SELECCIÓN DE LEVADURAS DE *DEBARYOMYCES HANSENII* Y DETERMINACIÓN DE SU EFECTO EN LA CALIDAD AROMÁTICA DE LOS EMBUTIDOS CRUDOS CURADOS

TESIS DOCTORAL

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Hace constar:

Que el trabajo de investigación titulado "Selección de levaduras de *Debaryomyces hansenii* y determinación de su efecto en la calidad aromática de los embutidos crudos curados" presentado por Dña. Liliana Cano García para optar al grado de Doctor por la Universidad Politécnica de Valencia, ha sido realizado bajo su dirección y supervisión en el Instituto de Agroquímica Tecnología de los Alimentos (IATA-CSIC), reuniendo las condiciones exigidas para ser defendida por su autora.

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Resumen

El aroma de los embutidos crudos curados es uno de los atributos más valorados por los consumidores. Por este motivo, es de gran importancia para la industria cárnica conocer los principales compuestos aromáticos responsables de la mayor aceptación de los productos tradicionales, su evolución a lo largo del proceso de fabricación así como los factores que fomentan la generación de dichos compuestos. Una de las posibles causas de la formación de determinados compuestos volátiles en embutidos crudos curados tradicionales es la presencia de una microbiota autóctona cuyo metabolismo y actividad favorece el desarrollo y la mejora del aroma en dichos productos.

En la presente tesis se ha realizado un estudio sobre las levaduras presentes en embutidos crudos curados tradicionales y su contribución a la generación de compuestos volátiles con poder aromático.

Por un lado, se han aplicado técnicas moleculares para la identificación y caracterización de levaduras aisladas de embutidos crudos curados tradicionales. Por otro lado, se ha estudiado el potencial aromático de cada una de las levaduras caracterizadas mediante el análisis de los compuestos volátiles por microextracción en fase sólida (SPME) y cromatografía de gases acoplada a espectrómetro de masas (GC-MS). Además, esta técnica también se ha utilizado para estudiar el efecto de las cepas de levaduras seleccionadas por su potencial aromático sobre la generación de compuestos volátiles en un sistema modelo cárnico y en embutidos crudos curados tradicionales.

En esta Tesis Doctoral se ha demostrado la existencia de una gran variabilidad genética en las cepas de la especie *Debaryomyces hansenii*

aisladas de embutidos crudos curados tradicionales. Por otra parte, se ha verificado la contribución de dos de las cepas de *D. hansenii* seleccionadas al perfil aromático de los embutidos crudo curados. Por último, se ha propuesto utilizar la técnica de espectrometría de masas directa Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS) para evaluar la inoculación de levaduras en embutidos crudos curados y se ha demostrado la eficacia de esta técnica para relacionar la formación de ciertos compuestos volátiles con la población de las diferentes levaduras inoculadas.

Abstract

The aroma of dry fermented sausages is one of the most appreciated attributes by consumers. Therefore, one of the main objectives of the meat industry is to understand the highest acceptance of traditional meat products, the main aromatic compounds present and the factors contributing to their generation as well as their evolution along processing. Among the reasons for the generation of aromatic compounds in traditional dry fermented sausages is the presence of an autochthonous microbiota which metabolism and activity favours the generation and improvement of aroma in these products.

The present Thesis has studied the yeast isolated from traditional dry fermented sausages and their contribution to the generation of volatile compounds with aroma activity.

On the one hand, molecular techniques were applied to identify and characterize the isolated yeasts from traditional dry fermented sausages. On the other hand, the aromatic potential of the characterized yeasts was studied through the analysis of volatile compounds using Solid Phase Micro Extraction (SPME) and Gas Chromatography with Mass Spectrometry (GC-MS). In addition to that, this technique was also applied to study the effect of the yeast strains selected by their aromatic potential, in the generation of volatile compounds when inoculated in meat models system and in traditional dry fermented sausages.

This Doctoral Thesis confirmed the large genetic variability within strains of *D. hansenii* isolated from traditional dry fermented sausages and confirmed the contribution of two selected *D. hansenii* strains to the aroma profile of dry fermented sausages. Finally, a direct mass spectrometric

technique Selected Ion Flow Tube – Mass Spectrometry (SIFT-MS) was used to evaluate the yeast inoculation in dry fermented sausages and the results confirmed that this is a reliable technique for monitoring changes in the volatile compounds associated to yeast population.

Resum

L'aroma dels embotits crus curats és un dels atributs més valorats pels consumidors. Per aquest motiu, és de gran interés per a la indústria càrnica conéixer els principals compostos aromàtics responsables de la major acceptació dels productes tradicionals, la seua evolució al llarg del procés de fabricació així com els factors que fomenten la generació d'aquestos compostos. Una de les possibles causes de la formació de determinats compostos volàtils en embotits crus curats tradicionals és la presència d'una microbiota autòctona amb un metabolisme i activitat que afavorisen el desenvolupament i la millora de l'aroma en els productes anomenats.

En la present tesi es va realitzar un estudi dels llevats presents en embotits crus curats tradicionals i la seua contribució en la generació de compostos volàtils amb poder aromàtic.

Per una banda, es van aplicar tècniques moleculars per la identificació i caracterització dels llevats aïllats d'embotits crus curats tradicionals. Per altra banda, es va estudiar el potencial aromàtic de cadascun dels llevats caracteritzats mitjantçant l'anàlisi dels compostos volàtils per micro extracció en fase sòlida (SPME) i cromatografia de gasos acoplada a espectròmetre de masses (GC-MS). A més a més, aquesta tècnica també es va aplicar per estudiar l'efecte que tenen els ceps de llevats seleccionats pel seu potencial aromàtic en la generació de compostos volàtils en sistemes models càrnics i en embotits crus curats tradicionals.

En aquesta Tesi Doctoral es va demostrar l'existència d'una gran variabilitat genètica en els ceps de l'espècie *Debaryomyces hansenii* aïllats d'embotits crus curats tradicionals. Per altra banda, es va verificar la contribució de dos dels ceps de *D. hansenii* seleccionats al perfil aromàtic

dels embotits crus curats. Per últim, es va proposar utilitzar la tècnica de espectrometria de masses directa Selected Ion Flow Tube – Mass Spectrometry (SIFT-MS) per evaluar l'inoculació de llevats en embotits crus curats on es va demostrar l'eficàcia d'aquesta tècnica per relacionar la formació de determinats compostos volàtils amb la població dels diferents llevats inoculats.

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I. INTRODUCCIÓN

I.1. El sector cárnico

La industria cárnica es uno de los cinco sectores más importantes en nuestro país. El número de empresas de la industria alimentaria, según datos del INE, es de 29.190 de las que 4.144 pertenecen al sector de las industrias cárnicas solo superado por el sector del pan, pastelería y pastas alimenticias. En el año 2011, el 21,6% de las ventas de la industria de alimentación y bebidas fue facturado por las industrias cárnicas, ocupando el primer lugar por encima de otros sectores como el de la pesca, industrias lácteas, conservas de frutas y hortalizas, grasas y aceites, etc (MAGRAMA, 2012). Esta producción se concentra en tres sectores: aviar, vacuno y porcino cuyo volumen de producción representó en el 2012 el 82,5% de las carnes de ungulados producidas en nuestro país. Con este volumen de producción, España se consolida como el cuarto productor mundial de carne de porcino, por detrás de China, EE.UU. y Alemania (ANICE, 2013). No obstante, la importancia económica del sector cárnico en España no sólo se debe al consumo de carne fresca sino también al de los productos cárnicos derivados. Así, España es uno de los países con mayor tradición en la elaboración y consumo de productos cárnicos derivados con una producción anual de 1,3 millones de toneladas, ocupando el cuarto lugar en Europa en la elaboración de estos productos. Por tipos de productos, el consumidor español prefiere los jamones curados (84,5%), aunque los productos de charcutería como los fiambres (74,6%) y embutidos crudos curados (59,2%) han ido captando cuota de mercado (MAGRAMA, 2012; ANICE, 2013).

El proceso industrial de fabricación de productos cárnicos fermentados se está modificando con el objetivo de reducir los costes y estandarizar el proceso de fabricación. Las industrias están muy interesadas en llevar a cabo fermentaciones más cortas que permitan un mayor control de los microorganismos patógenos y una reducción del tiempo de fabricación pudiendo comercializar sus productos en pocas semanas. Sin embargo, estas nuevas tecnologías también afectan negativamente a las propiedades organolépticas típicas del producto (Flores y col., 1997). Esto se debe a que el tipo de microbiota que se desarrolla durante la fermentación está fuertemente relacionada con la tecnología empleada en esta etapa. En los embutidos crudos curados fabricados mediante estas técnicas se suele inocular una elevada población de bacterias ácido lácticas que provocan un drástico y rápido descenso de pH debido a la acumulación de ácido láctico y, en menor medida, de ácido acético generando un sabor y un aroma ácido en el producto final (Montel y col., 1998).

En cambio, en los embutidos crudos curados elaborados de forma tradicional con fermentaciones lentas existe una mayor actividad de las bacterias ácido lácticas así como de otros microorganismos como las micrococáceas y las levaduras capaces de producir importantes cantidades de compuestos volátiles con gran impacto aromático (Lücke, 2000; Cocolin y col., 2011). Por ello, recientemente, los consumidores han mostrado un creciente interés por los “productos típicos” los cuales pueden ser considerados como alimentos producidos en una región determinada, elaborados en ambientes caseros (no industrializados) y a pequeña escala, empleando una limitada mecanización en el proceso, y que presentan características auténticas que permiten la identificación de un producto

concreto entre otros productos similares (Iaccarino y col., 2006; Conter y col., 2008). Sin embargo, los consumidores siguen exigiendo al mismo tiempo rapidez en el servicio, es decir, el consumidor quiere disponer de un producto típico en el mercado sin tener que esperar los largos tiempos de la elaboración tradicional (Conter y col., 2008). Esta demanda abre un amplio campo de investigación para perfeccionar las técnicas de fermentación rápida sin renunciar a las características organolépticas presentes en los productos cárnicos tradicionales.

En este sentido, la elección del estárter es uno de los factores determinantes en las características y calidad final de los embutidos crudos curados (Toldrá, 2006a). Los microorganismos nativos y los cultivos iniciadores presentes en la fermentación cárnica participan en los procesos de lipólisis y proteólisis liberando precursores aromáticos que son empleados como sustratos en reacciones de transformación de aminoácidos, fermentación de carbohidratos y oxidación lipídica en las que también actúan los cultivos iniciadores (Talón y col., 2007). Los principales estárters utilizados en embutidos crudos curados pertenecen a distintas especies de bacterias ácido lácticas y estafilococos (Hugas & Monfort, 1997; Toldrá, 2006a). Sin embargo, en algunos embutidos crudos curados producidos en el sur de Europa se han aislado hongos y levaduras con actividad lipolítica y proteólitica capaces de influir en el desarrollo del aroma final de estos productos (Ravyts y col., 2012). Existe una gran diversidad de hongos y levaduras en los embutidos crudos curados pero los más predominantes son *Penicillium nalgiovense* en el caso de los hongos mientras que entre las levaduras la especie mayoritaria es *Debaryomyces hansenii* seguida de las diversas especies del género *Candida* (Leroy y col.,

2006). Algunos autores sugieren que la presencia de la especie *D. hansenii* en los productos cárnicos mejora sus características sensoriales y contribuye al aroma (Encinas y col., 2000; Flores y col, 2004). No obstante, hay que tener en cuenta que diferentes cepas de una misma especie pueden presentar distintas actividades bioquímicas que desarrollen perfiles sensoriales diferentes en el producto final (Cocolin y col., 2011). Por todo ello, y teniendo en cuenta que el aroma es uno de los atributos más valorado por los consumidores, es de gran interés para la industria cárnica conocer cuál es el papel de la especie *D. hansenii* en el desarrollo del aroma en embutidos crudos curados para evaluar su empleo como cultivo estárter en dichos productos adaptándose mejor a las preferencias de los consumidores.

I.2. Embutidos crudos curados

Los embutidos crudos curados son productos que se elaboran con carne y grasa animal picadas a las que se añaden sales, especias y aditivos autorizados; los cuales son mezclados y embutidos en tripas naturales o artificiales y sometidos a un proceso de desecación. El proceso de fermentación es una técnica empleada para prolongar la vida de los productos y mejorar sus propiedades organolépticas (Cocconcelli & Fontana, 2008). Durante la fermentación tienen lugar complejas reacciones físicas y bioquímicas que modifican las características organolépticas destacando la producción de compuestos aromáticos que se generan en esta etapa de la fabricación y que determinará las características sensoriales del producto final.

Estos productos se caracterizan porque se consumen crudos, se conservan sin necesidad de refrigeración y tienen un tiempo de vida útil

muy largo. Además, poseen unas características organolépticas muy apreciables destacando su color, consistencia, aroma y sabor típicos. No obstante, la elaboración de embutidos fermentados varía sustancialmente de unos países a otros e incluso existen diferencias entre regiones dentro del mismo país. En España existe una amplia variedad de embutidos crudos curados con características muy diversas debido a variaciones en la composición de los embutidos y a la tecnología de elaboración, dependiendo de las costumbres regionales y locales (Flores, 1999). Según Leistner (1986), en la producción de embutidos crudos curados se emplea una tecnología imprecisa que permite grandes variaciones siempre que se mantenga el concepto básico de proporcionar una adecuada reducción del pH y/o de la actividad de agua (Aw).

Lücke en 1985 propuso una clasificación basándose en criterios relacionados con la pérdida de peso, aplicación o no de humo y crecimiento superficial de mohos y levaduras. En 1990, Roca & Incze emplearon una clasificación basada en el tiempo de fermentación y maduración estableciendo dos tipos de embutidos crudos curados: de maduración corta con un contenido final de agua en torno al 30 – 40 % y de maduración larga, con un contenido final de agua del 20 – 30 %. En este último grupo se incluye al chorizo y salchichón españoles.

Sin embargo, fue la entrada de España en la Unión Europea en 1986 y la importancia económica de los productos curados dentro del sector cárnico español lo que hizo necesario la elaboración de una clasificación y tipificación de los embutidos curados típicos españoles. Flores (1999) proporcionó una clasificación basándose en las características de las carnes utilizadas, el proceso de fabricación, características morfológicas y origen

geográfico. Así, los embutidos crudos curados quedarían clasificados en: chorizos, sobrasadas, lomo embuchado, morcones y salchichones.

I.3. Fabricación de embutidos crudos curados

La mayoría de los embutidos crudos curados se elaboran siguiendo un proceso similar. La figura 1 muestra el diagrama de flujo empleado en la fabricación de los embutidos crudos curados y que abarca las distintas fases del proceso incluyendo la selección de materias primas, preparación de la mezcla y embutido, y por último estufaje, desecación y/o ahumado.

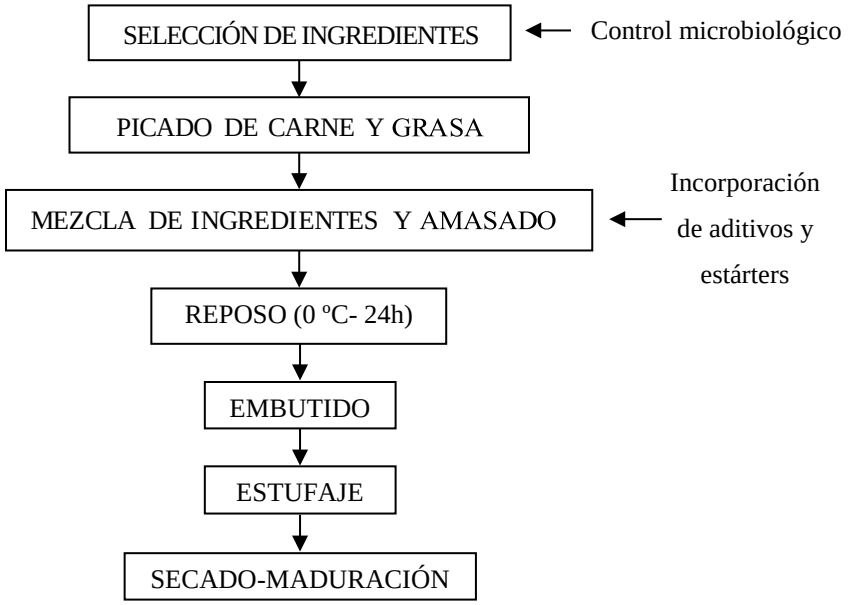

Figura 1.- Diagrama de flujo de la elaboración de embutidos crudos curados (adaptado de Toldrá & Flores, 2007).

I.3.1. Selección de materias primas

La calidad de las materias primas y la higiene del proceso son de vital importancia, por ello se debe llevar a cabo una buena selección de las materias primas empleadas así como un riguroso control microbiológico de las mismas (Solignat, 2002).

El proceso de fabricación se inicia con el picado de la carne magra (cerdo y/o vacuno) y la grasa de cerdo, empleando una temperatura de –2ºC para evitar la rotura de los glóbulos grasos que provocaría el embarrado de la masa cárnica (Olivares, 2011). La carne empleada debe estar sana, libre de coágulos de sangre, con un valor de pH entre 5,6 – 6,0 y ser conservada en refrigeración hasta el momento de la fabricación. La grasa seleccionada debe tener un alto grado de saturación para prevenir defectos de enranciamiento, por lo que se suele usar grasa de cerdo del depósito dorsal o panceta. La grasa influye de forma decisiva en la calidad sensorial del embutido condicionando la facilidad al corte, jugosidad, untuosidad y participando en el aroma y sabor del producto final (Olivares y col., 2010).

Por otra parte los aditivos que se añaden a los embutidos crudos curados son sustancias que regulan la maduración y la calidad microbiológica, modifican las características organolépticas e incrementan el periodo de conservación de los productos. Así, a los embutidos crudos curados se les añaden condimentos como la sal, nitratos, nitritos, azúcares y especias (pimentón, ajo, pimienta, etc.) (Ordóñez y col., 1999).

La sal se emplea como saborizante y por su acción conservadora ya que disminuye la actividad de agua inhibiendo el desarrollo de microorganismos patógenos y alterantes. Además, la sal favorece la

solubilización de las proteínas miofibrilares de la carne para lograr la ligazón de los embutidos formando un gel estable y consistente (Solignat, 2002; Honikel, 2007). El nitrato y nitrito juegan un papel fundamental en el desarrollo del color típico de los embutidos crudos curados mediante reacciones químicas y enzimáticas en las que participan microorganismos (micrococáceas) que reducen los nitratos a nitritos (Toldrá y col., 2001). Posteriormente, el nitrito se reduce a óxido nítrico que se combina con la mioglobina para formar nitrosomioglobina responsable del pigmento rojo del curado (Honikel, 2007). Otra función del nitrito es su acción bactericida sobre microorganismos indeseables como *Salmonella spp, Clostridium botulinum* y *Staphylococcus aureus* cuando se combina con bajos valores de actividad de agua y pH en el medio (Lücke, 1985). Los azúcares más utilizados en la elaboración de embutidos crudos curados son: glucosa, fructosa, sacarosa y lactosa. Todos ellos actúan como sustrato para los microorganismos responsables de la acidificación como son las bacterias ácido lácticas que reducen el pH de la masa cárnica a valores inferiores a 5,5. Esta bajada de pH reduce la capacidad de retención de agua favoreciendo la deshidratación del producto, inhibe el desarrollo de microorganismos patógenos y facilita la gelificación de las proteínas cárnicas, además la presencia de ácidos orgánicos contribuye al aroma del producto final (Ordóñez y col., 1999). En cuanto a las especias, su función es básicamente aromática y su empleo varía según el tipo de producto pero, en general, las más utilizadas son: pimentón, ajo, clavo, nuez moscada, cilantro, cebolla, orégano, pimienta y tomillo.

I.3.2. Preparación de la mezcla y embutido

A continuación, la carne picada se amasa a vacío con el resto de ingredientes y la masa cárnica resultante se deja reposar a 0 ºC durante 24 horas para favorecer la adaptación de la microbiota al medio y la ligazón de la masa cárnica debido a la solubilización de las proteínas por efecto de la sal (Solignat, 2002; Honikel, 2007). Transcurrida esta etapa, la masa cárnica es embutida en tripas, naturales o artificiales, permeables al agua.

I.3.3. Estufaje

Esta etapa de la fabricación se realiza en cámaras climáticas que permiten controlar las condiciones de temperatura y humedad relativa del ambiente, así como la velocidad del aire. Normalmente se emplean temperaturas comprendidas entre los 18 y 24 ºC durante 1 – 3 días con una humedad relativa inferior al 90% para evitar la condensación en la superficie del producto y la deshidratación excesiva de la tripa (Olivares, 2011).

Las piezas recién embutidas son sometidas a un estufaje cuyo objetivo es favorecer el crecimiento de las bacterias ácido lácticas responsables de la fermentación y de la acidificación del producto (Flores y col., 1997). Además, en esta fase las micrococáceas reducen los nitratos a nitritos iniciando el fenómeno de enrojecimiento.

I.3.4. Secado – maduración

En esta fase de la producción los embutidos crudos curados son sometidos a una fase de maduración – secado para deshidratarlos y alcanzar

así las características sensoriales típicas de estos productos (Olivares, 2011). Generalmente el secado se lleva a cabo a temperaturas inferiores a los 15 ºC y con humedades relativas comprendidas entre el 75 – 85% durante 30 – 60 días, además es muy importante controlar la velocidad y distribución del aire en los secaderos para alcanzar una deshidratación gradual en todo el producto. No obstante, hay que tener en cuenta que la correcta maduración y secado de estos productos también estará condicionada por otros factores como la composición de la masa original, el grado de picado, el pH y el calibre del embutido (Carballo y col., 2001). Existen dos técnicas claramente diferenciadas entre sí y que condicionan las características finales del producto final, así se puede hablar de fermentación industrial y fermentación tradicional. En las fermentaciones industriales, con la finalidad de acelerar la etapa de maduración, se aplican temperaturas que van desde los 22 ºC hasta los 26 ºC y se añaden cultivos iniciadores o estarters para conseguir un rápido descenso del pH, generar un suave sabor ácido y un color rojo vivo. La humedad relativa del aire se reduce paulatinamente del 95% al 75%, de forma que los productos crudos madurados presentan rápidamente una consistencia adecuada y se encuentran en condiciones para ser comercializados. Sin embargo, los efectos de la maduración rápida en las características organolépticas del producto final son muy variables y en ocasiones generan defectos que provocan el rechazo por parte del consumidor (Navarro y col., 2001).

Por otra parte, el proceso de fermentación tradicional se caracteriza por ser un proceso largo, con un descenso lento del pH y un secado gradual del producto empleando temperaturas inferiores a los 15 ºC y sin la adición de cultivos iniciadores. Así, esta fermentación tiene lugar de forma espontánea

producida por la microbiota endógena de las materias primas y del ambiente de la fabricación (Leroy y col., 2006; Talón y col., 2007). La diversidad de microorganismos presente en estas fermentaciones espontáneas es esencial para el desarrollo de las características organolépticas (Latorre-Moratalla y col., 2011). Numerosos estudios han aislado e identificado la microbiota endógena de los embutidos crudos curados tradicionales para determinar qué microorganismos son los responsables de esas cualidades tan apreciadas por los consumidores. Así, los principales microorganismos aislados en estos productos son las bacterias ácido lácticas (LAB) y los estafilococos coagulasa negativa (CSN) y que además, son muy activos participando en la acidificación y desnitrificación de los mismos así como en los procesos de lipólisis y proteólisis (Hammes & Hertel, 1998; Cocolin y col., 2001; Aymerich y col., 2003, 2006; Comi y col., 2005; Martín y col., 2006; García-Fontán y col., 2007). Sin embargo, en algunos embutidos crudos curados tradicionales, principalmente aquellos elaborados en Francia, Italia y España, las propiedades sensoriales de estos productos también se ven modificadas por el desarrollo de una microbiota superficial constituida principalmente por levaduras y algunos hongos filamentosos (Lücke, 2000; Andrade y col., 2010b; Mendonça y col., 2013). Las elevadas poblaciones de estos microorganismos sugieren que éstos juegan un importante papel en la maduración de estos productos cárnicos influyendo en su aroma y textura final (Durá y col., 2004b; Flores y col., 2004; Martín y col., 2006; Andrade y col., 2010b).

La gran variedad de embutidos crudos curados tradicionales encontrados en una región geográfica se debe a las diferentes materias primas e

ingredientes utilizados así como al proceso de fabricación empleado que depende de las tradiciones de cada zona (Toldrá, 2006a). Esto da lugar a embutidos crudos curados con características organolépticas muy diferentes entre sí, y especialmente frente a los embutidos crudos curados elaborados de forma industrial que presentan características sensoriales estandarizadas. Por ello, los embutidos crudos curados tradicionales son mejor valorados por los consumidores quienes aprecian una calidad superior en los mismos y unas cualidades que no son encontradas en los embutidos industriales (Moretti y col., 2004).

I.4. Microbiota cárnica

El importante papel que juegan los microorganismos en los procesos de curado de los productos cárnicos se traduce en el empleo de cultivos estárter que presenten las propiedades adecuadas al tipo de fermentación que se vaya a realizar. Estos cultivos iniciadores son dominantes frente a la microbiota espontánea, se adaptan al medio y deben cumplir cuatro objetivos: (1) favorecer el estado sanitario del producto final inhibiendo el desarrollo de microorganismos patógenos, (2) prolongar la vida útil del producto impidiendo cambios indeseables debido a las reacciones microbianas o abióticas durante la comercialización, (3) mejorar las características organolépticas y (4) aumentar los efectos beneficiosos del producto final (Lücke, 2000). Sin embargo, a la hora de adicionar el estárter hay que tener en cuenta la concentración adecuada para obtener los efectos deseados (Durá y col., 2004b) y conseguir una distribución homogénea del mismo en el producto (Katsaras & Leistner, 1991). La carne es un medio ideal para el crecimiento microbiano ya que presenta un pH entre 5,8 y 6,8,

es rica en componentes nitrogenados y posee factores de crecimiento así como minerales, vitaminas y pequeñas cantidades de carbohidratos (Dillon & Board, 1991). Los principales estárter utilizados en la elaboración de los productos cárnicos fermentados son las bacterias ácido lácticas y las micrococáceas, aunque también se han empleado levaduras y mohos como microbiota de superficie con la finalidad de aromatizar y mejorar la apariencia externa (Hammes y col., 1990).

I.4.1. Bacterias ácido lácticas

Las bacterias ácido lácticas son los microorganismos predominantes en los embutidos crudos curados debido a las condiciones de anaerobiosis, la adición de sales y azúcares, así como un valor de pH inicial bajo (< 5,8) (Lücke, 1998). Dentro de estos microorganismos se engloban cuatro géneros: *Lactobacillus*, *Lactococcus*, *Leuconostoc* y *Pediococcus*. Sin embargo, el género *Lactobacillus* es el que predomina de forma natural en los embutidos crudos curados por lo que las cepas aisladas de estos productos son empleadas como cultivos iniciadores debido a su buena adaptación e implantación en el medio (Hammes y col., 1990; Hammes & Knauf, 1994). En las pastas de los embutidos crudos curados se estima que existen entre 10^6 y 10^7 bacterias ácido lácticas por gramo (Carrascosa, 2001).

La principal función tecnológica de las bacterias ácido lácticas en los embutidos crudos curados se basa en su capacidad para producir ácido láctico a partir de los carbohidratos añadidos a la masa como son la galactosa, glucosa, sacarosa, maltodextrinas, etc (Cabeza, 2006). Este ácido láctico produce efectos beneficiosos sobre el sabor y el aroma (Mateo y

col., 1996; Montel y col., 1998), la textura y la conservación del embutido crudo curado (Bañón y col., 2011). La acumulación del ácido láctico provoca un descenso del pH que inhibe el crecimiento de microorganismos indeseables como *Salmonella* y *Staphylococcus aureus* en estos productos (Schillinger & Lücke, 1989), disminuye la población de *Listeria monocytogenes* (Campanini y col., 1993; Hugas y col., 1995) y *Escherichia coli* (Incze, 1998) hasta niveles inocuos para el consumidor, y aumenta la consistencia de los embutidos crudos curados. Ademas, algunas especies de bacterias ácido lácticas como son *Lactobacillus plantarum* y *Lactobacillus pentosus* poseen enzimas nitrato reductasas que participan en el proceso de enrojecimiento (Wolf & Hammes, 1988). Finalmente, distintos autores han defendido que la actividad lipolítica y proteolítica que pueden desarrollar ciertas bacterias ácido lácticas en los embutidos crudos curados favorece la formación del aroma (Demeyer, 1982; El Soda y col., 1986; Papon & Talón, 1988; Nielsen & Kemnner, 1989; Fadda y col., 1998, 1999a, 1999b, 2002; Pereira y col., 2001).

I.4.2. Micrococáceas

Las micrococáceas más empleadas como cultivos iniciadores en los embutidos crudos curados pertenecen a las especies *Staphylococcus xylosus*, *Staphylococcus carnosus* y *Kocuria varians*. Estos microorganismos se caracterizan por poseer enzima catalasa, ser sensibles a los ácidos y tolerar bien la baja Aw (Ravyts y col. 2012).

En cuanto a los beneficios tecnológicos que aporta la utilización de micrococáceas como cultivos iniciadores figura la contribución a la formación y estabilización del color y su participación en la formación del

aroma (Ravyts y col. 2012). Sus enzimas nitrato reductasas reducen los nitratos a nitritos que posteriormente se reduce a óxido nítrico el cual se combina con la mioglobina para formar nitrosilmioglobina o pigmento rojo del curado. Además su actividad catalasa desdobla el peróxido de hidrógeno responsable de los defectos de coloración y de sabores rancios. Su papel también es importante en el control de la oxidación lipídica así como en los procesos de lipólisis y proteólisis donde debido a su capacidad para generar péptidos, aminoácidos, aldehídos, aminas y ácidos grasos libres favorece la síntesis de aromas que mejoran la calidad organoléptica del producto final (Leroy y col., 2006). En la tabla 1 se muestran las especies de bacterias ácido lácticas y micrococáceas más utilizadas como estárter en productos cárnicos fermentados.

Tabla 1.- Estárters más utilizados en productos cárnicos fermentados (adaptado de Cocconcelli & Fontana, 2008).

Especie	Propiedades tecnológicas	Efecto sobre la calidad
Bacterias ácido lácticas ***Lactobacillus sakei*** ***Lactobacillus curvatus*** ***Lactobacillus plantarum*** ***Lactobacillus rhanmosus*** ***Pediococcus acidilactici*** ***Pediococcus pentosaceus***	Disminuye el pH Actividad catalasa Producen ácido láctico Producen bacteriocinas Actividad proteolítica Actividad antioxidante	Aumenta la conservación Aumenta la consistencia Facilita el secado Elimina bacterias patógenas Elimina bacterias indeseables Mejora el aroma
Micrococáceas ***Staphylococcus xylosus*** ***Staphylococcus carnosus*** ***Staphylococcus equorum*** ***Kocuria varians***	Actividad nitrato-reductasa Actividad catalasa Actividad proteolítica Actividad lipolítica	Reduce los nitratos a nitritos Mejora y evita defectos de color Degradación de peróxidos Evita el enranciamiento Mejora el sabor y el aroma

I.4.3. Levaduras

Las levaduras presentes en los productos cárnicos pueden ser ascomicetos como *Debaryomyces, Pichia, Yarrowia* y *Candida*, así como basidiomicetos como *Rhodotorula, Cryptococcus* y *Trichosporon* (Osei Abunyewa y col., 2000). En la carne fresca predominan los basidiomicetos pero a medida que avanza el proceso los ascomicetos van incrementando su número hasta imponerse totalmente. No obstante, existe una clara diferencia entre los géneros aparecidos en cada una de las etapas del proceso, así, durante las primeras etapas las levaduras mayoritarias pertenecen al género *Candida* mientras que al final de la fermentación y durante el curado predomina el género *Debaryomyces* (Encinas y col., 2000; Osei Abunyewa y col., 2000) especialmente en aquellos elaborados de forma tradicional (Mendonça y col., 2013).

I.5. Identificación y caracterización de *Debaryomyces hansenii*

La especie *Debaryomyces hansenii* presenta una alta flexibilidad en la utilización de fuentes de carbono y nitrógeno, lo que la ha convertido, junto a *Saccharomyces cerevisiae*, en una de las levaduras más ampliamente distribuidas en alimentos fermentados. Destacan, además de su importante contribución a las fermentaciones de alimentos carnicos, su dominancia en la fermentación de quesos y otros lácteos (Buchl & Seiler, 2011), alimentos en salmuera y productos cárnicos salados (Butinar y col., 2005). Por el contrario, algunos aislados han sido asociados con el deterioro de cervezas, mermeladas, frutas confitadas, aceitunas y mayonesa (Martorell, 2006).

La presencia de *D. hansenii* en la fermentación de alimentos tiene efectos beneficiosos, ya que participa en los procesos de maduración produciendo etanol, CO_2, y gran variedad de compuestos volátiles como alcoholes, esteres, cetonas, aldehídos y compuestos azufrados que contribuyen al sabor y al aroma final de los mismos (Boekhout & Phaff, 2003; Deák & Beuchat, 1996; Gardini y col., 2001; Martin y col., 2003). Sin embargo, el crecimiento excesivo de las mismas puede provocar cambios sensoriales indeseables dando lugar a una pérdida de aromas así como a la formación de sabores y olores desagradables (Diriye y col., 1993; Ismail y col., 2000; Martínez y col., 2004).

Tradicionalmente se han empleado técnicas microbiológicas clásicas de identificación y caracterización fenotípica, principalmente pruebas morfológicas, fisiológicas y bioquímicas, para la identificación y caracterización de *D. hansenii* aisladas de productos cárnicos crudos curados. Sin embargo, el empleo de estas técnicas tiene varios inconvenientes entre los que destacan la gran variabilidad entre aislados, la dependencia del estado fisiológico de la célula, el amplio número de pruebas a realizar y la elevada inversion en tiempo para la interpretación y análisis de los resultados (Kreger-Van Rij, 1984; Barnett y col., 1990; Deak & Beuchat, 1996). Por todo ello, desde hace algunos años se están desarrollando y aplicando técnicas moleculares que permiten la identificación y caracterización de los aislados con mayor precisión y en menor tiempo**.**

Entre las técnicas más utilizadas se encuentran las basadas en el estudio y comparación de ácidos nucleicos, generalmente ADN. Estas técnicas

además de ser precisas y rápidas, presentan además una elevada reproducibilidad ya que no dependen del estado fisiológico de la célula.

La aplicación de técnicas moleculares se inicia con la extracción del ADN, que en el caso de las levaduras requiere una rotura previa de la pared celular utilizando medios fisicos o químicos. Entre los métodos más utilizados estan los enzimáticos ya que producen una extracción suave del ADN manteniendo su estructura e integridad. Posteriormente, se realiza la purificación del ADN mediante el uso de diferentes enzimas y solventes orgánicos que rompen la membrana plasmática aislando el DNA y precipitando proteínas y otros restos celulares. El ADN aislado puede ser utilizado en las distintas técnicas moleculares para la identificación y caracterización de las levaduras aisladas.

