UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEPARTAMENTO DE CIENCIA ANIMAL

TESIS DOCTORAL

Material particulado y bioaerosoles en el aire de granjas de aves y conejos: cuantificación, caracterización y medidas de reducción

Airborne particulate matter and bioaerosols in poultry and rabbit houses: quantification, characterization and reduction measures

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Resumen

Los alojamientos ganaderos son una fuente importante de material particulado ("particulate matter", PM) y bioaerosoles. Estas sustancias tienen un efecto perjudicial tanto para la salud humana y animal como para el medio ambiente. Para reducir los niveles de PM y bioaerosoles en alojamientos ganaderos es necesario conocer el origen de los mismos y los factores que afectan a su generación y suspensión en el aire. Esta Tesis Doctoral aborda aspectos relacionados con la concentración, origen y propiedades físicas, químicas y biológicas del PM en el aire de granjas de conejos y aves, su relación con los bioaerosoles patógenos y técnicas para reducirlos. Este trabajo pretende contribuir a paliar los efectos negativos de estas sustancias tanto en el interior de los alojamientos ganaderos como en el exterior.

Los objetivos específicos planteados en la presente Tesis Doctoral fueron: *i).* caracterizar la morfología y la composición química del PM de distintos tamaños así como la concentración de bacterias en el aire de granjas de conejos, *ii).* cuantificar la concentración y emisión del PM de distintos tamaños en el aire e identificar las principales actividades que contribuyen a la generación del PM en granjas de conejos, *iii).* evaluar la distribución espacial de bacterias aerobias mesófilas en el aire durante un ciclo de producción de broilers y examinar su relación con la concentración y evolución del PM, *iv).* evaluar y comparar diferentes técnicas para muestrear y detectar el patógeno *Salmonella* spp. en el aire de granjas de broilers y *v).* evaluar la aplicación de desinfectantes en el aire como medida de reducción de los bioaerosoles en granjas de gallinas ponedoras con especial atención al patógeno *Mycoplasma gallisepticum*.

Los resultados de esta Tesis indican que en los alojamientos avícolas y cunícolas se generan y emiten cantidades importantes de PM y bioaerosoles, por encima de los valores límite de exposición que marca la Directiva 2008/50/CE relativa a la calidad del aire ambiente y a una atmósfera más limpia en Europa, sobre todo en granjas de aves. Estas sustancias deben ser controladas y reducidas para proteger el medio ambiente, la salud y bienestar de las personas y animales.

En alojamientos cunícolas, el PM mostró una morfología y composición química compleja, siendo las partículas irregulares y angulosas, ricas en S, Ca, Mg, Na y Cl, las más abundantes. La concentración de bacterias aerobias

mesófilas en el aire por metro cúbico varió entre $3,1x10^3$ y $1,6x10^6$ unidades formadoras de colonia (UFC). Las principales fuentes generadoras de PM fueron la piel, el pienso y las heces provenientes de las actividades de limpieza de la nave, sobre todo de barrer y de los propios animales. La concentración media de PM10 (partículas de 10 μm de diámetro o inferior) fue 0,08±0,06 mg/m^3 para conejos de cebo y 0,05±0,06 mg/m^3 para conejas y la concentración media de PM2,5 (partículas de 2,5 μm de diámetro o inferior) fue 0,01±0,02 mg/m^3 para conejos de cebo y 0,01±0,04 mg/m^3 para conejas. Las emisiones variaron entre 6 y 15 mg/plaza/día para PM10 y entre 0,2 y 3,0 mg/plaza/día para PM2,5.

En alojamientos de broilers, la concentración de bacterias varió entre 3,0 y 6,5 log UFC/m^3. La mayoría de bacterias se asociaron con partículas entre 3,3 y más de 7,0 μm de diámetro obteniéndose una correlación positiva entre las concentraciones de PM10 y PM2,5 y las de bacterias. Respecto a la detección de patógenos en el aire, no se detectó *Salmonella* spp. cultivable en una explotación de broilers infectados experimentalmente mediante el uso de borboteadores de aire y técnicas de cultivo tradicional. No obstante, se detectó este patógeno en el aire mediante impactación y técnicas moleculares. Por lo tanto, no se recomienda el uso de borboteadores y técnicas de cultivo para la detección y/o cuantificación de *Salmonella* spp. cultivable en el aire.

En alojamientos de gallinas, la concentración media de PM10 fue 0,55±0,38 mg/m^3 y 0,02±0,03 mg/m^3 para PM2,5. La concentración de bacterias varió entre 4,1 y 5,7 log UFC/m^3. La aplicación de un desinfectante químico de amplio espectro en el aire no fue efectiva ni para reducir los niveles de bacterias aerobias mesófilas en el aire ni de *Mycoplasma* spp. Es necesario estudiar diferentes productos, dosis o técnicas de aplicación.

En su conjunto, los resultados presentados en esta Tesis Doctoral proporcionan una información útil sobre el PM y los bioaerosoles en el aire de alojamientos ganaderos, que permitirá diseñar e implementar medidas de reducción prácticas y eficaces que mejoren la calidad del aire en los alojamientos ganaderos y reduzcan su emisión al exterior.

Abstract

Livestock houses are an important source of particulate matter (PM) and bioaerosols. These substances can have a detrimental effect on human and animal health and the environment, as well. Knowledge on the origin of PM and bioaerosols in livestock houses and the factors affecting their generation and aerosolization is necessary to reduce them. The present PhD thesis addresses issues related with the concentration, origin and physical, chemical and biological properties of airborne PM in rabbit and poultry farms, their relationship with pathogenic bioaerosols and techniques to reduce them. This work aims to contribute to alleviate the negative effects of these substances both indoor and outdoor livestock houses.

The specific objectives within this PhD thesis were *i).* to characterize airborne PM in rabbit farms in terms of morphology, chemical composition and bacterial concentration in different size fractions, *ii).* to quantify airborne PM10 and PM2.5 concentrations and emissions in rabbit farms, and to identify the main factors related with farm activities influencing PM generation, *iii).* to assess the spatial distribution of mesophilic aerobic bacteria in the air during a broiler cycle and examine their relationship with the concentration and evolution of PM, *iv).* to compare the performance of techniques to sample and detect airborne *Salmonella* spp. in broiler farms and *v).* to evaluate the application of an air disinfectant to reduce airborne microorganism in a commercial laying hen house, with focus on its effect on *Mycoplasma* spp.

The results of this thesis suggest that poultry and rabbit houses generate and emit relevant amounts of PM and bioaerosols which can exceed the limit values established in Directive 2008/50/EC on ambient air quality and cleaner air for Europe, especially in poultry farms. Therefore, these substances must be controlled and reduced to protect the environment and the health and welfare of humans and animals.

In rabbit houses, PM showed a complex morphology and chemical composition, being fragmentation type particles with irregular and acute edges, rich in S, Ca, Mg, Na and Cl the most abundant. The concentration of mesophilic aerobic bacteria per cubic meter in the air varied between $3.1x10^3$ and $1.6x10^6$ colony forming units, CFU. The main sources of PM were skin, animal feed and faeces from cleaning activities, specially sweeping and the animals themselves. The average concentration of PM10 (particles

smaller than 10 μm in diameter) was 0.08 ± 0.06 mg/m^3 for fattening rabbits and 0.05±0.06 mg/m^3 for reproductive does and the average concentration of PM2.5 (particles smaller than 2.5 μm in diameter) was 0.01±0.02 mg/m^3 for fattening rabbits and 0.01±0.04 mg/m^3 for reproductive does. Emissions ranged from 6 to 15 mg/animal/day for PM10 and from 0.2 to 3.0 mg/animal/day for PM2.5.

In broiler houses, the concentration of bacteria ranged from 3.0 to 6.5 log UFC/m^3. Most bacteria were associated with particles between 3.3 and 7.0 μm in diameter existing a positive correlation between the concentration of PM10 and PM2.5 and airborne bacteria. Regarding the detection of pathogens in the air of an experimentally infected broiler room, cultivable *Salmonella* spp. was not recovered using impingement and traditional culture methods, but could be detected with impaction and molecular techniques. Therefore, the use of impingers and traditional culture methods are not recommended for the detection and/or quantification of airborne cultivable *Salmonella* spp.

In laying hen houses, the average concentration of PM10 was 0.55±0.38 mg/m^3 and 0.02±0.03 mg/m^3 for PM2.5. The concentration of bacteria ranged from 4.1 to 5.7 log CFU/m^3. The application of a thermonebulized wide spectrum disinfectant in the air was not effective in reducing the concentration of airborne mesophilic aerobic bacteria nor *Mycoplasma* spp. It would be desirable to evaluate different air disinfection doses, products and application methods.

Overall, the results presented in this PhD Thesis provide necessary information about the PM and bioaerosols in the air of livestock housing and their relationship, which is useful to design and implement effective measures to reduce PM and bioaerosols to improve air quality in livestock housing and reduce their emissions to the outside.

Resum

Els allotjaments ramaders són una font important de material particulat ("particulate matter", PM) i bioaerosols. Aquestes substàncies tenen un efecte perjudicial tant per a la salut humana i animal com per al medi ambient. Per reduir els nivells de PM i bioaerosols en allotjaments ramaders cal conèixer l'origen dels mateixos i els factors que afecten la seva generació i suspensió a l'aire. Aquesta Tesi Doctoral aborda aspectes relacionats amb la concentració, origen i propietats físiques, químiques i biològiques del PM en l'aire de granges de conills i aus, la seva relació amb els bioaerosols patògens i tècniques per reduir-los. Aquest treball pretén contribuir a pal·liar els efectes negatius d'aquestes substàncies tant a l'interior dels allotjaments ramaders com a l'exterior.

Els objectius específics plantejats en la present Tesi Doctoral van ser: *i).* caracteritzar la morfologia i la composició química del PM en l'aire en diferents fraccions, així com la concentració de bacteris aerotransportades de granges de conills, *ii).* quantificar la concentració i emissió del PM en diferents fraccions en l'aire i identificar les principals activitats que contribueixen a la generació del PM en granges de conills, *iii).* avaluar la distribució espacial de bacteris aerobis mesòfils suspesos en l'aire durant un cicle de producció de broilers i examinar la seva relació amb la concentració i evolució del PM, *iv).* Avaluar i comparar diferents tècniques per mostrejar i detectar el patogen *Salmonella* spp. en l'aire de granges de broilers i *v).* avaluar l'aplicació de desinfectants en l'aire com a mesura de reducció dels bioaerosols en granges de gallines ponedores amb especial atenció al patogen *Mycoplasma gallisepticum*.

Els resultats d'aquesta Tesi indiquen que als allotjaments avícoles i cunícoles es generen quantitats importants de material particulat i bioaerosols, per sobre dels valors límit d'exposició que marca la Directiva 2008/50/CE, relativa a la qualitat de l'aire ambient i a una atmosfera més neta a Europa, sobre tot en granges d'aus. Estes substàncies han de ser controlades i reduïdes per protegir el medi ambient, la salut i benestar de les persones i animals.

