green, fresh, mushrooms | 5 |
| Octanal | 1046 | 1047 | a | Sweet, citrus, floral | 7 |
| Hexanoic acid | 1068 | 1064 | a | Cheese, unpleasant, floral fresh | 3 |
| Unidentified terpene | 1092 | | b | Spicy, fresh, floral | 3 |
| Nonanal | 1150 | 1151 | a | Fresh, herbaceous, green, unpleasant | 7 |
| 2-Acetyl-2-thiazoline | 1180 | 1178 | a | Toasted, fried corn, caramel, toasted corn, bread | 8 |
| 4-Methylphenol | 1198 | 1190 | a | Unpleasant, rotten, sulphur | 6 |
| Methyl-2-methyl-3-furyl disulfide | 1225 | 1225 | a | Meaty, unpleasant, wet wood, fermented, rotten | 9 |

[1]Linear retention index of the compounds eluted from the GC-FID-O using a DB-624 capillary column.
[2]Linear retention index of standard compounds in the GC-FID-O.
[3]Reliability of identification: a, identification by mass spectrum, coincidence with LRI of an authentic standard and by coincidence of the assesors's descriptors with those described in the Fenaroli's handbook of flavour ingredients (Burdok, 2002); b, tentative identification by mass spectrum.
[4]Detection frequency value.

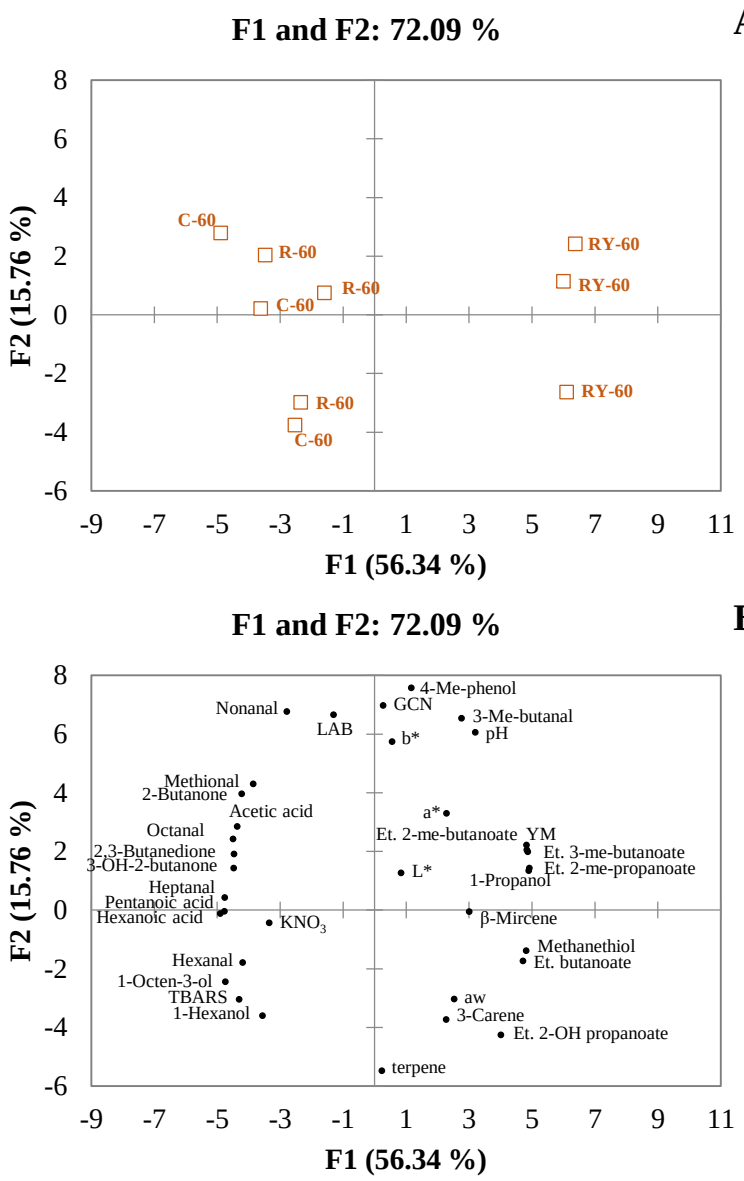


**Figure 3.** Loadings of the first two principal components (PC1-PC2) representing the variability (physicochemical, microbiological parameters and aroma compounds) of the three batches of dry fermented sausages at 60 d (A and B) and 83 d (C and D) of ripening. C (control), R (47% reduction of nitrate and nitrite), RY (47% reduction of nitrate and nitrite and inoculated with *D. hansenii*). YM: yeasts and moulds; LAB: lactic acid bacteria; GCN: Gram positive coagulase negative cocci.

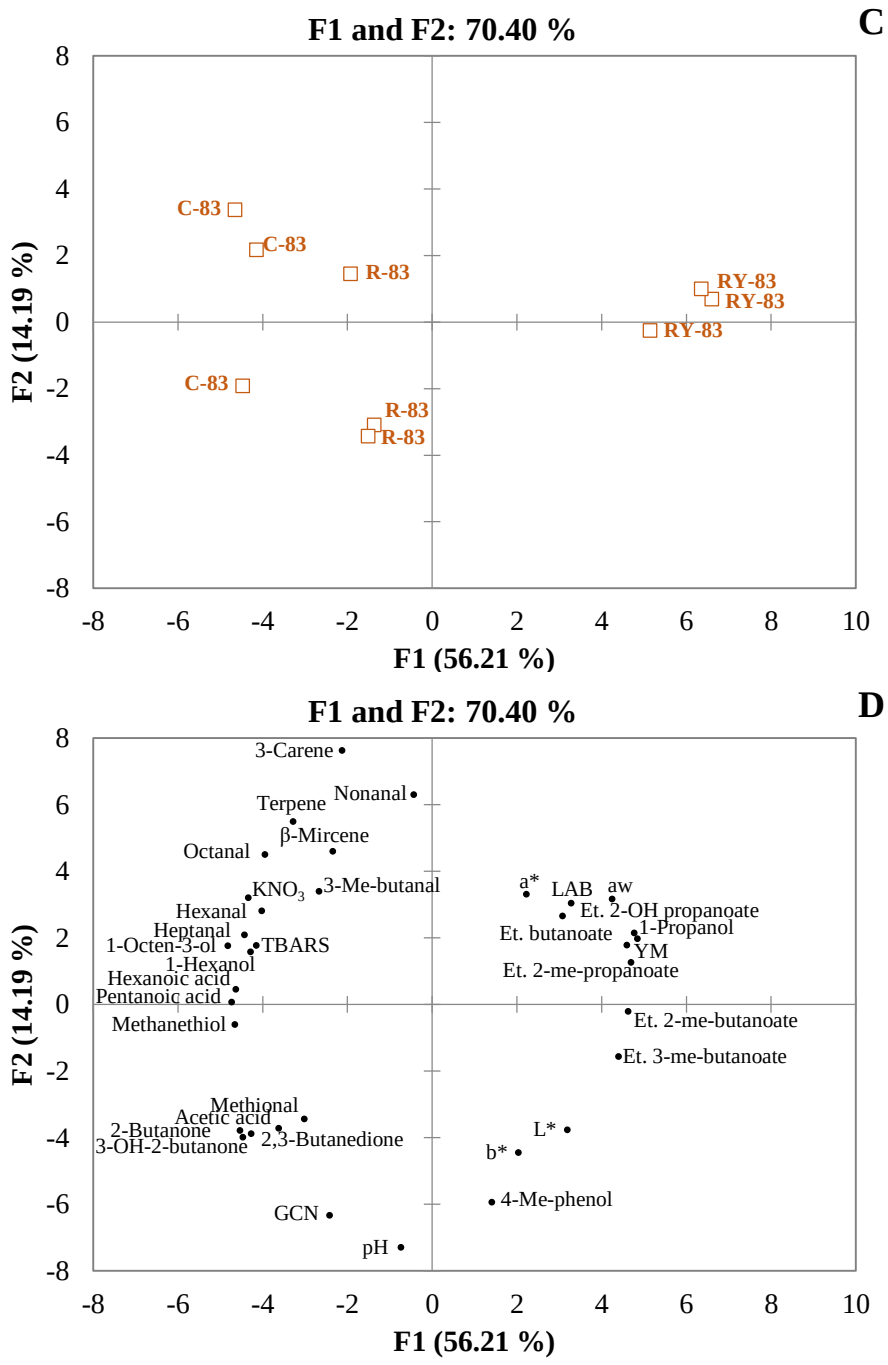


**Figure 3.** Continued.

## 4. Discussion

Physicochemical changes observed were similar to the ones reported for this type of product (Olivares et al., 2010; Perea-Sanz et al., 2018) (Table 1 and Fig. 1). The reduction in the ingoing amounts of nitrate and nitrite was confirmed. The lowest nitrate reduction observed during drying in C sausages respect to R and RY sausages may be due to the generation of nitrate from nitrite oxidation (Honikel, 2008). Moreover, other authors have shown that the use of nitrate and nitrite mixtures increases the amount of residual nitrate (Gratacós-Cubarsí et al., 2013). The lowest residual nitrate detected in inoculated sausages (RY) could be explained by the inhibition of nitrite oxidation by *D. hansenii* (Cano-Garcia et al., 2014).

Nitrate and nitrite reduction did not affect pH value however, Aquilani et al. (2018) and Gonzales-Barron et al. (2015) observed a small increase in pH values due to the absence of nitrate and/or nitrite in dry fermented sausages. These differences among studies can be due to differences in formulations as well as differences in process manufacture like long drying times that may cause a pH increase. In RY sausages, *D. hansenii* consumption of organic acids and ammonium production (Flores, Corral, Cano-García, Salvador & Belloch, 2015) increased pH at the end of the ripening.

Regarding lipid oxidation, a decrease in TBARS in R sausages was observed in previous studies (Perea-Sanz et al., 2018). However, this is not in agreement with the reported antioxidant ability of nitrite (Zanardi, Ghidini, Battaglia & Chizzolini, 2004). Similar reductions in TBARS were observed in nitrite reduced Pastrima although they were related to the stage of processing (Aksu, Erdemir & Çakici, 2016). The chemistry of nitrate and nitrite in cured meats is a complex subject (Majou & Christieans, 2018). The nitric oxide generated from nitrite can react with neutrophil-derived superoxide present in muscle tissues and generate peroxynitrite ($ONOO^-$), which has prooxidant activity and antimicrobial effects (Brannan, Connolly & Decker, 2001). Thus, in fermented sausages with high ingoing amounts of nitrate and nitrite, peroxynitrite formation might increase oxidation events including lipid oxidation. The nitrite prooxidant effect of peroxinitrite is directly related with its antimicrobial effect which is more harmful against Gram positive anaerobic bacteria, like *C. botulinum* (Majou & Christieans, 2018). On the contrary, the antioxidant capacity of *D. hansenii* limits lipid oxidation processes as shown by its great antioxidant capacity (Corral

et al., 2015) during the entire drying time and, moreover, prevents nitrite oxidation as observed by the lowest residual nitrate content in RY.

On the other hand, the reduction of the ingoing amounts of nitrate and nitrite did not have effect on the microbial counts. LAB counts at both drying times were stable as reported by Christieans et al. (2018), and GCN and yeasts and moulds counts (Fig. 1A, 1C) were comparable to those found by Cano-García et al. (2014). On the contrary, Hospital et al. (2015) reported an increase in GCN related to nitrite and nitrate reduction in dry-fermented sausages and a similar effect was observed in Pastrima (Aksu et al., 2016). Similar findings were reported by Gonzales-Barron et al. (2015) in dry-fermented sausages, although the presence in the formulation of polyphosphate, which produced a pH rise effect, was the main factor affecting the growth of acid sensitive microorganisms. In our formulations C and R, the absence of differences in pH due to the similar LAB growth corroborated that the reduction of nitrate and nitrite levels did not affect GCN growth. Regarding the absence of Enterobacteriaceae, this indicated that the process was carried out with good hygiene practices, which are important to assure microbial safety. The inoculation of *D. hansenii* in RY sausages was confirmed by the yeasts and moulds counts during the whole process and presence of M13 RAPD PCR pattern of *D. hansenii* L1 (Fig. 2). The presence of *D. hansenii* L1 patterns in yeasts isolated from C and R sausages could be due to yeast cross contamination in the drying chamber. Nevertheless, the cell load (Figure 1) is very different between samples, leading to a scarce impact of these isolates in R and C sausages.

The main mechanisms involved in generation of volatile compounds with aroma impact on fermented sausages are from microbial origin, chemical oxidation (Flores & Olivares, 2015) and thiamine degradation (Flores, 2018). Contrary to Hospital et al. (2015), total volatile compounds derived from amino acid degradation were not affected by the reduction of ingoing amounts of nitrate and nitrite. Besides, amino acid derived volatile compounds were in high abundance in sausages inoculated with *D. hansenii* strain L1 (Table 2) due to the yeast strain amino acid degradation capacity (Perea-Sanz et al., 2019a). On the other hand, our results indicated that *D. hansenii* might have consumed acetic acid and produced ethanol in contrast to what happened in C and R sausages (Table 2) (Cano-García et al., 2014). Esterase activity in dry-fermented sausages is carried out by *Staphylococcus* and *Debaryomyces* species (Sthanke, 1994; Flores, Dura, Marco & Toldra, 2004). Accordingly,

*D. hansenii* inoculation in RY sausages increased production of esters compounds (Flores et al., 2004). Morevoer, *D. hansenii* produced a strong decrease in lipid oxidation volatile compounds in agreement with the low TBARS values and the high antioxidant capacity of this yeast (Corral et al., 2015). Nitrate and nitrite reduction and *D. hansenii* did not affect volatile compounds derived from black pepper, which was used as spice in the formulations.

Generation of volatile compounds derived from amino acid degradation reactions in C and R sausages was not altered from 60 to 83 d (Table 2). However, RY sausages showed a decline in volatile compounds derived from amino acid degradation indicating a probable transformation of these into other compounds. The abundance of volatile compounds derived from lipid oxidation decreased in C and R sausages due to the further drying (Perea-Sanz et al., 2018).

Concerning volatile compounds with direct impact on the sausage aroma profile (Table 3) at 60 d of ripening, the reduction of the ingoing amounts of nitrate and nitrite produced few aroma differences in contrast to the highest effect produced by *D. hansenii* inoculation. The yeast prevented oxidation reactions and generation of aromas derived from lipid oxidation (Flores et al., 2004) which may produce unpleasant rancid odour notes in the sausages. In addition, it was responsible for the production of ester compounds which provided fruity and sweet odours to the sausages (Cano-García et al., 2014; Corral et al., 2015). The decrease on carbohydrate fermentation aromas caused by *D. hansenii* inoculation could be explained by the yeast consumption of acetic acid (Table 2). In general, *D. hansenii* inoculation decreased acid notes detrimental to sausage aroma (Flores, 1997). On the other hand, at 83 d of ripening the effect of nitrate and nitrite reduction between C and R on aroma profile is more clear (Fig. 3C and D) (Perea-Sanz, Montero, Belloch & Flores, 2019b) and *D. hansenii* yeast contributed to fruity notes (ester compounds) and prevented oxidation events. In addition, the relationship between nitrate/nitrite reduced fermented sausages (R) and the aroma compounds derived from carbohydrate fermentation (acetic acid, 2-butanone, 2,3-butanedione, 3-hydroxy-2-butanone, Fig. 3D) may be explained by the GCN metabolic activity, although no differences in GCN growth were observed between nitrate/nitrite reduced and no-reduced sausages.

## 5. Conclusions

Nitrite and nitrate reduction in fermented sausages did not affect microbial growth but decreased lipid oxidation and generation of derived volatiles. Microorganisms metabolic activity caused differences in the aroma profile at the longest drying time. The nitrite prooxidant and antimicrobial effects were confirmed in C sausages. *D. hansenii* inoculation limited lipid oxidation and increased generation of volatile compounds derived from amino acid degradation and esterase activity. The antioxidant capacity of *D. hansenii* during the entire drying time prevented nitrite oxidation as demonstrated by the lowest residual nitrate levels in RY sausages. In summary, yeast inoculation counteracted the negative impact of nitrite and nitrate reduction on aroma due to its antioxidant capacity during drying time, aroma production and hindered nitrite oxidation.

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# DISCUSIÓN

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Existe una gran variedad de embutidos curado-madurados en función del tipo de elaboración, cada uno con características sensoriales propias. En el área mediterránea, es común el empleo de maduraciones lentas (igual o superior a 30 días), en las cuales es habitual el uso exclusivo de nitrato como agente de curado (Toldrá & Flores, 2014). Este tipo de proceso da lugar a embutidos curado-madurados con características sensoriales especiales, diferentes a la de los embutidos con adición de nitrito (Marco, Navarro & Flores, 2008). Esto indicaría que el uso de nitrito o nitrato puede tener diferentes implicaciones en el desarrollo del aroma en embutidos curado-madurados (Olesen, Meyer & Stahnke, 2004; Marco, Navarro & Flores, 2006, 2008). Además, no hay que olvidar que la principal justificación del empleo de los agentes de curado (nitrato/nitrito) se debe a su efecto sobre la seguridad microbiológica (Rahman, 2007). Aun así, Christieans, Picgirard, Parafita, Lebert & Gregori (2018) indicaron que en los embutidos sometidos a largos procesos de maduración-secado, el empleo de nitrito es fundamental para garantizar la seguridad microbiológica del embutido. Es por ello que, en la presente tesis, se ha estudiado el impacto de la reducción de la cantidad añadida de nitrato (Capítulos 1 y 2) y de las mezclas de nitrato y nitrito (Capítulo 4) en el aroma de embutidos curado-madurados.

Con el fin de comparar el impacto en el aroma producido por las reducciones de agentes de curado (nitrito/nitrato) aplicadas, en la tabla 1 se recogen exclusivamente los compuestos aromáticos detectados de entre todos los compuestos volátiles identificados en los capítulos 1, 2 y 4. Los aromas detectados se han clasificado en cuanto a su origen más probable con el fin de facilitar la discusión. Así, los compuestos se clasifican en los que proceden de la actividad microbiana como son la fermentación de carbohidratos, degradación de aminoácidos, actividad esterasa y β-oxidación lipídica, de procesos químicos como la oxidación de lípidos y degradación de tiamina, del uso de especias y de origen desconocido. Es de destacar que cuando se emplea únicamente nitrato en la elaboración (Capítulo 1 y 2), se detectan algunos compuestos aromáticos que, sin embargo, no están presentes en el perfil aromático de embutidos curado-madurados elaborados con mezcla de nitrato y nitrito (Capítulo 4). Éstos son el dimetil disulfuro, octanoato de etilo, 2,3-pentanodiona, 2-heptanona, 2-metilfurano y 2-pentilfurano (Tabla 1). Por el contrario, los compuestos aromáticos 3-metilbutanal, dimetil trisulfuro, 2-metilbutanoato de etilo, 2-metilpropanoato

de etilo, 1-propanol, nonanal, 4-metilfenol, 2-metil-3-furantiol, 2-acetil-2-tiazoline, metil-2-metil-3-furildisulfuro y los derivados de especias se detectan exclusivamente en los embutidos elaborados con la mezcla de nitrato y nitrito (Tabla 1). Hay varias razones por las que se obtienen estas diferencias. En primer lugar, los terpenos detectados en el capítulo 4 proceden del uso de especias, las cuales no se utilizaron en los capítulos 1 y 2 con el fin de no interferir en la identificación de aromas (Marco, Navarro & Flores, 2004). Aunque las especias interfieren en la producción de aromas, también tienen un efecto positivo en la prevención de los procesos de oxidación del embutido (Ordoñez, Hierro, Bruna & de la Hoz, 1999), por lo que en el capítulo 4 se decidió emplear pimenta negra. Otra de las razones por las que se observan diferencias en los compuestos aromáticos detectados podría deberse a que dichos compuestos se encuentren a una concentración inferior a su umbral de detección y que, por ello, no hayan sido detectados como aromáticos (Tabla 1). De hecho, estudios previos han demostrado que no existen diferencias en cuanto a los compuestos aromáticos identificados en embutidos curado-madurados fabricados con nitrato o nitrito (Marco, Navarro & Flores, 2007), pero si en su impacto aromático, ya que presentan diferencias en los valores de frecuencia de detección de los aromas. Por otra parte, también existen diferencias debidas al proceso de fabricación realizado, así el 2-metilpropanoato de etilo produce un impacto aromático en los embutidos elaborados con nitrato y nitrito y no en los que solo se añadió nitrato, debido a que en los primeros se inoculó una levadura caracterizada por su alta producción de ésteres. Por lo tanto, los resultados obtenidos en los capítulos 1, 2 y 4 indican que el uso de diferentes sales nitrificantes, así como el proceso de fabricación, pueden contribuir modificando el perfil aromático en embutidos curado-madurados debido a cambios en la concentración de los compuestos volátiles aromáticos.

**Tabla 1. Compuestos aromáticos detectados en los embutidos curado-madurados fabricados con nitrato (Capítulos 1 y 2) o mezcla de nitrato/nitrito (Capítulo 4).**

| Compuesto | Descripción[1] | Cap. 1 y 2[2] ($NaNO_3$) | Cap. 4 ($NaNO_2$ /$NaNO_3$) | Umbral de detección[3] (µg/kg) |
|---|---|---|---|---|
| **Fermentación de carbohidratos** | | | | |
| *2,3-Butanodiona* | Queso, mantequilla | +[4] | + | 5 |
| *2-Butanona* | Fruta, mantequilla, fresco | + | + | 0,21 |
| *Ácido acético* | Vinagre | + | + | 25 |
| *3-OH-2-butanona* | Dulce, fruta, verde | + | + | - |
| **Degradación de aminoácidos** | | | | |
| *Metanotiol* | Podrido | + | + | 0,24 |
| *3-Metilbutanal* | Dulce, verde, picante | ND | + | 1.6 |
| *Dimetil disulfuro* | Tostado, ajo | + | ND | 0,050-0,078 |
| *Metional* | Patata cocida | + | + | 0,063 |
| *Dimetil trisulfuro* | Picante, podrido, vegetal | ND | + | 0,0073 |
| *2-Acetil-1-pirrolina* | Tostado, frutos secos, maíz frito | + | + | 0,00002-0,00004 |
| **Actividad esterasa** | | | | |
| *Butanoato de etilo* | Fruta, dulce | + | + | 0,017-0,023 |
| *2-OH-propanoato de etilo* | Fruta, dulce, floral, acido | + | + | 8 |
| *2-Metilbutanoato de etilo* | Piña, dulce, acido | ND | + | - |
| *3-Metilbutanoato de etilo* | Fruta, dulce, ácido | + | + | 4600 |
| *Octanoato de etilo* | Vegetal, fruta | + | ND | 0,022 |
| *2-Metilpropanoato de etilo* | Dulce, fruta | ND | + | 0,0003 |

**Tabla 1. Continuación.**

| Compuesto | Descripción[1] | Cap. 1 y 2[2] ($NaNO_3$) | Cap. 4 ($NaNO_2$ /$NaNO_3$) | Umbral de detección[3] (µg/kg) |
|---|---|---|---|---|
| **β-Oxidación** | | | | |
| *2,3-Pentanodiona* | Dulce, caramelo | + | ND | 63 |
| *2-Heptanona* | Rancio, fruta | + | ND | 45 |
| *1-Octen-3-ol* | Setas | + | + | 48 |
| **Lipid oxidation** | | | | |
| *2-Metilfurano* | Verde, ajo | + | ND | - |
| *1-Propanol* | Ácido, fermentado | ND | + | 27,5-35 |
| *Hexanal* | Hierba recién cortada | + | + | 330 |
| *1-Hexanol* | Grasa oxidada, fruta podrida, | + | + | 4 |
| *Heptanal* | Verde, ajo, curado, rancio, | + | + | 140 |
| *Ácido hexanoico* | Queso, desagradable, floral, | ND | + | 0,012 |
| *2-Pentilfurano* | Ajo, cebolla | + | ND | 270 |
| *Octanal* | Cítrico, dulce | + | + | 21 |
| *Nonanal* | Fresco, herbáceo, desagradable | ND | + | 20 |
| **Degradación de tiamina** | | | | |
| *2-Metil-3-furantiol* | Grasa, medicina, desagradable | ND | + | 0,000001-0,000002 |
| *2-Acetil-2-tiazolina* | Tostado, maíz frito, caramelo, pan | ND | + | 0,00002-0,00008 |
| *Metil-2-metil-3-furildisulfuro* | Carne, desagradable, madera húmeda, podrido, fermentado | ND | + | 0,00002-0,00008 |
| **Especias** | | | | |
| *β-Mirceno* | Verde | ND | + | 0,0445 |
| *3-Careno* | Setas, tierra, verde, fresco | ND | + | 9,3 |
| *Terpeno no identificado* | Picante, fresco, floral | ND | + | - |

**Tabla 1. Continuación.**

| Compuesto | Descripción[1] | C 1 y 2[2] ($NaNO_3$) | C 4 ($NaNO_2$ $NaNO_3$) | Umbral de detección[3] (µg/kg) |
|---|---|---|---|---|
| **Origen desconocido** | | | | |
| *4-Metilfenol* | Desagradable, podrido | ND | + | 0,00018 |

[1]Descripción olfativa de los compuestos aromáticos en los capítulos 1, 2 y 4 por GC-O. [2]Cap. 1 y 2 (Capítulos 1 y 2); Cap. 4 (Capítulo 4). [3]Umbral de detección en aire: van Gemert (2004). [4]+: detectado; ND: no detectado.

Con el fin de interpretar los resultados obtenidos en los capítulos 1, 2 y 4 de la presente tesis en cuanto a los compuestos aromáticos presentes en embutidos curado-madurados, se ha procedido a determinar la presencia relativa de dichos compuestos aromáticos mediante la representación en forma de mapas de calor (heat maps) utilizando el paquete estadístico XLSTAT 2018 (Addinsoft, Barcelona, Spain). Además, se ha determinado la correlación existente entre los compuestos aromáticos y la reducción de nitritificantes y la presencia de levadura mediante la determinación de la correlación de Pearson utilizando el entorno estadístico R (R Core Team, 2013).

En el capítulo 1, se ha estudiado el efecto de la reducción de la adición de nitrato en un 15% y 25% de la cantidad máxima permitida (250 ppm) en embutidos curado-madurados de maduración lenta. En el heat map de la figura 1 se representa el perfil de aromas de los embutidos fabricados con diferentes cantidades de nitrato añadido. Los colores indican la abundancia relativa de cada compuesto aromático: el color amarillo claro representa una abundancia relativa baja (<-1) y el color rojo una abundancia relativa alta (>1). El dendograma que se encuentra en la parte superior de la figura, agrupa los embutidos en función de su perfil aromático de forma que se obtienen dos grupos, el primero formado por los embutidos del lote control y el lote reducido en un 15% de nitrato, y el segundo grupo formado por los embutidos con un 25% de reducción de nitrato. La reducción de un 15% de nitrato añadido no supondría cambios significativos en el aroma de embutidos curado-madurados, mientras que reducciones superiores del 25% afectan principalmente a los aromas que proceden de la oxidación lipídica reduciendo su abundancia relativa (Figura 1). Además, en la figura 1 se observa que tal reducción aumenta la abundancia relativa de ésteres, aromas con notas a "fruta" y dulce". En este caso, la reducción podría resultar positiva en cuanto a las notas aromáticas aportadas por estos compuestos y a que se disminuiría algunas notas aromáticas a "verde" y a "grasa oxidada" (Tabla 1). Sin embargo, los compuestos derivados de la oxidación lipídica forman parte del perfil aromático característico de los embutidos curado-madurados (Ordoñez et al., 1999). Por otra parte, en la figura 1 se observan diferentes cambios en la abundancia relativa de aromas procedentes de la actividad microbiana (degradación de aminoácidos y fermentación de carbohidratos), destacando el aumento de la abundancia relativa del ácido acético como consecuencia de la reducción de la adición de nitrato en un 25%. Estos cambios se deben al incremento en el

crecimiento de las levaduras y mohos, y probablemente, a cambios en el metabolismo de otros microorganismos, además de la disminución de la oxidación lipídica por la reducción de nitrato en un 25%. Este hecho podría traducirse en un impacto en el aroma del embutido ya que contribuye con notas aromáticas a "vinagre" (Tabla 1), que podría perjudicar el perfil aromático final enmascarando el aroma típico a embutido curado-madurado.

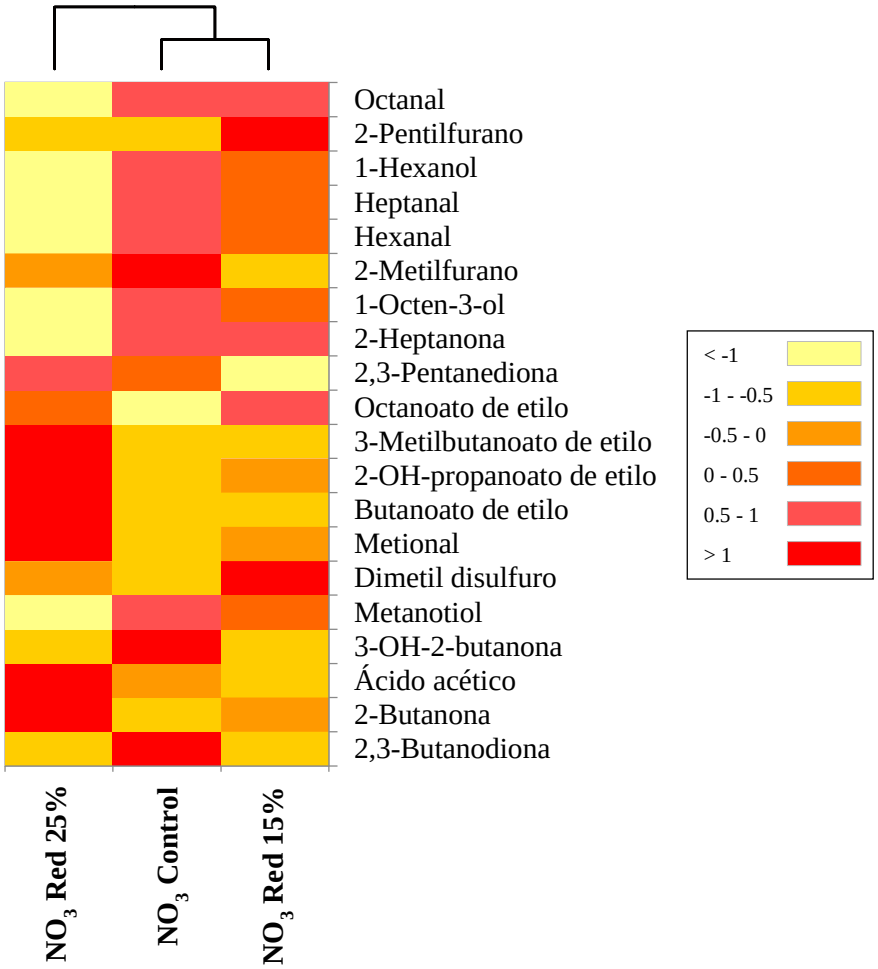


**Figura 1. Perfil aromático de embutidos curado-madurados de maduración lenta elaborados con diferentes cantidades de nitrato; $NO_3$ Control (250 ppm de $NaNO_3$), $NO_3$ Red 15% (15% de reducción, 212,5 ppm de $NaNO_3$), $NO_3$ Red 25% (25% de reducción, 187,5 ppm de $NaNO_3$).**

Por otra parte, en el capítulo 4 se ha estudiado el efecto de la reducción de una mezcla de nitrato y nitrito en el proceso de elaboración de embutidos curado-madurados. En este caso se ha estudiado una reducción del 47% de la cantidad máxima permitida (150 ppm) de ambas sales nitrificantes, ya que Christieans et al. (2018) determinaron la necesidad de la presencia de al menos 80 ppm de nitrito en la seguridad del embutido. En este capítulo además se evalúan 2 tiempos de proceso a 60 y 83 días. El heat map de la figura 2 representa el perfil de aromas de los embutidos fabricados con diferentes cantidades de mezclas de nitrato y nitrito, y también incluye un lote inoculado con la levadura *Debaryomyces hansenii (D. hansenii)*. En cuanto a la reducción de la mezcla de nitrato/nitrito, se observa una reducción de la oxidación lipídica responsable de la disminución en la producción de aromas procedentes de este proceso (Figura 2) siendo más apreciable a los 83 días de maduración. Estos resultados coinciden con los obtenidos al reducir la cantidad de nitrato (Capítulo 1), aunque la reducción de la mezcla de nitrato/nitrito afecta en menor medida a la generación de compuestos aromáticos que proceden del metabolismo microbiano (degradación de aminoácidos y fermentación de carbohidratos). Estos resultados indicarían que la presencia del nitrito en la mezcla (Capítulo 4) ejerce un mayor control de los microorganismos responsables de la producción de aromas, a diferencia de cuando se emplea exclusivamente nitrato (Capítulo 1). A pesar de las diferencias indicadas entre el uso de nitrato o nitrato/nitrito, la reducción de los agentes de curado en ambos casos parece producir un efecto en el metabolismo de los microrganismos presentes en los embutidos curado-madurados. Además, ese efecto es más notable cuando se emplea nitrato que cuando se emplean mezclas de nitrato/nitrito. Por su parte, Hospital, Hierro & Fernández (2012) y Hospital et al. (2015) indicaron que cuando se reduce en un 50% la mezcla añadida de nitrato y nitrito en embutidos curado-madurados se produce un aumento en el crecimiento de los cocos Gram positivos catalasa positivos junto con algunos cambios en compuestos volátiles procedentes de su metabolismo. Por el contrario, nuestros resultados (Capítulo 4) indican que no hay diferencias en el crecimiento de estos microorganismos debidos a la reducción de nitrato y nitrito. Estas diferencias entre estudios pueden deberse a las diferentes condiciones de procesado, que pueden afectar a los resultados (Marco et al., 2008).