Entre las técnicas moleculares más utilizadas para la identificación de *D. hansenii* están las basadas en el análisis de restricción (RFLPs o Restriction Fragment Length Polymorphism) y secuenciación de regiones y genes ribosomales (ADNr). Mientras que para la caracterización de *D. hansenii* se han utilizado metodos basados en la PCR como RAPD-PCR (Random Amplified Polymorphic DNA) o RFLPs del DNA mitocondrial (mtDNA). En la Tabla 2 se muestran las contribuciones más importantes a la identificación y caracterización de *D. hansenii* y otras levaduras aisladas de productos cárnicos.

Tabla 2.- Métodos de identificación y caracterización de levaduras aisladas de productos cárnicos

Técnica molecular	Tipo de análisis	Especie de levadura	Origen del producto cárnico	Referencia bibliográfica
PCR-DGGE	Identificación	*Debaryomyces hansenii, Candida krissii, Candida sake, Williopsis saturnus*	Salami italiano	Rantsiou y col., 2005
PCR-DGGE y secuenciación ITS-5.8S rRNA	Identificación	*Debaryomyces hansenii, Candida parapsilosis, Candida zeylanoides, Metschnikowia pulcherrima, Saccharomyces cerevisiae, Sterigmatomyces elviae, Pichia triangularis, Zygosaccharomyces bisporus*	Salami italiano	Cocolin y col., 2006
RFLP de regiones ribosómicas	Identificación	*Debaryomyces hansenii, Debaryomyces polymorphus, Candida zeylanoides, Saccharomyces cerevisiae, Pichia carsonii, Rhodotorula mucilaginosa, Yarrowia lipolytica*	Diversos productos cárnicos	Andrade y col., 2006
RFLP de regiones ribosómicas, secuenciación ITS-5.8S rRNA y PCR-DGGE	Identificación	*Debaryomyces hansenii, Trichosporon brassicae, Saccharomyces cerevisiae, Candida stellata, Rhodotorula mucilaginosa, Yarrowia lipolytica*	Salami italiano	Aquilanti y col., 2007
PCR-DGGE	Identificación	*Debaryomyces hansenii, Candida psyschrophila, Saccharomyces cerevisiae*	Salami italiano	Silvestri y col., 2007
Secuenciación ITS-5.8S rRNA	Identificación	*Debaryomyces hansenii, Candida zeylanoides*	Jamón Ibérico	Andrade y col.,2010a

Continúa en la página siguiente

Continuación de la Tabla 2.

Técnica molecular	Tipo de análisis	Especie de levadura	Origen del producto cárnico	Referencia bibliográfica
RFLP de regiones ribosómicas	Identificación	*Debaryomyces hansenii, Candida parapsilosis, Rhodotorula mucilaginosa Yarrowia lipolytica*	Salchichón español	Mendonça y col., 2013
RAPD-PCR	Caracterización	*Debaryomyces hansenii*	Salami italiano	Cocolin y col., 2006
RAPD-PCR y RFLP del mtADN	Caracterización	*Debaryomyces hansenii, Debaryomyces polymorphus, Candida zeylanoides Saccharomyces cerevisiae, Pichia carsonii Rhodotorula mucilaginosa, Yarrowia lipolytica*	Diversos productos cárnicos	Andrade y col., 2006
RAPD-PCR y RFLP del mtADN	Caracterización	*Debaryomyces hansenii, Candida zeylanoides*	Jamón Ibérico	Andrade y col., 2010a

I.5.1. Identificación de *D. hansenii* utilizando técnicas moleculares

La mayoría de técnicas utilizadas en la actualidad para la identificación de levaduras están basadas en los RFLPs y secuenciación de regiones y genes nucleares ribosomales (ADNr). En las levaduras estos genes están localizados en una región genómica con un tamaño aproximado de 9 kb y en un número aproximado entre 100 y 200 copias (White y col., 1990; Kurtzman, 1992). Esta región ribosómica incluye genes y zonas espaciadoras cuya estructura se muestra en la Figura 2.

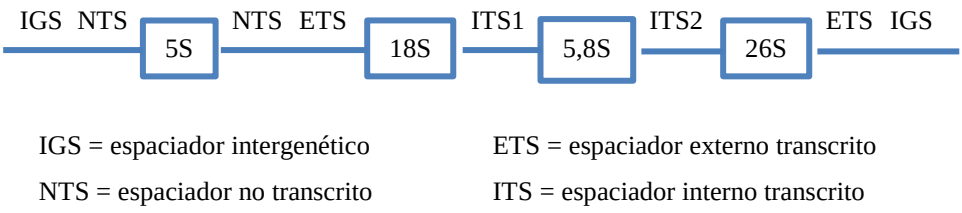

Figura 2.- Estructura de la región ITS.

Entre las regiones del ADNr más utilizadas para la identificación de levaduras están las regiónes ITS1-5.8S-ITS2 y los dominios D1 y D2 del gen ribosomal 26S rADN (Kurtzman & Robnett, 1998; Scorzetti y col., 2002). Otras regiones como las IGS (Quirós y col., 2006) y el gen 18S ADNr (Cappa & Cocconcelli, 2001) también se han empleado para la identificación de levaduras aunque su aplicación no ha sido tan amplia como las anteriores.

El análisis de restricción o RFLPs de la región ITS1-5.8S-ITS2 ha sido eficazmente utilizado desde la década de los 90 para la identificación de

levaduras (Molina y col., 1992). Esta técnica se basa en aprovechar la elevada tasa de sustitución nucleotídica que presentan las regiones espaciadoras intergénicas ITS para encontrar diferencias tras su restricción con las endonucleasas adecuadas. El resultado final es la identificación de las diferentes levaduras por generación de un perfil electroforético característico de cada especie. La técnica de los RFLPs ITS1-5.8S-ITS2 ha sido empleada en la mayoría de géneros y especies de levaduras aisladas de muy diversos ambientes, especialmente de alimentos (Esteve-Zarzoso y col., 1999). La mayoria de los patrones de restricción de la región ITS1-5.8S-ITS2 de las cepas tipo de especies de levaduras frecuentemente encontradas en alimentos están recogidos en la base de datos Yeast-Id (www.yeast-id.com).

De forma simplificada la técnica consiste inicialmente en la amplificación por PCR de la región ITS1-5.8S-ITS2 utilizando los cebadores ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') e ITS4 (5'-TCCTCCGCTTATTGA TATGC-3'). Posteriormente, el producto de amplificación se digiere directamente con varias endonucleasas para generar un perfil de restricción característico (Figura 3).

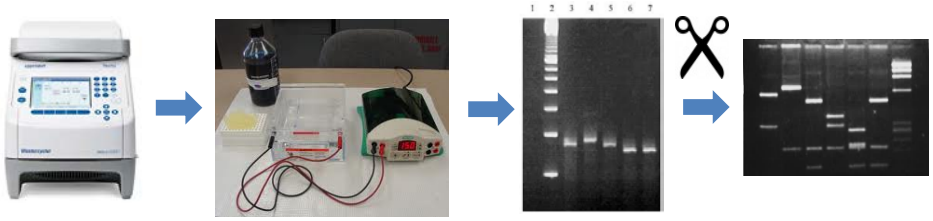

Figura 3.- Esquema de la técnica RFLPs ITS1-5.8S-ITS2.

Esta técnica se ha aplicado para identificar levaduras aisladas de embutidos crudos curados confirmando que la especie *D. hansenii* es la predominante en el proceso (Andrade y col., 2006; Mendonça y col., 2013).

La secuenciación de los dominios D1 y D2 del gen 26S ADNr se ha establecido como el estandard para la identificación de levaduras. En la actualidad se considera que la secuenciación es el método más robusto y fiable de identificación ya que la obtencion y comparación entre secuencias se puede realizar facilmente seleccionando los genes o regiones de genes apropiadas. La secuencia del fragmento D1/D2 del gen 26S ADNr, con una longitud de aproximadamente 600 nucleótidos, se ha comprobado que es capaz de identificar la mayoría de especies de levaduras (Kurtzman & Robnett, 1998).

La técnica consiste básicamente en la amplificación por PCR de la región D1/D2 26S ADNr utilizando los cebadores NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') y NL4 (5'-GGTCCGTGTT TCAAGACGG-3'). El producto de amplificación se secuencia y el resultado se compara con las secuencias disponibles en bases de datos electrónicas (GenBank y EMBL) (Figura 4). A una levadura se le puede asignar una especie conocida cuando el porcentaje de similitud entre su secuencia y la disponible en la base de datos es igual o superior al 99% (Kurtzman & Robnett, 1998). Esta técnica también permite la identificación tentativa de nuevas especies de levaduras cuando el nivel de similitud es inferior al 99%, aunque en estos casos se requiere la secuenciación de más genes para confirmar la existencia de la nueva especie.

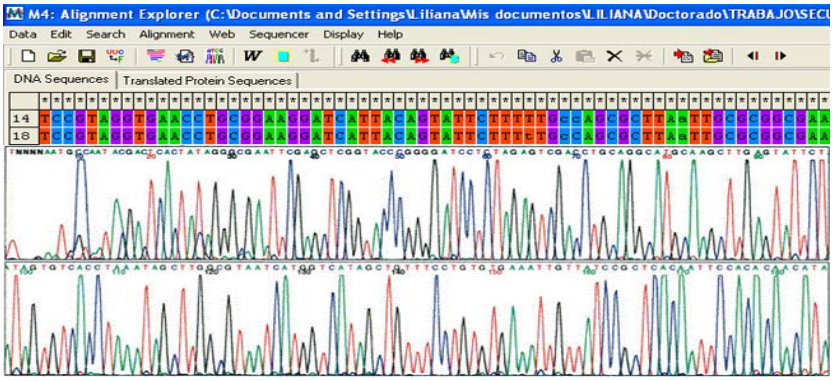

Figura 4.- Comparación de dos secuencias de los dominios D1/D2 del gen 26S ADNr.

De forma similar, en géneros de levaduras especialmente poco variables en la secuencia del gen 26S, entre los que se incluye el género *Debaryomyces* ya que la similitud de sus secuencias varia entre el 98.6 y el 99.5, se suelen secuenciar las regiones ITS anteriormente mencionadas u otros genes más variables como ACT1 (Martorell y col., 2005). Estas técnicas se han aplicado para la identificación de *D. hansenii* aislados de diversos productos cárnicos curados (Asefa y col., 2009; Nielsen y col., 2008).

I.5.2. Caracterización genética de la especie *D. hansenii*

La existencia de un gran número de cepas dentro de la especie *D. hansenii* con diferentes comportamientos y actividades metabólicas, que pueden afectar sensorialmente al producto cárnico elaborado, hace imprescindible la utilización de técnicas que nos permitan la diferenciación de dichas cepas. Entre las técnicas más estudiadas se encuentran los RFLPs

del ADN mitocondrial y la generación de RAPDs utilizando diversos cebadores.

El análisis de restricción (RFLP) del ADN mitocondrial (ADNmt) se ha utilizado desde principios de los 90 para diferenciar cepas vínicas de *Saccharomyces cerevisiae* (Querol y col., 1992a, 1992b) y su utilización se ha aplicado a numerosas especies de levaduras importantes en alimentos como las alterantes *Zygosaccharomyces rouxii* y *Z. bailii* (Guillamon y col., 1994), *Kluyveromyces* marxianus y *K. lactis* de productos lácteos (Belloch y col., 1997) y *Debaryomyces hansenii* de productos cárnicos (Petersen y col., 2001; Andrade y col., 2006; Andrade y col., 2010a).

Esta técnica se basa en poner de manifiesto la variabilidad presente en el ADNmt cortándolo con endonucleasas que permitan generar polimorfismos en el tamaño de los fragmentos de restricción (Figura 5). Esta técnica es relativamente rápida y eficaz, aunque el grado de polimorfismo generado depende de las endonucleasas de restricción utilizadas y de las especies de levaduras estudiadas.

Figura 5.- Esquema de los RFLPs del ADNmt.

Otra de las técnicas utilizadas para la diferenciación de levaduras pertenecientes a la misma especie es la generación de RAPDs utilizando la PCR y diferentes cebadores entre los que destacan el minisatelite M13 y los microsatélites $(GACA)_4$ y $(GAC)_5$ por ser los mayoritariamente utilizados en levaduras. Los RAPDs tambien se empezaron a aplicar en la decada de los 90 por Williams y col., (1990) y permiten detectar diferencias en la secuencia de ADN a partir de amplificaciones aleatorias múltiples por todo el genoma utilizando la reacción en cadena de la polimerasa (PCR). La diferencia más importante entre esta técnica y otras basadas en la PCR es que permite utilizar solo un cebador y no se necesita conocer la secuencia de ADN a amplificar. La técnica RAPD genera un producto de PCR que puede estar constituido por varias bandas en patrones muy complejos (Figura 6). El perfil de estos patrones depende de la secuencia del cebador, la temperatura y tiempo de amplificación, reactivos utilizados en la reacción de PCR, concentración de ADN molde, y otros (Ramos y col., 2008).

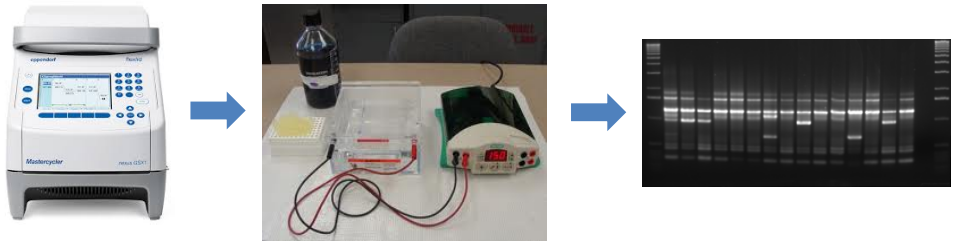

Figura 6.- Esquema de la técnica RAPD.

La optimización de todos estos parametros permite la obtencion de perfiles caracteristicos de cepa, que permiten establecer diferencias incluso entre levaduras procedentes de la misma fuente de aislamiento. Se trata de una técnica muy rápida, que requiere de poco ADN molde y, además, no necesita que el ADN esté muy puro a diferencia de los RFLPs del ADNmt.

Los RAPDs han sido empleados en la caracterización de levaduras de origen vínico (Pérez y col., 2001; Succi y col., 2003; Howell y col., 2004), lácteo (Andrighetto y col., 2000; Fadda y col., 2004) así como en levaduras de origen cárnico (Cocolin y col., 2006; Andrade y col. 2010a). Esta técnica también ha sido empleada para caracterizar cepas de *D. hansenii* aisladas en diferentes etapas de fabricación de salami italiano mostrando la existencia de distintos patrones moleculares en los diferentes estados del proceso de fabricación (Cocolin y col., 2006).

I.6. El aroma de los embutidos

Los embutidos crudos curados típicos españoles presentan un aroma característico que se debe a la naturaleza y cantidad de especias usadas en su elaboración dependiendo de la tradición de cada zona geográfica (Toldrá y col., 2006a). No obstante, también existen cambios químicos, bioquímicos y microbiológicos dentro de la matriz del producto que provocan el desarrollo de compuestos volátiles responsables del aroma de los embutidos crudos curados y que dependen de las condiciones de temperatura y humedad relativa empleadas durante el proceso de elaboración (Franco y col., 2002; García-Fontán y col., 2007), así como del tipo de microorganismo empleado como estárter que actuará sobre los componentes de la masa cárnica modificando el perfil aromático del

producto final (Tjener & Stahnke, 2007). Estos compuestos son principalmente cetonas, ácidos, alcoholes y ésteres (Stahnke, 1994; Hinrinchsen & Pedersen, 1995) que se generan durante la fermentación y maduración – secado de los embutidos crudos curados mediante la lipólisis y proteólisis, donde se generan los precursores, y también a través de la oxidación lipídica y de las reacciones de degradación de aminoácidos (descarboxilación, desaminización y transaminación) así como de carbohidratos (Toldrá, 2008) (Tabla 3).

Tabla 3.- Principales compuestos generados durante la elaboración de embutidos crudo curados y sus efectos sensoriales (adaptada de Toldrá, 2008).

Tipo de reacción	Producto final	Efecto sensorial
Glucólisis homofermentativa	Ácido láctico	Sabor
Glucólisis heterofermentativa	Ácido láctico, diacetilo, acetaldehído, acetoína, ácidos grasos de cadena corta, dióxido de carbono	Sabor y aroma
Proteólisis	Péptidos y aminoácidos libres	Sabor
Degradación de aminoácidos	Aldehídos lineales y ramificados, ácidos lineales y ramificados, alcoholes, cetonas, aminas, amonio y aminoácidos libres	Aroma y sabor
Lipólisis	Ácidos grasos libres	Sabor
Oxidación lipídica	Hidrocarbonos lineales, alcoholes, aldehídos, cetonas, esteres	Aroma

I.6.1. Precursores del aroma

Los procesos más importantes en la producción de precursores aromáticos son la lipólisis y proteólisis cuyos productos son utilizados como sustratos en reacciones químicas y microbianas para formar compuestos volátiles (Toldrá y col., 2001).

I.6.1.1. Lipólisis

La lipólisis consiste en la hidrólisis enzimática de origen endógeno y microbiano de los lípidos presentes en el músculo y el tejido adiposo rompiendo los enlaces éster de los tri, di y monoglicéridos así como de los fosfolípidos para producir ácidos grasos libres (Hierro y col., 1997). Estos ácidos grasos libres tienen un efecto directo sobre el sabor, contribuyendo ligeramente a la acidez, e indirecto sobre el aroma (Toldrá, 2008).

Según Montel y col., (1993), la lipólisis en embutidos crudos curados se debe principalmente a la acción de las enzimas endógenas pero otros autores han indicado que los cultivos iniciadores tienen un efecto definitivo en la formación del aroma en los productos cárnicos crudos curados (Berdagué y col., 1993). Así, las micrococáceas son consideradas los principales microorganismos responsables de la lipólisis en embutidos crudos curados (Ordóñez y col., 1999). No obstante, en otros estudios en los que se emplearon micrococáceas del género *Staphylococcus* aislados de productos crudos curados también se detectó una importante actividad lipolítica (Nielsen & Kemmer, 1989; Nieto y col., 1989; Talón y col., 1992). También, se han detectado lipasas intracelulares y extracelulares en bacterias ácido lácticas aisladas de embutidos crudos curados que mostraron actividad lipolítica actuando sobre mono-, di- y triglicéridos (Sanz y col., 1988). Además, muchas levaduras aisladas de embutidos crudos curados presentaron actividad lipolítica actuando sobre el tejido adiposo y contribuyendo al desarrollo del aroma y sabor en el producto final (Sorensen & Samuelsen, 1996; Sorensen, 1997; Hammes & Knauf, 1994).

I.6.1.2. Proteólisis

La proteólisis es la hidrólisis enzimática de las proteínas sarcoplásmicas y miofibrilares por parte de las proteasas endógenas y microbianas. En primer lugar actúan las endopeptidasas para generar polipéptidos que posteriormente son degradados a péptidos y aminoácidos por la acción de las exopeptidasas (Toldrá, 2006c). Estos aminoácidos libres producidos durante la proteólisis contribuyen de forma importante al sabor y también al aroma de los embutidos crudos curados tras ser transformados en compuestos volátiles por el metabolismo microbiano (Talón y col., 2004). Sin embargo, la contribución de cada grupo de enzimas depende del tipo de producto cárnico y de los microorganismos empleados como cultivos iniciadores (Toldrá, 2006c).

Así, los lactobacilos han mostrado una buena habilidad para degradar proteínas sarcoplásmicas y miofibrilares (Fadda y col., 1999a, 1999b). Además, estudios recientes han indicado que la inoculación de *Lactobacillus curvatus* y *Staphylococcus xylosus* en productos cárnicos crudos curados produce un incremento de la concentración de aminoácidos libres (Casaburi y col., 2007, 2008).

I.6.2. Oxidación lipídica

Los ácidos grasos libres generados durante la lipólisis actúan como sustrato de las reacciones oxidativas que dan lugar a la formación de compuestos volátiles de gran importancia en los embutidos crudos curados como son los alcoholes, aldehídos y cetonas (Johanson y col., 1994; Toldrá,

1998; Marco y col., 2004), así como ésteres cuando los alcoholes reaccionan con los ácidos grasos carboxílicos libres (Toldrá, 2006b).

La oxidación puede ser iniciada a través de catalizadores externos como luz, calor, presencia de humedad y/o cationes metálicos, o mediante enzimas oxidativas musculares como peroxidasas y ciclooxigenasas (Toldrá y col., 2001). Esta oxidación inicial genera hidroxiperóxidos muy reactivos que participan en una oxidación secundaria donde se producen compuestos que contribuyen al aroma final del producto. Sin embargo, una oxidación excesiva da lugar al enranciamiento del producto (Skibsted y col., 1998).

El uso de ciertos microorganismos como cultivos iniciadores en embutidos crudos curados ha demostrado una mayor producción de compuestos volátiles deseables y una reducción en el enranciamiento de dichos productos. Este es el caso de ciertas especies de *Staphylococcus* que consumen el oxígeno reduciendo la excesiva oxidación lipídica, y por tanto el enranciamiento del producto final, y que además presentan actividad catalasa que favorece la estabilidad del color (Berdagué y col., 1993).

I.6.3. Reacciones de transformación de aminoácidos

Los aminoácidos libres producidos durante la proteólisis generan aldehídos ramificados y productos secundarios como ácidos, alcoholes y ésteres a través del metabolismo microbiano de aminoácidos (Toldrá, 2006b). Estos resultados fueron demostrados por Olesen y col., (2004b) quienes indicaron un aumento en la generación de ácido 3-metilbutanoico y α-hidroxi-isocaproico al incubar leucina con cepas de *S. xylosus* y *S. carnosus*. Sin embargo, en otros estudios en los que se emplearon extractos celulares de *L. sakei* y *B. pumilis* sobre embutidos crudo curados en los que

se detectó una mayor rotura de los aminoácidos (valina, isoleucina y leucina), no se observaron mejoras en la calidad sensorial del producto final (Herranz y col., 2006). En cuanto al papel de las levaduras, Demeyer y col., (2000) determinaron que la especie *Debaryomyces hansenii* era capaz de transformar los aminoácidos mediante su actividad microbiana. Estos resultados fueron confirmados por Durá y col., (2004a) al inocular cepas de *D. hansenii* en un sistema *in vitro* donde estas levaduras fueron capaces de generar alcoholes y aldehídos ramificados, compuestos ácidos y ésteres a partir del metabolismo de tres aminoácidos (leucina, isoleucina y valina), aunque la evolución de estos compuestos volátiles se vió influenciada por el pH del medio, la fase de crecimiento en la que se encontraban las levaduras y la presencia de sal y lactato en el sistema.

I.6.4. Fermentación de los hidratos de carbono

Los hidratos de carbono sirven como sustrato en el crecimiento de los cultivos iniciadores inoculados en la masa cárnica de los embutidos crudos curados. Sin embargo, la generación de compuestos volátiles durante la fermentación de los carbohidratos depende del tipo de estárter utilizado en el proceso de elaboración de productos cárnicos crudos curados. Por ello, existen dos tipos de fermentaciones de los hidratos de carbono.

Por un lado, la vía homofermentativa en la que se genera ácido láctico como producto final, el cual se acumula en el medio dando lugar a una bajada de pH en el embutido. Este tipo de fermentación la llevan a cabo las bacterias ácido lácticas empleadas como estárter (Demeyer & Toldrá, 2004) y está condicionada por el tipo de bacterias ácido lácticas empleadas, la cantidad de carbohidratos adicionados, la temperatura de fermentación, el

contenido en sal así como el tiempo y condiciones de procesado (Demeyer, 1992; Demeyer y col., 2000).

En cambio si el estárter empleado es heterofermentativo la transformación de los carbohidratos puede generar otros productos. Esta vía heterofermentativa puede estar causada por la presencia de bacterias heterolácticas, levaduras (vía alcohólica) o por enterobacterias en cuyo caso siguen la vía de los ácidos mixtos. Así, el proceso heterofermentativo puede generar productos como acetato, formato, etanol y acetoína que afectan a la calidad sensorial de los productos cárnicos crudos curados (Demeyer & Stahnke, 2002). Por otro lado, la generación de compuestos como el diacetilo, acetoína y butanodiol aportan aromas a mantequilla y yogur a los embutidos crudos curados (Montel y col., 1998). Además, en la vía heterofermentativa también se forman ácidos grasos de cadena corta como el ácido fórmico, acético, propiónico y butanoico que tienen un fuerte impacto aromático en el producto final (Toldrá, 2008).

I.7. Efecto de la especie *D. hansenii* en el aroma de los productos cárnicos

La relación entre el aroma de los embutidos crudos curados y los diferentes cultivos iniciadores empleados ha sido muy estudiada pero casi siempre se ha atribuido al uso de especies de *Lactobacillus* y *Staphylococcus* (Berdagué y col., 1993; Montel y col., 1998; Olesen y col., 2004a, 2004b). Sin embargo, en algunos embutidos crudos curados elaborados en Italia, Francia y España se observó que la microbiota superficial (hongos y levaduras) influía en las propiedades sensoriales de estos productos (Lücke, 2000).

En los últimos años, con el objetivo de estudiar los efectos de la especie *D. hansenii* en el aroma de los productos cárnicos crudos curados, se han empleado sistemas modelos cárnicos inoculados con este microorganismo y donde se imita la composición y las condiciones de elaboración de estos productos. De esta forma, se ha evaluado la actividad lipolítica y proteolítica de las levaduras mencionadas para conocer su capacidad de generación de precursores aromáticos y su posterior empleo para producir compuestos volátiles que influyan en el aroma del producto final. No obstante, en estos estudios el efecto de la especie *D. hansenii* ha mostrado resultados contradictorios. Así, algunos autores observaron que cepas de *D. hansenii* eran incapaces de hidrolizar la trioleína *in vitro* (Nuñez, 1995) mientras que otros estudios sí detectaron actividad lipolítica por parte de estas levaduras. Sorensen & Samuelsen, (1996) demostraron la presencia de actividad lipolítica al inocular extractos celulares de *D. hansenii* y *S. xylosus* en un sistema modelo cárnico durante los primeros estadíos del proceso cuando los valores de pH son más elevados. Estos resultados fueron confirmados por Sorensen (1997), al inocular levadura viva de *D. hansenii* sobre grasa de cerdo donde se liberó gran cantidad de ácidos grasos sin que este proceso se viese afectado por la presencia de sal y valores bajos de pH en el medio. En cuanto a la actividad proteolítica de la especie *D. hansenii*, son numerosos los estudios en los que se demostró una mayor proteólisis cuando esta levadura era inoculada en sistemas modelo en los que se emplearon sustratos específicos como la miosina (Núñez, 1995; Rodríguez y col., 1998; Sosa, 2005). Además, existen trabajos en los que el uso de extractos celulares de *D. hansenii* como fuente adicional de enzimas en embutidos crudos curados favoreció la hidólisis de las proteínas

sarcoplásmicas y generó péptidos polares y apolares así como aminoácidos libres (Santos y col., 2001). Bolumar y col., (2003a, 2003b, 2005 y 2008) purificaron y caracterizaron enzimas endo y exoproteolíticas procedentes de una cepa de la especie *D. hansenii* aislada de embutidos de fermentación tradicional por Mendonça y col., (2013). Por otra parte, Durá y col., (2002) demostraron la capacidad de la levadura *D. hansenii* para transformar los aminoácidos mediante la purificación y caracterización de una glutaminasa que generó amonio y α-glutamato y neutralizó el sabor ácido.

Tras estudiar la capacidad lipolítica y proteolítica de estas levaduras también se emplearon sistemas modelo para evaluar el efecto de la especie *D. hansenii* sobre el aroma. Olesen & Stahnke, (2000) determinaron que la inoculación de cepas *D. hansenii* en un sistema modelo cárnico no tenía efectos sobre los compuestos volátiles generados en el medio, sin embargo apuntaron a que este hecho podría deberse al empleo de esencia de ajo como condimento en el ensayo y al uso de unas inadecuadas condiciones para el crecimiento de levaduras. Teniendo en cuenta estos resultados, otros autores desarrollaron nuevos sistemas cárnicos en los que se controlaron las condiciones para un correcto crecimiento de los microorganismos inoculados. Así, en todos los estudios en los que se inocularon cepas de la especie *D. hansenii* se observó un aumento en el contenido de los compuestos volátiles. Durá y col., (2004b) estudiaron las reacciones de transformación de aminoácidos (isoleucina, leucina y valina) en un sistema *in vitro* inoculado con *D. hansenii* en el que detectaron un aumento gradual de los compuestos volátiles iniciándose con un aumento del contenido de aldehídos (3-metil butanal y 2-metil butanal) y alcoholes (2-metil-1-propanol, 3-metil-1-butanol y 2-metil-1-butanol), y terminando con un

incremento en compuestos ácidos (ácido 2-metil propanoico y ácidos 2-metil y 3-metil butanoico) y ésteres (3-metil butanoato de etilo y 3-metil butanoato de metilo), aunque la evolución de estos compuestos se vio afectada por la presencia de sal y de lactato en el medio así como por el descenso del pH. La generación de estos compuestos también fue detectada por Andrade y col., (2009a, 2009b) al inocular cepas de *D. hansenii* en un sistema modelo que simulaba las condiciones de procesado del jamón curado y que contenía sal, ácido oleico, ribosa, creatina y aminoácidos. En estos estudios, observaron además un aumento en la cantidad de compuestos azufrados, cetonas e hidrocarbonos aunque las concentraciones de estos compuestos dependían del tipo de cepa de *D. hansenii* utilizada en el sistema modelo cárnico (Andrade y col., 2009a, 2009b).

A pesar de todos estos trabajos donde se demuestra la actividad proteolítica, lipolítica y la capacidad para transformar los aminoácidos que presenta la especie *D. hansenii* así como su contribución a la formación de compuestos volátiles, son pocos los estudios en los que se ha empleado esta levadura como cultivo iniciador en productos cárnicos. La tabla 4 recoge los trabajos más recientes en los que se ha estudiado el efecto de la especie *D. hansenii* en productos cárnicos.

Tabla 4.- Efecto de la inoculación de la especie *D. hansenii* en productos cárnicos.

Producto cárnico	Efecto en el producto cárnico	Referencia bibliográfica
Embutido crudo curado	Generación de compuestos volátiles	Olesen & Stahnke (2000)
Lomo	Generación de compuestos volátiles	Martín y col., (2003)
Productos cárnicos madurados	Generación de compuestos volátiles	Alonso (2004)
Embutido crudo curado	Actividad antioxidante Generación de compuestos volátiles	Flores y col., (2004)
Embutido crudo curado	Presencia de actividad proteolítica Liberación de aminoácidos Disminución del contenido de amoniaco Aumento del contenido de ácido acético y D-láctico	Durá y col., (2004a)
Embutido crudo curado (Extractos celulares)	Generación de compuestos volátiles	Bolumar y col., (2006)
Jamón curado	Generación de compuestos volátiles Mejora del aspecto y de la textura	Martín y col., (2006)

Continúa en la página siguiente

Continuación de la Tabla 4.

Producto cárnico	Efecto en el producto cárnico	Referencia bibliográfica
Embutido crudo curado	Disminución de la Aw Presencia de actividad lipolítica Liberación de ácidos grasos Presencia de actividad proteolítica	Patrignani y col., (2007)
Embutido crudo curado	Generación de compuestos volátiles Mejora del aspecto final del producto Aumenta el grado de aceptación del consumidor	Iucci y col., (2007)
Salchichón	Generación de compuestos volátiles	Andrade y col., (2010b)
Lacón	Generación de compuestos volátiles	Purriños y col., (2013)

La inoculación de cepas de *D. hansenii* en embutidos crudos curados ocasiona una degradación más rápida de las proteínas miofribrilares al principio del secado y aumenta el contenido de aminoácidos libres (Durá y col., 2004a). En este mismo estudio, Durá y col., (2004a) observaron una disminuación en el contenido de amoniaco y un incremento en la concentración de ácido láctico y acético sin que este hecho tuviese un efecto sobre el pH final. Además, el empleo de estas levaduras como cultivo iniciador favorece el descenso de la Aw de una forma más acusada y rápida, la proteólisis y la lipólisis liberando aminoácidos y ácidos grasos (Patrignani y col., 2007).

Sin embargo, al igual que ocurría en el caso de los estudios realizados en sistemas modelos cárnicos, al inocular cepas de la especie *D. hansenii* para evaluar su efecto sobre el aroma de los productos cárnicos crudos curados los resultados obtenidos muestran conclusiones contradictorias. Así, en el año 2000, Olesen & Stahnke detectaron un aumento en el contenido de aldehídos ramificados, compuestos ácidos, cetonas y algunos ésteres en embutidos crudos curados inoculados con cepas de *D. hansenii* aunque este incremento en los compuestos volátiles no mostró diferencias significativas respecto a los lotes control debido a la inhibición de la levadura en presencia de ajo y a la interferencia de los aromas de las especias empleadas en el análisis de los compuestos volátiles. Resultados similares fueron indicados por Selgas y col., (2003) quienes inocularon cepas seleccionadas de la especie *D. hansenii* con capacidad catalasa, nitrato-reductasa, aminopeptidasa y proteolítica, en la superficie de embutidos crudos curados tradicionales para evaluar su efecto sobre las características sensoriales de dicho producto donde no detectaron diferencias significativas

respecto a los lotes control. Por el contrario, en otros estudios se observó que estos microorganismos presentan actividad lipolítica y proteolítica y que además son capaces de utilizar los productos obtenidos en estos procesos para generar compuestos volátiles y contribuir al aroma característico de estos productos. Así, estudios realizados en productos cárnicos crudos curados como lacón (Purriños y col., 2013), lomo (Martín y col., 2003; Alonso, 2004) o jamón curado (Martín y col., 2006) mostraron un aumento en el contenido de compuestos volátiles aunque se detectaron resultados contradictorios en cuanto al tipo de compuesto volátil generado al inocular levaduras de la especie *D. hansenii*. De esta forma Purriños y col., (2013) detectaron un aumento de la cantidad de ésteres, alcoholes, ácido acético y cetonas, mientras que los estudios realizados en lomo indicaron además un incremento en el contenido de aldehídos (Martín y col., 2003; Alonso, 2004). Por el contrario, Martín y col., (2006) observaron un aumento en la cantidad de ésteres y una disminución en el contenido de alcoholes, aldehídos y cetonas así como de compuestos azufrados y ácidos, en el análisis de los compuestos volátiles de jamón curado inoculado con cepas de *D. hansenii*. El estudio realizado por Bolumar y col., (2006) en el que se evaluó el efecto producido por la adición de extractos de *D. hansenii* y *L. sakei* en embutidos crudos curados detectó un aumento en la generación de compuestos volátiles. De esta forma, la combinación de estos dos extractos de microorganismos favoreció la generación de compuestos volátiles derivados de la oxidación lipídica y de la fermentación de los carbohidratos mientras que el empleo del extracto de levadura procedente únicamente de la especie *D. hansenii* aumentó la producción de compuestos volátiles originados por el catabolismo de los aminoácidos y de la

fermentación microbiana. Flores y col., (2004) inocularon diferentes concentraciones de una misma cepa de *D. hansenii* en embutidos crudos curados tradicionales detectando un aumento en la cantidad de ésteres etílicos, una disminución en la concentración de alcoholes así como aldehídos y cetonas generados durante la oxidación lipídica. Sin embargo, ellos observaron una mayor abundancia de compuestos ácidos en aquellos lotes inoculados con mayor concentración de inóculo, lo que podría enmascarar el efecto de los ésteres generados. Resultados similares fueron indicados por Iucci y col., (2007) quienes inocularon cepas de *D. hansenii* en embutidos crudos curados de diferente diámetro detectando un aumento en el contenido de alcoholes y compuestos azufrados en embutidos crudos curados de pequeño calibre y un incremento en la cantidad de aldehídos y terpenos en embutidos crudos curados de gran calibre. Por otra parte, Andrade y col., (2010b) inocularon embutidos crudos curados con diferentes cepas de *D. hansenii* observando un aumento en el contenido en alcoholes, aldehídos, compuestos ácidos, compuestos azufrados, cetonas e hidrocarburos en todos los lotes inoculados aunque el incremento de cada uno de estos compuestos volátiles resultó distinto para cada cepa inoculada. Así pues, tanto la concentración de inóculo como la cepa inoculada influyen en el tipo y cantidad de compuesto volátil que se genera.