En allotjaments cunícoles, el PM va mostrar una morfologia i composició química complexa, les partícules mostraren una morfología irregular i angular, riques en S, Ca, Mg, Na y Cl. La concentració de bacteris aerobis mesòfils en l'aire per metre cúbic va variar entre $3{,}1x10^3$ i $1{,}6x10^6$ unitats

formadores de colònia (UFC). Les principals fonts generadores de PM van ser la pell, el pinso i els excrements provinents de les activitats de neteja de la nau, sobretot agranar, i dels propis animals. La concentració mitjana de PM10 (partícules de 10 μm de diàmetre o inferior) va ser 0,08±0,06 mg/m^3 per conills d'engreix i 0,05±0,06 mg/m^3 per conilles i la concentració mitjana de PM2,5 (partícules de 2,5 μm de diàmetre o inferior) va ser 0,01±0,02 mg/m^3 per conills d'engreix i 0,01±0,04 mg/m^3 per conilles. Les emissions van variar entre 6 i 15 mg/plaça/dia per PM10 i entre 0,2 i 3,0 mg/plaça/dia per PM2,5.

En allotjaments de broilers, la concentració de bacteris va variar entre 3,0 i 6,5 log UFC/m^3. La majoria de bacteris es van associar amb partícules entre 3,3 i més de 7 micres de diàmetre i es va obtindre una correlació positiva entre les concentracions de PM i les de bacteris. Pel que fa a la detecció de patògens en l'aire, no es va detectar *Salmonella* spp. cultivable en una explotació de broilers infectats experimentalment mitjançant l'ús de borboteadores d'aire i tècniques de cultiu tradicional. No obstant això, es va detectar aquest patogen en l'aire mitjançant impactació i tècniques moleculars. Per tant, no es recomana l'ús de borbollejadors i tècniques de cultiu per a la detecció i/o quantificació de *Salmonella* spp. cultivable en l'aire.

En allotjaments de gallines, la concentració mitjana de PM10 va ser 0,55±0,38 mg/m^3 i 0,02±0,03 mg/m^3 per PM2, 5. La concentració de bacteris va variar entre 4,1 i 5,7 log UFC/m^3. L'aplicació d'un desinfectant químic d'ampli espectre en l'aire no va ser efectiva ni per reduir els nivells de bacteris aerobis mesòfils en l'aire ni de *Mycoplasma* spp. Cal estudiar diferents productes, dosis o tècniques d'aplicació.

En conjunt, els resultats presentats en aquesta Tesi Doctoral proporcionen una informació útil sobre el PM i els bioaerosols en l'aire d'allotjaments ramaders, que permetrà dissenyar i implementar mesures de reducció practiques i eficaços que millorin la qualitat de l'aire en els allotjaments ramaders i redueixin la seva emissió a l'exterior.

Listado de abreviaturas

ADFI	Consumo Medio Diario, del inglés "Average Daily Feed Intake"
ADG	Ganancia Media Diaria, del inglés "Average Daily Gain"
ADN	Ácido Desoxirribonucleico
ANOVA	Análisis de la Varianza
BGA	Agar Verde Brillante, agar selectivo para *Salmonella* spp., del inglés "Brilliant Green Agar"
bp	Pares de Bases, del inglés "Base pairs"
CECT	Colección Española de Cultivos Tipo
CFU	Unidades Formadoras de Colonias, del inglés "Colony Forming Unit"
CMD	Consumo Medio Diario
DNA	Ácido Desoxirribonucleico, del inglés "Deoxyribonucleic acid"
EDX	Análisis por dispersión de energías de rayos-X, del inglés "energy-dispersive X-ray analysis"
GMD	Ganancia Media Diaria
HR	Humedad Relativa
IC	Índice de Conversión
LM	Microscopía Óptica, del inglés "Light Microscopy"
ME	Mycoplasma Experience, agar selectivo para *Mycoplasma* spp.
PBS	Tampón Fosfato Salino, del inglés "Phosphate Buffered Saline"
PCA	Recuento en Placa de Agar, agar para bacterias aerobias mesófilas, del inglés "Plate Count Agar"
PCR	Reacción en Cadena de la Polimerasa, del inglés "Polymerase Chain Reaction"
qPCR	Análisis cuantitativo de la Reacción en Cadena de la Polimerasa, del inglés "quantitative Polymerase Chain Reaction"
RV	Rappaport Vassiliadis, agar selectivo para *Salmonella* spp.
PM	Material Particulado, del inglés "Particulate Matter"
PM2,5	Partículas suspendidas en el aire con un diámetro inferior a 2,5 μm
PM10	Partículas suspendidas en el aire con un diámetro inferior a 10 μm
SEM	Microscopio Electrónico de Barrido, del inglés "Scanning Electron Microscope"
T	Temperatura
TSP	Partículas suspendidas en el aire de entre 30 y 100 μm de diámetro, del inglés "Total Suspended Particles"

TEOM	Tapered Element Oscillating Microbalance
UFC	Unidades Formadoras de Colonias
VBNC	Viable Pero No Cultivable, del inglés "Viable But Non-Culturable"
XLD	Agar de Xilosa, lisina, desoxicolato; agar selectivo de *Salmonella* spp. y *Shigella* spp., del inglés "Xilose lysine deoxicholate"

Índice

1

Introducción general

Una versión de este capítulo titulado "Bioaerosoles en alojamientos ganaderos: un problema emergente ambiental y sanitario" (Autores: Elisa Adell Sales y María Cambra-López) ha sido galardonado con el segundo premio en el XIII Premio Cristóbal de la Puerta. Editorial Agrícola Española. Diciembre 2013.

1.1. Contexto: El material particulado y los bioaerosoles en el aire de las explotaciones ganaderas

En las explotaciones ganaderas se generan gases de efecto invernadero (óxido nitroso, metano, dióxido de carbono) gases acidificantes y eutrofizantes del medio como el amoniaco, además de sustancias no gaseosas como el material particulado (del inglés, Particulate Matter; PM) y los bioaerosoles. Estas sustancias se encuentran generalmente suspendidas en el aire.

En los años 90, debido al endurecimiento de la legislación ambiental y al creciente interés científico en torno a la calidad del aire en las explotaciones ganaderas, las investigaciones europeas y norteamericanas se centraron en sustancias gaseosas (Gustaffson, 1997; Christensen and Thorbek, 1987; Krylova et al., 1997). Desde entonces, se ha avanzado en la cuantificación y caracterización de los distintos gases (fundamentalmente amoniaco) en el interior y exterior de explotaciones ganaderas (Groot Koerkamp et al., 1998; Hansen et al., 1998). No obstante, la información sobre gases de efecto invernadero, incluso sobre sustancias no gaseosas es todavía escasa. Esta Tesis Doctoral se centra en el estudio de dos sustancias no gaseosas que se encuentran suspendidas en el aire en explotaciones avícolas y cunícolas: el PM y los bioaerosoles.

El PM del aire se caracteriza por ser una mezcla compleja y heterogénea de partículas de diferente origen, composición química, forma, tamaño y densidad que determinan tanto su comportamiento atmosférico como sus efectos sobre la salud y el medio ambiente (EPA, 2004). A la asociación entre el PM y los microorganismos suspendidos en el aire se le denomina bioaerosol. Éstos se definen como aerosoles que comprenden partículas de origen o actividad biológica (esporas, hongos, virus, bacteria, toxinas y alérgenos) que pueden afectar a seres humanos causándoles algún tipo de patología, ya sea de tipo alérgico, tóxico o infeccioso (Cox and Wathes, 1995). Los bioaerosoles generados en el interior de las explotaciones ganaderas contienen un 90% de polvo orgánico (e.g. proteínas y carbohidratos), microorganismos (e.g.

bacterias, hongos y virus) y componentes biológicos activos que se relacionan con reacciones tóxicas y procesos inflamatorios intensos (e.g. endotoxinas y glucanos) (Aarnink et al., 1999).

Las emisiones totales de PM a la atmósfera en España representan el 6% de las emisiones globales de la Unión Europea (CEIP, 2013). A nivel nacional, el sector del transporte y las plantas de combustión son los principales responsables de la emisión de PM. Cada uno de estos sectores representa entre el 30 y 40%, según el tamaño de partícula, del total de las emisiones de PM en España (Ministerio de Agricultura, Alimentación y Medio Ambiente). La ganadería contribuye entre un 4 y un 17%, según tamaño de partícula, al total de las emisiones de PM de España. A pesar de la menor aportación de las actividades ganaderas a la emisión total de PM en España comparada con el sector transporte, por ejemplo, estas sustancias son una fuente de contaminación ambiental relevante que afecta tanto a los ecosistemas como a la salud humana y animal (Harrison and Yin, 2000; Crook et al., 1991; Grantz et al., 2003). Hasta la fecha, no existe un inventario similar para bioaerosoles.

Los estudios realizados hasta el momento sobre la cuantificación y caracterización del PM y bioaerosoles en el aire de explotaciones ganaderas se han centrado en aves y porcino (Cambra-López et al., 2010). No obstante, la información relativa a los procesos y factores que determinan la generación, emisión y dispersión de PM y transmisión de bioaerosoles en este tipo de alojamientos es escasa (Vucemilo, 2007; Cambra-López et al., 2011b). En cuanto al conocimiento sobre el PM y bioaerosoles en la producción cunícola, ésta es muy limitado (Navarotto et al., 1995). A pesar de que el conejo es una especie ganadera minoritaria comparado con la producción avícola o porcina a nivel europeo, la producción cunícola en España representa aproximadamente el 12% de la producción cunícola europea y el 5% de la producción mundial (FAO, 2013). Es por ello que es fundamental conocer las características del PM y bioaerosoles, sus concentraciones en el interior de las granjas de conejos, así como su emisión al medio ambiente externo.

Por otro lado, el PM procedente de explotaciones ganaderas se ha estudiado tradicionalmente de forma aislada, sin atender a su relación

con otras sustancias que se encuentran suspendidas en el aire. La mayoría de trabajos han tratado sobre su concentración (Gallmann et al., 2002; Li et al., 2011), composición o caracterización (Cambra-López et al., 2011a), sin prestar atención a su posible relación con partículas de origen biológico como microorganismos (Nimmermark et al., 2009). La presencia de microorganismos tales como bacterias, hongos y virus asociados al PM en alojamientos ganaderos ha sido confirmada por varios estudios en la literatura (Curtis et al., 1975; Thorne et al., 2009; Schulz et al., 2011). Aunque los microorganismos pueden existir suspendidos libremente en el aire, se acepta por consenso que éstos suelen estar unidos al PM. El estudio de forma conjunta de estas dos sustancias es fundamental, sobre todo para evaluar de forma integrada medidas efectivas para reducirlos y sus efectos colaterales.

