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TIPIFICACIÓN Y SUSCEPTIBILIDAD A ANTIMICROBIANOS NATURALES EN CEPAS DE *STAPHYLOCOCCUS AUREUS* DE CERDOS Y CARNE DE CERDO

(TYPING AND SUSCEPTIBILITY TO NATURAL ANTIMICROBIALS IN *STAPHYLOCOCCUS AUREUS* STRAINS FROM PIGS AND PORK MEAT)

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ALCIDES MORAIS SATUALA LOFA
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Profesor Guía: Valeria Velasco Pizarro
Dpto. de Producción Animal, Facultad de Agronomía
Universidad de Concepción

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Aprobada por:

Valeria Velasco Pizarro
Ingeniero en Alimentos, Ph.D. _____
Profesora Guía

María Dolores López Belchí
Licenciado en Química, Ph.D. _____
Evaluadora Interna

Macarena Gerding González
Ingeniero Agrónomo, Ph.D. _____
Evaluadora Interna

Inés Figueroa Cares
Ingeniero Agrónomo, Mg. Cs., Dr. Cs _____
Directora de Programa

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(TYPING AND SUSCEPTIBILITY TO NATURAL ANTIMICROBIALS IN *STAPHYLOCOCCUS AUREUS* STRAINS FROM PIGS AND PORK MEAT).

RESUMEN

Staphylococcus aureus es un patógeno oportunista presente comúnmente en las fosas nasales, piel y pelo de personas y animales, y es responsable de varias enfermedades. El objetivo de este estudio fue caracterizar las cepas de *S. aureus* de origen porcino y determinar la susceptibilidad a antimicrobianos naturales (NA). Un total de veinte cepas de *S. aureus* de origen porcino se sometieron a tipificación molecular a través de electroforesis en gel de campo pulsado (PFGE), de las cuales se seleccionaron seis cepas (piel, n = 1, nasal, n = 2, canal, n = 1 y carne, n = 2) para un ensayo de antibióticos y ensayo de susceptibilidad a NA [aceite esencial de orégano (EO), timol, carvacrol y extracto de maqui], utilizando el método de difusión en disco. También se usaron dos cepas de referencia, ATCC 43300 *S. aureus* resistente a meticilina (MRSA) y ATCC 25923 susceptible a meticilina (MSSA). Se observó diversidad genética entre las cepas de *S. aureus* con seis cepas clasificadas en dos clusters. Todas las cepas fueron resistentes al menos a una clase de antibiótico. Todas las cepas fueron sensibles a NA, excepto al extracto de maqui. La concentración mínima inhibitoria (CIM) varió de 0,01% a 0,04%, y la concentración bactericida mínima (CBM) varió de 0,02% a 0,08%, sin diferencias significativas entre las cepas MRSA y MSSA. Por lo tanto, los antimicrobianos naturales extraídos de plantas representan una herramienta promisoria para el control de cepas de *S. aureus* resistentes a antibióticos.

SUMMARY

Staphylococcus aureus is an opportunistic pathogen commonly carried on the nares, skin and hair of people and animals, and is responsible of various diseases. The aim of this study was to characterize *S. aureus* strains of swine origin, and to determine the susceptibility to natural antimicrobials (NA). A total of twenty *S. aureus* strains of swine origin were subjected to molecular typing by pulsed-field gel electrophoresis (PFGE), of which six strains (skin, n=1, nasal, n=2, carcass, n=1, and meat, n=2) were selected for an antibiotic and NA [oregano essential oil (EO), thymol, carvacrol and maqui extract] susceptibility testing assay, using the disk diffusion method. Two reference strains, ATCC 43300 methicillin-resistant (MRSA) and ATCC 25923 methicillin-susceptible *S. aureus* (MSSA) were also used. Genetic diversity among *S. aureus* strains was observed, with six strains classified in two clusters. All strains were resistant to at least one class of antibiotic. All strains were sensitive to NA, except to maqui extract. The minimum inhibitory concentration (MIC) ranged from 0.01% to 0.04%, and the minimum bactericidal concentration (MBC) ranged from 0.02% to 0.08%, with no significant differences between MRSA and MSSA strains. Natural antimicrobials extracted from plants represent a promising strategy for controlling antibiotic-resistant *S. aureus* strains.

CAPTULO 1

INTRODUCCIÓN GENERAL

1.1. Características generales de *Staphylococcus aureus*.

Staphylococcus aureus es una bacteria que se encuentra en la microbiota normal del ser humano, y alrededor de 30% de los seres humanos sanos la porta en sus fosas nasales (Gorwitz et al. 2008). Hay más de 30 especies de estafilococos, entre los cuales *S. aureus* es el más patogénico para los humanos (Wendlandt et al., 2013).

El nombre del género -*Staphylococcus*- proviene del griego *staphylé* que significa racimo de uvas, y que presentan forma esférica (cocos), con un diámetro de 0,5 a 1,5 µm y se agrupan de forma irregular. Éstas son bacterias gram positivas, inmóviles, no forman esporas y, generalmente, no poseen cápsula y son anaerobias facultativas. Se diferencian de los géneros *Streptococcus* y *Enterococcus* por producir la enzima catalasa, la cual hidroliza el peróxido de hidrógeno (H_2O_2) en oxígeno (O_2) y agua (H_2O). Presentan metabolismo oxidativo y fermentativo de la glucosa, a diferencia del género *Micrococcus*, el cual no la fermenta (de Cueto y Pascual, 2009).

El *S. aureus* es la principal causa de las enfermedades piógenas en piel, osteomielitis, otros padecimientos como artritis séptica, infecciones profundas, abscesos, neumonía, empiema, endocarditis, pericarditis, meningitis y enfermedades mediadas por sus toxinas, incluyendo intoxicación alimentaria, fiebre escarlatina, síndrome de piel escaldada y síndrome de choque tóxico (Shiroma et al., 2015).

El genoma de *S. aureus* es aproximadamente de 2,8 Mb. El cromosoma contiene genes de patogenicidad y genes de resistencia a antibióticos adquiridos a través de la transferencia horizontal de elementos genéticos móviles (EGMs) (Katayama et al., 2000). Los EGMs ocupan 15 - 20 % del cromosoma. Durante los brotes de enfermedades, el genoma de *S. aureus* puede desarrollar polimorfismos de nucleótido único y pequeños reordenamientos genéticos y adquieren o pierden EGMs que contienen genes de resistencia o de virulencia (Lindsay, 2014). Además, esta bacteria tiene la capacidad de adherirse no solo a los tejidos vivos, sino también a otras superficies, donde es capaz de crecer y persistir, llegando a formar biopeliculas (Angelica y Fong, 2008).

1.2. Mecanismos de resistencia de *Staphylococcus aureus*.

La mortalidad de los pacientes con bacteriemia por *S. aureus* en la era preantibiótica superó el 80%, la introducción de penicilina G a principios de la década de 1940 mejoró significativamente el pronóstico, pero pronto se registaron cepas resistentes a penicilina en 1942 (Rammelkamp and Maxon, 1942).

Su mecanismo de resistencia consiste en una enzima penicilinasa (β-lactamasa) que hidroliza el anillo β-lactamico e inactiva el fármaco (Kirby, 1944). Esta enzima está codificada por el gen *blaZ*, que típicamente reside en un transposón grande en un plásmido. La tasa de resistencia a la penicilina es ahora superior al 90% en aislados humanos de *S. aureus*, lo que hace que el uso de la penicilina sea esencialmente inútil para tratar estas infecciones (Peacock and Paterson 2015).