Además, en la mayoría de estos estudios no se ha estudiado la cinética de crecimiento e implantación de las cepas inoculadas para comprobar su efecto. Así, sólo Andrade y col., (2010b) caracterizaron las levadura presentes en el producto terminado para comprobar la implantación de las cepas inoculadas aunque no determinaron su cinética de implantación ni su actividad en cada una de las etapas del proceso. Por todo ello, es necesario

un estudio más exhaustivo que permita una buena caracterización molecular de las cepas de *D. hansenii* inoculadas en embutidos crudos curados tradicionales ya que, tal y como se ha indicado previamente, diferentes cepas de una misma especie pueden generar distintos compuestos volátiles dando lugar a diversos perfiles aromáticos. De esta forma se podrían diferenciar y seleccionar aquellas cepas con mayor potencial aromático para emplearlas como coadyuvantes de la fabricación comprobando su adaptación e implantación al medio a lo largo del proceso y favorecer así la generación de compuestos volátiles que mejoren el aroma del producto final.

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II. OBJETIVOS

II.Objetivos

Teniendo en cuenta los antecedentes expuestos en la introducción de esta Tesis y la controversia en cuanto al papel de las levaduras en el aroma de los embutidos crudos curados se propuso utilizar embutidos crudo curados tradicionales caracterizados por un gran desarrollo del aroma. Estos embutidos se emplearon para el aislamiento y selección de levaduras que pudieran emplearse como coadyuvantes del curado.

Para ello se plantearon los siguientes objetivos de investigación:

- Identificación y caracterización molecular de cepas de la especie *Debaryomyces hansenii* aisladas de embutidos crudo curados tradicionales.
- Caracterización del potencial aromático de las levaduras de la especie *D. hansenii* aisladas de los embutidos crudos curados tradicionales con el fin de seleccionar cepas que puedan actuar como coadyuvantes en la fabricación de embutidos crudos curados.

Con el fin de llevar a cabo los objetivos propuestos se diseñó el siguiente plan de trabajo:

1. Identificación y caracterización molecular de levaduras aisladas de embutidos crudo curados tradicionales y estudio del potencial aromático de las levaduras aisladas de la especie *D. hansenii* en un medio de cultivo de composición definida.
2. Selección de cepas que puedan actuar como coadyuvantes en la fabricación de embutidos crudo curados mediante el estudio de la

generación de compuestos aromáticos en un sistema modelo cárnico inoculado con *D. hansenii*.

3. Estudio del efecto de la inoculación de cepas seleccionadas de la especie *D. hansenii* en embutidos crudos curados de fermentación lenta.

4. Aplicación de la técnica de espectrometría de masas directa Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS) para evaluar la inoculación de levaduras en embutidos crudos curados mediante la producción de compuestos volátiles.

III. RESULTADOS

Capítulo 1

Caracterización molecular y potencial aromático de cepas de *D. hansenii* aisladas de embutidos crudos curados tradicionales.

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Molecular characterization and aromatic potential of *Debaryomyces hansenii* strains isolated from naturally fermented sausages

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Abstract

Twenty two *Debaryomyces hansenii* strains isolated from naturally fermented sausages were characterized by molecular methods and their contribution to sausage aroma measured by volatile compounds production on a defined culture media. The *D. hansenii* isolates were identified by RFLPs and sequencing of the ITS-5.8S rDNA region. Genetic characterization of strains was achieved by RFLPs of mitochondrial DNA and minisatellite M13 PCR amplification. The UPGMA dendrogram based on molecular patterns revealed an important genetic heterogeneity within the new sausage isolates of *D. hansenii*. Generation of ester compounds was tested on culture media supplemented with methanol or ethanol and 2-methyl butanoic acid. Only seven *D. hansenii* strains were able to produce differences in the aroma profiles detected by sensory and GC-MS analysis. The volatile compounds produced were mainly ester compounds, ethyl and methyl esters, sulfur, alcohols, aldehydes and ketones. The results of our study revealed no correspondence between genetic patterns and aroma profiles within *D. hansenii* strains.

Keywords: *D. hansenii*, sausages, genetic diversity, aroma, volatile compounds.

INTRODUCCTION

Fermentation is an ancient technology used to extend the shelf life of raw meat. Essential chemical and physical changes take place during the process and the organoleptic and sensory characteristics of the final product are completely different from those of the starting materials. An important contribution to this transformation comes from the action of microorganisms present on the raw materials such as meat, salt, spices and casings. Together with the endogenous enzymes of the meat, they are responsible for the aroma formation (Lücke, 2000). The main microbial groups in fermented sausages are Lactobacilli and coagulase-negative cocci which are essential to achieve the sensory characteristics. However, other microbial groups such as yeast may also play a role in the final characteristics of fermented meat products (Lücke, 2000; Rantsiou & Cocolin, 2008).

Yeasts are considered to affect sausage colour and flavor due to their oxygen-scavenging and lipolytic activities. They may also delay rancidity and further catabolize products of fermentation, such as lactate produced by meat lactobacilli, to other by-products, thereby increasing the pH and contributing to the development of less tangy and more aromatic sausages (Hammes & Knauf, 1994).

Regarding yeast ecology in fermented sausages, several studies have documented the dominance of *Debaryomyces hansenii* (Baruzzi, Matarante, Caputo, & Morea, 2006; Cocolin, Urso, Rantsiou, Cantoni, & Comi, 2006). *D. hansenii* has been extensively studied because of its ability to hydrolyze pork muscle sarcoplasmic proteins, thereby influencing the aroma

formation and sensory quality of dry-fermented sausages (Durá, Flores, & Toldrá, 2004a, 2004b; Flores, Durá, Marco, & Toldrá, 2004).

Identification and characterization of *D. hansenii* isolates using conventional morphological and physiological test is complex as many of the results obtained are variable. Recently, methods based on DNA have been applied on strains of this species to study its dynamics during natural fermentation of Italian sausages (Cocolin et al., 2006). Among other techniques, LTR fingerprinting, RAPDPCR, DNA probes, IGS fingerprints and gene sequencing have demonstrated substantive strain heterogeneity in the strains of *D. hansenii* (Corredor, Davila, Gaillardin, & Casaregola, 2000; Nguyen *et al*., 2009; Prillinger, Molnar, Eliskases-Lechner, & Lopandic, 1999; Sohier *et al*., 2009). Previous studies had demonstrated that minisatellite M13 and RFLPs of mtDNA allowed good differentiation of genotypes within *D. hansenii* isolated from different environments (Andrade, Rodríguez, Sánchez, Aranda, & Córdoba, 2006; Groenewald, Daniel, Robert, Poot, & Smith, 2008).

The contribution of *D. hansenii* starter cultures to the aroma of meat products has been investigated by several authors. In 2000, Olesen and Stahnke reported a very low effect of *D. hansenii* on dry fermented sausage aroma attributed to a low survival of the yeast produced by the fungistatic effect of the garlic used as a spice in the formulation. Further studies done in our laboratory have proved the contribution of *D. hansenii* to sausage aroma by inhibiting the lipid oxidation phenomenon and promoting the generation of ethyl esters (Flores *et al*., 2004). Recently, Andrade, Rodriguez, Casado, Bermudez, and Cordoba (2009a, 2010a) showed the contribution of *D. hansenii* to the aroma of meat products by the increase in

methyl-branched aldehydes and few ester compounds. Moreover, *D. hansenii* has shown proteolytic and lipolytic activities which could have and important effect on flavor (Aquilanti *et al*., 2007; Baruzzi *et al*., 2006).

The development of the final sensory characteristics of fermented sausages depends on the different processing conditions, fermentation process and raw materials used in formulation, because they can also determine the selection of specific microbial populations (Rantsiou & Cocolin, 2008). In our study, naturally fermented sausages produced without the use of starter culture and at low fermentation temperatures produced a limited rate of acid formation turning out in low acid sausages. Naturally fermented sausages have been selected by consumers who appreciate products with singular attributes that distinguished them from others of the same category (Conter *et al*., 2008). Aroma analysis of naturally fermented sausages has revealed the presence of numerous esters, both ethyl and methyl esters contributing positively to the aroma (Olivares, Navarro, & Flores, 2010). It is generally accepted that coagulase-negative cocci are the most important group in the production of flavor compounds because of its capacity to generate high amounts of branched aldehydes, methyl ketones and ester compounds (Montel, Masson, & Talon, 1998). However, the origin of ester compounds is not well established as lactic acid bacteria, coagulase-negative cocci, yeast and molds are able to produce them (Tjener & Stanhke, 2007).

The aim of this study was to associate genetically diverse strains of *D. hansenii* with their different production of ethyl and methyl esters found in naturally fermented sausages.

MATERIALS AND METHODS

Sausage sampling and yeasts isolation

Naturally fermented sausages from eight different manufacturers (Requena, Valencia, Spain) were used to isolate *D. hansenii* yeasts. Fermented sausages were manufactured using lean pork, pork back fat, sodium chloride, nitrite, nitrate, sugar and species using traditional practices, without the addition of starter cultures and naturally ripened at 10–12 °C during 1 month. At the end of the process, a cylindrical sausage sample of 10 g was aseptically taken from each sausage. The sample was finely minced and homogenized (Ultra-Turax, Spain) in tubes containing 10 mL of saline solution (0.9% sodium chloride). A sample, 50 μL, of the homogenate was spread onto GYPA medium plates (glucose 2%, peptone 0.5%, yeast extract 0.5% and agar 2%, pH 6.0, chloramphenicol 100 mg/L) and incubated 4 days at 25 °C. Up to 6 colonies were selected from each plate, further streaked on GYPA medium and incubated for 2 days at 25 °C. The isolated yeasts were codified using the manufacture initials (M, P, T, V, EV, S, EN and I). Yeasts were conserved at −80 °C using 15% glycerol as cryoprotectant. In addition to the sausage isolates, three strains from fermented sausages and six strains from the CECT (Spanish Type Culture Collection) were used as reference strains (Table 1).

Identification of yeasts by molecular techniques

The isolated yeasts were cultured overnight on GPY medium (2% glucose, 0.5% peptone and 0.5% yeast extract) at 25 °C and shaking at 200 rpm. DNA was extracted as described in Querol, Barrio, and Ramón (1992). The ribosomal DNA region spanning the ITS1, 5.8S rDNA and ITS2 (ITS-

5.8S rDNA) was amplified by PCR using the primer pairs its1 (5′-TCCGTAGGTGAACCTGCGG) and its4 (5′-TCCTCCGCTT ATTGATATGC) following the methodology of Esteve-Zarzoso, Belloch, Uruburu, and Querol (1999). The PCR product was digested with endonucleases *Hae*III, *Hinf*I and *Cfo*I according to the supplier's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Restriction fragments were separated by electrophoresis on 3% agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8), stained with RedSafe (INtRON Biotech., Spain) and visualized under UV light. DNA fragment sizes were determined using a 100-bp DNA ladder. Fragment sizes were compared with patterns in the Yeast-id database (http:// www.yeast-id.com) and assigned to a known yeast species.

Table 1. List of *Debaryomyces hansenii* yeast investigated in this study

Species	**strain**	**isolation source**
D. hansenii	CECT 11369^{T}	unknown
	CECT 10026	salted cod, Spain
	CECT 10352	tomato, Spain
	CECT 11363	chilled beef, Australia
	CECT 11365	dry white wine, South Africa
	C 1, C3, C7[a]	fermented sausages, Spain
	M 1, M 2, M 3, M 4, M 5, M 6, P 2, P 3, T 3, T 4, V 1, V 2, EV 1, EV 2, S 1, S 2, EN 2, EN 3, EN 4, I 1, I 2, I 3	natural fermented sausages, Spain
D. fabryii	CECT 11370^{T}	interdigital mycotic lesion, Germany

CECT, Spanish Type Culture Collection, University of Valencia, Spain
[a] C1and C3 were isolated in a previous study by Bolumar *et al.* (2003, 2006) and C7 was isolated by Durá *et al.* (2004).

Characterization of yeast strains

Characterization of yeast strains was achieved using ITS-5.8S rRNA sequencing, RFLPs of mitochondrial DNA (mtDNA) and minisatellite PCR amplification using the M13 primer (Belloch, Barrio, Uruburu, García, & Querol, 1997; Fadda, Mossa, Pisano, Deplano, & Cosentino, 2004).

Sequences of ITS-5.8S rDNA were analyzed with MEGA 4 (Tamura *et al*., 2011). The sequences were compared with the sequences from the GenBank database using BlastN (NCBI).

The RFLPs of mtDNA were carried out as described elsewhere (Belloch *et al*., 1997). DNA was digested with the restriction enzyme *Hinf*I (Roche Molecular Biochemicals, Mannheim, Germany) according to the supplier's instruction. Restriction fragments were separated on 1% agarose gels in 1× TAE buffer, stained with ethidium bromide (100 μg/mL) and visualized under UV light. Band sizes were compared against fragment sizes of lambda phage DNA digested with HindIII and PstI.

Minisatellite PCR amplification using the M13 primer (5′GAGGGTGGCGGTTCT3′) was performed as described in Fadda *et al*. (2004) using 50 μL reaction volume containing 0.3 μL rTaq (5U) DNA polymerase, 4 μL dNTP mix (2.5 mM), 5 μL buffer, 3 μL MgCl2 (1.5 mM), 1 μL M13 minisatellite primer and 25 μL solution containing 80–100 ng of genomic DNA. PCR amplification conditions were as follow: 95 °C for 5 min followed by 40 cycles of 93 °C for 45 s, 44 °C for 1 min and 72 °C for 1 min with a final extension step at 72 °C for 6 min. The PCR products (10 μL) were resolved by electrophoresis on 2% agarose gel in 1× TAE buffer at 90 V for 3 h, stained with RedSafe (INtRON Biotech., Spain) and

visualized under UV light. DNA fragment sizes were determined using a 100-bp DNA ladder.

Yeast growth on defined culture media

Yeasts were grown for 24 h in 5 mL GPY medium at 25 °C and 200 rpm shaking. Overnight cultures were adjusted to an absorbance of 0.3 at 655 nm (Biophotometer, Eppendorf) by dilution using fresh GPY medium and further incubated for 4 h at 25 °C and 200 rpm shaking. Cells were collected by centrifugation (3000 rpm for 10 min) and washed three times with 0.9% saline solution. Cell suspensions were adjusted to a concentration of 10^6 cell mL^{-1} using saline solution and 100 μL of each yeast suspension was used to inoculate the aroma producing media.

Two different aroma producing media containing 2-methyl-butanoic acid and methanol or ethanol were prepared. Media consisted of filter sterilized YNB with amino acids (histidine, methionine, tryptophan) (Difco-BD, Spain) and supplemented with 100 ppm 2-methylbutanoic acid and 20,000 ppm ethanol (YNB + B + E) or methanol (YNB + B + M). Yeasts suspensions were inoculated in 10 mL of each media and cultured at 24 °C and 200 rpm shaking for 15 days. Control tubes of each media without yeast were incubated under the same conditions. Growth experiments were done in triplicate.

After 15 days of incubation, eight expert panelists evaluated yeast growth and aroma of each tube using the non inoculated control tubes as reference. The method was used to estimate the aromatic impact of each yeast in the media. A total of eight assessments were done per inoculated yeast. The aroma descriptors were recorded and the results expressed as the

number of times a descriptor was detected by the assesors (Pollien *et al*., 1997).

Analysis of the volatile compounds produced in the culture media

Only those inoculated tubes showing different aroma than the control tube were selected for volatile compound analysis. Selected culture tubes were centrifuged at 4000 rpm for 10 min and supernatant separated. Seven microliters of supernatant was added to a 15 mL headspace vial and stored at −20 °C until analysis. Volatile compounds in the headspace of supernatant samples (YNB + B + E or YNB + B + M media) were extracted by Solid Phase Microextraction (SPME) device. The extraction was done using a 85 μm CAR/PDMS sf fiber (Supelco, Bellefonte, PA, USA) by headspace exposure during 2.5 h at 37 °C in a water bath while stirring (Durá *et al*., 2004b).

The identification and quantification of volatile compounds extracted by SPME from the supernatants was done using a gas chromatograph (GC HP 7890 series II) equipped with an HP 5975C mass selective detector (MS Hewlett Packard, Palo Alto, CA, USA). The compounds absorbed by the fiber were desorbed in the injection port of the GC-MS for 15 min at 220 °C with the purge valve off (splitless mode) using He as carrier gas at a flow rate of 34 cm s−1. Compounds were separated on a DB-624 capillary column (J&W Scientific, 30 m × 0.25 mm × 1.4 μm) and analyzed as described Flores *et al*. (2004). Volatile compounds were identified by comparison with mass spectra from the library database (Nist'05) and with Kovats retention indices of authentic standards (Kovats, 1965). Quantification of each volatile compound was based on the abundance of

total extracted area (TIC) or the area of a target ion when different compounds coeluted. The results were expressed in percentage as the relation between area in inoculated and control tubes.

Statistical analysis

In order to estimate the similarity between electrophoretic patterns, DNA markers were scored for the presence (1) and absence (0) of homologous bands. A dendrogram was constructed using the UPGMA (unweighted pair-group method using arithmetic averages) method using Jaccard Similarity Index in the NTSYS package version 2.21p (Numerical Taxonomy and Multivariate Analysis System, Exeter Publishing Ltd.).

The effect of yeasts inoculation on the generation of volatile compounds in the defined culture media was tested by one-way analysis of variance (ANOVA) using the statistic software XLSTAT, 2009.4.03 (Addinsoft, Barcelona, Spain). Differences between sample means were analyzed according to Fisher's least significant difference (LSD) test.

RESULTS AND DISCUSSION

Genetic characterization of yeast isolates

D. hansenii isolates were identified using RFLPs of PCR amplified rDNA region ITS1-5.8S-ITS2 and presence of different genetic groups within isolates revealed by RFLPs of mitochondrial DNA, PCR amplification of minisatellite M13 and sequencing of the ITS-5.8S rDNA region.

A total of 22 yeasts were isolated from eight naturally fermented sausages (Table 1). PCR amplification and restriction of the ITS-5.8S

rDNA region resulted in a pattern (PCR product: 650 bp; *Cfo*I: 300 + 300 + 50; *Hae*III 420 + 150 + 90; *Hinf*I 325 + 325) (Fig. 1) matching with the yeast species *D. hansenii* as deduced by comparison with www.yeast-id.com database and collection strains used as references in this study.

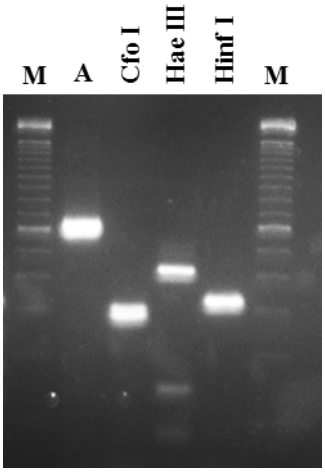

Figure 1. Electrophoretic patterns showing PCR amplification of the rDNA region ITS1-5.8S-ITS2 (lane 2) and restriction using enzymes *Cfo*I (lane 3), *Hae*III (lane 4) and *Hinf*I (lane 5).

RFLPs of mtDNA and minisatellite PCR amplification allowed detection of DNA polymorphisms among 31 strains of *D. hansenii* (Figures 2 and 3). RFLPs of mtDNA revealed 17 bands distributed in 9 different restriction patterns, whereas a total of 23 bands distributed in 8 different profiles were obtained using minisatellite M13 PCR amplification. Band presence or absence was recorded in a 0/1 matrix (Table 1 Supplementary Material) and used to construct an UPGMA dendrogram based on RFLP mtDNA and minisatellite M13 combined patterns (Figure 4). The primary trend observed in the dendrogram was separation of most *D. hansenii* new isolates and reference strains CECT 11369T, CECT 10026 and CECT

10352 into group G1 at approximately 85% similarity. The remaining new *D. hansenii* isolates and CECT 11363 appear in groups G2 and G3 at 65% and 60% similarity respectively. Reference strains CECT 11365 *D. hansenii* and CECT 11370T *D. fabryii* appear in a separated branch of the dendrogram.

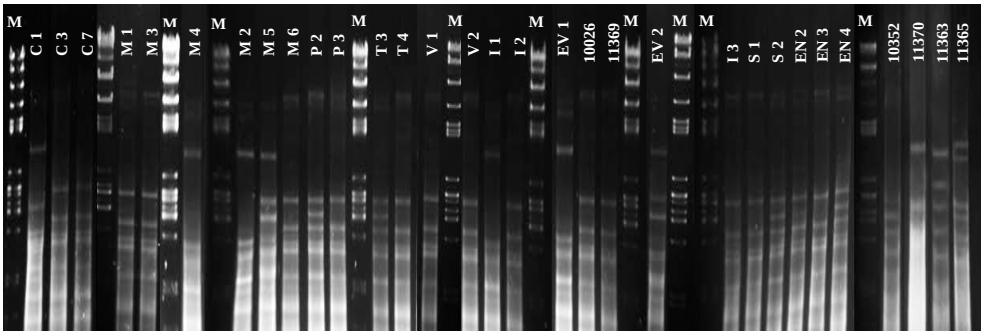

Figure 2. Patterns of mtDNA RFLPS after DNA digestion with enzyme *Hinf*I. Lanes are labeled with the strains names or with an "M" showing DNA markers (lambda DNA digested with *Hind*III and *Pst*I).

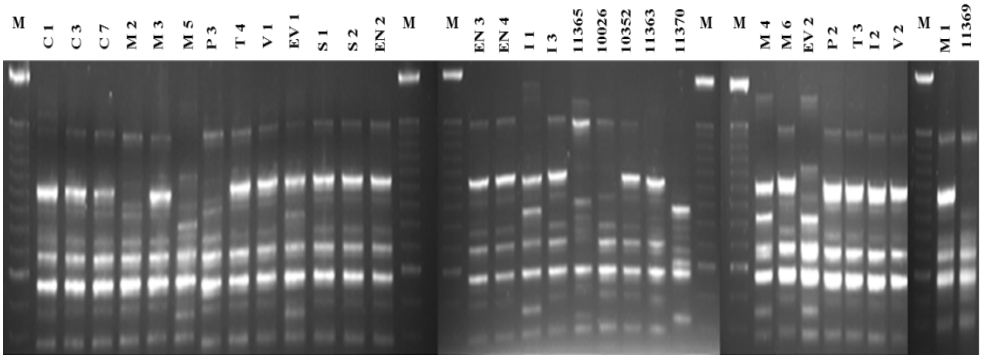

Figure 3. Electrophoretic patterns of minisatellite M13 PCR amplification. Lanes are labeled with the strains names or with an "M" showing the 100 pb ladder (Invitrogen, Carlsbad, CA, USA).

Alignment of ITS1-5.8S-ITS2 rDNA sequences from the new sausage isolates and reference strains showed only one nucleotide substitution in position 430 within 639 bp (GenBank ACCN JX982234) respect to the sequence of the type strain of *D. hansenii* CECT 11369T (GenBank ACCN AJ586526). The strains of *D. hansenii* showing this variation in the ITS1-5.8S-ITS2 are labeled as ITS-5.8S II on the UPGMA dendrogram. Sequence type ITS-5.8S II is also present in *D. fabryi* CECT 11370. An additional nucleotide substitution in position 448 (GenBank ACCN JX982235), labeled ITS-5.8S III, appeared in reference strain CECT 11363.

Among the yeast found in fermented meat products *D. hansenii* has been recognized as the predominant species by culture and culture-independent-DNA analyses (Aquilanti *et al*., 2007; Cocolin *et al*., 2006; Rantsiou *et al*., 2005). This yeast species displays significant metabolic properties including, hydrolysis of sarcoplasmic proteins and generation of several polar and nonpolar peptides and free amino acids, lipolysis of pork fat, as well as production of flavor volatiles such as alcohols, acids, esters and various carboxyls (Sorensen, 1997).

The species *D. hansenii* displays a high intraspecific physiological variability (Suzuki, Prasad, & Kurtzman, 2011), therefore recent studies have focused on genetic differentiation of the strains. Molecular analyses using inter-LTR fingerprinting, RAPD-PCR, RFLP of mtDNA and DNA probes have demonstrated substantive strain heterogeneity in the strains of *D. hansenii* (Corredor *et al*., 2000; Prillinger *et al*., 1999; Sohier *et al*., 2009). A similar level of strain heterogeneity within *D. hansenii* strains isolated from naturally fermented sausages produced in the same locality can be also deduced from our study. This is plainly observed in case of *D.*

hansenii new isolates within groups G2 and G3 on the UPGMA dendrogram. This is in agreement with similar studies using RAPDs and minisatellite M13 where comparable levels of heterogeneity between strains of *D. hansenii* were found (Andrade *et al*., 2006; Groenewald *et al*., 2008).

The large genetic heterogeneity observed within *D. hansenii* difficults its separation from the type strain of *D. fabryi* isolated from human interdigital mycosis. As can be observed in Figure 4, taxonomic affiliation of strain *D. hansenii* CECT 11365 could not be accurately resolved. The lowest similarity value between CECT 11365 and other *D. hansenii* strains (around 20%) confirms the complex population structure within the species pair *D. hansenii* - *D. fabryi*. Previous taxonomic studies demonstrated that PCR based techniques allowed good differentiation of genotypes (Andrade *et al*., 2006; Groenewald *et al*., 2008; Petersen, Lange Møller, & Jespersen, 2001; Prillinger *et al*., 1999; Sohier *et al*., 2009). Moreover, Andrade *et al*. (2009a) and Andrade, Rodríguez, Casado, & Córdoba, (2010b) revealed the presence of different groups within *D. hansenii* strains isolated from dry-cured Iberian hams on dendrograms based on RFLPs of mitochondrial DNA.

The UPGMA dendrogram in this study (Figure 4) based on the combined patterns of minisatellite and RFLPs of mtDNA indicates the separation of *D. fabryi* from *D. hansenii* although leaving strain CECT 11365 in a middle position. The large genetic variability found within the strains analyzed could be interpreted as intraspecific variation or presence of cryptic and hybrid strains within *D. hansenii* as already described (Jacques, Mallet, & Casaregola, 2009; Jacques *et al*., 2010). The lowest similarity values found in the Jaccar Similarity Matrix (data not shown) for

strains in UPGMA groups G2 and G3 would point out to new varieties or species within *D. hansenii*.

Evaluation of the genetic diversity within *D. hansenii* strains using ITS-5.8S rDNA sequences demonstrated that the only difference between *D. hansenii* and *D. fabryi* was at position 430 due to a replacement of a thymine by a cytosine (Petersen *et al*., 2001). Our study shows that several *D. hansenii* sausage isolates present the same ITS-5.8S rDNA sequence than *D. fabryi* (ITS-5.8S II). Moreover, reference strain CECT 11363 presents an additional nucleotide substitution (ITS-5.8S III) which is present in several ITS-5.8S rDNA sequences of *D. hansenii* sausage isolates found in GenBank (data not shown).

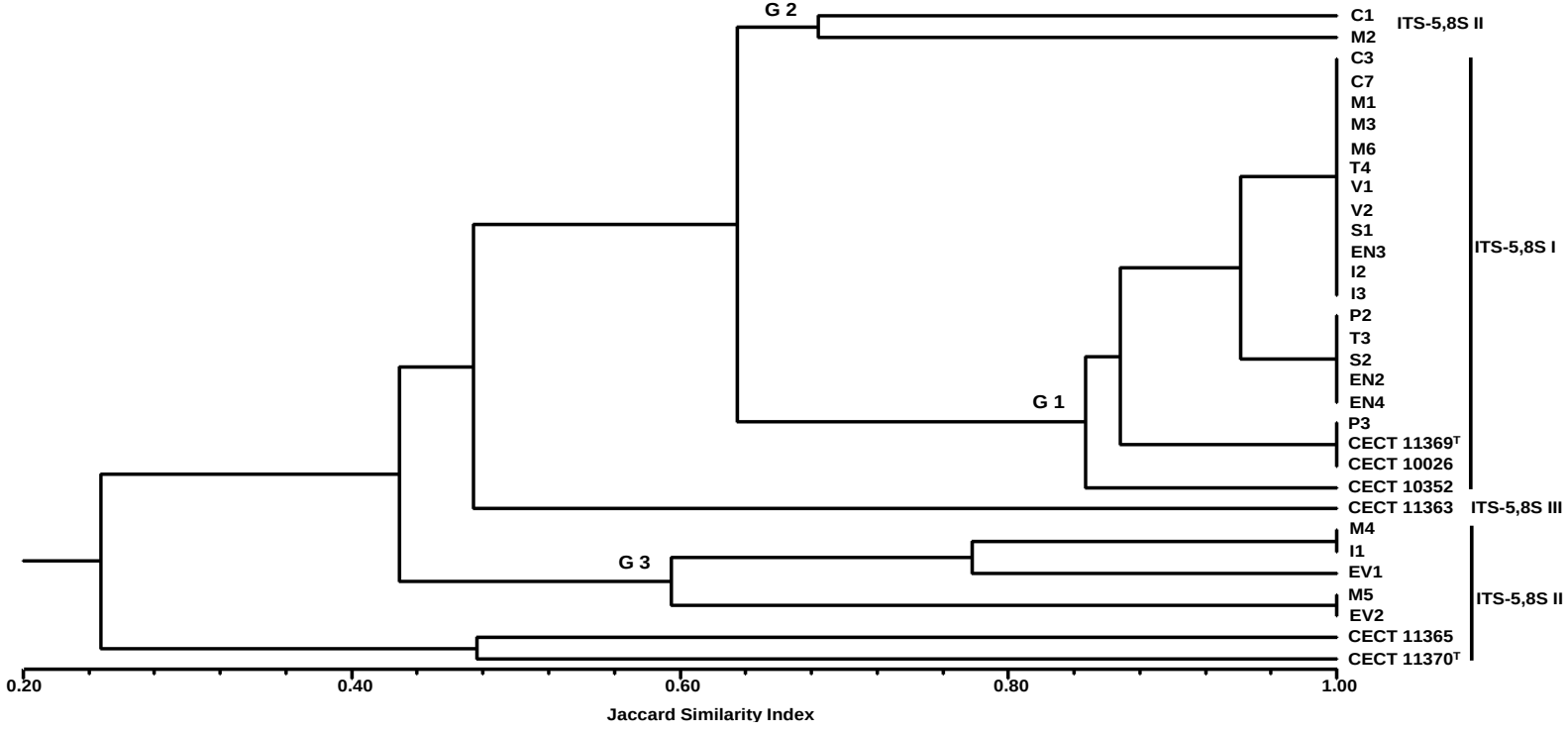

Figure 4. UPGMA dendrogram analysis showing the relationships among *D. hansenii* strains isolated from fermented sausages and CECT reference strains based on mtDNA restriction and M13 patterns analysis. Three different ITS1-5.8S-ITS2 rDNA region sequence types are indicated as ITS-5.8S I, ITS-5.8S II and ITS-5.8S III.

Aroma production of isolated yeast in “in vitro” media

All yeasts were able to grow at 25 °C on both defined media (YNB + B + E and YNB + B + M) containing 2-methyl butanoic acid and ethanol or methanol. After 15 days of incubation, the supernatant was sensory evaluated and compared to the non inoculated media. For most of the 30 strains assayed, the panelist did not observe differences in aroma between inoculated and non inoculated tubes. However, the panelist detected different aroma descriptors in seven isolates as shown in Table 2. *D. hansenii* yeasts M4, M6 and P2 produced fruity and floral aroma notes, whereas T3, V2 and EV2 strains produced sulfur, feet and cooked vegetables notes (Table 2). On the other hand, I2 strain displays an intermediate behavior regarding aroma descriptors as contribute to fruity and sulfur notes in both media.

Only those isolates that were able to produce different aroma than the control tube on both media (Table 2) were analyzed by SPME – GC – MS analysis. Table 3 shows the volatile compounds identified in both inoculated media, YNB + B + E and YNB + B + M. The compounds ethanol or methanol and 2-methyl butanoic acid were detected in the control tubes as they were added. Also in the YNB + B + M control media several volatile compounds were detected although they represented less than 2% of the total extracted area. The inoculated tubes contained different volatile compounds, such as aldehydes, ketones, alcohols, esters and sulfur compounds, in addition to the supplemented compounds (Table 3). YNB + B + E medium was characterized by the presence of ethanol and ethyl esters while YNB + B + M medium presented methanol and methyl esters (Table 3).

Table 2. Aroma descriptors detected in YNB media supplementedwith 2-methyl butanoic acid and ethanol (YNB + B + E) or methanol (YNB + B + M) after incubation with *D. hansenii* yeasts.

Yeast strains	Media	Aroma descriptors*				
M4	YNB+B+E	Fruity (6)	Floral (1)	Sweet (1)		
	YNB+B+M	Fruity (4)	Floral (2)	Cheese (2)		
M6	YNB+B+E	Fruity (5)	Floral (2)	Sweet (1)		
	YNB+B+M	Fruity (4)	Feet (2)	Cheese (1)		
P2	YNB+B+E	Fruity (5)	Leather (2)	Roasted nuts (1)		
	YNB+B+M	Roasted nuts (3)	Feet (1)	Leather (1)	Mustier (1)	
T3	YNB+B+E	Roasted nuts (2)	Cheese (2)	Cooked vegetables (2)	Fruity (1)	Feet (1)
	YNB+B+M	Feet (3)	Mustier (2)	Cooked vegetables (1)	Cheese (1)	
V2	YNB+B+E	Cooked vegetables (5)	Cheese (2)			
	YNB+B+M	Cheese (3)	Mustier (3)	Asparagus (1)	Fruity (1)	
EV2	YNB+B+E	Sulphur (3)	Mustier (2)	Bitter almond (1)	Cheese (1)	Fruity (1)
	YNB+B+M	Mustier (3)	Cooked vegetables (2)	Herbaceous (1)	Cheese (1)	
I2	YNB+B+E	Fruity (3)	Sulphur (3)	Feet (1)	Cheese (1)	
	YNB+B+M	Mustier (6)	Sulphur (1)	Feet (1)		

* Numbers in parentheses indicate the number of times a descriptor was detected by assessors.