1.2. Riesgos del material particulado y los bioaerosoles para la salud humana y animal

Las sustancias que se encuentran suspendidas en el aire de explotaciones ganaderas pueden afectar de forma distinta a la salud humana y animal. Según Wathes (1998), los mecanismos por los cuales la salud de los animales puede verse afectada dependen de las interacciones entre el huésped animal, los microorganismos y la calidad del aire. Las variables que intervienen en estos mecanismos son: la concentración de contaminantes aéreos, la presencia de microorganismos patógenos respiratorios específicos, la microflora comensalista (positiva) en el tracto respiratorio y el huésped animal. Estas interacciones y variables se esquematizan en la Figura 1.1.

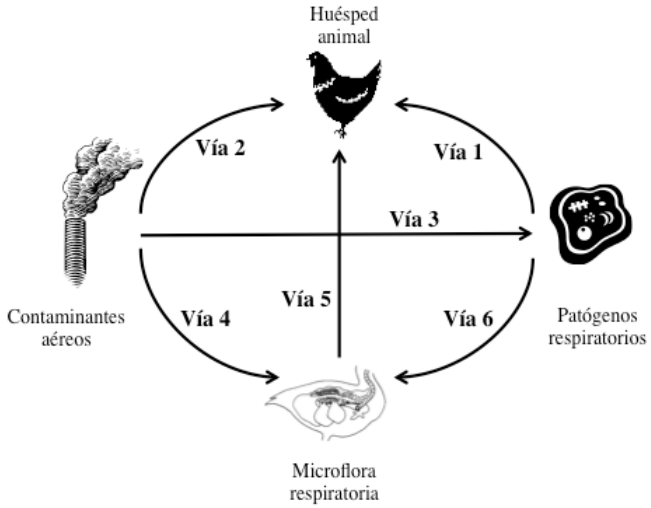

Figura 1.1. Interacciones entre el huésped animal, los microorganismos y la calidad del aire y vías de acción. Fuente: Adaptado de Wathes (1998).

Mediante estas interacciones se explica el fundamento de cómo la calidad del aire influida por las distintas sustancias que se encuentran suspendidas en el aire puede afectar a los animales. El fundamento reside en que la incidencia y gravedad de un patógeno respiratorio específico es mayor cuando se combina con la exposición crónica a contaminantes aéreos (principalmente gaseosos en forma de amoniaco y particulados).

Atendiendo a la Figura 1.1, las seis vías de acción que participan en las interacciones entre el huésped animal, los microorganismos y la calidad del aire, según Wathes (1998), son:

Vía 1: Los patógenos respiratorios específicos pueden deprimir la función y el rendimiento animal en condiciones de calidad de aire buenas.

Vía 2: En ausencia de patógenos, la exposición a contaminantes aéreos reduce el rendimiento y productividad animal.

Vía 3: Los contaminantes aéreos pueden afectar a la patogenicidad, multiplicación y supervivencia de patógenos respiratorios.

Vía 4: Los contaminantes aéreos pueden afectar a la multiplicación y supervivencia de la microflora comensalista del tracto respiratorio del animal huésped.

Vía 5: La microflora comensalista del tracto respiratorio afecta de forma beneficiosa al animal huésped.

Vía 6: Los patógenos respiratorios específicos pueden interaccionar con la microflora comensalista del animal huésped y aumentar la supervivencia de una o más especies.

En este sentido, Al Homidan et al. (2003) describió los efectos perjudiciales de contaminantes aéreos sobre el rendimiento de las aves, y señaló que los efectos sobre la salud animal y los rendimientos productivos están sujetos a las interrelaciones entre las distintas sustancias en el aire. En los trabajos realizados por Van Wicklen et al. (2001), sin embargo, no encontraron diferencias en lesiones del aparato respiratorio de broilers con combinaciones de 24 ppm de amoniaco y 46,6 partículas/mL y atribuyeron los efectos perjudiciales a la combinación no sólo de amoniaco, sino de otros gases nocivos (dióxido de carbono o sulfuro de hidrógeno) y de bioaerosoles patógenos. Es por eso que Donham (1991) observó que el efecto de la calidad del aire sobre las posibles enfermedades infecciosas y productividad en porcino, depende de una mezcla de agentes: PM, dióxido de carbono, amoniaco y bioaerosoles. Consecuentemente, es necesario implementar medidas que mejoren la calidad del aire en explotaciones ganaderas y desarrollar estrategias combinadas que contribuyan a reducir la concentración de estas sustancias gaseosas y particuladas en el aire.

Respecto a los bioaerosoles y al PM, los problemas de salud más frecuentes relacionados con la exposición a estas sustancias son los problemas respiratorios y las enfermedades infecciosas. Estos problemas pueden afectar no sólo a la salud y bienestar de los animales, sino también a los rendimientos productivos y consecuentemente se convierte en una cuestión con importancia económica. Los problemas respiratorios causados por la inhalación de PM y bioaerosoles varían desde enfermedades subclínicas con síntomas poco evidentes, hasta enfermedades respiratorias severas (Douwes et al., 2003). Generalmente se traducen en síntomas como la inflamación de las vías respiratorias (rinitis, inflamación de la tráquea y enfermedades pulmonares) causada por las propias partículas en sí o por exposiciones a determinadas toxinas, agentes inflamatorios o alérgenos asociados. Crook et al. (1991)

evaluaron la salud de 29 trabajadores de granjas de cerdos expuestos a ambientes cargados de partículas (entre 1,7 y 21 mg/m^3) y bioaerosoles (entre 10^5 y 10^7 unidades formadoras de colonia, UFC/m^3). La mayoría de los trabajadores presentaron síntomas respiratorios como tos además de irritación nasal y ocular, indicando irritación de la membrana mucosa. Además algunos de los trabajadores mostraron respuestas alérgicas a las partículas de la piel de los animales, orina y componentes del pienso.

En cuanto a las enfermedades infecciosas, la mayoría de virus, bacterias, hongos o protozoos que encontramos en los alojamientos ganaderos pueden provocar infecciones. La transmisión de estos agentes ya sea por inhalación o por contacto puede provocar serios problemas de salud. Entre ellos, zoonosis como campilobacteriosis, salmonelosis, fiebre aftosa, colibacilosis, influenza y enfermedad de Newscastle (Chinivasagam et al., 2009; Davies and Breslin, 2001; Zhao et al., 2011).

El tamaño del PM asociado a los bioaerosoles es el factor que más influye en los efectos sobre la salud humana y animal ya que determina el lugar del tracto respiratorio en el que se depositará la partícula una vez inhalada. La norma europea UNE EN 481 (UNE-EN, 1993) sobre "Atmósferas en los puestos de trabajo: definición de las fracciones por el tamaño de las partículas para la medición de aerosol" clasifica el PM en "inhalable", "torácico" y "respirable". En función de la mayor profundidad de entrada en el tracto respiratorio, define las partículas inhalables, como aquellas susceptibles de inhalación a través de la nariz y de la boca; las partículas torácicas, como aquellas susceptibles de inhalación que pueden penetrar en la laringe; y las partículas respirables, como aquellas que pueden atravesar la laringe y penetrar en el sistema respiratorio no ciliado (UNE-EN, 1993).

La exposición a bioaerosoles no es exclusiva de animales y ganaderos ya que los bioaerosoles pueden emitirse al exterior de los alojamientos ganaderos y ser transportados por el aire (Dungan, 2010; Schulz et al., 2011; Thorne et al., 2009; Takai et al., 1998). La dispersión del PM y bioaerosoles, especialmente patógenos en el aire ambiente, puede suponer un riesgo para el medio ambiente y para la salud de las poblaciones vecinas, sobre todo en ciertos segmentos de la población, como niños, ancianos o personas con trastornos pulmonares crónicos.

Se ha demostrado que el nivel de bioaerosoles en áreas con alta densidad ganadera es superior que en áreas no ganaderas (Hartung, 1992). Sin embargo, a pesar de la evidencia de los riesgos relacionados con la exposición a bioaerosoles de las poblaciones cercanas, su impacto en el medio social todavía no ha sido estudiado en profundidad.

Un mayor conocimiento de la calidad del aire de las explotaciones ganaderas es fundamental para mejorar la salud de los ganaderos y animales. Identificar las principales fuentes generadoras del PM y bioaerosoles es necesario para caracterizar las partículas y desarrollar medidas de reducción eficientes que puedan contribuir a mejorar la calidad del aire y la salud respiratoria humana y animal. Asimismo, conocer las interacciones entre PM y bioaerosoles y los efectos sobre la salud resulta imprescindible.

1.3. Origen y transmisión del material particulado y los bioaerosoles en alojamientos ganaderos

La presencia del PM y bioaerosoles en alojamientos ganaderos y su emisión al exterior depende de diversos procesos que se extienden desde su generación en el interior hasta su emisión y dispersión al exterior. Estos procesos que se pueden clasificar en: generación, suspensión al aire-sedimentación, emisión y dispersión (Figura 1.2), definen las rutas de transmisión vía aérea del PM y bioaerosoles. Cada uno de estos procesos está influenciado por una serie de factores relacionados con la especie animal, el tipo de alojamiento y las condiciones ambientales, entre otros (Takai et al., 1998), además de factores relacionados con las partículas (Figura 1.2).

La Figura 1.2 describe los procesos y factores que intervienen en la transmisión del PM y los bioaerosoles. Por tanto, conocer las propiedades de los bioaerosoles y comprender la interacción del PM con los bioaerosoles y qué papel desempeña el PM en la transmisión de éstos, es esencial para identificar el origen y el comportamiento de los bioaerosoles. Esta información contribuirá a desarrollar técnicas efectivas de reducción en el origen y a disminuir la concentración de bioaerosoles tanto en el interior como en el exterior de los alojamientos

ganaderos. En este sentido, el principal objetivo es evitar la generación y suspensión en el aire de los bioaerosoles y la proliferación de microorganismos en el interior de las naves, especialmente patógenos, para controlar su dispersión al exterior y el potencial riesgo de contagio entre naves o explotaciones.

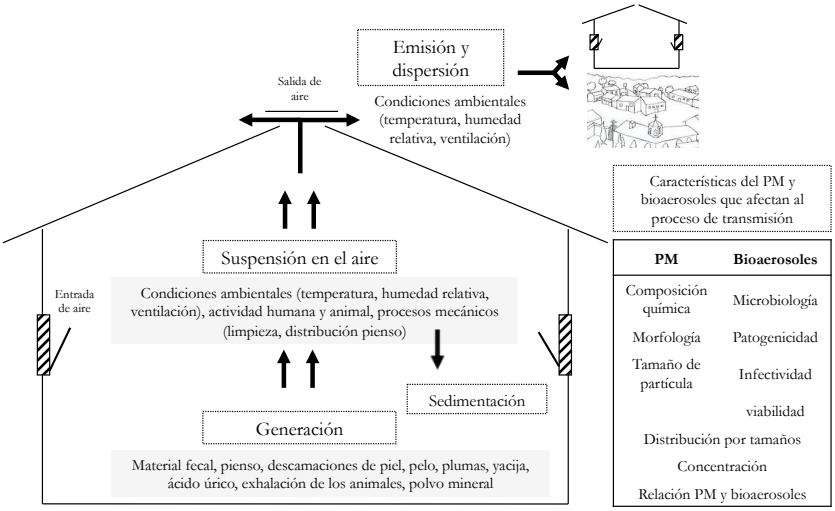

Figura 1.2. Rutas en la transmisión vía aérea del PM y de los bioaerosoles en alojamientos ganaderos y características relacionadas que afectan a la transmisión. Fuente: Autor (2012).