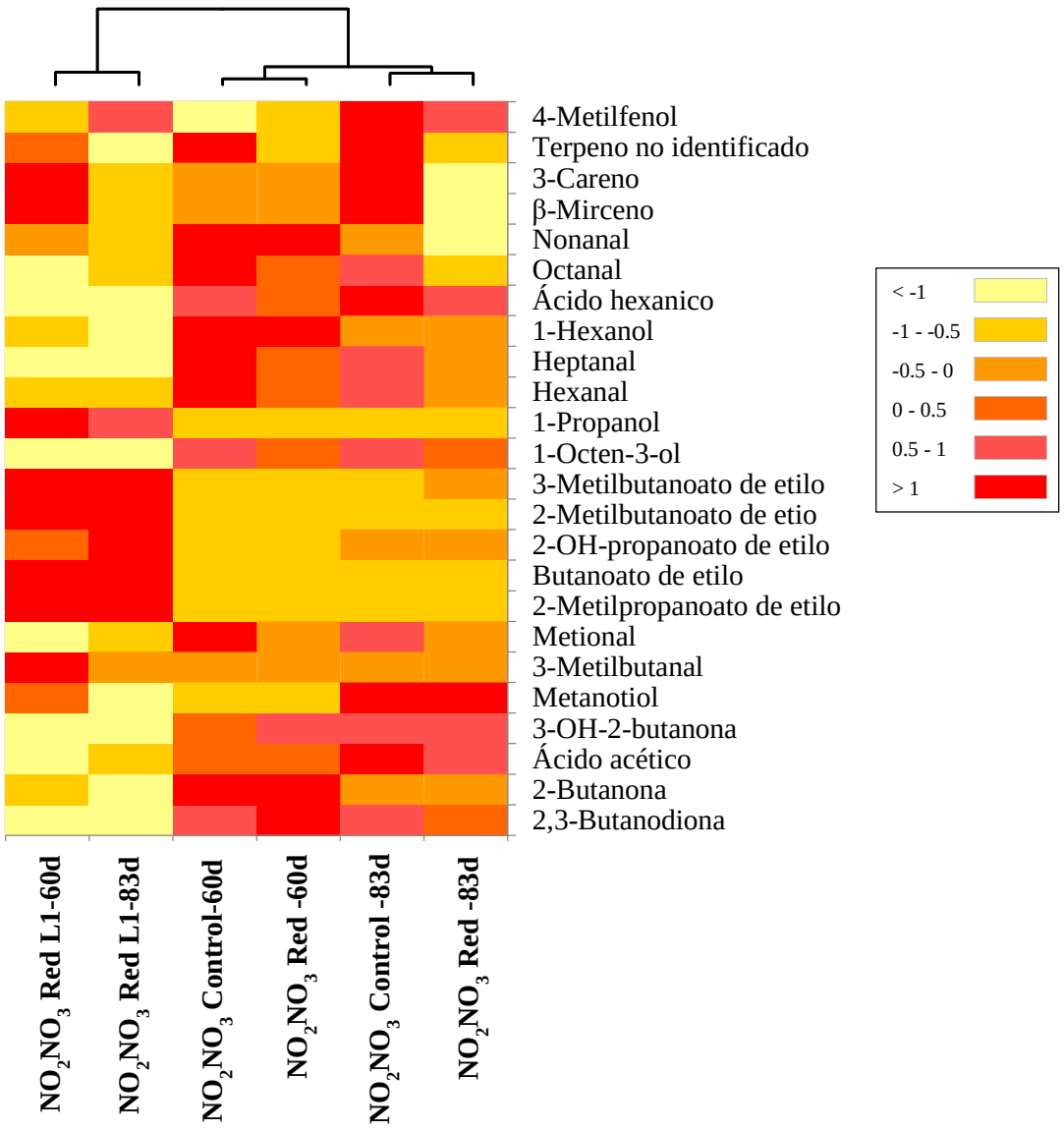


**Figura 2. Perfil aromático de embutidos curado-madurados elaborados con diferentes cantidades de nitrato y nitrito y con o sin inoculación de *D. hansenii* L1 durante dos tiempos de maduración (62 y 83 días). $NO_2NO_3$ Control (150 ppm de $NaNO_2$ y 150 ppm de $NaNO_3$), $NO_2NO_3$ Red (47% de reducción de $NaNO_2$ y $NaNO_3$), $NO_2NO_3$ Red L1 (47% de reducción, con inoculación de L1).**

Teniendo en cuenta que los embutidos curado-madurados poseen una larga vida útil, es importante conocer la implicación de la reducción de la cantidad añadida de agentes de

curado durante su vida útil. Para ello, en el capítulo 2 se estudia el efecto de la reducción de nitrato sódico (15 y 25%) durante 1, 2 y 3 meses de almacenamiento a vacío y 18-20 °C. El perfil de aromas durante el almacenamiento de dichos embutidos se muestra en el heat map de la figura 3. En el cual se observan mayores diferencias a mayor tiempo de envasado (3 meses), ya que en el dendograma detecta la presencia de dos grupos, los embutidos de 3 meses frente a los de 1 y 2 meses de envasado a vacío. Como se observa en esta figura, a mayor tiempo de almacenamiento se produce una reducción de la abundancia relativa de los compuestos aromáticos tales como ésteres y otros procedentes de la fermentación de carbohidratos, lo que produciría una reducción de las notas a "fruta", "dulce" y "vinagre" (Tabla 1). En cambio, aumenta la de compuestos de la oxidación lipídica produciendo notas "verdes" y a "grasa oxidada" (Tabla 1). Además de estos cambios principales, el almacenamiento a vacío y 18-20 ℃ parece provocar un aumento de la abundancia relativa de los compuestos azufrados metional y metanotiol, tras 2 y 3 meses. Estos compuestos poseen umbrales de detección muy bajos y notas aromáticas que, en el caso del metanotiol, pueden llegar a ser desagradables (Tabla 1). El metanotiol, procedente de la degradación del aminoácido metionina, es muy reactivo dando lugar a otros compuestos azufrados también aromáticos como son el dimetil disulfuro, metionol, metil tioacetato y también el metional (Landaud, Helinck & Bonnarme, 2008), lo que cabria esperar que estos compuestos se encuentren presentes en los embutidos estudiados. De hecho, el metional y dimetil disulfuro se detectan en la fracción volátil del embutido y participan en su perfil aromático (Figura 3). Estos cambios podrían ser el resultado de la disminución del crecimiento microbiano general a los 3 meses de envasado y al aumento de los procesos de oxidación lipídica. Estos resultados también se han descrito en estudios previos (Summo, Caponio, Pasqualone & Gomes, 2011), aunque con algunas diferencias debidas al empleo de una etapa de ahumado en la fabricación.

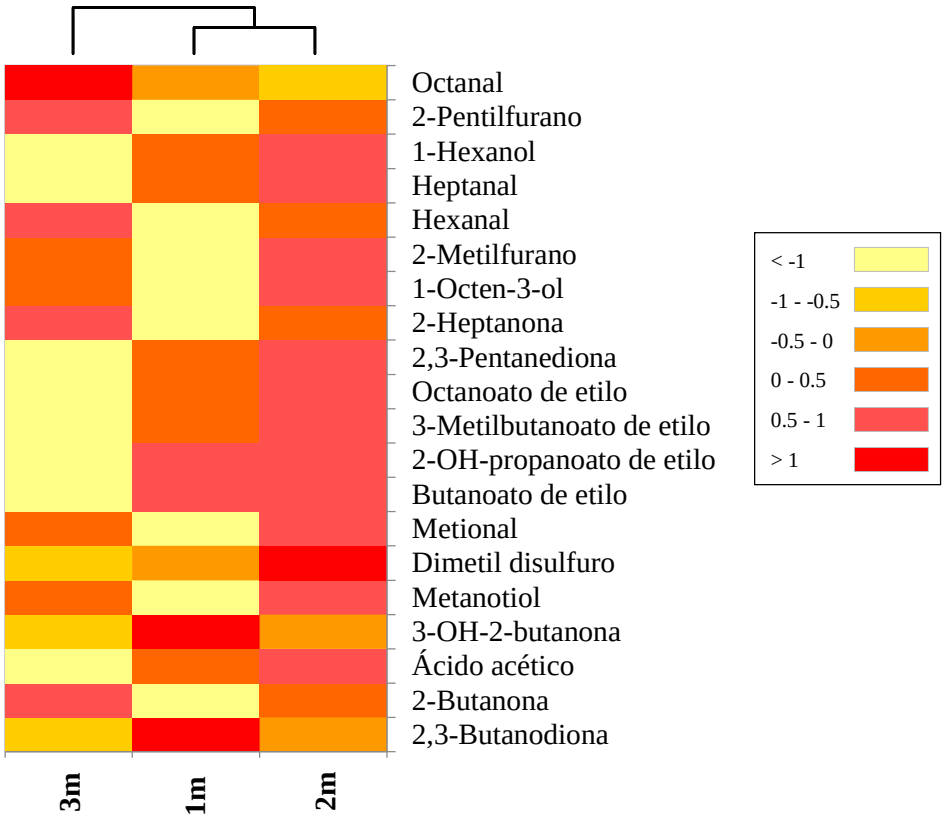


**Figura 3. Perfil aromático de embutidos curado-madurados de maduración lenta (control y reducidos) durante su almacenamiento a vacío y 18-20 °C; 1m (98 días), 2m (132 días), 3m (162 días).**

Cuando se tienen en cuenta de manera conjunta los efectos de la reducción de la cantidad añadida de nitrato y del almacenamiento, se observa que a los 3 meses aparece el mayor efecto de la reducción (Figura 4). El dendograma situado en la parte superior de la figura 4 muestra que, a los 3 meses de almacenamiento, el embutido control (no reducido en nitrato) se diferencia de los embutidos reducidos en nitrato en función de su perfil aromático. La diferencia se debe principalmente a una menor abundancia relativa de compuestos aromáticos derivados de la oxidación y β-oxidación lipídica en los embutidos reducidos en nitrato. También se observa que la reducción en un 25% de nitrato aumenta la abundancia relativa de ésteres y de algunos compuestos azufrados como el metional y dimetil disulfuro,

ambos con umbrales de detección muy bajos (Tabla 1), que junto con la reducción de aromas con notas agradables debido a tiempos largos de almacenamiento (Figura 3), podrían modificar el aroma del embutido.

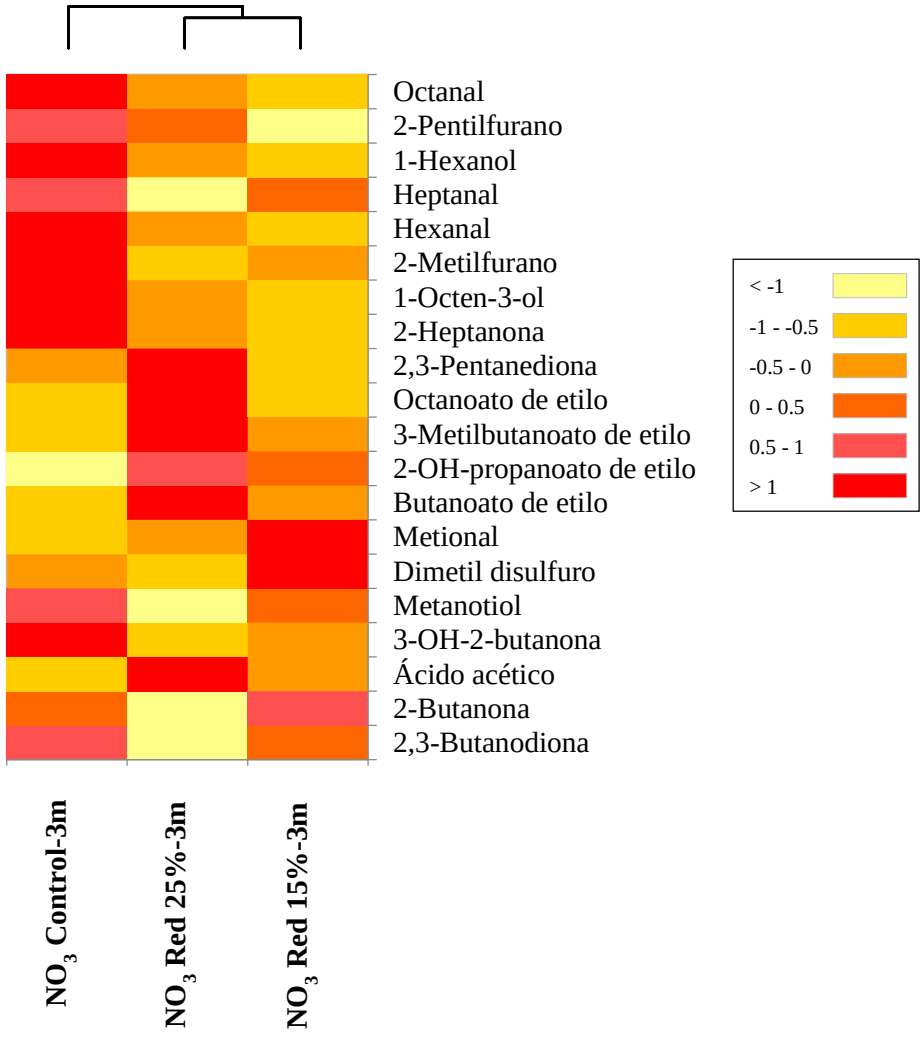


**Figura 4. Perfil aromático de embutidos curado-madurados de maduración lenta elaborados con diferentes cantidades de nitrato tras 3 meses de almacenamiento a vacío y 18-20 °C; $NO_3$ Control (250 ppm de $NaNO_3$), $NO_3$ Red 15% (15% de reducción, 212,5 ppm de $NaNO_3$), $NO_3$ Red 25% (25% de reducción, 187,5 ppm de $NaNO_3$).**

Los resultados obtenidos respecto a los fenómenos de oxidación observados en los lotes reducidos en nitrificantes (Capítulos 1, 2 y 4), presentan un escenario complejo en cuanto a

la química del nitrito en productos cárnicos (Majou & Christieans, 2018). Según la hipótesis planteada al inicio de esta tesis doctoral, los embutidos fabricados con cantidades reducidas en nitrificantes podrían presentar valores de oxidación superiores a los no reducidos. Sin embargo, no solo los valores de TBARS son inferiores en embutidos reducidos, sino que también los compuestos volátiles resultantes de la oxidación (aldehídos lineales, entre otros) muestran valores inferiores, lo que demuestra una menor oxidación. De hecho, nuestra presente hipótesis es que el óxido nítrico generado a partir del nitrito también actúa como oxidante mediante la reacción con el anión superóxido o con el peróxido de hidrógeno presentes en los tejidos de la carne, generando peroxinitrito (ONOO-) que posee actividad prooxidante además de antimicrobiana (Brannan, Connolly & Decker, 2001).

Los efectos en el aroma de los embutidos producidos por la reducción de nitrificantes, han dirigido nuestro interés en la búsqueda de alternativas que contrarrestasen dichos efectos. Teniendo en cuenta el potencial de las levaduras en la potenciación del aroma (Cano-García, Rivera-Jiménez, Belloch & Flores, 2014b; Corral, Belloch, López-Díez, Salvador & Flores, 2017) en esta tesis nos hemos planteado seleccionar levaduras capaces de transformar aminoácidos en compuestos aromáticos potentes. Según se indica en la tabla 1, los compuestos azufrados poseen umbrales de detección muy bajos y se caracterizan por contribuir con notas cárnicas al aroma de los productos cárnicos (Mottram & Madruga, 1994). Por ello, en el capítulo 3 se estudia la capacidad de producción de compuestos volátiles azufrados por parte de diferentes cepas de *D. hansenii*. Los compuestos aromáticos azufrados pueden provenir de la degradación de los aminoácidos azufrados presentes y, además son de interés por ejercer un papel importante en el perfil aromático de embutidos curado-madurados (Corral, Leitner, Siegmund & Flores, 2016). La capacidad de producción de estos aromas por parte de diferentes cepas de *D. hansenii* aisladas de quesos madurados que emplean metionina como precursor principal ha sido previamente estudiada (López Del Castillo-Lozano, Delile, Spinnler, Bonnarme & Landaud, 2007).

Las cepas seleccionadas se obtuvieron de diferentes alimentos; embutidos curado-madurados de cerdo (L1 y L5) y de llama (L12, L21, L25), queso curado (L66) y altramuces (L74). La capacidad de producción de aromas azufrados de estas levaduras se evaluó en medio de cultivo suplementado con metionina, cisteína o cistina, para determinar el aminoácido principal precursor. De los tres aminoácidos azufrados estudiados, la metionina

es la que produce una mayor abundancia de aromas azufrados, como indican otros autores (Bonnarme, Lapadatescu, Yvon & Spinnler, 2001; López Del Castillo-Lozano et al., 2007). Las cepas estudiadas son capaces de producir diferentes compuestos aromáticos algunos de los cuales encontramos en los embutidos, como el metanotiol, dimetil disulfuro y dimetil trisulfuro (Capítulos 1, 2 y 4), en diferentes proporciones y con diferencias significativas entre ellas. Sin embargo, otros compuestos como el metil tioacetato y el etil tioacetato también se producen, pero no han sido descritos en embutidos curado-madurados. Por otra parte, los resultados ponen de manifiesto que la producción de metional es principalmente por mecanismos químicos (probablemente por degradación de Strecker) (Escudero, Hernández-Orte, Cacho & Ferreira, 2000). Las cepas L1, L5 y L74 de *D. hansenii* son las que consumen mayor cantidad de metionina, lo que permite que las cepas procedentes de embutidos de cerdo (L1 y L5) sean las que produzcan un perfil más complejo de los compuestos azufrados estudiados y que a su vez, demuestren un mayor consumo del resto de aminoácidos, lo que aumenta el interés para su empleo como cultivos iniciadores en la elaboración de embutidos curado-madurados reducidos en nitrificantes.

A partir de estos resultados, se planteó la hipótesis de que el conocimiento de las rutas metabólicas y los genes implicados en la formación de los aromas azufrados, permitiría controlar los factores que afectan la generación de aromas azufrados en planta piloto. Inicialmente se seleccionaron dos cepas de *D. hansenii,* L1 por la producción en metil tioacetato y L74 en dimetil disulfuro, para someter a un análisis de expresión génica (transcriptómico) que permitiera dilucidar la ruta metabólica implicada en la formación de estos compuestos. De entre todos los genes estudiados (12), ortólogos de los implicados en la ruta de degradación de metionina en *S. cerevisiae* (Cholet, Hénaut, Hébert & Bonnarme, 2008; Lessard, Viel, Boyle, St-Gelais & Labrie, 2014), solo el gen ATF2 (acetil transferasa) aparece sobreexpresado en la cepa L1 y en medio de cultivo con elevada concentración de metionina. La sobreexpresión de este gen se correlaciona con la producción de metil tioacetato a partir del alcohol metanotiol. Estas enzimas son responsables de producir otros ésteres con notas aromáticas agradables que recuerdan a frutas y dulces (Pires, Teixeira, Brányik & Vicente, 2014, Tabla 1). La generación de ésteres en embutidos curado-madurados por la actividad de *D. hansenii* ha sido objeto de varios estudios previos del grupo (Flores, Durá, Marco & Toldrá, 2004; Bolumar et al., 2006; Cano-García, Belloch & Flores,

2014a; Corral et al., 2015, 2017). Por lo tanto, la cepa L1 de *D. hansenii* aislada de embutidos curado-madurados de cerdo, fue la elegida para ser utilizada como cultivo iniciador en embutidos curado-madurados reducidos en nitrificantes en el capítulo 4.

La inoculación de la cepa L1 de *D. hansenii* en embutidos curado-madurados fabricados con cantidades reducidas de una mezcla de nitrato y nitrito, produce efectos significativos tanto a nivel físico-químico (aumento del pH, actividad antioxidante) (Corral, Salvador, Belloch & Flores, 2015; Flores, Corral, Cano-García, Salvador & Belloch, 2015), como microbiológico (Yepez, Neef, Flores & Belloch, 2020), que afectan el aroma final del embutido (Figura 2). El dendograma de la figura 2 diferencia los lotes inoculados con levadura de los no inoculados en función del perfil aromático, seguido de la diferencia según el tiempo de maduración (60 y 83 días). La inoculación de *D. hansenii* L1 produce un aumento de la abundancia relativa de ésteres y una disminución de la abundancia relativa de los aromas procedentes de la oxidación lipídica. Además, también disminuye la abundancia relativa del metional y de algunos compuestos aromáticos derivados de la fermentación de carbohidratos. Por lo tanto, la inoculación de *D. hansenii* L1 seleccionada en el capítulo 3 en los embutidos reducidos en agentes de curado, muestra un potencial de mejora del aroma del producto final mediante el aporte de notas aromáticas "dulces" y a "fruta" y por la disminución de notas "verdes" y a "vinagre" (Tabla 1).

Uno de los efectos adicionales de la inoculación de *D. hansenii* (L1) en embutidos reducidos en nitrificantes es que evita la oxidación del nitrito a nitrato como se observa por la menor presencia de niveles residuales de nitrato en el embutido. Puesto que el empleo de mezclas de nitrato/nitrito aumenta la cantidad de nitrato residual (Gratacós-Cubarsí et al., 2013), la inoculación de *D. hansenii* L1 en embutidos curado-madurados elaborados con estas mezclas supondría una mejora por la disminución de la cantidad de nitrato residual.

No todos los aromas azufrados producidos por *D. hansenii* L1 (Capítulo 3) se detectan en los embutidos curado-madurados inoculados con esta levadura (Figura 5). Los compuestos metanotiol, metional y dimetil disulfuro se detectan, e incluso el metanotiol y metional contribuyen al aroma del embutido. Sin embargo, no se observan cambios en la abundancia relativa de dichos compuestos en los embutidos inoculados respecto a los no inoculados. Hay que destacar que ambos compuestos, metanotiol y metional, se encuentran a niveles

bajos en los embutidos y su umbral de detección es muy bajo (Tabla 1). Por otro lado, la presencia de metional en los embutidos está relacionada con la presencia del aminoácido metionina (Corral et al., 2016), pero no está claro si su origen en los embutidos es debido al metabolismo microbiano o por reacciones químicas. En el capítulo 3, donde se realizaron estudios en sistemas modelo con el aminoácido metionina y diversas levaduras, entre ellas *D. hansenii* L1, se observó que el metional se produce principalmente por fenómenos oxidativos. Así, si tenemos en cuenta que *D. hansenii* L1 posee actividad antioxidante, la inoculación de esta levadura en el embutido podría evitar la formación de metional en el embutido. Utilizando este mismo argumento, el dimetil disulfuro tampoco se vería afectado por la inoculación de *D. hansenii* L1 ya que procede de la oxidación del metanotiol, un compuesto muy lábil, difícil de analizar por su alta volatilidad y que rápidamente se degrada en otros compuestos aromáticos (Landaud et al., 2008) y que, por ello, no se detecta como compuesto aromático. En cuanto a la ausencia de metil tioacetato en los embutidos inoculados con *D. hansenii* L1 (Capítulo 4) podría explicarse por la actividad ATF2 para generar esteres de otros alcoholes mas abundantes, esterificándolos y produciendo otros ésteres aromáticos que también son de interés (Flores et al., 2004, Figura 5).

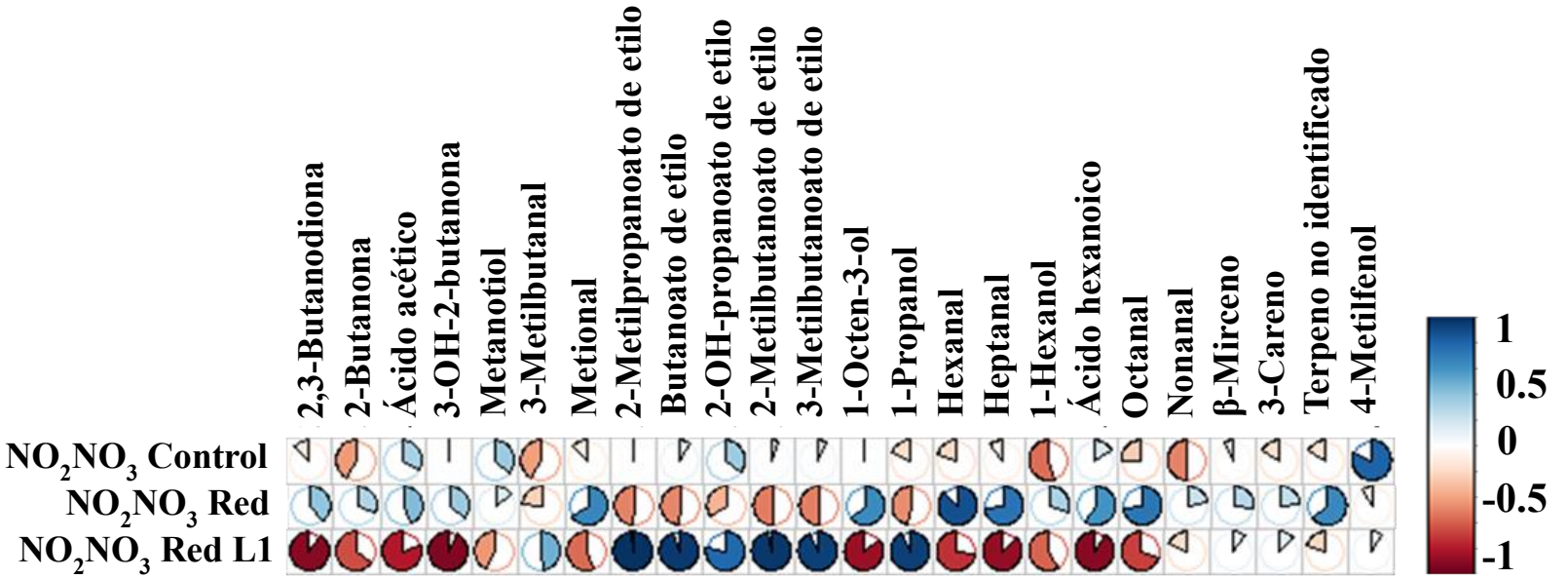


**Figura 5. Correlograma que muestra la clasificación del índice de Pearson entre las distintas formulaciones y los aromas generados. $NO_2NO_3$ Control (150 ppm de $NaNO_2$ y 150 ppm de $NaNO_3$), $NO_2NO_3$ Red (47% de reducción de $NaNO_2$ y $NaNO_3$), $NO_2NO_3$ Red L1 (47% de reducción, con inoculación de *D. hansenii* L1).**

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# CONCLUSIONES

## CONCLUSIONES

1. La reducción de la cantidad máxima permitida de agentes de curado (reducción del 25% de nitrato y del 47% de nitrato/nitrito) en embutidos curado-madurados modifica el perfil aromático del producto, principalmente por la disminución de los compuestos aromáticos procedentes de la oxidación lipídica, reduciendo notas a "verde" y a "grasa oxidada". Cuando se usa únicamente nitrato como agente de curado, su reducción (25%) también modifica los aromas procedentes de la actividad microbiana, principalmente el ácido acético lo que afecta al aroma del producto con notas a "vinagre". Sin embargo, el uso de nitrito como agente de curado en las mezclas, ejerce un mayor control del crecimiento y metabolismo de los microorganismos responsables de la producción de aromas, minimizando los cambios en el perfil aromático causadas por su reducción.
2. El almacenamiento a vacío y 18-20 °C de embutidos curado-madurados durante 3 meses afecta el perfil aromático del producto al disminuir los aromas procedentes del metabolismo microbiano, lo que reduciría notas a "fruta", a "dulce" y también a "vinagre". Además, aumenta los procedentes de la oxidación lipídica y algunos aromas azufrados (metional y metanotiol).
3. La reducción de la cantidad de nitrato añadida en embutidos curado-madurados durante su vida útil a vacío y 18-20 °C, tiene mayor repercusión sobre el aroma tras 3 meses de almacenamiento. Además de la reducción de los compuestos procedentes de procesos de oxidación lipídica, aumenta otros compuestos como los ésteres y algunos compuestos azufrados (metional y dimetil disulfuro).
4. El uso de de cepas de *Debaryomyces hansenii* como alternativa a los cambios que se observan en embutidos reducidos en nitrificantes, requiere de un estudio de su potencial para generar compuestos con impacto aromático. Las cepas procedentes de embutidos curado-madurados de cerdo (L1 y L5) son las que presentan gran potencial para producir compuestos azufrados y mayor consumo de metionina y del resto de aminoácidos. El gen ATF2 tiene un papel relevante en el metabolismo de la metionina, que en el caso de la cepa *D. hansenii* L1 da lugar a metil tioacetato, lo que aumenta el interés de esta cepa como cultivo iniciador en la elaboración de embutidos curado-madurados reducidos en nitrificantes.

5. La inoculación de la cepa L1 de *Debaryomyces hansenii* en embutidos curado-madurados demuestra su capacidad para contrarrestar los cambios en el aroma por la reducción de nitrificantes (mezclas de nitrato y nitrito), debido al aumento de ésteres con notas a "fruta" y a "dulce", y por la disminución en la producción de ácido acético. También evita la producción de aromas procedentes de la oxidación lipídica, además de la oxidación de nitrito a nitrato disminuyendo su contenido residual. Sin embargo, la inoculación de esta levadura no aumenta la generación de aromas azufrados de interés que cabría esperear por su potencial de degradación de metionina.

# ANEXO

Autorización para la publicación en la Tesis Doctoral.

**Nitrate reduction in the fermentation process of salt reduced dry sausages: Impact on microbial and physicochemical parameters and aroma profile**

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# Nitrate reduction in the fermentation process of salt reduced dry sausages: Impact on microbial and physicochemical parameters and aroma profile

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ABSTRACT

Slow fermented sausages with reduced ingoing amounts of sodium nitrate were manufactured: control (250 ppm), 15% (212.5 ppm) and 25% (187.5 ppm) reduction. The effect of nitrate reduction on microbiology and chemical parameters, volatile compounds and aroma production was studied. Parameters like, pH, $a_w$ and colour decreased during ripening, without being affected by nitrate reduction. Lipid oxidation increased during ripening and it was higher in control sausages due to fat content. Residual nitrite was below the detection limit during the whole process and residual nitrate decreased during ripening, with higher reduction in RN25 sausages. Lactic acid bacteria, total mesophilic bacteria and yeasts and moulds increased during ripening but Gram positive cocci decreased. Microbial counts from nitrate reduced sausages at the end of the manufacturing process were not statistically different from the control sausages with nitrate. Regarding volatile compounds formation, compounds derived from amino acid degradation were increased by nitrate reduction. Aroma compounds derived from amino acid degradation and responsible for strong odours, dimethyl disulphide (toasted, garlic) and methional (cooked potato) and, to a lesser extent, compounds derived from esterase activity producing fruity odours (ethyl acetate, ethyl butanoate, ethyl-2-hydroxypropanoate, ethyl-2-methylbutanoate and ethyl-3-methylbutanoate) and several compounds from carbohydrate fermentation acetic acid (vinegar odour) and 2-butanone (fruity) were related to the high nitrate reduction (25%). Despite nitrate reduction up to 25% produced minor effect on microbial growth, their metabolism is regulated by nitrate content and therefore by nitrite generation affecting the production of key aroma compounds that alter the sausage aroma profile.

## 1. Introduction

Nitrite has several important functions in the manufacturing of meat products. Among them, colour improvement through its reduction to nitric oxide and development of cured flavour, are the most important for consumer acceptability (Sindelar and Milkowski, 2011). Moreover, nitrite is able to control the oxidative stability by preventing lipid oxidation (Berardo et al., 2016) and plays an important role inhibiting the growth and toxin production of *Clostridium botulinum* (Rahman, 2007). On the other hand, generation of nitrosamines in meat products by reactions between nitrosating agents, derived from nitrite, and secondary amines, derived from protein and lipid degradation, has been of safety concern due to their carcinogenic potential (De Mey et al., 2015). Generally, intensive heat treatments are necessary to obtain carcinogenic nitrosamines and in case of fermented sausages, the mild acidic conditions and low water activities prevent their formation (De Mey et al., 2015). The search for alternatives to nitrite have focused on the use of spices and fruits (Gassara et al., 2016), organic acids, antimicrobial peptides, etc. (Alahakoon et al., 2015). However, none of them fulfils all technological needs (colour, flavour, microbiological safety and antioxidant activity) and the actual trend is directed to reduce the ingoing amount of nitrite and nitrate in meat products (EFSA, 2010; FCEC, 2016).