The generation of volatile compounds by the seven inoculated yeasts on both media was expressed as a percentage relative to the abundance found in the control tube as shown in Table 4 and 5, respectively. Significant differences were found among yeasts and control tubes in the generation of volatile compounds in YNB + B + E medium (Table 4). The volatile compounds produced by the yeasts in highest abundance were ethanol, ethyl acetate, ethyl 2-methylbutanoate, dimethyl disulfide, dimethyl trisulfide, 2-methyl and 3-methyl butanol, 2-ethyl 1-hexanol and in less abundance aldehydes and ketones (Table 4). However, there were significant differences in the volatile production among yeasts. M4 was the yeasts that produce the highest significant proportion of ester compounds and it produced the highest abundance of ethyl acetate and ethyl 2-methyl butanoate. M4 strain could be responsible for the fruity and floral notes detected in the sensory odor analysis (Stahnke, 1994).

M4 was the only yeast able to consume ethanol and the lowest producer of 2-methyl butanoic acid. In addition, M4 yeast also produced sulfur compounds (dimethyl disulfide and dimethyl trisulfide) but in significant lower proportion than V2 yeast. This V2 yeast was the main significant producer of sulfur compounds, it showed the highest significant abundance in comparison to control and other yeasts in the three sulfur compounds detected (methanethiol, dimethyl disulfide and dimethyl trisulfide). V2 also showed significant abundance of ester compounds (ethyl acetate and ethyl 2-methyl butanoate) in comparison to control tubes but in significant less proportion than M4 yeast. A similar yeast that was able to produce sulfur compounds was EV2 strain as it showed a highest significant abundance of dimethyl disulfide and dimethyl trisulfide but in this case, the production of

methanethiol was not different to the control tube. These sulfur compounds have been identified as essential contributors to sausage aroma due to their low detection threshold and their sensory notes (Schmidt & Berger, 1998; Söllner & Schierberle, 2009).

The remaining yeasts displayed an intermediate behavior in the production of ethyl esters and sulfur compounds respect to M4, V2 and EV2. M6, P2, T3 and I2 yeasts produced significant proportions of ester compounds (ethyl acetate and ethyl 2-methyl butanoate) and dimethyl disulfide but they did not show significant differences in the production of dimethyl trisulfide in comparison to control tubes. Moreover, V2, EV2 and T3 yeasts that showed unpleasant aromas (cooked vegetables, mustier, sulfur) in the sensory analysis produced the highest proportion of sulfur compounds. Finally, T3 yeasts showed a significant different generation of 2-butanone, ethyl hexanoate, ethyl octanoate in comparison to control and other yeasts. Nevertheless, all the yeasts showed a significant generation of methyl-branched alcohols but few significant differences were detected among them. The incubation of yeasts in the culture media containing 2-methyl butanoic acid and methanol (YNB + B + M) showed also significant differences in the generation of volatile compounds (Table 5).

Table 3. Volatile compounds identified in YNB media supplemented with 2-methyl butanoic acid and ethanol (YNB + B + E) or methanol (YNB + B + M) after incubation with *D. hansenii* yeasts.

KI*	R**	Compounds	YNB+B+E		YNB+B+M	
			Control	Inoculated	Control	Inoculated
462	a	Acetaldehyde		+		
472	a	Methanethiol (47)[C]		+		
473	a	Methanol			+	+
509	a	Ethanol	+	+		
552	a	Methyl acetate				+
633	a	2-Butanone (72)		+	+	+
634	a	Ethyl acetate		+		
734	a	2-Pentanone (86)		+	+	+
741	a	3-Pentanone				+
745	a	Ethyl propanoate		+		
755	a	Methyl butanoate (74)			+	+
772	a	Dimethyl disulfide		+	+	+
794	a	3-methyl 1-butanol		+		+
797	a	2-methyl 1-butanol		+		+
805	b	Methyl 2-methyl butanoate			+	+
831	a	Ethyl butanoate		+		
833	b	Methyl 2-Pentenoate (83)				+
878	a	Ethyl 2-methyl butanoate		+		+
882	a	Ethyl 3-methyl butanoate		+		+
899	b	Methyl 2-methyl 2-butenoate (55)				+
918	a	Styrene		+		+
934	a	2-Heptanone		+		
949	a	2-methyl butanoic acid	+	+	+	+
971	b	Ethyl 2-methyl 2-butenoate		+		

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Table 3. (continued)

KI*	R**	Compounds	YNB+B+E		YNB+B+M	
			Control	Inoculated	Control	Inoculated
1002	a	Dimethyl trisulfide (126)		+		+
1015	a	Benzaldehyde (77)		+		+
1029	a	Ethyl hexanoate (99)		+		
1071	b	4-Nonanone (71)		+	+	+
1082	a	2-ethyl 1-hexanol,		+		+
1127	a	Ethyl heptanoate (88)		+		
1149	a	Nonanal (95)		+		+
1229	a	Ethyl octanoate (127)		+		
1256	a	Decanal		+		+
1260	a	Methyl nonanoate (141)				+
1327	a	Ethyl nonanoate		+		
1358	a	Methyl decanoate				+
1425	a	Ethyl decanoate (101)		+		
1553	b	Methyl dodecanoate (143)				+

KI*: Kovats Index calculated for DB-624 capillary column (J & W Scientific: 30 m, 0.25 mm i.d., 1.4 μm film thickness) installed on a gas chromatograph equipped with a mass-selective detector. R*: Reliability of identification a: mass spectrum and retention time identical with an authentic standard, b: tentative identification by mass spectrum. c: Target ion used to quantify the compoundwhen the peakwas not completely resolved.

Table 4. Abundance of volatile compounds (expressed in percentage as the relation between area in inoculated and control tubes) in YNB media supplemented with 2-methyl butanoic acid and ethanol (YNB+B+E) after incubation with *D. hansenii* yeasts.

Volatile compound	CONTROL		M4		M6		P2		T3		V2		EV2		I2		SEM[2]	P -Value[1]
	Mean		Mean		Mean		Mean		Mean		Mean		Mean		Mean			
Aldehydes																		
Acetaldehyde	100	a[3]	2363	b	2637	b	2921	b	100	a	1434	ab	1537	ab	1784	ab	507	*
Benzaldehyde	100	a	2802	c	354	ab	100	a	100	a	3030	c	373	ab	687	b	147	**
Nonanal	100	b	44	a	100	b	100	b	100	b	100	b	100	b	45	a	7	**
Ketones																		
2-Butanone	100	a	100	a	100	a	100	a	1313	b	284	a	434	a	424	a	196	**
2-Pentanone	100	a	2887	c	2304	b	405	a	2597	bc	2245	b	2476	bc	2199	b	148	**
4-Nonanone	100		100		100		100		100		430		100		306		91	ns
Sulfur compounds																		
Methanethiol	100	a	100	a	100	a	100	a	147	b	224	c	100	a	100	a	9	**
Dimethyl disulfide	100	a	14219	bc	10259	b	9493	b	7737	ab	20241	c	19631	c	12311	bc	2923	**
Dimethyl trisulfide	100	a	6721	b	494	a	466	a	410	a	8184	c	6350	b	1077	a	435	**
Alcohols																		
Ethanol	100	a	2	a	953625	c	518298	bc	510021	b	577879	bc	594057	bc	724193	bc	145987	**
3-Methyl 1-Butanol	100	a	7757	b	8651	bc	9904	bc	11604	c	8706	bc	9832	bc	7274	b	1055	**
2-Methyl 1-Butanol	100	a	3175	b	3187	b	4698	c	4625	c	3130	b	3519	bc	3523	bc	438	**
2-Ethyl 1-Hexanol	100	a	17954	c	10901	bc	8786	b	13143	bc	7581	b	14758	bc	10140	bc	2685	*

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Table 4. (Continued)

Volatile compound	CONTROL		M4		M6		P2		T3		V2		EV2		I2		SEM[2]	P -Value [1]
	Mean		Mean		Mean		Mean		Mean		Mean		Mean		Mean			
Ester compounds																		
Ethyl acetate	100	a	21473	d	10711	bc	19670	cd	8060	ab	11210	bc	11065	bc	15708	bcd	3347	**
Ethyl 2-methyl butanoate	100	a	32545	d	19111	c	7323	b	17194	c	17474	c	17443	c	13806	bc	2250	**
Ethyl hexanoate	100	a	436	a	413	a	254	a	4495	b	399	a	100	a	301	a	900	*
Ethyl heptanoate	100	a	225	b	100	a	100	a	207	ab	100	a	100	a	261	b	139	*
Ethyl octanoate	100	a	2483	b	58	a	53	a	3310	b	65	a	100	a	809	a	346	**
Ethyl nonanoate	100	a	1648	b	100	a	100	a	100	a	100	a	100	a	100	a	10	**
Ethyl decanoate	100	a	100	a	100	a	100	a	100	a	100	a	100	a	134	b	4	**
Other compounds																		
2-Methyl butanoic acid	100	a	440	a	8261	b	126292	c	70337	b	94448	bc	83880	b	56127	b	11641	**
Styrene	100		100		100		510		459		751		596		992		227	ns

[1], ns = $p > 0.05$, * = $p < 0.05$; ** = $p < 0.01$; [2] SEM: standard error of the mean. [3] Identical letters in each row indicate the absence of significant differences at $p>0.05$.

Table 5. Abundance of volatile compounds (expressed in percentage as the relation between area in inoculated and control tubes) in YNB media supplemented with 2-methyl butanoic acid and methanol (YNB+B+ME) after incubation with *D. hansenii* yeasts.

Volatile Compounds	CONTROL	M4	M6	P2	T3	V2	EV2	I2	SEM[2]	P –Value[1]
	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean		
Aldehydes										
Benzaldehyde	100	100	100	100	100	100	147	100	23	ns
Nonanal	100 a[3]	12320 b	100 a	100 a	100 a	100 a	100 a	36 a	895	**
Decanal	100 a	100 a	140 b	142 b	1220 c	100 a	100 a	100 a	42	**
Ketones										
2-Butanone	100 a	1404 b	1143 b	1090 b	195 a	1480 b	1144 b	1341 b	156	**
2-Pentanone	100 a	3939 c	1333 b	1360 b	1122 b	1376 b	1336 b	1437 b	113	**
3-pentanone	100 a	1533 d	1177 bc	1379 bcd	1080 b	1216 bc	1405 cd	1588 d	105	**
2-Nonanone	100 a	100 a	100 a	399 b	100 a	100 a	100 a	100 a	9	**
4-Nonanone	100	100	100	332	100	100	100	100	82	ns
Sulfur compounds										
Dimethyl disulfide	100 a	1376 a	4027 b	7717 c	14507 d	5283 b	6039 bc	4589 b	700	*
Dimethyl trisulfide	100 a	10832 b	259 a	875 b	246 a	172 a	100 a	100 a	643	**
Alcohols										
Methanol	100 a	1067 b	897 b	742 b	1037 b	2611 c	1246 b	1008 b	199	**
3-Methyl 1-butanol	100 a	323 b	119 a	124 a	89 a	84 a	119 a	127 a	34	**
2-Methyl 1-butanol	100 a	1792 c	1169 b	1135 b	815 b	815 b	901 b	1186 b	161	**
2-Ethyl 1-hexanol	100 a	9779 b	15398 bcd	13633 bcd	12334 bc	14565 bcd	21412 d	17297 cd	2678	**

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Table 5. (Continued)

Volatile Compounds	CONTROL		M4		M6		P2		T3		V2		EV2		I2		SEM[2]	P –Value[1]
	Mean		Mean		Mean		Mean		Mean		Mean		Mean		Mean			
Ester compounds																		
Methyl acetate	100	a	1930	d	1521	bcd	911	b	1736	cd	947	b	1193	bc	1330	bcd	247	**
Methyl butanoate	100	abc	438	d	56	a	176	c	89	ab	100	abc	170	bc	100	abc	32	**
Methyl 2-methyl butanoate	100	a	2386	a	15097	c	7891	b	8644	b	16422	c	8433	b	14554	c	1590	**
Methyl 2-Pentenoate	100	a	100	a	100	a	100	a	100	a	100	a	349	b	100	a	27	**
Methyl 2-methyl 2-butenoate	100	a	49673	d	7913	c	408	a	487	a	5861	bc	100	a	3645	b	701	**
Methyl nonanoate	100	a	100	a	100	a	100	a	100	a	100	a	133	b	100	a	9	ns
Methyl decanoate	100	a	100	a	96	a	409	a	100	a	77	a	169	a	1077	b	133	**
Methyl dodecanoate	100	a	1144	c	89	a	51	a	100	a	234	b	45	a	68	a	52	**
Other compounds																		
2-Methyl butanoic acid	100	a	53	a	100	a	847	b	418	ab	1043	b	757	ab	1006	b	120	**
Styrene	100	a	25197	d	3181	c	2982	c	770	b	773	b	2357	c	3282	c	489	**

[1], ns = $p > 0.05$; ** = $p < 0.01$; * = $p < 0.05$. [2] SEM: standard error of the mean. [3] Identical letters in each row indicate the absence of significant differences at $p>0.05$.

The volatile compounds produced in highest abundance by the yeasts were methyl acetate, methyl 2-methylbutanoate, methyl 2-methyl 2-butenoate, dimethyl disulfide, dimethyl trisulfide, 2-ethyl 1-hexanol, styrene and in less abundance aldehydes and ketones (Table 5). As observed previously, there were also significant differences in the generation of volatile compounds among yeasts. M4 yeast was the strain with the highest significant production of volatile compounds and it showed the highest significant production of nonanal, 2-pentanone, dimethyl trisulfide, 3-methyl and 2-methyl butanol, methyl butanoate, methyl 2-methyl butenoate, methyl dodecanoate and styrene. M6 yeast produced a significant increase of methyl 2-methyl butanoate and methyl 2-methyl 2-butenoate esters; P2 was the yeast that produced the highest significant 2-nonanone abundance. T3 significantly produced decanal and dimethyl disulfide and V2 was the main producer of methyl 2-methyl-butanoate and methanol. I2 yeast showed a significant highest production of methyl decanoate and methyl 2-methyl-butanoate whereas EV2 yeast generated the highest abundance of 2-ethyl 1-hexanol and it was the only one that produced methyl 2-pentenoate.

As observed in both media, the generation of volatile compounds by yeasts depends on the culture media. In the inoculated media three amino acids (histidine, methionine, tryptophan) were present as a nitrogen source, and their degradation generates histamine, methional and methanethiol and indol compounds, respectively (Yvon & Rijnen, 2001). Several of these compounds were detected in the inoculated media such as methanethiol although the yeasts were capable to produce many other volatile compounds.

In addition, the present study has shown the ability of the isolated yeasts to generate ester compounds apart from other volatile compounds. Until now it was not possible to attribute the ester compound generation in fermented sausages to a specific microbial group (Tjener & Stanhke, 2007). The inoculated media were supplemented with 2-methyl butanoic acid and an alcohol (ethanol or methanol) and it was confirmed the generation of the corresponding ethyl or methyl 2-methyl butanoate ester in high abundance respect to the control tube. Therefore, this study has confirmed the esterase activity in the species *D. hansenii* and the ability to produce ethyl and methyl esters from simple media containing an alcohol and an organic acid (Besançon, Ratomahenina, & Galzy, 1995).

Previous studies done by Andrade *et al*. (2009b) also reported the generation of similar volatile compounds from *D. hansenii* yeasts isolated from dry cured ham and inoculated in a culture media resembling the conditions of the process. They found a high generation of branched alcohols, aldehydes, ketones and also sulfur compounds as we have observed under our experimental conditions. However, we detected important differences such as an absence of branched aldehydes and a high generation of ester compounds in our culture media. In addition, in 2004, Flores *et al*., applied a *D. hansenii* strain to a fermented sausage and the increase in ester compounds in the inoculated batches was small, in contrast to the results reported by Andrade *et al*. (2010a) who observed a higher generation of ester compounds in *D. hansenii* inoculated batches. Therefore, it is essential to select the appropriate *D. hansenii* strain with the ability to form ester compounds that can provide fruity notes to the fermented sausages increasing the sensory quality (Stahnke, 1994).

This study confirms the large genetic variability within strains of *D. hansenii* isolated from naturally fermented sausages. Our results indicate that depletion or production of alcohols and production of esters and sulfur compounds seems to be a strain trait not corresponding with M13 and mtDNA profiles. Therefore selection of a unique representative strain from each *D. hansenii* UPGMA group for testing of aroma production on methanol and ethanol media might have lead to different conclusions. Finally, the differences detected in the aroma profiles produced by the selected *D. hansenii* isolates have to be confirmed in real fermented sausages.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2013.02.047.

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Supplementary material

"Data matrix to test UPGMA D hansenii"
"40 characters and 31 OTUs"
"columns labeled no missing values"

1	40	31	0																										
	C1	C3	C7	M1	M2	M3	M4	M5	M6	P2	P3	T3	T4	V1	V2	EV1	EV2	S1	S2	EN2	EN3	EN4	I1	I2	I3	11369	10026	10352	11363	11365	11370
a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
b	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0
c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
d	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
e	0	1	1	1	0	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0
f	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
g	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	0	0	0
h	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
i	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
j	0	1	1	1	0	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	0	0	0	0
k	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
l	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
m	0	1	1	1	0	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	0	0	0
n	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
o	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
p	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
q	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
r	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
s	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
t	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	0	0	0
u	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
v	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
w	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
x	1	1	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	0	1	0	0	0
y	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
z	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0
ab	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	1	1
ac	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
ad	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
ae	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
af	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ag	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
ah	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ai	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1
aj	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
ak	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	0	0
al	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
am	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
an	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
ao	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0

Capítulo 2

Generación de compuestos aromáticos producidos por cepas de *Debaryomyces hansenii* en un sistema modelo cárnico similar a un embutido crudo curado.

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Generation of aroma compounds in a fermented sausage meat model system by *Debaryomyces hansenii* strains.

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Abstract

The ability of seven *Debaryomyces hansenii* strains to generate aroma compounds in a fermented sausage model system was evaluated. The presence of the yeast in the inoculated models was confirmed by PCR amplification of M13 minisatellite. Volatile compounds production was analyzed using Solid Phase Micro-Extraction and gas chromatography/mass spectrometry. Forty volatile compounds were detected, quantified and their odour activity values (OAVs) calculated. All volatile compounds increased during time in the inoculated models although significant differences were found among them. Ester and sulfur production was strongly dependent on the strain inoculated. *D. hansenii* P2 and M6 strains were the highest producers of sulfur compounds being dimethyl disulfide and dimethyl trisulfide the most prominent aroma components identified by their OAVs whereas, M4 showed the highest OAVs for ester compounds followed by P2 strain. The meat model system has been useful to show the real ability of yeast strains to produce aroma compounds.

Keywords: yeast, *D. hansenii*, fermented sausage, flavour, aroma, volatile, odour activity values.

INTRODUCCTION

Yeasts are one of the predominant groups within the microbiota present in dry fermented sausages (Selgas, Ros, & García, 2003). The yeast genera frequently found include *Candida, Cryptococus, Debaryomyces, Kluyveromyces, Pichia, Rhodotorula, Saccharomyces, Trichosporon* and *Yarrowia,* although among them, *Debaryomyces hansenii* is the predominant yeast species (Aquilanti, Santarelli, Silvestri, Osimani, Petruzzelli, & Clementi, 2007). Recently, Mendonça, Gouvêa, Hungaro, Sodré, & Querol-Simón (2013) reported the predominance of *Debaryomyces* during the manufacturing process of artisanal and industrial style Spanish dry-cured sausages. Studies carried out with *D. hansenii* showed that it can positively contribute to the stabilization of the reddening reaction by its ability to degrade peroxides and development of flavour in dry fermented sausages favoured by its lipolytic and proteolytic activities (Olesen & Stahnke, 2000; Durá, Flores, & Toldrá, 2004; Flores, Durá, Marco, & Toldrá, 2004).

Yeast growth dynamics and physico-chemical parameters have been previously studied using different *in vitro* meat model systems. Olesen *et al*. (2000) studied the aroma formation in a model system resembling fermented sausage composition and inoculated with *D. hansenii* and other starter cultures. They reported a very limited production of volatile compounds by *D. hansenii* attributed to unfavourable growth conditions, growth inhibition by spices (garlic powder) and finally, to the low aromatic potential of the strain used. In 2009, Andrade, Córdoba, Sánchez, Casado, & Rodríguez, studied the aroma contribution of yeasts isolated from dry-

cured Iberian hams (*D. hansenii* and *C. zeylanoides)* using a culture medium containing compounds representative of dry-cured ham composition. The authors reported differences in volatile compounds generation among the yeast biotypes studied. Therefore, the selection of a model system resembling the real sausage/meat fermentation conditions and the inoculation of an appropriated yeast strain seem to be a key point to understand the effects of *D. hansenii* on the production of aroma compounds.

Although different meat models have been used for simulation of the fermentation process in sausages, an acidification stage is always included. The acidification due to the activity of lactic acid bacteria produces coagulation of meat proteins and later, during drying the formed protein gel provides the necessary firmness to the final product (Demeyer, Hoozee, & Mesdom, 1974). This gelification process can be done by using glucono-δ-lactone (Ngapo, Wilkinson, & Chong, 1995) and avoid the addition of lactic acid bacteria which could interfere in the analysis of the volatile compounds.

In a previous study, seven strains pertaining to the species *D. hansenii* were selected due to their ability to produce aroma compounds such as aldehydes, ketones, alcohols, esters and sulfur compounds in a defined culture media (Cano-García, Flores, & Belloch, 2013). Among them, esters and sulfur compounds have been identified as essential contributors to sausage aroma due to their low detection threshold and their sensory notes, fruity and feet or cooked vegetables, respectively (Schmidt & Berger, 1998; Söllner & Schierberle, 2009; Olivares, Navarro, & Flores, 2010). However, the use of a model system resembling the fermented sausage is necessary to

select the appropriate yeast to be used at pilot plant scale. Therefore, the main objective of our study was to evaluate the aroma produced by seven *D. hansenii* strains in a model system with conditions resembling those used in fermented sausages.

MATERIALS AND METHODS

Reagents and standards

The chemical compounds used for volatile compound identification and quantification were all obtained from Sigma-Aldrich (Steinheim, Germany).

Preparation of yeast strains

Debaryomyces hansenii var. *hansenii* strains M4, M6, P2, T3, V2, EV2 and I2, isolated from naturally fermented sausages "salchichón de Requena" (Requena, Valencia, Spain) were cultured overnight in GPY medium (glucose 2%, yeast extract 0.5%, peptone 0.5%) at 25 ºC (Cano-García *et al*., 2013). Cells were collected by centrifugation and suspended in physiological saline solution. OD at 655 nm (Biophotometer AG eppendorf 22331, Hamburg, Germany) was measured and cell suspensions adjusted to 10^8 cell mL^{-1} for inoculation of the meat model system.

Preparation of fermented meat model system

The fermented meat model system was prepared using fresh minced pork muscle *longissimus dorsi* with no visible fat or connective tissue. The meat was homogenized (1:2 w/v) with 0.1 M Tris-HCl, 20 mM EDTA at pH 7.0 by using a mixer (Krups 577, Germany). The homogenate was centrifuged at 10,000 rpm for 30 min at 4 ºC and the supernatant was discarded. The

process was repeated three times to remove the supernatant containing sarcoplasmic proteins and the pellet containing myofibrillar proteins was stored at – 20 ºC until used.

The meat model system was prepared using the extracted myofibrillar proteins, pork fat (pork lard, El Pozo, Murcia, Spain) and a buffer solution containing a similar composition of the additives used in fermented sausage processing and including free amino acids at a concentration similar to fresh meat. The prepared solution contained (g/L): sodium chloride (30), glucose (10), sodium nitrite (0.15), potassium nitrate (0.15) and a solution of free amino acids according to Flores, Sanz, Spanier, Aristoy, & Toldrá (1998). This solution was sterilized by vacuum-driven filtration system (0.22 μm). The meat model system was prepared by homogenization of the thawed myofibrillar proteins (175 g), pork fat (75 g) and 500 mL of the solution (additives and free amino acids) using a mixer (Krups type 577, México). Eight batches of meat model system, 500 g each, were prepared. Seven batches were used to inoculate the *D. hansenii* strains (10^6 per g) while one batch was not inoculated and used as control. Each batch was separated in five 100 g portions. Gel formation was produced by acidification adding 7.5 mL of 56 mM glucono-δ-lactone to each batch portion (Ngapo *et al*., 1995). Model systems were incubated at 12 ºC and 100 g portion samples were taken at 0, 7, 15, 30 and 45 days for further analyses. The incubation experiments were done in triplicate.

Yeast counts

One gram of sample from each meat model system was transferred into a tube containing 9 mL of sterile saline solution (0.9 % salt) and

homogenized for 2 min in a Stomacher (IUL Instrument, Barcelona, Spain). Decimal dilutions, from 10^{-1} to 10^{-5} were inoculated (100 µL) into agar plates. Yeasts were counted in GPYA medium (2 % glucose, 0.5 % peptone, 0.5 % yeast extract and 2 % agar) and incubated at 28 ºC for 4 to 5 days. Tests were carried out in triplicate. Ten colonies from each sample were isolated and stored in glycerol at -80 ºC for further characterization by molecular methods.

Strain typing by molecular methods

Yeast isolates from each meat model sample were subjected to genotyping by minisatellite M13 PCR amplification. DNA was extracted as described in Querol, Barrio & Ramón, (1992) and M13 minisatellite PCR amplification was carried out as described elsewhere (Cano-García *et al.*, 2013). PCR products were separated by electrophoresis on 2 % agarose gel in 1xTAE buffer at 90 V for 3 h, stained with Red Safe nucleic acid staining solution 20,000x (Intron biotechnology, Kyunggi-do, Korea) and visualized under UV light. DNA fragment sizes were determined by comparison to a 100-bp DNA ladder. Minisatellite PCR patterns were compared with those previously observed for these strains (Cano-García *et al*., 2013).

Measurement of lipolytic activity in yeast strains

Lipolytic activity of the yeast strains was evaluated using the method described by Sierra (1957) using lauric acid ester (T20), palmitic acid ester (T40), stearic acid ester (T60) and oleic acid ester (T80) at 1 % final concentration. Yeast strains were streaked and incubated at 28 ºC for 15 days or until lipolytic activity was detected as an opaque halo around the colonies caused by precipitation of the respective fatty acid calcium salt.

pH measurement

Protein, fat and yeast cells were removed from the meat model sample by centrifugation at 10,000 rpm during 10 min at 4 ºC. The supernatant was filtered through glass wool and pH measured using pH–meter (Orion EA 920, Boston, USA). Supernatants were stored at -20 ºC for further analysis.

Analysis of volatile compounds

Seven mL aliquots of the supernatant obtained from the model systems as described above were introduced into 20 mL headspace vial (Gerstel, Germany) for volatile analysis. Volatile compounds in supernatant samples were extracted by a Solid Phase Microextraction (SPME) device using an automatic injector Gerstel MPS2 (Gerstel, Germany). The vial was previously equilibrated for 15 min at 37 ºC. Then, the extraction was done using a 85 μm carboxen/polydimethylsiloxane StableFlex fiber (Supelco, Bellefonte, PA, USA) by headspace exposure during 2 h at 37ºC while shaking at 250 rpm.

Identification and quantification of volatile compounds was done using a gas chromatograph (GC HP 7890 series II) equipped with an HP 5975C mass selective detector (MS Hewlett Packard, Palo Alto, CA, USA). The compounds absorbed by the fiber were desorbed in the injection port of the GC-MS for 15 min at 220 ºC with the purge valve off (splitless mode) using He as carrier gas with a linear velocity of 34.3 cm sec^{-1}. Compounds were separated on a DB-624 capillary column (J&W scientific, 30 m x 0.25 mm x 1.4 μm) and analyzed as described by Marco, Navarro, & Flores (2006). The GC oven temperature program began when the fibre was inserted and was held at 38°C for 13 min, ramped to 100° C at 3°C per min and held at

100°C for 5 min, then to 150°C at 4°C per min and to 210°C at 10°C per min and finally, held at 210°C for 5 min. The total run time was 62.17 min and the GC-mass spectrometer interface was maintained at 240°C. Mass spectra were obtained by electron impact at 70 eV, and data were acquired across the range 29–400 amu. Source and quadrupole temperatures of the MS were maintained at 230° and 150°C. Volatile compounds were identified by comparison with mass spectra from the library database (NIST'05) and with Kovats retention indices of authentic standards (Kovats, 1965).

The quantification of volatile compounds was done by using the MS detector in SCAN mode and by the external standard method. Stock standard solutions of pure compounds were prepared in methanol and serial dilutions were analyzed by GC – MS under the same chromatographic conditions. A linear calibration was obtained for each compound and used to measure the concentration in the headspace (HS) extracted by the SPME fiber in each analysis (Table 1 supplementary material). The quantification was expressed in ng of volatile compound extracted by the SPME fiber per g of meat model and subtracting the concentration detected in the control tube.

The odour-activity values (OAVs) in air of each volatile compound were calculated as the ratio of concentration in HS to the odour threshold in air obtained from Van Gemert & Nettenbreijer (2004).

Statistical analysis

The effect of each yeast strain and incubation time on the generation of volatile compounds in the fermented meat model were tested by two-factor

analysis of variance (ANOVA) using the statistic software XLSTAT 2009.4.03 (Addinsoft, Barcelona, Spain). Differences between samples means were analyzed according to Fisher's Least Significant Difference test (LSD).

RESULTS

The growth kinetics of yeast strains inoculated in the meat model is shown in Figure 1. Yeast counts increased in the models from day 0 until day 30, except in case of P2, M6 and M4. In case of P2 and M6 a slight decrease was observed after day 15, whereas M4 population suffered a noticeable decrease since day 15. After day 30, T3 and EV2 counts displayed a decrease, whereas I2 and V2 maintained its growth trend until the last day. The M13 minisatellite patterns of the inoculated *D. hansenii* strains showed no change from day 0 until day 45 (Figure 1 supplementary material).

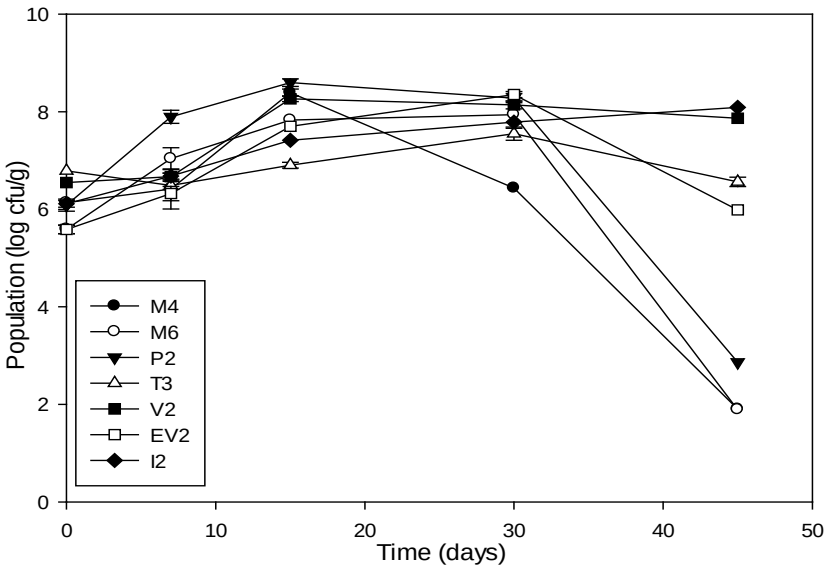

Figure 1. Yeast evolution during incubation in the meat model system.

Moreover, all isolated yeast strains showed lipolytic activity (Table 2 supplementary material). M4 yeast displayed the highest lipolytic activity against the different substrates and it was the only one capable to hydrolyze lauric and oleic acid esters. The other yeast strains only showed lipolytic activity against palmitic and steraric acid esters. On the other hand, the pH measured in the models ranged from 3.1 to 3.5 during all the incubation process (data not shown).

Regarding the production of volatile compounds a total of 40 volatile compounds were identified in the meat model system (Table 1). These volatile compounds included aldehydes (10), sulfur (4), ketones (4), alcohols (10), esters (7) and acids (5) compounds. However, twenty four volatile compounds, mostly aldehydes and alcohols, were also identified in the control batch (not inoculated). These volatiles were probably produced by chemical degradation of lipids and amino acids present in the meat model system during the fermentation time. Among the volatile compounds produced by the inoculated yeasts in each meat model (Table 1), the main differences were detected in the production of ester compounds as only M4 strain, was able to produce all of them. Production of sulfur compounds was also different among yeasts as only M4 and V2 strains, generated methionol.

Table 1.- Volatile compounds detected in fermented meat model systems inoculated with selected yeast.

Compounds	KI[1]	R[2]	C[3]	Yeast							Threshold (ng/g)[4]
				M4	M6	P2	T3	V2	EV2	I2	in Air
Aldehydes											
2-Methyl propanal	594	a	+	+	+	+	+	+	+	+	15-410
3-Methyl butanal	689	a	+	+	+	+	+	+	+	+	2-4
2-Methyl butanal	700	a	+	+	+	+	+	+	+	+	100
Hexanal	840	a	+	-	+	-	-	-	-	-	20-330
Heptanal	940	a	+	-	+	-	-	-	-	-	60-260
(Z)-2-Heptenal	1010	a	+	+	+	-	-	-	-	-	34-2800
Benzaldehyde	1017	a	+	+	+	-	-	-	+	+	10-3.4x10^{6}
Octanal	1048	a	+	+	+	-	+	+	-	-	5-20
Nonanal	1149	a	+	+	+	+	+	-	+	-	5-230
Decanal	1256	a	+	-	+	+	+	+	+	-	0.62-3800
Sulfur compounds											
Methanethiol	472	a	+	+	+	+	+	+	+	+	1x10^{-9}-1100
Dimethyl disulfide	772	a	+	+	+	+	+	+	+	+	1.1-3500
Dimethyl trisulfide	1002	a	-	-	+	+	-	+	+	+	0.06-14
Methionol	1062	a	-	+	-	-	-	+	-	-	1710

Continued on next page

Table 1. (Continued)

Compounds	KI[1]	R[2]	C[3]	Yeast							Threshold (ng/g)[4] in Air
				M4	M6	P2	T3	V2	EV2	I2	
Ketones											
2,3-Butanedione	626	a	-	+	+	+	+	+	+	+	5 - 20
2-Butanone	631	a	+	-	+	+	+	+	+	-	750-2.5x10^5
3-Hydroxy-2-butanone	780	a	-	-	+	+	+	+	+	+	800[c]
2-Heptanone	933	a	+	+	+	+	+	-	+	+	45-330
Alcohols											
Ethanol	509	a	+	+	+	+	+	+	+	+	640-7.6x10^7
1-Propanol	613	a	-	+	+	+	+	+	+	+	75-2.5x10^7
2-Methyl propanol	682	a	+	+	+	+	+	+	+	+	200-5x10^5
3-Methyl butanol	795	a	+	+	+	+	+	+	+	+	19-6300
2-Methyl butanol	797	a	-	+	+	+	+	+	+	+	0.9 - 1.0
1-Pentanol	827	a	+	+	+	+	+	+	+	-	20-1.1x10^6
1-Hexanol	922	a	+	+	+	+	+	+	+	+	10-6.5x10^4
1-Heptanol	1023	a	-	+	-	-	+	-	-	-	50-1.7x10^4
1-Octen-3-ol	1030	a	+	+	+	+	+	+	+	-	12-110
1-Octanol	1123	a	-	+	-	-	-	-	-	-	5-9000

Continued on next page

Table 1. (Continued)

Compounds	KI[1]	R[2]	C[3]	Yeast							Threshold (ng/g)[4]
				M4	M6	P2	T3	V2	EV2	I2	in Air
Ester compounds											
Ethyl acetate	635	a	+	+	+	+	+	+	+	+	340-6.23x10^5
Ethyl propanoate	744	a	-	+	-	-	-	-	-	-	200-1000
Propyl acetate	748	a	-	+	-	-	-	-	-	-	200-1.1x10^5
Ethyl 2-methyl propanoate	788	a	-	+	-	-	-	-	-	-	3.5-1060
Isobutyl acetate	805	b	-	+	-	-	-	-	-	-	420-34000
3-Methylbutyl acetate	906	a	-	+	-	+	-	-	-	-	15-8000
2-Methylbutyl acetate	909	a	-	+	-	-	-	-	-	-	140-210
Acids											
Acetic acid	714	a	+	+	+	+	+	+	+	+	25-5x10^5
Propanoic acid	806	a	-	-	+	+	+	+	+	+	3-14100
2-Methyl propanoic acid	864	a	+	+	+	+	-	+	+	+	5-240
3-Methyl butanoic acid	888	a	-	-	+	+	-	-	-	+	0.22-14
Hexanoic acid	1074	a	+	+	-	-	-	-	-	-	12-3500

[1]: Kovats Index calculated for DB-624 capillary column (J & W Scientific: 30 m, 0.25 mm i.d., 1.4 µm film thickness) installed on a gas chromatograph equipped with a mass-selective detector. [2]: Reliability of identification: a = mass spectrum and retention time identical with an authentic standard. b = Tentative identification by mass spectrum. [3]: Control batch without inoculated yeast. [4]: threshold values obtained from Van Gembert and Nettenbreijer (2004) except compound marked as [c] that was obtained from Buttery, Teranishi, Ling and Turnbaugh (1990) in water.