Por esta razón se crearon nuevas penicilinas (semi sintéticas), destacándose la meticilina (Bustos-Martínez et al., 2006). Tras la introducción de la meticilina (penicilina semi sintética resistente a penicilinasa) surgió *S. aureus* resistente a meticilina (MRSA del inglés Methicillin-Resistant *S. aureus*), al principio, asociado a infecciones adquiridas en instalaciones hospitalarias (MRSA-AH). A fines de la década de los 90s, emergieron en todo el mundo cepas de MRSA asociados a infecciones adquiridos en la comunidad (MRSA-AC) (Lowy, 2003; Deurenberg y Stobberingh, 2008). Otro tipo de cepas emergentes se han asociado con infecciones en animales de abasto, denominadas MRSA-AV, (del inglés MRSA-LA: Livestock-associated). Estas cepas se han detectado en cerdos (Voss et al., 2005), en pollos (Feßler et al., 2011), en vacuno y ovejas (Fessler et al., 2012). Otra cepa emergente está asociada a intoxicaciones alimentarias (FBA-MRSA, FoodBorne Associated-MRSA). Waters et al. (2011) y Ogata et al. (2012) afirman que MRSA en los alimentos podría ser de origen animal o humano. Aunque la prevalencia es baja, la presencia de cepas MRSA resistentes a múltiples fármacos y enterotoxigénicos en los alimentos representa una amenaza potencial para los consumidores y hace hincapié en la necesidad de un mejor control de las fuentes de contaminación (Wang et al., 2014).

A partir del análisis comparativo del genoma, MRSA-AV ha evolucionado desde el *S. aureus* susceptible a la meticilina asociado al ganado, y la transmisión al hombre se asoció con varios cambios genéticos. Esto conlleva a un riesgo potencial para la salud y requiere una estrecha vigilancia. Aunque la mayoría de MRSA-AV (> 80%) es resistente a varios antibióticos, todavía hay suficientes

opciones de tratamiento (Cuny et al. 2015). La presencia de clon de *S. aureus* característico de seres humanos en animales sugiere la posibilidad de una transmisión de seres humanos a animales (Hata, 2016).

Desde el punto de vista genómico, la resistencia se produce por selección natural (Zendejas-Manzo et al., 2014). Sin embargo, existen otros factores que pueden aumentar la presencia de cepas MRSA en los alimentos de origen animal, como es el mal uso de antibióticos en terapia, profilaxis, y promoción del crecimiento en producción animal (Dupont et al. 1987; FAO. 2016). Esto induce la selección de cepas resistentes y su posterior introducción en la cadena alimentaria (Intrakamhaeng et al., 2012). La detección de MRSA en alimentos de origen animal es un riesgo potencial para la salud humana (Basanisi et al. 2017), por tanto, se demuestra la necesidad de tipificar las cepas de *S. aureus.*

La resistencia meticilina generalmente es conferida mediante la adquisición del gen *mecA* que codifica la proteína de unión a penicilina modificada PBP2a o PBP2', la cual presenta una baja afinidad por antibióticos β-lactámicos. Esta resistencia permite que la biosíntesis de la pared celular continúe incluso en presencia de concentraciones inhibidoras de antibiótico (Peacock and Paterson. 2015). El gen *mecA* es parte de un elemento genético móvil denominado "cassette cromosómico estafilocócico (SCC) mec". SCCmec está flanqueado por genes de cassette cromosoma recombinasa (ccrA/ccrB o ccrC) que permiten la transmisión horizontal intra e interespecífica de SCCmec. El reservorio inicial de SCCmec no está claro, pero puede haber sido una especie de estafilococo coagulasa negativa (Angelica y Fong, 2008)

1.3. Tipificacion molecular de *Staphylococcus aureus*

Los métodos moleculares de tipificación se han empleado principalmente en estudios de epidemiología molecular de *S. aureus* y MRSA, lo que ha permitido entender mejor las relaciones evolutivas de estos clones (Shopsin y Kreiswirth, 2001). Entre los métodos empleados para tipificar MRSA se encuentran los siguientes:

a) Electroforesis en gel de campo pulsado (PFGE, del ingles pulsed-field gel electrophoresis), basado en modelos de macrorrestricción de ADN genómico.

Este método es el más utilizado para estudios de la epidemiología molecular de *S. aureus* y ha sido de gran valor en estudios de enfermedades nosocomiales (Mcdougal *et al.*, 2003). Inicialmente esta técnica estuvo restringida a estudios locales y de corto plazo, debido a la falta de reproducibilidad entre laboratorios. Sin embargo, estudios de Tenover y colaboradores permitieron la estandarización de la metodología para hacer la electroforesis e interpretación de los patrones producidos (Tenover *et al.*, 1995). En la actualidad se cuenta con un software que permite comparar los perfiles obtenidos con PFGE e identificar el linaje de MRSA de las cepas en estudio (Mcdougal *et al.*, 2003). Frecuentemente se usan seis clones epidémicos provenientes de varias regiones del mundo como referentes de tipificación en estudios epidemiológicos (Moreno *et al.*, 2005).

b) Tipificación de secuencia de multilocus (MLST, del ingles, multilocus sequence typing) que determina el perfil alélico de siete genes conservados (Enright *et al.*, 2000).

Este método se basa en la amplificación y secuenciación de fragmentos internos de siete genes (*arcC*, *aroE*, *glpF*, *gmK*, *pta*, *tpi*, y *yqiL*) conservados (housekeeping), su principal ventaja es ser una técnica estandarizada y cuenta con un sitio web que contiene información de más de 1.500 cepas aisladas de humanos y animales (Malachowa *et al.*, 2005).

c) Tipificación *Spa* (del inglés *Staphyloccocus* protein A).

Tipificación del gen de la proteína A involucra la amplificación y secuenciación de la región polimórfica X, una secuencia corta repetida del gen de la proteína A (constituyente de la pared celular), un superantígeno de *S. aureus* (Shopsin *et al.*, 1999). Esta región posee un alto grado de polimorfismo y, por consiguiente, es potencialmente adecuada para estudiar brotes por su alto poder de discriminación (Harmsen et al., 2003).

d) Tipificación por técnicas basadas en PCR para evaluar diferentes tipos de SCCmec del inglés (Staphylococcal Chromosome Cassette mec) (Amirkhiz et al. 2015).

Para una mejor caracterización de cepas se requiere definir además de su perfil genético, los tipos estructurales del cassette cromosómico SCCmec (Zhang *et al.*,

2005). Estudios realizados por Ito y colaboradores (Ito *et al.*, 2001) para tipificar cepas de MRSA, permitieron dilucidar las diferencias en la estructura del SCCmec; estos autores diseñaron una PCR basada en las variaciones de tamaño y composición genética de los complejos *mecA* y *ccr*, la cual permitió identificar los cinco tipos de SCCmec. La PCR ofrece ventajas como rapidez, sensibilidad, especificidad y menor costo, al compararla con métodos basados en secuenciación; además, permite detectar MRSA directamente de muestras que contienen una mezcla de microorganismos (Huletsky *et al.*, 2004).

Shopsin et al. (1999) ha demostrado que PFGE tiene un mayor poder discriminatorio. Sin embargo, una combinación de dos métodos puede proporcionar una mayor precisión en los estudios epidemiológicos (Malachowa et al., 2005).

La aplicación de técnicas de biología molecular también ha demostrado que algunos linajes no están restringidos y pueden colonizar o causar infecciones en una amplia variedad de especies animales, incluyendo seres humanos. Un ejemplo es la cepa ST398, originalmente denominada como "clon cerdo", la cual se ha aislado también de otros animales de abasto y de seres humanos (Voss et al. 2005; Witte et al. 2007). La tasa de colonización de MRSA ST398 ha sido alta en cerdos y la reciente aparición de infecciones en humanos asociadas a esta cepa es un problema de salud pública (Golding et al. 2010). Además, se ha encontrado similitud genética entre cepas de *S. aureus* aisladas de los seres humanos, animales y carne, lo que sugiere la contaminación de la carne durante la faena, manipulación o procesamiento, siendo un riesgo para la transmisión a los seres humanos (Velasco et al. 2015).