Previous studies have demonstrated that the reduction of nitrate and nitrite in dry fermented sausages produces changes in quality (Hospital et al., 2015) and microbial safety (Hospital et al., 2012, 2014, 2016). Dry sausages with reduced amounts of nitrate and nitrite contained the highest amounts of volatile compounds derived from carbohydrate fermentation and amino acid degradation (Hospital et al., 2015) which could be attributed to increased counts of Gram-positive catalase-positive cocci and Enterobacteriaceae at the end of the ripening. Furthermore, the presence of nitrite is essential to control *Salmonella typhimurium* growth in fermented sausages; although, reduced concentrations of the compound are sufficient to provide equal protection (Hospital et al., 2014). Regarding *Listeria*, nitrite reduction increased the growth of inoculated L. *innocua* in the final sausage.

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Production of *C. botulinum* toxin has not been detected in nitrate and nitrite reduced fermented sausages, although this could depend on existing hurdles and specific ripening conditions (Hospital et al., 2016).

The effect of reduced ingoing amounts of nitrate and nitrite in fermented meat products seems to depend on the presence of both agents (nitrite and nitrate) and specific ripening conditions. Nevertheless, the specific provision concerning nitrites and nitrates (Regulation (EC) no 1333/2008) indicates the possibility of the exclusively use of nitrates (250 ppm) without added nitrite in traditional slow ripened sausages such as "salchichón" and "chorizo" with maturation period of at least 30 days. Presently, nothing is known about the effect of nitrate reduction on these traditionally cured meat products where nitrate is used exclusively as curing agent.

Slow-fermented sausages are typical Mediterranean products subjected to a first ripening step at low temperatures and a second step at higher temperatures in order to develop special sensory characteristics (Toldrá and Flores, 2014). These sausages have a mild pH decline, although starters are added in the process to improve the microbial safety of the product. The long process allows the exclusively use of nitrate as a nitrite reservoir by the activity of acid sensitive bacteria from the *Staphylococcaceae* family (Sánchez Mainar and Leroy, 2015). Nitrate slow fermented sausages have a preferred flavour than those made with nitrite and provide better organoleptic characteristics (Marco et al., 2008). In fact, the use of only nitrate instead of nitrite in dry fermented sausages produces the inhibition of lipid oxidation and affects the generation of volatile compounds derived from microbial metabolism, but as Marco et al. (2006) indicated it depends greatly on the fermentation process applied.

The longer ripening times employed in traditional dry sausages and the use exclusively of nitrates without added nitrite can produce different effects on the quality and safety of reduced nitrate sausages. Factors such as final pH, temperature and $a_w$ are closely interrelated with microbial growth. Moreover, the actual trends to reduce sodium in meat products may also affect microbial growth and sausage safety (Aaslayng et al., 2014; Laranjo et al., 2016). In view of these actual trends, the aim of this study was to evaluate the reduction of nitrate used exclusively without added nitrite on the overall quality of traditional dry sausages with reduced sodium content.

## 2. Materials and methods

### 2.1. Dry fermented sausage manufacture

Three different formulations (6 kg/formulation) of dry fermented sausages were manufactured: sodium nitrate at 250 ppm (C), 15% reduction (RN15) and 25% reduction (RN25). Three replicates of each formulation were carried out. Pork's lean and belly fat were purchased from a local producer (Cárnicas La Cope, Torrente, Spain).

For each manufacture batch, lean (50% lean pork meat) and fat (50% pork belly) were ground through a 10 mm diameter mincing plate and mixed with the following ingredients: 20 g/kg lactose, 20 g/kg dextrin, 7 g/kg glucose, 20.25 g/kg sodium chloride (NaCl), 6.75 g/kg potassium chloride (KCl), 0.5 g/kg sodium ascorbate, and sodium nitrate at 250 ppm (C), 212.5 ppm (RN15) or 187.5 ppm (RN25) depending on the batch. Also, a commercial starter culture (0.125 g/kg) TRADI-302 (Danisco, Cultor, Madrid, Spain) containing *Lactobacillus sakei*, *Staphylococcus xylosus* and *Staphylococcus carnosus* was added.

Each formulation was kept at 3–5 °C for 24 h and then stuffed into 95 mm diameter collagen casings (Fibran, S.A., Girona, Spain), being the final weight of each sausage approximately 500 g. Sausages were dried for 62 days at 10 °C and 75–90% relative humidity (RH). In order to control the ripening process, temperature and RH of the ripening chambers were continuously recorded. Two sausages from each batch were weighed almost every day to control weight losses. Also, one sausage from each batch was used to control the pH by introducing a pH meter HI 99163 (Hanna Instruments Inc., Hoonsocket, USA) into the centre of the sausage as described by Olivares et al. (2010).

A total of 9 batches were produced and approximately 10 sausages were obtained from each batch. Before stuffing, approximately 200 g of the minced meat mixture were collected for further analysis. At the end of the process, two sausages from each batch were randomly chosen for analysis. A slice of approximately 25 g was taken for microbial analyses. Several slices were taken, wrapped in aluminium foil, vacuum packed and storage at −80 °C for volatile analysis. Sausage colour was measured and approximately 100 g of sausage were minced and used for moisture, water activity ($a_w$) and pH analysis. The remaining minced sausage was vacuum packed and frozen at −20 °C for subsequent physicochemical analyses (TBARS, lipid, protein, residual nitrite and nitrate).

### 2.2. Physicochemical analysis

#### 2.2.1. pH, aw, colour and moisture

pH was measured with a pH meter HI 99163 (Hanna Instruments Inc.) using minced sausage and distilled water (1:1, p/v) (ISO 2917:, 1999). Water activity was measured with a Fast-lab water activity meter (Gbx, Romans sur Isère Cédex, France) and colour was analysed with a colorimeter (CR-400/410, Konica Minolta Sensing Inc., Japan) with D65 illuminant (Olivares et al., 2010). Moisture was determined by drying at 100 °C until constant weight (BOE, 1979).

#### 2.2.2. Lipid and protein content

Lipid content was determined by organic extraction with $Cl_2CH_2$:$CH_3OH$ (2:1) (Folch et al., 1957) as described Olivares et al. (2010). Nitrogen content was determined by the Kjeldahl method and protein estimated by multiplying the nitrogen content by 6.25.

#### 2.2.3. Oxidation and nitrifying agents

Lipid oxidation was evaluated using the thiobarbituric acid reactive substances test (TBARS) as described Corral et al. (2013). Values were expressed as μg of malonaldehyde per gram of dry matter (μg MDA/g dm). Nitrate and nitrite contents were extracted with hot water (Mohamed et al., 2008) and determined using an enzymatic kit (Boehringer) (Arneth and Herold, 1988).

### 2.3. Microbiological analysis

Microbial counts were done on 25 g of dry fermented sausage. Samples were finely sliced, blended with 225 ml of buffered peptone water (Pronadisa, Spain) and homogenized in a Pulsifier (Microgen Biotech, Spain). Homogenates were used to prepare decimal dilutions which were spread on appropriate media plates. Microbial counts were determined on the following media: bacterial starter containing lactic acid bacteria (LAB) on MRS Agar (Scharlau, Spain) at 30 °C for 3 days and Gram positive cocci on Mannitol Salt Agar (MSA) (CN-M) (Scharlau, Spain), at 30 °C for 3 to 5 days and Baird Parker Agar (BP) (CN-BP) (Pronadisa, Spain) at 37 °C for 48 h. Coagulase test was carried out in Gram positive cocci isolates from BP (EN ISO 6888–1) using lyophilised rabbit plasma (Scharlau, Spain). Mesophilic bacteria (TMB) were determined on Plate Count Agar (Pronadisa, Spain) at 30 °C for 3 days, yeasts and moulds on Rose Bengal Agar with chloranphenicol (Scharlau, Spain) at 30 °C for 5 to 7 days. Enterobacteriaceae were counted on Violet Red Bile Agar with Glucose (VRBG) (Pronadisa, Spain) at 37 °C for 24 h in anaerobiosis. Sulphite reducing clostridia were determined from 1 ml homogenate sample inoculated in freshly prepared Lactose Sulphite Broth supplemented with sodium metabisulfite and ferric ammonium citrate (Pronadisa, Spain) dispensed into tubes with Durham gas collecting tubes and incubated in anaerobiosis (bioMerieux, Spain) at 46 °C for 48 h. Twenty-five ml of homogenated sample were used for enrichment of *Yersinia enterolytica* in Sorbitol Peptone Broth and Bile Salts (PBS) (Pronadisa, Spain) at 25 °C for 5 days and subsequently plated on Yersinia Selective Agar (YSA) (Pronadisa,

**Table 1**
Effect of nitrate reduction on physicochemical parameters of dry fermented sausages at initial and end of process.

| | 0 days | | | 62 days | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | C[a] | RN15 | RN25 | C | RN15 | RN25 | RMSE[c] | $P_t$[d] | $P_n$ | $P_{txn}$ |
| pH | 5.85 a[b] | 5.83 a | 5.88 a | 4.99 b | 4.95 b | 4.93 b | 0.038 | *** | ns | ns |
| Aw | 0.981 a | 0.974 a | 0.975 a | 0.890 b | 0.894 b | 0.895 b | 0.004 | *** | ns | * |
| Moisture (%) | 63.12 a | 64.18 a | 64.88 a | 40.33 b | 41.03 b | 42.61 b | 2.091 | *** | ns | ns |
| Proteins (% dm) | 49.11 d | 49.37 cd | 50.05 cd | 54.58 bc | 56.15 b | 61.23 a | 2.409 | *** | ** | ns |
| Fat (% dm) | 35.97 ab | 34.07 abc | 31.68 bc | 36.30 a | 33.15 abc | 30.57 c | 2.052 | ns | *** | ns |
| L* | 64.92 a | 61.15 a | 61.04 a | 50.50 b | 49.84 b | 49.27 b | 1.716 | *** | * | ns |
| a* | 26.05 a | 27.14 a | 26.47 a | 17.66 b | 18.21 b | 17.88 b | 0.994 | *** | ns | ns |
| b* | 15.73 a | 15.69 a | 15.32 a | 6.59 b | 6.76 b | 6.26 b | 0.538 | *** | ns | ns |
| TBARS[e] | 0.56 b | 0.46 b | 0.41 b | 0.93 a | 0.69 ab | 0.61 b | 0.139 | *** | ** | ns |
| $NO_3$ (ppm dm) | 445.21 a | 378.58 ab | 338.50 bc | 299.79 c | 231.81 d | 163.52 e | 27.214 | *** | *** | ns |

[a] C: control batch. RN15: 15% sodium nitrate reduction batch. RN25: 25% sodium nitrate reduction batch.
[b] Different letters in the same row indicate significant differences at $p < 0.05$ among batches.
[c] RMSE: Roost mean square error.
[d] *P*: *P* value of time (t), nitrate content (n) and time and nitrate content (txn) effect, ns: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
[e] TBARS expressed as μg malonaldehyde/g dm.

Spain) at 30 °C for 48 h. Twenty-five ml of homogenated sample were used for enrichment of *Listeria* spp. in 1/2 Fraser and Fraser Broth supplemented with ferric ammonium citrate (Pronadisa, Spain) at 30 °C for 24 and 48 h, respectively. Dilutions of the *Listeria* enriched Fraser medium were inoculated onto Listeria Chromogenic Agar (Pronadisa, Spain) and incubated at 37 °C for 24 h.

The remaining homogenate was incubated at 37 °C during 16–20 h for Salmonella pre-enrichment. One millilitre of the incubated homogenate was used for enrichment of *Salmonella* spp. in Rappaport Soy Broth (Pronadisa, Spain) at 41.5 °C for 24 h and Muller Kauffmann Broth Base w/Brilliant Green and Novobiocin supplemented with iodine and potassium iodide solution (MKTTN) (Pronadisa, Spain) at 37 °C for 24 h. Enriched cultures were plated on Xylose Lysine Desoxycholate Agar (XLD) (Pronadisa, Spain) and incubated at 37 °C for 24 h.

### 2.4. Volatile compound analysis

#### 2.4.1. Gas chromatography-mass spectrometry

The analysis of headspace (HS) volatile compounds was carried out by solid phase micro extraction (SPME) with an 85 μm Carboxen/Polydimethylsiloxane (CAR/PDMS) fibre (Supelco, Bellefonte, PA) using a gas chromatograph (Agilent HP 7890 series II (Hewlett-Packard, Palo Alto, CA) with a mass detector (HP 5975C (Hewlett-Packard) equipped with an autosampler (Gerstel MPS2 multipurpose sampler (Gerstel, Germany), as described Corral et al. (2015) with minor modifications. Briefly, 5 g of sausage with 0.75 mg BHT to avoid oxidation, was weighed into a headspace vial. The vial was incubated at 37 °C for 30 min. Then, the fibre was exposed into the headspace vial for 120 min while maintaining the sample at 37 °C. The compounds adsorbed by the fibre were desorbed in the injection port of the GC–MS for 5 min at 240 °C in splitless mode. The compounds were identified by comparison with mass spectra from the library database (Nist'05), with linear retention indices (Van Den Dool and Kratz, 1963) and with authentic standards. The results were expressed as abundance units (AU) $10^5$.

#### 2.4.2. Gas chromatography-olfactometry/FID

The analysis of aroma compounds was carried out by a gas chromatograph (Agilent 6890, USA) equipped with a FID detector and sniffing port (ODP3, Gerstel, Mülheim an der Ruhr, Germany) as described Corral et al. (2015). Each assessment was carried out with 5 g of sample using the detection frequency method (Pollien et al., 1997). Four trained panellists evaluated the odours from the GC-effluent of the sausages at the end of the maturation (62 days). A total of 12 assessments were carried out. The aroma compounds were identified by comparison with mass spectra, with linear retention indices of authentic standards injected in GC–MS and GC-O and by the coincidence of the assessor's descriptors with those in the Fenaroli's handbook of flavour ingredients (Burdok, 2002).

### 2.5. Statistical analysis

Data were analysed using the Generalized Linear Model (GLM) procedure of statistical software (XLSTAT 2011, v5.01, Addinsoft, Barcelona, Spain). Data was analysed using the linear mixed model and included nitrate reduction and ripening time as fixed effects, and replicates as random effect. When significant effect of the treatment group was detected ($P < 0.05$), least squares means (LSM) were compared using Tukey test. Principal component analysis (PCA) was done to evaluate the relationships among sausage formulation and different parameters (pH, water activity, TBARS, protein and fat content, nitrate residual, colour, microbiota and aroma compounds).

## 3. Results and discussion

### 3.1. Physicochemical analyses

Physicochemical parameters are shown in Table 1. Observed differences between batches in fat and protein content were due to random formulation variations which resulted in a higher fat content in control sausages and small differences in weight losses (C 39.78%, RN15 41.97%, RN25 42.58%). Final dry fermented sausages had an average pH value of 4.9, as expected for this type of product (Marco et al., 2008), and the $a_w$ decreased from 0.977 to 0.893 (average values). These results guarantee the stability and safety of the sausages and, therefore, nitrate reduction did not seem to affect them, as observed in nitrite and nitrate reductions by Hospital et al. (2015). The colour parameters evaluated (L*, a* and b* values) decreased during ripening due to drying with values within the expected range (Olivares et al., 2010). Nitrate reduction did not affect the colour of sausages neither at the beginning nor at the end of process, although a higher L* value was detected in the control raw sausage than in nitrate reduced ones (RN15 and RN25). Regarding lipid oxidation, it increased with ripening in all sausages, although at the end of process C batch had a significant higher TBARS value than RN15 and RN25. The antioxidant activity of nitrite is well known (Zanardi et al., 2004), therefore, the higher lipid oxidation in C batch could be due to its higher fat content (Olivares et al., 2011).

At the beginning of the process, nitrite was not detected because

**Table 2**
Effect of nitrate reduction on microbial counts (log cfu/g) of dry fermented sausages at initial and end of process.

| | Culture medium[e] | 0 days | | | 62 days | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C[a] | RN15 | RN25 | C | RN15 | RN25 | RMSE[c] | $P_t$[d] | $P_n$ | $P_{txn}$ |
| Total mesophilic bacteria (TMB) | PCA | 6.84 b[b] | 6.88 b | 6.87 b | 8.89 a | 8.83 a | 8.96 a | 0.12 | *** | ns | ns |
| *Lactobacillus* (LAB) | MRS | 5.85 b | 5.83 b | 5.75 b | 6.98 a | 6.94 a | 6.96 a | 0.07 | *** | ns | ns |
| Yeasts and moulds (YM) | RB | – | – | – | 2.32 b | 3.24 a | 3.33 a | 0.20 | ** | ** | ns |
| Enterobacteriaceae (EB) | VRBGA | 4.84 a | 4.83 a | 4.81 a | 2.39 b | 2.34 b | 2.33 b | 0.03 | *** | ns | ns |
| Gram positive cocci CN (CN-M) | MSA | 5.47 a | 5.41 a | 5.28 a | 4.49 b | 4.33 b | 4.39 b | 0.13 | *** | ns | ns |
| Gram positive cocci CN (CN-BP) | BP | 4.86 b | 4.84 ab | 4.78 ab | 4.45 c | 4.56 bc | 4.64 abc | 0.10 | *** | ns | ns |

[a] C: control batch. RN15: 15% sodium nitrate reduction batch. RN25: 25% sodium nitrate reduction batch.
[b] Different letters in the same row indicate significant differences at $p < 0.05$ among batches.
[c] RMSE: Roost mean square error.
[d] *P*: *P* value of time (t), nitrate content (n) and time and nitrate content (txn) effect, ns: $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.
[e] PCA: Plate Count Agar, RB: Rose Bengal Agar, VRBGA: Violet Red Bile Agar with Glucose, MSA: Mannitol Salt Agar, BP: Baird Parker Agar.

only nitrate was added and there was not enough time for its reduction. At the same time, residual nitrate content confirmed the different ingoing amounts added to each batch. At the end of the process, the nitrate decline was 33, 39 and 52% respect to the initial amount (C, RN15 and RN25). Nitrite was never detected; this compound was always below the detection limits. Marco et al. (2006, 2008) also observed a decrease of nitrate concentration during ripening of slow fermented sausages with only nitrate added. These authors found a nitrate decline of approximately 30% in sausages with a final pH value of 4.8 (Marco et al., 2006), however, higher nitrate drops were observed in sausages with pH slightly above 5 (Marco et al., 2008). In both cases, residual nitrate amounts were close to 100 ppm dm. In contrast, the residual amounts of the manufactured fermented sausages were above 100 ppm dm but the differences may be due to the fermentation process and its effect on the nitrate reductase enzyme (Cachaldora et al., 2013).

### 3.2. Microbiological analyses

Microbial counts are shown in Table 2. At the beginning of the process, total mesophilic bacteria (TMB) and lactic acid bacteria (LAB) had mean counts of 6.8 and 5.8 log cfu/g respectively, in accordance with the amount of starter inoculated. Moreover, the increase by 2 log in TMB and 1 log in LAB during ripening is in the range expected for this type of sausage (Fontana et al., 2005). The population of Gram positive cocci that include staphylococcal starters numerated either on MSA (CN-M) or on BP (CN-BP) decreased during the process. This could be due to the pH decrease produced by LAB which is affecting coagulase negative staphylococci (Leroy et al., 2006) as well as NaCl reduction and replacement by KCl as observed by Corral et al. (2014). Coagulase test on CN-BP cocci isolates (about 400 studied) classified all of them as coagulase negative and probably representative of *Staphylococcus* species also included in the bacterial starter. Enterobacteria counts decreased by 2 log from the beginning to the end of the process. Yeasts and moulds increased until $10^3$ cfu/g in final sausages. Sulphite reducing clostridia, *Salmonella* spp. and *Yersinia enterolitica* were not present during the whole process. Listeria counts were also negative in all samples except for two positive colonies grown on LCA media in a replicate of RN25 reduced nitrate sausages at the end of the fermentative process.

In summary, microbial counts in control samples were in the range expected and followed the dynamics observed in slow fermented sausages (Rantsiou et al., 2005; Samelis et al., 1998; Di Luccia et al., 2016; Tabanelli et al., 2016). Hospital et al. (2015) studied the effect of nitrate and nitrite reduction on the microbial growth of dry fermented sausages. In agreement with our results, these authors did not find differences in LAB counts at the beginning of the process, but neither in Gram positive catalase positive cocci. The differences between results in the present study and those of Hospital et al. (2015) can also be attributed to the initial addition of nitrite together with nitrate and the kind of fermentation (Marco et al., 2008; Sanz et al., 1997). Nitrate reduction appeared to affect mostly growth of yeasts and moulds which counts were significantly higher in nitrate reduced samples.

### 3.3. Volatile compound analysis

The effect of nitrate reduction on the volatile compounds of dry fermented sausages was studied through the analysis of the volatile compounds present in the headspace (HS) of the different sausages analysed by SPME-GC–MS (Table 3). Fifty-three volatile compounds were identified and quantified in the HS of dry fermented sausages using a CAR/PDMS fibre. The volatile compounds identified and quantified in the HS can be compared with previous studies using the same extraction technique and fibre. The volatile compounds present in the HS of sausages were derived from chemical and metabolic reactions during ripening. Table 3 shows the volatile compounds classified by their possible origin: microbial activity: amino acid degradation (14), carbohydrate fermentation (9), esterase activity (6) and β-oxidation (3), and from lipid oxidation reaction (20) and unknown origin (1). All of these compounds have been previously identified in slow fermented sausages (Corral et al., 2013; Marco et al., 2006; Olivares et al., 2011).

Volatile compounds derived from amino acid degradation were significantly affected by nitrate reduction. In RN25 sausages a significant increase was observed for 2-methylpropanal, 2-methylpropanol, 3-methylbutanol, 2-methylbutanol, and 2,6-dimethylpyrazine, which come mainly from branched chain amino acids (Søndergaard and Stanhke, 2002). However, only benzene was significantly reduced in nitrate reduced sausages ($p < 0.01$). Thus, amino acid degradation took place in more abundance when nitrate was reduced. This fact can be related to the growth of LAB, *Staphylococcaceae*, yeasts and moulds as responsible for the degradation of amino acids (Toldrá, 2008) although only yeasts and moulds were significantly high in RN25 sausages (Table 2). Hospital et al. (2012, 2015) also found an increase in volatile compounds derived from amino acid degradation in reduced nitrite and nitrate sausages. These authors also observed an increase on 3-methylbutanol in reduced nitrite and nitrate sausages. However, it is important to notice that differences among studies are due to the use of different curing agents, since different effect is produced by the use of nitrate versus nitrite in the generation of volatile compounds as already observed by Marco et al. (2006), specially volatiles from high branched chain amino acid degradation (Olesen et al., 2004).

Carbohydrate fermentation was the group with the highest proportion of volatile compounds in all formulations (75–80%) (Corral et al., 2013) being acetic acid, ethanol and 3-hydroxy-2-butanone the most abundant compounds in this group. Nitrate reduction did not affect the total abundance of compounds derived from carbohydrate fermentation. As observed in Table 2, starter culture (LAB and Gram

**Table 3**
Effect of nitrate reduction on volatile compounds generated (expressed as AU × $10^5$) in dry fermented sausages at the end of process.

| | LRI[a] | RI[b] | C[c] | RN15 | RN25 | RMSE[d] | $P_n$[e] |
|---|---|---|---|---|---|---|---|
| Microbial origin | | | | | | | |
| Amino acid degradation | | | 400.589 b | 392.157 b | 534.289 a | 72.31 | ** |
| 2-Methylpropanal | 594 | a | 4.852 ab | 4.428 b | 7.655 a | 1.75 | * |
| Benzene | 676 | a | 2.011 a | 1.982 a | 1.034 b | 0.47 | ** |
| 2-Methyl-1-propanol | 683 | a | 12.449 ab | 9.707 b | 17.083 a | 4.22 | * |
| 3-Methylbutanal | 691 | a | 34.320 | 27.215 | 39.208 | 8.98 | ns |
| Dimethyl disulfide | 773 | a | 2.439 | 3.904 | 3.148 | 1.38 | ns |
| Toluene | 788 | a | 27.531 | 101.371 | 70.830 | 58.49 | ns |
| 3-Methyl-1-butanol | 795 | a | 227.773 ab | 176.059 b | 286.989 a | 57.99 | * |
| 2-Methyl-1-butanol | 797 | a | 49.137 b | 40.051 b | 68.571 a | 12.58 | ** |
| 2,6-Dimethylpyrazine | 945 | a | 3.230 b | 3.509 b | 4.586 a | 0.61 | ** |
| Methional | 968 | a | 2.186 | 3.145 | 5.137 | 1.24 | ns |
| Benzaldehyde | 1020 | a | 8.538 | 11.533 | 11.050 | 3.45 | ns |
| Benzene acetaldehyde | 1110 | a | 3.253 | 2.295 | 3.686 | 1.14 | ns |
| Phenol | 1114 | a | 17.529 | 17.635 | 16.779 | 1.55 | ns |
| Methanethiol | 473 | a | 12.395 | 10.746 | 8.2407 | 2.62 | ns |
| Carbohydrate fermentation | | | 6845.361 | 8221.824 | 7426.739 | 1448.965 | ns |
| Acetaldehyde | 466 | a | 75.874 | 72.257 | 61.998 | 17.915 | ns |
| Ethanol | 507 | a | 1858.751 | 2126.592 | 2326.289 | 565.348 | ns |
| Acetone | 529 | a | 31.026 b | 51.857 a | 38.867 ab | 8.163 | ** |
| 2,3-Butanedione | 627 | a | 78.890 a | 47.539 b | 45.482 b | 18.699 | * |
| 2-Butanone | 631 | a | 41.494 b | 47.258 b | 101.245 a | 20.633 | ** |
| Acetic acid | 718 | a | 2685.579 b | 2601.611 b | 3031.631 a | 233.298 | * |
| 3-hydroxy-2-butanone | 781 | a | 1333.706 | 1021.151 | 1025.607 | 448.975 | ns |
| 2,3-Butanediol | 888 | a | 702.097 | 2259.924 | 1449.120 | 1172.704 | ns |
| Butanoic acid | 896 | a | 55.761 b | 50.373 b | 95.959 a | 15.088 | ** |
| Esterase activity | | | 718.76 | 713.97 | 1093.10 | 304.53 | ns |
| Ethyl acetate | 635 | a | 571.12 b | 704.31 b | 1049.16 a | 136.75 | *** |
| Ethyl butanoate | 832 | a | 47.01 | 46.30 | 68.99 | 14.16 | ns |
| Ethyl 2-hydroxypropanoate | 867 | a | 45.84 b | 55.66 ab | 67.80 a | 13.87 | * |
| Ethyl 2-methylbutanoate | 878 | a | 22.84 b | 28.77 ab | 40.31 a | 9.44 | * |
| Ethyl 3-methylbutanoate | 882 | a | 29.34 b | 27.45 b | 62.31 a | 14.84 | * |
| Ethyl octanoate | 123 | a | 7.51 | 10.75 | 9.60 | 2.60 | ns |
| β-oxidation | | | 43.95 | 38.70 | 38.14 | 10.46 | ns |
| 2,3-Pentanedione | 745 | a | 16.64 | 11.84 | 19.44 | 4.51 | ns |
| 2-Heptanone | 935 | a | 11.41 | 11.35 | 10.40 | 1.97 | ns |
| 1-Octen-3-ol | 1033 | a | 17.49 | 16.58 | 12.18 | 4.20 | ns |
| Chemical origin | | | | | | | |
| Lipid oxidation | | | 831.43 | 805.59 | 560.18 | 186.39 | ns |
| Pentane | 500 | a | 51.79 | 55.47 | 37.52 | 17.295 | ns |
| Propanal | 524 | a | 8.74 | 6.40 | 7.65 | 2.043 | ns |
| Hexane | 600 | a | 19.80 | 20.40 | 15.01 | 4.808 | ns |
| 1-Propanol | 612 | a | 11.88 ab | 10.71 b | 16.55 a | 3.038 | * |
| 2-Methylfuran | 616 | a | 1.80 a | 1.25 b | 1.36 ab | 0.317 | * |
| Butanal | 622 | a | 2.11 a | 0.48 b | 0.93 b | 0.703 | ** |
| Heptane | 700 | a | 94.23 | 94.22 | 78.82 | 24.149 | ns |
| Pentanal | 739 | a | 55.17 | 48.45 | 36.12 | 12.601 | ns |
| Octane | 800 | a | 126.91 | 121.26 | 93.47 | 22.281 | ns |
| 1-Pentanol | 827 | a | 34.12 | 30.49 | 21.56 | 7.228 | ns |
| Hexanal | 842 | a | 355.29 a | 322.76 ab | 183.14 b | 93.305 | * |
| 2-Hexenal | 907 | a | 1.55 b | 1.78 b | 2.96 a | 0.520 | ** |
| 1-Hexanol | 924 | a | 57.22 | 53.23 | 46.12 | 13.174 | ns |
| Heptanal | 941 | a | 34.88 a | 31.88 ab | 22.97 b | 6.441 | * |
| Decane | 1000 | a | 2.59 b | 3.19 ab | 3.63 a | 0.603 | * |
| 2-Pentylfuran | 1010 | a | 7.16 | 8.50 | 7.08 | 1.516 | ns |
| (*E*)-2-Heptenal | 1013 | a | 3.41 a | 2.88 ab | 1.93 b | 0.835 | * |
| Octanal | 1049 | a | 21.69 | 22.12 | 16.26 | 4.872 | ns |
| Hexanoic acid | 1079 | a | 14.34 a | 12.27 ab | 9.53 b | 2.164 | * |
| Nonanal | 1151 | a | 24.45 | 26.76 | 21.95 | 5.178 | ns |
| Unknown compounds | | | | | | | |
| Carbon disulfide | 537 | a | 57.80 b | 73.28 ab | 104.65 a | 20.871 | * |

[a] LRI: Linear retention index of the compounds eluted from the GC–MS.
[b] RI: Reliability of identification: a, identification by mass spectrum, coincidence with the LRI of an authentic standard; b, tentatively identification by mass spectrum.
[c] C: control batch, RN15: 15% sodium nitrate reduction batch, RN25: 25% sodium nitrate reduction batch.
[d] RMSE: Roost mean square error.
[e] $P_n$: *P* value of nitrate content effect, ns: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, different letters in the same row indicate significant differences at $p < 0.05$ among batches.

**Table 4**
Odour active compounds identified in dry fermented sausages at the end of process.

| | LRI GC-O[a] | LRI std GC-O[b] | RI[c] | Descriptor | DF[d] |
|---|---|---|---|---|---|
| Methanethiol | 472 | 471 | a | Rotten, unpleasant | 8 |
| 2-Methylfuran | 615 | 619 | a | Green, garlic, toasted, yeast, malt | 4 |
| 2,3-Butanedione | 629 | 632 | a | Cheese, butter, floral, fresh | 4 |
| 2-Butanone | 636 | 638 | a | Fruity, sweet, cheese, butter, dairy | 8 |
| Acetic acid | 699 | 700 | a | Vinegar, acid, unpleasant | 8 |
| 2,3-Pentanedione | 740 | 739 | a | Sweet, candy, fruit, glue | 4 |
| Dimethyl disulfide | 769 | 774 | a | Toasted, garlic | 3 |
| 3-Hydroxy-2-butanone | 782 | 777 | a | Strawberry, sweet, fruity, green | 9 |
| Ethyl butanoate | 824 | 825 | a | Sweet, fruity, floral | 10 |
| Hexanal | 834 | 836 | a | Fresh cut grass, vegetable, fresh | 10 |
| Ethyl 2-hydroxypropanoate | 865 | 859 | a | Cheese, fruit, strawberry, sweet | 11 |
| Ethyl 3-methylbutanoate | 874 | 876 | a | Strawberry, fruit, glue, sweet | 9 |
| 1-Hexanol | 920 | 919 | a | Cheese, oxidized fat, humidity | 11 |
| 2-Heptanone | 931 | 931 | a | Rancid, burnt, strawberry, fruit | 4 |
| Heptanal | 938 | 937 | a | Green, unpleasant, toasted | 5 |
| 2-Acetyl-1-pyrroline | 960 | 960 | a | Toasted, fried corn, bread | 12 |
| Methional | 965 | 969 | a | Cooked potato, roast meat | 10 |
| 2-Pentylfuran | 1008 | 1011 | a | Garlic, onion, unpleasant, grass | 8 |
| 1-Octen-3-ol | 1023 | 1028 | a | Mushrooms, humidity, spicy | 11 |
| Unknown | 1031 | – | c | Burnt, mushrooms, humidity, herbs | 8 |
| Unknown | 1037 | – | c | Green, grass, earth, burnt, herbs | 6 |
| Octanal | 1045 | 1047 | a | Orange, sweet | 4 |
| Unknown | 1162 | – | c | Spices, fried corn, unpleasant | 5 |
| Unknown | 1178 | – | c | Fried corn, toasted | 10 |
| Ethyl octanoate | 1223 | 1226 | a | Cooked vegetable, onion, fruity | 7 |

[a] Linear retention index of the compounds eluted from the GC-FID-O.
[b] Linear retention index of standard compounds in the GC-FID-O.
[c] Reliability of identification: a, identification by mass spectrum, coincidence with LRI of an authentic standard and by coincidence of the assesors's descriptors with those described in the Fenaroli's handbook of flavour ingredients (Burdok, 2002); b, tentatively identification by mass spectrum; c, unknown compounds.
[d] Detection frequency value.