Table 2. Quantification (ng of volatile compounds/g of meat model) of alcohol, esters and acid compounds in the headspace of meat model systems inoculated with selected yeast at different incubation times.

YEAST	TIME (Days)	ALCOHOLS								ESTERS	ACIDS		
		Ethanol [1]	1-Propanol	2-methyl propanol	3-methyl butanol	2-methyl butanol	1-Pentanol	1-Hexanol	1-octen-3-ol	Ethyl acetate	Acetic acid	Propanoic acid	2-methyl propanoic acid
M4	0						2.6 f						
	7	0.9 g[3]						23.9 hij			50.8 m		
	15	15.3 fg			13.8 j	18.9 mn	21.3 c	101.6 c	17.5 cdef		54 m		
	30	107.2 e		82.7 jk	69.5 ij	61.1 jkl	18.8 cd	160.9 b	17 cdef	34.1 c	57.9 lm		
	45	462.5 a	40 cde	119.9 hi	681.8 cd	516.8 b	13.1 de	222.5 a	12.1 b	907.7 a	472.9 jkl		38 fg
M6	0									1.4 c			
	7	2.1 g			3 j	7.3 n	5.8 ef	24.7 j	7.3 f	2.3 c	134.4 lm		
	15	8 g			11.1 j	17.6 mn	20.8 c	35.6 ghij	16.2 def	4.7 c	144.6 lm		
	30	75.3 ef	18.8 e	187.3 ef	345.9 f	433.9 cd	9.1 ef	44.1 fghi	26.1 bcd		2438.2 cd		30.9 g
	45	218.7 cd	60.3 bcd	185.8 efg	595.8 cde	492.3 bc	7.8 ef	42.9 fghi	11 b	3.2 c	3210.2 b	48.5 c	68.3 cd
P2	0						2.2 f						
	7	16.4 fg		73.2 jk	16.1 j	27.1 klmn	9.7 ef	31.9 ghij	7.9 f	0.8 c	247.1 klm		
	15	102.6 e	22.7 e	240.3 de	317.1 fg	365.5 de	30.7 ab	97.9 cd	19.2 cde		1569 ef		
	30	214.1 cd	66.1 bc	342.1 b	565 de	523.8 bc		37.9 fghij	7.5 f	177.1 b	3577.8 b	58.3 bc	44 efg
	45	221.5 cd	44.7 cde	489.2 a	876.6 b	660.6 a		50 hij	17.5 a	201.3 b	5632.4 a	85.8 a	89.8 b

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Table 2. (Continued)

YEAST	TIME (Days)	ALCOHOLS								ESTERS	ACIDS		
		Ethanol [1]	1-Propanol	2-methyl propanol	3-methyl butanol	2-methyl butanol	1-Pentanol	1-Hexanol	1-octen-3-ol	Ethyl acetate	Acetic acid	Propanoic acid	2-methyl propanoic acid
T3	0												
	7	1.9 g		52.6 k	8.4 j	26.5 lmn	4.7 f			7.3 c	198.4 klm		
	15	6 g		60.2 ijk	28 j	97.3 ijk	17.2 cd	160.2 b	8.4 ef		518.6 jk		
	30	39.7 fg	42.8 cde	116.1 hi	185.8 hi	233.1 gh	31.8 a	218.6 a	29.4 b		908.5 hi		
	45	65.2 ef	31 de	122.8 h	264.8 fgh	271.4 gh	24 bc	219.3 a	27.4 bc		1816 e	19 d	
V2	0												
	7	22.3 fg	16.1 e	52.4 jk	13.7 j	49 klm		33.2 ghij		3.3 c	455.5 jkl		
	15	74.7 ef	40.9 cde	130.7 gh	200 gh	226.5 h	4.9 ef	66.2 def			1172.8 gh		
	30	105.4 ef	38.9 cde	207.6 e	385 f	299 efg	4.4 f	46.3 fghi			1441.2 fg		
	45	2158 cd	78.3 b	235.6 de	514.5 e	358.9 ef	4.1 f	58.1 efg	16.8 cdef	8 c	2795.1 c	43.8 c	50.3 ef
EV2	0												
	7	4.7 g		20.2 k	21.7 j	21.3 mn		38.9 fghij		6.4 c	136.1 lm		
	15	36.4 fg		84.4 hij	333.6 f	128.4 ij	5.5 ef	82.6 cde		6.4 c	749.2 ij		
	30	259.9 bc	124 a	303.8 bc	566.8 de	260.5 gh	1.8 f	42.1 fghi	11.5 ef	14.3 c	1516.4 efg	20.5 d	55.8 de
	45	228.1 cd	86 b	262.1 cd	713.5 c	292.9 fgh	3.7 f	48.5 fgh	11.6 ef	6.6 c	2319.8 d	45.7 c	79.1 bc

Continued on next page

Table 2. (Continued)

YEAST	TIME (Days)	ALCOHOLS								ESTERS	ACIDS		
		Ethanol [1]	1-Propanol	2-methyl propanol	3-methyl butanol	2-methyl butanol	1-Pentanol	1-Hexanol	1-octen-3-ol	Ethyl acetate	Acetic acid	Propanoic acid	2-methyl propanoic acid
I2	0												
	7	28.5 fg		61.3 jk	42 j	32.9 lmn		31 ij			400 jklm		
	15	63.4 efg	33.4 de	139.4 fgh	353.9 f	137.3 i		44.9 hij			1470.6 efg		
	30	184.1 d	83 b	306.1 bc	551.4 e	272.2 gh		19.5 j			1466.4 efg	42.7 cd	82 bc
	45	293.6 b	74.4 b	479.5 a	1267.5 a	513 b		15.3 j	9.6 ef	7 c	1238.1 fgh	79.5 ab	216.6 a
SEM [2]		76.39	7.01	26.53	62.1	37.94	2.08	12.35	1.63	53.68	249.23	7.61	16.88
Pt [4]		***	***	***	***	***	***	***	***	***	***	***	***
Pc		***	***	***	***	***	***	***	***	***	***	***	***
Ptxc		***	**	***	***	***	***	***	***	***	***	ns	***

[1]Ethanol: abundance expressed as area units from TIC X 10^6. [2]SEM: Standard error of the mean. [3]Different letters in the same columns means significant differences at p value. [4] Pt: p value of ripening time effect; Pc: p value of inoculated yeast effect; Ptxc: p value of interaction between inoculated yeast and ripening time effects. ***: $p<0.001$, **: $p<0.01$, ns: $p>0.05$.

Table 3. Quantification (ng of volatile compounds/g of meat model) of sulfur compounds, aldehydes and ketones in the headspace of meat model systems inoculated with selected yeast at different incubation times.

YEAST	TIME (Days)	SULFUR COMPOUNDS			ALDEHYDES					KETONES			
		Methanethiol [1]	Dimethyl disulfide	Dimethyl trisulfide	2-methyl propanal	3-methyl propanal	2-methyl butanal	Octanal	Nonanal	2,3-butanedione	2-butanone	3-hydroxy-2-butanone	2-heptanone
M4	0								3.9 cde				
	7		5.8 d					4.4 c	7.7 a				
	15		11.7 d		17.3 lm	20.1 ij	16.5 ijklm		3.7 de				3.9 d
	30	79.7 cd[3]			15.1 m	12.7 j	16.7 ijklm						
	45				113.9 b	111.6 ab	105.1 ab			14.2 ijkl			14.8 ab
M6	0												
	7		18.8 d		47.6 fghi	16.2 j	46.3 fgh				1.8 e		
	15		14.3 d		61.2 ef	46.1 efgh	109.1 a	5.9 c	5 cd	9.4 kl	5.1 ab		4 cd
	30	65.4 cd	169.1 cd	10.6 b	77.2 de	23.4 hij	96.9 abc	5 c	4.6 cde	41.8 d	5.9 a	101.1 a	11.7 bc
	45		908.7 b	10.7 b	38.6 ghijk	35.1 ghij	42.7 fgh		7.1 a	79.5 bc	4.3 abc	53.4 fghi	20.4 a
P2	0						2.4 m		3.6 de				
	7	120 abc	115.2 cd		13.6 m		10.3 klm		4.2 cde			10.7 i	4 d
	15	155.7 a	224.9 cd		50.1 fgh	30 hij	31.4 hi			39.5 fg		66.1 efg	
	30	87.9 bcd	227.7 cd	8.1 b	29.3 jklm	32.1 hij	30.4 hij			21.4 hijk		84.4 bc	
	45	147.9 ab	1730.8 a	104.1 a	33.2 ijkl	50.5 efgh	23 ijk			19.4 ijkl	3.6 bcd	16.7 hi	

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Table 3. (Continued)

YEAST	TIME (Days)	SULFUR COMPOUNDS			ALDEHYDES								
		Methanethiol [1]	Dimethyl disulfide	Dimethyl trisulfide	2-methyl propanal	3-methyl propanal	2-methyl butanal	Octanal	Nonanal	2,3-butanedione	2-butanone	3-hydroxy-2-butanone	2-heptanone
T3	0												
	7				24.5 klm	11.5 j	9.9 klm		4.2 cde		3.1 cde		
	15	114.4 abc	71.2 d		16.6 lm	14.1 j	14.4 jklm		5.3 bc	8.9 l		15.9 hi	
	30		102 d		23.9 klm	17.4 j	19.9 ijklm		6.5 ab	22.8 hij		45.7 fghi	
	45				27.9 klm	15.3 j	20.7 ijkl	7.1 bc	7.6 a	34 fg		56.4 fgh	9.7 bcd
V2	0						lm						
	7				88.7 d	46.1 efgh	69.1 e			12.3 jkl	3 cde		
	15	151.6 a	196 cd		90.5 cd	66.3 de	80.3 de	4.5 c		42 ef		45.3 fghi	
	30	67 cd	393.2 c		85.6 d	54.7 efg	50.3 fg	12.7 b		87.9 b		76 cd	
	45	65.2 cd	385.8 c	13.3 b	39.6 ghijk	32.9 ghij	15.3 ijklm	19.6 a		126.1 a	3.3 bcde	100.2 a	4.6 cd
EV2	0						3						
	7		33.3 d		45.5 fghij	63.6 def	51.4 f		3.5 e		4 bc		
	15	168.7 a	103.8 d		117.2 ab	123.7 a	81 cde			30.9 gh		40.6 fghi	
	30	69.4 cd	273.5 cd	10.6 b	84.7 d	66.1 de	44.8 fgh			68 c		75.4 cd	
	45		16.4 d	10.6 b	36.1 hijk	43.4 fghi	32.6 ghi			79.4 bc	2.1 de	13.6 i	3.3 d

Continued on next page

Table 3. (Continued)

YEAST	TIME (Days)	SULFUR COMPOUNDS			ALDEHYDES								
		Methanethiol [1]	Dimethyl disulfide	Dimethyl trisulfide	2-methyl propanal	3-methyl propanal	2-methyl butanal	Octanal	Nonanal	2,3-butanedione	2-butanone	3-hydroxy-2-butanone	2-heptanone
I2	0						3.1 lm						
	7		31.9 d		131.6 a	92.1 bc	82.7 cde			16.5 ijkl		18.5 hi	
	15	156.6 a	187.3 cd		107.3 bc	80.8 cd	90.7 bcd			51.8 de		71 ef	
	30	30.5 d	298.5 cd	13.6 b	80.8 d	83 cd	48.4 fg			35.7 d		94.7 ab	
	45		45.2 d	11.8 b	53 fg	128.3 a	51.4 f			23.1 hi		22.8 ghi	4.3 cd
SEM [2]		11.94	79.42	10.34	6.77	6.54	5.93	2.16	0.45	6.74	0.4	7.09	1.86
Pt [4]		***	***	***	***	***	***	**	***	***	**	***	***
Pc		ns	***	***	***	***	***	**	*	***	ns	***	**
Ptxc		ns	***	***	***	***	***	*	***	***	**	***	ns

[1] Methanethiol: abundance expressed as area units from TIC X 10^4. [2]SEM: Standard error of the mean. [3]Different letters in the same columns means significant differences at p value. [4] Pt: p value of ripening time effect; Pc: p value of inoculated yeast effect; Ptxc: p value of interaction between inoculated yeast and ripening time effects. ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$, ns: $p>0.05$.

The concentration of volatile compounds produced by the yeasts was expressed as the concentration detected in the headspace of each inoculated meat model minus the one found in the headspace of the control batch (Tables 2 and 3). Figure 2 shows the quantification of the volatile compounds classified by chemical group. The production level of all chemical groups increased during time in the different inoculated models (Figure 2). The generation of aldehyde compounds was higher in the M6 strain than in the other strains studied (Figure 2a) while the generation of ketone compounds was similar among inoculated models except for M4 strain that did not produce ketones (Figure 2b). The generation of sulfur compounds increased significantly in P2 and M6 inoculated models at 45 days (Figure 2c). The production of acid compounds was very different among inoculated yeasts as shown in Figure 2d.

On the other hand, alcohol compounds were produced at similar quantities in almost all the inoculated yeast models, only V2 model showed a highest concentration at the end of the incubation time (Figure 2e). However, the most remarkable result was the significant production of ester compounds in the inoculated M4 and P2 models, after 30 and 45 days of incubation (Figure 2f).

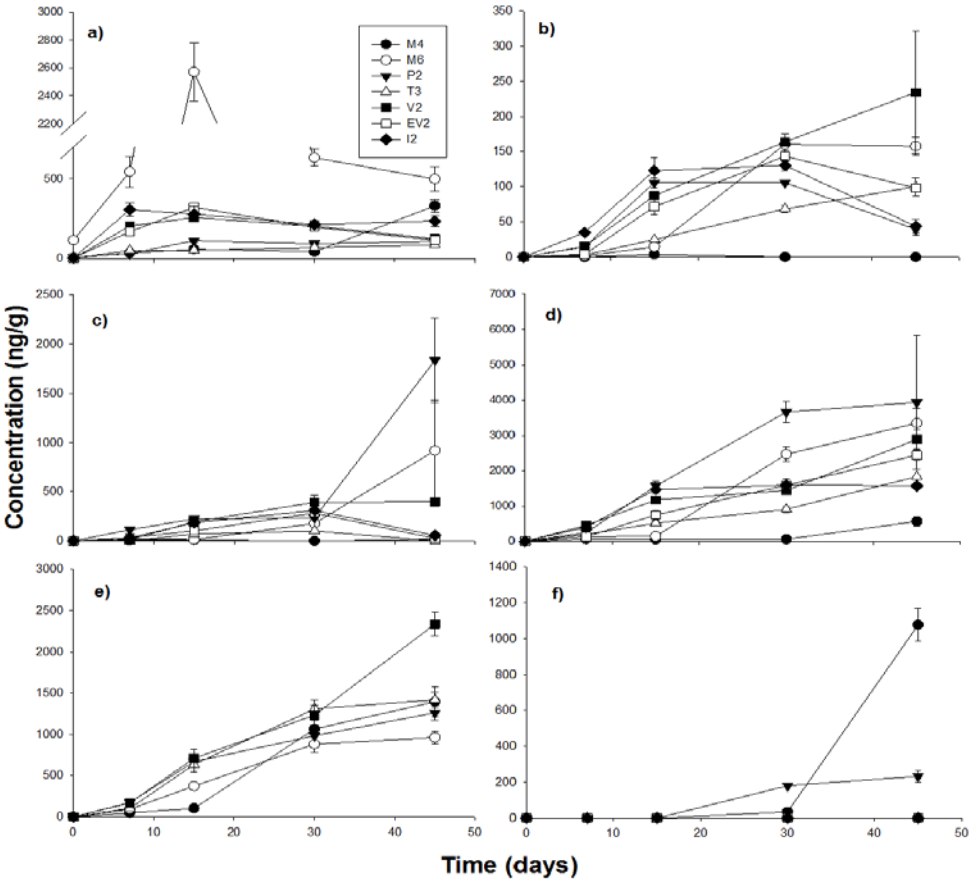

Figure 2. Quantification of volatile compounds classified according to chemical group. a) aldehydes, b) ketones, c) sulfur compounds, d) acids, e) alcohols, and f) ester compounds.

The effect of inoculated yeasts and incubation time was significant in all volatile compounds analyzed except for methanethiol and 2-butanone that were not affected by the inoculated yeast (Table 3). The ethanol abundance was different among the inoculated models and a significant increase was observed during time (Table 2). The V2 and T3 inoculated models showed the highest and lowest ethanol abundances, respectively. Among the other

detected alcohol compounds detected it was remarkable the highest concentration of 3-methylbutanol, 2-methylbutanol and 2-methylpropanol that showed significant increases in all inoculated models during time. Although different ester compounds were detected (Table 1), ethyl acetate was the only one produced in highest concentration by all inoculated models (Table 2). In addition, M4 model was the highest significant producer of ethyl acetate followed by P2 inoculated model. The acid compound produced in highest concentration was acetic acid and it was different among inoculated models (Table 2). P2 inoculated model showed the highest acetic acid concentration while M4 presented the lowest production.

The concentration of sulfur compounds in the headspace of meat models was significantly different between inoculated batches (Table 3). Strain P2 was the highest producer of sulfur compounds showing the highest concentration of dimethyl disulfide and dimethyl trisulfide and being the only one able to produce methanethiol along the incubation time. In contrast, strain M4 showed a lowest generation of sulfur compounds with a low production of dimethyl disulfide and absence of dimethyl trisulfide while T3 inoculated model also did not generate dimethyl trisulfide (Table 3). Respect to aldehydes compounds, 2-methylpropanal, 3-methylbutanal and 2-methylbutanal were detected in highest concentrations in the M4 followed by I2 inoculated models. However, compounds such as hexanal and heptanal are not shown in Table 3 as few differences were observed among inoculated batches being generated only by strain M6 in large amounts (data not shown). For this reason, M6 model showed the highest concentration in aldehyde compounds (Figure 2a). The effect of yeast and

incubation time was also significant in the production of ketones (Table 3). 2,3-Butanedione and 3-hydroxy-2-butanone were the ketones detected in higher concentrations. V2 strain was the highest producer of 2,3-butanedione and 3-hydroxy-2-butanone while M4 strain showed a lowest generation of ketones being unable to produce 2-butanone and 3-hydroxy-2-butanone.

DISCUSION

In the present study each meat model system was inoculated with a unique yeast strain of *D. hansenii* without the addition of bacterial starters which might interfere in the analysis of the volatile compounds produced by the yeasts. The low pH values observed were due to the artificial acidification produced by the addition of glucono–δ–lactone (Ngapo *et al*., 1995) and not by lactic acid bacteria growth. In addition, the M13 minisatellite patterns of the inoculated *D. hansenii* strains were followed during the incubation time and they were identical to the ones showed by the original selected strains (Cano-García *et al*., 2013). This fact confirmed the presence in each meat model of the inoculated yeast strain.

All volatile compounds identified in the inoculated meat model systems have been previously reported in fermented sausages (Ansorena, Gimeno, Astiasarán, & Bello, 2001; Olivares *et al*., 2010). The high production of volatile compounds by the inoculated *D. hansenii* in the meat model systems is in contrast to the results reported by Olesen & Stahnke (2000). These authors indicated that *D. hansenii* had a limited production of volatile compounds and suggested that the volatile compounds detected were due to the presence of other microorganisms. On the contrary, Andrade *et al*.

(2009) identified many different volatile compounds in a meat model inoculated with different yeast strains isolated from dry-cured Iberian ham. However, these authors did not detect differences in the production of ester and sulfur compounds among yeast strains. In our study, important amounts of ester and sulfur compounds were detected in the inoculated meat model systems. Moreover, different concentrations of these compounds were detected in the meat models inoculated with different *D. hansenii*, even for strains displaying the same M13 pattern, as in case of P2 and T3 yeasts (Table 2 and 3 and Figure 2).

The ability to produce flavour compounds by different microbial groups during sausage fermentation has been associated to coagulase-negative cocci. Mainly due to its capacity to generate high amounts of branched aldehydes, methyl ketones and ester compounds (Montel, Masson, & Talon, 1998). However, other microbial groups such as yeast may generate ester compounds in fermented sausages (Tjener & Stanhke, 2007). Therefore, the presence of lipolytic activities in *D. hansenii* strains may have an important effect on flavour. In this sense, the high lipolytic activity shown by all *D. hansenii* strains (Mendonça *et al*., 2013) can be related to the volatile compounds detected in the models. The highest lipolytic activity was detected in M4 strain that at the same time was the highest producer of ester compounds (ethyl esters and acetate esters) and the lowest producer of acetic acid. On the contrary, the other *D. hansenii* strains produced higher quantities of acetic acid and lower ester compounds than M4. These observations would indicate that ester production is strongly dependent on the strain, as already reported Procopio, Quian, & Becker (2011). It is worth to note that strain M4 displayed an M13 pattern clearly different from the

other *D. hansenii* strains used in this study (Figure 1 supplementary material); although, as pointed out in previous studies (Cano-García *et al*., 2013) correspondence between a particular aroma profile and a singular M13 pattern is not possible.

On the other hand, the production of sulfur compounds by yeasts has been studied in culture media and dairy models (López del Castillo-Lozano, Delile, Spinnler, Bonnarme, & Landaud, 2007; Liu & Crow, 2010). Liu & Crow (2010) indicated that the profile and composition of minor sulfur compounds vary with yeast species and strains, although yeast may not be responsible of the direct production of sulfur compounds but can supply substrates or intermediates for these compounds. In our study, only two of the yeasts studied, M4 and V2, were able to produce methionol in the meat models. However, production of methionol from L-methionine metabolism has been described in few studies done in synthetic media by *D. hansenii* strains (López del Castillo-Lozano *et al*., 2007, Liu & Crow, 2010). Our meat model system contained free amino acids, including methionine, in a concentration similar to fresh meat (Flores *et al*., 1998) and it has been recently indicated the importance of the proteinogenic amino acids in the production of aroma compounds by yeasts during fermentation (Procopio, Krause, Hofmann, & Becker, 2013). However, as observed in the meat models not only meat medium composition is essential for aroma production but it is yeast dependent also, as only M4 and V2 strains were able to produce the aromatic compound methionol (Table 1).

In order to select the yeast strain to be used in pilot plant for the production of fermented sausages, its aromatic impact should be considered. Thus, the calculation of the Odour Activity Value (OAV) of

each volatile compound produced by the yeast strains in the model system can be an objective measurement of their aromatic potential. OAVs can be easily calculated if the concentration and the odour threshold of the aroma compounds are known and the compound contributes to the aroma in the sample when its OAVs is equal or higher than 1 (De Roos, 2007). In the meat model the volatile concentration was calculated in the HS above the model; therefore, the threshold values in air reported in Table 1 for each volatile were used to calculate the OAV. A total of 40 volatile compounds were quantified at 45 days of incubation (Table 3 supplementary material) and the OAVs were calculated for 37 of them (Figure 3) except for ethanol, methanethiol and (Z)-2-heptenal. Many of the OAVs calculated in the inoculated meat model systems showed values higher than 1 (Figure 3) with dimethyl disulfide and dimethyl trisulfide having the highest OAVs.

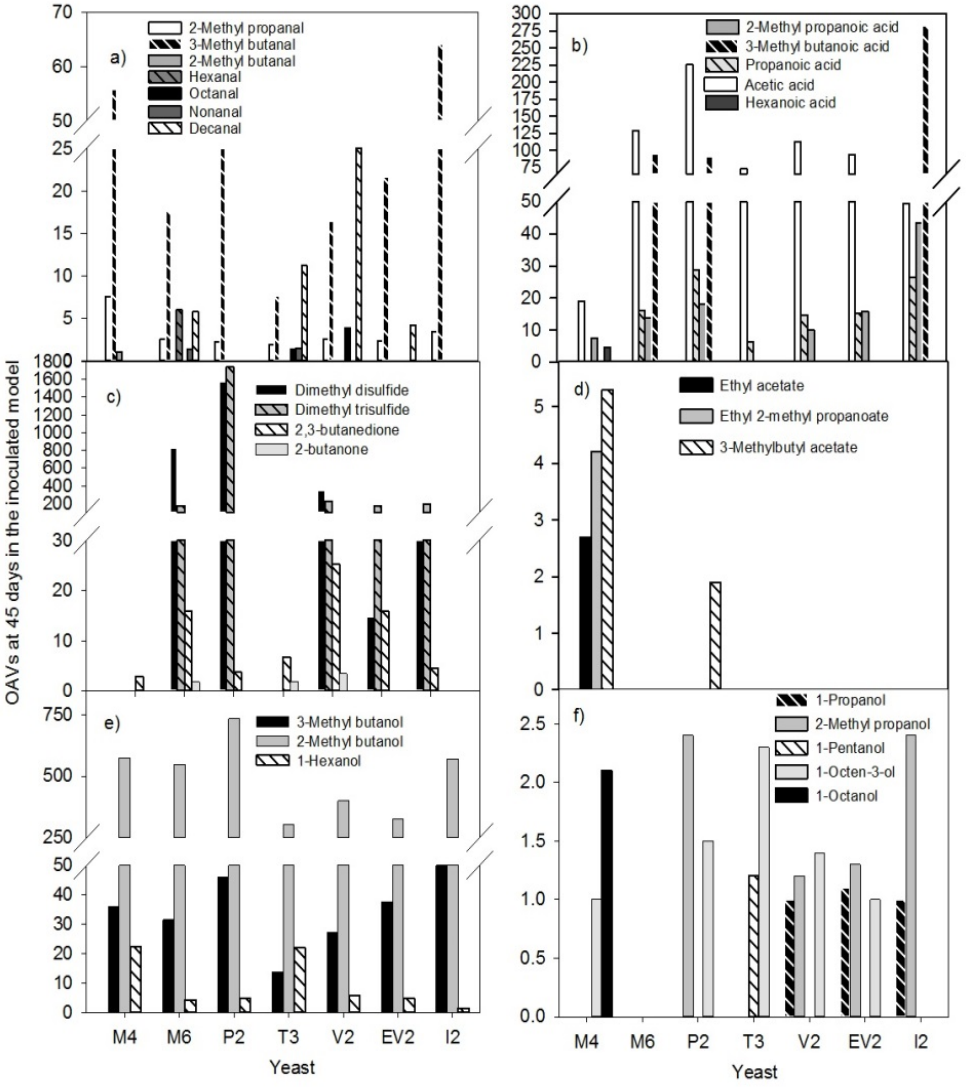

Figure 3.- Odour-activity values (OAVs) in air of volatile compounds detected in the headspace of fermented meat model after 45 days of incubation time. a) aldehydes, b) acids, c) ketones and sulfur compounds, d) ester compounds, e) and f) alcohols.

This fact means that many of the volatile compounds generated by the yeasts in the models will have an aroma impact. However, the highest differences among the inoculated meat model systems were found when M4, M6 and P2 strains were used. M4 strain showed the highest OAVs for ester compounds followed by P2 strain (Figure 3d), while M6 and P2 strains showed the highest OAVs for sulfur compounds (Figure 3c).

These volatiles sulfur and ester compounds, have been identified in fermented sausages where they were described as important flavour contributors due to their low threshold values. Previous studies reported that sulfur compounds could afford flavours of boiled cabbage and cauliflower, garlic, onion and eggs (Schmidt & Berger, 1998; Meynier, Novelli, Chizzolini, Zanardi, & Gandemer, 1999; Marco, Navarro, & Flores, 2007) while ester compounds are responsible for fruity notes and contribute to mask rancid and vegetable cooked odours (Stahnke, 1994). Therefore, the selected yeast strains M4, M6 and P2 with their highest production of esters and sulfur compounds could have an important aromatic impact on fermented sausages. In addition, the origin of ester compounds in fermented sausages was attributed to different microbial groups, lactic acid bacteria, coagulase-negative cocci, yeast and mould (Tjener & Stanhke, 2007). This is the first time that a specific *D. hansenii* strain (M4) has been reported as an ester aroma contributor in meat. It is essential to bear in mind that this yeast was originally isolated from naturally fermented sausages (Cano-García *et al*., 2013) which showed a high consumer acceptability related to the high abundance of methyl and ethyl esters (Olivares *et al*., 2010). In addition, these results are partially in accordance with previous studies using defined culture media (in vitro)

where M4 strain was the highest producer of ester compounds, although the production of sulfur compounds was similar among all assayed strains (Cano-García *et al*., 2013). Therefore, the use of a meat model system resembling the real fermentation conditions has been useful to show the real ability of yeast strains to produce aroma compounds.

CONCLUSION

In summary, the addition of selected *D. hansenii* strains in the meat model system affected flavour development, as wide differences were found among M4, M6 and P2 strains in the production of volatile compounds. M4 strain generated mainly ester compounds, whereas P2 and M6 strains produced highest amounts of sulfur compounds. A correspondence between the M13 pattern and the aromatic profile of the meat medium fermented with the different yeast was not found, although the most singular strain M4 showed a characteristic M13 pattern. Moreover, different fermentation parameters like temperature and substrate composition play a major role on aroma production of the *D. hansenii* strains studied. For this reason, it is necessary to investigate the effect of *D. hansenii* strains M4, M6 and P2, on the production of volatile compounds in real dry fermented sausages.

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Supplementary material

Table 1 Suppl.- Linearity and correlation coefficients of the standard compounds analyzed by HS-SPME**.**

Compounds	Measure range (ng)	Correlation coefficient (r^2)	Slope (A/ng x 10^4)*	Intercept (10^4)
Aldehydes				
2-Methyl propanal	4.5 - 121.4	0.9921	19.99	-100
3-Methyl butanal	5.5 - 148.2	0.9959	23.43	-66.61
2-Methyl butanal	3.8 - 101.8	0.9948	26.79	2.47
Hexanal	3.8 - 366.7	0.9811	6.88	100
Heptanal	0.5 - 4.9	0.998	100	-349.29
(Z)-2-Heptenal	7.4 - 64.8	0.9921	9.61	-76.19
Benzaldehyde	0.7 - 12.9	0.9975	200	-100
Octanal	2.7 - 13.6	0.9939	36.53	-75.86
Nonanal	1.7 - 8.6	0.9962	100	-200
Decanal	1.6 - 7.4	0.9908	30.86	-43.78
Sulfur compounds				
Dimethyl disulfide	1.6 - 180	0.9901	38.82	-500
Dimethyl trisulfide	3.2 - 47.6	0.991	5.21	-6.91
Methionol	3.5 - 55	0.999	5.33	16.62
Ketones				
2,3-Butanedione	0.5 - 180	0.9977	22.67	1,000.00
2-Butanone	0.4 - 28.5	0.9986	63.33	13.42
3-Hydroxy-2-butanone	5.8 - 660	0.9905	87.23	-3,000.00
2-Heptanone	1.9 - 29.1	0.9922	17.72	-25.62

Continued on next page

Table 1 Suppl. (Continued)

Compounds	Measure range (ng)	Correlation coefficient (r^2)	Slope (A/ng x 10^4)*	Intercept (10^4)
Alcohols				
1- Propanol	8.3 - 74.1	0.99	8.98	-54.57
2-Methyl propanol	7.5 - 220	0.9916	33	1.03
3-Methyl butanol	0.4 - 450	0.9383	44.79	2,000.00
2-Methyl butanol	0.4 - 500	0.9484	41.28	2,000.00
1-Pentanol	0.5 - 36.7	0.9967	56.53	-67.52
1-Hexanol	7.5 - 220	0.9914	38.61	-200
1-Heptanol	3.4 - 100	0.9915	62.31	-400
1-Octen-3-ol	2.7 - 80	0.9916	69.47	-100
1-Octanol	2.5 - 60	0.9934	19.42	1.16
Ester compounds				
Ethyl acetate	0.2 - 100	0.9974	100	-100
Ethyl propanoate	2.7 - 180.6	0.9956	28.43	33.21
Propyl acetate	1.9 - 27.2	0.9902	43.12	58.92
Ethyl 2-methyl	2.5 - 166.7	0.9957	42.43	62.16
Isobutyl acetate	1.6 - 5.9	0.9941	200	17.08
3-Methylbutyl acetate	4.1 - 61.7	0.9933	9.63	-26.19
2-Methylbutyl acetate	5.1 - 15.4	0.9903	39	-100
Acids				
Acetic acid	26.5 - 466.7	0.9953	11.88	-500
Propanoic acid	13.3 - 120.2	0.9912	16.5	-200
2-Methyl propanoic	17.3 - 100	0.9912	35.92	-500
3-Methyl butanoic acid	14.1 - 65.7	0.9961	17.53	-200
Hexanoic acid	5.2 - 125	0.9913	4.72	-18.4

* A is area of compound

Table 2 Suppl.- Lipolytic activity of selected yeast strains.

	T20[a]	T40	T60	T80
M4	++++	++++	++++	+[b]
M6	-	+++	++	-[c]
P2	-	++	+++	-
T3	-	+	++	-
V2	-	++	+++	-
EV2	-	++	++	-
I2	-	++	++	-

[a] T20: Lauric acid ester, T40: palmitic acid ester, T60: stearic acid ester, T80: oleic acid ester. [b] +: Lipolytic activity shown as positive marks. [c] -: Absence of lipolytic activity.

Table 3 Suppl.- Concentration (ng/g) of volatile compounds in the headspace of fermented meat model after 45 days of incubation time.