A continuación se detallan las características y factores que afectan a la transmisión del PM y bioaerosoless en explotaciones ganaderas, que son: generación, suspensión en el aire y emisión y dispersión (Figura 1.2).

1.3.1. Generación

El PM asociado a los bioaerosoles está básicamente compuesto por material fecal, pienso, descamaciones de piel, pelo, y polvo mineral (Figura 1.2) (Cambra-López et al., 2010). Las fuentes generadoras del PM varían de una especie animal a otra dependiendo del sistema de alojamiento. Por ejemplo, en aves, las fuentes generadoras del PM más importantes son las plumas, la yacija y los cristales de ácido úrico (Aarnink et al., 1999). En porcino las fuente más abundante son las heces (Cambra-López et al., 2011a) y en conejos, el pelo, descamaciones

de piel, material fecal, ácido úrico, pienso, yacija y desinfectantes son fuentes generadoras del PM.

Estas partículas suspendidas del interior de los alojamientos ganaderos muestran diferentes morfologías dependiendo de su origen (Cambra-Lopez et al., 2011b). Por ejemplo, las partículas de plumas son generalmente puntiagudas, estrechas y largas. Las partículas procedentes de material fecal pueden ser o bien, partículas fragmentadas, rugosas y angulares o bien, partículas esféricas lisas compuestas por partículas de ácido úrico. En la Figura 1.3 se muestran imágenes de muestras del aire de una nave de gallinas ponedoras (Figura 1.3a) y una nave de conejos (Figura1.3b). Las imágenes fueron tomadas al microscopio electrónico de barrido y muestran la composición heterogénea del PM que se encuentran suspendidas en el aire.

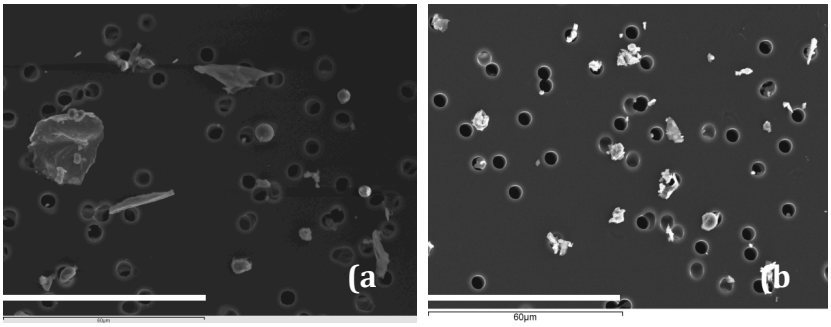

Figura 1.3. Muestras del aire recogidas en filtros de policarbonato en una nave de gallinas ponedoras (a) y una nave de conejos de cebo (b). Imágenes tomadas con el microscopio electrónico de barrido (1000x). Los círculos negros corresponden a poros del filtro de 5 µm de diámetro. Barra de escala: 60 µm. Fuente: Autor (2013).

El PM en alojamientos de aves y cerdos está compuesto de elementos químicos como el nitrógeno, sodio, magnesio, aluminio, silicio, azufre, cloro, potasio y calcio. Muchos de estos elementos son comunes a estructuras biológicas (plumas y piel), otros elementos como el aluminio y silicio son comunes en el polvo mineral (Cambra-López et al., 2011b).

La principal fuente generadora de bioaerosoles en alojamientos ganaderos son los propios animales. Los microorganismos se generan principalmente a partir del material fecal (Chien et al., 2011; Zucker et al., 2000) y en menor medida a través de la exhalación de los animales

infectados. Mediante la respiración, la tos o estornudos, los microorganismos de las vías respiratorias pueden liberarse al aire.

Otras fuentes importantes generadoras de bioaerosoles son materiales orgánicos como el pienso y la cama (Cox and Wathes, 1995). Se ha detectado bacterias en el pienso, incluso microorganismos patógenos como *Salmonella* spp. o bacterias coliformes (Hofacre et al., 2001; Kinley et al., 2010). En los alojamientos en los que hay cama, la concentración de microorganismos en aire es superior que en aquellos en los que no existe material de cama (Madelin T. and Wathes, 1989; Vucemilo, 2007).

En cuanto a la composición microbiológica, los microorganismos más comúnmente aislados en los alojamientos ganaderos son las bacterias Gram positivas, siendo las bacterias de los géneros *Staphylococcus* y *Streptococcus* las que predominan, aunque se han identificado *Bacillus*, *Micrococcus*, *Proteus* y *Pseudomonas*, entre otras, en alojamientos ganaderos (Matkovic et al., 2007). Estas bacterias han sido reconocidas como responsables de diferentes infecciones humanas (Gunn and Davis, 1988; Degener et al., 1994; Razonable et al., 2001). Entre las bacterias Gram negativas, las bacterias de la familia *Enterobacteriaceae* son las más abundantes.

El tamaño medio del PM y bioaerosoles en alojamientos ganaderos varía generalmente entre 2 y 18 μm y depende fundamentalmente de su origen mineral u orgánico. Las estructuras biológicas como por ejemplo la piel o el pelo tienen un mayor tamaño que otras partículas como por ejemplo las procedentes del pienso y las minerales que suelen ser las de menor tamaño. El tamaño de los bioaerosoles varia generalmente entre 0,5 y 100 μm y está limitado por el propio tamaño del microorganismo, sin embargo puede adherirse a partículas de mayor tamaño alcanzando mayores tamaños de partículas.

1.3.2. Suspensión en el aire

Tras la generación de las partículas y bioaerosoles en el interior de los alojamientos ganaderos, éstas pueden suspenderse al aire. Estas partículas suspendidas pueden salir al exterior a través de los ventiladores y dispersarse en el medio ambiente o pueden sedimentar en el interior

sobre las superficies o sobre los animales (Figura 1.2). La suspensión en el aire tanto del PM como de bioaerosoles está muy relacionada con la actividad humana y animal, los procesos mecánicos y las condiciones ambientales (Figura 1.2).

La actividad animal es el principal factor de la suspensión del PM y bioaerosoles. Por ejemplo, el aleteo de las gallinas crea turbulencias a su alrededor que conllevan a una mayor generación y suspensión de PM (Qi et al., 1992). Durante las horas de luz, la concentración de PM y bioaerosoles en el aire es superior a la de las horas de oscuridad debido a una mayor actividad animal (Li et al., 2008). Esta diferencia se hace más evidente sobre todo en alojamientos de gallinas ponedoras, en las que la concentración del PM durante las horas de luz duplica los niveles del PM en horas de oscuridad y los niveles de bacterias totales en el aire pueden variar hasta un orden de magnitud (Takai et al., 1998).

La suspensión en el aire del PM y bioaerosoles está también influenciada por las diferentes actividades relacionadas con las actividades rutinarias de las granjas (Ni et al., 2012). Una de las actividades que más influye es el reparto de pienso, momento en el que se incrementa la concentración del PM y los bioaerosoles puntualmente (Bundy and Hazen, 1975; Honey and McQuitty, 1979). Además de la variación diaria, puede existir una variación semanal del nivel de partículas suspendidas relacionada también con las distintas actividades de una granja, como por ejemplo la extracción de la gallinaza en granjas de gallinas ponedoras o barrer. El efecto sobre la suspensión del PM y bioaerosoles en este sentido no ha sido estudiado hasta el momento.

Las condiciones ambientales como la humedad relativa y la temperatura son también factores relacionados con la suspensión del PM y los bioaerosoles. Una humedad relativa en el ambiente elevada reduce la concentración de PM y bioaerosoles en el aire, el contenido de agua de las propias partículas previene que se suspendan al aire, mientras que bajas humedades propician altas concentraciones (Vucemilo et al., 2008).

La tasa de ventilación es un factor determinante que favorece la suspensión a través de una mayor velocidad de aire y turbulencias sobre las fuentes generadoras del PM y bioaerosoles (Lin et al., 2012). La tasa

de ventilación se relaciona con la estación del año. Durante el verano, las concentraciones del PM tienden a ser menores que en invierno ya que la temperatura es muy elevada y es necesaria una alta tasa de ventilación, que ayuda a la extracción de las partículas suspendidas; mientras que en invierno, las concentraciones son superiores debido a una menor tasa de ventilación. Por otro lado, la ventilación está muy relacionada con la humedad relativa, ya que en alojamientos con menor ventilación la humedad relativa es mayor y por lo tanto la sedimentación del PM y los bioaerosoles asociados a éste es mayor (Yao et al., 2010).

1.3.3. Emisión y dispersión

Los compuestos que se encuentran en suspensión en el interior de los alojamientos ganaderos pueden ser emitidos al exterior de las naves a través de las salidas de ventilación (cuando se trata de alojamientos con ventilación forzada) o por las ventanas (en alojamientos con ventilación natural) (Figura 1.2).

La emisión del PM y bioaerosoles al exterior de las naves varía de una especie animal a otra, además, está determinada por la concentración de estas sustancias en el interior del alojamiento y por la tasa de ventilación. Los alojamientos de broilers son los que mayor cantidad de PM y bioaerosoles emiten al exterior (Lacey et al., 2003; Takai et al., 1998; Seedorf et al., 1998).

De la misma manera que ocurre en el interior de los alojamientos, las partículas emitidas al exterior tienden a sedimentar más o menos rápido dependiendo de sus características, sobre todo de su tamaño. La velocidad de sedimentación de las partículas es el principal factor que afecta a la distancia de transmisión del PM y bioaerosoles. El tamaño de partícula determina el tiempo de permanencia en el aire, de modo que partículas más finas permanecen mayor tiempo en el ambiente y partículas más gruesas sedimentan más rápidamente (Tegen and Lacis, 1996), por tanto, las partículas más grandes permanecen más cerca de los alojamientos (Tegen and Lacis, 1996). Las partículas finas pueden tener mayores repercusiones sobre la salud y el ambiente ya que alcanzan mayores distancias y pueden alcanzar poblaciones cercanas (Seinfeld and Pandis, 1998).

Los bioaerosoles, durante el tiempo que permanecen suspendidos en el aire, podrían ser transportados por la acción del viento a distancias que pueden variar desde unos pocos metros hasta varios kilómetros, dependiendo del tamaño del microorganismo o del tamaño del PM al cual vaya unido. Aunque se ha confirmado una fuerte disminución exponencial a los 100 m de distancia de granjas de aves (Schulz et al., 2011; Müller and Wieser, 1987), algunos autores han encontrado un número significativo de bacterias viables procedentes del alojamiento ganadero a 160 metros de distancia en la dirección del viento en una granja porcina (Thorne et al., 2009). Del mismo modo, Herber et al. (2001) detectó bacterias viables a 800 metros de distancia en la dirección del viento bajo condiciones favorables en una granja de broilers.