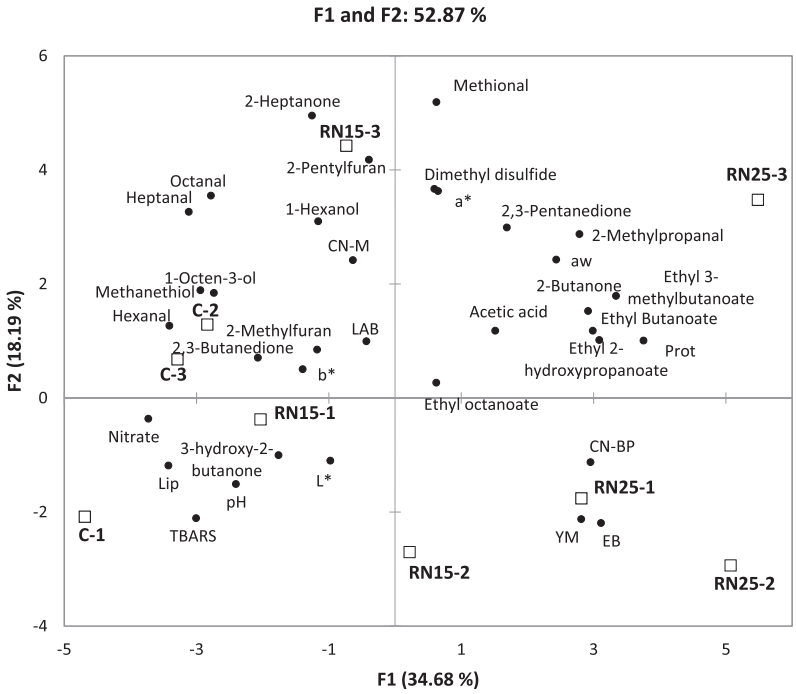


**Fig. 1.** Loadings of the first two principal components (PC1-PC2) of dry fermented sausages with different nitrate ingoing amounts: C (control batch, 250 ppm), RN15 (15% sodium nitrate reduction, 212.5 ppm), RN25 (25% sodium nitrate reduction, 187.5 ppm) and instrumental variables (physico-chemical, microbiological parameters and aroma compounds). Abbreviations are indicated in Tables 1 and 2.

positive cocci) were not affected by nitrate reduction, being these microorganisms responsible for the generation of these volatile compounds (Toldrá, 2008). Nevertheless, the effect of nitrate reduction affected the abundance of several compounds especially at the highest reduction (RN25) producing an increase in acetone, 2-butanone and, acetic and butanoic acids. Similar increases in acid compounds were also reported in nitrite and nitrate reduced dry fermented sausages (Hospital et al., 2012, 2015).

Among volatile compounds derived from esterase activity, the most abundant was ethyl acetate as showed by other authors in similar sausages (Corral et al., 2013). The total abundance of compounds derived from esterase activity was higher in RN25 batch, although the increase was not significant. The abundance of compounds ethyl acetate, ethyl butanoate, ethyl-2-hydroxypropanoate, ethyl-2-methylbutanoate and ethyl-3-methylbutanoate was significantly higher in nitrate reduced than in C sausages. However, Gram positive cocci (CN-M and CN-BP) growth was not significantly different among sausages (Table 2). In contrast, Hospital et al. (2012, 2015) reported a reduction of ethyl acetate in dry fermented sausages containing reduced nitrite and nitrate levels. These differences among studies can be due to the exclusive use of nitrate (Marco et al., 2008) and for the differences in ethanol abundance, which is a substrate in esterase activity (Talon et al., 1998).

Compounds 2,3-pentanedione, 2-heptanone and 1-octen-3-ol were identified as volatiles derived from β-oxidation (Corral et al., 2013). These compounds, found in the smallest proportion (< 1%), seemed not to be affected by nitrate reduction. Although high fat content has been related to high abundance of these compounds (Olivares et al., 2010), no differences were observed among nitrate reduced sausages nor due to different fat content. Hospital et al. (2012) found significantly higher amounts of 2-heptanone and 1-octen-3-ol in absence of nitrite and nitrate, demonstrating the antioxidant activity of curing agents.

Compounds derived from lipid oxidation reactions have an important role in dry fermented sausages aroma. The highest abundance of volatiles from lipid oxidation was detected in the control batch, although the differences with nitrate reduced sausages were not significant. This is in accordance with the high TBARS values detected in control sausages (Table 1). Among the lipid oxidation compounds, 2-methylfuran, butanal, 1-pentanol, hexanal, heptanal, 2-heptenal and hexanoic acid were produced in significantly increased amounts in control sausages. On the other hand, 1-propanol, 2-hexenal and decane appeared in significantly higher amounts in nitrate reduced sausages. The antioxidant activity of nitrite (Berardo et al., 2016) was difficult to appreciate in the nitrate reduced fermented sausages because the higher fat content in control sausages produced a higher susceptibility to oxidation. In addition, the nitrate reduced sausages had the lowest residual nitrate content, indicating a higher conversion of nitrate to nitrite and probably a higher antioxidant effect.

### 3.4. Aroma analysis

Aroma compounds were analysed by gas chromatography-olfactometry (Table 4). Twenty-five aroma active zones were detected. From these, 21 compounds were identified by mass spectra, liner retention indices and by comparison with the odour description in literature. Four odour zones were not identified and were labelled as unknown compounds. The most important aroma compounds with highest DF values were ethyl butanoate (sweet and fruity odour), hexanal (fresh cut grass), ethyl 2-hydroxypropanoate (cheese and fruity odour), 1-hexanol (cheese and rancid odour), 2-acetyl-1-pyrroline (popcorn odour), methional (cooked potato odour), 1-octen-3-ol (mushrooms odour) and 1 unknown aromatic compound (cooked potato, fried corn, toasted and dried fruit odour) as already observed by Corral et al. (2013) and Olivares et al. (2011).

To examine the relationship of the instrumental variables with the ingoing amounts of nitrate, a principal component analysis was done using the following parameters; chemical composition, TBARS values, pH, colour parameters, $a_w$, residual nitrate concentration, aroma active volatile compounds and microorganism's groups (Fig. 1). Two principal components were able to explain the 52.87% of the total variance. PC2 accounted for 18.19% of the variance and showed the variability among replicates of the same formulation. PC1 accounted for 34.68% of the variance and distinguished sausages by nitrate ingoing amounts placing C sausages on the left quadrant and nitrate reduced sausages, RN15 and RN25, on the centre and right quadrant, respectively. C batch was related to lipid oxidation and aroma compounds derived from oxidation reactions (hexanal, octanal, heptanal and 2-methylfuran) and carbohydrate fermentation (2,3-butanedione and 3-hydroxy-2-butanone). Other aroma impact compounds, methanethiol and 1-octen-3-ol, were also related to the control sausages. In contrast, nitrate reduced sausages (RN25) were related to aroma compounds derived from amino acid degradation (dimethyl disulphide and methional), esterase activity (ester compounds) and several compounds from carbohydrate fermentation (acetic acid and 2-butanone). Moreover, nitrate reduced sausages (RN25) were related to Enterobacteriaceae, Gram positive cocci (CN-BP) and yeast and moulds growth indicating the presence of other microorganisms which can be responsible for the high volatile compounds derived from microorganism's metabolism. In summary, nitrate reduction affected the aroma profile of fermented sausages due to their effect on microbial metabolism.

## 4. Conclusion

The reduction of in going amounts of nitrate when used exclusively without nitrite addition in slow fermented sausages produced changes in the production of volatile compounds affecting the aroma profile. Microbial counts dynamics were mostly influenced by fermentation time and only slightly by nitrate reduction in case of yeasts and moulds. Manufacturing with reduced amount of nitrate up to 15% did not have a significant impact on microbial counts. Nitrate reduction resulted in an increase of volatile compounds derived from amino acid degradation and, to a lesser extent, from esterase activity, affecting the production of key aroma compounds that alter the sausage aroma profile. Our results indicate that nitrate reduction does not seem to affect directly microbial growth but affects microbial metabolism. Moreover, microbial metabolism can affect nitrate reduction into nitrite and hence its effect on lipid oxidation. More studies are necessary to know the extent and significance of nitrate reduction into the manufacturing process of traditional dry sausages.

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# Microbial changes and aroma profile of nitrate reduced dry sausages during vacuum storage

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ABSTRACT

Slow fermented sausages with reduced ingoing amounts of sodium nitrate (control, 15% and 25% reduction) were stored under vacuum up to three months. Changes in microbiology, chemical parameters and volatile compounds were studied. Residual nitrate was not affected by vacuum storage and its reduction resulted in a reduction of sausage redness. General microbial counts decreased during vacuum storage, though nitrate reduction increased the growth of total mesophilic bacteria and Gram positive cocci. Long storage time and 25% nitrate reduction affected microbial activity and sausage aroma profile. Short vacuum storage times and moderate nitrate reduction (15%) were related to compounds producing pleasant odours (3-hydroxy-2-butanone, ethyl octanoate, ethyl-3-methylbutanoate and 2,3-pentanedione) and cheesy/buttery odour (2,3-butanedione and ethyl-2-hydroxypropanoate). In contrast, 25% nitrate reduction increased compounds like heptanal (green, unpleasant odour) and those related to unpleasant odours, methanethiol (rotten odour) and methional (cooked potato).

## 1. Introduction

Consumers demands healthier meat products reduced in additives such as nitrite due to the generation of nitrosamines with carcinogenic potential (De Mey, De Maere, Paelinck, & Fraeye, 2015). However, nitrate and nitrite are used in fermented sausage manufacture as curing salts due to the nitrite effect on the control of *Clostridium botulinum* and its toxin production (Sindelar & Milkowski, 2011). Moreover nitrite influences several technological parameters like colour development, typical cured flavour and antioxidant effect (Honikel, 2008). In this term, the interest of producers is directed to the knowledge of the reasonable nitrite/nitrate reduction to operate with safety warrant and maintain the high organoleptic properties of traditional meat products. Recently, Christieans, Picgirard, Parafita, Lebert, and Gregori (2018) have demonstrated the impact of reducing the ingoing amount of nitrate/nitrite in dry fermented sausages manufacture and its effect on the growth of pathogens like *Salmonella* and *Listeria*. However, scientific studies should provide information not only regarding microbial risks but also on organoleptic properties like aroma and the changes that may be produced during the long shelf life of this type of products.

Different storage conditions are used depending on product type to extent their shelf-life while maintaining quality and safety. Dry fermented sausages can be kept unpackaged or packaged as whole or slices pieces under modified atmospheres or under vacuum conditions. Among these, vacuum packed is widely used to extend the shelf-life of dry sausages. Therefore, many studies have reported the changes observed during storage under vacuum conditions (Ansorena & Astiasarán, 2004; Dos Santos, Campagnol, Fagundes, Wagner, & Pollonio, 2015, 2017; Kim et al., 2012; Rubio et al., 2007; Rubio, Martinez, Garcia-Cachan, Rovira, & Jaime, 2008; Ščetar, Kovacic, Kurek, & Galic, 2013; Summo, Caponio, Paradiso, Pasqualone, & Gomes, 2010; Summo, Caponio, & Pasqualone, 2006; Summo, Caponio, Pasqualone, & Gomes, 2011; Zanardi, Dorigoni, Badiani, & Chizzolini, 2002), modified atmospheres (Rubio et al., 2007, 2008; Ščetar et al., 2013; Tabanelli, Montanari, Grazia, Lanciotti, & Gardini, 2013; Viallon et al., 1996; Zanardi et al., 2002;) and perforated packages (Bañon, Serrano, & Bedia, 2014; Lorenzo, Bedia, & Bañon, 2013). Changes in pH, water activity ($a_w$), red colour (a*) and oxidation parameters (TBARS) during shelf-life have been reported. Moreover, microbiology counts show a general decrease, except for LAB (Kim et al., 2012; Rubio et al., 2007). Overall, sausage acceptability decreases during storage due to colour, aroma, and taste deterioration. The most common changes are the decrease in red intensity, ripened flavour and firmness and the increase in rancid aroma and hardness (Kim et al., 2012; Rubio et al., 2007; Summo et al., 2010; Zanardi et al., 2002) which is apparently accentuated by vacuum storage versus modified atmosphere

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(Rubio et al., 2008) and unpackaged storage (Summo et al., 2006).

In addition to the physicochemical, microbiological and sensory changes attributed to storage, several studies have dealt with the effect on volatile compounds responsible for ripened aroma (Summo et al., 2011; Tabanelli et al., 2013; Viallon et al., 1996). Viallon et al., (1996) described the variation in sausage volatile profile with packaging under modified atmosphere as an increase of compounds derived from carbohydrate and amino acid degradations. Recent studies revealed that microbial and endogenous enzyme activities during modified atmosphere packaging depended on the initial sausage water activity and, therefore, changes in the latter affected the aroma profile (Tabanelli et al., 2013). Regarding the effect of vacuum storage on aroma profile, differences in lipid oxidation compounds like aldehydes (Ansorena & Astiasarán, 2004) and a significant increase of volatile compounds derived from carbohydrate and amino acid degradation reactions have been described in dry fermented sausages (Marco, Navarro, & Flores, 2006). Moreover, recent studies have shown a general increase in volatile compounds derived from lipid oxidation reactions (Dos Santos et al., 2015; Summo et al., 2011) and a decrease of those derived from spices under vacuum storage (Dos Santos et al., 2015). In summary, reported volatile changes during storage are highly dependent on sausage properties like $a_w$ (Tabanelli et al., 2013), lipid profile (Ansorena & Astiasarán, 2004), curing agents (Marco et al., 2006) and salt substitutes (Dos Santos et al., 2015) in addition to packaging conditions like temperature and time (Ščetar et al., 2013).

The latest trends in fermented sausage composition are directed to the reduction of additives such as nitrifying agents (EFSA, 2010; FCEC, 2016). Until now, only Hospital, Hierro, and Fernández (2014) have studied the effect of nitrate and nitrite reduction in rapid fermented sausages on microbial evolution during 1 month of vacuum storage. These authors reported changes in microbial counts but the impact of nitrifying agents and storage conditions on aroma was not investigated. Furthermore, the possibility of the exclusive use of nitrates (250 ppm) without added nitrite in traditional slow ripened sausages such as “salchichón” and “chorizo” with maturation period of at least 30 days is indicated in a specific provision concerning nitrites and nitrates (EC Regulation no 1129/2011). Therefore, the aim of the present study is to determine the effect of vacuum storage and nitrate reduction on the aroma quality and microbial counts of slow fermented sausages manufactured with reduced sodium content.

## 2. Materials and methods

### 2.1. Dry fermented sausages manufacture

Three replicates of the experiment were performed. In each replicate, three different formulations of dry fermented sausages were manufactured: Control with 250 ppm sodium nitrate (C) and two formulations with a reduction of 15% (RN15) and 25% (RN25) of ingoing amounts of sodium nitrate. Lean pork meat (50%) and pork fat (bellies boneless and skinless) (50%) were minced with the following ingredients (g/kg): lactose (20), dextrin (20), glucose (7), sodium chloride (NaCl) (20.25), potassium chloride (KCl) (6.75), sodium ascorbate (0.5), sodium nitrate at 250 ppm (C), 212.5 ppm (RN15) or 187.5 ppm (RN25) depending on the batch. A commercial starter culture (0.125) TRADI-302 containing *Lactobacillus sakei*, *Staphylococcus xylosus* and *Staphylococcus carnosus* (Danisco, Cultor, Madrid, Spain) was added. The mixture was stuffed into 95 mm diameter collagen casings (Fibran, S.A., Girona, Spain). After ripening for 62 d, dry sausages were vacuum packaged and stored at 18–20 °C. For each of three replications, two sausages per batch were randomly taken after 1, 2 and 3 months of storage. In each sausage, colour was measured and a portion of 100 g was minced and used for moisture, water activity ($a_w$) and pH analyses. The remaining minced sausage was vacuum packed and frozen at −20 °C for physicochemical analyses (TBARS, lipid, protein and residual nitrite and nitrate). A slice of approximately 25 g was taken for microbial analyses. Several slices were wrapped in aluminium foil, vacuum packed and stored at −80 °C for volatile analysis.

### 2.2. Physicochemical analysis

pH was measured with a pH meter HI 99163 (Hanna Instruments Inc.) with an electrode including built-in temperature sensor and calibration was performed automatically at two points (4 and 7) using standard buffers. Water activity was measured with a Fast-lab water activity meter (Gbx, Romans sur Isère Cédex, France). Colour (CIE L*a*b* system) was analysed with a portable colorimeter (CR-400/410, Konica Minolta Sensing Inc., Japan) with a fixed aperture (8 mm diameter diaphragm with optical glass) and measurements were made with a D65 iluminant and 0° viewing angle. Three colour measurements were made on each sausage. Moisture was determined by the dehydration method until constant weight (BOE, 1979).

Lipid content was determined by organic extraction (Folch, Lees, & Stanley, 1957), lipid oxidation was evaluated using the thiobarbituric acid reactive substances test (TBARS) and protein content was determined by the Kjeldahl method as described in Olivares, Navarro, Salvador, and Flores (2010). Residual nitrate and nitrite contents were extracted with hot water (Mohamed, Mubarak, Fawy, & El-Shahat, 2008) and determined using an enzymatic kit (Boehringer) (Arneth & Herold, 1988).

### 2.3. Microbiological analysis

Microbial counts were done on 25 g of dry fermented sausage. Samples were finely sliced, blended with 225 ml of buffered peptone water (Pronadisa, Spain) and homogenized in a Pulsifier (Microgen Biotech, Spain). Homogenates were used to prepare decimal dilutions which were spread on appropriate media plates. Microbial counts were determined on the following media: bacterial starter containing lactic acid bacteria (LAB) on MRS Agar (Scharlau, Spain) at 30 °C for 3 days and Gram positive cocci on Mannitol Salt Agar (MSA) (CN-M) (Scharlau, Spain), at 30 °C for 3 to 5 days and Baird Parker Agar (BP) (CN-BP) (Pronadisa, Spain) at 37 °C for 48 h. Gram positive cocci isolates from BP were tested for coagulase activity (EN ISO 6888-1) using lyophilised rabbit plasma (Scharlau, Spain). Mesophilic bacteria (TMB) were determined on Plate Count Agar (Pronadisa, Spain) at 30 °C for 3 days, yeasts and moulds on Rose Bengal Agar with chloramphenicol (Scharlau, Spain) at 30 °C for 5 to 7 days. Enterobacteriaceae were counted on Violet Red Bile Agar with Glucose (VRBG) (Pronadisa, Spain) at 37 °C for 24 h in anaerobiosis. Sulphite reducing clostridia were determined from 1 ml homogenate sample inoculated in freshly prepared Lactose Sulphite Broth supplemented with sodium metabisulfite and ferric ammonium citrate (Pronadisa, Spain) dispensed into tubes with Durham gas collecting tubes and incubated in anaerobiosis (bioMerieux, Spain) at 46 °C for 48 h. Twenty-five ml of homogenated sample were used for enrichment of *Yersinia enterocolytica* in Sorbitol Peptone Broth and Bile Salts (PBS) (Pronadisa, Spain) at 25 °C for 5 days and subsequently plated on Yersinia Selective Agar (YSA) (Pronadisa, Spain) at 30 °C for 48 h. Twenty-five ml of homogenated sample were used for enrichment of *Listeria* spp. in ½ Fraser and Fraser Broth supplemented with ferric ammonium citrate (Pronadisa, Spain) at 30 °C for 24 and 48 h, respectively. Dilutions of the *Listeria* enriched Fraser medium were inoculated onto Listeria Chromogenic Agar (Pronadisa, Spain) and incubated at 37 °C for 24 h.

The remaining homogenate was incubated at 37 °C during 16–20 h for Salmonella pre-enrichment. One millilitre of the incubated homogenate was used for enrichment of *Salmonella* in Rappaport Soy Broth (Pronadisa, Spain) at 41.5 °C for 24 h and Muller Kauffmann Broth Base w/Brilliant Green and Novobiocin supplemented with iodine and potassium iodide solution (MKTTN) (Pronadisa, Spain) at 37 °C for 24 h. Enriched cultures were plated on Xylose Lysine Desoxycholate Agar (XLD) (Pronadisa, Spain) and incubated at 37 °C for 24 h.

### 2.4. Volatile compound analysis

The analysis of headspace (HS) volatile compounds was carried out by solid phase micro extraction (SPME) with an 85 μm Carboxen/Polydimethylsiloxane (CAR/PDMS) fibre (Supelco, Bellefonte, PA) using a gas chromatograph Agilent 7890 series II with a mass spectrometer detector MS 5975C, (Agilent, Palo Alto, CA) equipped with an autosampler (Gerstel MPS2 multipurpose sampler, Gerstel, Mülheim an der Ruhr, Germany), as described Corral, Salvador, Belloch, and Flores (2015) with minor modifications. Sausage sample (5 g) was weighed into a 20 ml headspace vial and 0.75 mg BHT was added. The vial was incubated at 37 °C for 30 min and volatile compounds were extracted by exposing the fibre to the headspace for 120 min at 37 °C. The fibre was desorbed in the injection port of the GC–MS for 5 min at 240 °C in splitless mode. The compounds were identified by comparison with mass spectra from the library database (Nist'05), by comparison to linear retention indices (Van Den Dool & Kratz, 1963) and using authentic standards. Quantification was based on the total extracted area (TIC). The results were expressed as abundance units (AU × $10^6$).

### 2.5. Statistical analysis

Data were analysed using the Generalized Linear Model (GLM) procedure of statistical software (XLSTAT 2011, v5.01, Addinsoft, Barcelona, Spain). The data was analysed using the linear mixed model and included nitrate reduction and storage time as fixed effects, and replicates as random effect. The interaction between fixed effects was tested, and it was not significant and was excluded from the model. The replication was not significant ($P > .10$) for any of the traits. When significant effect of the treatment group was detected ($P < .05$), least squares means (LSM) were compared using Tukey test. Principal component analysis (PCA) was done to evaluate the relationships among sausage formulation (nitrate reduction), storage time and different parameters (pH, water activity, TBARS, protein and fat content, nitrate residual, colour, microbiota and volatile compounds).

## 3. Results

The results of the statistical analysis on physicochemical, microbiology and volatile compounds are shown in Tables 1 to 3 and supplementary tables have been included reporting the results of all nitrate groups at each storage time.

### 3.1. Physicochemical analyses

Physicochemical parameters were analysed taking into account the two factors vacuum storage time and nitrate reduction (Table 1). During vacuum storage, pH and water activity values suffered a significant decrease, as well as the redness parameter (a*) which decreased significantly after 3 months of storage. In addition, lipid oxidation values (TBARS) showed a significant decrease after the second month of vacuum storage that was maintained up to the third month of storage. About residual nitrite and nitrate levels, residual nitrite was below the detection limits while residual nitrate was not affected by vacuum storage.

Variation in sausage composition (protein and fat content) among nitrate batches was attributed to variations in the trimming of the pork meat. Control sausages (C) had the highest fat content. Sausages with the smallest ingoing amount of nitrate (RN25) presented a slightly low pH value. Similarly, the redness parameter (a*) was lower in reduced sausages (RN25) than in C batch. Regarding lipid oxidation, TBARS values were lower in reduced nitrate sausages than in C sausage. Residual nitrate was lower in nitrate reduced sausages and confirmed the reduced ingoing amount used in formulation.

### 3.2. Microbiology analyses

The changes in microbiota are shown in Table 2. Total mesophilic bacteria (TMB) and lactic acid bacteria (LAB) decreased a logarithm cycle ($p < .001$) after three months, whereas Gram positive cocci (CN-M and CN-BP) decreased between 1.5 and 2 logarithm cycles. Coagulase test on CN-BP cocci isolates (about 200 isolates) classified all of them as coagulase negative suggesting that they are probably *Staphylococcus* from the bacterial starter.

In the case of Enterobacteriaceae, yeast and moulds, sulphite reducing clostridia, *Salmonella* spp. and *Yersinia enterocolytica* no counts were detected in the whole vacuum storage period. *Listeria* counts were also negative in all samples except for one positive (blue-green) colony found in a LCA replicate of a RN25 sample at one month of vacuum storage. No positive colonies were found in the equivalent sample in successive months of vacuum storage. On the other hand, nitrate reduction (Table 2) produced a general increase in microbial counts, especially in case of Gram positive cocci, and for TMB only when nitrate was 15% reduced.

### 3.3. Volatile compound analysis

Volatile compounds were analysed in the headspace of sausages by

**Table 1**
Effect of vacuum storage time and nitrate reduction on physicochemical parameters of dry fermented sausages. Values are presented as least squares means.

| | Vacuum storage time | | | | | | Nitrate reduction | | | | | | $P_t$[2] | $P_n$ | RMSE[3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 m | | 2 m | | 3 m | | C[1] | | RN15 | | RN25 | | | | |
| pH | 5.09 | a | 5.03 | b | 4.96 | c | 5.05 | a | 5.04 | a | 5.00 | b | *** | ** | 0.05 |
| Aw | 0.887 | a | 0.883 | b | 0.876 | c | 0.883 | | 0.881 | | 0.881 | | *** | ns | 0.01 |
| Moisture (%) | 40.3 | b | 42.4 | a | 42.3 | a | 40.6 | b | 41.9 | a | 42.5 | a | *** | ** | 1.38 |
| Protein (% dm) | 55.4 | ab | 56.4 | a | 54.0 | b | 51.7 | c | 55.5 | b | 58.5 | a | ** | *** | 2.00 |
| Fat (% dm) | 30.7 | b | 33.8 | a | 33.8 | a | 36.4 | a | 32.3 | b | 29.6 | c | *** | *** | 2.01 |
| L* | 48.1 | | 48.1 | | 47.8 | | 48.4 | | 48.2 | | 47.5 | | ns | ns | 1.27 |
| a* | 18.3 | ab | 18.6 | a | 17.9 | b | 18.5 | a | 18.4 | ab | 17.9 | b | * | * | 0.68 |
| b* | 6.7 | b | 7.1 | a | 7.3 | a | 7.2 | a | 7.2 | a | 6.7 | b | *** | *** | 0.36 |
| TBARS[4] | 0.99 | a | 0.75 | b | 0.74 | b | 1.02 | a | 0.86 | b | 0.62 | c | *** | *** | 0.18 |
| $NO_3$ (ppm dm) | 197.8 | | 190.2 | | 196.1 | | 235.7 | a | 185.7 | b | 162.6 | b | ns | *** | 35.0 |

[1] C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm).
[2] *Pt*: *P* value of storage time effect and *Pn*: *P* value of nitrate reduction effect. Different letters in the same row of each group indicate significant differences at *** $P < .001$, ** $P < .01$, * $P < .05$. ns: $P > .05$.
[3] RMSE: root mean square error.
[4] TBARS expressed as μg malonaldehyde/g dm.

**Table 2**
Effect of vacuum storage time and nitrate reduction on microbial counts (log cfu/g) of dry fermented sausages. Values are presented as least squares means.

| | Culture medium | Vacuum storage time | | | | | | Nitrate | | | | | | $P_t$[2] | $P_n$ | RMSE[3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 m | | 2 m | | 3 m | | C[1] | | RN15 | | RN25 | | | | |
| Total mesophilic bacteria (TMB) | PCA[4] | 7.6 | a | 7.5 | a | 6.5 | b | 7.1 | b | 7.3 | a | 7.2 | ab | *** | * | 0.2 |
| *Lactobacillus* (LAB) | MRS | 6.6 | a | 6.5 | a | 5.6 | b | 6.2 | | 6.3 | | 6.2 | | *** | ns | 0.2 |
| Gram positive cocci (CN-M) | MSA | 3.7 | a | 2.0 | b | 1.6 | b | 1.4 | b | 2.9 | a | 2.9 | a | *** | *** | 0.7 |
| Gram positive cocci (CN-BP) | BP | 4.1 | a | 3.4 | b | 2.6 | c | 3.2 | b | 3.5 | a | 3.4 | a | *** | *** | 0.2 |

[1] C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm).
[2] *Pt*: *P* value of storage time effect and *Pn*: *P* value of nitrate reduction effect. Different letters in the same row of each group indicate significant differences at *** $P < .001$, ** $P < .01$, * $P < .05$. ns: $P > .05$.
[3] RMSE: root mean square error.
[4] PCA: Plate Count Agar, MRS: Man Rogosa Sharpe agar, MSA: Mannitol Salt Agar, BP: Baird Parker Agar.

SPME-GC–MS. Fifty-three volatile compounds were identified and quantified (Table 3) using the CAR/PDMS fibre. These volatile compounds were classified by their possible origin: microbiota activity (amino acid degradation (14), carbohydrate fermentation (9), lipid β-oxidation (3) and esterase activity reactions (6)), lipid oxidation reaction (20) and unknown origin (1). Fig. 1 shows the abundance of volatile compounds groups according to storage time and nitrate reduction.

Volatile compounds derived from amino acid degradation were affected by storage time producing a decrease after 3 months of storage (Fig. 1a). This might be due to the significant decrease of benzene, 2-methyl-1-propanol, toluene, 3-methyl-1-butanol, and 2-methyl-1-butanol (Table 3). However, other compounds (2,6-dimethyl-pyrazine, methional and 3-methylbutanal) increased with vacuum storage time. In contrast, nitrate reduction did not affect the total abundance of volatiles derived from amino acid degradation, except for two compounds. An increase of 2,6-dimethyl-pyrazine and a decrease of benzene could be observed as nitrate concentration diminished.

Carbohydrate fermentation was the group who represented the highest proportion of volatile compounds throughout vacuum storage (70–75%). Among them, acetic acid and ethanol were the most abundant compounds. Volatile compounds from carbohydrate fermentation decreased significantly after 3 months of storage (Fig. 1b). Ethanol, acetic acid and 2,3-butanediol were less abundant after 3 months, while a reduction in butanoic acid was observed since the second month (Table 3). On the contrary, acetone and 2-butanone increased with vacuum storage. Regarding nitrate reduction, an increase in the compounds generated by carbohydrate fermentation was observed (Fig. 1b). Acetone, acetic acid and 2,3-butanediol were more abundant in RN25 sausages. In contrast, 2,3-butanedione and butanoic acid were more abundant in C batch.

Volatile compounds derived from esterase activity decreased after 3 months of storage (Fig. 1c). Ethyl acetate, ethyl butanoate, ethyl 2-hydroxypropanoate, ethyl-3-methylbutanoate and ethyl-2-methylbutanoate decreased at the third month of storage. However, nitrate reduction had not impact on production of these volatile compounds, except for ethyl octanoate which was less abundant in RN25 sausages (Table 3).

Regarding volatile compounds derived from lipid β-oxidation, only nitrate reduction produced a significant effect on the total abundance (Fig. 1d). The effect of vacuum storage time was only seen in few compounds such as 2-heptanone and 1-octen-3-ol which concentration increased and 2,3-pentanedione which showed the opposite effect (Table 3). Moreover, the highest reduction in nitrate (RN25) produced the decrease of 2-heptanone and 1-octen-3-ol.

In the same way, lipid oxidation volatile compounds increased with storage time (Fig. 1e). This is the case of pentane, butanal, pentanal, 1-pentanol, hexanal, 2-pentylfuran, and (E)-2-heptenal (Table 3). However, several compounds decrease after 3 months of vacuum storage (propanal, 1-propanol, 2-hexenal and nonanal). Regarding the effect of nitrate content, only the highest nitrate reduction (RN25) produced a significant reduction of the total abundance (Fig. 1e). The strongest decrease in concentration was observed in pentane, heptane, octane, hexanal, hexanoic acid and octanal.

Carbon disulphide was identified as an unknown compound which increased with storage time and nitrate reduction (Table 3).

Among the 53 volatile compounds present in the sausages, 20 of them were identified as potential aroma contributors by gas chromatography-olfactometry (Perea-Sanz, Montero, Belloch, & Flores, 2018). These compounds contribute to specific aroma notes as indicated in Table 3. In order to examine the relationship of the chemical and microbiological parameters with the aroma compounds a principal component analysis (PCA) was performed (Fig. 2). Two principal components were able to explain the 55.34% of the total variability. PC1 accounts for 32.87% of the variability and distinguishes samples by vacuum storage time as seen by the time progression from right to left quadrant. First months of storage (1 and 2 months, right upper quadrant) were related to aromatic volatile compounds derived from microorganism metabolism (LAB and CN-BP, CN-M): carbohydrate fermentation (2,3-butanedione, 3-hydroxy-2-butanone and acetic acid), esterase activity (ethyl octanoate, ethyl butanoate, ethyl-2-hydroxypropanoate and ethyl-3-methylbutanoate), one compound from lipid β-oxidation (2,3-pentanedione) and amino acid degradation (dimethyl disulphide) as well as to aroma compounds derived from lipid oxidation (1-hexanol and heptanal). In contrast, longer vacuum storage times (3 months) were related to lipid oxidation volatile compounds (2-pentylfuran, 2-methylfuran, octanal, and hexanal), lipid β-oxidation (1-octen-3-ol and 2-heptanone) and compounds from sulphur amino acid degradation (methanethiol and methional) and one from carbohydrate fermentation (2-butanone). PC2 accounts for 22.46% of the variability and distinguishes samples by nitrate content. As can be observed, C and RN15 sausages are placed on the upper quadrant, and RN25 sausages on the bottom quadrant. In addition, C and RN15 sausages appeared related to most of the aromatic volatile compounds analysed derived from microbial metabolism and lipid oxidation reactions. However, volatile compounds derived from sulphur amino acid degradation (methanethiol and methional) and 2-butanone were related to sausages with 25% nitrate reduction (RN25).