VOLATILE COMPOUNDS	M4	M6	P2	T3	V2	EV2	I2
Aldehydes							
2-Methyl propanal	113.9	38.6	33.2	27.9	39.6	36.1	53
3-Methyl butanal	111.6	35.1	50.5	15.3	32.9	43.4	128.3
2-Methyl butanal	105.1	42.7	23	20.7	15.3	32.6	51.4
Hexanal	-[a]	362.82	-	-	-	-	-
Heptanal	-	6.99	-	-	-	-	-
Benzaldehyde	-	2.72	-	-	-	-	2.83
Octanal	-	-	-	7.1	19.6	-	-
Nonanal	-	7.1	-	7.6	-	-	-
Decanal	-	3.6	-	6.96	16.34	2.58	-
Sulfur compounds							
Dimethyl disulfide	-	908.7	1730.8	-	385.8	16.4	45.2
Dimethyl trisulfide	-	10.7	104.12	-	13.3	10.6	11.8
Methionol	18.68	-	-	-	-	-	-
Ketones							
2,3-Butanedione	14.2	79.5	19.4	34	126.1	79.4	23.1
2-Butanone	-	53.4	16.7	56.4	100.2	13.6	22.8
3-Hydroxy-2-butanone	-	53.4	-	56.4	100.2	13.6	-
2-Heptanone	14.8	20.4	-	9.7	4.6	3.3	4.3
Alcohols							
1-Propanol	40	60.3	44.7	31	78.3	86	74.4
2-Methyl propanol	119.9	185.8	489.2	122.8	235.6	262.1	479.5
3-Methyl butanol	681.1	595.8	876.6	264.8	514.5	713.5	1267.5
2-Methyl butanol	516.8	492.3	660.6	271.4	358.9	292.9	513
1-Pentanol	13.1	7.8	-	24	4.1	3.7	-
1-Hexanol	222.5	42.9	50	219.3	58.1	48.5	15.3
1-Heptanol	20.07	-	-	-	-	-	-
1-Octen-3-ol	12.1	11	17.5	27.4	16.8	11.6	9.6
1-Octanol	19.16	-	-	-	-	-	-

Continued on next page

Table 3 Suppl. (Continued)

VOLATILE COMPOUNDS	M4	M6	P2	T3	V2	EV2	I2
Ester compounds							
Ethyl acetate	907.7	3.2	201.3	-	8	6.6	7
Ethyl propanoate	40.81	-	-	-	3.2	-	-
Propyl acetate	18.97	-	-	-	-	-	-
Ethyl 2-methyl propanoate	14.57	-	-	-	-	-	-
Isobutyl acetate	4.53	-	-	-	-	-	-
3-Methylbutyl acetate	78.93	-	28.54	-	-	-	-
2-Methylbutyl acetate	11.62	-	-	-	-	-	-
Acids							
Acetic acid	472.9	3210.2	5632.4	1816	2795.1	2319.8	1238.1
Propanoic acid	-	48.5	85.8	19	43.8	45.7	79.5
2-Methyl propanoic acid	38	68.3	89.8	-	50.3	79.1	216.6
3-Methyl butanoic acid	-	20.91	19.83	-	-	-	62.02
Hexanoic acid	55.62	-	-	-	-	-	-

[a] (-):not detected.

Capítulo 3

Impacto de la inoculación de cepas de *Debaryomyces hansenii* en la calidad de embutidos crudos curados de fermentación lenta.

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Impact of *Debaryomyces hansenii* strains inoculation on the quality of slow fermented sausages

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Abstract

Debaryomyces hansenii strains, M4 and P2, isolated from natural fermented sausages were inoculated in slow fermented sausages to study their effect on processing parameters, microbial population, volatile compound and sensory characteristics. The inoculation of *D. hansenii* strains, M4 and P2, did not affect the ripening process as no differences in pH and Aw were detected. The dominance of the inoculated yeast strains along the process was followed by RAPDs of M13 minisatellite. The inoculated yeasts, P2 and M4, were recovered at the end of the ripening process although P2 appeared in higher counts than M4. The sausages inoculated with P2 resulted in a decrease in lipid oxidation values (TBARS) and a reduction of lipid-oxidation derived aldehydes in addition to a highest acid compound abundance. M4 inoculated sausages resulted in highest sulphur containing compounds abundance. However, no differences in consumer acceptance were detected. Moreover, both yeast strains were responsible for the generation of ethyl methyl-branched ester compounds in the dry-cured sausages.

Keywords: yeast, *D.hansenii*, dry-cured sausages, flavour, aroma, volatile compound.

INTRODUCCTION

Slow fermentation of large diameter fermented sausages is a process that requires long ripening times (Marco, Navarro, & Flores, 2008). These traditional low acid dry-cured sausages are much appreciated in Southern European countries due to their moderate pH decrease produced by microbial activity and by the development of specific sensory characteristics (Ravyts, De Vuyst & Leroy, 2012).

The improvement and diversification of fermented flavour by selecting microorganism with unique flavour-developing capabilities is an opportunity to increase the competitiveness of the final dry-cured sausage. In this case, the contribution of yeast strains to the sensory characteristics of dry-cured fermented sausages has been mainly attributed to the stabilization of the reddening reaction and development of a characteristic yeast flavour, due to their ability to degrade peroxides and the presence of lipolytic and proteolytic activities (Olesen & Stahnke, 2000, Durá, Flores & Toldrá, 2004). Among spontaneous fermented sausages, *Debaryomyces hansenii* is often the dominated yeast strain in fermented sausages (Cocolin, Urso, Rantsiou, Cantoni & Comi, 2006; Mendonça, Gouvêa, Hungaro, Sodré, & Querol-Simón, 2013).

The selection of yeast strains to improve sausage fermentation with respect to flavour may be affected due to yeast growth inhibition by ingredients, antifungal compounds or technological parameters (Olesen & Stahnke, 2000). Also, yeast metabolic capabilities to produce flavour compounds may differ among strains. The addition of *D. hansenii* yeast strains in dry-cured fermented sausages has been related to the increase in

esters and acids compounds (Flores, Durá, Marco, & Toldrá, 2004; Andrade, Córdoba, Casado, Córdoba, & Rodríguez, 2010; Purriños, Carballo, & Lorenzo, 2013) an increase in branched alcohols and aldehydes (Andrade *et al*., 2010) a decrease in linear aldehydes (Flores *et al*., 2004; Purriños *et al*., 2013) although Olesen & Stahnke, (2000) did not find any volatile compound effect.

The differences in flavour development in dry-cured fermented sausages associated with a particular yeast strain have been followed throughout the ripening process using yeast media (Olesen & Stahnke, 2000; Flores *et al*., 2004), morphological and biochemical yeast characteristics (Flores *et al*., 2004; Lucci, Patrignani, Belletti, Ndagijimana, Guerzoni, Gardini, & Lanciotti, 2007) but few studies have demonstrated the presence of the inoculated yeast strains by molecular characterization (Andrade *et al*., 2010; Mendonça *et al*., 2013). Also, the implantation of the inoculated yeast strains throughout the ripening process has not been followed.

The molecular characterization of the different yeast strains of *D. hansenii* isolated from traditional fermented sausages have demonstrated that there is a large variability within *D. hansenii* (Cano-García, Flores, & Belloch, 2013; Mendonça *et al*., 2013). Also, the aromatic potential is different among selected *D. hansenii* strains as it was shown by Cano-García, Rivera-Jiménez, Belloch, & Flores, (2014) in a meat model system. In this study, two *D. hansenii* strains, M4 and P2, showed a remarkable aroma potential due to their ester and sulphur production capabilities. However, the metabolic activity of these yeasts might be affected by meat ingredients and technological parameters and also microorganism present

since competition between microbial populations is occurring throughout the ripening process (Ravyts *et al*., 2012).

Therefore, our aim was to evaluate the effect of *D. hansenii* yeast strains, M4 and P2, on the sensory quality of slow dry-cured fermented sausages. In addition, the implantation of the inoculated *D.hansenii* strains was checked by molecular techniques throughout the whole ripening process.

MATERIALS AND METHODS

Preparation of yeast strains starter

Two *Debaryomyces hansenii* strains, M4 and P2, previously isolated from naturally fermented sausages "salchichón de Requena" (Requena, Valencia, Spain) were selected by their aromatic potential (Cano-García *et al*., 2013, 2014) to be inoculated in slow dry-cured fermented sausages. The yeasts were grown on GPY medium (glucose 2%, peptone 0.5%, yeast extract 0.5%, pH 6.0) to a concentration of 10^8 cfu mL^{-1}. The cells were separated by centrifugation (7000 rpm for 10 min at 4 ºC) and washed with sterile saline solution (0.9 % salt). The collected cells were concentrated in 1 mL of saline solution per gram of the cells (wet weight) and the suspensions stored at – 80 ºC until further use.

Preparation of dry-cured fermented sausages

Three different batches of dry-cured fermented sausages were manufactured; a control batch without yeasts (batch C) and two batches inoculated with M4 (batch M4) and P2 (batch P2) yeast strains, respectively. Twenty two sausages were manufactured in each batch.

Sausage batches were prepared with lean pork (75%) and pork back fat (25%) and the following additives (g/kg): NaCl (27), lactose (20), dextrin (20), sodium caseinate (20), glucose (7), sodium ascorbate (0.5), sodium nitrite (0.15), potassium nitrate (0.15). The meat was ground through a 10 mm hole diameter plate, vacuum minced with the remaining ingredients and inoculated with a starter culture C-P-77 S bactoferm (Chr. Inc., Hansen, Denmark) containing *Lactobacillus pentosus* and *Staphylococcus carnosus*. For the yeast inoculated batches, appropriate volumes of P2 or M4 yeast strains suspension were added to the meat batter at final concentration of 10^6 cfu g^{-1} of yeast strain. The meat mixture was stuffed into collagen casings of 9.5 cm diameter (FIBRAN, S.A., Girona, Spain) making sausages of approximately 0.7 kg as the final weight. All sausages batches were ripened in the same drying chamber and subjected to an initial stage at 15-20 ºC and 75-85% HR for 22 h, followed by drying at 9 ºC and 75-85% HR for 43 days. In order to control the ripening process, weight losses and pH were measured during processing (Olivares, Navarro, & Flores, 2010).

From each batch, a 500 g portion of meat mixture (0 days) and three sausages at 10, 27 and 43 days were randomly collected to study the effect of inoculated yeast strains and ripening time. 150 g portion of each sausage was minced and used for moisture, water activity and pH tests and a 10 g portion was taken for microbiological analysis. In addition, the remaining minced sausage was vacuum packaged and frozen at -20 ºC for subsequent analyses (TBARS) after measuring sausage colour. Also, several sausage slices were wrapped in aluminium foil, vacuum packaged and stored at -80 ºC for volatile compounds analysis. Finally, sensory analysis was carried

out at 43 d of the drying process. Results were expressed as the mean of three replicates per 100 g of dry matter at each processing time and batch.

Chemical analysis

The measurement of pH, water activity, colour evaluation (CIE L*, a*, b*), and moisture was performed as described Olivares *et al*. (2010). Thiobarbituric acid reactive substances (TBARS) were quantified to determine the degree of lipid oxidation (Olivares, Navarro, & Flores 2011) using trichloroacetic acid as solvent instead of perchloric acid. The results were expressed as mg malonaldehyde (MDA)/kg in dry matter.

Microbial analysis

For microbial analysis, minced sausage samples were homogenized with sterile saline solution (1/10) in a Stomacher (IUL Instruments, Barcelona, Spain) for 1 min. Further decimal dilutions of the homogenate were made in sterile saline solution and 0.1 mL of each dilution were spread onto the surface of appropriate agar media. Rose Bengal Agar with chloramphenicol (RBA) (Conda SA, Madrid, Spain) incubated at 28 ºC for 72 h was used for yeast counts. Lactobacilli counts were done on (MRS) Man Rogosa Sharpe Agar (Scharlau Chemie SA, Barcelona, Spain) in anaerobic jars while staphylococci were counted by surface plating on (MSA) Mannitol Salt Agar (Scharlau Chemie SA, Barcelona, Spain) and both were incubated at 37 ºC for 48 h.

Strains characterization by molecular methods

Ten yeast strains were isolated from each inoculated batch at the different ripening times and then subjected to molecular characterization by

minisatellite PCR amplification using the M13 primer. DNA was extracted as described in Querol, Barrio, & Ramón, (1992) and M13 minisatellite PCR amplification was carried out as described elsewhere (Cano-García *et al.*, 2013a). PCR products (10 μL) were resolved by electrophoresis on 2 % agarose gel in 1xTAE buffer at 90 V for 3 h, stained with Red Safe nucleic acid staining solution 20,000x (Intron biotechnology, Kyunggi-do, Korea) and visualized under UV light. DNA fragment sizes were determined using 100-bp DNA ladder. Minisatellite PCR patterns were compared with those previously obtained for these strains (Cano-García *et al*., 2013a).

Analysis of volatile compounds.

Frozen sausage slices were minced with 0.75 mg of BHT and 5 g weighted into 20 mL headspace vial (Gerstel, Germany). The vial was maintained at 37 ºC during 30 min to equilibrate its headspace. Then, volatile compounds in samples were extracted by a Solid Phase Microextraction (SPME) device using an automatic injector Gerstel MPS2 (Gerstel, Germany). The extraction was done using a 85 μm CAR/PDMS sf fiber (Supelco, Bellefonte, PA, USA) by headspace exposure during 3 h at 37ºC and a previous vial equilibration for 30 min at 37°C.

Identification and quantification of volatile compounds was done using a gas chromatograph (GC HP 7890 series II) equipped with an HP 5975C mass selective detector (MS Hewlett Packard, Palo Alto, CA, USA). The compounds absorbed by the fiber were desorbed in the injection port of the GC-MS for 15 min at 220 ºC with the purge valve off (splitless mode) using He as carrier gas with a linear velocity of 34.3 cm sec^{-1}. Compounds were separated on a DB-624 capillary column (J&W scientific, 30 m x 0.25 mm

x 1.4 μm) and analyzed as described Cano-García *et al*., (2013a). Volatile compounds were identified by comparison with mass spectra from the library database (Nist'05) and with Kovats retention indices of authentic standards (Kovats, 1965) calculated using the series of n-alkanes (Aldrich, Germany). Quantification of each volatile compound was based on the abundance of total extracted area (TIC) or the area of a target ion when different compounds coeluted.

Sensory analysis

A consumer sensory analysis was performed at the end of the ripening process (43 d). Testing was carried out in a sensory laboratory equipped with individual booths (Olivares *et al*., 2011). Data acquisition was performed using Compusense *five* release 5.0 software (Compusense Inc., Guelph, Ontario, Canada). The casing was removed and sausages cut into slices of 4 mm thickness. Sausages from each batch (C, M4, P2 batches) were labelled with random three-digit codes and presented on a plate at room temperature with water and unsalted toasts to cleanse the palate among samples. An acceptability test was carried out using 9-box hedonic scale (1 extremely dislike – 9 extremely like). A panel of 80 untrained consumers was used. The consumers were asked to evaluate each sausage based on appearance (evaluated inside a camera with D65 illuminant), flavour, taste, hardness, juiciness and overall quality.

Statistical analysis

The effect of inoculated yeast strains and processing time on chemical, microbial and volatile compounds was tested by two-factor analysis of variance (ANOVA) using the statistic software XLSTAT 2009.4.03

(Addinsoft, Barcelona, Spain). Differences between samples means were analyzed according to Fisher's Least Significant Difference (LSD) test. The effect of inoculated yeast strain on sensory parameters was done by one factor ANOVA analysis.

RESULTS AND DISCUSSION

Microbial analysis

Figure 1 shows the results concerning the viable counts of lactic acid bacteria, staphylococci and yeasts during ripening of control and inoculated dry-cured sausages (M4 and P2 batches). The population of lactic acid bacteria and staphylococci was within the range expected in dry-cured fermented sausages (figure 1A and 1B) (Durá *et al*., 2004).

A slight decrease in the number of staphylococci was observed during processing in all batches although a highest staphylococci population was detected in M4 sausages ($p < 0.001$) (Figure 1b). The presence of a highest staphylococci population may modulate the dry-cured sausage aroma through the conversion of branched chain amino acids and free fatty acids (Leroy, Verluyten & De Vuyst, 2006).

Yeast population during dry-cured sausage processing is reflected in figure 1C. After 10 days of processing M4 and P2 batches showed an increase in the yeast population up to 6 and 8 logarithmic cycles, respectively, which were maintained along the ripening process. This is in contrast to the slight reduction observed in other yeast inoculated fermented sausages (Dura *et al*., 2004, Andrade *et al*., 2010) or the completely disappearance reported by Olesen & Stahnke (2000). Nevertheless, control

sausages showed a low yeast population similar to those reported in other non yeast inoculated sausages (Dura *et al*., 2004, Andrade *et al*., 2010).

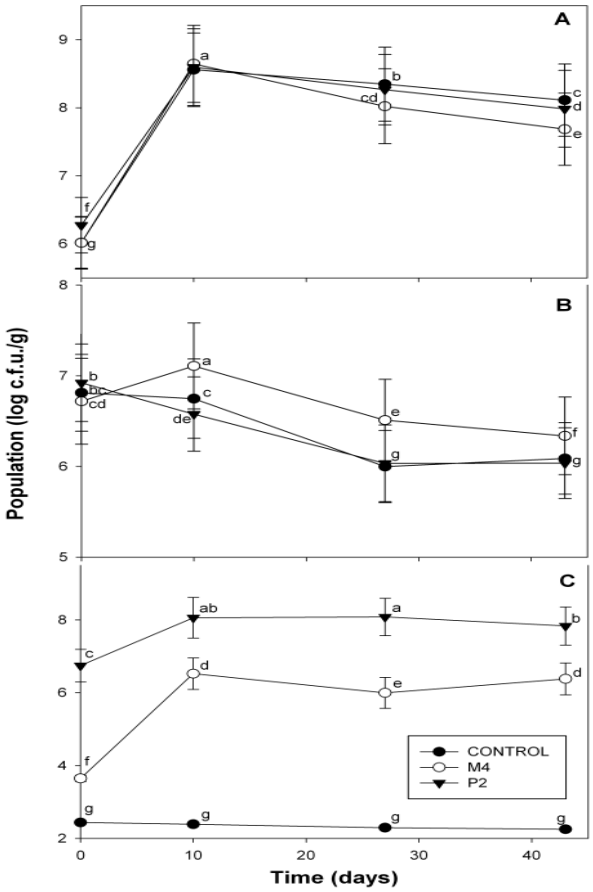

Figure 1.- Evolution of microbial counts during ripening of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage). a) Lactic acid bacteria, b) staphylococci and c) Yeasts.

The implantation of the inoculated yeast strains was followed by comparison of the M13 minisatellite PCR amplification patterns with those obtained from the original yeast strains (Cano-García *et al*., 2013). At the beginning of the process, all yeasts isolated from batches M4 and P2 displayed their original M13 minisatellite PCR amplification pattern (Figure 2A). In case of P2 batch all isolated yeasts (100%) along the ripening process showed the same pattern as the originally inoculated (Figures 2B, 2C and 2D). However, in case of batch M4 approximately 70 % and 50 % of the isolated yeasts at 10 (Figure 2B) and 27 (Figure 2C) days respectively, were ascribed to the original pattern. Nevertheless, at the end of the process dominance of M4 was 100% (Figure 2D).

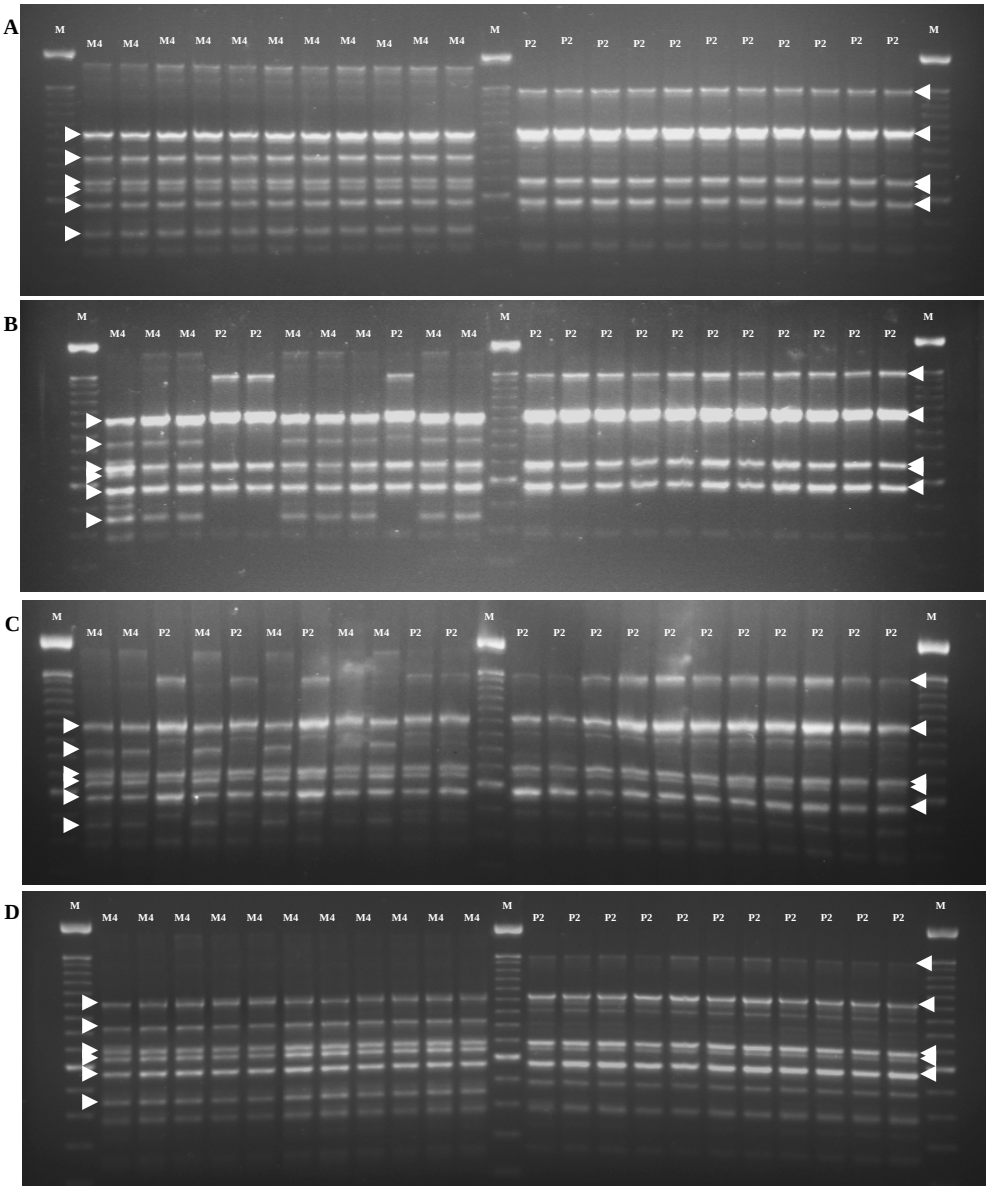

Figure 2.- Electrophoretic patterns of minisatellite M13 PCR amplification of strains isolated at 0 d (A), 10 d (B), 27 d (C) and 43 d (D). The arrows indicate the bands present in the strains. In each photograph: first lane represents "M" 100 pb ladder (Invitrogen, Carlsbad, CA, USA) followed by the original inoculated strain M4 or P2 yeast and the 10 isolated strains in M4 or P2 inoculated batches, respectively.

Chemical parameters

The evolution of the pH, Aw and TBARS values during ripening time are shown in figure 3. The pH was very similar in all batches (figure 3A) but for M4 sausages the pH was slightly highest. These results are in agreement with previous studies, as few changes in pH have been reported in yeast inoculated dry-cured fermented sausages (Selgas, Ros & García, 2003, Dura *et al*., 2004, Andrade *et al*., 2010, Patrignani, Iucci, Vallicelli, Guerzoni, Gardini & Lanciotti, 2007). About water activity Aw, there were not relevant differences among dry-cured sausages (Figure 3B). In addition, moisture decreased significantly ($p<0.05$) in all the samples, from initial values of about 65% to 44% at the end of ripening. Sausage colour was also measured along the process in the three batches, but not differences in colour parameters L* (Lightness), a* (redness) and b* (yellowness) were detected among them (data not shown). The lipid oxidation (TBARS values) in dry-cured fermented sausages was measured along ripening time (Figure 3C). It was significant the increase in TBARS values during the drying process in the three batches especially after 30 days of processing. However, at the end of the process TBARS values were significantly highest in control sausages followed by M4 sausages and finally, the lowest TBARS values were detected in P2 sausages. This inhibition of the lipid oxidation was previously reported by Flores *et al*., (2004) when they detected a reduction in the generation of volatile compounds derived from lipid oxidation reactions in fermented sausages inoculated with *Debaryomyces* spp.

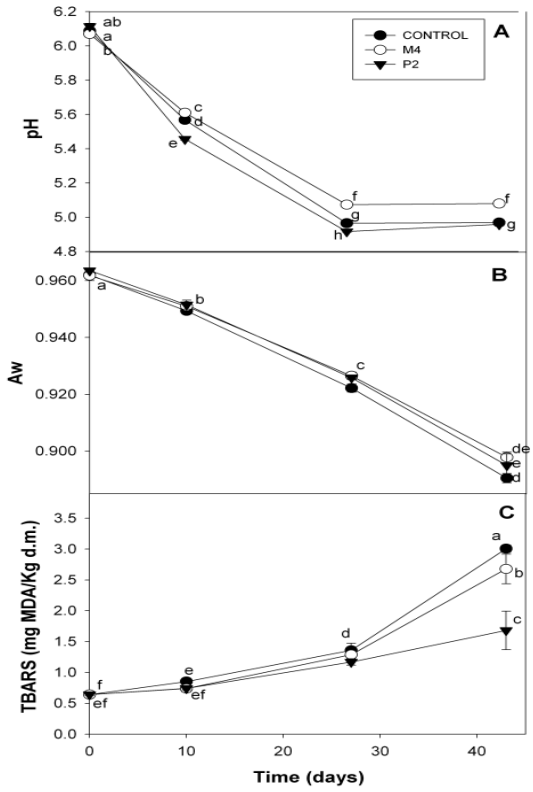

Figure 3.- Evolution of pH, Aw and lipid oxidation values (TBARS) during ripening of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage).

Volatile compound analysis

Table 1 shows the quantification of volatile compounds by SPME-GC-MS in control and inoculated dry-cured sausages throughout the processing time (0, 10, 27 and 43 days). A total of 53 volatile compounds were identified in the dry-cured fermented sausages, all of them were confirmed using authentic standards except 4-hexen-1-ol that was tentatively identified (Table 1).

Table 1.- Volatile compounds identified and quantified (expressed as AU x 10^4 extracted by HS-SPME) by GC-MS in the headspace of dry fermented sausages inoculated with *D.hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage).

VOLATILE COMPOUNDS	KI [1]	0 Days			10 Days			27 Days			43 Days			P [4]			
		C	M4	P2	C	M4	P2	C	M4	P2	C	M4	P2	SEM [3]	Pt	Pc	Ptxc
Aldehydes																	
Acetaldehyde	466				289 abcd	385 ab	56 cd	396 abc	405 a	67 de	266 bcd	432 a	140 de	50	n.s.	***	n.s.
2-Methylpropanal	592				97 ab	164 a			90 ab			48 b		24	**	n.s.	n.s.
3-Methylbutanal	689				1306 a	693 ab	1142 a	295 b	272 b	717 ab	232 b	340 b	360 b	122	***	n.s.	n.s.
Pentanal	737						148 d	214 d	810 bc	274 d	1588 a	1001 b	272 d	253	***	***	***
Hexanal	840				187 d			2498 d	7091 c	1256 d	12799 b	17236 a	2550 d	2458	***	***	**
Heptanal	940							193 d	462 cd	285 d	833 bc	1431 a	911 b	211	***	*	n.s.
Octanal	1047						133 c	119 c	216 bc	296 bc	354 b	555 a	180 c	78	**	*	n.s.
Nonanal	1149				109 e	84 e	129 e	258 cd	314 c	156 d	444 b	563 a	324 c	55	***	**	**
Sulfur compounds																	
Methanethiol (47) [2]	472	5 de	5 de	6 de	5 e	6 de	4 e	13 bc	9 cde	18 ab	22 a	17 ab	14 bc	2	***	n.s.	n.s.
Carbon disulfide	537	297 a	225 abc		233 ab	205 abc	28 bc	261 ab	195 abc	31 bc	192 abc	61 c	70 c	29	**	*	n.s.
Dimethyl disulfide	771	3 c	4 c	2 c	2 c	1 c	2 c	5 c	1 c	7 c	8 bc	24 a	15 b	2	***	n.s.	**
Dimethyl trisulfide (126)	1002	2 b			3 b		1 c	2 b		1 c		93 a	2 b	13	**	***	***
Methionol	1062				15 d		24 d	50 cd	71 bc	20 d	65 bc	260 a	59 bc	28	***	***	***

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Table 1 (continued)

VOLATILE COMPOUNDS	KI [1]	0 Days			10 Days			27 Days			43 Days			P [4]		
		C	M4	P2	C	M4	P2	C	M4	P2	C	M4	P2	SEM [3]	Pt	Pc	Ptxc
Ketones																	
Acetone	528	242 bc	199 cd	207 cd	259 bc	98 de	229 bc	764 a	93 de	403 ab	232 bc	138 de	352 bc	55	***	***	***
2,3-Butanedione (43)	625		364 a		164 bc	224 b	294 ab	118 c	195 b	218 b		106 c	97 c	35	***	**	n.s.
2-Butanone (72)	631	286 a	211 b	197 b	87 d		103 d	92 d	126 bc	61 d	114 bc	205 b	168 bc	24	***	n.s.	***
2-pentanone (86)	732				196 c	1140 a	62 c	185 c	508 b	68 c	90 c	224 bc	155 c	115	***	***	***
3-Hidroxy-2-butanone	780		3480 a	1445 bc	2120 b	4114 a	2056 b	1515 bc	2006 b	1438 bc	817 c	1067 bc	1407 bc	375	***	***	*
2-Heptanone	933		126 e		862 b	1025 a	42 ef	462 c	765 b	41 ef	193 de	44 cd	103 e	116	***	**	n.s.
Alcohols																	
Ethanol	509				4645 cd	5934 abc	4169 cd	5441 abc	6242 abc	4152 cd	5406 abc	5262 bc	3495 e	629	***	**	**
1-Propanol	613				122 a	142 a	49 c	93 a	118 a	82 b	79 b	111 a	119 a	11	***	***	**
2-Methylpropanol	682				399 a	489 a	185 d	386 a	360 b	188 d	267 c	253 c	457 a	37	***	***	***
1-Butanol	725				117 c			251 a	138 bc		171 b	133 bc		24	***	***	*
3-Methylbutanol	795			4383 e	9263 a		9191 a	8224 b	7144 c	5961 d	5258 d	3979 ef	4241 e	1012	***	n.s.	n.s.
2-Methylbutanol	797			351 e	950 b	1364 a	1843 a	914 b	823 b	1118 a	621 c	587 d	725 c	113	***	*	**
1-Pentanol	827				112 c			291 c	1115 ab		680 b	1662 a	190 c	249	*	*	*
1-Hexanol	922				945 c	676 cd	103 d	1784 b	3843 a	138 d	1820 b	4051 a	262 d	507	***	***	***
4-Hexen-1-ol	917TI				4142 a	246 c	869 bc	2570 a	181 c	341 bc	1604 b		59 c	545	n.s.	***	n.s.
2-heptanol	947							575 ab	517 bc	191 e	580 ab	504 cd	454 de	64	***	***	n.s.
1-Octanol	1123				126 ab			135 a	88 c		120 ab	83 c		10	n.s.	*	n.s.
Phenylethyl alcohol (91)	1192				71 cd	176 ab	106 b	59 d	70 cd	202 a	60 d	72 cd	94 b	14	***	***	**

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Table 1 (continued)

VOLATILE COMPOUNDS	KI [1]	0 Days			10 Days			27 Days			43 Days			P [4]			
		C	M4	P2	C	M4	P2	C	M4	P2	C	M4	P2	SEM [3]	Pt	Pc	Ptxc
Ester compounds																	
Ethyl acetate	635				247 c	775 c	1288 c	2441 ab	3144 a	1241 b	2923 ab	3018 ab	3159 a	380	***	n.s.	n.s.
Ethyl 2-methylpropanoate	788					108 e	217 de	289 de	297 de	594 b	363 d	460 c	731 a	63	***	***	*
Ethyl butanoate	830				322 d	526 cd	366 d	934 bc	1204 ab	541 cd	1538 a	1367 ab	667 cd	164	***	***	**
Ethyl 2-hydroxypropanoate	865				95 e	128 de	114 de	385 c	410 c	256 c	858 a	651 b	433 c	87	***	***	**
Ethyl 2-methylbutanoate	877					118 d		121 d	257 ab	214 ab	166 cd	293 a	257 ab	30	***	***	n.s.
Ethyl 3-methylbutanoate	881					394 d		311 d	618 b	488 c	448 c	813 a	652 b	76	***	***	**
3-Methylbutyl acetate	906				87 f	184 de	83 f	283 c	369 b	156 de	469 a	486 a	243 cd	55	***	***	**
Ethyl hexanoate (99)	1028				135 d	179 d	156 d	386 c	610 b	136 d	956 a	1057 a	379 c	119	***	***	***
Ethyl 2,4-hexadienoate	1144				194 b	287 a		74 c	206 b		72 c	97 c		36	***	***	n.s.
Ethyl octanoate (127)	1229				87 de	114 de	112 de	229 c	323 b	135 d	397 a	416 a	124 d	49	***	***	***
Ethyl decanoate (101)	1425							41 d	56 c		76 b	91 a	28 d	10	***	***	n.s.
Acids																	
Acetic acid	737				662 e		2618 bc	2392 cd	2192 cd	2405 bc	3061 b	2990 b	6392 a	571	***	***	**
Propanoic acid	806						89 b			116 ab			198 a	40	**	**	*
2-Methylpropanoic acid	864						271 b	79 c	71 c	469 ab	115 c	113 c	659 a	79	***	***	***
Butanoic acid	891	192 d	177 d	178 d	402 b	425 b	390 c			692 ab			949 a	93	***	n.s.	n.s.
3-Methylbutanoic acid	949					146 b			178 b		124 b	225 a		22	***	*	**
Hexanoic acid	1074				110 c	92 c	80 c	157 bc	161 bc	211 b	294 ab	350 a	288 ab	33	**	***	n.s.
Octanoic acid	1264				70 c	58 cd	68 c	97 ab	90 b	118 a	107 a	108 a	109 a	9	***	n.s.	n.s.

(continued on next page)

Table 1 (continued)

VOLATILE COMPOUNDS	KI [1]	0 Days			10 Days			27 Days			43 Days			P[4]			
		C	M4	P2	C	M4	P2	C	M4	P2	C	M4	P2	SEM[3]	Pt	Pc	Ptxc
Alkanes																	
Pentane	500							122 c	416 b	106 c	474 b	848 a	201 c	116	***	***	n.s.
Hexane	600								72 b	20 d	72 b	93 a	53 c	12	n.s.	n.s.	n.s.
Heptane (71)	700				186 e	317 cd	99 f	273 d	569 c	96 f	705 b	899 a	242 d	94	***	**	**
Octane	800				103 d		107 d	360 c	699 b	293 cd	1084 ab	1956 a	393 c	225	**	*	n.s.