La viabilidad de los bioaeoroles en el aire está condicionada por diferentes factores, en primer lugar dependerá de la forma y tamaño del PM al cual van unidos, éste puede proteger al microorganismo de condiciones climáticas adversas (Cox and Wathes, 1995). En segundo lugar, el micro-ambiente que se genera en la partícula podría favorecer la supervivencia de los microorganismos (White, 1993). En tercer lugar, las condiciones ambientales como la temperatura, humedad relativa y radiación solar del exterior influirán en la supervivencia de bioaerosoles y su estabilidad durante el proceso de dispersión (Bateman et al., 1962; Cox and Wathes, 1995). Por tanto, la relación e interacción entre el PM y los microorganismos en el aire es clave para la supervivencia de éstos.

El grado de supervivencia de las bacterias en el aire es variable, debido a su diversidad estructural y metabólica. En general, las bacterias Gram positivas son más resistentes que las Gram negativas ya que su pared celular es más gruesa. Por ejemplo, en aire seco algunas especies de *Bacillus* y *Clostridium* son capaces de sobrevivir más de 200 años, y otras como por ejemplo *Mycobacterium*, un mes.

Debido a la complejidad de los factores que intervienen en el proceso de dispersión, el grado de supervivencia e infectividad de los bioaerosoles no ha sido suficientemente descrito aún para comprender el alcance de los problemas sanitarios y de salud que pudieran ocasionar al ser emitidos al exterior, sobre todo los bioaerosoles patógenos.

1.4. Marco legal y valores límite de exposición del material particulado y los bioaerosoles suspendido en el aire

Las Directivas Europeas que establecen los niveles máximos de exposición del PM, clasifican el PM según su "diámetro aerodinámico" y distinguen dos tamaños de partículas: PM10 y PM2,5. Partículas de un tamaño similar pero de diferente forma y densidad se comportan de manera diferente en el aire. Por ello se utiliza el término "diámetro aerodinámico" para describir el tamaño de partícula. El diámetro aerodinámico se define como el diámetro de una partícula esférica con una densidad de 1 g/cm^3 que tendría la misma velocidad de sedimentación que la partícula en cuestión (Baron and Willeke, 1993).

En estas directivas se define la fracción PM2,5 como "las partículas que pasan a través de un cabezal de tamaño selectivo para un diámetro aerodinámico de 2,5 μm con una eficiencia de corte del 50%" y la fracción PM10 como "las partículas que pasan a través de un cabezal de tamaño selectivo para un diámetro aerodinámico de 10 μm con una eficiencia de corte del 50%". Esta nomenclatura se utiliza preferentemente para referirse a calidad del aire. Estas fracciones podrían asimilarse a las fracciones relativas a la salud laboral y ocupacional referidas en la norma europea UNE EN 481 (UNE-EN, 1993). De este modo, las partículas respirables serían comparables al PM2.5 y las partículas torácicas comparables al PM10 aunque el rango de tamaños no coincida exactamente (Cambra-López et al., 2010). El término TSP (del inglés, Total Suspended Particles) se utiliza para referirse al total del PM que se encuentra suspendido en el aire. Generalmente incluye partículas entre 30 y 100 μm de diámetro, comparables a las partículas inhalables.

Los niveles máximos de concentración del PM en el aire ambiente para la protección de la salud humana están regulados por la "Directiva 2008/50/CE relativa a la calidad del aire ambiente y a una atmósfera más limpia en Europa". Esta Directiva recoge el valor límite diario para PM10 de 25 $\mu g/m^3$, que no debe excederse más de 35 días por año y el límite medio anual de 20 $\mu g/m^3$. Para PM2,5 establece un valor límite medio anual inferior de 12 $\mu g/m^3$.

En ambientes ganaderos, la naturaleza heterogénea y la variabilidad de compuestos que forman parte del PM y bioaerosoles complica la tarea de establecer límites de exposición de estos dos compuestos. La Tabla 1.1 recoge a nivel descriptivo niveles de concentraciones de PM habituales en alojamientos de aves y conejos según el sistema de alojamiento, el método de muestreo utilizado y por países. El método de muestreo de referencia del PM es el método gravimétrico.

Los niveles del PM más elevados se encuentran en alojamientos de broilers con yacija, alcanzando valores máximos para PM10 de 11,4 mg/m^3 (Redwine et al., 2002) y para PM2,5 de 1,9 mg/m^3 (Ellen et al., 1999). Esto es debido probablemente a la presencia de la cama y al contacto de los animales no solo con el material de cama sino con las deyecciones (Cambra-López et al., 2011a), a las altas tasas de ventilación y a la elevada densidad animal. Las gallinas alojadas en aviario se exponen también a concentraciones elevadas (0,25-0,40 mg/m^3 para PM2,5 y 2,3-2,8 mg/m^3 para PM10) (Hayes et al., 2013; Wathes et al., 1997) en comparación a las gallinas alojadas en batería (0,04-0,27 mg/m^3 para PM2,5 y 0,39-2,8 mg/m^3 para PM10) (Li et al., 2011; Wathes et al., 1997). Los alojamientos de conejos son los que presentan menores concentraciones de partículas en el aire (0,19-0,90 mg/m^3 para PM10 y 0,02 y 18,05 mg/m^3 para partículas totales) comparado con las aves (Navarotto et al., 1995; Ooms et al., 2008; Ribikauskas et al., 2006; Sancilio et al., 1999).

Tabla 1.1. Concentración del PM en el aire aire de granjas de aves y conejos según sistema de alojamiento y método de muestreo.

Especie animal	Sistema de alojamiento	Partículas totales (mg/m³)	PM10 (mg/m³)	PM2,5 (mg/m³)	Método de muestreo	País	Referencia
Broilers	Cría con cama	-	10,1	1,2	Gravimetría	Reino Unido	Wathes et al. (1997)
		-	9,2-11,1	-		Escocia	Al Homidan et al. (1998
		-	3,8-10,4	0,4-1,1	Gravimetría	Norte Europa	Takai et al. (1998)
		-	8,2-9,0	1,4-1,9	Gravimetría	Holanda	Ellen et al (1999)
		-	2,3-8,6	0,3-1,8	Gravimetría	Australia	Banhazi et al. (2008)
		-	2,0-4,9	-	Gravimetría	Croacia	Vucemilo et al. (2008)
		-	0,7-11,4	-	Gravimetría	EEUU	Redwine et al. (2002)
		-	1-14	-	Gravimetría	Alemania	Hinz and Linke (1998)
		-	0,03-4,5	-	Gravimetría	Polonia	Lawniczek-Walczyk et al (2013)
Gallinas	Batería	0,8-1,6	-	-	Gravimetría	Norte Europa	Takai et al. (1998)
		-	1,7	0,3	Gravimetría	Reino Unido	Wathes et al. (1997)
		2,4	0,6	-	TEOM	EEUU	Lim et al. (2007)
		-	0,4	0,04	TEOM	EEUU	Li et al. (2011)
	Aviario	2,2	-	-	Gravimetría	Norte Europa	Takai et al. (1998)
		-	2,8	0,4	Gravimetría	Reino Unido	Wathes et al. (1997)
		-	2,3	0,3	TEOM	EEUU	Hayes et al. (2013)
Conejos	-	0,1	-	-	Gravimetría	Italia	Kaliste et al. (2002)
	-	0,4	0,2	-	Gravimetría	Italia	Navarotto et al. (1995)
	-	0,8-3,6	0,2-0,9	-	Gravimetría	-	Sancilio et al. (1999)
	-	8,5-18,1	-	-	-	Lituania	Ribikauskas et al. (2010)
	-	0,02	-	-	Gravimetría	EEUU	Ooms et al. (2008)

TEOM: Tapered Element Oscillating Microbalance

La Tabla 1.2 recoge los niveles de concentración de bacterias aerobias mesófilas cultivables en el aire de granjas avícolas y cunícolas, según el sistema de alojamiento, el método de muestreo utilizado y por países.

El rango de concentraciones de bacterias aerobias mesófilas varía desde 10^1 hasta 10^7 UFC/m^3 según la especie. Las concentraciones más elevadas se encuentran de nuevo en general en alojamientos de broilers y las más bajas en alojamientos de conejos. No obstante, la comparación de los valores de concentración de bioaerosoles debe realizarse con cautela por la variabilidad que puede resultar de utilizar diferentes métodos de muestreo.

Actualmente no existe un marco legal específico para ambientes agrícolas o para alojamientos ganaderos referido al PM o bioaerosoles. Como consecuencia de la falta de legislación respecto a los niveles máximos de exposición del PM y bioaerosoles en ambientes ganaderos, se han propuesto distintas recomendaciones, sobre todo para el PM, ya que establecer límites de exposición para los bioaerosoles es más complicado por la falta de un método estandarizado de medición y la gran variabilidad de microorganismos que se encuentran suspendidos en el aire. Como regla general, los niveles máximos admisibles de PM recomendables para proteger a los animales son 3,7 mg/m^3 para partículas totales y 1,7 mg/m^3 (0,23 mg/m^3 para porcino) para partículas respirables (comparables a PM2,5) (CIGR, 1992; CIGR, 1994).

Tabla 1.2. Concentración de bacterias aerobias mesófilas en el aire de granjas de aves y conejos según sistema de alojamiento y método de muestreo.

Especie animal	Sistema de alojamiento	Concentración bacterias aerobias mesófilas (UFC/m^3)	Método de muestreo	País	Referencia
Broilers	Cría con cama	$7{,}7x10^6$	Filtración	Alemania	Wiegand et al.(1993)
		$2{,}7x10^3$	Muestreador automático de bacterias	Norte de Europa	Seedorf et al. (1998)
		$5{,}3x10^7$	Impactación	Suiza	Oppliger (2008)
		$10{,}5x10^2$-$2{,}9x10^6$	Impactación	Polonia	Lawniczek-Walczyk et al (2013)
Gallinas	Bateria	$4{,}2x10^5$	Impactación	Suecia	Clark et al. (1983)
		$1{,}7x10^5$	Filtración	Alemania	Saleh et al. (2007)
		$1{,}6x10^4$-$2{,}5x10^4$	Impactación	Croacia	Vucemilo et al. (2010)
	Aviario	$6{,}2x10^4$-$8{,}9x10^4$	Impactación	Croacia	Vucemilo et al. (2010)
	-	$1x10^4$-$1x10^5$	Muestreador automático de bacterias	Norte de Europa	Seedorf et al. (1998)
		$7{,}1x10^3$-$2{,}9x10^4$	Borboteador de aire	EEUU	Woodward et al. (2004)
Conejos	-	$1{,}0x10^2$	Impactación	Italia	Kaliste et al. (2002)
	-	$7{,}8x10^2$-$2{,}0x10^5$	Impactación	-	Duan et al. (2006)
	-	$4{,}9x10^1$-$2{,}7x10^1$	Impactación	Lituania	Ribikauskas et al. (2010)

Estudios como el de Donham (1991) basados en ensayos experimentales, establecieron concentraciones máximas recomendadas del PM y bacterias suspendidas en el aire en estudios dosis-respuesta en ganado porcino y humanos, resultando en los valores que se muestran en la Tabla 1.3.