## 4. Discussion

During vacuum storage, dry fermented sausages underwent changes on physicochemical and microbiological characteristics as reported by Kim et al. (2012) and Rubio et al. (2007). The general decrease of microbial counts during vacuum storage (Table 2) might have an impact on organoleptic sausage quality. This general decline in microbial counts during vacuum storage appears to be the main consequence of low pH and $a_w$, which act as hurdles for microbial growth (Christieans et al., 2018; Leistner, 2000). The slight but continuous pH decrease observed at successive months of storage (Table 1) may be due to the

**Table 3**
Effect of vacuum storage time and nitrate reduction on volatile compounds generated (expressed as AU × $10^6$) in dry fermented sausages. Values are presented as least squares means.

| | LRI[1] | RI[2] | Aroma[6] | Vacuum Storage time | | | | | | Nitrate | | | | | | $P_t$[4] | *Pn* | RMSE[5] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 m | | 2 m | | 3 m | | C[3] | | RN15 | | RN25 | | | | |
| Amino acid degradation | | | | | | | | | | | | | | | | | | |
| 2-Methylpropanal | 594 | a | | 0.49 | | 0.48 | | 0.61 | | 0.51 | | 0.59 | | 0.50 | | ns | ns | 0.17 |
| Benzene | 676 | a | | 0.19 | a | 0.17 | a | 0.13 | b | 0.19 | ab | 0.18 | a | 0.11 | b | ** | *** | 0.04 |
| 2-Methyl-1-propanol | 683 | a | | 1.44 | a | 0.68 | b | 0.99 | b | 0.87 | | 1.25 | | 0.99 | | *** | ns | 0.48 |
| 3-Methylbutanal | 691 | a | | 2.23 | b | 3.98 | a | 4.20 | a | 3.44 | | 3.36 | | 3.77 | | *** | ns | 0.87 |
| Dimethyl disulfide | 773 | a | Toasted, garlic | 0.45 | | 0.55 | | 0.39 | | 0.41 | | 0.47 | | 0.49 | | ns | ns | 0.15 |
| Toluene | 788 | a | | 3.44 | a | 3.78 | a | 2.64 | b | 3.55 | | 3.09 | | 3.22 | | *** | ns | 0.73 |
| 3-Methyl-1-butanol | 795 | a | | 10.87 | a | 9.89 | a | 5.81 | b | 9.12 | | 8.64 | | 8.96 | | *** | ns | 2.41 |
| 2-Methyl-1-butanol | 797 | a | | 2.11 | a | 1.86 | a | 1.11 | b | 1.68 | | 1.67 | | 1.72 | | *** | ns | 0.51 |
| 2,6-Dimethylpyrazine | 945 | a | | 1.97 | b | 2.65 | a | 2.29 | b | 1.79 | c | 2.23 | b | 2.88 | a | *** | *** | 0.41 |
| Methional | 968 | a | Cooked potato | 0.65 | b | 1.31 | a | 1.17 | a | 0.88 | | 0.86 | | 1.31 | | * | ns | 0.39 |
| Benzaldehyde | 1020 | a | | 1.23 | | 1.39 | | 1.40 | | 1.24 | | 1.34 | | 1.43 | | ns | ns | 0.37 |
| Benzeneacetaldehyde | 1110 | a | | 0.81 | | 0.86 | | 0.80 | | 0.77 | | 0.87 | | 0.84 | | ns | ns | 0.17 |
| Phenol | 1114 | a | | 2.56 | | 2.45 | | 2.38 | | 2.41 | | 2.45 | | 2.53 | | ns | ns | 0.29 |
| Methanethiol | 473 | a | Rotten | 0.99 | | 1.14 | | 1.11 | | 1.01 | | 1.10 | | 1.13 | | ns | ns | 0.3 |
| Carbohydrate fermentation | | | | | | | | | | | | | | | | | | |
| Acetaldehyde | 466 | a | | 5.10 | | 5.56 | | 4.64 | | 5.36 | | 5.03 | | 4.91 | | ns | ns | 1.1 |
| Ethanol | 507 | a | | 291.12 | a | 309.72 | a | 228.76 | b | 263.73 | | 300.37 | | 265.50 | | ** | ns | 66.11 |
| Acetone | 529 | a | | 5.40 | b | 7.37 | a | 7.40 | a | 5.41 | b | 6.28 | b | 8.47 | a | ** | *** | 1.84 |
| 2,3-Butanedione | 627 | a | Cheese, butter | 1.25 | | 1.13 | | 1.01 | | 1.33 | a | 0.90 | b | 1.16 | ab | ns | * | 0.46 |
| 2-Butanone | 631 | a | Fruity, butter | 1.99 | b | 3.13 | a | 3.43 | a | 2.93 | | 2.44 | | 3.18 | | *** | ns | 0.9 |
| Acetic acid | 718 | a | Vinegar | 290.55 | a | 300.94 | a | 247.70 | b | 260.05 | b | 278.82 | ab | 300.31 | a | *** | * | 38.54 |
| 3-Hydroxy-2-butanone | 781 | a | Sweet, fruity | 21.67 | | 18.23 | | 15.52 | | 21.74 | | 15.36 | | 18.31 | | ns | ns | 8.36 |
| 2,3-Butanediol | 888 | a | | 72.17 | a | 70.39 | a | 45.03 | b | 53.98 | b | 62.97 | ab | 70.64 | a | *** | ** | 12.61 |
| Butanoic acid | 896 | a | | 8.51 | a | 5.97 | b | 3.23 | c | 7.09 | a | 5.18 | b | 5.44 | b | *** | *** | 0.68 |
| Esterase activity | | | | | | | | | | | | | | | | | | |
| Ethyl acetate | 635 | a | | 112.65 | a | 122.35 | a | 69.68 | b | 94.63 | | 109.45 | | 100.62 | | *** | ns | 28.73 |
| Ethyl butanoate | 832 | a | Fruity | 9.95 | a | 9.58 | a | 6.57 | b | 8.84 | | 9.54 | | 7.71 | | *** | ns | 2.41 |
| Ethyl-2-hydroxypropanoate | 867 | a | Fruity, sweet | 9.54 | a | 9.70 | a | 6.09 | b | 7.45 | | 9.31 | | 8.57 | | *** | ns | 2.2 |
| Ethyl-2-methylbutanoate | 878 | a | | 4.31 | a | 4.46 | a | 2.73 | b | 3.54 | | 4.09 | | 3.87 | | *** | ns | 1.16 |
| Ethyl-3-methylbutanoate | 882 | a | Fruity, sweet | 9.17 | a | 10.67 | a | 5.67 | b | 7.76 | | 9.13 | | 8.63 | | *** | ns | 3.17 |
| Ethyl octanoate | 123 | a | vegetable, fruity | 5.07 | | 5.10 | | 4.14 | | 4.60 | b | 5.93 | a | 3.78 | b | ns | *** | 1.48 |
| Lipid β-oxidation | | | | | | | | | | | | | | | | | | |
| 2,3-Pentanedione | 745 | a | Sweet, candy | 2.17 | ab | 2.47 | a | 1.66 | b | 2.19 | | 2.19 | | 1.92 | | ** | ns | 0.65 |
| 2-Heptanone | 935 | a | Rancid, fruity | 1.59 | b | 1.99 | a | 2.09 | a | 1.97 | a | 2.01 | a | 1.70 | b | *** | ** | 0.31 |
| 1-Octen-3-ol | 1033 | a | Mushroom | 2.31 | b | 2.77 | a | 2.75 | ab | 2.75 | a | 2.92 | a | 2.16 | b | * | *** | 0.55 |
| Lipid oxidation | | | | | | | | | | | | | | | | | | |
| Pentane | 500 | a | | 3.40 | b | 5.28 | a | 4.49 | ab | 4.63 | a | 5.40 | a | 3.14 | b | ** | *** | 1.43 |
| Propanal | 524 | a | | 0.73 | ab | 0.91 | a | 0.66 | b | 0.76 | ab | 0.93 | a | 0.61 | b | * | ** | 0.22 |
| Hexane | 600 | a | | 1.77 | | 1.85 | | 1.61 | | 1.86 | b | 2.30 | a | 1.07 | c | ns | *** | 0.5 |
| 1-Propanol | 612 | a | | 2.32 | a | 1.37 | b | 1.02 | b | 1.09 | b | 2.36 | a | 1.26 | b | * | ** | 0.8 |
| 2-Methylfuran | 616 | a | Green, garlic | 0.14 | | 0.20 | | 0.17 | | 0.20 | | 0.17 | | 0.15 | | ns | ns | 0.06 |
| Butanal | 622 | a | | 0.06 | b | 0.13 | a | 0.15 | a | 0.14 | a | 0.11 | ab | 0.08 | b | *** | *** | 0.04 |
| Heptane | 700 | a | | 15.36 | | 16.81 | | 17.45 | | 19.65 | a | 20.18 | a | 9.77 | b | ns | *** | 4.67 |
| Pentanal | 739 | a | | 2.62 | c | 4.43 | b | 5.68 | a | 4.74 | a | 4.91 | a | 3.07 | b | *** | *** | 0.92 |
| Octane | 800 | a | | 22.36 | | 21.94 | | 24.11 | | 26.15 | a | 28.54 | a | 13.71 | b | ns | *** | 6.95 |
| 1-Pentanol | 827 | a | | 2.20 | b | 3.20 | a | 2.97 | a | 3.07 | a | 3.38 | a | 1.92 | b | ** | *** | 0.87 |
| Hexanal | 842 | a | Fresh cut grass | 25.90 | b | 33.44 | ab | 40.19 | a | 35.03 | a | 40.30 | a | 24.20 | b | *** | *** | 9.41 |
| 2-Hexenal | 907 | a | | 0.24 | a | 0.21 | a | 0.14 | b | 0.18 | | 0.22 | | 0.20 | | *** | ns | 0.05 |
| 1-Hexanol | 924 | a | Oxidized fat | 6.70 | | 7.11 | | 5.35 | | 6.17 | | 7.34 | | 5.65 | | ns | ns | 2.08 |
| Heptanal | 941 | a | Green | 7.61 | | 7.92 | | 7.28 | | 5.69 | b | 8.21 | a | 8.92 | a | ns | *** | 2.15 |
| Decane | 1000 | a | | 0.40 | | 0.43 | | 0.42 | | 0.39 | | 0.45 | | 0.42 | | ns | ns | 0.09 |
| 2-Pentylfuran | 1010 | a | Garlic, onion | 1.42 | b | 1.65 | ab | 1.75 | a | 1.60 | ab | 1.82 | a | 1.40 | b | * | *** | 0.32 |
| (E)-2-Heptenal | 1013 | a | | 0.15 | b | 0.24 | a | 0.20 | ab | 0.19 | | 0.20 | | 0.19 | | ** | ns | 0.07 |
| Octanal | 1049 | a | Orange, sweet | 3.30 | | 3.25 | | 3.42 | | 3.23 | ab | 3.77 | a | 2.98 | b | ns | * | 0.81 |
| Hexanoic acid | 1079 | a | | 3.46 | | 3.59 | | 3.67 | | 3.76 | a | 4.10 | a | 2.85 | b | ns | *** | 0.83 |
| Nonanal | 1151 | a | | 5.70 | a | 5.34 | ab | 4.83 | b | 4.59 | b | 5.70 | a | 5.58 | b | * | *** | 0.92 |
| Unknown compound | | | | | | | | | | | | | | | | | | |
| Carbon disulfide | 537 | a | | 3.65 | b | 6.58 | a | 4.12 | b | 4.16 | b | 4.56 | b | 5.65 | a | *** | ** | 1.18 |

[1] LRI: Linear retention index of the compounds eluted from the GC–MS.
[2] RI: Reliability of identification: a, identification by mass spectrum, coincidence with the LRI of an authentic standard; b, tentatively identification by mass spectrum.
[3] C: control batch (250 ppm sodium nitrate). RN15: 15% sodium nitrate reduction (212.5 ppm). RN25: 25% sodium nitrate reduction (187.5 ppm).
[4] *Pt*: *P* value of storage time effect and *Pn*: *P* value of nitrate reduction effect. Different letters in the same row of each group indicate significant differences at *** $P < .001$, ** $P < .01$, * $P < .05$. ns: $P > .05$.
[5] RMSE: root mean square error.
[6] Compounds detected as aroma active compound by GC-olfactometry (Perea-Sanz et al., 2018).

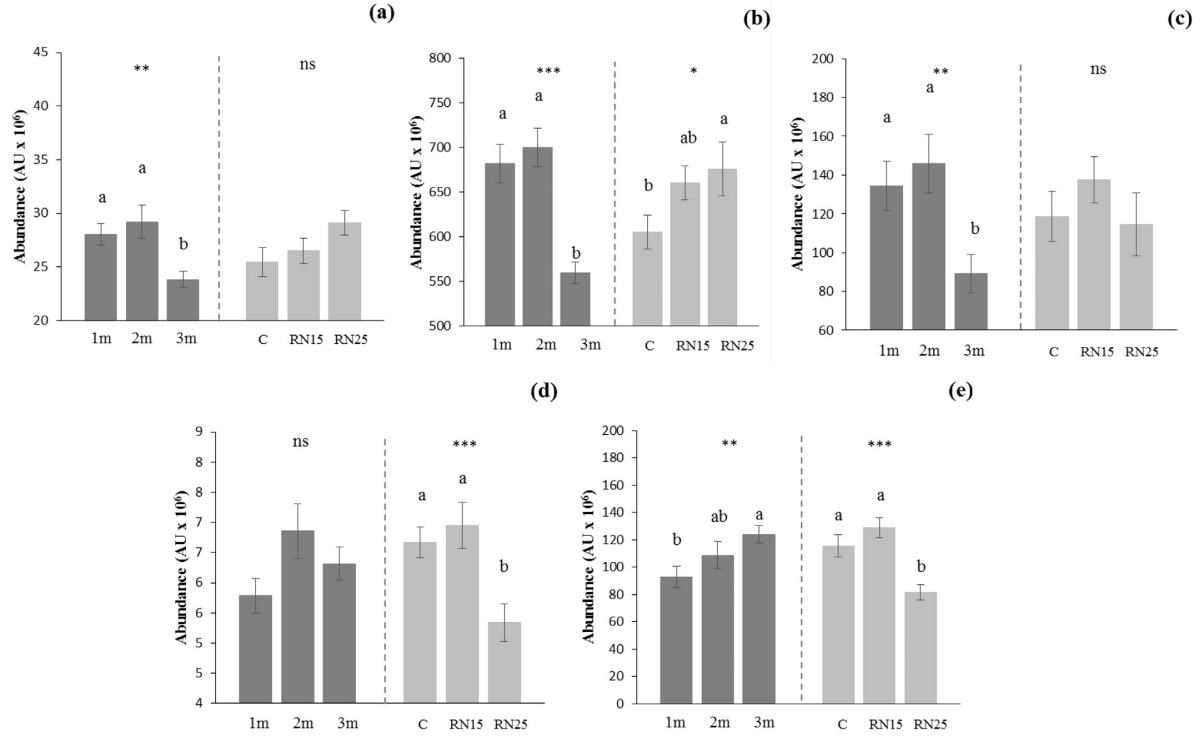


**Fig. 1.** Abundance of volatile compounds (Au × $10^6$) according to storage time (1, 2 or 3 m of vacuum storage) and nitrate reduction (C: control batch 250 ppm sodium nitrate; RN15: 15% reduction 212.5 ppm; RN25: 25% reduction 187.5 ppm). Different letters in each group indicate significant differences: *** $P < .001$, ** $P < .01$, * $P < .05$. ns: $P > .05$. Volatile compounds grouped according to origin: derived from bacterial metabolism (a: amino acid degradation; b: carbohydrate fermentation; c: esterase activity, d: lipid β-oxidation reactions) and chemical reactions (e: lipid oxidation).

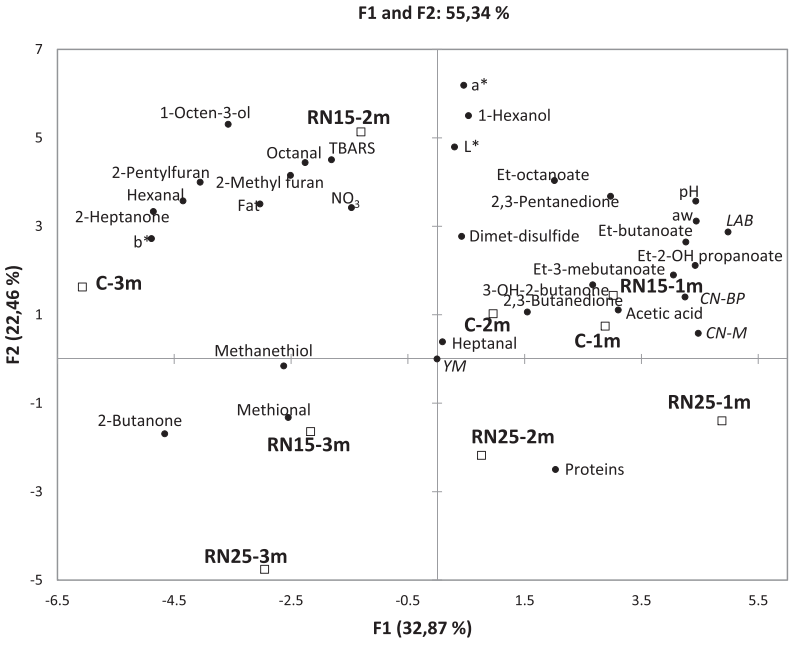


**Fig. 2.** Loadings of the first two principal components (PC1-PC2) of the analysed parameters (physicochemical and microbiological parameters and aroma volatile compounds) in dry fermented sausages based on nitrate content: C: control batch 250 ppm sodium nitrate; RN15: 15% reduction 212.5 ppm; RN25: 25% reduction 187.5 ppm, and vacuum storage (1, 2 and 3 m). Abbreviations are indicated in Tables 1 and 2.

metabolic activity of LAB, which are still active although to a lesser extent. Similar results were reported by Rubio et al. (2007) in sliced sausages under vacuum storage and modified atmospheres, despite no changes in $a_w$ were seen. In agreement with our results, Bañon et al. (2014) and Tabanelli et al. (2013) reported a general microbial growth inhibition possibly due to the $a_w$ decrease. Other authors have described few changes in microbial counts and no effect on pH values (Hospital et al., 2014; Kim et al., 2012). On the contrary, an increase in sausage pH under vacuum conditions (Ščetar et al., 2013), modified atmospheres (Ščetar et al., 2013; Tabanelli et al., 2013) and perforated packages (Bañon et al., 2014) has been demonstrated in other studies.

Regarding sausage colour, a redness decrease (Table 1) was reported in sausages storage under vacuum (Summo et al., 2006; Summo et al., 2010) and in entire sausages stored in perforated packages (Bañon et al., 2014). In fact, several authors indicated that vacuum packaged produce less redness intensity than modified atmosphere packaging (Rubio et al., 2008; Zanardi et al., 2002) or perforated packaging (Summo et al., 2006). On the contrary, other authors indicated an increase in redness (a*) under vacuum packed storage (Kim et al., 2012; Rubio et al., 2008).

Concerning lipid oxidation, different results have been reported during vacuum storage of dry fermented sausages. Rubio et al. (2008) observed a decrease on lipid oxidation value in agreement with our results (Table 1), while others did not observe changes (Summo et al., 2010). However, many studies have reported an increase in lipid oxidation values during vacuum storage (Dos Santos et al., 2017; Kim et al., 2012; Summo et al., 2006, 2010; Ščetar et al., 2013; Zanardi et al., 2002) and under modified atmosphere (Ščetar et al., 2013; Zanardi et al., 2002;). Different patterns of lipid oxidation can be explained by different ingredients, such as spices with antioxidant activity (Yashin, Yashin, Xia, & Nemzer, 2017), in addition to the manufacture process. Moreover, the low specificity of the TBARS test contributes to the observed differences since malonaldehyde is an unstable molecule and could react with other compounds present in the meat matrix (Janero, 1990).

The general decrease observed in microbial counts is in agreement with previous studies (Bañon et al., 2014; Rubio et al., 2007). Despite the decrease in LAB and Gram positive cocci inoculated with the bacterial starter, pH decreased slightly during storage suggesting the existence of bacteria metabolic activity. The low pH and $a_w$ effectively prevented growth of pathogenic bacteria as *Salmonella* spp., *Listeria* spp., Gram positive coagulase positive cocci and *Clostridium* spp. even in RN25 sausages (Bañon et al., 2014). Therefore, our results suggest that no apparent risk regarding microbial safety can be attributed to sausages stored in the conditions utilised in our study.

Microbial growth is related to volatile compounds production through their metabolism. LAB generate volatile compounds from amino acid degradation and carbohydrate fermentation reactions together with staphylococci, which also generate ethyl esters with fruity notes through their esterase activity (Flores & Olivares, 2015). During vacuum storage a general decrease of volatile compounds derived from microbial activity was observed (Fig. 1). Similar results under vacuum storage were reported by Summo et al. (2011). These authors found a decrease of volatile compounds derived from carbohydrate fermentation during its shelf-life under vacuum storage, in addition to an increase of volatile compounds derived from lipid oxidation (Fig. 1). On the contrary, Dos Santos et al. (2015) observed an increase of volatile compounds derived from amino acid degradation and carbohydrate fermentation in addition to those from lipid oxidation. Differences between studies can be due to different sausage manufacture process, use of spices and smoking process (Summo et al., 2011).

Nitrate residual content was not affected by the storage time under vacuum although the residual concentration detected declined between 44 and 51% respect to the initial amount measured in the minced meat in C, RN15 and RN25 sausages (Perea-Sanz et al., 2018). The absence of nitrate reduction during vacuum storage could be due to a low nitrate reductase activity available during storage due to the low Gram positive cocci counts (Table 2) and the pH value close to 5.0 that inhibit this activity (Sánchez Mainar & Leroy, 2015). Nevertheless, the reduction of nitrate ingoing amounts in fermented sausages produced changes in the production of volatile compounds although nitrate reduction did not affect directly microbial growth but affected microbial metabolism (Perea-Sanz et al., 2018). Nitrate reduced sausages had less nitrite available and therefore, lowest antioxidant activity, but the highest oxidation reactions were detected in control sausages due to its high fat content (Olivares et al., 2010). This fact is in accordance with volatile compounds derived from lipid oxidation and lipid β-oxidation, which were in high abundance in control sausages. Moreover, reduced nitrite antimicrobial activity in nitrate reduced sausages may be the reason for high Gram positive cocci counts (CN-M and CN-BP) as observed by Hospital et al. (2014) after thirty days of vacuum storage. The higher counts of Gram positive cocci detected in nitrate reduced sausages (RN15 and RN25) would be responsible for the high amount of volatile compounds derived from carbohydrate fermentation. Similarly, the increment in the generation of volatile compounds from amino acid degradation and ester compounds observed in nitrate reduced sausages would be the result of high counts of Staphylococci (Flores & Olivares, 2015), as LAB were insignificantly affected by nitrate reduction.

Changes in volatile compounds produced by vacuum storage of slow fermented sausages (Table 3) affected the aroma profile of the product (Fig. 2). Under vacuum storage, several authors observed a decline of the characteristic sausage aroma and quality as reported by Kim et al., (2012), Rubio et al., (2007) and Summo et al., (2006). Packaging under modified atmosphere altered the sausage volatile profile and produced a more intense “raw meat” aroma and a less distinct “dry sausage” aroma (Viallon et al., 1996). The effect on the volatile profile was related to the increase in ethanol, diacetyl, acetoin and restriction of acetic acid, 1,3-butanediol and 2,3-butanediol (Viallon et al., 1996). Moreover, packaging under vacuum storage produced a limited number of lipid oxidation compounds as reported by Viallon et al., (1996), in opposition to the results observed in Fig. 2. The present results demonstrate the relationship of microbiological and physicochemical characteristics and the effect of factors, vacuum storage and nitrate reduction, on sausage aroma. The compounds with pleasant and sweet aroma (3-hydroxy-2-butanone, ethyl octanoate, ethyl-3-methylbutanoate and 2,3-pentanedione) and with cheesy/buttery odour (2,3-butanedione and ethyl-2-hydroxypropanoate) were related to short vacuum storage times and to control and 15% reduced nitrate sausages. In contrast, the characteristic “dry sausage” aroma loss might be the result of the increase of volatile compounds such as heptanal (green, unpleasant odour) and compounds related to unpleasant odours, methanethiol (rotten odour) and methional (cooked potato) (Perea-Sanz et al., 2018). In summary, small nitrate reductions of 15% did not produce a significant effect on aroma profile in slow fermented sausages in contrast to the more negative effect produced by a reduction of 25% nitrate.

## 5. Conclusion

Vacuum storage and reduced amounts of ingoing nitrate influenced the shelf-life of slow fermented sausages in terms of microbial and organoleptic characteristics. Microbial growth was affected mainly by vacuum storage and to a lesser extent by nitrate content, leading to changes in the profile of volatile compounds. On the one hand, vacuum storage time produced a decrease in volatile compounds derived from amino acid degradation, carbohydrate fermentation and esterase activity after three months under vacuum. On the other hand, the reduction of ingoing nitrate amounts caused a decrease of volatile compounds derived from lipid oxidation and β-oxidation reactions. These changes affected the production of key aroma compounds and sausage aroma. More studies are necessary to elucidate the mechanism involved in the effect of nitrate reduction during vacuum storage in slow

fermented sausages to determine the appropriate sausage shelf-life.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meatsci.2018.08.026.

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**Debaryomyces hansenii Metabolism of Sulfur Amino Acids As Precursors of Volatile Sulfur Compounds of Interest in Meat Products**

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# *Debaryomyces hansenii* Metabolism of Sulfur Amino Acids As Precursors of Volatile Sulfur Compounds of Interest in Meat Products

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Supporting Information

**ABSTRACT:** The ability of *Debaryomyces hansenii* to produce volatile sulfur compounds from sulfur amino acids and the metabolic pathway involved have been studied in seven strains from different food origins. Our results proved that L-methionine is the main precursor for sulfur compound generation. Crucial differences in the sulfur compound profile and amino acid consumption among *D. hansenii* strains isolated from different food sources were observed. Strains isolated from dry pork sausages displayed the most complex sulfur compound profiles. Sulfur compound production, such as that of methional, could result from chemical reactions or yeast metabolism, while according to this study, thioester methyl thioacetate appeared to be generated by yeast metabolism. No relationship between sulfur compounds production by *D. hansenii* strains and the expression of genes involved in sulfur amino acid metabolism was found, except for the *ATF2* gene in the L1 strain for production of methyl thioacetate. Our results suggest a complex scenario during sulfur compound production by *D. hansenii*.

**KEYWORDS:** *Debaryomyces hansenii, L-methionine, volatile sulfur compounds, sulfur amino acids*

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## INTRODUCTION

A wide range of volatile compounds is responsible for meat product aromas, among which volatile sulfur compounds contribute to the characteristic meaty notes.[1] The main source of sulfur compounds in fermented meat is the transformation from sulfur amino acids. During meat processing for manufacturing dry fermented sausages, sulfur amino acids transformation is mainly conducted by microorganisms and, to a lesser extent, chemical reactions like Maillard and Strecker degradation.[2] L-Methionine, L-cysteine, and L-cystine are the main sulfur amino acids present in dry fermented sausages, with L-methionine being the most abundant followed by L-cysteine.[3] Microbial transformation of sulfur amino acids into volatile sulfur compounds is conducted by bacteria (lactic acid bacteria and staphylococci) and yeasts present in the meat product.[4]

The generation of volatile sulfur compounds from sulfur amino acids by yeasts metabolism has been thoroughly investigated in cheese.[5−7] The main sulfur compound precursor in yeasts seems to be L-methionine.[8−10] Methanethiol is believed to result from direct L-methionine demethiolation by $\beta/\gamma$-lyases activity (demethiolation pathway) or by a two-step transformation carried out by an aminotransferase, giving $\alpha$-keto-$\gamma$-methylthio-oxobutyric acid (KMBA), followed by a demethiolase activity in the Ehrlich pathway. Further transformation of methanethiol leads to generation of other sulfur compounds such as dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide generated by an oxidation reaction, while thioester methyl thioacetate is generated by a chemical or enzymatic reaction. KMBA can be transformed directly to methional through decarboxylation, and methional can be subsequently reduced to methionol.

Among the yeasts isolated from dry fermented sausages, *Debaryomyces hansenii* is the dominant yeast species.[11] Addition of a *D. hansenii* starter has potential functionalities in the manufacture of dry fermented sausages, contributing to the proteolytic activity[12] and producing free amino acids which act as precursors of volatile compounds.[3,13] The ability of *D. hansenii* to generate ester and sulfur compounds among other volatiles and to reduce the production of oxidation derived compounds in dry fermented sausages has been demonstrated.[14,15] Moreover, addition of *D. hansenii* improves the sensory quality of the final dry fermented sausages product.[16,17] However, the metabolic pathways involved in sulfur compounds generation in dry fermented sausages have not yet been elucidated. The aim of this study was to compare the ability of several *D. hansenii* strains to produce volatile sulfur compounds from sulfur amino acids and to obtain insight into the metabolic pathway involved in the generation of L-methionine-derived volatile compounds.

## MATERIALS AND METHODS

**Chemicals.** The following compounds were commercially purchased from Sigma-Aldrich (Missouri, USA): dimethyl sulfide, methyl thioacetate, dimethyl disulfide, ethyl thioacetate, methional, dimethyl trisulfide, methionol, L-methionine, L-cysteine, and L-cystine.

**Yeast Strains.** *D. hansenii* strains used in this study (Table 1) were isolated from different dry fermented pork and llama sausages, cheese, and vegetables.

**Growth of *D. hansenii* on Sulfur Amino-Acid-Supplemented Media.** *D. hansenii* strains were precultured during 48 h on a GPY

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**Table 1. *D. hansenii* Strains Used in This Study**

| yeast strain | source | ref |
|---|---|---|
| L1 | pork dry fermented sausage | Bolumar et al.[18] |
| L5 | pork dry fermented sausage | Cano-García et al.[14] |
| L12 | llama dry fermented sausage | Mendoza et al.[19] |
| L21 | llama dry fermented sausage | Mendoza et al.[19] |
| L25 | llama dry fermented sausage | Mendoza et al.[19] |
| L66 | ewe's cheese | Padilla et al.[20] |
| L74 | lupine | Flores et al.[13] |

liquid medium (2% glucose, 0.5% peptone, and 0.5% yeast extract) at 25 °C. After growth, each culture was centrifuged and washed three times with saline solution (0.9% NaCl). Cell suspensions were adjusted for inoculation in culture media at a final concentration of $10^6$ cells/mL. A control medium (C) composition was 0.5% yeast extract (amino acids content in Table S1), 10 g/L glucose, 30 g/L NaCl, 127.5 mg/L $NaNO_2$, and 127.5 mg/L $NaNO_3$. Composition of the sulfur amino-acids-supplemented media were the same as the control medium, except for the addition of 100 mg/L L-methionine (M), 250 mg/L L-cysteine (Cy), and 50 mg/L L-cystine (Ci), respectively. The media were adjusted to pH 6.5 and sterilized using a vacuum-driven filtration system of 0.22 μm. Experiments were conducted in 100 mL flasks containing 50 mL of C, M, Cy, and Ci media. Seven flasks were inoculated with each *D. hansenii* strain while a noninoculated (NI) flask of each medium was the control. Each experiment was conducted in triplicate (96 experiments in total). Flasks were incubated at 25 °C for 15 days for sulfur compounds analysis. After incubation, optical density (OD) was measured at 600 nm in a BioPhotometer (Eppendorf, Germany). The supernatant was recovered by centrifugation at 4000 rpm for 5 min at 20 °C, cell-free filtered (0.22 μm), and frozen at −20 °C until the volatiles and amino acid analyses. Additional experiments in media C and M inoculated with yeasts L1 and L74 were carried out in triplicate (12 in total) for studying gene expression. After 2 days of incubation, cells were collected by centrifugation at 4000 rpm for 5 min at 20 °C. The resulting yeast pellet was resuspended in sterile Milli-Q water and frozen at −80 °C until RNA isolation.