KI[1]: Kovats Index calculated for DB-624 capillary column (J & W Scientific: 30 m, 0.25 mm i.d., 1.4 µm film thickness) installed on a gas chromatograph equipped with a mass-selective detector. Identification of compoundswas confirmed by mass spectrum and retention time with an authentic standard except marked with (TI) that was tentatively identified by mass spectrum. [(2)]: Target ion used to quantify the compound when the peak was not completely resolved. [(3)]: SEM: Standard error of the mean. [(4)]:Pt: p value of ripening time effect; Pc: p value of inoculated yeast effect; Ptxc: p value of interaction between inoculated yeast and ripening time effects. ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$, n.s.: $p>0.05$.

The volatile compounds were grouped in chemical families including aldehydes (8), ketones (6), alcohols (12), acids (7), esters (11), sulfur (5), and alkanes (4) compounds. All of them have been previously reported in fermented sausages using the same extraction technique (Marco, Navarro, & Flores, 2007; Olivares *et al*., 2011; Corral, Salvador, & Flores, 2013). The effect of inoculated yeasts and ripening time was significant in all volatile compounds analyzed except for acetaldehyde, 4-hexen-1-ol and 1-octanol that were not affected by the ripening time and 2-methylpropanal, 3-methylbutanal, methanethiol, dimethyl disulfide, 2-butanone, 3-methylbutanol, ethyl acetate, butanoic acid and octanoic acids that were not affected by the inoculated yeast. Moreover, hexane was not significantly affected by inoculated yeast and ripening time (Table 1).

Figure 4 shows the abundance of volatile compounds classified by chemical groups at the end of the ripening process in all batches (C, M4 and P2). The inoculation with P2 yeast produced a significant decrease in the abundance of aldehydes, sulphur containing compounds, alcohols, esters and alkanes. However, the M4 inoculated sausages did not show this decrease indicating different behaviour between P2 and M4 *D. hansenii* strains.

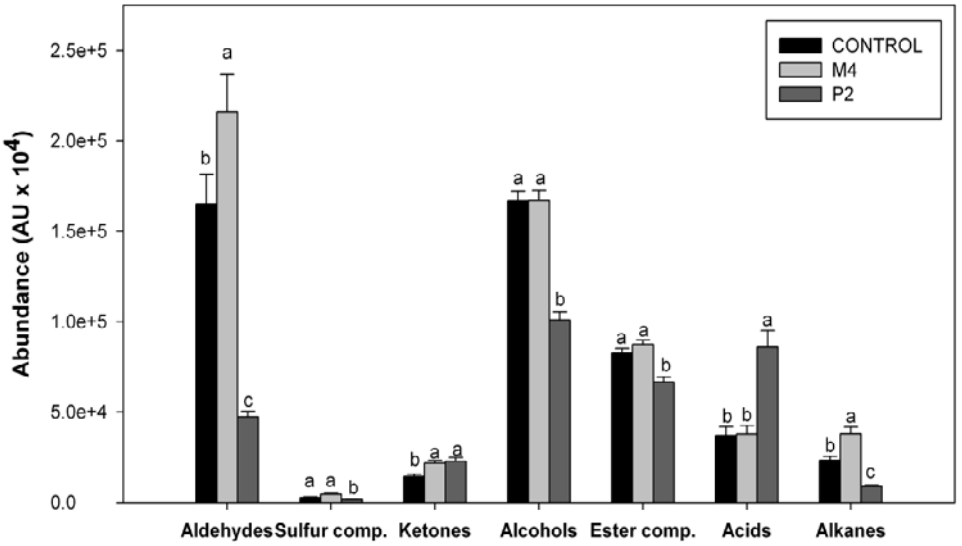

Figure 4.- Total volatile compounds abundance (expressed as AU x 10^4) in the headspace of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage) at the end of the ripening process (43 d). Different letters in the same chemical group indicate significant differences ($p<0.05$) among sausages.

The generation of aldehydes during ripening showed an increase during the whole process except for the branched aldehydes (2-methylpropanal and 3-methylbutanal) which presented a decrease along time in all dry-cured sausages studied (Table 1). This fact could be due to the conversion of these aldehydes to their corresponding alcohols (Table 1) as this has been favoured by the presence of high levels of yeast in inoculated fermented sausages (Andrade *et al.*, 2010). However, the main differences were detected at the end of the process in hexanal, octanal and nonanal, these compounds derived from lipid oxidation reactions were significantly highest in M4 sausages followed by control sausages and they were the lowest in P2 sausages. The generation of these compounds can be related to

the TBARS values measured and therefore, Pearson correlation coefficients were calculated (Table 2). In fact, positive and significant correlations were obtained between aldehydes (nonanal) and TBARS values in the three batches. However, hexanal, the aldehyde significantly related to the lipid oxidation phenomenon (Gandemer, 2002, Olivares *et al*., 2011), was only significantly correlated in control and M4 batches probably due to the low hexanal concentration detected in P2 batch (Table 1). As seen in table 1, the two inoculated yeast showed a different behaviour; P2 inhibited the lipid oxidation process while M4 was not able to do it. This fact agrees with other studies who have indicated an inhibition of the lipid oxidation (Flores *et al*., 2004) while other did not find it (Andrade *et al*., 2010).

Table 2.- Pearson correlation coefficients between aldehydes measured by SPME-GC-MS and lipid oxidation values (TBARS) during the processing of dry fermented inoculated with *D. hansenii* strains; (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage).

	CONTROL		**M4**		**P2**	
	r	**P**	**r**	**P**	**r**	**P**
Acetaldehyde	0.468	0.290	0.267	0.488	0.771	0.439
2-methyl-propanal	0.635	0.249	0.699	0.036	-*	-
3-methyl-propanal	0.473	0.141	0.465	0.176	0.650	0.550
Pentanal	0.956	0.003	0.593	0.292	0.254	0.836
Hexanal	0.940	0.000	0.850	0.007	0.412	0.730
Heptanal	0.890	0.017	0.725	0.166	0.232	0.851
Octanal	0.923	0.009	0.718	0.108	0.999	0.035
Nonanal	0.939	0.000	0.920	0.000	1.000	0.000

*: (-) not detected

Ketone abundance was the highest in yeast inoculated dry-cured fermented sausages (Figure 4). 2,3-butanedione was the ketone compound

with the highest abundance in both inoculated sausages and acetone was highest in P2 inoculated sausages. Both compounds are generated from carbohydrate fermentation and contribute to the aroma in dry-cured fermented sausages (Marco *et al*., 2007).

As observed in figure 4, sausages inoculated with P2 strain generated the lowest alcohol abundance. In addition, ethanol was the compound detected in highest abundance among the alcohols reported however, it was generated in lowest abundance in P2 sausages (Table 1). Although, ethanol is not an essential aroma contributor (Marco *et al*., 2007) it is a precursor of ester compounds (Molimard & Spinnler, 1996) that are responsible of fruity odours in fermented sausages (Marco *et al*., 2007, Olivares, Navarro & Flores, 2013). Other alcohols derived from the lipid oxidation reactions, 1-pentanol and 1-hexanol (Shahidi, Rubin, & D´Souza, 1986), were also in lowest abundance in P2 sausages indicating an inhibition of the lipid oxidation process in this P2 inoculated sausage as seen previously (Table 1). In addition, the effect of the inoculated yeast on the production of alcohols derived from the amino acid catabolism, 2-methylpropanol, 3-methyl and 2-methylbutanol, was not clear (Table 1).

Acid compound generation increased during ripening process and it was the highest in P2 inoculated sausages (Figure 4). As seen in table 1, acetic acid was the compound present in the highest abundance and it was responsible for the differences among sausages. Not only acetic acid was increased in P2 inoculated sausages but also 2-methyl-propanoic, propanoic and butanoic acids were significantly produced in these P2 sausages. In contrast, 3-methyl butanoic and hexanoic acids were mainly produced in M4 inoculated sausages (Table 1). This highest production of acid

compounds has been reported in other *Debaryomyces spp*. inoculated fermented sausages (Flores *et al*., 2004, Andrade *et al*., 2010).

Ester compounds increased along the ripening time in all batches although the highest amounts were detected in control and M4 sausage (Figure 4) due to the highest abundance of ethyl acetate (Table 1). However, other differences among batches were detected such as the highest production of ethyl 2-methylpropanoate and ethyl 2-methyl- and 3-methyl butanoate in the two M4 and P2 inoculated batches (Table 1), confirming the presence of ester activity in the inoculated *Debaryomyces* yeasts (Olesen & Stahnke, 2000, Flores *et al*., 2004 and Andrade *et al*., 2010). These ester compounds have a high impact on sausage aroma contributing to fruity notes and masking rancid odours (Schmidt & Berger, 1998, Olivares *et al*., 2013). Moreover, the abundance of these ester compounds in inoculated dry-cured sausages confirms our previous results obtained in a meat model system that reported a high ester production capability of M4 yeast strain followed by P2 yeast strain (Cano-García *et al*, 2014).

The abundance of sulphur containing compounds in the headspace of sausages increased during the ripening time (Table 1). At the end of the process, control and M4 batches showed a higher abundance of sulphur containing compounds than P2 inoculated sausage (Figure 4). The main difference in the production of sulphur containing compounds was due to the highest abundance of methionol, dimethyl disulfide and dimethyl trisulfide in M4 inoculated sausages. On the contrary, P2 inoculated sausages did not show an increase in these sulphur containing compounds (Table 1). In our previous studies, a similar sulphur capability of M4 and P2

yeast strains was detected in a defined culture media (Cano-García *et al*., 2013) although in a meat model system, P2 showed a higher sulphur capability than M4 yeast strain (Cano-García *et al*, 2014). However, in a real dry-cured fermented sausage opposite results have been obtained being M4 the highest sulphur containing compounds producer, even though M4 yeast counts were lower than those found in P2 inoculated sausages (Figure 1c). These results indicate that yeast strains are strongly influenced by the composition of the media (meat, fat, salt, and additives) and environmental processing conditions (temperature and relative humidity). Probably, this is the reason for low sulphur containing compounds production detected in other dry-cured fermented sausages inoculated with *Debaryomyces spp* strains (Andrade *et al*., 2010; Flores *et al*., 2004; Olesen & Sthanke, 2000). Furthermore, Andrade *et al*., (2010) indicated a low sulphur containing compounds production in fermented sausages inoculated with *D. hansenii* individually but an absence of sulphur production when three different strains were combined and inoculated.

Respect to alkanes, its abundance increased during the ripening time (Table 1). Significant differences were detected at the end of the process ($p < 0.05$) among dry-cured sausages obtaining the highest levels in M4 sausage while P2 sausage was the lowest producer of these volatile compounds (Table 1 and Figure 4). However, the contribution of linear hydrocarbons to the aroma is low due to their high odour threshold (Shahidi *et al*., 1986).

Sensory analysis

The sensory attributes evaluated in the dry-cured fermented sausages were appearance, aroma, taste, tenderness, juiciness and overall quality (Table 3). The consumer sensory panel only detected significant differences in appearance ($p<0.001$) among sausages being the P2 inoculated sausages preferred by the panel. A higher taste preference of the P2 inoculated sausages was obtained but the results were not significant.

Table 3.- Sensory acceptability of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage)

	CONTROL			M4			P2			P[1]
	Mean	SD		Mean	SD		Mean	SD		
Appearance	5.86	1.58	a[2]	5.69	1.80	a	6.71	1.46	b	***
Aroma	6.71	1.67		6.86	1.31		6.74	1.35		n.s.
Taste	6.21	1.78		6.28	1.49		6.58	1.40		n.s.
Tenderness	6.59	1.46		6.51	1.41		6.38	1.45		n.s.
Juiciness	6.66	1.34		6.53	1.38		6.48	1.38		n.s.
Overall quality	6.39	1.65		6.24	1.57		6.53	1.35		n.s.

[1]P value of inoculated yeast effect: *** $p<0.001$, ** $p<0.01$, * $p<005$, n.s.: $p>0.05$. [2]Different letters in each row indicate differences at $p<0.05$ (Fisher test). The values represent the mean and standard deviation (SD).

Therefore, under the ripening conditions used, the inoculated yeast strains P2 and M4, did not affect the sensory characteristic. Few authors have reported a significant effect on the sensory characteristics of fermented sausages inoculated with *Debaryomyces* spp (Flores *et al*., 2004, Iucci *et al*., 2007) while other studies reported absence of sensory effects (Olesen & Stahnke, 2000, Selgas *et al*., 2003). Nevertheless, these studies did not confirm the implantation of the inoculated yeasts during the

ripening process. Moreover, it has been reported that the effect on the sensory characteristics depends on the selected *Debaryomyces* strain (Olesen & Stahnkle, 2000) and their inoculated quantity (Flores *et al*., 2004). In addition, the presence of other starters may affect their grow (Flores *et al*., 2004) and results in different sensory characteristic such as the presence of staphylococci which can modulate the sausage aroma through the conversion of branched chain amino acids and free fatty acids (Leroy *et al*., 2006). In the present study, the starter used was a combination of *Lactobacillus pentosus* and *Staphylococcus carnosus* and a significant highest concentration of staphylococci was detected in M4 inoculated sausages (Figure 1b) that could have affected the final sensory characteristic perceived. However, further studies with more samples should be done to evaluate if those defferences found in volatile compounds are really detected in sensory analysis.

Conclusion

The inoculation of selected *Debaryomyces hansenii* strains, M4 and P2, in slow dry-cured fermented sausages did not affect the ripening process as no changes in pH and Aw were detected. The inoculated yeast strains were recovered at the end of the process although a greatest implantation degree was observed for P2 than M4. The inoculated *D. hansenii* strains affected the lipid oxidation process and the generation of volatile compounds. However, the changes depended on the yeast strain inoculated as a decrease in lipid oxidation values (TBARS) and a reduction of lipid oxidation derived aldehydes were observed in P2 inoculated sausages in addition to a highest acid compound abundance. On the other hand, M4 inoculated

sausages resulted in highest sulphur containing compounds abundance while both yeast strains were responsible for the generation of ethyl methyl-branched ester compounds in the dry-cured fermented sausages. Although, significant results were obtained for production of volatile compounds, differences between the inoculated dry-cured fermented sausages were not sensory appreciated by the consumer panel probably due to the interference of the bacterial starter used in the ripening process. Nevertheless, the aroma potential shown by M4 and P2 *D.hansenii* strains in dry-cured fermented sausages may be used to improve and diversify the flavour of low aroma fermented sausages.

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Capítulo 4

Evaluación de la inoculación de levaduras en embutidos crudos curados mediante la producción de compuestos volátiles utilizando la técnica de espectrometría de masas directa por selección de iones en tubo de flujo (SIFT-MS).

Evaluation of yeast inoculation in fermented sausages by their volatile compound production using Selected ion Flow tube mass spectrometry technique (SIFT-MS)

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Abstract

The meat industry is interested in the application of rapid quality control techniques allowing fine discrimination between meat products. For this purpose, a direct mass spectrometry technique (SIFT-MS) was used to quantify volatile compounds present in the headspace of dry fermented sausages inoculated with two different *D. hansenii* yeast strains (M4 and P2 yeasts). SIFT-MS was able to monitor 30 volatile compounds. P2 batch showed the lowest concentrations of aldehydes, ketones, alcohols, acids, esters and sulphur compounds. M4 sausages had the highest ester compound concentrations. The volatile concentrations were correlated to the yeast population in each batch by Pearson correlation analysis. Both yeasts M4 and P2 were correlated to sulphur compounds and ethanol production. However, the high correlation observed between yeast populations and concentration of aldehydes and longer chain alcohols respectively, was probably due to the generation of linear and methyl branched compounds. SIFT-MS has proven to be a reliable technique for monitoring changes in the volatile compounds associated to yeast population.

Keywords: SIFT-MS, dry fermented sausages, yeast, *D. hansenii*, flavour, aroma and volatile compounds.

INTRODUCTION

The food industry demands rapid analysis techniques, sensitive and reliable, such as direct mass spectrometric techniques. These techniques are based on a mass spectrometric analysis without chromatographic separation in which the mixture of the emitted volatile compounds from a food matrix is sampled directly into a mass spectrometer and the compounds are immediately detected and quantified (Španěl & Smith, 1999). During the last years, several direct mass spectrometry techniques have been used including atmospheric pressure chemical ionization (APCI), proton transfer reaction mass spectrometry (PTR-MS), and selected ion flow tube mass spectrometry (SIFT-MS) (Smith & Španěl, 2011). In the field of meat products, PTR-MS has been applied to study the volatile profile and quality of dry-cured ham (Sánchez del Pulgar *et al*., 2011; Sánchez del Pulgar *et al*., 2013) and the quality changes observed in cooked meat products during storage (Holm *et al*., 2013). In addition, SIFT-MS was applied to control the generation of volatile compounds in meat products (Flores, Olivares, Dryahina, & Španěl, 2013) specially in dry fermented sausages (Olivares *et al*., 2010b; Olivares *et al*., 2011) and more recently in beef muscle packed under modified atmosphere (Olivares, Dryahina, Španěl, & Flores, 2012).

In fermented sausages, the main groups of microorganism used as starters are Lactobacilli and Micrococcaceae although yeast can be considered habitual components of the microbiota growing on dry fermented sausage as the presence of the lactic acid, low water activity and high concentration of salts favour their development (Dillon & Board, 1991; Hammes & Knauf, 1994). The most commonly isolated yeast in dry

fermented sausages is the specie *Debaryomyces hansenii* (Mendonça, Gouvêa, Hungaro, Sodré, & Querol-Simón, 2013; Cano-García, Flores, & Belloch, 2013). The contribution of this yeast to the typical aroma of fermented sausages is based on their proteolytic activity (Bolumar, Sanz, Aristoy, & Toldrá, 2003a, 2003b, 2005; Durá, Flores, & Toldrá, 2004b), lipolytic activity (Cano-García, Rivera-Jiménez, Belloch, & Flores, 2014a) and their capacity to generate flavour volatile compounds from branched-chain amino acids (Durá, Flores, & Toldrá, 2004a). Recently, Cano-García, Belloch, & Flores (2014b) inoculated dry fermented sausages with different strains of *D. hansenii* and obtained significant differences in the generation of volatile compounds analyzed by SPME-GC-MS. However, the application of SIFT-MS as a fast and reliable technique to follow the generation of volatile compounds derived from yeast metabolism can be an interesting approach to confirm the aroma impact of these inoculated yeasts in fermented sausages. Therefore, the aim of the present study was to apply SIFT-MS to the measurement of volatile compounds generated during the processing of fermented sausages inoculated with different *D. hansenii* yeast strains.

MATERIALS AND METHODS

Preparation of dry fermented sausages

Fermented sausages were manufactured as described Cano-García *et al*., (2014b). Three different batches including a control and two yeast inoculated batches (M4 and P2 *D. hansenii* yeasts) were manufactured. Three sausages from each batch (C, M4 and P2) were randomly chosen at day 0 (immediately following production) and then, at days 10, 27 and 43

of processing after which they were sliced, vacuum packaged, and frozen at -80 ºC to await further analysis.

SIFT-MS analyses

A SIFT-MS *Profile* 3 instrument (Instrument Science Limited, Crewe, U.K.) was used to measure the volatile compounds present in the headspace (HS) of dry fermented sausages. For each measurement, 5 g of crushed sausage was weighed into a 15 mL headspace vial, together with 0.75 mg of BHT used as antioxidant and its volatile compounds analyzed as described by Olivares *et al*., (2010b, 2011). The emitted volatiles were allowed to develop in the HS of the sealed vial (initially purged with laboratory air) at 37 ºC for 1 h. After this time, the HS air/volatile compounds of the sealed vial were sampled directly by piercing the septum with a stainless steel needle connected directly to the SIFT-MS sampling line. Then, the sample entered the helium carrier gas via a heated (70 ºC) capillary tube at a measured rate of 0.45 Torr L/s while a second syringe pierced the septum to maintain the pressure in the vial at atmospheric pressure by introducing laboratory air compensating for the small loss rate produced during the sampling into the SIFT-MS instrument. The chemical reaction took place into flow tube that was 4 cm long and 1 cm of diameter, at 26 º C and 1.0 Torr pressure. Background (laboratory air) concentrations of all the volatile compounds included in the analysis were routinely recorded before and after the analysis of each sample. For accurate quantification, the multiple ion monitoring (MIM) mode was used to target specific volatile compounds (Spanel & Smith, 2007). In this mode, the analytical mass spectrometer is rapidly switched between selected m/z values of both the precursor ions

(H_3O^+, NO^+ and O_2^+) and the characteristic product ions. The characteristic product ions for each precursor ion were chosen from a kinetic library compiled from results obtained in previous ion chemistry studies. Thus the count rates of the precursor and product ions were recorded for each sample for all three precursor ions and integrated for a period of 200 s. So, the known rate coefficients for the ion molecular reactions were then used to calculate the absolute HS concentrations of the compounds using the standard SIFT-MS data analysis software and the general method of quantification (Španěl, Dryahina, & Smith, 2006). The mean values of concentrations over the sampling time were recorded in the units of parts per billion by volume of the headspace (ppbv; nL of volatile compound/L of air). Note that due to the overlaps between the product ions of isobaric compounds the calculated concentrations correspond to the total of all possible isomers. In Table 1 the volatile compounds quantified are listed together with the precursor ion and product ions used for analysis. The measuring order of the samples was randomized and the headspace of sausages was analyzed in duplicate for each batch. Thus, a total of six measurements were obtained for each batch at each processing stage.

Yeast counts

Yeast counts in sausages were measured as described in Cano-García *et al*. (2014b) using Rose Bengal Agar with chloramphenicol (RBA) (Conda SA, Madrid, Spain) incubated at 28 ºC for 72 h and expressed as log cfu/g.

Table 1.- The molecular formula and the mass-to-charge ratios, m/z values of the characteristic product ions of the aroma compounds shown analyzed by SIFT-MS using H_3O^+, O_2^+ and NO^+ precursor ions.

Compound	Molecular formula	Precursor ion	m/z	Characteristic product ions
Aldehydes				
Acetaldehyde	C_2H_4O	H_3O^+	45, 81	$C_2H_4OH^+(H_2O)_{0,2}$
Butanal and methylpropanal	C_4H_8O	NO^+	71	$C_4H_7O^+$
Pentanal and methylbutanal	$C_5H_{10}O$	NO^+	85	$C_5H_9O^+$
Hexanal	$C_6H_{12}O$	NO^+	99	$C_6H_{11}O^+$
Heptanal	$C_7H_{14}O$	NO^+	113	$C_7H_{13}O^+$
Nonanal	$C_9H_{18}O$	NO^+	141	$C_9H_{17}O^+$
Pentenal	C_5H_8O	NO^+	83,114	$C_5H_7O^+$, $C_5H_8O{\cdot}NO^+$
Heptenal	$C_7H_{12}O$	NO^+	111,142	$C_7H_{11}O^+$,
Ketones				
Acetone	C_3H_6O	H_3O^+	59, 77	$C_3H_6OH^+(H_2O)_{0,1}$
Butanone	C_4H_8O	NO^+	102	$NO^+{\cdot}C_4H_8O$
Pentanone	$C_5H_{10}O$	NO^+	116	$NO^+{\cdot}C_5H_{10}O$
Heptanone	$C_7H_{14}O$	NO^+	144	$NO^+{\cdot}C_7H_{14}O$
2,3-butanedione	$C_4H_6O_2$	NO^+	86	$C_4H_6O_2^+$
Alcohols				
Methanol	CH_4O	H_3O^+	33, 51, 69	$CH_4OH^+(H_2O)_{0,1,2}$
Ethanol	C_2H_6O	H_3O^+	47, 65, 83	$C_2H_6OH^+(H_2O)_{0,1,2}$
Propanol	C_3H_8O	H_3O^+	43	$C_3H_7^+$
Butanol and methylpropanol	$C_4H_{10}O$	H_3O^+	57, 75, 93	$C_4H_{10}OH^+(H_2O)_{0,1,2}$
Pentanol and methylbutanol	$C_5H_{12}O$	H_3O^+	71, 89, 107, 125	$C_5H_{11}^+(H_2O)_{0,1,2,3}$
Esters				
Methyl formate	$C_2H_4O_2$	H_3O^+	115	$C_2H_4O_2H^+(H_2O)_3$
Ethyl formate	$C_3H_6O_2$	NO^+	104	$C_3H_6O_2.NO^+$
Ethyl acetate	$C_4H_8O_2$	NO^+	118	$C_4H_8O_2.NO^+$
Ethyl propionate	$C_5H_{10}O_2$	NO^+	132	$C_5H_{10}O_2.NO^+$

(continued on next page)

Table 1 (continued)

Compound	Molecular formula	Precursor ion	m/z	Characteristic product ions
Acids				
Acetic acid	$C_2H_4O_2$	NO^+	90,108	$C_2H_4O_2 \cdot NO^+(H_2O)_{0,1}$
Propanoic acid	$C_3H_6O_2$	NO^+	104,122	$C_3H_6O_2 \cdot NO^+(H_2O)_{0,1}$
Butyric and methylpropanoic acid	$C_4H_8O_2$	NO^+	118,136	$C_4H_8O_2 \cdot NO^+(H_2O)_{0,1}$
Sulphur compounds				
Hydrogen sulfide	H_2S	H_3O^+	35, 53	$H_2SH^+(H_2O)_{0,1}$
Methanethiol	CH_4S	H_3O^+	49, 67, 85	$CH_4SH^+(H_2O)_{0,1,2}$
Dimethyl disulfide	$C_2H_6S_2$	NO^+	94	$C_2H_6S_2{}^+$
Dimethyl sulfide	C_2H_6S	$O_2{}^+$	62, 80	$C_2H_6S^+(H_2O)_{0,1}$
Carbon disulfide	CS_2	$O_2{}^+$	76	$CS_2{}^+$

Statistical analysis

The effect of inoculated yeast strains and processing time on volatile compounds measured by SIFT-MS was tested by two-way analysis of variance (ANOVA) using the statistic software XLSTAT 2009.4.03 (Addinsoft, Barcelona, Spain). Differences between sample means were analyzed according to Fisher's Least Significant Difference (LSD) test. Pearson correlation analysis was performed to correlate SIFT-MS results and yeast growth in sausages (Cano-García *et al*., 2013).

RESULTS AND DISCUSSION

Volatile compounds analysis of each batch by SIFT-MS yielded a total of 30 compounds which were identified and quantified. Table 1 shows the volatile compounds identified together with the characteristic products ion generated in the acid-basic reaction that is produced between the precursor ion and the volatile compounds present in the headspace.

As can be seen in Table 1, NO^+ was the most suitable precursor ion for quantify these volatile compounds. However, the use of various precursor ions resulted useful to identify and quantify the volatile compounds when in the samples exist a potential overlap of characteristic ions. This is the case of volatile compounds as ethyl acetate and methyl formate that were analyzed using both precursor ions $H3O^+$ and NO^+ to avoid overlapping carboxylic acids (Pysanenko, Španěl, & Smith, 2009).

Eight aldehydes were detected by SIFT-MS although two of them, heptanal and nonanal, presented concentrations close to the quantification limit (10 ppb), and were not quantified (Španěl, Doren, & Smith, 2002). Figure 1 represents the generation of the aldehyde compounds quantified during ripening in the different sausage batches.

Significant differences were observed in the headspace concentration of all aldehydes due to the effect of the inoculated yeast strains and ripening process ($p < 0.001$) except for butanal that was not affected by the inoculated yeast strains (Figure 1B) ($p > 0.05$). Acetaldehyde (Figure 1A) was the most abundant aldehyde followed by hexanal (Figure 1D) and pentenal (Figure 1E). Generally, hexanal is reported as the major aldehyde released in dry fermented sausages when measured using SPME-GC-MS technique (Marco, Navarro, & Flores, 2006; Olivares, Navarro, & Flores, 2010a; Cano-García *et al*., 2014b). In fact, aldehyde concentrations, except for butanal isomers including 2-methyl-propanal, were the lowest in the P2 yeast inoculated batches especially after 27 and 43 d of ripening. This can be related to the lower oxidation values (TBARS) also reported in this sausages (Cano-García *et al*., 2014b) and attributed to the ability of *D.*

hansenii yeast to inhibit the lipid oxidation process in fermented sausages (Flores, Durá, Marco, & Toldrá, 2004).

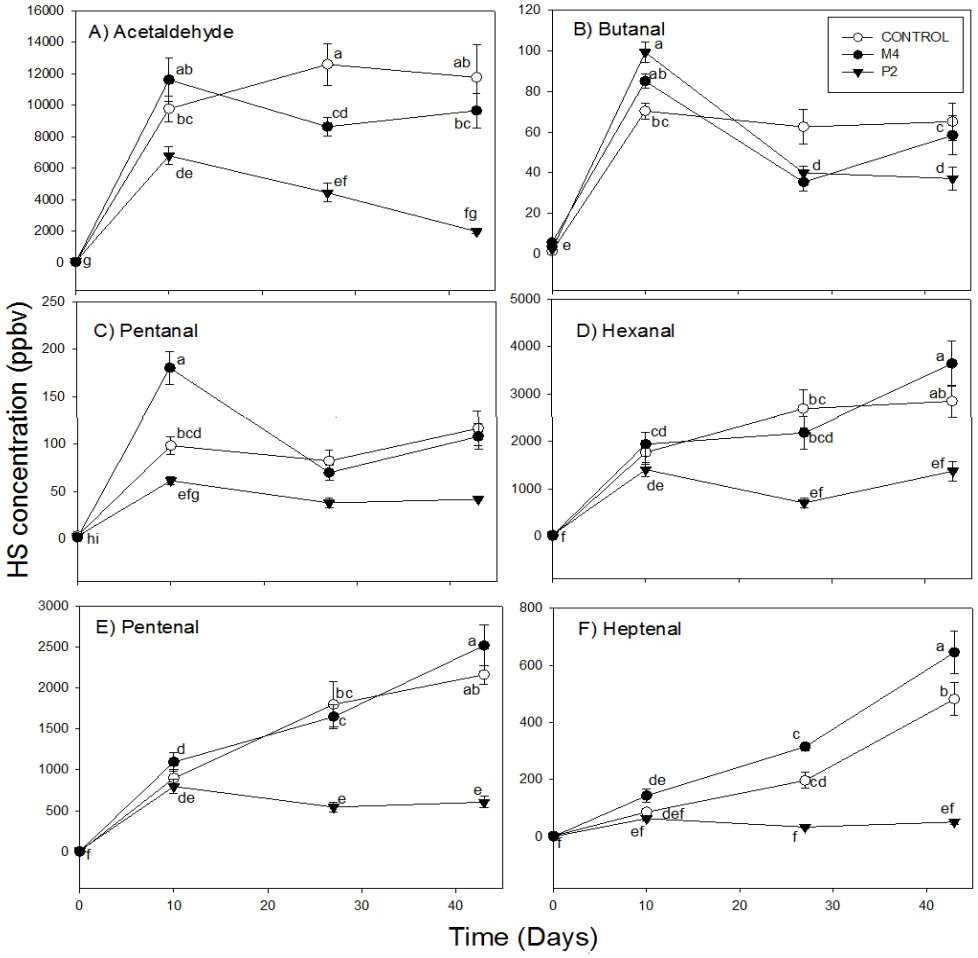

Figure 1.- Aldehyde concentration in the headspace of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage) along the process. A) Acetaldehyde, B) Butanal, C) Pentanal, D) Hexanal, E) Pentenal and F) Heptenal. Different letters indicate significant differences at $p < 0.05$.

Ketones were quantified in the headspace of the different sausage batches (Figure 2). The concentration of butanone, pentanone, heptanone, 2,3-butanedione and acetone increased significantly along the ripening time ($p < 0.001$) (Figures 2A, 2B, 2C, 2D and 2E, respectively). Acetone was the most abundant ketone in the headspace (Olivares *et al*., 2010), although its concentration was not affected by the yeast strains inoculated (Figure 2E). In contrast, butanone, heptanone and 2,3-butanedione (Figures 2A, 2C and 2D, respectively) were affected by the inoculated yeast strains ($p < 0.001$) as the lowest concentrations were generally found in the P2 yeast inoculated batch. Concentration of various alcohols in the headspace of sausages was increased during ripening in control and M4 yeast inoculated batches however the concentration of ethanol, butanol and pentanol remained constant in P2 yeast inoculated sausages during all ripening process (Figures 2F, 2I and 2J, respectively). Nevertheless, the generation of methanol and propanol (2G and 2H) was not affected by the inoculated yeasts ($p > 0.05$). Ethanol was the most abundant alcohol detected (Figure 2F) in accordance with previous studies (Olivares *et al*., 2010a; Cano-García *et al*., 2014b). In addition, ethanol has been associated with bacterial metabolism in dry fermented sausages (Marco *et al*., 2006). However all batches were inoculated with the same bacterial starter culture; therefore, the ethanol differences among batches could be due to yeast metabolism (Figure 2F). Methanol was also detected in all batches and increased during processing (Figure 2G) although this compound has only been detected previously in the headspace of fermented sausages using SIFT-MS (Olivares *et al*., 2010b).

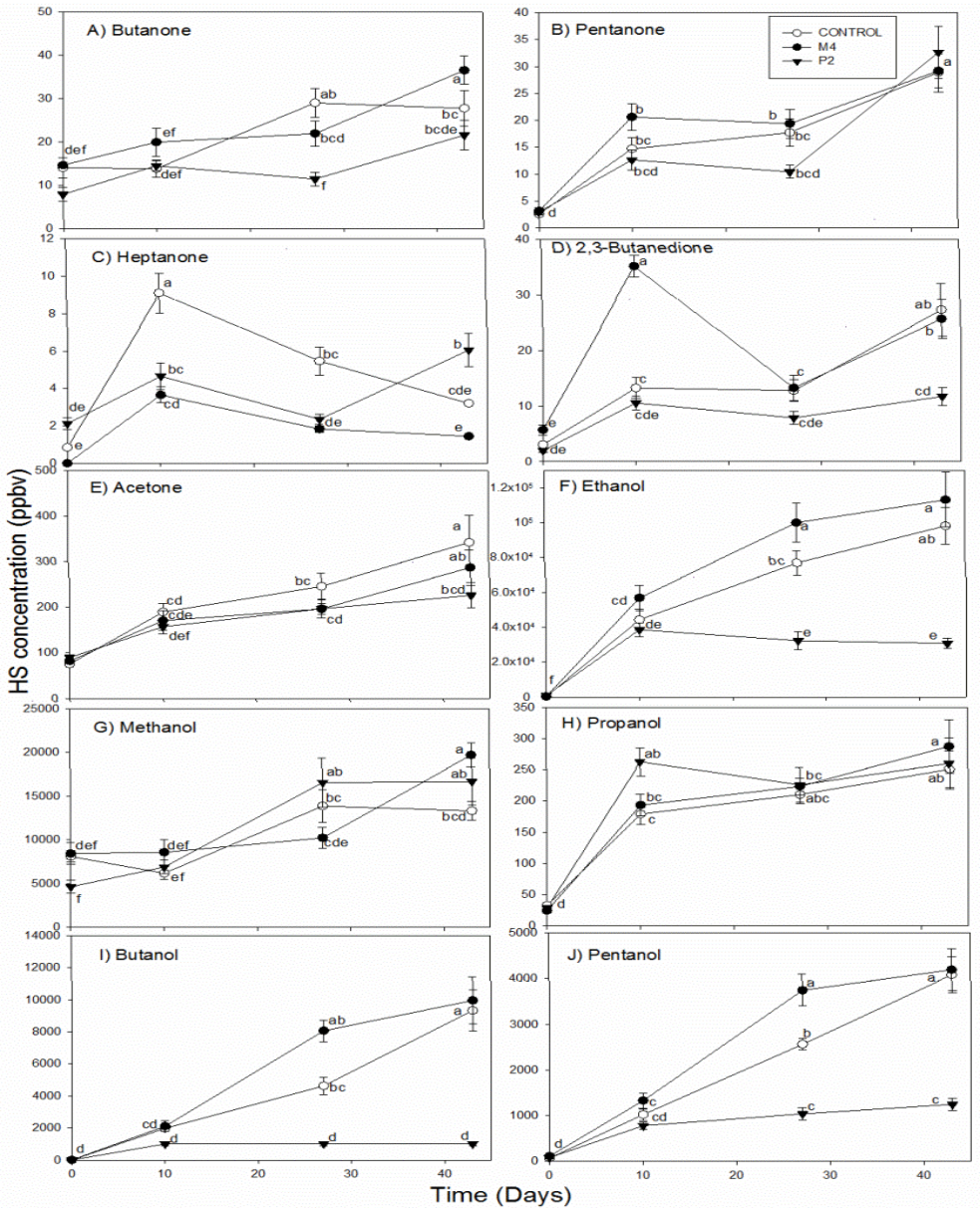

Figure 2.- Ketones and alcohols concentration in the headspace of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage) along the process. A) Butanone, B) Pentanone, C) Heptanone, D) 2,3-Butanedione, E) Acetone, F) Ethanol, G) Methanol, H) Propanol, I) Butanol and J) Pentanol. Different letters indicate significant differences at $p < 0.05$.