Vega et al. (2015), indican que es fundamental mantener programas de vigilancia que permitan detectar la presencia de cepas con este fenotipo en pacientes con factores de riesgo como tratamiento prolongado con antibióticos, para predecir la efectividad de estos fármacos y optimizar la terapia antimicrobiana.

La evidencia acumulada respecto de los aspectos negativos del uso indiscriminado de antibacterianos en terapia y en profilaxis en medicina humana y veterinaria ha llevado a que en la mayoría de los países de Europa y de Norteamérica se restrinja y se regule su uso (Basanisi et al. 2017).

En Chile no se permite el uso de antibióticos como promotores del crecimiento en producción animal. Sólo se permiten para fines terapéuticos (SAG. ley N° 18.755

de 13 de octubre de 2015; Zambrano 2011). La Resol. SAG N°1992 de 2006, establece una nueva nómina de aditivos y elimina los antibióticos de la lista de aditivos autorizados.

1.4. Antimicrobianos Naturales

La aparición frecuente de cepas resistentes a los antibióticos preocupa la comunidad científica y ha estimulado la prohibición del uso de antibióticos en la alimentación animal (Guerra et al., 2008); por tanto, la búsqueda de nuevos agentes antibióticos naturales o sintéticos, es una necesidad apremiante (Oyarzabal et al., 2011). Para reemplazarlos, entre las diferentes alternativas se encuentra el uso de metabolitos secundarios provenientes de plantas. Los metabolitos secundarios son producidos por la planta para multiples funciones, como mecanismo de defensa, para la inhibición del crecimiento de la planta, atracción de insectos para la reproducción, entre otras funciones, también tienen la capacidad de regular los pasos claves del crecimiento celular y la diferenciación en eucariotas. Los flavonoides tienen funciones reguladoras, que comparten las plantas y los animales, que son de gran valor en la fotoprotección contra radiaciones UV-B (Agati *et al*., 2013).

Muchos compuestos naturales que se encuentran en las plantas han demostrado que poseen funciones antimicrobianas y podrían utilizarse como fuente de agentes antimicrobianos naturales (AN) contra patógenos (Kim el al.,1995). Los compuestos químicos de los aceites esenciales (AE) se plantean como una alternativa para mejorar la eficiencia de utilización de alimentos y reducir las pérdidas de nutrientes, así mismo mejorar los parámetros productivos (Guerra et al., 2008).

Diferentes plantas han demostrado contener compuestos con bioactividad como el timol y carvacrol del *Origanum vulgare* L. subsp *vulgare* (Acevedo et al., 2013; Moon y Rhee, 2016), y compuestos fenólicos del maqui [*Aristotelia chilensi*s (Molina) Stuntz] (Genskowsky et al., 2015).

El orégano (*Origanum vulgare* subespecie *vulgare*) se caracteriza por la presencia de AE, con efecto antimicrobiano. Según Sarikurkcu et al. (2015), el AE del orégano está compuesto por timol (58,31%), carvacrol (16,11%), p-cimeno (13,45%) y alpha-terpineno (4,64%) como los principales compuestos. En otro estudio los principales componentes identificados en AE de orégano fueron:

carvacrol (67%), timol (16,22%), p-cimeno (3,88%), linalol (2,73%), α-pineno (1,17%) y mirceno (1,02%) (Rodrigues et al., 2017). Bisht et al. (2009) notó diferencias significativas en la composición del aceite, lo que sugiere que factores extrínsecos e intrínsecos/genéticos juegan un papel en la composición del aceite. Estos compuestos le otorgan múltiples propiedades antioxidantes, microbiológicas y conservantes de alimentos, entre otras (Acevedo et al., 2013).

El Maqui es una planta endémica de Chile y Argentina. En Chile crece entre las latitudes 31^{o} y 43^{o}, principalmente en los bosques higrófilos templados de Valdivia (Araneda et al., 2014). Es una planta de gran interés científico por las propiedades de sus compuestos químicos (Mølgaard et al., 2011). La fruta, semilla, hojas y tallos de *A. chilensis* demostraron experimentalmente bioactividad, la cual puede ser atribuida a ácidos fenólicos, flavonoides o antocianinas, presentes en sus extractos (Genskowsky et al., 2015) y otros como los ácidos cafeico y ferúlico (Alonso, 2012).

1.5. Mecanismos de accion de los Antimicrobianos Naturales

Existen diferentes mecanismos por los cuales, los compuestos polifenólicos de bayas y principalmente las antocianinas pueden conducir a la toxicidad de microorganismos (Genskowsky et al., 2015). Las antocianinas y los compuestos fenólicos han demostrado una acción permeativa desestabilizando la membrana del lipopolisacárido y aumentando el eflujo de ATP del citoplasma (Puupponen-Pimiä et al. 2005). Genskowsky et al. (2015) evaluaron las propiedades antibacterianas de los extractos de bayas de maqui frente a seis cepas bacterianas Gram negativa, presentando el extracto actividad biológica frente a estas cepas. En general, los extractos de bayas inhibieron el crecimiento de bacterias Gram negativa, pero no Gram positiva. Estas variaciones pueden reflejar diferencias en las estructuras de la superficie celular entre bacterias Gram negativas y Gram positivas. El ácido gálico permeabilizó la membrana externa de cepas de *Salmonella*. Después de la exposición a los ácidos fenólicos, las células se cargan menos negativamente. Este cambio de carga superficial fue particularmente verificado para las bacterias Gram negativas y podría ser la razón de su mayor susceptibilidad en comparación con las bacterias Gram positivas (Borges et al. 2013).

La exposición de cepas de *S. aureus* a timol a bajas concentraciones puede inducir a alteraciones evidentes en la composición de ácidos grasos de las membranas. Las células tratadas con timol a 0,5 de la concentración inhibitória mínima se volvieron rugosas y se distorsionaron e incluso se colapsaron (1,0 de la misma concentración), y parecían tener un alto grado de lisis celular después de tratarse con timol a 2,0 de la concentración inhibitória mínima (Wang et al., 2017).
El carvacrol ejerce su efecto antibacteriano al aumentar la permeabilidad de la membrana celular bacteriana y unirse directamente al ADN genómico. La exposición de cepas de *S. aureus* a carvacrol daña severamente la integridad y morfología de la membrana celular de *S. aureus*, se une al ADN a través en el surco menor, desestabiliza la estructura secundaria del ADN e induce la agregación de moléculas de ADN (Wang et al., 2016).

S. aureus es un patógeno oportunista que se encuentra normalmente en las fosas nasales, piel y pelo de personas y animales. Puede transmitirse por contacto directo entre personas y animales portadores. Los alimentos de origen animal pueden contaminarse a lo largo de la cadena productiva, ya sea por contacto con animales o manipuladores. En Chile no se tiene información de la existencia de cepas emergentes de *S. aureus* en la cadena productiva de carne de cerdo. Por esa razón es necesario conocer los tipos de cepas existentes y la susceptibilidad a antimicrobianos naturales extraídos de plantas como el orégano (*O. vulgare*) y maqui (*A. chilensis)*, que podrian utilizarse para el control de la propagación de estas cepas.

HIPÓTESIS.

Entre de las cepas de *S. aureus* presentes en la cadena productiva de carne de cerdo en Chile, existen cepas emergentes susceptibles a antimicrobianos naturales.

OBJETIVO GENERAL.

Tipificar y caracterizar cepas de *S. aureus* en la cadena productiva de carne de cerdo y determinar su susceptibilidad a antimicrobianos naturales.