**Volatile Sulfur Compounds Analysis.** The sulfur compounds analyzed (Figure 1) were methanethiol (**1**), dimethyl sulfide (**2**), dimethyl disulfide (**3**), dimethyl trisulfide (**4**), methional (**5**), methionol (**6**), methyl thioacetate (**7**), and ethyl thioacetate (**8**). Analysis was carried out by headspace (HS) solid-phase microextraction (SPME) with an 85 μm carboxen/polydimethylsiloxane (CAR/PDMS) fiber (Supelco, Bellefonte, PA) using a gas chromatograph (Agilent HP 7890 series II (Hewlett-Packard, Palo Alto, CA)) with a quadrupole mass detector (HP 5975C (Hewlett-Packard)) and equipped with an autosampler (MPS2 multipurpose sampler (Gerstel, Germany)). Supernatants (7 mL) were placed into 20 mL headspace vials containing 2.37 g of NaCl to produce a salting out effect. To prevent oxidation, the vials were purged with nitrogen gas for 5 s before sealing. The internal standard 2-methyl-3-heptanone (256 ng) was added, and the vials were incubated at 37 °C for 30 min at 250 rpm for equilibration. Then, the fiber was exposed to the headspace for 30 min at 250 rpm while maintaining the sample at 37 °C, after it was desorbed in the injection port of the GC-MS for 5 min at 240 °C in splitless mode. The volatile compounds were separated using a DB-624 capillary column (30 m, 0.25 mm i.d., film thickness 1.4 μm, (J&W Scientific, Agilent Technologies, USA)). Helium was used as a carrier gas with a linear velocity of 34.3 cm/s. The GC oven temperature was held at 38 °C for 13 min, ramped to 100 °C at 3 °C/min, held at 100 °C for 5 min, ramped to 150 °C at 4 °C/min and to 210 °C at 10 °C/min, and held at 210 °C for 5 min. The MS interface temperature was set to 240 °C. Sulfur compounds were identified in full scan mode and by their retention time according to authentic standards. The identified volatile compounds were quantified in SIM mode using specific $m/z$ ions: 48 for (**1**), 62 for (**2**), 94 for (**3**), 126 for (**4**), 104 for (**5**), 106 for (**6**), 90 for (**7**), and 104 for (**8**). Calibration curves for each sulfur compound (except for methanethiol (**1**), relative to the internal standard, were obtained using the same SPME conditions in water. The peak areas of the compounds were compared to their respective standard and expressed as nanograms per milliliters of supernatant. Sulfur compounds were counted based on the normalized area and using the response factors shown in Table 2. The calibration curve for methanethiol (**1**) was not obtained, and it was expressed as nanogram equivalents of dimethyl disulfide (**3**) per milliliters of supernatant. Experimental triplicates were analyzed in duplicate.

**Table 2. Response Factors for the Analysis of Sulfur Compounds in Selected-Ion-Monitoring (SIM) Mode and using 2-Methyl-3-heptanone as the Internal Standard**

| compound | monitored ion ($m/z$) used for quantitation | response factor |
|---|---|---|
| dimethyl sulfide | 62 | 0.0941 |
| methyl thioacetate | 90 | 0.0589 |
| dimethyl disulfide | 94 | 1.2547 |
| ethyl thioacetate | 104 | 0.0723 |
| methional | 104 | 0.0370 |
| dimethyl trisulfide | 126 | 1.0625 |
| methionol | 106 | 0.0603 |

**Amino Acid Analysis.** The analysis of free amino acids was done using the EZ-Faast kit bought from Phenomenex (Torrance, CA, USA). Media supernatants were diluted 1:5 (v/v) with distilled water and analyzed using the kit. The derived amino acids were analyzed using GC-FID. A gas chromatograph (Agilent Technologies 7890B) with a flame ionization detector (FID) equipped with an autosampler G4513A and a ZB-AAA 10 m × 0.25 mm GC column (Phenomenex) was used. The injection volume was 2.5 μL at 250 °C in split mode (15:1). Helium was used as a carrier gas at a constant flow of 27 mL/min during the run, and the column head pressure was 8.78 psi. The GC oven temperature was initially held at 110 °C and then raised to 320 °C at 32 °C/min; the inlet temperature was 250 °C, and the detector was set at 320 °C. Identification and quantitation was based on retention times and peak area integration of the reference amino acids (Phenomenex). Norleucine was the internal standard. Calibration curves for each amino acid were obtained with the standard amino acids solutions (Phenomenex). Results were expressed in milligrams per 100 mL of supernatant. Consumption of

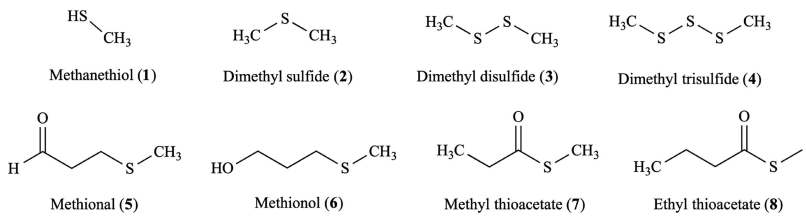


**Figure 1.** Chemical structures of the volatile sulfur compounds studied.



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**Table 3. Primers Used in RT-qPCR**

| *D. hansenii* open reading frame | *D. hansenii* gene name | enzyme | direction | sequence |
|---|---|---|---|---|
| DEHA2D05412g | *DhACT1* | actine | F | GGTAACATTGTTATGTCTGGTG |
| | | | R | TACTTTCTTTCTGGAGGAGC |
| DEHA2C05236g | *DhAAT2* | cytosolic aspartate aminotransferase | F | AACACCGTCAGAACCAAG |
| | | | R | CAATTCAATAACTTGTTCAGC |
| DEHA2A06886g | *DhARO8* | methionine aminotransferase | F | CAAGGTTGTTTGATGATCYCC |
| | | | R | AACGGCAGCATATGTACCTC |
| DEHA2C09724g | *DhATF1* | alcohol acetyltransferase | F | CTGGTGCAGCATTAGGAC |
| | | | R | AAATGGCTTYAATCTGTCTC |
| DEHA2D14762g | *DhATF2* | alcohol acetyltransferase | F | GCCTCAACTTGCTGRC |
| | | | R | GTTCCAAGAGTTTTGTAGTAAAC |
| DEHA2D06952g | *DhBAT2* | cytosolic branched-chain amino acid transferase | F | TTTAGAAGGTGTCACCAGAG |
| | | | R | CYCTTTCTTCAATTTCGTGG |
| DEHA2A04818g | *DhBNA3* | arylformamidase | F | CCYTATACATCTGCTCAAGG |
| | | | R | TCCGATTTCYTTTATCAACCAG |
| DEHA2C15686g | *DhCYS3* | cystathionine $\gamma$-lyase | F | CACGGTGGTATTCCAAAAG |
| | | | R | AAGCTTGTCTGACATCTTCG |
| DEHA2E21604g | *DhILV6* | acetolactate synthase | F | GTTGAYATTGCTGATAGAAACG |
| | | | R | CTTGGTAAGCCATCATACC |
| DEHA2C09152g | *DhPDB1* | E1 beta subunit of pyruvate dehydrogenase complex | F | ACGGTGTTAAGGCTGAAGTTAT |
| | | | R | ATGATTTGGGCACAGATTTC |
| DEHA2B03872g | *DhPDC1* | pyruvate decarboxylase | F | GGTACATCAGCATTTGGRATTG |
| | | | R | CCATKACTGCTCCTAATGTAG |
| DEHA2G18348g | *DhPDC6* | pyruvate decarboxylase | F | GATTAATTCATGGTGAAAATGCC |
| | | | R | AGCATCGTAATCCTCAGCAC |
| DEHA2A06798g | *DhSTR3* | cystathionine $\beta$-lyase | F | TATCTTTGGAATTGCCGTTTC |
| | | | R | TTCTTCTCTGGTCTTGGCATC |
| DEHA2G21032g | *DhADH1* | alcohol dehydrogenase | F | GGGCACATGGAGTWATTAATG |
| | | | R | CACKACAGTACCACGAGATC |

amino acids was analyzed for each yeast strain in each medium, subtracting the concentration measured in the respective NI medium, at the end of incubation time. The method used did not allow for detection of L-cysteine in liquid media.

**RNA Extraction and cDNA Copy.** About $10^8$ yeast cells were used for RNA isolation. Total RNA was extracted as described by Sanvisens et al.[21] with modifications. Cell pellets were suspended in 400 $\mu$L of LETS buffer (2 M LiCl, 1 M EDTA, 1 M Tris-HCl pH 7.4, SDS 10%) and 400 $\mu$L of phenol, chloroform, and isoamyl alcohol (120:24:1); glass beads were added, and cells were lysed using a Millmix 20 Bead Beater (Tehtnica, Slovenia). The supernatant was collected by centrifugation, and RNA was precipitated in two steps with 5 M LiCl and 96% ethanol at −20 °C overnight and with 3 M sodium acetate and 96% ethanol at −80 °C for 2−3 h. RNA was isolated by centrifugation and washed with 70% ethanol. Isolated RNA was dried and suspended in 200 $\mu$L of RNase-free Milli-Q water (VWR, USA). RNA concentration and purity were determined using a NanoDrop (Thermo Scientific, USA) instrument, and DNA was removed using the DNA-free Kit (DNase Treatment and Removal, Invitrogen, USA). Reverse transcription of cDNA was made with Reverse Transcriptase SuperScript III (Invitrogen) with the Oligo-$(dT)_{12-18}$ primer, using a Protector RNase inhibitor kit (Roche, Switzerland) according to the manufacturer's recommendations. DNA-free RNA and cDNA concentration and purity were determined using a NanoDrop (Thermo Scientific) instrument.

**Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) Experiments.** Expression of *S. cerevisiae* orthologous genes in L1 and L74 *D. hansenii* strains *ARO8*, *CYS3*, *AAT2*, *BNA3*, *ADH1*, *ILV6*, *ATF1*, *ATF2*, *PDC1*, *PDC6*, *STR3*, *PDB1*, and *BAT2* related to sulfur compounds generation[7,22] was studied. Primers, listed in Table 3, were designed using the OligoAnalyzer tool on the IDT webpage (https://eu.idtdna.com/pages/tools/oligoanalyzer). We searched close to the 3′ end of the orthologous gene sequences for conserved regions using available *D. hansenii* genome sequences, i.e., NRRLY-7426T (syn. CBS767), MTCC234, and J6. Our PCR and Sanger-sequencing of genes *PDB1* and *STR3* helped redesign the pairs of primers which failed in our first approach.

The RT-qPCR was performed using a LightCycler (Roche) and LightCycler 480 SYBR Green I Master Kit (Roche), according to the manufacturer's recommendations. The *ACT1* gene was used as the reference gene. The quantity of cDNA for each gene was normalized to the quantity of the *ACT1* cDNA in each sample. The E-method (LightCycler 480, Roche) was used for accurate relative quantitation data analysis. The amplification efficiency of the reference and target genes was calculated using LinRegPCR 2017.[23] The relative change in the expression of each gene was described as the ratio of normalized quantity of cDNA for each gene studied under different conditions low L-methionine content in control medium (C) and high L-methionine content in (M) medium. A gene was considered overexpressed when the ratio of its transcriptomic response in treatment M and C conditions (M/C) was >2.

**Statistical Analysis.** Data were analyzed using the generalized linear model (GLM) procedure in XLSTAT 2018.4 (Addinsoft, Paris, France). Sulfur compounds and amino acids consumption data were analyzed using the linear mixed model including the yeast strain as the fixed effect and replicates as the random effect. Differences in gene expression between L1 and L74 strains growing on C and M media were analyzed using the same model including replicates as the random effect. In case an effect because of yeast inoculation or media was detected ($P < 0.05$), least-squares mean (LSM) were compared using the Tukey test. Heatmaps of amino acid consumption in each medium were calculated, relative to the concentration in the control medium without inoculation (XLSTAT).



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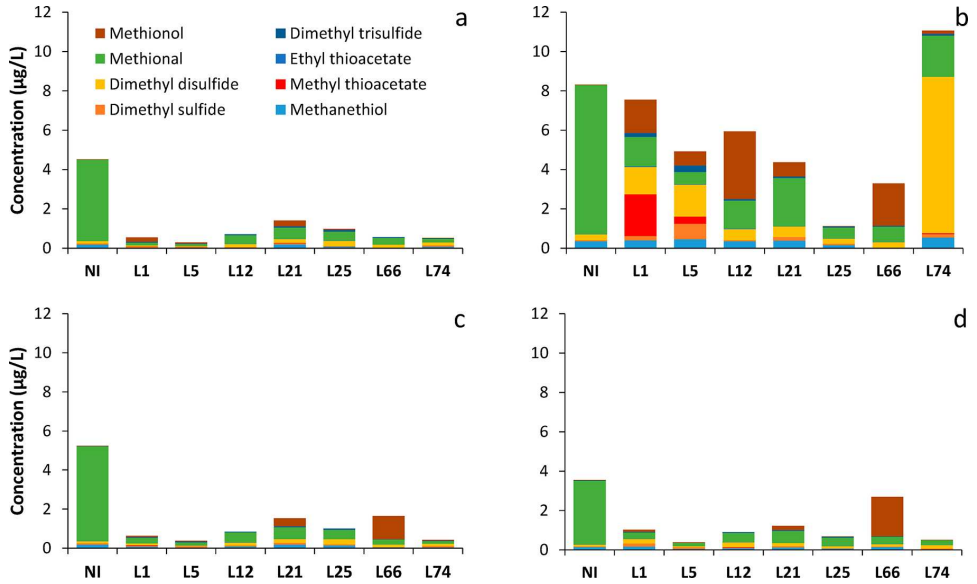


**Figure 2.** Volatile sulfur compounds generated in sulfur amino-acids-supplemented media noninoculated (NI) and inoculated with *D. hansenii* (Table 1) in different media: (a) control (C); (b) L-methionine-supplemented medium (M); (c) L-cysteine-supplemented medium (Cy); and (d) L-cystine-supplemented medium (Ci).

**Table 4. Concentration of Sulfur Compounds (μg/L) in L-Methionine Medium (M) after 15 d Incubation with *D. hansenii* Strains**

| | NI[a] | L1 | L5 | L12 | L21 | L25 | L66 | L74 | RMSE[b] | P[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| methanethiol | 0.349 ab | 0.402 ab | 0.453 a | 0.346 ab | 0.382 ab | 0.161 bc | 0.005 c | 0.546 a | 0.078 | *** |
| dimethyl sulfide | 0.069 b | 0.216 b | 0.792 a | 0.072 b | 0.180 b | 0.074 bc | 0.034 b | 0.175 b | 0.066 | *** |
| methyl thioacetate | n.d.[5] | 2.116 a | 0.366 b | n.d. | n.d. | n.d. | n.d. | 0.051 b | 0.309 | * |
| dimethyl disulfide | 0.284 bc | 1.391 b | 1.621 b | 0.556 bc | 0.539 bc | 0.253 c | 0.268 c | 7.935 a | 0.370 | *** |
| ethyl thioacetate | 0.009 b | 0.029 a | 0.026 a | 0.027 a | 0.025 ab | 0.007 b | 0.006 b | 0.012 b | 0.002 | *** |
| methional | 7.585 a | 1.505 bc | 0.620 c | 1.428 bc | 2.438 b | 0.550 c | 0.781 bc | 2.079 bc | 0.617 | *** |
| dimethyl trisulfide | n.d. | 0.204 ab | 0.330 a | 0.072 c | 0.090 c | 0.059 c | 0.051 c | 0.094 bc | 0.025 | ** |
| methionol | 0.004 b | 1.680 ab | 0.711 ab | 3.447 a | 0.707 ab | 0.004 b | 2.149 ab | 0.153 b | 0.849 | * |
| total | 8.300 | 7.543 | 4.919 | 5.948 | 4.361 | 1.108 | 3.294 | 11.045 | | |

[a]NI: medium noninoculated. [b]RMSE: root-mean-square error. [c]P: P value of inoculation effect of different yeasts (Table 1). Different letters in the same row indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. ns: $p > 0.05$. [d]n.d.: not detected.

## RESULTS

**Volatile Sulfur Compounds Analysis.** The sulfur compounds methanethiol (**1**), dimethyl sulfide (**2**), dimethyl disulfide (**3**), dimethyl trisulfide (**4**), methional (**5**), methionol (**6**), methyl thioacetate (**7**), and ethyl thioacetate (**8**) were identified and quantified in all experiments. The highest generation of sulfur compounds was seen on the M medium (Figure 2b). Comparisons between culture media show that the concentration of volatile sulfur compounds in the sulfur amino-acids-supplemented media inoculated with yeasts (Figure 2b–d) was higher than in their respective C flasks (Figure 2a). The amount of sulfur compounds produced in sulfur amino acids rich media (Ci, Cy, and M) inoculated with yeasts were similar except for medium M. Regarding the sulfur compounds profile, dimethyl trisulfide (**4**), methionol (**6**), and methyl thioacetate (**7**) were not produced in media Ci or Cy (Tables 4 and S2–S4). Generation of sulfur compounds occurred in NI flasks of all media. Methional (**5**) was exceptionally abundant in all NI flasks while methyl thioacetate (**7**) was absent in NI flasks of sulfur amino-acids-supplemented media. The compound dimethyl trisulfide (**4**) appeared only in NI flasks from Ci and Cy media. Significant differences were observed between *D. hansenii* strains regarding sulfur compounds generation on all media (Tables 4 and S2–S4). However, differences between replicates were not significant ($P > 0.10$). In medium M (Table 4), strain L74 was the highest producer of sulfur compounds in contrast to strain L25, which was the lowest. The most abundant sulfur compounds in medium M were methyl thioacetate (**7**) by strain L1, methionol (**6**) by strain L12, dimethyl disulfide (**3**) by strain L74, and methional (**5**) by strain L21. It is worth noting that methyl thioacetate (**7**) was only produced by L1, L5, and L74. The volatile profile between strains was also different. Strains isolated from pork sausages, L1 and L5, produced the same sulfur compounds but in different amounts. Between strains isolated from llama sausages we found larger differences in the production of methionol (**6**) and methional (**5**) in strains L12



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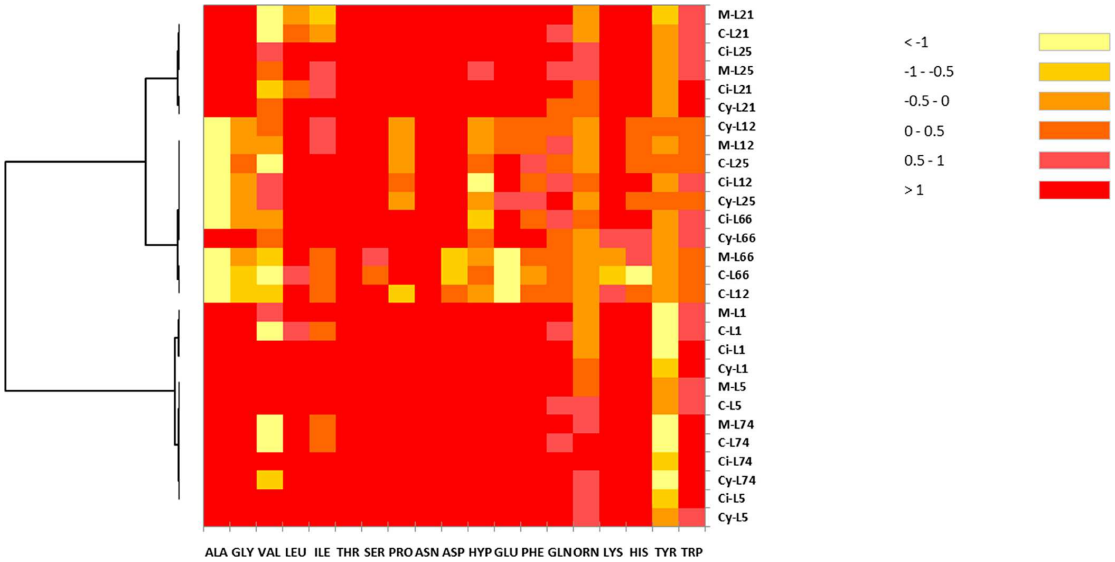


**Figure 3.** Heatmap showing the consumption of non sulfur amino acids by the metabolic activity of *D. hansenii* strains (Table 1) in all media. C (control medium), M (L-methionine-supplemented medium), Cy (L-cysteine-supplemented medium), and Ci (L-cystine-supplemented medium). Red color indicates relatively high amino acid consumption (>1), and the yellow color indicates the low amino acid consumption (>−1); the orange color indicates no difference.

**Table 5. L-Methionine and L-Cystine Consumption (mg/100 mL) Conducted by the *D. hansenii* Yeast Strains in Each Medium**

| medium | L1 | L5 | L12 | L21 | L25 | L66 | L74 | RMSE[a] | P[b] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | L-Methionine Consumption | | | | | |
| C[c] | 3.27 a | 3.11 a | 1.14 bc | 1.57 b | 1.13 bc | 0.84 c | 3.27 a | 0.24 | *** |
| M | 9.24 a | 6.04 ab | 1.13 c | 3.55 bc | 3.24 bc | 0.82 c | 5.5 b | 1.81 | *** |
| Cy | 2.99 a | 2.86 a | 0.92 b | 1.4 b | 0.92 b | 1.12 b | 2.66 a | 0.39 | *** |
| Ci | 3.4 a | 3.23 a | 1.08 bc | 1.49 bc | 2 b | 0.96 c | 3.18 a | 0.54 | *** |
| | | | | *L-Cystine Consumption* | | | | | |
| Cy | 2.81 bc | 2.05 c | −0.28 d | 3.89 ab | −0.7 d | −1.4 d | 4.83 a | 0.87 | *** |
| Ci | 4.93 ab | 3.4 bc | 1.22 c | 6.53 a | 3.44 bc | 0.93 c | 6.53 a | 1.7 | ** |

[a]RMSE: root-mean-square error. [b]*P*: *P* value of inoculation effect of different yeasts (see Table 1). Different letters in each row indicate significant differences at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. ns: $p > 0.05$. [c]Control medium (C), supplemented medium with 100 mg/L L-methionine (M), 250 mg/L L-cysteine (Cy), and 50 mg/L L-cystine (Ci).

and L21. Strain L25 differed from the other strains from llama sausage, regarding its sulfur compound profile. Cheese strain L66 showed a sulfur compound profile like the strains isolated from meat. The most different strain regarding sulfur compounds profile was L74, isolated from lupine. For media Cy and Ci, inoculation of strain L66 had a remarkable effect on the generation of methionol (**6**) which was not produced in C (Tables S3 and S4).

**Amino Acid Analysis.** Amino acids asparagine (ASN), glutamine (GLN), and ornithine (ORN) were chemically produced after 15 days incubation in NI media (Tables S5–S8). Data on consumption of amino acids by each yeast strain was represented in a heatmap with hierarchical clustering (Figure 3) and in Table 5 for sulfur amino acids. Concentration of the remaining amino acids in C, M, Cy, and Ci media are shown in Tables S5–S8. In the heatmap, consumption of each amino acid is relative to the total consumption of amino acids. The highest consumption is given a red color, while the lowest consumption is given a yellow color. Hierarchical clustering is done on the rows of the data matrix and is represented in the left part of the figure. Clustering in Figure 3 divided *D. hansenii* strains into two groups of yeasts, the low amino acid consumption group (top) and the high amino acid consumption group (bottom). The principal differences between both groups was consumption of tyrosine. Strains L66 and L12 showed the lowest amino acid consumption, while strains L21 and L25 displayed an intermediate amino acid consumption. The main differences between those two groups was the consumption of alanine, glycine, proline, hydroxyproline, glutamic acid, phenylalanine, and histidine. Strains L1, L5, and L74 were in the highest amino acid consumers group, where the consumption of ornithine was the main difference between L1 and the other strains.

Yeast consumption of individual amino acids was different depending on the culture media (Tables S5–S8). The most consumed amino acids in all media were glutamic acid and threonine, and the least were valine and tyrosine. Biogenic amine production was not observed in any of the strains assayed in the amino acid medium following the method of



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Aslankoohi et al.[24] In individual sulfur amino acids, L1, L5, and L74 consumed the highest amount of L-methionine in all media (Table 5). L-Cystine was only detected in media supplemented with L-cystine or L-cysteine (Ci and Cy), and L1, L5, L21 and, L74 were the highest consumers of this sulfur amino acid.

**Gene Expression.** Expression of genes related to generation of sulfur compounds derived from L-methionine in media containing low L-methionine concentration (C) and high L-methionine concentration (M) was analyzed in RNA extracted from yeasts cultivated for 2 days (Table 6).

**Table 6. Transcriptomic Response of *D. hansenii* L1 and L74 to L-Methionine**

| | L1 | | L74 | |
|---|---|---|---|---|
| *D. hansenii* gene name | ratio M/C | $p^a$ | ratio M/C | $p$ |
| *DhARO8* | 1.73 | ns | 1.25 | ns |
| *DhCYS3* | 1.13 | ns | 0.89 | ns |
| *DhAAT2* | 0.95 | ns | 0.71 | * |
| *DhBNA3* | 0.71 | ns | 1.27 | ns |
| *DhADH1* | 1.59 | ns | 0.97 | ns |
| *DhILV6* | 1.56 | ns | 0.91 | ns |
| *DhATF1* | 0.76 | ns | 1.36 | ns |
| *DhATF2* | 2.33 | ** | 1.51 | ns |
| *DhPDC1* | 1.57 | ns | 1.73 | * |
| *DhPDC6* | 1.49 | ns | 1.06 | ns |
| *DhBAT2* | 1.56 | ns | 0.80 | ns |
| *DhSTR3* | 0.79 | ns | 1.32 | ns |
| *DhPDB1* | 1.52 | ns | 0.94 | ns |

[a]$p$: significant differences between transcriptomic response in C and M treatment at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. ns: $p > 0.05$. Abbreviations are indicated in Table 3. Ratio above 2 means it was overexpressed; a ratio below 0.5 means repressed.

Differential expression of few genes between M and C culture media (M/C ratio) was significant in strains L1 and L74. Expression of gene *DhATF2* (alcohol acetyltransferase) was overexpressed in L1 on media supplemented with L-methionine ($p < 0.01$). Expression of genes *DhAAT2* (cytosolic aspartate aminotransferase) and *DhPDC1* (pyruvate decarboxylase) was significantly different ($p < 0.05$) in C and M media in L74. However, none of these genes could be considered overexpressed or repressed.

## DISCUSSION

*D. hansenii* is added as a starter in the production of many Mediterranean meat products contributing to the generation of volatile compounds and overall quality of the products.[17] During the dry curing process many amino acids are released by different proteolytic activities.[25] The amounts of amino acids in the media used in this study are like the quantities found in dry fermented meat products.[3] The free amino acids are potential sulfur compound precursors, and especially, L-methionine catabolism has been investigated as the source of methanethiol (**1**). Methanethiol (**1**) is the first-step degradation product of L-methionine, is the main precursor for many sulfur compounds,[9] and appeared in medium M in very small amounts. The highest production of sulfur compounds by yeast was observed in the medium supplemented with L-methionine (M) confirming that methionine is the main precursor of sulfur compounds in *D. hansenii*, as observed by other authors in cheese yeasts.[5,26] *D. hansenii* strain L1 consumed the highest amount of L-methionine and produced the most complex sulfur compound profile. Overexpression of genes involved in sulfur compounds generation from L-methionine has been revealed in several cheese yeast species including *Geotrichum candidum, Yarrowia lipolytica, Kluyveromyces lactis*, and *Saccharomyces cerevisiae*.[27−29] Among the genes involved in conversion of L-methionine to methanethiol (**1**), we tested the expression of *DhAAT2* (cytosolic aspartate aminotransferase), *DhARO8* (aromatic amino acid transferase), *DhBAT2* (cytosolic branched-chain amino acid transferase), *DhBNA3* (arylformamidase), *DhCYS3* (cystathionine gamma-lyase), *DhSTR3* (cystathionine beta-lyase), and *DhILV6* (acetolactate synthase). However, we did not find any of these genes overexpressed in the strains and media evaluated, although *DhARO8* M/C ratios in L1 indicate high levels of expression of this gene in medium M respective to medium C.

Alternatively, chemical oxidation plays a key role in the generation of sulfur compounds. Dimethyl sulfide (**2**), dimethyl disulfide (**3**), and dimethyl trisulfide (**4**) are considered the result of the chemical oxidation of methanethiol (**1**).[9] These sulfur compounds appear in lesser amounts in media inoculated with *D. hansenii*, except for dimethyl disulfide (**3**) by L74. Strong chemical transformations of methional (**5**) into methanethiol (**1**) do not occur in NI M medium, therefore enzymatic production of methanethiol (**1**) is the most probable explanation for their presence in inoculated M media. Comparison between inoculated M media revealed that L25 M medium was like the NI M medium, except for the number of compounds, indicating that strain L25 is preventing oxidation of L-methionine. The large amount of methionol (**6**) and methyl thioacetate (**7**) in L1 and L5 M media indicates that the L-methionine catabolic pathway plays an important role in their generation. The large amount of dimethyl disulfide (**3**), produced by oxidation, indicates that strain L74 is not preventing oxidation of L-methionine or any other sulfur compound. Taking into account that the precursor of dimethyl disulfide (**3**) is methanethiol (**1**), the presence of methyl thioacetate (**7**) indicates that its precursor is also methanethiol (**1**). The most probable explanation is that methanethiol (**1**) is being produced by catabolism of L-methionine and rapidly oxidized to dimethyl disulfide (**3**).

Chemical oxidation of amino acids and nonbiological generation of sulfur compounds take place in NI medium.[2] In addition, conversion from L-cysteine to L-methionine in the medium could have happened.[30] Methional (**5**) was the only sulfur compound whose generation in NI was always higher than in media inoculated with yeasts. The most probable explanation could be that methional (**5**) is the consequence of an oxidative chemical reaction from L-methionine (Strecker reaction).[31,32] Escudero et al.[31] established the relationship between oxidized wines and the generation of methional (**5**), but this compound was not found in not oxidized wines. Similarly, our results show that in NI, methional (**5**) generation is the consequence of chemical oxidation, while in the inoculated media, chemical oxidation was not prevalent. This would agree with earlier studies reporting the role of *D. hansenii* in prevention of lipid oxidation in meat products.[14] However, our study did not reveal changes in the expression of pyruvate decarboxylase genes (*PDC*) responsible for the conversion of KMBA into methional (**5**). On the contrary, Cholet et al.[22] found *PDC* expression of *DhPDC1* and *DhPDC6* genes in *Yarrowia lipolytica*, although the authors


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did not detect generation of methional (**5**). This might indicate that methional (**5**) is not the result of pyruvate decarboxylase activity from KMBA. Another explanation could be the rapid conversion of methional (**5**) into methionol (**6**), which would prevent accumulation of the former in inoculated media. This conversion occurred in some of our *D. hansenii* yeasts in M medium. Conversion of methionol (**6**) from methional (**5**) is conducted by alcohol dehydrogenases (ADH); however, these genes (*DhADH1*) were also not overexpressed in our *D. hansenii* strains. Moreover, the lower amounts of methional (**5**) found in the inoculated media could result from methional oxidation and generation of methyl thiopropionic acid.[32] Nevertheless, this compound was not analyzed in our study. Thioesters generated from alcohol acetyltransferases activity (ATF), methyl thioacetate (**7**), and ethyl thioacetate (**8**) were detected in media inoculated with *D. hansenii* strains, although in very low amounts among the sulfur compounds analyzed. Generation of ethyl thioacetate (**8**) is absent in sulfur compounds produced by cheese yeasts.[6,8,27−29] Although, in most studies, including ours, methyl thioacetate (**7**) is the most produced thioester from L-methionine. *D. hansenii* strain L1 produced the highest methyl thioacetate (**7**) amount in medium M, and the *DhATF2* gene was overexpressed in this yeast strain.