Tabla 1.3. Parámetros de calidad del aire máximos recomendados en ganado porcino y humanos. Fuente: Donham (1991)

Partículas totales	2,4 mg/m^3
Partículas respirables	0,16 mg/m^3
Bacterias totales	10^5 UFC/m^3

Otras recomendaciones como la del Instituto de Investigación de Salud Laboral y Seguridad (IRSST) de Canadá, sobre la exposición a bacterias recomienda no sobrepasar 10^4 UFC/m^3 en ambientes agrícolas (Goyer et al, 2001). En Polonia, el límite propuesto por Karwowska (2004) es $2x10^5$ en alojamientos de aves y cerdos.

1.5. Bioaerosoles patógenos: Problemática y medición

La presencia de patógenos en las explotaciones ganaderas es habitual y puede suponer un riesgo importante para la salud humana y animal (Eduard et al., 2012). Numerosos patógenos como *Escherichia coli*, *Enterococus faecalis, Campylobacter* spp. o *Salmonella* spp. han sido identificados en granjas como los bioaerosoles patógenos más habituales (Brodka et al., 2012; Davis and Morishita, 2005; Chinivasagam et al., 2009). La presencia de algunos de estos patógenos, como por ejemplo *Salmonella* spp. en granjas de broilers, presenta un riesgo para el consumidor. Otros patógenos como por ejemplo *Mycoplasma* spp. provoca un descenso importante en el rendimiento productivo de gallinas ponedoras.

En qué medida se produce la transmisión de microorganismos patógenos desde los alojamientos ganaderos hacia las poblaciones vecinas o hacia otras explotaciones a través del aire es una cuestión que permanece sin clarificarse. Se han detectado patógenos en el exterior de los alojamientos ganaderos. Davis y Morishita (2005) aislaron *Salmonella* spp. y *E. coli* a 12 metros de una explotación de gallinas ponedoras y algunos patógenos han sido detectados hasta 9 kilómetros de distancia de la granja (Otake et al., 2010). Incluso se ha confirmado la infección de granja a granja. Dee et al. (2010) detectaron *Mycoplasma* spp. en una granja porcina proveniente de otra granja situada a 120 metros en la dirección del viento. Aunque se conoce la distancia de transmisión de

algunos patógenos en determinadas condiciones, no se conoce ciertamente el riesgo de infección de estos patógenos emitidos.

En todo caso, la presencia de un patógeno no implica que vaya a causar infección. Para que se produzca infección es necesario que un humano o animal susceptible se exponga a una cantidad de organismos suficientes capaces de causar la enfermedad (dosis infectiva) (Blackall et al., 2010). La dosis infectiva es la cantidad de organismos necesarios para producir reacción en el 50% de los individuos expuestos. La relación dosis-respuesta no ha sido establecida para muchos agentes biológicos. Por ejemplo, la dosis infectiva para *Salmonella* spp. se ha establecido entre 10^3 y 10^5 organismos, dependiendo de la cepa y de la edad y condiciones físicas de los individuos susceptibles a ser infectados (Blaser and Newman, 1982; Kothary and Babu, 2001). Para *Campylobacter* spp. se ha establecido una dosis infectiva de $5x10^2$ organismos (Blackall et al., 2010). Por tanto, además de conocer la presencia o no de patógenos en el ambiente, es importante conocer la cantidad de organismos viables que alcanzan una determinada distancia que puede poner en peligro la salud de las personas de poblaciones vecinas o de animales de otras explotaciones cercanas.

El muestreo de PM y bioaerosoles en alojamientos ganaderos es cada vez más frecuente para evaluar la calidad del aire y diseñar posibles medidas de reducción. Sin embargo, al contrario que para el PM, no existe un muestreador de referencia para bioaerosoles ya que todavía no se ha desarrollado un muestreador que tenga una eficiencia del 100% en la recogida de microorganismos. Este hecho dificulta la evaluación del riesgo de los bioaerosoles procedentes de fuentes ganaderas, ya que la falta de un método de referencia dificulta la comparación de resultados obtenidos con métodos diferentes.

Los equipos de muestreo más frecuentemente utilizados para bioaerosoles se basan en tres principios: impactación, borboteo o filtración (EN 13098:2001). Cada uno de ellos presentan ventajas e incovenientes para el muestreo de microorganismos relacionados sobre todo con el tiempo de muestreo y la supervivencia de los mismos. Los equipos basados en la impactación permiten discriminar las partículas según su tamaño (Andersen, 1958). Además, las bacterias impactan

directamente en placas de agar que son incubadas directamente. Sin embargo, el tiempo de muestreo debe ser corto para no sobresaturar las placas debido a la elevada concentración de bacterias en los alojamientos ganaderos, por lo que podrían obtenerse muestras no representativas. Los equipos basados en el borboteo, no tienen problemas con la saturación ya que la muestra de aire se recoge en un medio líquido del que se pueden hacer diluciones, sin embargo, no se puede muestrear durante largos periodos de tiempo debido a la evaporación del medio líquido (Lin et al., 1997). La filtración es un método práctico, sin embargo, no es adecuado para muestrear microorganismos que son vulnerables a la deshidratación.

A pesar de que distintos autores han comparado la eficacia de los métodos de muestreo utilizados para la cuantificación de microorganismos específicos en el aire (Juozaitis, 1994; Li et al., 1999; Terzieva et al., 1996; Thorne et al., 1992), no existe consenso sobre el método más adecuado para muestrear un amplio espectro de microorganismos. Algunos autores concluyeron que para el muestreo de bacterias totales el muestreador más eficaz fue el basado en los borboteadores (Li et al., 1999; Thorne et al., 1992). Sin embargo, métodos basados en la filtración o impactación han mostrado mayor eficiencia en otros estudios (Juozaitis, 1994; Zhao et al., 2011). El muestreo de bioaerosoles patógenos presenta mayores dificultades ya que se encuentran en bajas concentraciones. Por tanto, es necesario desarrollar técnicas de muestreo con menores límites de detección para detectar bioaeoroles patógenos y asegurar de esta forma la evaluación de la exposición a patógenos en alojamientos ganaderos.

Además de la elección del método de muestreo más eficaz en cada ocasión, la variabilidad de agentes biológicos, sus comportamientos y sus requerimientos para sobrevivir y crecer dificultan el estudio de la transmisión de bioaerosoles. Por esta razón, recientemente muchos autores han utilizado técnicas moleculares, como la PCR (del inglés, Polymerase Chain Reaction) (Chang et al., 2010; Eriksson and Aspan, 2007; Fallschissel et al., 2009). Esta técnica consiste en la amplificación de una región específica de la cadena del ADN que identifica el microorganismo en cuestión y de esta forma se detecta más fácilmente

su presencia. La técnica de PCR permite detectar y/o cuantificar bacterias, incluso muertas. El uso de PCR para detectar patógenos suspendidos en el aire proporciona resultados rápidos y sensibles en la detección de niveles bajos de patógenos específicos (Álvarez et al., 1995). El límite de detección de la PCR es menor que las técnicas de cultivo ya que se puede detectar una única célula de la muestra (Álvarez et al., 1995). Estudios recientes muestran que las técnicas de cultivo subestiman los niveles de bacterias totales en comparación con las técnicas moleculares (Nehme et al., 2008; Zhao et al., 2011).

Además de las ventajas que presenta el método molecular de PCR para la detección de patógenos, su utilización presenta algunas limitaciones ya que no aporta información sobre la viabilidad del patógeno. Conocer si el patógeno está vivo o muerto es clave para conocer si podría poner en peligro la salud humana y animal (Keer and Birch, 2003).

1.6. Medidas para reducir el material particulado suspendido y los bioaerosoles en explotaciones ganaderas

Existe una necesidad de reducir el PM y los bioaerosoles de las explotaciones ganaderas tanto por los problemas de salud que genera en humanos y animales como por la disminución del rendimiento productivo y las consecuencias económicas que ello implica, así como por los problemas medioambientales que pudiera ocasionar. En la actualidad se están estudiando y mejorando diferentes técnicas de reducción, sobre todo del PM.

Las medidas para reducir el PM y los bioaerosoles en alojamientos ganaderos deben adaptarse al sistema de producción animal (e.g. porcino de engorde en suelo emparrillado, broilers sobre cama, gallinas ponedoras en batería) y a las condiciones ambientales en el interior de estos alojamientos, especialmente al caudal de ventilación, a la velocidad de aire cerca de las fuentes generadoras de bioaerosoles, a la humedad relativa elevada y a los rangos variables de temperatura.

Teniendo en cuenta la Figura 1.2, las estrategias de reducción se pueden dividir en: medidas que evitan la generación y suspensión en el aire

(reducción en origen) y medidas que evitan su emisión a la atmósfera y dispersión (purificación y limpieza de aire).

La mayoría de las medidas de reducción del PM y bioaerosoles en origen implican modificaciones en el pienso y el uso de aditivos. Normalmente se basan en cambios a dietas más húmedas (aplicable en porcino donde se puede cambiar a alimentación líquida) y en aumentar el contenido en grasa del pienso mediante la adición de una cobertura de grasa o aceite.

La medidas cuyo objetivo es evitar que las partículas y/o bioaerosoles una vez ya generados, puedan suspenderse en el aire han demostrado ser efectivas, sobre todo para reducir las concentraciones de PM, ya que su efecto sobre bioaerosoles ha sido poco investigado. Estas técnicas se basan en favorecer la agregación de las partículas y así su sedimentación. Algunos ejemplos son la pulverización con agua y aceite (Takai and Pedersen, 2000; Zhang et al., 1995) o desinfectantes (Zheng et al., 2014) y la ionización del aire (Cambra-López et al., 2009).

La técnica de pulverización de aceites o mezclas de agua y aceites vegetales se basa en crear una capa fina de aceite sobre las superficies (cama, suelo y animales) y así evitar que las partículas se desprendan. Trabajos recientes han demostrado que se trata de una medida práctica para reducir el PM en alojamientos para broilers (Aarnink et al., 2011) y gallinas (Winkel et al., 2010), aunque todavía se desconoce la dosis y la frecuencia óptima de aplicación. Además, existe poca información de su efecto sobre las concentraciones de bioaerosoles y patógenos concretos, algunos estudios en porcino han demostrado una reducción de hasta un 53% en las bacterias totales y un 30% en el PM suspendido total (Griffin and Vardaman, 1970; Rule et al., 2005).