OBJETIVOS ESPECÍFICOS.

(i) Caracterizar de forma molecular las cepas de *S. aureus*, aisladas de cerdos y carne de cerdo.

(ii) Determinar la susceptibilidad a antimicrobianos naturales en cepas de *S. aureus* aisladas de cerdos y carne de cerdo.

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Capitulo 2

Molecular typing and susceptibility to natural antimicrobials of *Staphylococcus aureus* from swine origin.

Molecular typing and susceptibility to natural antimicrobials of *Staphylococcus aureus* from swine origin.

Alcides Lofa[1,3], Valeria Velasco[1], Macarena Gerding[2], María D. López[2], Diego Vallejos[1], Alejandra Mallea[1], Ana María Bonilla[1]

[1]Department of Animal Sciences, Faculty of Agronomy, University of Concepción, Chillán, Chile. [2]Department of Plant Sciences, Faculty of Agronomy, University of Concepción, Chillán, Chile. [3]Department of Sciences, Faculty of Veterinary Medicine, University José Eduardo Dos Santos, Huambo, Angola.

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Abstract

Staphylococcus aureus is an opportunistic pathogen commonly carried on the nares, skin of people and animals, and can cause various medical problems. The aim of this study was to characterize *S. aureus* strains of swine origin, and to determine the susceptibility to natural antimicrobials (NA). A total of twenty *S. aureus* strains of swine origin were subjected to molecular typing by pulsed-field gel electrophoresis (PFGE), of which six strains (skin, n=1, nasal, n=2, carcass, n=1, and meat, n=2) were selected for an antibiotic and NA [oregano essential oil (EO), thymol, carvacrol and maqui extract] susceptibility testing assay, using the disk diffusion method. Two reference *S. aureus* strains, ATCC 43300 methicillin-resistant (MRSA) and ATCC 25923 methicillin-susceptible *S. aureus* (MSSA) were also used.Genetic diversity among *S. aureus* strains was observed, with six strains classified in two clusters. All strains were resistant to at least one class of antibiotic. All strains were sensitive to NA, except to maqui extract. The minimum inhibitory concentration (MIC) ranged from 0.01% to 0.04%, and the minimum bactericidal concentration (MBC) ranged from 0.02% to 0.08%, with no significant differences between MRSA and MSSA strains. Natural antimicrobials represent a promising strategy for controlling antibiotic-resistant *S. aureus* strains.

Key words: *Staphylococcus aureus*, pigs, pork meat, natural antimicrobials; susceptibility testing.

Introduction

The coagulase-positive staphylococci, *Staphylococcus aureus*, causes a wide variety of hospital and community acquired infections (Casey et al. 2007).

This bacterium exhibits antibiotic resistance, such as methicillin-resistant *S. aureus* (MRSA) which has been a serious health problem (Zendejas-Manzo et al. 2014). Methicillin resistance in *S. aureus* is primarily mediated by the production of an altered penicillin-binding protein, PBP2' (also called PBP2a, encoded by the *mecA* gene), which has a lower affinity for β-lactam antibiotics (Alipour et al. 2014).

After the introduction of methicillin, MRSA strains emerged, initially associated with hospital-acquired infections (HA-MRSA). At the end of the 1990s, community-associated MRSA strains (CA-MRSA) emerged around the world (Deurenberg and Stobberingh 2008). Other emerging strains have been called Livestock-associated MRSA strains (LA-MRSA). These strains have been detected in pigs (Voss *et al.* 2005), chickens (Feßler et al. 2011), cattle and sheep (Fessler et al. 2012). Another emerging strain is Foodborne associated-MRSA (FBA-MRSA) related to enterotoxins production. The presence of staphylococcal enterotoxin genes has been involved in staphylococcal food poisoning outbreak (Bastos et al. 2017).

Staphylococcus aureus can colonize the nares, skin and hair of animals, and humans (Voss et al. 2005). Therefore, there is a risk of transmission of MRSA strains to humans and food-producing animals (Pantosti. 2012) with the consequent contamination of food.The genetic similarity between clones from humans and meat suggests the risk of spread of *S. aureus* in the food chain (Velasco et al. 2015).

Different techniques have been used for molecular typing of *S. aureus* strains to identify clones. It has been shown that the discriminatory power of pulsed-field gel

electrophoresis (PFGE) is greater than multilocus sequence typing (MLST) and *spa* typing (Shopsin et al., 1999). However, a combination of two methods may provide greater precision in molecular typing (Malachowa et al., 2005).

The increasing prevalence of antibiotic-resistant strains progressively concerns the scientific community (Kunin 2008; FAO 2016). Therefore, the search for new antimicrobial agents is a pressing need (Oyarzabal et al. 2011).

Some compounds from different plants may serve as natural antimicrobial (NA), such as thymol and carvacrol from oregano (*Origanum vulgare* L. subsp *vulgare*) (Moon and Rhee 2016), and phenolic compounds from maqui [*Aristotelia chilensis* (Molina) Stuntz] (Genskowsky et al. 2015).

Oregano is characterized by the presence of essential oil (EO), with antimicrobial effect. Oregano EO belongs to the chemo type thymol or carvacrol, depending on the main compound, followed by p-cymene, γ-terpinene, caryophyllene, caryophyllene oxide, trans-α-bergamotene, eugenol, and α-bergamotene (Sarikurkcu et al. 2015). Analysis of Maqui showed polyphenolic compounds identified as anthocyanins, flavonols and ellagic acid (Genskowsky *et al.*, 2016), with bioactivity properties (Mølgaard et al. 2011).

In Chile, there is no information about the characteristics and the presence of emerging *S. aureus* strains in the pork chain supply. For this reason, it is necessary to characterize the strains and to determine the susceptibility to NA agents extracted from plants such as oregano and maqui, which could be used to control the spread of these strains in the pork production chain. Therefore, the aim of this study was to characterize *S. aureus* strains of swine origin, and to determine the susceptibility to natural antimicrobials.

Materials and methods

Staphylococcus aureus isolates

A total of twenty *S. aureus* strains were isolated from pork chain supply, between August 2015 and January 2016, and were subjected to molecular typing. Six strains isolated from skin, n=1, nasal, n=2, carcass, n=1, and meat, n=2 (Table 1) were selected for susceptibility testing assays. Two reference strains American Type Culture Collection (ATCC) ATCC 43300 and ATCC 25923 were used as methicillin-resistant (MRSA) and methicillin-susceptible *S. aureus* (MSSA), respectively.

Table 1 - Characteristics and resistance profile of *Staphylococcus aureus* strains of swine origin.

	Characteristics					
Strains	Api® Staph	*nuc* gen	*mec A* gen	PBP2a	Enterotoxin B	Resistance profile
1 (Skin)	+	+	-	-	-	PEN-TET-ERY-VAN-CIP
2 (Nasal)	+	+	-	-	-	PEN-TET-ERY-CIP
3 (Nasal)	+	+	-	-	-	PEN-TET
4 (Carcass)	+	+	-	-	-	PEN-OXA-CEF
5 (Meat)	+	+	-	-	-	PEN-OXA-CEF-GEN-KAN
6 (Meat)	+	+	-	-	+	PEN

PEN: Penicillin; OXA: Oxacillin; CEF: Cefoxitin; GEN: Gentamicin; KAN: Kanamycin; ERY: Erythromycin; CIP: Ciprofloxacin; TET: Tetracycline.