Five sulfur compounds were quantified by SIFT-MS in the headspace of the sausages (Figure 3, panels A to E). Significant changes ($p < 0.001$) occurred in the headspace along the ripening time. Hydrogen sulfide decreased after 10 days of processing while the abundance of methanethiol, carbon sulfide, dimethyl sulfide and dimethyl disulfide increased along the time except in P2 yeast inoculated batch where the concentration of these compounds remained constant during processing (Figure 3). The rapid decrease of hydrogen sulfide has been related to its high reactivity with other compounds present in meat (Olivares *et al*., 2010). In addition, the lower sulfur generation in P2 inoculated sausages was also observed previously using SPME-GC-MS (Cano-García *et al*., 2014b).

Figures 3F, 3G and 3H show generation of acetic, propanoic, and butanoic acids quantified by SIFT-MS. All acid compounds increased ($p < 0.001$) during process. However, higher concentrations were detected in control and M4 yeast inoculated batches, mainly in propanoic and butanoic acids (figures 3G and 3H). These results are in contrast with those reported by SPME-GC-MS where the highest production of acid compounds was detected in P2 yeast batch (Cano-García *et al*., 2014b). Nevertheless, acetic acid concentration followed the same trend as observed previously Olivares *et al*., (2010) using SIFT-MS in fermented sausages.

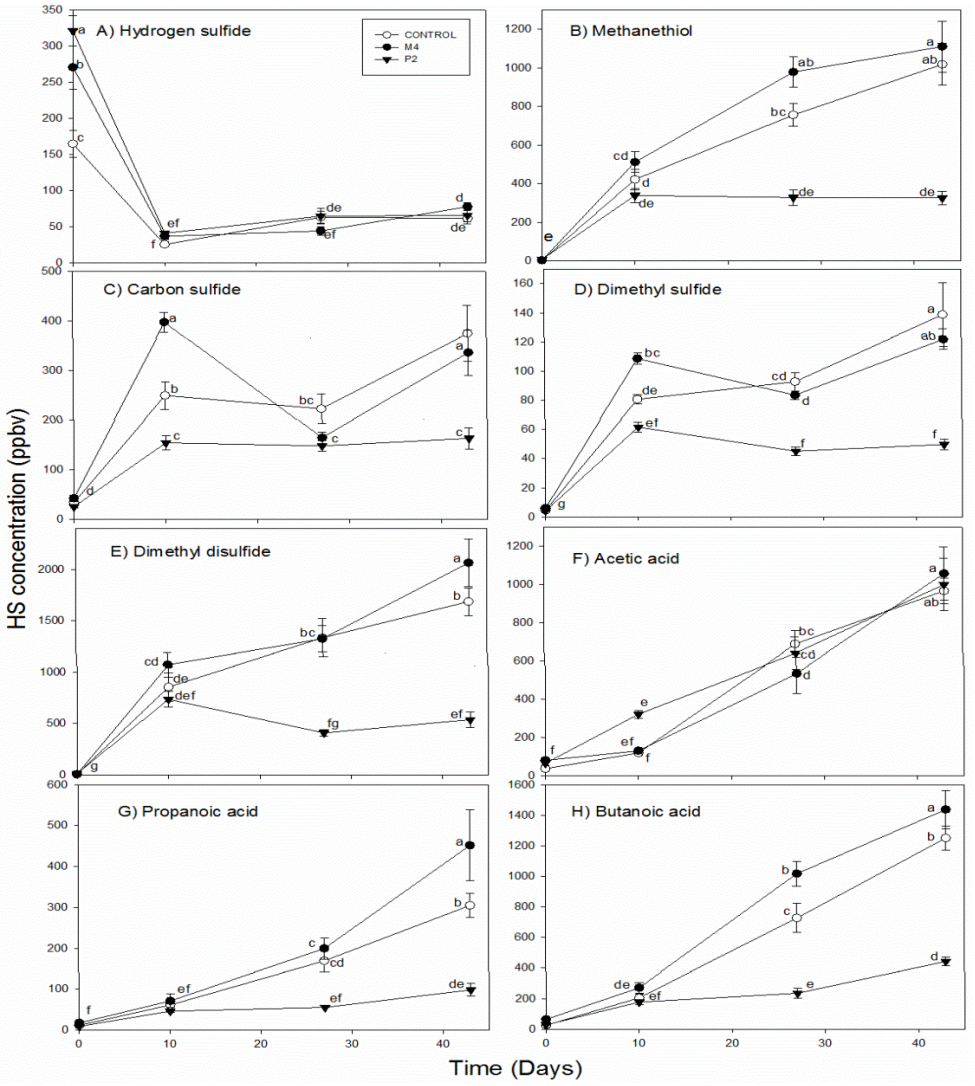

Figure 3.- Sulphur and acid compounds concentration in the headspace of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage) along the process. A) Hydrogen sulfide, B) Methanethiol, C) Carbon sulfide, D) Dimethyl sulfide, E) Dimethyl disulfide, F) Acetic acid, G) Propanoic acid and H) Butanoic acid. Different letters indicate significant differences $p < 0.05$.

The evolution of ester compounds along the ripening time is displayed in Figure 4 where an increase in their concentration during process was observed. Methyl formate and ethyl acetate were the most abundant ester compounds (Figures 4A and 4C, respectively) while ethyl formate and ethyl propanoate were detected in minor concentrations (Figures 6B and 6D, respectively). The yeast strains inoculated had a significant effect in the ester concentration ($p < 0.001$). *D. hansenii* M4 strain was the highest producer of ester compounds while the lowest concentration were detected in the batch inoculated with P2 yeast strain confirming the previous studies using SPME-GC-MS technique (Cano-García *et al*., 2014a,b).

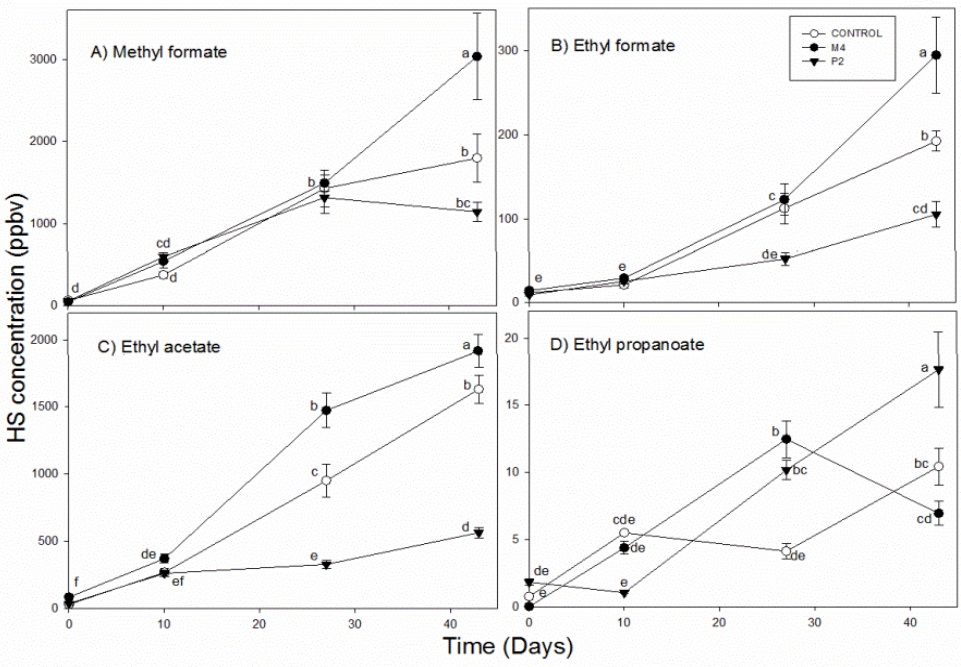

Figure 4.- Ester compounds concentration in the headspace of dry fermented sausages inoculated with *D. hansenii* strains (C: Control sausage; M4: M4 inoculated sausage; P2: P2 inoculated sausage) along the process. A) Methyl formate, B) Ethyl formate, C) Ethyl acetate and D) Ethyl propanoate. Different letters indicate significant differences $p < 0.05$.

Mainly all the SIFT-MS quantified volatile compounds were detected previously by SPME-GC-MS (Cano-García *et al*., 2014b) except for several volatile compounds such as aldehydes (butanal, pentanal and heptanal), sulfur compounds (hydrogen sulfide and dimethyl sulfide), ester compounds (methyl formate, ethyl formate and ethyl propionate) and methanol. Probably these compounds were not detected by SPME-GC-MS due to their low concentration in the HS or to the low affinity by the fiber used in the analysis (Olivares *et al*., 2010, 2012).

In order to determine which volatile compounds were produced by the activity of the inoculated yeast strains, a Pearson correlation analysis was done between the analyzed compounds and yeast population. As reported in Cano-García *et al*., (2014b) yeast population in the Control batch was low during the process (around 2 log ufc/g), while the yeast population in M4 and P2 batches at the beginning of process (4 and 7 log ufc/g respectively) increased up to 6 and 8 log ufc/g during the first 10 days and was maintained stable until the end of the process. As pointed out in Table 2 no significant correlations were obtained in the Control batch while several compounds were correlated to yeast population in M4 and P2 inoculated batches specially, sulphur compounds and ethanol. These findings confirm our previous results using meat model systems that showed the ability of M4 and P2 to produce sulphur compounds and ethanol (Cano-García *et al*., 2014a).

Table 2.- Pearson correlation coefficient between yeast population and volatile compounds monitored by SIFT-MS during the manufacture of dry fermented sausages.

COMPOUNDS	CONTROL		M4		P2	
	r	*P*	r	*P*	r	*P*
Acetaldehyde	0.336	0.3120	0.934	**< 0.0001**	0.891	**0.0000***
Butanal and methylpropanal	0.287	0.3920	0.842	**0.0010**	0.695	**0.0180**
Pentanal and methylbutanal	0.381	0.2470	0.824	**0.0020**	0.722	**0.0120**
Hexanal	0.457	0.1580	0.783	**0.0040**	0.653	**0.0300**
Heptanal	0.194	0.5680	0.702	**0.0160**	0.403	0.2190
Nonanal	0.496	0.1200	0.891	**0.0000**	0.458	0.1560
Pentenal	0.407	0.2140	0.73	**0.0110**	0.859	**0.0010**
Heptenal	0.16	0.6380	0.563	0.0720	0.696	**0.0170**
Hydrogen sulfide	0.339	0.3080	0.959	**< 0.0001**	0.981	**< 0.0001**
Methanethiol	0.388	0.2380	0.713	**0.0140**	0.914	**< 0.0001**
Dimethyl sulfide	0.441	0.1740	0.964	**< 0.0001**	0.778	**0.0050**
Carbon disulfide	0.467	0.1470	0.812	**0.0020**	0.703	**0.0160**
Dimethyl disulfide	0.37	0.2630	0.789	**0.0040**	0.785	**0.0040**
Acetone	0.304	0.3630	0.711	**0.0140**	0.559	0.0740
2,3-butanedione	0.353	0.2860	0.629	**0.0380**	0.651	**0.0300**
2-butanone	0.082	0.8100	0.481	0.1340	0.361	0.2760
2-pentanone	0.515	0.1050	0.778	**0.0050**	0.411	0.2090
2-heptanone	0.197	0.5620	0.675	**0.0230**	0.156	0.6470
Methanol	0.126	0.7130	0.36	0.2760	0.499	0.1180
Ethanol	0.416	0.2040	0.729	**0.0110**	0.902	**0.0000**
Propanol	0.438	0.1780	0.806	**0.0030**	0.865	**0.0010**
Butanol and methylpropanol	0.355	0.2840	0.535	0.0900	0.888	**0.0000**
Pentanol and methylbutanol	0.371	0.2610	0.596	0.0530	0.752	**0.0080**
Methyl formate	0.21	0.5350	0.499	0.1190	0.51	0.1090
Ethyl formate	0.103	0.7620	0.395	0.2290	0.342	0.3030
Ethyl acetate	0.243	0.4710	0.512	0.1080	0.595	0.0530
Ethyl propionate	0.118	0.7310	0.549	0.0800	0.265	0.4310
Acetic acid	0.317	0.3420	0.468	0.1460	0.509	0.1100
Propanoic acid	0.073	0.8300	0.482	0.1340	0.562	0.0720
Butanoic and methylpropanoic acid	0.243	0.4720	0.512	0.1070	0.596	0.0530

*P value in bold indicate a significant effect.

Nevertheless, it is important to remark that yeasts may not be directly responsible of sulphur compounds production but can supply intermediates. In addition, M4 population showed the highest significant correlation to aldehydes production such as acetaldehyde, butanal, pentanal and pentenal ($r > 0.8$), while P2 population had a highest correlation to alcohols production ($r > 0.7$) (Table 2). The correlation of both yeast populations to butanal and pentanal was probably due to the overlapping of 2-methyl-propanal and 2 and 3-methyl-butanal, respectively as SIFT-MS is not able to distinguish between linear and branched volatile compounds (Španěl *et al.*, 2002). Similarly, the analysis of butanol and pentanol might include the presence of 2-methyl-propanol and 2 and 3-methyl-butanol, respectively, which are highly produced by P2 yeast (Cano-García *et al.*, 2014a).

CONCLUSION

SIFT-MS was able to monitor the volatile compounds generated during the process and to detect significant differences in volatile production due to yeast activity. *D. hansenii* P2 inoculated sausages showed the lowest concentrations by SIFT-MS of aldehydes, ketones, alcohols, acids, esters and sulphur compounds while *D. hansenii* M4 sausages had the highest ester compound concentrations. However, the correlation of volatile compounds to yeast population showed that both yeasts M4 and P2 produced sulphur compounds and ethanol. In addition, the high correlation observed between M4 and P2 yeast populations and the concentration of aldehydes and longer chain alcohols respectively, was probably due to the generation of linear and methyl branched compounds. In summary, SIFT-MS can be used to monitor yeast activity in a complex matrix like dry

fermented sausages and it is a reliable technique for monitoring changes in the volatile compounds associated to yeast population. This study also reveals that the overlaps between isomeric compounds occur in yeast generated volatile compounds and that further research will be needed to facilitate their independent quantification by SIFT-MS.

Acknowledgments

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IV. DISCUSIÓN GENERAL

IV. Discusión general:

En esta tesis doctoral se han planteado 2 objetivos principales que han sido alcanzados en los resultados descritos en 4 capítulos. Sin embargo, con la finalidad de ofrecer una visión global del trabajo realizado se ha redactado esta discusión general donde se recogen los resultados más relevantes.

Uno de los aspectos más valorados por los consumidores en los embutidos crudos curados es el aroma, formado por una mezca compleja de compuestos volátiles. Estos compuestos se generan como resultado de multiples reacciones bioquímicas que tienen lugar durante el proceso de fabricación y que dependen de las condiciones del proceso, de las materias primas empleadas y también de los microorganismos que mediante su metabolismo participan en dichas reacciones bioquímicas (Talon y col., 2007; Leroy y col., 2006; Tjener & Stahnke, 2007).

Dentro de los embutidos crudos curados aquellos fabricados mediante técnicas tradicionales presentan un aroma característico muy apreciado por los consumidores (Moretti y col., 2004). Este hecho puede deberse a que el empleo de largos procesos de elaboración produce un mayor desarrollo de los microorganismos presentes en el producto ya sean los propios cultivos iniciadores como la microbiota autócotona (Cocolin y col., 2011). Por ello, la primera parte de este estudio se ha centrado en identificar y caracterizar levaduras aisladas de embutidos crudos curados elaborados de forma tradicional (Capítulo 1).

La identificación y caracterización de estas levaduras se llevó a cabo utilizando diferentes técnicas moleculares previamente utilizadas con éxito

en la caracterización de especies de levaduras procedentes de embutidos crudos curados (Iucci y col., 2007; Patrignani y col., 2007; Andrade y col., 2010; Mendonça y col., 2013). El análisis de restricción y secuenciación de la región ITS1-5.8S-ITS2 ADNr agrupó a todas las levaduras aisladas de embutidos crudos curados tradicionales dentro de la especie *Debaryomyces hansenii* confirmando los resultados obtenidos por otros autores (Mendonça y col., 2013). En cuanto a la caracterización molecular de las levaduras aisladas realizada mediante el análisis de restricción del ADN mitocondrial y de la técnica RAPD-M13 se encontró que la última permitía una mejor diferenciación a nivel de cepa. Además también presentó otras ventajas frente a los RFLPs del ADNmt, como una mayor rapidez de análisis y una menor exigencia en cuanto a la cantidad y pureza de ADN necesario.

La caracterización molecular permitió diferenciar cepas de *D. hansenii* procedentes de distintos embutidos crudos curados tradicionales, así como cepas de *D. hansenii* aisladas de un mismo producto. Esto nos llevó a pensar en la posibilidad de que el aroma final de estos productos se deba a la actividad conjunta de varias cepas de *D. hansenii*.

Con el objetivo de estudiar el potencial de las diferentes cepas de *D. hansenii* para producir aromas característicos en los embutidos tradicionales, se llevó a cabo un estudio en medio definido, en presencia del ácido 2-metil butanoico y de un alcohol (etanol o metanol). En este estudio se observó que, aunque todas las levaduras fueron capaces de crecer en el medio y generar compuestos volátiles, sólo algunas eran capaces de producir perfiles aromáticos distintos. Las cepas que presentaron mayor potencial aromático (M4, M6, P2, T3, V2, EV2 e I2) fueron seleccionadas

para ser inoculadas en un sistema modelo cárnico similar a un embutido crudo curado.

El potencial aromático de las siete cepas seleccionadas fue evaluado en un sistema modelo cárnico de composición similar a un embutido en el que la bajada de pH y la gelificación del medio fueron inducidas empleando glucono-δ-lactona (Capítulo 2). De esta forma se evitó el uso de bacterias ácido lácticas que podrían interferir en el análisis de los compuestos volátiles generados durante el ensayo. Las cepas seleccionadas fueron inoculadas de forma individual empleando la misma población de levadura en todos los medios inoculados llevándose a cabo un análisis de crecimiento e implantación de cada una de las levaduras así como de los compuestos volátiles generados en cada uno de los lotes a distintos tiempos.

Con este estudio se pudo comprobar el crecimiento y la implantación de las levaduras inoculadas a lo largo del proceso. Además, se detectó la capacidad de todas las cepas para generar compuestos volátiles en un medio similar a un embutido crudo curado sin la presencia de bacterias ácido lácticas o *Staphylococcus* a los que generalmente se les atribuye el papel de contribuir al aroma final de estos productos (Montel y col., 1996, 1998). Las diferentes cepas seleccionadas presentaron distintos perfiles aromáticos destacando las cepas P2 y M6 por su gran capacidad para generar compuestos azufrados y las cepas M4 y P2 por la producción de elevadas concentraciones de ésteres. Dado que las concentraciones de los compuestos volátiles en el sistema modelo fueron calculadas, se pudo determinar la contribución de estos compuestos al aroma mediante el cálculo de la actividad aromática (OAV). Tomando como referencia los umbrales de percepción en aire (Van Gembert & Nettenbreijer, 2004) se

concluyó que varios compuestos volátiles presentaron OAV positivos aunque las mayores diferencias entre los sistemas modelos inoculados fueron detectadas en los modelos inoculados con las cepas M4, M6 y P2. Así, el modelo inoculado con la cepa M4 se caracterizó por tener los OAVs más elevados en ésteres (acetato de etilo, 2-metilpranoato de etilo y acetato de 3-metilbutilo) seguido en menor medida por el modelo P2 (3-metilbutilo), mientras que los lotes inoculados con las cepas M6 y P2 mostraron los mayores OAVs en compuestos azufrados (dimetil disulfuro y dimetil trisulfuro). Estos compuestos volátiles han sido descritos como importantes contribuidores del aroma debido a sus bajos límites de detección y en consecuencia, a su gran impacto aromático (Marco y col., 2007; Meynier y col., 1999; Schmidt & Berger, 1998; Stahnke, 1994). Por tanto, tras caracterizar las cepas aisladas de embutidos crudos curados tradicionales y estudiar su potencial aromático en un medio definido y en un sistema modelo cárnico de características similares a un embutido crudo curado, dos cepas (M4 y P2) de entre 22 aislados de *D. hansenii*, fueron seleccionadas para ser utilizadas como estárter en embutidos crudos curados de fermentación lenta con la finalidad de estudiar su capacidad para producir compuestos volátiles en dichos productos (Capítulo 3). A modo de resumen, en la Tabla 1 quedan reflejados los distintos perfiles aromáticos que cada una de las cepas presentaron en los diferentes medios ensayados. Así, en el estudio realizado en el medio definido las cepas M6 e I2 se caracterizaron por su gran capacidad para producir alcoholes, las cepas V2 y EV2 destacaron por generar importantes cantidades de compuestos azufrados mientras que la cepa P2 fue la mayor productora de compuestos ácidos y la cepa M4 fue la que mostró mayor potencial aromático

generando grandes cantidades de cetonas, aldehídos y ésteres. Sin embargo, en el sistema modelo cárnico las cepas presentaron comportamientos diferentes a los esperados ya que sólo cuatro de las siete cepas empleadas mostraron una mayor concentración en compuestos volátiles detectados habitualmente en este tipo de productos. De esta forma, destacaron las cepas M4 por su producción de ésteres, la M6 por su capacidad para generar aldehídos, la P2 por su elevada síntesis de compuestos azufrados, ésteres y ácidos, y finalmente la cepa V2 por producir alcoholes y cetonas. De esta forma se puso de manifiesto que el empleo de unas determinadas condiciones de proceso y el uso de diferentes sustratos condiciona la actividad de las levaduras afectando al aroma final del producto.

Durante el proceso de elaboración de los embutidos crudos curados de fermentación lenta (Capítulo 3), la población de levaduras fue incrementando aunque la implantación de cada una de las cepas inoculadas fue muy diferente. De este modo, la cepa P2 fue capaz de implantarse desde el momento inicial mientras que la cepa M4 tuvo una implantación más lenta imponiéndose completamente sobre la microbiota competitiva al final del proceso. No obstante, quedó demostrado que en los dos casos la inoculación de levadura en este tipo de producto favorece la protección del mismo frente a la oxidación lipídica y que además generan compuestos volátiles que contribuyen al aroma final del producto.

Tabla 5.- Principales volátiles, clasificados en grupos químicos, producidos por las levaduras aisladas de embutidos crudos curados tradicionales.

Estudio	Cepas empleadas						
	M4	**M6**	**P2**	**T3**	**V2**	**EV2**	**I2**
Capítulo 1. Medio definido	**Aldehídos**(*) **Cetonas** **Ésteres** Comp. azufrados	**Alcoholes** Ésteres	**Comp. ácidos** Aldehídos Alcoholes Ésteres	Cetonas Alcoholes Ésteres	**Comp. azufrados** Cetonas Comp. ácidos Alcoholes Ésteres	**Comp. azufrados** Cetonas Comp. ácidos Alcoholes Ésteres	**Alcoholes** Comp. azufrados Ésteres
Capítulo 2. Sistema modelo cárnico	**Ésteres** Aldehídos Alcoholes	**Aldehídos** Cetonas Comp. ácidos Comp. azufrados	**Comp. azufrados** **Comp. ácidos** **Ésteres**	Comp. ácidos Alcoholes	**Cetonas** **Alcoholes** Comp. ácidos	Cetonas Comp. ácidos	Alcoholes
Capítulo 3. Salchichón tradicional	**Aldehídos** **Comp. azufrados** **Ésteres**		**Comp. ácidos** **Cetonas**				

(*) Los grupos químicos que se encuentran en negrita son aquellos generados en mayor medida por cada una de las cepas estudiadas en los diferentes medios ensayados.

Observándose que aquellos lotes inoculados con levadura presentaron valores de TBARS inferiores a los detectados en el lote control, aunque al comparar los resultados obtenidos entre los lotes inoculados se pudo observar que los embutidos crudos curados inoculados con la cepa P2 fueron los que menor grado de oxidación lipídica mostraron. Además, la cepa P2 se caracterizó por una mayor producción de compuestos ácidos y cetonas mientras que la cepa M4 generó mayores cantidades de aldehídos, ésteres y compuestos azufrados. Este hecho confirma la capacidad de protección frente a la oxidación por parte de la cepa P2 puesto que los aldehídos, compuestos volátiles relacionados con la oxidación lipídica, fueron detectados en mayor abundancia en los lotes inoculados con la cepa M4 y en el lote control.

En cuanto al análisis sensorial realizado al final de la fabricación los catadores detectaron diferencias significativas en la apariencia de los distintos lotes estudiados, siendo el lote P2 el mejor valorado. Además, se detectó una mayor aceptación en el sabor de los lotes inoculados con la cepa P2 posiblemente debido a sus bajos valores en TBARS por lo que presentaba un menor grado de oxidación del producto, aunque estas diferencias no fueron significativas.

Teniendo en cuenta el volumen de trabajo necesario para seleccionar las cepas utilizadas y el tiempo empleado en los diferentes análisis de los compuestos volátiles, se planteó la posibilidad de buscar una técnica alternativa al análisis de compuestos volátiles mediante micro-extracción en fase sólida (SPME) y cromatografía de gases con detector de espectrometría de masas (CG – MS). Con este objetivo, en el Capítulo 4 se aplicó la técnica de espectrometría de masas por selección de iones en tubo de flujo

(SIFT – MS). Esta técnica fue desarrollada inicialmente para el estudio de las cinéticas de reacción ión-molécula por Adams & Smith (1976) aunque posteriormente Smith & Španěl (2005) mejoraron el sistema al acoplar un detector selectivo de masas al instrumento. La técnica SIFT – MS permite analizar los compuestos volátiles de una muestra en tiempo real sin extracción ni separación cromatográfica previa basándose en la tranferencia de protones que tiene lugar en la ionización química donde, a partir de reacciones ácido-base entre los compuestos de una muestra y un gas reactivo en fase gaseosa, se forman iones productos característicos. Así, basándose en la reacción por transferencia de protones y conociendo tanto los iones producto característicos como los coeficientes de reacción a un tiempo de reacción dado, se puede llevar a cabo la cuantificación de los compuestos volátiles. Esta técnica ha sido empleada recientemente para estudiar la generación de compuestos volátiles en embutidos crudos curados de fermentación lenta elaborados con diferentes porcentajes de grasa revelándose como una técnica rápida de detección y cuantificación de dichos compuestos volátiles (Olivares y col., 2010) así como una buena herramienta para la evaluación del estado oxidativo de estos productos cárnicos (Olivares y col., 2011). De esta forma, la técnica SIFT – MS permitió calcular rápidamente la concentración absoluta de los compuestos volátiles presentes en los embutidos crudos curados de fermentación lenta inoculados con cepas de *D. hansenii* (M4 y P2). Para ello se midió el ratio ion producto/ion precursor específico de cada compuesto volátil y se consiguió aportar información similar a la obtenida con la técnica SPME – GC – MS pero en menor tiempo.

La técnica SIFT – MS fue capaz de detectar los cambios producidos en la concentración de los compuestos volátiles a tiempo real a lo largo del proceso de fabricación. Sin embargo, con la técnica convencional SPME – GC – MS se analizaron 53 compuestos volátiles presentes en el espacio de cabeza de los diferentes embutidos crudos curados estudiados mientras que al aplicar la técnica SIFT – MS fueron 30 los compuestos volátiles detectados y cuantificados. La diferencia en la detección de compuestos volátiles al emplear estas dos técnicas pone de manifiesto la existencia de ciertas limitaciones en cada una de ellas. Así, la baja concentración de algunos compuestos volátiles en el espacio de cabeza o bien el uso de una fibra con una escasa afinidad por los mismos, impide la detección de estos compuestos volátiles al emplear la técnica convencional SPME – GC – MS tal y como indicaron Olivares y col., (2012) en su estudio de compuestos volátiles orgánicos presentes en carne de ternera envasada en atmósfera modificada.

En cambio, la técnica SIFT – MS permitió detectar ésteres y compuestos azufrados, presentes en baja concentración en el espacio de cabeza, con un gran impacto aromático en el producto final y además, resultó ser una técnica eficaz para relacionar los cambios en la concentración de los compuestos volátiles con la presencia de las diferentes cepas de levadura inoculadas. De esta forma, el aumento de la población de levadura M4 se correlacionó significativamente con un incremento en la concentración de aldehídos como acetaldehído, butanal, pentanal, hexanal, nonanal y pentenal mientras que en el caso de la levadura P2 su población se correlacionó con una mayor producción de alcoholes como etanol, propanol, butanol y pentanol. Sin embargo, las altas correlaciones obtenidas

entre la poblacion de M4 y P2 y la concentración de aldehídos y alcoholes no solo se debieron a compuestos lineales sino también a metil-ramificados, compuestos típicamente producidos por el metabolismo de levaduras (Olesen & Stahnke, 2000; Durá y col., 2004). Estos resultados demuestran que la técnica SIFT – MS puede emplearse como técnica de detección rápida de la presencia de levadura en los embutidos crudos curados siendo capaz de distinguir la cepa responsable de cada perfil aromático. Resultados similares fueron obtenidos por Mayr y col., (2003) quienes emplearon la técnica de espectrometría de masas directa PTR – MS, basada en la transferencia de protones, para estudiar la correlación entre la población bacteriana y diversos compuestos volátiles en carne (ternera y cerdo) envasada a vacio y en atmósfera modificada evaluando el deterioro de la misma durante el almacenamiento. Estos autores observaron altas correlaciones entre algunos compuestos volátiles y la contaminación microbiana como en el caso de compuestos azufrados que aumentaron enormemente durante el periodo de almacenamiento. Sin embargo, al igual que ocurre al aplicar la técnica SIFT – MS, la técnica PTR – MS también presentó problemas de solapamiento debido a una excesiva fragmentación de los iones lo que dificultó la interpretación de los espectros obtenidos ya que un mismo ion producto puede proceder de distintos compuestos volátiles (Blake y col., 2009). Así pues, teniendo en cuenta las limitaciones de las técnicas de espectrometría directa (SIFT – MS) sigue siendo necesario el empleo de la técnica SPME – GC – MS para evitar interferencias y poder detectar y cuantificar los compuestos volátiles ramificados relacionados con el metabolismo de las levaduras. Por este motivo, la combinación de las dos técnicas es adecuada para completar el

perfil aromático de cada una de las cepas empleadas en la fabricación de los embutidos crudos curados.

En resumen, los resultados obtenidos demuestran la existencia de una microbiota autóctona en los embutidos crudos curados de fermentación tradicional con gran potencial aromático que contribuye al aroma final del producto. No obstante, existen otros factores tecnológicos que condionan el desarrollo y el metabolismo de estas levaduras dando lugar a la generación de distintos perfiles aromáticos. Por último, se ha confirmado que el empleo de cepas seleccionadas de levaduras de la especie *D. hansenii* puede prevenir la oxidación lipídica de estos productos y mejorar su aspecto final.

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V. CONCLUSIONES

V. Conclusiones:

- Los perfiles de RAPD – M13 han demostrado la existencia de una gran variabilidad genética en las cepas de la especie *Debaryomyces hansenii* aisladas de embutidos crudos curados tradicionales. Todas las cepas de *D. hansenii* aisladas fueron capaces de proliferar y generar compuestos volátiles en medio definido. La producción de los distintos grupos químicos de compuestos volátiles no pudo relacionarse con el patrón de RAPD – M13.
- La selección de siete cepas de *D. hansenii* se basó en su capacidad para producir compuestos aromáticos como aldehídos, cetonas, alcoholes, ésteres y compuestos azufrados en medio definido.
- La implantación de las siete cepas de *D. hansenii* en el sistema modelo cárnico en ausencia de otros estárters, demostró la capidad de M4, M6 y P2 para producir compuéstos volátiles con un elevado impacto aromático. La cepa M4 produjo principalmente ésteres, mientras que M6 y P2 resaltaron por su gran capacidad para producir compuestos azufrados.
- Las cepas *D. hansenii* M4 y P2 fueron capaces de implantarse en el proceso de fermentación de los embutidos crudo curados en presencia de bacterias ácido lácticas y micrococáceas. El empleo de M4 y P2 tuvo un efecto protector frente a la oxidación lipídica. Esta protección fue mayor en los lotes inoculados con la cepa P2 presentando los valores más bajos de TBARS y una menor abundancia en compuestos derivados de la oxidación lipídica (aldehídos).
- Las cepas de *D. hansenii* seleccionadas, M4 y P2, generaron perfiles aromáticos distintos en los embutidos crudo curados sin embargo estas

diferencias no fueron apreciadas por los consumidores. Ambas cepas destacaron por la producción de estéres metil-ramificados y M4, además, por la producción de compuestos azufrados.

- La técnica de espectrometría directa SIFT – MS fue capaz de detectar diferencias significativas en la producción de compuestos volátiles debido a la actividad de las levaduras. La relación entre la población de levaduras y la producción de compuestos volátiles fue significativa en el caso de compuestos azufrados y etanol.
- La selección de cepas de *D. hansenii* para su utilización como coadyuvantes en la fabricación de embutidos crudos curados no puede basarse en sus diferencias en el patrón RAPD-M13, ya que cepas con patrones idénticos pueden producir diferentes perfiles aromáticos que aumentan y/o diversifican la generación de compuestos volátiles que contribuyan al aroma de los embutidos crudo curados.