La desinfección del aire mediante la aplicación de sustancias desinfectantes a través de la pulverización o nebulización es otra técnica en vías de estudio. Mediante la aplicación de desinfectantes se reduce la viabilidad de los bioaerosoles. El agua electrolizada, utilizada recientemente en la industria alimentaria para la desinfección de los alimentos por su poder antimicrobiano, ha demostrado ser eficaz, seguro, fácil de manipular, relativamente barato y ecológico en otros ámbitos (Huang et al., 2008). En el ámbito de la ganadería, se ha

estudiado diferentes aplicaciones del agua electrolizada ácida como agente de limpieza y desinfectante de superficies, higienizante del agua de bebida o desinfección aérea (Tabernero de Paz et al., 2013). Trabajos realizados en alojamientos porcinos demuestran una reducción de bacterias totales aerotransportadas de hasta un 59% (Zheng et al., 2013). El uso de desinfectantes químicos también ha demostrado reducir la concentración de bacterias totales aerotransportadas hasta un 49% (Zheng et al., 2013). Sin embargo, del mismo modo que en el resto de técnicas de reducción de bacterias en el aire, todavía se desconoce la dosis y la frecuencia óptima de aplicación.

En último lugar, en cuanto a medidas de reducción en origen destaca la ionización del aire. Esta técnica se basa en la atracción electrostática de las partículas suspendidas en el aire mediante un generador de electrones. Las partículas cargadas negativamente por los iones circulantes, son atraídas por las superficies cargadas positivamente y especialmente diseñadas para recoger el polvo. Se trata de una medida efectiva, ya que alcanza reducciones medias del 10% de partículas finas y del 36% de partículas gruesas en alojamientos de broilers (Cambra-López et al., 2009) y entre 23% de partículas finas y 38% de partículas gruesas en gallinas en aviario (Winkel et al., 2010). La información respecto al efecto de la ionización del aire sobre las concentraciones de microorganismos es contradictoria. A pesar de que se ha descrito el potencial de la ionización negativa para matar microorganismos (Holt et al., 1999), Cambra-López et al. (2009) no observaron variación en las concentraciones de bacterias totales, enterobacterias ni hongos y mohos durante dos ciclos de producción en broilers utilizando este sistema.

Las medidas destinadas a la purificación y limpieza del aire son útiles en zonas con elevada densidad ganadera, y pueden utilizarse para evitar tanto la entrada de patógenos como su emisión y dispersión entre naves y/o complejos. Se trata de mecanismos que generalmente se instalan bien a la salida de los ventiladores para reducir la emisión o a la entrada del aire para evitar la penetración de patógenos. Son pues medidas de prevención y limpieza tanto del aire de entrada como de salida de las naves. Carpenter (1986) clasificó estos sistemas en: sistemas centrífuga (ciclones), filtros húmedos o lavadores, precipitadores electrostáticos y

filtros secos. Todos estos sistemas se comercializan actualmente, sin embargo la eficacia de reducción de estos equipos son variables y todavía es necesario optimizarlas para los diferentes sistemas productivos.

Para la elección de una técnica u otra de reducción del PM y/o microorganismos es necesario conocer el proceso de transmisión, desde su generación hasta su emisión al exterior, para detectar el momento del proceso de transmisión más adecuado en cada ocasión para actuar. Además, es necesario ensayar y evaluar medidas de reducción en origen para bioaerosoles patógenos de interés comercial en explotaciones ganaderas.

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2

Objetivos y estructura de la Tesis

2.1. Objetivos

Esta Tesis Doctoral aborda aspectos relacionados con la concentración, origen y propiedades físicas, químicas y biológicas del material particulado (del inglés, Particulate Matter; PM) suspendido en el aire de granjas de aves y conejos, su relación con los bioaerosoles patógenos y técnicas para reducirlos. Este trabajo pretende contribuir a paliar los efectos negativos de estas sustancias tanto en el interior de los alojamientos ganaderos como en el exterior.

Para ello, se han planteado cinco objetivos generales:

1. Caracterizar la morfología y la composición química del PM de distintos tamaños así como la concentración de bacterias en el aire de granjas de conejos.
2. Cuantificar la concentración y emisión del PM de distintos tamaños e identificar las principales actividades que contribuyen a la generación del PM en granjas de conejos.
3. Evaluar la distribución espacial de bacterias aerobias mesófilas en el aire durante un ciclo de producción de broilers y examinar su relación con la concentración y evolución del PM.
4. Evaluar y comparar diferentes técnicas para muestrear y detectar el patógeno *Salmonella* spp. en el aire de granjas de broilers.
5. Evaluar la aplicación de desinfectantes en el aire como medida de reducción de los bioaerosoles en granjas de gallinas ponedoras con especial atención al patógeno *Mycoplasma gallisepticum*.

2.2. Estructura de la Tesis

La presente Tesis Doctoral se estructura en nueve capítulos. El primer capítulo abarca la introducción general y el contexto de esta Tesis. En el segundo capítulo se plantean los objetivos generales. Los dos últimos capítulos abarcan la discusión general, conclusiones y líneas de trabajo

futuras. Los cinco capítulos intermedios corresponden con cada uno de los objetivos generales descritos anteriormente.

En el primer capítulo se ha analizado la información existente sobre la relación entre el PM y los bioaerosoles en explotaciones ganaderas, así como las rutas de transmisión, desde su generación en el interior de los alojamientos hasta su emisión al exterior, con especial interés en los bioaerosoles patógenos. Además se han revisado las medidas disponibles para reducir estas sustancias en el aire en explotaciones ganaderas y sus limitaciones. Asimismo, se han identificado los vacíos del conocimiento ("gaps of knowledge") que pudieran existir en este contexto.

En este capítulo 2 se plantean los objetivos generales de la presente Tesis Doctoral y se relaciona cada objetivo con cada uno de los capítulos.

En el capítulo 3 se caracterizan las fuentes del PM en explotaciones cunícolas desde el punto de vista morfológico y químico y se cuantifica la concentración de bacterias suspendidas en el aire. Este capítulo corresponde con el objetivo 1.

En el capítulo 4 se amplía la información existente sobre los niveles de concentración y emisión de PM en explotaciones cunícolas. Además, se estudian posibles factores que influyen en la generación del PM relacionados con la actividad humana y otras actividades diarias de manejo de los animales. Este capítulo corresponde con el objetivo 2.

En el capítulo 5 se evalúa la relación entre los niveles del PM y de bacterias aerobias mesófilas suspendidos en el aire de una explotación de broilers a lo largo de un ciclo productivo. Además se estudia la distribución espacial de las bacterias aerobias mesófilas en el aire y en distintos tamaños de partículas. Este capítulo corresponde con el objetivo 3.

En el capítulo 6 se aborda la problemática relacionada con el muestreo y la detección de patógenos en el aire. Se evalúan diferentes métodos de muestreo para detectar y/o cuantificar *Salmonella* spp. en una nave de broilers así como diferentes técnicas de análisis en el laboratorio basada en métodos de cultivo y técnicas moleculares. Este capítulo corresponde con el objetivo 4.

En el capítulo 7 se evalúa la aplicación de una técnica de reducción de bioaerosoles en el aire de una granja de gallinas ponedoras, con especial atención a sus posibles efectos sobre el patógeno respiratorio *Mycoplasma gallisepticum*. Este capítulo corresponde con el objetivo 5.

En el capítulo 8 se discute de forma general los resultados obtenidos en esta Tesis en el marco de las investigaciones recientes.

El capítulo 9 cierra esta Tesis Doctoral con las conclusiones más relevantes obtenidas de este trabajo, seguidas de unas recomendaciones de líneas de investigación futuras.

Los capítulos del 3 al 7 son artículos publicados en revistas indexadas internacionales y nacionales. A pesar de que el idioma principal de esta Tesis Doctoral es el castellano, se ha mantenido el idioma original de publicación de cada uno de los artículos, por lo que algunos de ellos están en castellano y otros en inglés.

3

Morphology, chemical composition, and bacterial concentration of airborne particulate matter in rabbit farms

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Abstract. Livestock houses are major sources of airborne particulate matter (PM) which can originate from manure, feed, feathers, skin and bedding; and contain and transport microorganisms. Improved knowledge on particle size, morphology, chemical and microbiological composition of PM in livestock houses, can help to identify major sources of PM and contribute to develop adequate source-specific reduction techniques. In rabbit production systems, however, there is limited information on specific particle characteristics. The objective of this study was to characterize airborne PM in rabbit farms in terms of morphology, chemical compositions and bacterial concentration in different size fractions. Size-fractioned PM was sampled in the air of a fattening rabbit and a reproductive doe farm with a virtual cascade impactor, which simultaneously collected total suspended PM (TSP), PM10 and PM2.5 size fractions. Airborne PM samples were examined using light microscopy and scanning electron microscopy combined with energy dispersive X-ray analysis. Representative samples from potential sources of PM were also collected and examined. Additionally, a methodology to extract bacteria from the collected samples of airborne PM was developed to determine the bacterial concentration per PM size fraction. Results showed that airborne PM in rabbit farms is highly complex in particle morphology, especially in size. Broken skin flakes, disintegrated particles from feed or faecal material from mechanical fracture are the main sources of airborne PM in rabbit farms. Major elements found in rabbit airborne PM were S, Ca, Mg, Na and Cl. Bacterial concentrations ranged from $1.7x10^4$ to $1.6x10^6$ colony forming unit, CFU/m^3 (TSP); from $3.6x10^3$ to $3.0x10^4$ CFU/m^3 (PM10); and from $3.1x10^3$ to $1.6x10^4$ CFU/m^3 (PM2.5). Our results will improve the knowledge on essential particle characteristics necessary to understand PM's origin in rabbit farms and contribute to its reduction.

Keywords. Air quality, animal housing, bioaerosol, characterization, dust, SEM-EDX.

3.1. Introduction

Livestock houses are major sources of airborne particulate matter (PM) which can originate from several sources: manure, feed, feathers, skin and bedding (Donham et al., 1986; Cambra-López et al., 2011a). The heterogeneous nature of PM in livestock houses comprises particles of different morphology and chemical composition (Cambra-López et al., 2010). Moreover, particle size is one of the most relevant properties related to the potential health and the environment hazards of PM (Harrison and Yin, 2000). In livestock environments, airborne PM includes size fractions ranging from fine (PM which passes through a size-selective inlet with a 50% efficiency cut-off at 2.5 μm, PM2.5), coarse (PM which passes through a size-selective inlet with a 50% efficiency cut-off at 10 μm, PM10), and total suspended particles (all airborne particles, TSP). Furthermore, particle size and morphology are very closely related to lung deposition (Zhang, 2004). Consequently, high concentrations of PM can cause detrimental effects on animal performance and efficiency (Donham and Leininger, 1984; Donham, 1991; Al Homidan and Robertson, 2003), and on the health and welfare of farmers (Andersen et al., 2004; Donham et al., 1984). Emitted PM can also cause detrimental effects on the environment (Grantz et al., 2003).

The morphology and chemical composition of PM depends on livestock species and housing systems. In poultry, Cambra-López et al. (2011a) reported that the most abundant sources of airborne PM are feathers and uric acid crystals; whereas in pigs, the most abundant sources are manure and pig's skin. In addition, PM can contain and transport microorganisms (fungi, viruses, bacteria, toxins and allergens), some of them pathogenic (Bakutis et al., 2004; Adell et al., 2011) which can cause direct harm to humans and animals. In rabbit production systems, however, there is limited information on specific particle characteristics such as morphology (i.e. shape, size and texture), chemical composition, and microbiological components of PM.