Pulsed-field gel electrophoresis of *S. aureus* isolates

The PulseNet protocol with slight modifications was used to assess the relatedness between *S. aureus* isolates (McDougal et al. 2003). Briefly, frozen isolates were struck to Trypticase Soy Agar (TSA) plates and incubated at 37°C for 18–24h. A single colony was inoculated onto a second TSA plate and incubated at 37°C for 18–24h. Colonies were transferred to 5mL polystyrene round-bottom

tubes containing 2mL of cell suspension buffer (100mM Tris HCl [pH 8.0], Invitrogen; and 100mM EDTA [pH 8.0], Gibco), adjusting the concentrations to an absorbance of 0.9–1.1 in a spectrophotometer at 610nm. After that, the preparation, lysis, and washes of plugs, and then the *Sma*I enzyme restriction digestion were performed. *Salmonella* Branderup H9812 was used as a DNA marker (Ribot et al. 2006). The electrophoresis was carried out in a Chef Mapper PFGE rig, with an initial switch time of 5 s, a final switch time of 40 s, and a total running time of 17h 45min. After staining the gels with ethidium bromide (1.5 µg/mL), they were visualized using a UVP imager. Macro restriction patterns were compared using the BioNumerics Fingerprinting software. The similarity index was calculated using the Dice coefficient, a band position tolerance of 1%, and an optimization of 0.5%. The unweighted pair group method with arithmetic averages was used to construct a dendrogram, and clusters were selected using a cut off at 80%.

Antibiotic susceptibility test of *S. aureus*

Minimum Inhibitory Concentration (MIC) of oxacillin, tetracycline, erythromycin, vancomycin, gentamicin, kanamycin, ciprofloxacin, quinupristin/dalfopristin, and penicillin was determined by the epsilon test (Etest) (Liofilchem SRL, Italy), a quantitative method consisting in plastic strips with a gradient concentrations (0.016 – 256 mg/L). Disk diffusion method was used to test the susceptibility to cefoxitin with disks containing 30 µg (CLSI, 2014). Briefly, 0.1 mL of bacterial solution (saline solution 0.85% of NaCl in water) with concentration equivalent to 0.5 McFarland was uniformly distributed on the surface of Mueller-Hinton agar plates (supplemented with 2% NaCl) using a drigalski spatula. The antibiotic strips and disks were deposited on the surfaces, and the plates were incubated at 35 ± 2°C during 24 h.

Natural antimicrobials.

Five NA were used: thymol (Thy) (2-isopropyl-5-methylphenol) (Sigma Aldrich; purity, ≥99.0%), carvacrol (Carv) (5-isopropyl-2-methylphenol) (Sigma Aldrich; purity, ≥98.0%), mixture of thymol + carvacrol (1:1) (Thy + Carv), oregano EO (R.C. TREATT & CO, LTD) and Maqui berries extract (berries were harvested in Los Angeles, Region of Biobío, Chile in December 2015, and stored at –80^{o}C). The maqui berries extract and oregano EO were analyzed in order to evaluate their main compounds.

Fresh maqui berries extraction:

Maqui berries were placed at room temperature and then milled with a mortar to obtain a homogeneous size with a diameter less than 0.8 mm. A total of 0.5 g of the ground sample was mixed with 5 mL of water:methanol:formic acid (24:25:1). The mixture was placed in an ultrasonic bath (Brason models 5800 Virginia Corporation) for 1 h and then stored in darkness for 24 h at 4^{o} C. After that, samples were placed back in the ultrasonic bath for 1 h and finally centrifuged (centrifuge L-535R) at 5,000 x *g* for 15 min. The supernatant was recovered and stored at 4º C until use (Gironés-Vilaplana et al. 2014).

Anthocyanin identity assignment and quantification by HPLC-DAD-ESI-MS/MS

Identification and quantification of the main compounds of maqui berries extract were performed on a chromatograph (Shimadzu VP class) and a mass spectrophotometer (Applied Biosystem triple quadruple mass), using the method described by Ruiz et al. (2013). Samples were filtered before injection (Pes 0.22 µm). The chromatographic analyses for the identification and quantification were carried out on a Kromasil C-18 (250x4.6 mm, 5 µm at 40^{o}C). Water, formic acid

and acetonitrile were used as the mobile phases A and B, respectively A: 87:10:3 % (Agua: HCOOH: Acetonitrilo) B: 40:10:50 % (Agua: HCOOH: Acetonitrilo), with a flow rate of 0.8 mL/min. The linear gradient started with 6% solvent B, reaching 30% solvent B at 15 min, 50% at 30 min, 60% at 35 min, 6% at 41 min, and 6% at 46 min. The injection volume was 10 μL, chromatograms were recorded at 518 nm.

Identity assignment was carried out considering the retention times and by analysis of DAD and ESI-MS/MS spectra. In the last case, positive ionization mode was required under the following parameters: 5 V of collision energy, 4000 V of ionization voltage, capillary temperature at 450°C, nebulizer gas 40 psi and auxiliary gas 50 psi. Quantification was carried out at 518 nm by external calibration using delphinidin-3-glucoside; the amount of anthocyanins was expressed in μmol g^{-1}. No absorptivity correction was made for individual anthocyanins. DAD data collection was carried out using a Shimadzu Chromatography Data System CLASS-VP software, a HPLC-MS/MS control system and data collection by the Analyst 1.5.2. software.

GC-MS Analysis of Oregano Essential oil.

The GC/MS analyses were carried out using methodologies according to Teixeira et al. (2012). The *O. vulgare* EO was analysed on an Agilent 6890 gas chromatograph interfaced to an Agilent 5973N mass selective detector (Agilent Technologies, Palo Alto). A vaporisation injector operating in the split mode (1:50) at 250°C was used, into which a fused silica capillary column (30 m length×0.32 mm internal diameter×0.25 μm film thickness; HP-5MS; 5% diphenyl–95% dimethyl polydimethylsiloxane; Agilent Technologies) was installed. The oven temperature was programmed at 45°C for 1 min, raised to 250°C at 5°C min^{-1}, and maintained at 250°C for 5 min. Helium was used as carrier gas at 30 cm s^{-1} and

the injection volume was 1 µL. The transfer line, ion source, and quadrupole analyser temperatures were maintained at 280°C, 230°C and 150°C, respectively, and a turbo molecular pump (10^{-5} Torr) was used. In the full-scan mode, electron ionisation mass spectra in the range 40–400 Da were recorded at 70 eV electron energy. A solvent delay of 3 min was selected. The acquisition data and instrument control were performed by the MSD Chem Station software (G1701CA, version C.00.00; Agilent Technologies, Santa Clara, CA, USA). The identity of each compound was assigned by comparison of their retention index relative to a standard mixture of n-alkanes, as well as by comparison with the mass spectra characteristic features obtained with the Wiley's library spectral data bank (G1035B, Rev D.02.00; Agilent Technologies, Santa Clara).

Natural antimicrobial susceptibility test.

The solutions used as NA were: oregano EO, Thy, Carv and Thy + Carv, which were prepared in ethanol absolute (99.8%, Merck, Germany) to obtain the concentration of 9%. The ethanol absolute was used as control. The NA were filtered using a 0.22 µm filter (EDLAB CA Syringe Filter).

To assess the antimicrobial activity of NA, the disk diffusion method was carried out using sterile paper disks (Whatman No. 1, with 6 mm diameter) according to Rota et al. (2008). Bacterial suspensions were prepared in tripticase soy broth (TSB) with an optic density of 0.1 (OD_{600}=0.1) measured in a UV/VIS spectrophotometer (Korea, model Optizen POP BIO). A volume of 0.1 mL of this suspension was inoculated on TSA plates. Sterile disks impregnated with 15 µL of solutions of oregano EO, Thy, Carv, and Thy + Carv at a concentration of 9% and maqui extract at a concentration of 9%, 12.5% and 100% were placed in inoculated plates and were incubated at 37 °C for 24h. The inhibition criteria for the NA susceptibility assay (disk diameter included) was the following: ≥20 mm

zone of inhibition is strongly inhibitory; <20–12 mm zone of inhibition is moderately/mildly inhibitory; and <12 mm is not inhibitory (Rota et al. 2008).