Gene expression was similar in *D. hansenii* strains growing on M medium respective to C medium at 48 h except for *DhATF2*. Comparable experiments of gene expression were also conducted at 4 and 15 days (data not shown); however, the expression of all genes evaluated did not change between media M and C. Moreover, sulfur compounds were analyzed at 15 days because previous studies show a measurable concentration at this time.[15] However, after 15 days incubation yeasts are in stationary phase of growth and none of the selected genes must be overexpressed at this late time in the growth curve. Most authors used 48 and 96 h incubation to test overexpression of genes and its relationship with sulfur compounds production in other yeast species.[8,27−29] *D. hansenii* strains L1 and L74 were selected for gene expression analysis because of their great ability to generate sulfur compounds from amino acids, plus their different volatile production pattern. Medium M was selected for gene expression analysis because L-methionine is the precursor of most sulfur compounds. Other genes tested in previous studies dealing with sulfur compounds production by Ehrlich or demethylation pathways such as *ARO9*, *ARO10*, and *BAT1* were overexpressed in *S. cerevisiae* and *Y. lipolytica*[22,28,33] and assayed in high L-methionine medium. Liu et al.[28] found overexpression of the *STR3* gene (demethiolase activity) and *ADH4* and *ADH5* genes (alcohol dehydrogenase activity) in *S. cerevisiae*, although other studies did not find these genes overexpressed in *K. lactis* and *Y. lipolytica*.[27,34]

Our results showed that C, Cy, and Ci media contained all of the necessary amino acids but generated low amounts of sulfur compounds, in agreement with previous studies.[6,8,26,27] One explanation could be that L-cysteine is preferably consumed by yeasts producing $H_2S$. However, $H_2S$ is highly volatile and reactive, preventing the production of its derived sulfur compounds, and is difficult to detect by GC-MS.[26]

Regarding the relationship between amino acid consumption and sulfur compounds generation in the *D. hansenii* strains tested, a clear link between these variables was found in strains L1 and L5, isolated from pork dry fermented sausages, and L74 isolated from lupine. Moreover, the high consumption of amino acids, besides L-methionine, by these yeast strains indicates the probable production of other volatile compounds plus other sulfur compounds, which can provide an interesting flavor profile to the product.[13] Nevertheless, the performance of *D. hansenii* strains in amino-acid-supplemented media for production of sulfur compounds could change with the composition of the media and environmental conditions in production of dry cured meat products.[14]

In summary, L-methionine-supplemented media (M) was the most efficient for production of sulfur compounds by yeasts. Methional (**5**) was preferably generated by chemical oxidation of L-methionine, while methyl thioacetate (**7**) was solely produced by yeasts. Profiles of sulfur compounds generated by yeast were different, and strains from pork meat origins presented the most complex sulfur compound profiles. Expression of genes in the metabolic pathway of L-methionine, for generation of sulfur compounds, could not be directly related to sulfur compound production in *D. hansenii*, except in case of methyl thioacetate (**7**), strain L1, and overexpression of gene *DhATF2*. Sulfur compounds produced by yeast were detected long after genes involved in the metabolic pathway of sulfur compounds generation were expressed. This could explain the absence of overexpression of most genes in M media. Nevertheless, this study has deeply investigated the metabolic pathway involved in the generation of L-methionine-derived volatile compounds in *D. hansenii* isolated from meat products. It has subsequently shown their relevance as a producer of volatile sulfur compounds with aroma impacts in meat products. The results have shown that different metabolic pathways are expressed in *D. hansenii* from fermented pork and llama sausages, and their impact on the final meat product aroma is different. However, the expression of the metabolic pathways depends on many processing conditions that affect the production of sulfur compounds from their precursor sulfur amino acids. Therefore, it is necessary to continue to elucidate in real conditions (sausage manufacture) that the expression of genes impacts the produced sulfur compounds and, subsequently, the meat product aroma.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b03361.

(Table S1) Initial amino acids concentration in control medium and (Tables S2−S8) concentrations of sulfur compounds and amino acids in the control medium, the L-cysteine medium, L-cystine, and the L-methionine medium inoculated with different *D. hansenii* strains (PDF)

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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS USED

ATF, alcohol acetyltransferases activity; KMBA, α-keto-γ-methylthio-oxobutyric acid; HS, headspace; SPME, solid-phase microextraction; CAR/PDMS, carboxen/polydimethylsiloxane

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# Counteracting the effect of reducing nitrate/nitrite levels on dry fermented sausage aroma by *Debaryomyces hansenii* inoculation

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ABSTRACT

The reduction of ingoing amounts of nitrate and nitrite in dry fermented sausages was studied together with the impact of *Debaryomyces hansenii* inoculation on aroma generation. Three different formulations of sausages were manufactured: control (C), reduced in nitrate and nitrite ingoing amounts (R) and reduced R inoculated with *D. hansenii* (RY). Changes in physicochemical and microbiological parameters, volatile compounds and aroma were investigated at different drying times. Nitrite/nitrate reduction did not seem to affect microbial growth but affected their metabolic activity. Moreover, nitrite/nitrate reduction decreased lipid oxidation and generation of derived volatile compounds. Yeast inoculation limited lipid oxidation and prevented nitrite oxidation. Sausage aroma profile was positively affected by *D. hansenii* inoculation which contributed to the generation of potent aroma compounds like ethyl ester compounds and 3-methylbutanal. Long drying time impacted sausage aroma profile as well as yeast metabolism. Yeast inoculation counteracted the negative influence of nitrite/nitrate reduction due to its antioxidant capacity, aroma generation and hindered nitrite oxidation.

## 1. Introduction

The use of nitrite and nitrate as curing agents in dry fermented sausages have important technological functions. Nitrite has antioxidant and antimicrobial activities being the most important its activity against the pathogen *Clostridium botulinum* and control of toxin production. Furthermore, nitrite facilitates the generation and stabilization of the typical colour and enhances cured flavour (Honikel, 2008; Sindelar & Milkowski, 2011). Nitrate acts as nitrite reservoir in dry cured products with a long ripening time (Sánchez Mainar & Leroy, 2015). However, the controversy about the adequate amounts of nitrite used as additive in meat products processing is under concern (CODEX, 2019). Nitric oxide, from nitrite, can react with biogenic amines present in meat under heating conditions to form N-nitrosamines, with carcinogenic potential (De Mey, De Maere, Paelinck, & Fraeye, 2015). Therefore, the actual trend is to reduce the use of nitrite and nitrate in cured meat products (EFSA, 2010), although nitrite is considered beneficial for improving the microbial safety of the products. Up to now, several alternatives to nitrite addition have been studied. Among them, plant derivatives are one of the most used, followed by organic acids, bacteria and bacteriocins (Alahakoon, Jayasena, Ramachandra, & Jo, 2015). However, these alternatives do not provide all nitrite functions in dry cured fermented sausages. Nitrate and nitrite reduction below their allowed maximum levels in European legislation (150 mg/kg of sodium nitrate and 150 mg/kg of sodium nitrite, (Regulation (EC) No 1333 (2008)) have safety and sensory consequences. The reduction up to a 50% of the allowed limits affects growth of pathogenic microorganisms (Hospital, Hierro, & Fernández, 2014), volatile compounds production (Hospital, Hierro, Stringer, & Fernández, 2016) and aroma profile (Perea-Sanz, Montero, Belloch, & Flores, 2018). A recent study (Christieans, Picgirard, Parafita, Lebert, & Gregori, 2018) has shown that the use of a mixture of sodium nitrate and sodium nitrite at a concentration of 80 mg/kg, was able to control the growth of *Listeria monocytogenes* and *Salmonella typhimurium*. Nevertheless, the importance of nitrite presence at the beginning of the process to control these pathogens was remarked due to the slow conversion of nitrate into nitrite (Christieans et al., 2018).

The consequences of the use of reduced nitrate and nitrite mixtures on aroma profile of dry cured meat products have been scarcely studied. The inoculation of yeasts such as *Debaryomyces (D.) hansenii* with potential to generate desirable aromas in dry fermented sausages is a suitable alternative. This yeast has been shown to produce esters and sulfur compounds as cured aroma contributors to the aroma profile of dry fermented sausages (Cano-García, Belloch, & Flores, 2014; Perea-Sanz, Peris, Belloch, & Flores, 2019b). The production of aroma compounds by *D. hansenii* strains is influenced by the concentration of

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curing agents (Flores, Moncunill, Montero, López-Díez, & Belloch, 2017), as well as by the availability of specific amino acids used as volatile precursors (Perea-Sanz, Montero, Belloch, & Flores, 2019a). A recent study regarding the production of volatile sulfur compounds that contribute to meat aroma notes by *D. hansenii* strains revealed that one yeast strain may have an impact on dry fermented sausage aroma due to its ability to express a gene directly related to sulfur compound production (Perea-Sanz, Montero, et al., 2019a). Consequently, the aim of the present study is to evaluate the effect of the inoculation of a *D. hansenii* strain on dry fermented sausages manufactured with reduced ingoing amounts of nitrite and nitrate and its effect on aroma production during the ripening time.

## 2. Materials and methods

### 2.1. Dry fermented sausages manufacture

Three different formulations of dry fermented sausages were manufactured. Control (C), containing sodium nitrite at 150 ppm and potassium nitrate at 178 ppm, and two formulations with a 47% reduction of both nitrite and nitrate (80 and 95 mg/kg, respectively) ingoing amounts (R) as proposed by Christieans et al. (2018), but one of them inoculated with *D. hansenii* strain L1 (RY). Three replicates of the experiment were performed. Pork's lean meat and belly fat (boneless and skinless) were purchased from a local producer (Cárnicas La Cope, Torrente, Spain). Lean (50% lean pork meat) and fat (50% pork belly) were ground through a 10 mm diameter minced plate and mixed with the following ingredients: 20 g/kg lactose, 20 g/kg dextrin, 6 g/kg glucose, 20.25 g/kg sodium chloride (NaCl), 6.75 g/kg potassium chloride (KCl), 0.5 g/kg sodium ascorbate and 1.5 g/kg black pepper. Commercial starter culture TRADI-302 (Danisco, Cultor, Madrid, Spain) containing *Lactobacillus sakei, Staphylococcus xylosus* and *Staphylococcus carnosus* was added to the meat (0.125 g/kg). In the three replications, each formulation was supplemented with the different ingoing amounts of nitrate/nitrite mixtures. Then, *D. hansenii* L1 was inoculated using 10 ml saline suspension containing approximately $6 \times 10^9$ cells/g. This yeast suspension was added to 6 kg of meat batter to reach a final concentration of $10^6$ cells/g. To homogenize all ingredients into the meat batter, a mixer-kneading machine provided with rotating blades was used. Formulations were kept at 3–5 °C for 24 h to rest after yeast inoculation and then, stuffed into 95 mm diameter collagen casings (Fibran, S.A., Girona, Spain), being the final weight of each sausage approximately 500 g. All sausages were submitted to a slow ripening process and dried at 10 °C and 75–90% relative humidity (RH). In order to control the ripening process, temperature and RH of the ripening chambers were continuously recorded. A total of 9 batches ($3 \times 3$) were produced. One sausage from each formulation was weighed regularly to control weight losses. Also, one sausage per formulation was used to control the pH by introducing a pH meter HI 99163 (Hanna Instruments Inc., Hoonsocket, USA) into the sausage centre as described by Olivares, Navarro, Salvador, and Flores (2010). Sausages from the different formulation and replicates were sampled for analyses at 0, 60 and 83 d of process. At 0 d, approximately 200 g of the minced meat mixture were collected for analysis. After 60 d of ripening (44–45% weight loss), three sausages from each formulation and replicate ($3 \times 3$ sausages) were randomly chosen for analyses. The remaining sausages were ripened for 23 additional days (50% weight loss), and afterwards they were collected as above. Slices were taken for microbial analyses (25 g) and other slices for volatile analysis were vacuum packed and stored at −80 °C. Sausage colour and physicochemical analysis (moisture, water activity aw, pH, TBARS, protein and residual nitrite and nitrate content) were measured on minced sausage.

### 2.2. Physicochemical parameters measurement

Measurements of pH, $a_w$, weight loss, colour (CIE L*, a*, b*), moisture, and protein was done as described in Perea-Sanz et al. (2018). Lipid autoxidation was measured by the thiobarbituric acid reactive substances (TBARS) method and results expressed as μg malonaldehyde (MDA)/g in dry matter (Perea-Sanz et al., 2018). Residual nitrate and nitrite contents were extracted by Carrez precipitation and determined after zinc reduction and Griess reaction as reported in Merino (2009). Measurements were expressed as mg/kg of sodium nitrite and potassium nitrate in dry matter.

### 2.3. Microbiological analysis

Microbial counts of lactic acid bacteria (LAB), gram positive coagulase negative cocci (GCN), yeasts and moulds and enterobacteria were carried out as described in Perea-Sanz et al. (2018).

### 2.4. D. hansenii L1 monitoring by M13 RAPD PCR

Fifteen yeast colonies were isolated (Perea-Sanz et al., 2018) from each sausage batch (C, R and RY) at the three ripening times (0, 60 and 83 d). Colonies were tested for purity and freshly isolated colonies selected and cultured overnight at 25 °C in 5 ml of GPY (glucose 2%, peptone 0.5% and yeast extract 0.5%). DNA extraction and M13 minisatelite PCR amplification were carried out as described in Cano-García, Flores, and Belloch (2013). PCR products were separated by electrophoresis on 2% agarose gel in 1× TAE buffer at 90 V for 3 h, stained with Red safe nucleic acid staining solution 20,000× (Intron biotechnology, Korea) and visualized under UV light. DNA fragment sizes were determined using 100 bp DNA ladder (Invitrogen, USA).

### 2.5. Volatile compound analysis

Analysis of volatile compounds was carried out by headspace (HS) solid-phase microextraction (SPME) with an 85 μm carboxen/polydimethylsiloxane (CAR/PDMS) fibre (Supelco, Bellefonte, USA) using a gas chromatograph (Agilent HP 7890 series II, Hewlett-Packard, Palo Alto, USA) with a quadrupole mass detector (HP 5975C, Hewlett-Packard, Palo Alto, USA) and equipped with an autosampler (MPS2 multipurpose sampler (Gerstel, Germany). In summary, 5 g of sausage supplemented with 0.75 mg BHT to avoid oxidation, were placed into a headspace vial and incubated at 37 °C for 30 min. The extracted volatile compounds were adsorbed in the fibre for 90 min at 37 °C and desorbed in the injection port of the GC–MS for 5 min at 240 °C in splitless mode. The volatile compounds were separated using a DB-624 capillary column (30 m, 0.25 mm i.d., film thickness 1.4 μm, (J&W Scientific, Agilent Technologies, USA)) using the conditions described by Corral, Salvador, Belloch & Flores. (2015). The MS interface temperature was set to 240 °C. The compounds were identified in full scan mode and by comparison with mass spectra from the library database (Nist'05), with linear retention indices (Van Den Dool & Kratz, 1963) and with authentic standards. The quantitation was done in SCAN mode using either total or extracted ion current (TIC or EIC) on an arbitrary scale and expressed as abundance units (AU) x $10^5$.

### 2.6. Olfactometric analysis

The analysis of aroma compounds was carried out in a gas chromatograph (Agilent 6890, USA) equipped with a FID detector and a sniffing port (ODP3, Gerstel, Mülheim an der Ruhr, Germany) as described in Corral, Salvador, Belloch, and Flores (2015). Each assessment was carried out on 5 g of sausage using the detection frequency method (Pollien et al., 1997). The olfactometry analysis was done in sausages ripened for 60 d. Five trained panellists evaluated the odours from the GC-effluent. A total of 12 assessments were carried out. Aroma compounds were identified by comparison with mass spectra, using linear retention indices of authentic standards injected in GC–MS and GC-O and by the coincidence of the assessor's descriptors with those in the

Fenaroli's handbook of flavour ingredients (Burdok, 2002).

### 2.7. Statistical analysis

Data were analysed using the Generalized Linear Model (GLM) procedure of statistical software (XLSTAT 2011, v5.01, Addinsoft, Barcelona, Spain). Data were analysed using the linear mixed model including sausage formulation and ripening time as fixed effects, and replicates as random effect. The interaction between fixed effects was tested and evaluated as not significant, therefore it was excluded from the model. When a significant effect of the treatment group was detected ($P < .05$), least squares means (LSM) were compared using Tukey test. Principal component analysis (PCA) was plotted to evaluate the relationships among sausage formulations and parameters (physicochemical, microbiological and aroma compounds) measured at each sampling time.

## 3. Results

### 3.1. Physicochemical characteristics of the sausages

Measurements of physicochemical parameters from samples of the three formulations at the three sampling times are shown in Table 1. At the initial time (0 d), the three formulations were very similar regarding all parameters except nitrite and nitrate concentration. Residual nitrite differences between C and reduced R and RY formulations was 45–52%, whereas residual nitrate differences were about 31–33%. After 60 d of ripening, no residual nitrite levels were detected in any formulation, while residual nitrate levels in R and RY respect to C were 40% and 46%, respectively. These differences in residual nitrate concentrations were maintained after 83 d of ripening. During the ripening time, residual nitrate decreased around 17%, 36% and 48% within C, R and RY sausages, respectively. Colour parameter a* increased after 60 d of ripening. Lipid oxidation (TBARS) evolution was different among the formulations. C and R showed the same trend, an initial increase at 60 d and a slow decline after 83 d, while RY sausages did not show lipid oxidation maintaining low TBARS values during the ripening time. In addition, the highest pH value was reached at 60 d in RY sausages. These differences between RY and the other two formulations could be observed also in water activity and moisture at 83 d of ripening time.

### 3.2. Microbiological counts

Results on microbial counts are shown in Fig. 1. *Enterobacteriaceae* were not found in any sausage batch at any sampling time (data not shown). LAB counts (Fig. 1A) increased from 0 d to 60 d and decreased slightly at 83 d. Significant differences between LAB counts were found at 0 d in RY respect to C and R batches, but at 60 and 83 d LAB counts were indistinguishable between batches. GCN counts (Fig. 1B) decreased constantly from 0 d and no significant differences were found among sausages at any ripening time. Yeast counts (Fig. 1C) were lower than 10 cfu/g in C (2 colonies) and R (5 colonies) sausages at 0 d, whereas the RY showed a yeast count in accordance with *D. hansenii* L1 inoculation. Yeast counts increased from 0 d to 60 d in all sausages and decreased slightly from 60 to 83 d, however counts in RY were higher

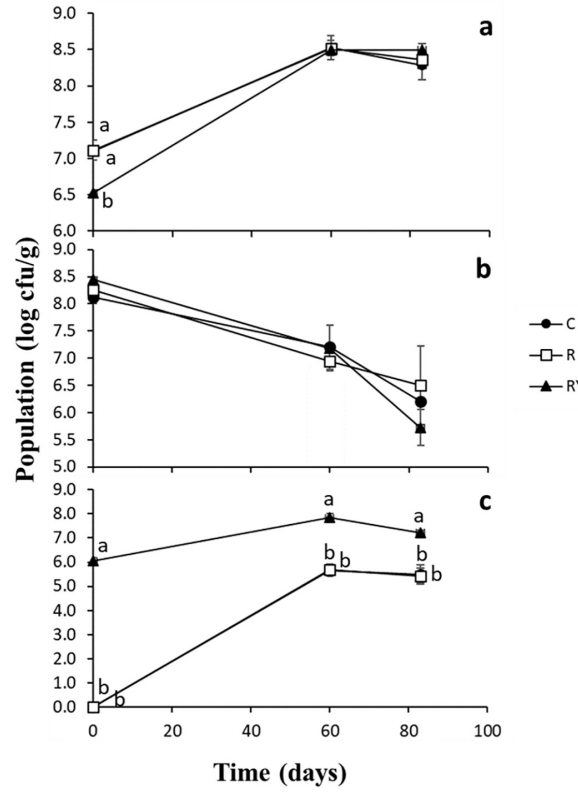


**Fig. 1.** Changes in microbial populations during the ripening of dry fermented sausages in Control (●), R (□) and RY (▲) formulations. Lactic acid bacteria (LAB) (a), Gram positive coagulase negative cocci (GCN) (b) and yeasts and moulds (c). Different letters in the same ripening time indicate mean differences at *** $P < .001$ among sausages.

**Table 1**
Effect of nitrite/nitrate reduction and *D. hansenii* inoculation on physicochemical parameters of dry fermented sausages.

| | C[1] | | | | | | R | | | | | | RY | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0d | | 60d | | 83d | | 0d | | 60d | | 83d | | 0d | | 60d | | 83d | | RMSE[2] | $P_t$[3] | $P_f$ |
| pH | 5.79 | a | 4.85 | d | 4.94 | bc | 5.79 | a | 4.86 | d | 4.94 | bc | 5.80 | a | 4.99 | b | 4.92 | c | 0.05 | *** | ** |
| aw | 0.973 | a | 0.894 | b | 0.809 | d | 0.973 | a | 0.897 | b | 0.807 | d | 0.969 | a | 0.899 | b | 0.837 | c | 0.006 | *** | *** |
| Moisture % | 64.15 | a | 42.44 | b | 29.69 | d | 64.19 | a | 42.28 | b | 29.77 | d | 63.48 | a | 41.92 | b | 32.58 | c | 1.18 | *** | ns |
| Protein (% dm) | 49.42 | d | 52.89 | cd | 54.14 | bc | 50.71 | cd | 58.08 | a | 53.23 | bcd | 49.97 | d | 56.22 | ab | 53.25 | bcd | 2.81 | *** | ns |
| L* | 59.81 | a | 52.85 | b | 44.76 | cd | 59.91 | a | 52.21 | b | 44.34 | d | 60.26 | a | 53.45 | b | 46.64 | c | 1.71 | *** | ns |
| a* | 13.79 | c | 16.81 | a | 14.40 | bc | 14.49 | bc | 16.76 | a | 14.24 | bc | 12.64 | d | 17.04 | a | 14.78 | b | 0.51 | *** | ns |
| b* | 11.00 | a | 6.07 | b | 5.11 | c | 11.28 | a | 5.67 | b | 5.07 | c | 11.72 | a | 6.03 | b | 5.59 | bc | 0.47 | *** | * |
| TBARS[4] | 0.59 | d | 3.62 | a | 2.21 | b | 0.48 | d | 2.23 | b | 1.27 | c | 0.47 | d | 0.63 | d | 0.47 | d | 0.32 | *** | *** |
| $NaNO_2$ mg/kg dm | 217.40 | a | 0.00 | c | 0.00 | c | 102.32 | b | 0.00 | c | 0.00 | c | 119.10 | b | 0.00 | c | 0.00 | c | 11.20 | *** | *** |
| $KNO_3$ mg/kg dm | 533.05 | a | 396.77 | bc | 442.44 | b | 363.81 | c | 235.32 | d | 233.20 | d | 364.66 | c | 212.67 | d | 188.39 | d | 42.37 | *** | *** |

[1]C: control batch; R: 47% reduction in nitrite and nitrate; RY: 47% reduction in nitrite and nitrate and *D. hansenii* inoculated. [2]RMSE: root mean square error. [3]$P_t$: *P* value of time effect, $P_f$: *P* value of formulation effect. Different letters in the same row of each group indicate significant differences at *** P < .001, ** $P < .01$, * $P < .05$. ns: $P > .05$. [4]TBARS expressed as μg malonaldehyde/g dm.

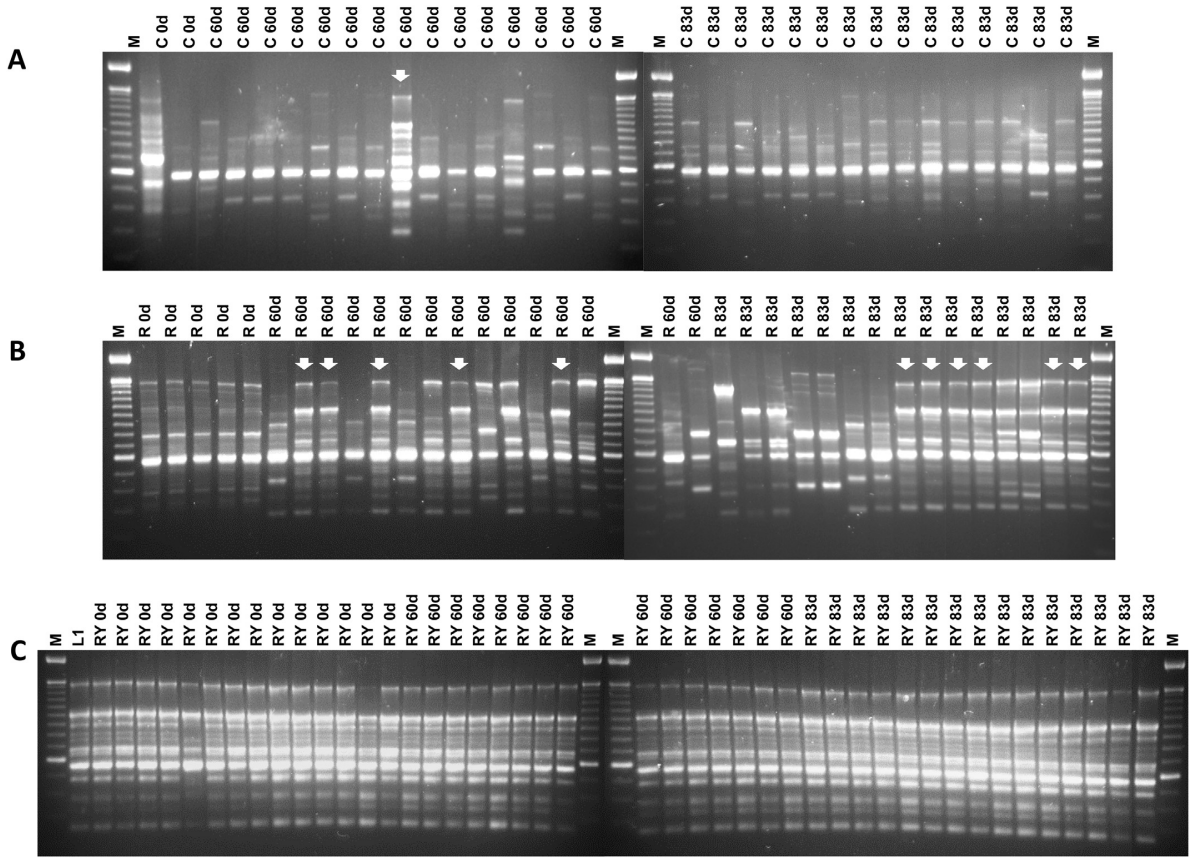


**Fig. 2.** Electrophoretic patterns of minisatellite M13 PCR amplification of yeast isolates at 0, 60 and 83 d of ripening in C (A), R (B) and RY (C) formulations. The arrow indicates bands of isolated yeast in C and R sausages matching the pattern of the inoculated strain L1. Lane M is the 100 bp ladder. Lane L1 in panel C represents the pattern of the inoculated *D. hansenii* strain.

than C and R.

### 3.3. *D. hansenii* monitoring by M13 RAPD-PCR along sausage ripening

The implantation of the inoculated *D. hansenii* strain on RY sausages, as well as its presence in C and R sausages, was followed by comparison of M13 minisatellite PCR patterns of yeast isolates with the inoculated *D. hansenii* L1 strain (Fig. 2). M13 patterns of yeast isolates from RY sausages at any ripening time were identical to the pattern of the inoculated *D. hansenii* L1 strain. On the other hand, several yeast isolates from C sausages (Fig. 2A) and R sausages (Fig. 2B) showed M13 patterns different from the pattern of *D. hansenii* L1 strain. In C sausages, only one isolate at 60 d and in R batch 5 isolates at 60 d and 6 isolates at 83 d were found with identical pattern of the inoculated *D. hansenii* L1 strain.

### 3.4. Volatile compounds generation

Eighty volatile compounds were identified and quantified in the HS of dry fermented sausages using a CAR/PDMS fibre (Table 2). The volatile compounds were classified by their most probable origin. Within the bacterial metabolism origin, four subgroups were done: amino acid degradation (17), carbohydrate fermentation (8), esterase activity (10) and β-oxidation (1)), from the chemical reaction origin two subgroups: lipid oxidation reaction (22) and thiamine degradation (2)); and finally, from spices (17) and from unknown origin (3). Among the 80 volatiles identified, 61 compounds were confirmed while 11 were tentatively identified.

The RY sausages showed the highest concentration of volatile compounds from amino acid degradation at both drying times. The most abundant compounds were 3-methyl-1-butanol, 2-methyl propanoic acid and 2-methyl and 3-methyl butanoic acids, whereas dimethyl disulphide, toluene, 3-(methylthio)propanal, benzeneacetaldehyde, 2,6-dimethylpyrazine and phenol were the less abundant. On the other hand, methanethiol and 3-methylbutanal were the most abundant compounds found in C and R sausages. Comparison between C and R sausages revealed that nitrite and nitrate reduction produce significant differences only in two compounds; 3-(methylthio)propanal and benzeneacetaldehyde which were more abundant in C sausages. During the ripening period between 60 and 83 d, the total volatile compounds derived from amino acid degradation diminished in RY sausages. This decrease was significant in methanethiol, 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, and 3-methyl-1-butanol concentrations. Only benzeneacetaldehyde increased after the 60 d of ripening in RY sausages. Three volatile compounds derived from amino acid degradation were identified and only traces were detected of 2-acetyl-2-thiazoline, 2-acetyl-1-pyrroline and dimethyl trisulfide.

The sum of carbohydrate fermentation volatiles was significantly higher in C and R sausages than in RY sausages at both drying times. The most abundant compounds in C and R sausages respect to RY sausages were 2,3-butanedione, 2-butanone, acetic acid and 3-hydroxy-2-butanone. On the contrary, acetaldehyde and ethyl alcohol were

**Table 2**
Effect of nitrite/nitrate reduction and *D. hansenii* inoculation on volatile compounds generated in dry fermented sausages (expressed as abundance (AU) of total ion current (TIC) or area of the target ion shown in parenthesis as AU × $10^5$).