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined according to Rota et al. (2008), with slight modifications. The appropriate amount of NA was dissolved in pure ethanol and added to TSB. Serial dilutions were prepared from this broth in triplicate. Well-isolated, single bacterial colonies from TSA plates were transferred into solutions to a final concentration of 10^6 cells mL^{-1}. Eleven concentrations were used with 4.5% (v/v) as the highest concentration, and 0.005% (v/v) as the lowest concentration. The growth of each culture was observed by plating suitable diluted aliquots of the culture (viable counts), on TSA at specific time intervals. In each case a broth containing 3% (v/v) solvent (pure ethanol) with NA, and controls broths containing 3% (v/v) solvent (pure ethanol) with bacterial strains were also prepared.

After incubation at 37°C for 24h, the MIC was read. The MIC was the lowest concentration at which bacteria failed to grow in tube (TSB), but bacterial growth was observed after transferring 100 µL to TSA. Similarly, the MBC was the lowest concentration at which bacteria failed to grow in TSB, however a growth of 0.1% of the initial inoculum is observed (Yadav et al. 2015). The evaluation of the MIC and MBC assay were carried out in triplicate.

Statistical analysis.

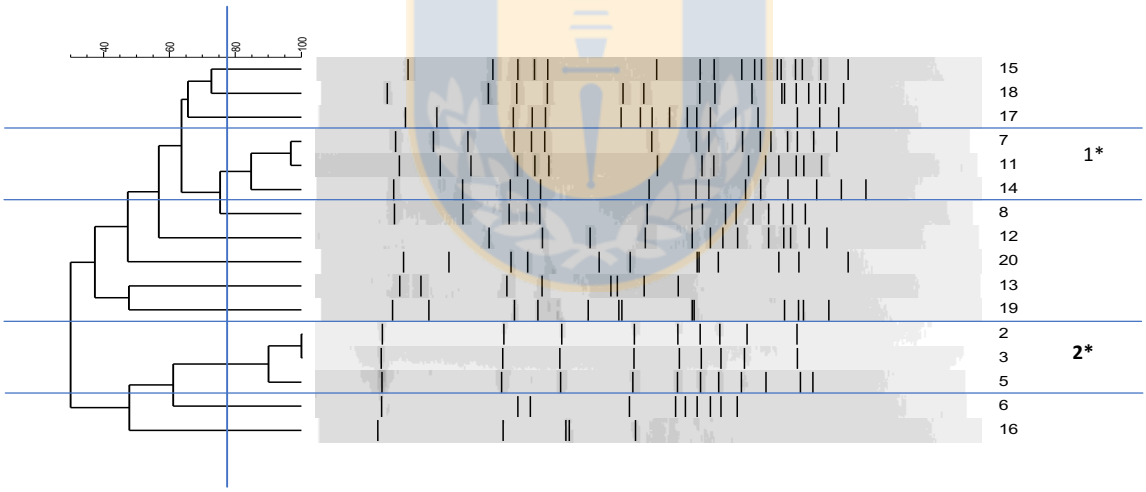
A completely randomized design with a factorial arrangement of 4x5x8 (four NA, five concentrations, eight bacterial strains) was used. The effect of the treatments was interpreted using analysis of variance ANOVA, with statistical software INFOSTAT version 2016. Tukey test was used to determine the significant difference between the means, with a level of significance of 0.05.

Results and Discussion

Molecular typing

The *Sma*I macrorestriction fragment profiles of *S. aureus* isolates are shown in Figure 1. The dendrogram revealed some genetic similarity between some strains, which were classified in two clusters: cluster 1 contains *S. aureus* strains from natural breeding pigs, and cluster 2 includes strains from meat from butchers with different origins. However, most of the strains were genetically diverse, included the six strains that were selected for susceptibility testing assays.

Figure 1. Dendrogram showing the genetic similarity between *S. aureus* isolates from pork production chain.



* Clusters formed; 6, 12, 15, 19 Strains used in antimicrobials test.

Genetic diversity among *S. aureus* strains of swine origincould suggest different source of contamination at different stages of the pork chain supply. These results agreed with Normanno et al. (2015), who found a great genetic diversity of MRSA strains in slaughtered pigs and in abattoir employees in Italy. However, Buyukcangaz et al. (2013) found that MRSA isolates had the same allelic profile

and indistinguishable PFGE patterns than some methicillin-susceptible *S. aureus* strains, all obtained from pork.

Antibiotic susceptibility

The MIC of antibiotics shown that, quinupristin/dalfopristin was the most effective antibiotic, with none resistant strain, followed by vancomycin and gentamicin with one resistant strain. The less effective antibiotic was penicillin, in which all strains were resistant. Hata (2016) found similar results in the mastitis outbreak of cows when treated with penicillin. The low effectiveness of that antibiotic could be due to their mechanism of resistance, consisting in the enzyme penicillinase that hydrolyses the β-lactam ring and inactivates the drug (Peacock and Paterson. 2015).

Four strains were resistant to more than one class of antibiotic. Waters et al. (2011) defined multidrug resistance, as intermediate or complete resistance to 3 or more antimicrobial classes. Therefore, two strains were multidrug resistant, 1(Skin) and 2(Nasal).

CLSI (2014) evaluated methicillin resistance using disk diffusion test with 30 µg cefoxitin disk (≤ 21 mm). Cefoxitin MIC results for the *S. aureus* showed a clear separation of *mecA*-positive and *mecA*-negative strains between 4 and 6 µg/mL; most isolates with cefoxitin MICs of 6 µg/mL contained the *mecA* gene (Swenson et al. 2009). Among the six strains of swine origin, the strains 4(Carcass) and 5(Meat) were resistant to oxacillin and cefoxitin. However, these strains were *mecA*-negative *S. aureus* strains (Table 1) but with greater resistance to β-lactam antibiotics. The resistance to methicillin could also be due to production of modified PBPs or normal PBPs with reduced affinity for β-lactams (Haberberger et al. 1998).

Chemical composition of maqui extract and oregano EO.

The main compounds identified in maqui extract were anthocyanins: delfinidine-3-sambubioside-5-glucoside (329.57 ± 59.11 mg L^{-1}), delfinidine-3,5-diglucoside (319.10 ± 26.44 mg L^{-1}), cyanidin-3-sambubioside-5-glucoside + cyanidin-3,5-diglucoside (242.60 ± 19.63 mg L^{-1}), delfinidin-3-glucoside (253.67 ± 21.6 mg L^{-1}), cyandine-3-sambubioside + cyanidin-3-glucoside (86.11 ± 7.11 mg L^{-1}).

Also, Brauch et al. (2016) detected eight individual anthocyanins by HPLC-DAD-MS in maqui berries and juice, and Schreckinger et al. (2010) found seven main anthocyanin structures in maqui berries.

Eleven compounds were identified in the oregano EO, which can be classified into three groups: alcohol (benzyl alcohol), esters, (ethyl hexanoate, carvacryl acetate o thymyl acetate, benzyl acetate) and terpenes (limonene, p-cymene, E-limonene oxide, eucarvone dihydrocarvone Z or E, thymol and carvacrol). The oregano EO used in this study was carvacrol chemo type, since the content of carvacrol was 90%.

Rota et al. (2008) reported that the oregano EO carvacrol and thymol chemotypes, show bactericidal and bacteriostatic properties. Pesavento et al. (2015) identified in oregano EO, 77.2% oxygenated monoterpenes, principally represented by carvacrol (71.8%). The differences noted in oregano EO composition, suggest that extrinsic and intrinsic genetic factors play a role in determining the oil composition (Bisht et al. 2009), and the characteristic chemical profile differs from one to another year due to the rainfall and the temperature (Mechergui et al. 2016).

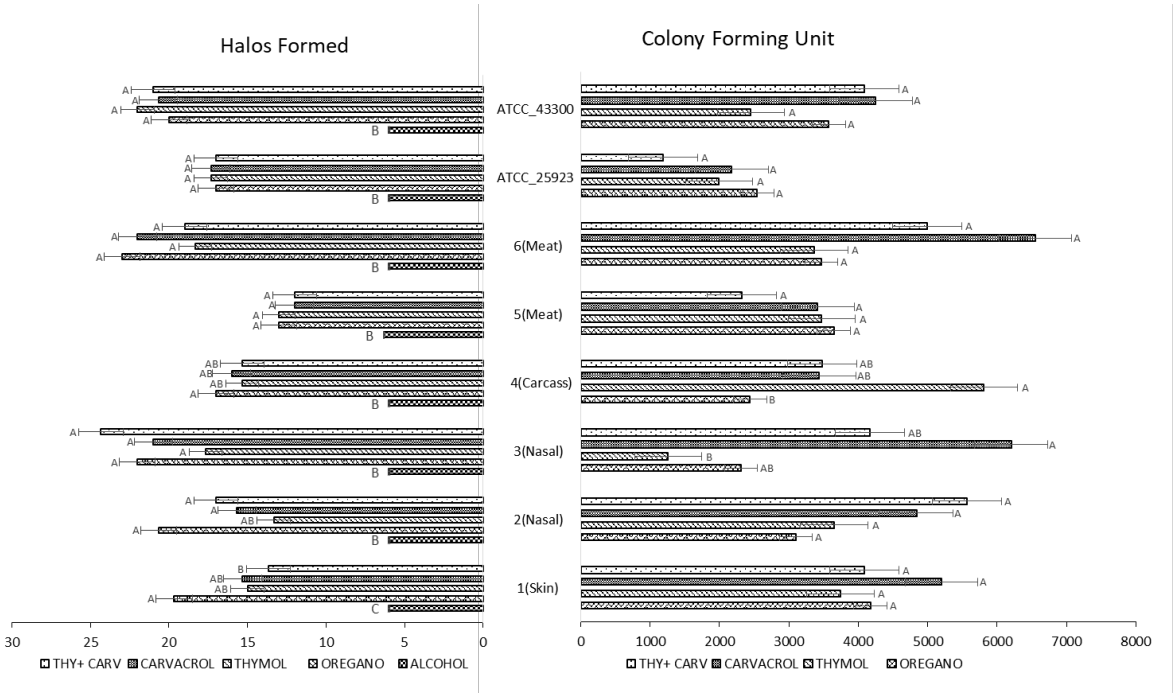
Antimicrobial activity of natural compounds.

The results of the biological activity of NA showed that maqui (*Aristotelia chilensis*) extract at different concentration (9% to 100% v/v) did not present any biological activity against *S. aureus* strains of swine origin.

The absence of biological activity of maqui extracts against gram-positive bacteria has been reported by different authors. Puupponen-Pimia et al. (2001) proposed that the extracts with high content of anthocyanins, obtained from berries, inhibited the growth of gram-negative bacteria, while gram-positive bacteria were quite resistant. In addition, Genskowsky et al. (2015) found an inhibitory effect when incorporating maqui berry extract in the chitosan edible film on 7 of the 8 gram-negative bacteria, but not in gram-positive bacteria. After phenolic acids exposure, the cells become less negatively charged. This surface charge change was particularly verified for the gram-negative bacteria and could be the reason of their higher susceptibility when compared with the gram-positive bacteria (Borges et al. 2013). The mechanisms behind the antimicrobial activity of phenolics acids, flavonoids or anthocyanins are not completely known (Genskowsky et al. 2015).

Oregano EO, Carv, Thy and Thy + Carv, presented biological activity against all strains and were classified as moderately inhibitory according to Rota et al. (2008), with significant differences with the control (alcohol) (Figure 2). There was only inhibition of bacterial growth in the paper disk area (6 mm) impregnated with alcohol, which was not considered as inhibitory activity according to Rota et al. (2008).

Figure 2- Biological activity (halos formed) and Bactericidal activity (Colony Forming Unit) of natural antimicrobials against *Staphylococcus aureus* strains of swine origin.



The NA had the same inhibitory activity against 2(Nasal), 3(Nasal), 4(Carcass), 5(Meat), 6(Meat), and reference strains (P>0.05). In strain 1(Skin), oregano EO was more effective than Thy + Carv (P≤0.05) (Figure 2). Strain 5(Meat) was the less sensitive to oregano EO with no significant differences with the strains 4(Carcass) and ATCC 25923 (P>0.05). In addition, the strain 5(Meat) was the less sensitive to Carv with no significant differences with strains 1(Skin), 2(Nasal), 4(Carcass) and ATCC 25923 (P>0.05). The strains 1(Skin) and 5(Meat) were more resistant to Thy + Carv than the strain 3(Nasal) (P≤0.05). In Thy and control the strains did not present any difference (P>0.05).

Strong inhibitory activity was found with all NA against ATCC 43300; oregano EO against 2(Nasal), 3(Nasal) and 6(Meat) strains; Carv against 3(Nasal) and 6(Meat); and Thy + Carv against 3(Nasal) (Figure 2). The results of this study are according to Nostro et al. (2004), who reported that all *S. aureus* strains were susceptible to oregano EO, carvacrol and thymol without significant differences between MRSA and MSSA strains.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration.

The lowest concentration that inhibited the growth of the strains in the broth (MIC) varied from 0.01% to 0.04% (v/v) (Table 2). There were no significant differences between reference strains and strains of swine origin in MIC values (P>0.05). In combination, Thy + Carv, did not show significantly lower MIC values compared to the single compounds (P>0.05). These results are in disagreement with Lambert et al.(2001) who reported that mixing carvacrol and thymol at proper amounts may improve bacterial inhibition.

Table 2- Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of natural antimicrobials in *Staphylococcus aureus* strains of swine origin.

Strains	**MIC (%)**				**MBC (%)**			
	OREGANO EO*	THY	CARV	THY+ CARV	OREGANO EO	THY	CARV	THY+ CARV
1 (Skin)	0.02	0.01	0.02	0.02	0.04	0.08	0.08	0.08
2 (Nasal)	0.02	0.01	0.02	0.02	0.04	0.04	0.04	0.08
3 (Nasal)	0.01	0.01	0.02	0.01	0.08	0.02	0.08	0.08
4 (Carcass)	0.01	0.02	0.02	0.01	0.08	0.08	0.08	0.08
5 (meat)	0.02	0.02	0.04	0.04	0.04	0.08	0.08	0.08
6 (meat)	0.02	0.02	0.02	0.02	0.08	0.08	0.04	0.08
ATCC25923	0.02	0.02	0.02	0.01	0.04	0.04	0.04	0.04
ATCC43300	0.02	0.01	0.04	0.04	0.04	0.08	0.08	0.08

EO: Essential oil; THY: Thymol; CARV: Carvacrol; THY+CARV: Thymol + Carvacrol.