| | | | C[3] | | | | R | | | | RY | | | | RMSE[4] | $P_t$[5] | $P_f$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LRI[1] | RI[2] | 60 d | | 83 d | | 60 d | | 83 d | | 60 d | | 83 d | | | | |
| Microbial origin | | | | | | | | | | | | | | | | | |
| **Amino acid degradation** | | | **155.29** | **c** | **202.53** | **c** | **135.14** | **c** | **210.42** | **c** | **457.22** | **a** | **355.54** | **b** | **51.70** | **ns** | *** |
| Methanethiol | 473 | a | 28.43 | c | 60.35 | a | 30.51 | c | 64.79 | a | 43.02 | b | 16.27 | d | 5.59 | *** | *** |
| 2-Methylpropanal | 593 | a | 7.36 | b | 5.25 | b | 5.76 | b | 4.46 | b | 26.54 | a | 5.78 | b | 8.17 | ** | ** |
| 3-Methylbutanal | 690 | a | 35.96 | ab | 32.74 | b | 32.29 | b | 26.29 | b | 55.26 | a | 24.05 | b | 12.61 | ** | ns |
| 2-Methylbutanal (58) | 698 | a | 1.94 | b | 1.73 | b | 1.93 | b | 1.61 | b | 6.26 | a | 1.62 | b | 1.72 | ** | ** |
| Dimethyl disulfide | 773 | a | 18.21 | a | 5.73 | b | 9.61 | ab | 5.77 | b | 4.66 | b | 7.60 | ab | 6.14 | * | ns |
| Toluene (91) | 788 | a | 10.62 | a | 8.98 | ab | 8.14 | ab | 7.04 | b | 7.80 | b | 6.79 | b | 1.47 | * | *** |
| 3-Methyl-1-butanol (55) | 795 | a | 2.66 | c | 2.14 | c | 2.85 | c | 2.52 | c | 33.68 | a | 16.31 | b | 1.78 | *** | *** |
| 2-Methyl propanoic acid | 864 | a | 14.80 | b | 32.68 | b | 11.84 | b | 48.07 | b | 142.41 | a | 161.01 | a | 30.95 | * | *** |
| 3-Methyl butanoic acid (60) | 941 | a | ND | b | ND | b | ND | b | ND | b | 93.81 | a | 127.11 | a | 26.67 | ns | *** |
| 2-Acetyl-1-pyrroline | 961 | a | tr[6] | | tr | | tr | | tr | | tr | | tr | | | | |
| 2,6-Dimethylpyrazine (108) | 944 | a | 2.88 | b | 21.26 | a | 3.63 | b | 18.85 | a | 0.54 | b | 2.54 | b | 4.11 | *** | *** |
| 2-Methyl butanoic acid (74) | 946 | a | 6.54 | b | 8.63 | b | 8.59 | b | 11.38 | b | 29.36 | a | 29.98 | a | 5.46 | ns | *** |
| 3-(Methylthio)propanal (104) | 968 | a | 1.45 | a | 1.06 | ab | 0.79 | bc | 0.68 | bc | 0.33 | c | 0.49 | c | 0.27 | ns | *** |
| Dimethyl trisulfide | 1003 | a | tr | | tr | | tr | | tr | | tr | | tr | | | | |
| Benzeneacetaldehyde | 1110 | a | 10.93 | a | 9.99 | ab | 7.11 | bc | 7.89 | abc | 5.11 | c | 8.73 | ab | 1.84 | ns | *** |
| Phenol | 1114 | a | 13.53 | a | 13.42 | a | 12.09 | a | 12.97 | a | 8.44 | b | 10.91 | ab | 1.90 | ns | *** |
| 2-Acetyl-2-thiazoline | 1175 | a | tr | | tr | | tr | | tr | | tr | | tr | | | | |
| **Carbohydrate fermentation** | | | **5631.62** | **b** | **7405.09** | **a** | **6280.08** | **ab** | **6877.28** | **ab** | **3473.90** | **c** | **3777.01** | **c** | **983.47** | ** | *** |
| Acetaldehyde | 467 | a | 25.14 | b | 20.89 | b | 18.13 | b | 16.98 | b | 73.09 | a | 28.18 | b | 13.21 | *** | *** |
| Ethyl alcohol | 507 | a | 887.72 | b | 739.85 | b | 670.60 | b | 854.35 | b | 2294.16 | a | 2192.11 | a | 151.08 | ns | *** |
| Acetone | 529 | a | 69.34 | a | 47.78 | ab | 61.17 | ab | 42.92 | b | 49.51 | ab | 54.99 | ab | 13.49 | ** | ns |
| 2,3-Butanedione | 627 | a | 129.85 | a | 129.93 | a | 160.14 | a | 119.13 | a | 19.36 | b | 9.69 | b | 27.37 | ns | *** |
| 2-Butanone | 631 | a | 72.68 | a | 48.17 | b | 79.28 | a | 43.59 | b | 28.79 | b | 26.80 | b | 13.22 | *** | *** |
| Acetic acid | 717 | a | 2805.24 | b | 4435.95 | a | 3122.49 | b | 3822.32 | ab | 813.68 | c | 1343.73 | c | 651.28 | *** | *** |
| 3-Hydroxy-2-butanone | 781 | a | 1586.48 | a | 1914.66 | a | 2109.15 | a | 1890.61 | a | 150.70 | b | 46.42 | b | 400.29 | ns | *** |
| Butanoic acid | 891 | a | 55.16 | de | 67.85 | bc | 59.12 | cd | 87.39 | a | 44.60 | e | 75.09 | b | 6.81 | *** | *** |
| **Esterase activity** | | | **404.73** | **b** | **364.80** | **b** | **415.44** | **b** | **423.13** | **b** | **1077.97** | **a** | **1134.48** | **a** | **112.28** | **ns** | *** |
| Ethyl acetate | 635 | a | 229.96 | bc | 178.56 | c | 283.17 | b | 199.47 | bc | 430.45 | a | 462.49 | a | 58.33 | ns | *** |
| Ethyl 2-methylpropanoate (71) | 788 | a | ND | b | ND | b | ND | b | ND | b | 30.70 | a | 31.56 | a | 4.13 | ns | *** |
| Ethyl butanoate | 831 | a | 29.65 | b | 29.57 | b | 27.13 | b | 32.82 | b | 69.91 | a | 77.54 | a | 7.21 | ns | *** |
| Ethyl 2-hydroxypropanoate | 867 | a | 40.96 | bc | 46.99 | c | 26.86 | d | 51.52 | c | 81.60 | b | 143.68 | a | 10.39 | *** | *** |
| Ethyl 2-methylbutanoate (57) | 878 | a | 2.41 | b | 3.26 | b | 2.60 | b | 5.20 | b | 31.95 | a | 32.78 | a | 5.84 | ns | *** |
| Ethyl 3-methylbutanoate | 882 | a | 33.96 | b | 55.91 | b | 32.89 | b | 94.84 | b | 387.96 | a | 386.12 | a | 84.17 | ns | *** |
| 3-Methylbutyl acetate (70) | 907 | a | ND | b | ND | b | ND | b | ND | b | 1.00 | a | 1.03 | a | 0.10 | ns | *** |
| Ethyl pentanoate | 928 | a | 21.53 | a | 12.56 | bc | 13.65 | b | 10.93 | bcd | 8.44 | cd | 6.76 | d | 2.82 | *** | *** |
| Ethyl hexanoate | 1030 | a | 45.43 | a | 37.45 | ab | 28.48 | b | 27.88 | b | 32.58 | b | 36.30 | ab | 6.71 | ns | *** |
| Ethyl octanoate (88) | 1230 | a | 0.83 | c | 0.50 | c | 0.66 | c | 0.46 | c | 3.37 | b | 4.10 | a | 0.28 | ns | *** |
| β-Oxidation | | | | | | | | | | | | | | | | | |
| 1-Octen-3-ol | 1032 | a | 30.49 | a | 29.09 | a | 25.23 | a | 24.13 | a | 10.04 | b | 12.43 | b | 5.00 | ns | *** |
| **Chemical origin** | | | | | | | | | | | | | | | | | |
| **Lipid oxidation** | | | **3441.38** | **a** | **2502.57** | **b** | **1768.25** | **bc** | **1192.48** | **cd** | **539.38** | **d** | **603.53** | **d** | **499.39** | ** | *** |
| Pentane | 500 | a | 31.95 | | 17.41 | | 28.03 | | 28.22 | | 32.05 | | 35.80 | | 11.09 | ns | ns |
| Hexane | 600 | a | 8.28 | | 5.01 | | 8.38 | | 7.40 | | 9.35 | | 7.43 | | 2.62 | * | ns |
| 1-Propanol | 312 | a | 2.02 | c | 1.99 | c | 1.95 | c | 1.74 | c | 21.69 | a | 13.16 | b | 1.66 | *** | *** |
| Butanal | 622 | a | 3.68 | a | 2.41 | b | 1.91 | bc | 1.51 | bcd | 0.77 | d | 1.22 | cd | 0.62 | ns | *** |
| Heptane | 700 | a | 9.49 | | 6.94 | | 9.76 | | 8.63 | | 6.92 | | 7.06 | | 3.20 | ns | ns |
| Pentanal | 739 | a | 136.60 | a | 88.75 | b | 69.27 | bc | 49.72 | bcd | 19.23 | d | 31.16 | cd | 24.34 | * | *** |
| Octane | 800 | a | 135.27 | a | 128.26 | ab | 120.72 | abc | 102.54 | abc | 74.20 | c | 82.53 | bc | 29.58 | ns | *** |
| Propanoic acid | 811 | a | 14.80 | bc | 21.55 | a | 12.39 | c | 18.80 | ab | 12.72 | c | 15.09 | bc | 2.77 | *** | ** |
| 1-Pentanol (70) | 827 | a | 4.45 | a | 2.57 | b | 3.83 | a | 2.02 | bc | 1.82 | bc | 1.15 | c | 0.65 | *** | *** |
| Hexanal | 842 | a | 2774.14 | a | 1969.97 | b | 1223.18 | c | 768.59 | cd | 201.53 | d | 270.65 | d | 410.63 | ** | *** |
| 1-Hexanol | 924 | a | 81.87 | a | 39.79 | ab | 80.92 | ab | 37.78 | bc | 30.23 | c | 11.20 | c | 24.98 | *** | *** |
| Heptanal (70) | 941 | a | 17.36 | a | 17.18 | ab | 13.11 | ab | 8.69 | b | ND | b | ND | b | 5.62 | ns | ** |
| Pentanoic acid (60) | 980 | a | 4.00 | ab | 4.96 | a | 3.29 | b | 4.23 | ab | 1.08 | c | 1.30 | c | 0.73 | ** | *** |
| 2-Pentylfuran | 1010 | a | 25.20 | a | 25.22 | a | 15.42 | b | 17.78 | b | 3.79 | c | 4.28 | c | 3.51 | ns | *** |
| (*Z*)- 2-Heptenal | 1012 | a | 10.45 | a | 8.20 | ab | 5.90 | bc | 5.65 | c | 1.89 | d | ND | | 1.47 | * | *** |
| Octanal (56) | 1049 | a | 7.14 | a | 6.27 | a | 5.71 | ab | 3.71 | bc | 3.02 | c | 3.12 | c | 1.33 | * | *** |
| Hexanoic acid (60) | 1077 | a | 22.26 | a | 25.95 | a | 19.09 | a | 22.42 | a | 5.27 | b | 6.88 | b | 3.98 | 0.038 | *** |
| (E)-2-Octenal | 1117 | a | 13.03 | a | 11.01 | ab | 7.45 | bc | 7.76 | bc | 1.37 | d | 5.17 | cd | 2.25 | ns | *** |
| Nonanal | 1151 | a | 122.97 | | 95.75 | | 122.58 | | 75.31 | | 97.86 | | 87.20 | | 30.58 | ** | ns |
| (E)-2-Nonenal | 1224 | a | 3.69 | a | 3.59 | a | 2.36 | b | 2.15 | b | 1.11 | c | 1.92 | bc | 0.49 | ns | *** |
| Octanoic acid | 1267 | a | 11.02 | d | 18.10 | a | 11.42 | cd | 16.30 | ab | 12.29 | bcd | 16.16 | abc | 2.76 | *** | ns |
| Nonanoic acid (60) | 1359 | a | 1.70 | | 1.70 | | 1.56 | | 1.53 | | 1.51 | | 1.03 | | 0.96 | ns | ns |
| **Thiamine degradation** | | | | | | | | | | | | | | | | | |
| 2-Methyl-3-furanthiol | 896 | a | tr | | tr | | tr | | tr | | tr | | tr | | | | |
| Methyl-2-methyl-3-furyldisulfide | 1221 | a | tr | | tr | | tr | | tr | | tr | | tr | | | | |
| **Spices** | | | **3105.15** | | **3210.12** | | **3087.19** | | **2804.85** | | **3299.25** | | **2941.91** | | **376.00** | **ns** | **ns** |
| O-Xylene (91) | 917 | a | 0.84 | | 0.72 | | 0.70 | | 0.67 | | 0.69 | | 0.59 | | 0.14 | ns | ns |

(*continued on next page*)

**Table 2** (*continued*)

| | | | C[3] | | | | R | | | | RY | | | | RMSE[4] | $P_t$[5] | $P_f$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LRI[1] | RI[2] | 60 d | | 83 d | | 60 d | | 83 d | | 60 d | | 83 d | | | | |
| Styrene (104) | 919 | a | 0.57 | c | 0.78 | c | 0.51 | c | 0.64 | c | 3.17 | a | 2.53 | b | 0.19 | ns | *** |
| Unidentified terpene | 934 | c | 165.77 | | 141.31 | | 168.37 | | 138.55 | | 160.73 | | 141.74 | | 20.13 | *** | ns |
| β-Phellandrene | 988 | b | 460.31 | | 419.40 | | 486.90 | | 407.33 | | 490.25 | | 440.91 | | 66.65 | * | ns |
| β-Myrcene (93) | 1003 | a | 29.80 | | 33.77 | | 29.68 | | 28.42 | | 33.01 | | 29.16 | | 4.26 | ns | ns |
| Unidentified terpene | 1019 | c | 113.30 | | 121.94 | | 120.30 | | 105.53 | | 126.23 | | 110.98 | | 19.27 | ns | ns |
| 3-Carene | 1022 | a | 571.43 | | 630.26 | | 555.16 | | 515.62 | | 638.08 | | 540.43 | | 100.34 | ns | ns |
| Unidentified terpene | 1034 | c | 51.50 | ab | 48.03 | b | 55.76 | ab | 48.49 | b | 61.38 | a | 55.69 | ab | 6.19 | * | ** |
| D-limonene | 1046 | a | 1081.58 | | 1170.91 | | 1063.91 | | 1004.14 | | 1133.01 | | 1022.63 | | 129.31 | ns | ns |
| Unidentified terpene | 1075 | c | 68.52 | | 68.29 | | 68.15 | | 61.41 | | 83.39 | | 66.98 | | 17.45 | ns | ns |
| Unidentified terpene (93) | 1099 | c | 0.99 | | 0.98 | | 0.80 | | 0.81 | | 0.90 | | 0.78 | | 0.14 | ns | ns |
| Terpinolene (93) | 1101 | a | 3.70 | | 3.86 | | 3.75 | | 3.31 | | 4.08 | | 3.48 | | 0.50 | ns | ns |
| Unidentified terpene | 1121 | c | 15.05 | ab | 19.32 | a | 14.83 | ab | 17.49 | ab | 14.48 | b | 19.14 | a | 2.57 | *** | ns |
| Unidentified terpene | 1366 | c | 26.89 | a | 26.61 | a | 22.58 | ab | 20.12 | b | 23.74 | ab | 21.74 | ab | 3.62 | ns | ** |
| Unidentified terpene | 1377 | c | 8.24 | | 9.49 | | 6.77 | | 7.60 | | 7.66 | | 8.29 | | 2.50 | ns | ns |
| Unidentified terpene | 1407 | c | 71.50 | ab | 77.51 | a | 64.64 | ab | 59.95 | b | 66.96 | ab | 64.48 | ab | 9.45 | ns | ** |
| Caryophyllene | 1470 | a | 435.18 | | 436.96 | | 424.39 | | 384.77 | | 451.48 | | 412.34 | | 49.79 | ns | ns |
| **Unknown origin** | | | **27.74** | **b** | **29.19** | **c** | **34.36** | **ab** | **39.25** | **a** | **63.34** | **ab** | **64.12** | **ab** | **13.80** | **ns** | *** |
| 2-Methyl-1-propene (41) | 459 | b | 4.87 | a | 4.08 | ab | 2.80 | bc | 2.13 | c | 1.42 | c | 1.61 | c | 0.88 | ns | *** |
| Carbon disulfide | 537 | a | 21.97 | b | 23.95 | b | 30.58 | b | 36.01 | b | 66.95 | a | 61.37 | a | 13.87 | ns | *** |
| 4-Methylphenol (107) | 1197 | a | 0.90 | b | 1.16 | a | 0.98 | b | 1.11 | a | 0.97 | b | 1.14 | a | 0.07 | *** | ns |

[1]LRI: Linear retention index of the compounds eluted from the GC–MS using a DB-624 capillary column. [2]RI: Reliability of identification: a, identification by mass spectrum and by coincidence with LRI of authentic standard; b, tentative identification by mass spectrum; c: unknown. [3]C: control batch; R: 47% reduction in nitrite and nitrate; RY: 47% reduction in nitrite and nitrate and *D. hansenii* inoculated. [4]RMSE: Roost mean square error. [5]$P_t$: *P* value of ripening time effect, $P_f$: *P* value of formulation effect. Different letters in the same row indicate significant differences at *** P < .001, ** P < .01, * P < .05. ns: *P* > .05.[6]tr: traces, not quantified. Bold indicates the group of origin of the volatile compounds

present in significant high proportions in RY sausages. During the ripening time between 60 and 83 d, the total abundance of volatile compounds derived from carbohydrate fermentation increased exclusively in C sausages due to the increase in acetic acid.

Ethyl acetate was the most abundant volatile compound derived from esterase activity in all sausage types, followed by ethyl-3-methylbutanoate in RY sausages. The total abundance of volatile compounds derived from esterase activity was significantly higher in RY sausages than in C and R sausages at both drying times. This difference was observed in all ester compounds except for ethyl pentanoate and ethyl hexanoate. In addition, 3-methylbutyl acetate and ethyl-2-methylpropanoate were exclusively present in RY sausages. Nitrate and nitrite reduction in R produced a decrease in two ester compounds, ethyl hexanoate and ethyl 2-hydroxypropanoate at 60 d although the additional drying time increased its abundance.

Among volatile compounds derived from chemical origin only those derived from lipid oxidation were analysed being the most abundant hexanal in all sausages. These compounds were the lowest in RY sausages at both ripening times. This was due to a significant decrease of most volatiles except for 1-propanol. The last step of ripening from 60 to 83 d, caused a decrease in the total abundance of volatile compounds derived from lipid oxidation although pentanoic and octanoic acids increased during this period.

Only two volatile compounds from thiamine degradation (2-methyl-3-furanthiol and methyl-2-methyl-3-furyldisulfide) were identified as traces. Finally, volatile compounds derived from spices were abundant in all sausage formulations but most of them were not affected by either formulation or drying time.

### 3.5. Aroma characteristics of the sausages

Gas chromatography-olfactometry (Table 3) revealed the presence of 29 aroma active zones which were identified except for 1 odour zone attributed to an identified terpene. The most important aroma compounds with the highest DF (detection frequency) values (DF > 10) were 2,3-butanedione, acetic acid, hexanal, ethyl 2-methylbutanoate, 1-hexanol, 2-acetyl-1-pyrroline, methional, dimethyl trisulfide and 3-carene. The assessors were able to detect five odour zones that corresponded to compounds 2-methyl-3-furanthiol, 2-acetyl-1-pyrroline, dimethyl trisulfide, 2-acetyl-2-thiazoline and methyl-2-methyl-3-furyldisulfide which were not analysed by mass spectra due to their presence as trace levels.

The relationship between the instrumental variables and the ingoing amounts of nitrate and nitrite and *D. hansenii* inoculation at each ripening time, were examined by a principal component analysis (Fig. 3). Comparisons were done using the parameters (physicochemical, microbiological parameters and aroma compounds) measurements at 60 (Fig. 3 A and B) and 83 d (Fig. 3C and D) of ripening. Regarding results at 60 d (Fig. 3A), the first two principal components were able to explain the 72.09% of the total variance. PC1 accounted for 56.34% of the variance and distinguished sausages by *D. hansenii* inoculation, placing C and R sausages on the left quadrant and RY sausages on the right quadrant. Sausages inoculated with *D. hansenii* RY, were associated with higher water activity, pH, yeast and moulds and colour parameters. Regarding aroma compounds, RY sausages were related to those derived from esterase activity, as well as 3-methylbutanal and methanethiol resulting from amino acid degradation. On the other hand, C and R sausages were related to an increase in TBARS and aroma compounds derived from lipid oxidation, β-oxidation and carbohydrate fermentation, as well as methional resulting from amino acid degradation. PC2 represented 15.76% of the variance corresponding to the variability among replicates.

Regarding results at 83 d of drying (Fig. 3C and D), two principal components were able to explain the 70.40% of the total variance. PC1 accounted for 56.21% of the variance and distinguished sausages by *D. hansenii* inoculation and nitrate and nitrite reduction, placing C sausages on the left quadrant, R sausages on the centre and RY sausages on the right quadrant. Sausages inoculated with *D. hansenii* RY, were again related to higher water activity, yeast and moulds, colour parameters and aroma compounds derived from esterase activity. Moreover, C sausages were again related to TBARS and aroma compounds derived from lipid oxidation, while the R sausages were related to GCN counts and aroma compounds derived from amino acid degradation and carbohydrate fermentation. PC2 represented 14.19% of the variance corresponding to the variability among replicates.

**Table 3**
Odour active compounds identified in dry fermented sausages.

| Compounds | IRL GC-O[1] | IRL std GC-O[2] | RI[3] | GC-O description | DF[4] |
|---|---|---|---|---|---|
| Methanethiol | 474 | 471 | a | Rotten, unpleasant | 9 |
| 1-Propanol | 611 | 614 | a | Acid, fermented | 4 |
| 2-Butanone | 628 | 632 | a | Wet, fresh, sweet | 3 |
| 2,3-Butanedione | 633 | 638 | a | Cheese, butter, dairy, fruit | 11 |
| 3-Methylbutanal | 690 | 691 | a | Sweet, green, spicy | 5 |
| Acetic acid | 701 | 700 | a | Acid, fermented, vinegar | 11 |
| 3-Hydroxy-2-butanone | 779 | 777 | a | Cardboard, green | 3 |
| Ethyl 2-methylpropanoate | 783 | 789 | a | Sweet, fruity | 4 |
| Ethyl butanoate | 824 | 825 | a | Sweet, fruity, fresh | 5 |
| Hexanal | 835 | 836 | a | Green, fresh cut grass, fatty | 12 |
| Ethyl 2-hydroxypropanoate | 861 | 859 | a | Fresh, floral, acid | 3 |
| Ethyl 2-methylbutanoate | 872 | 872 | a | Pineapple, sweet, acid, unpleasant, | 10 |
| Ethyl 3-methylbutanoate | 875 | 876 | a | Sweet, fruity, unpleasant, acid, | 7 |
| 2-Methyl-3-furanthiol | 899 | 897 | a | Fatty, medicinal, unpleasant, sulfur | 3 |
| 1-Hexanol | 925 | 919 | a | Fatty, rancid, rotten fruit, unpleasant | 12 |
| Heptanal | 942 | 939 | a | Cured, rancid, unpleasant | 6 |
| 2-Acetyl-1-pyrroline | 962 | 963 | a | Roasted, fried corn, roasted nuts, | 11 |
| Methional | 969 | 969 | a | Cooked potatoes, vegetable, meaty, sulfur | 11 |
| β-Myrcene | 1002 | 1003 | a | Green | 3 |
| Dimethyl trisulfide | 1011 | 1009 | a | Spicy, pungent, sulfur, rotten, vegetable | 11 |
| 3-Carene | 1021 | 1026 | a | Mushrooms, earthy, green, fresh | 12 |
| 1-Octen-3-ol | 1032 | 1028 | a | Green, fresh, mushrooms | 5 |
| Octanal | 1046 | 1047 | a | Sweet, citrus, floral | 7 |
| Hexanoic acid | 1068 | 1064 | a | Cheese, unpleasant, floral fresh | 3 |
| Unidentified terpene | 1092 | | b | Spicy, fresh, floral | 3 |
| Nonanal | 1150 | 1151 | a | Fresh, herbaceous, green, unpleasant | 7 |
| 2-Acetyl-2-thiazoline | 1180 | 1178 | a | Toasted, fried corn, caramel, toasted corn, bread | 8 |
| 4-Methylphenol | 1198 | 1190 | a | Unpleasant, rotten, sulfur | 6 |
| Methyl-2-methyl-3-furyl disulfide | 1225 | 1225 | a | Meaty, unpleasant, wet wood, fermented, rotten | 9 |

[1] Linear retention index of the compounds eluted from the GC-FID-O using a DB-624 capillary column.
[2] Linear retention index of standard compounds in the GC-FID-O
[3] Reliability of identification: a, identification by mass spectrum, coincidence with LRI of an authentic standard and by coincidence of the assesors's descriptors with those described in the Fenaroli's handbook of flavour ingredients (Burdok, 2002); b, tentative identification by mass spectrum.
[4] Detection frequency value.

## 4. Discussion

Physicochemical changes observed were similar to the ones reported for this type of product (Olivares et al., 2010; Perea-Sanz et al., 2018) (Table 1 and Fig. 1). The reduction in the ingoing amounts of nitrate and nitrite was confirmed. The lowest nitrate reduction observed during drying in C sausages respect to R and RY sausages may be due to the generation of nitrate from nitrite oxidation (Honikel, 2008). Moreover, other authors have shown that the use of nitrate and nitrite mixtures increases the amount of residual nitrate (Gratacós-Cubarsí et al., 2013). The lowest residual nitrate detected in inoculated sausages (RY) could be explained by the inhibition of nitrite oxidation by *D. hansenii* (Cano-García et al., 2014).

Nitrate and nitrite reduction did not affect pH value however, Aquilani et al. (2018) and Gonzales-Barron et al. (2015) observed a small increase in pH values due to the absence of nitrate and/or nitrite in dry fermented sausages. These differences among studies can be due to differences in formulations as well as differences in process manufacture like long drying times that may cause a pH increase. In RY sausages, *D. hansenii* consumption of organic acids and ammonium production (Flores, Corral, Cano-García, Salvador, & Belloch, 2015) increased pH at the end of the ripening.

Regarding lipid oxidation, a decrease in TBARS in R sausages was observed in previous studies (Perea-Sanz et al., 2018). However, this is not in agreement with the reported antioxidant ability of nitrite (Zanardi, Ghidini, Battaglia, & Chizzolini, 2004). Similar reductions in TBARS were observed in nitrite reduced Pastrima although they were related to the stage of processing (Aksu, Erdemir, & Çakici, 2016). The chemistry of nitrate and nitrite in cured meats is a complex subject (Majou & Christieans, 2018). The nitric oxide generated from nitrite can react with neutrophil-derived superoxide present in muscle tissues and generate peroxynitrite ($ONOO^-$), which has prooxidant activity and antimicrobial effects (Brannan, Connolly, & Decker, 2001). Thus, in fermented sausages with high ingoing amounts of nitrate and nitrite, peroxynitrite formation might increase oxidation events including lipid oxidation. The nitrite prooxidant effect of peroxinitrite is directly related with its antimicrobial effect which is more harmful against Gram positive anaerobic bacteria, like *C. botulinum* (Majou & Christieans, 2018). On the contrary, the antioxidant capacity of *D. hansenii* limits lipid oxidation processes as shown by its great antioxidant capacity (Corral et al., 2015) during the entire drying time and, moreover, prevents nitrite oxidation as observed by the lowest residual nitrate content in RY.

On the other hand, the reduction of the ingoing amounts of nitrate and nitrite did not have effect on the microbial counts. LAB counts at both drying times were stable as reported by Christieans et al. (2018), and GCN and yeasts and moulds counts (Fig. 1A, C) were comparable to those found by Cano-García et al. (2014). On the contrary, Hospital et al. (2015) reported an increase in GCN related to nitrite and nitrate reduction in dry-fermented sausages and a similar effect was observed in Pastrima (Aksu et al., 2016). Similar findings were reported by Gonzales-Barron et al. (2015) in dry-fermented sausages, although the presence in the formulation of polyphosphate, which produced a pH rise effect, was the main factor affecting the growth of acid sensitive microorganisms. In our formulations C and R, the absence of differences in pH due to the similar LAB growth corroborated that the reduction of nitrate and nitrite levels did not affect GCN growth. Regarding the absence of *Enterobacteriaceae*, this indicated that the process was carried out with good hygiene practices, which are important to assure microbial safety. The inoculation of *D. hansenii* in RY sausages was confirmed by the yeasts and moulds counts during the whole process and presence of M13 RAPD PCR pattern of *D. hansenii* L1 (Fig. 2). The

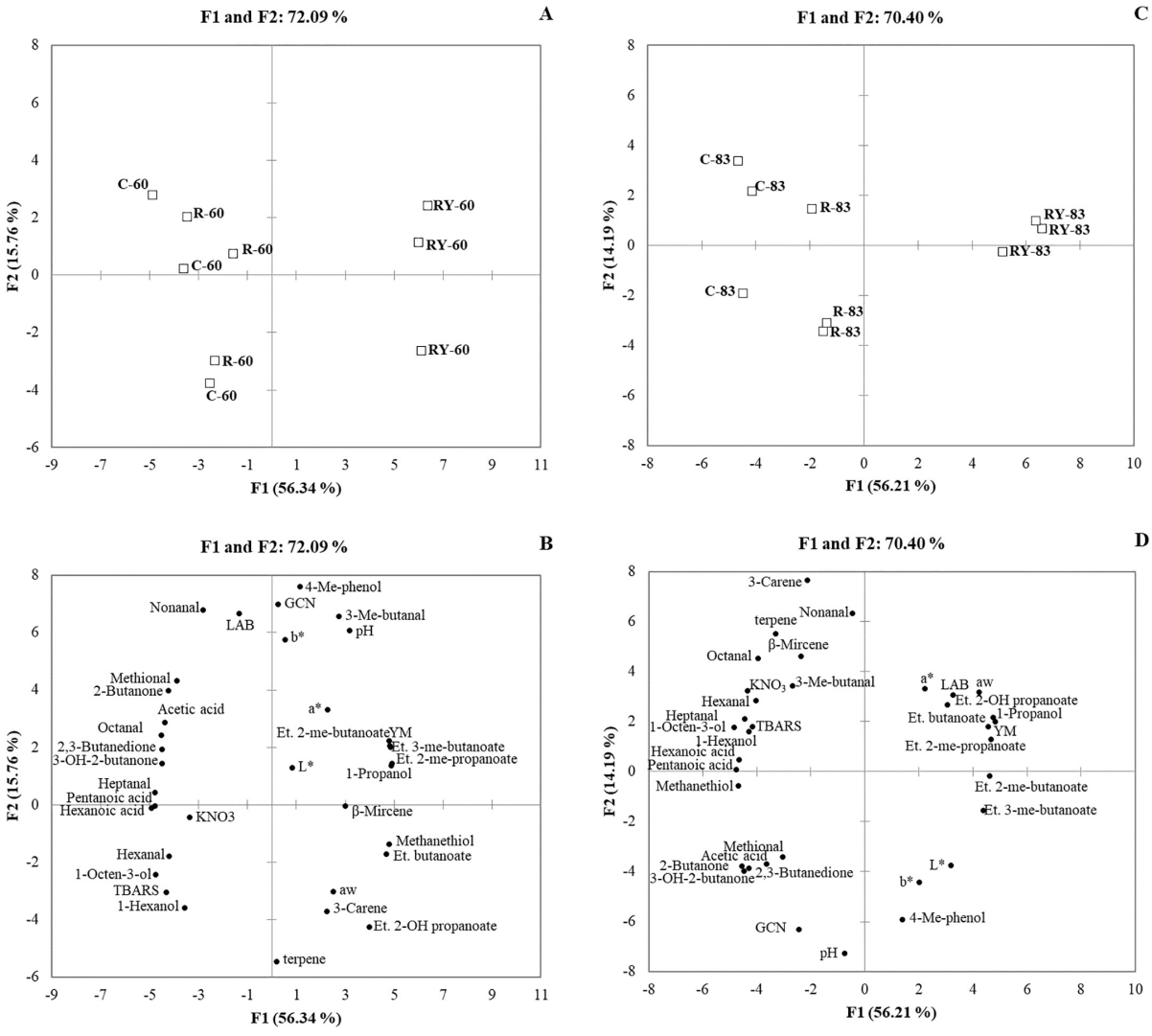


**Fig. 3.** Loadings of the first two principal components (PC1-PC2) representing the variability (physicochemical, microbiological parameters and aroma compounds) of the three batches of dry fermented sausages at 60 d (A and B) and 83 d (C and D) of ripening. C (control), R (47% reduction of nitrate and nitrite), RY (47% reduction of nitrate and nitrite and inoculated with *D. hansenii*). YM: yeasts and moulds; LAB: lactic acid bacteria; GCN: Gram positive coagulase negative cocci.

**Fig. 3.** (*continued*)

presence of *D. hansenii* L1 patterns in yeasts isolated from C and R sausages could be due to yeast cross contamination in the drying chamber. Nevertheless, the cell load (Fig. 1) is very different between samples, leading to a scarce impact of these isolates in R and C sausages.

The main mechanisms involved in generation of volatile compounds with aroma impact on fermented sausages are from microbial origin, chemical oxidation (Flores & Olivares, 2015) and thiamine degradation (Flores, 2018). Contrary to Hospital et al. (2015), total volatile compounds derived from amino acid degradation were not affected by the reduction of ingoing amounts of nitrate and nitrite. Besides, amino acid derived volatile compounds were in high abundance in sausages inoculated with *D. hansenii* strain L1 (Table 2) due to the yeast strain amino acid degradation capacity (Perea-Sanz, Montero, et al., 2019a). On the other hand, our results indicated that *D. hansenii* might have consumed acetic acid and produced ethanol in contrast to what happened in C and R sausages (Table 2) (Cano-García et al., 2014). Esterase activity in dry-fermented sausages is carried out by *Staphylococcus* and *Debaryomyces* species (Sthanke, 1994; Flores, Dura, Marco, & Toldrá, 2004). Accordingly, *D. hansenii* inoculation in RY sausages increased production of esters compounds (Flores et al., 2004). Morevoer, *D. hansenii* produced a strong decrease in lipid oxidation volatile compounds in agreement with the low TBARS values and the high antioxidant capacity of this yeast (Corral et al., 2015). Nitrate and nitrite reduction and *D. hansenii* did not affect volatile compounds derived from black pepper, which was used as spice in the formulations.

Generation of volatile compounds derived from amino acid degradation reactions in C and R sausages was not altered from 60 to 83 d (Table 2). However, RY sausages showed a decline in volatile compounds derived from amino acid degradation indicating a probable transformation of these into other compounds. The abundance of volatile compounds derived from lipid oxidation decreased in C and R

sausages due to the further drying (Perea-Sanz et al., 2018).

Concerning volatile compounds with direct impact on the sausage aroma profile (Table 3) at 60 d of ripening, the reduction of the ingoing amounts of nitrate and nitrite produced few aroma differences in contrast to the highest effect produced by *D. hansenii*, inoculation. The yeast prevented oxidation reactions and generation of aromas derived from lipid oxidation (Flores et al., 2004) which may produce unpleasant rancid odour notes in the sausages. In addition, it was responsible for the production of ester compounds which provided fruity and sweet odours to the sausages (Cano-García et al., 2014; Corral et al., 2015). The decrease on carbohydrate fermentation aromas caused by *D. hansenii* inoculation could be explained by the yeast consumption of acetic acid (Table 2). In general, *D. hansenii* inoculation decreased acid notes detrimental to sausage aroma (Flores, 1997). On the other hand, at 83 d of ripening the effect of nitrate and nitrite reduction between C and R on aroma profile is more clear (Fig. 3C and D) (Perea-Sanz, Montero, et al., 2019a) and *D. hansenii* yeast contributed to fruity notes (ester compounds) and prevented oxidation events. In addition, the relationship between nitrate/nitrite reduced fermented sausages (R) and the aroma compounds derived from carbohydrate fermentation (acetic acid, 2-butanone, 2,3-butanedione, 3-hydroxy-2-butanone, Fig. 3D) may be explained by the GCN metabolic activity, although no differences in GCN growth were observed between nitrate/nitrite reduced and no-reduced sausages.

## 5. Conclusions

Nitrite and nitrate reduction in fermented sausages did not affect microbial growth but decreased lipid oxidation and generation of derived volatiles. Microorganisms metabolic activity caused differences in the aroma profile at the longest drying time. The nitrite prooxidant and antimicrobial effects were confirmed in C sausages. *D. hansenii* inoculation limited lipid oxidation and increased generation of volatile compounds derived from amino acid degradation and esterase activity. The antioxidant capacity of *D. hansenii* during the entire drying time prevented nitrite oxidation as demonstrated by the lowest residual nitrate levels in RY sausages. In summary, yeast inoculation counteracted the negative impact of nitrite and nitrate reduction on aroma due to its antioxidant capacity during drying time, aroma production and hindered nitrite oxidation.

## Credit author statement

L. Perea-Sanz carried out the experiments and wrote the manuscript with support from C. Belloch and M. Flores. JJ López-Díez assisted in technical experiments. C. Belloch and M. Flores supervised the project and contributed to the design and analysis of the results.

## Declaration of Competing Interest

None.

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