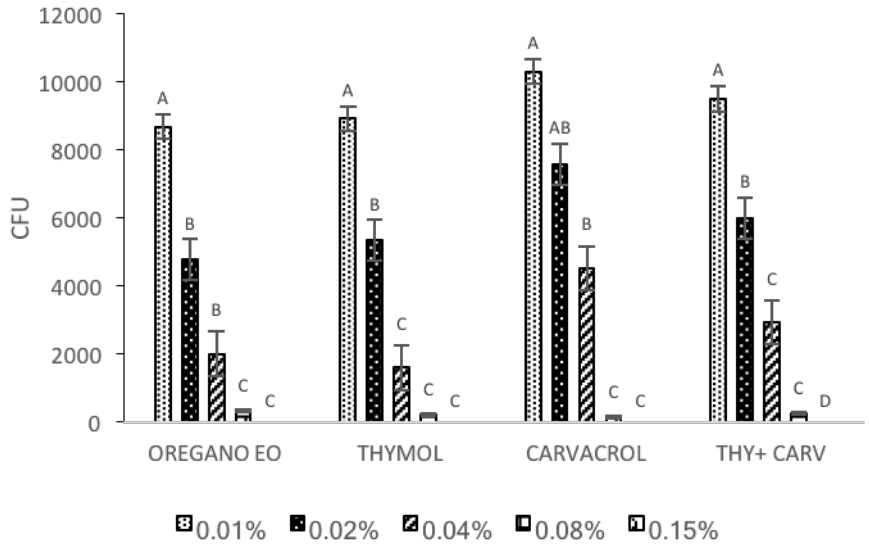
The lowest concentration at which the bactericidal (MBC) effect was recorded in the strains varied from 0.02 to 0.08% (v/v) (Table 2).

The reference strain ATCC 25923 was the most susceptible to all NA, forming less colonies (Figure 2). The strains ATCC 43300, 1(Skin), 2(Nasal), 5(Meat), and 6(Meat), did not show significant differences among the NA. However, in strain

4(Carcass), Thy, Carv and Thy + Carv were less effective compared with oregano EO. In the strain 3(Nasal) Thy was the most effective with significant differences with Carv, which was the less effective (Figure 2).

In general, Carv was the less effective NA, followed by Thy + Carv, Thy and oregano EO (Figure 3). There were no significant differences among strains when treated with Carv and oregano EO, however, for Thy + Carv, the strain 2(Nasal) was the most resistant and ATCC 25923 the most susceptible, and the strain 4(Carcass) was more resistant to Thy with significant difference with the strain 3(Nasal) and ATCC 25923. The NA have different actions mechanisms, oregano EO reduces lipase and coagulase activity of *S. aureus*, carvacrol can cause the disruption of the membrane, inhibition of ATPase activity, and thymol can also cause the disruption of the membrane with potential intracellular targets, and citrate metabolic pathway disruption (Langeveld et al. 2013).

Figure 3- Bactericidal activity of natural antimicrobials at different concentrations on *S. aureus* strains of swine origin.



The effectiveness of NA decreased when concentrations varied from 0.04%, to 0.01% with statistical differences between them (Figure 3). Different concentrations affected differently the strains. The concentration 0.01% did not inhibit cell growth of the strains in agar plates (Figure 3). The concentration of 0.02% (v/v) of thymol had great bactericidal effect against the strain ATCC 25923 with no statistical difference with the concentration 0.15%, inhibiting the growth of this strain. The concentration of 0.04% (v/v) was the minimum concentration that showed effective bactericidal effect against the strains ATCC 43300, 2(Nasal), 3(Nasal) and 6(Meat). For strains 1(Skin) and 4(Carcass) the concentration 0.04% showed bactericidal effect, and inhibited the growth of the strains with significant difference with concentration 0.02%.

The lowest concentration of NA that inhibited the growth of the strains in the broth (MIC) was 0.01% and the greater was the 0.04% (Table 2). The MIC of Thy varied from 0.01% to 0.02% and Carv from 0.02% to 0.04%, including MRSA strain, which differed with Engel et al. (2017) who reported a MIC of thymol and carvacrol of 0.66 $mg\ mL^{-1}$ and with Rodrigues et al. (2017) who reported a MIC of carvacrol of 0.0025% (v/v) against *S. aureus*. The exposure to carvacrol at low concentrations induces an increase of the level of unbranched fatty acids and at higher levels severely damages the integrity and morphology of *S. aureus* cell membrane. In addition, carvacrol can bind to DNA via minor groove mode, mildly perturbs the DNA secondary structure, and induces DNA molecules to be aggregated (Wang et al. 2016).

For bactericidal effects (MBC), the effectiveness of NA was measured by the number of colonies after the exposure of bacteria to different treatments, i.e. the greater the number of colonies, the lower effectiveness of NA. In general, oregano

EO and Thy were more effective on all strains compared to Carv and Thy + Carv. These results agreed with Miladi et al. (2017), who noted also that thymol was more effective than carvacrol.

Different concentrations of NA applied affected the bacterial growth, the highest concentrations were most effective. The concentration of 0.08% was the lowest concentration that inhibited cell growth in plates (MBC) on all strains (Figure 3). In this study, MBC for oregano EO ranged from 0.04% to 0.08%, due to the potential bactericidal activity at low concentration (Figure 3). Scandorieiro et al. (2016) showed previously a MIC and MBC of oregano EO of 0.596 and 1.193 mg mL^{-1}, respectively against *S. aureus*. The efflux of potassium ions from *S. aureus* cells occurred immediately after the addition of the oregano EO following a steady loss along the evaluated intervals. In many cases the biological activity of NA is related to the complex interaction between the different classes of compounds such as phenols, aldehydes, ketones, alcohols, esters, ethers or hydrocarbons found in EO (Bassolé and Juliani, 2012).

Conclusions

Resistant *S. aureus* strains to β-lactam antibiotics and multidrug-resistant strains were identified in samples of swine origin. All strains showed sensitivity to oregano EO, thymol, carvacrol and the mix of thymol + carvacrol. Maqui extract did not exhibit an inhibitory activity against *S. aureus*.

Natural antimicrobials represent a promising strategy for controlling antibiotic-resistant *S. aureus* strains.

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Capitulo 3

Conclusiones generales y Proyecciones

Conclusiones generales:

- Las cepas de origen porcino presentan diversidad genética, lo que indica diferentes orígenes de las cepas bacterianas en el proceso de producción de carne de cerdo.
- Quinupristina fue el antibiótico más eficaz, pues no hubo cepas resistentes a este antibiótico, seguido de vancomicina y gentamicina con una cepa resistente a cada antibiótico. Se encontraron dos cepas que presentaron resistencia a oxacilina y cefoxitina.
- El maqui (*Aristotelia chilensis*), en diferentes concentraciones no presentó actividad biológica. El aceite esencial de oregano, carvacrol, timol, y mezcla de carvacrol y timol, presentaron actividad antimicrobiana contra todas las cepas. La concentración mínima inhibitoria (MIC) fue de 0,01%-0,04%, y la concentración mínima bactericida (CMB) de 0.02%-0.08%, sin diferencia entre las cepas de referencia, y las cepas aisladas de la carne.

Proyecciones.

La diversidad genética de las cepas encontrada en las diferentes etapas del proceso de producción de carne de cerdo sugiere diferentes fuentes de contaminación, por lo tanto, es necesario evaluar las características moleculares de estas cepas a través de otros métodos complementarios para una mayor descripción.

Estudiar la presencia del gen *mecC* en las cepas bacterianas que fueron negativas al gen *mecA*, pero que presentaron resistencia a la oxacilina y a cefoxitin, y determinar otros mecanismos de resistencia a esos antimicrobianos.

Conocer con mayor precisión el mecanismo de acción de los antimicrobianos naturales utilizados en las cepas de *S. aureus*.

Las cepas de *Staphylococcus aureus*, especialmente asociadas a formación de biofilm, pueden presentar mayor resistencia antimicrobiana y ser resistentes a múltiples fármacos. Por esta razón, es interesante conocer la capacidad de las cepas aisladas de la cadena de producción de cerdo de formar biofilms.