Improved knowledge on particle size, morphology, chemical and microbiological composition of PM in livestock houses, can help to identify major sources of PM. The best approach to reduce PM in and from livestock houses seems to be to prevent it from being generated directly from its source. Consequently, the characterization of PM in

livestock houses is essential to develop adequate reduction techniques. This better understanding would contribute to develop efficient and practical source-specific reduction techniques to comply with European thresholds set in air quality regulations (Directive 1999/30/EC and Directive 2008/50/EC), and to protect the environment, and human and animal health and welfare in and around rabbit farms.

The aim of this study was to characterize airborne PM in rabbit farms in terms of morphology, chemical compositions and bacterial concentration in different size fractions. Our results will improve the knowledge on essential particle characteristics necessary to understand PM's origin in rabbit farms and contribute to its reduction.

3.2. Material and methods

3.2.1. Housing and animals

Two rabbit farms were surveyed in this study: one rearing fattening rabbits, and another rearing reproductive does. Animals were reared in cages in both farms. Manure was accumulated in pits below the cages for 3-4 weeks. Both farms were located in the region of Valencia (East of Spain) and were surveyed during summer.

Average indoor temperatures in the surveyed farms were 27.8ºC and 19.7ºC for fattening rabbits and reproductive does respectively. Regarding relative humidity, average values inside both buildings were 66.5% for fattening rabbits and 61.4% for reproductive does. In the outdoors, temperature was 22.7ºC for fattening rabbits and 15.6ºC for reproductive does and relative humidity was 62.2% for fattening rabbits and 58.6% for reproductive does. Table 3.1 describes both surveyed farms in terms of housing and animals.

Table 3.1. Description of the surveyed rabbit farms

	Fattening rabbits	Reproductive does
Length x width (m)	30 x 6	45 x 7
Animal places	2100 fattening rabbits	400 reproductive does
Feed distribution	Manually distributed pellets	Manually distributed pellets
Ventilation	Tunnel mechanical 2 fans	Tunnel mechanical 1 fan

3.2.2. Particulate matter sampling

To characterize PM in rabbit farms, firstly, airborne PM was sampled in each farm. Secondly, additional samples from potential known sources of PM were collected and examined to compare airborne samples against a reference of each PM sources.

3.2.2.1. Airborne PM sampling

A virtual cascade impactor (RespiCon, Wetzlar, Germany) was used in each farm to sample PM2.5, PM10 and TSP in the air. Each PM size fraction was collected onto separate filters. Two types of filters were used: glass fibre filters (37 mm Ø, Helmut Hund, Wetzlar, Germany), for chemical, morphological and bacterial concentration analysis; and polycarbonate filters (37 mm Ø, 5 μm pore size), to examine in more depth PM characteristics and confirm previous results obtained using glass fibre filters. Portable pumps (Genie VSS5, Buck Inc, U.S.) were used to draw air through each virtual cascade impactor at a constant flow of 3.11 L/min.

Sampling was conducted inside each farm, in the centre of the building, at 1.5 m height. Sampling frequency and time was adjusted to obtain sufficient particles for morphological and chemical composition examinations, on the one hand; and bacterial concentration analysis, on the other. No gravimetric analyses were subsequently performed with filter samples. Samples used for morphological and chemical composition analyses were collected weekly, during 5 weeks for fattening rabbits and 2 weeks for reproductive doe buildings. Sampling duration was 6 days. Samples used for bacterial concentration examinations were collected twice in each facility. Sampling duration was 15 minutes, to minimize dehydration of bacteria. In this case, the virtual cascade impactor was disinfected with 96% alcohol prior to sampling and sterile

glass fibre filters were used. After sampling, filters were transported to the laboratory under refrigeration (4ºC).

3.2.2.2. Sampling for known sources of PM

Representative samples from potential sources of PM were obtained by randomly sampling at different locations in each building for feed, manure, hair, and powdered disinfectants normally used in rabbit farms (calcium superphosphate and sulphur). Composite samples were collected directly from farm surfaces, avoiding contamination among them. Each sample was then homogenized in the laboratory to achieve a uniform sample, and then dried in the oven for 12 h at 70 ºC. Dried samples were crushed manually in a mortar.

To obtain size-segregated PM samples from the different know sources, a dust generator was used to aerosolize PM. The dust generator consisted of a stainless steel cylinder of 20 cm diameter and 30 cm height with an airtight lid, which had a mechanical agitation system and rotating blades at the end. The aerosolization process of potential PM sources was conducted following the methodology and set-up described in Cambra-López et al. (2011b). The mass of sample and the dust generation time was adjusted depending on the sample. Approximately 40 g of feed, 3 g of manure, 0.4 g of hair, 1.2 g of sulphur, and 1 g of calcium superphosphate were introduced in the agitation system. Sampling time varied from 1 minute (sulphur and calcium superphosphate), 2 minutes (manure), 2 hours (hair) and 12 hours (feed). The generated PM during aerosolization was collected in TSP, PM10 and PM2.5 size fractions, using a virtual cascade impactor (RespiCon, Helmunt Hunt GmbH, Wetzlar, Germany) and a portable pump, same as for airborne PM sampling, using polycarbonate filters.

3.2.3. Particulate matter characterization

3.2.3.1. Morphology

Particle's morphology was studied using two microscopic techniques: light microscopy (LM) and scanning electron microscopy (SEM).

Major PM components in airborne PM collected on glass fibre filters were qualitatively and quantitatively analyzed using LM. Qualitative

analysis was conducted with direct observations using a Nikon Eclipse E400 microscope at 10x and 20x magnification, and photomicrographs were taken with a Nikon Ds-5M Camera, coupled to the microscope. A representative area of the glass fibre filter collected in the air of each farm was cut and mounted on a glass slide. At least four views (spots) per filter were examined. The different identified components in PM were described in terms of their size and morphology. Iodine (dilution 1:10 of iodine in distilled water) was used to stain starch granules and identify feed particles, by directly pipetting 1 to 3 mL of dilution onto the filter, following Donham et al. (1986). Quantitative analysis of the different components found in the airborne PM fractions was performed, as well. The PM components were counted in each examined view per filter.

Furthermore, samples of airborne PM collected on polycarbonate filters were analyzed for particle morphology per size fraction using a high-resolution SEM (JEOL, JSM-5410). The SEM was used to support and complete LM analysis. The main advantages of using SEM were viewing particles at higher magnifications than using LM. Moreover, SEM was also used to morphologically examine samples from known sources of PM generated in the laboratory using the dust generator.

A small section (approximately 1 cm^2) of each polycarbonate filter from each size fractions was cut and mounted on a 12 mm carbon stub with a double-sided carbon adhesive tape. Each sample was then coated with carbon using a vacuum evaporator to create a conductive coating to the SEM electron beam. Photomicrographs of each field of view were taken at varying magnifications ranging from 600x to 2500x.

As regards morphology using LM and SEM, particle components were identified compared to published photographs of known particles (McCrone, 1992; Cambra-López et al., 2011b). Particle types were qualitatively analyzed and morphologically described in terms of shape (rounded, spherical, fibrous, flake, angular, aggregate, irregular, flattened, long-thin), surface (layered, smoothed, cracked), edges and borders (sharpness), texture (smooth, grape-like, and rough), and opacity, amongst others (McCrone, 1992; NIST, 2010).

3.2.3.2. Chemical composition

Samples of airborne PM collected on glass fibre filters were analyzed for particle chemical composition, per size fraction, using high resolution SEM (JEOL, JSM-5410) combined with energy-dispersive X-ray analysis (EDX) (Link Tetra Oxford Analyzer). Preparation of samples was the same as for morphological analysis using SEM with polycarbonate filters. The SEM/EDX was conducted manually to obtain particle-by-particle element chemical composition.

Elements with atomic number ≥ 11 (sodium) were detected from the element X-ray spectra. At least three fields of view (spots) per filter sample were analyzed. On each analyzed field, the elemental spectra of every particle found were analyzed. For quantitative element analyses, EDX spectrograms were recorded and analyzed using Oxford INCA Software (Oxford Instruments, Abingdon, U.K.).

The effect of PM size fraction on element chemical composition in the analyzed particles was tested with one-way ANOVA using SAS (2001), with size fraction as source of variance, and the individual particle element composition as the experimental unit in the ANOVA analyses.

3.2.3.3. Bacterial concentration

A methodology to extract bacteria from the collected samples of airborne PM on glass fibre filters was developed to determine the microbiological content of the different PM size fraction.

Each sample collected on glass fibre filters was eluted in 25 mL of Nutrient Broth, adding 0.05% Tween 20, and shaken for 90 min at 200 rpm at room temperature. One-mL samples were transferred from the suspension on duplicate plates directly on Compact Dry TC (Hyserve GmbH & Co., Uffing, Germany). Plates were incubated at 37ºC for 72 hours under aerobic conditions. Airborne concentrations of aerobic bacteria were determined by multiplying the colony forming units (CFU) by the elute volume, and divided by the volume plated (1 mL) and the volume of sampled air.

3.3. Results

3.3.1. Particulate matter characterization

3.3.1.1. Morphology

Qualitative LM analysis of the different components found in the PM from fattening rabbit and reproductive does revealed that PM from rabbit farms was highly diverse and comprised heterogeneous particles in size, morphology and origin. Seven different particle components were identified in PM using LM:

i. Feed: Starch granules from feed appeared as round, smooth and flattened particles. These could be stained with iodine, turning into a violet blue colour. Feed particles were highly agglomerated, but individual particles ranged from 3 to 30 μm in diameter (Figure 3.1a).

ii. Faecal material: Faecal particles were irregular in shape and size, and included heterogeneous components such as undigested feed residues. Faecal particles showed rounded edges in some cases, and acute edges in others. Particles were quite rough, showing a dark yellow to brown colour (Figure 3.1b). Generally, these were darker in colour than feed particles, although discrimination between them was complicated.

iii. Dander and skin cells: Dander and skin particles were flat, smooth, and transparent compared with other components in PM, and irregular in size. Particles from skin showed a relatively platy or flake-like morphology, with folded up edges (Figure 3.1c).

iv. Hair: Rabbit hair was easily detected and identified as long-thin structures, generally 5 to 30 μm in diameter, with a central canal characterized by a ladderlike chain of patches, similar to a string of pearls (Figure 3.1d).

v. Mould and fungus (hyphae and spores): Spores from mould and fungal conidia were also identified. Spores were transparent, colourless, smooth and oval bodies 3 to 5 μm in diameter (Figure 1e). Conidia were transparent, dark brown and walled

structures, forming 2 to 4 chambers of approximately 5 to 20 μm wide and 12 to 40 μm long (Figure 3.1f). Hyphae were also identified as individual fibres, transparent, colourless or yellowish walled structures.

vi. Insect parts: Insect parts such as insect wings were easily identified (Figure 3.1g).

vii. Sulphur particles: Sulphur particles were round, smooth, yellow in colour, and varied in size from 20 to 100 μm (Figure 3.1h).

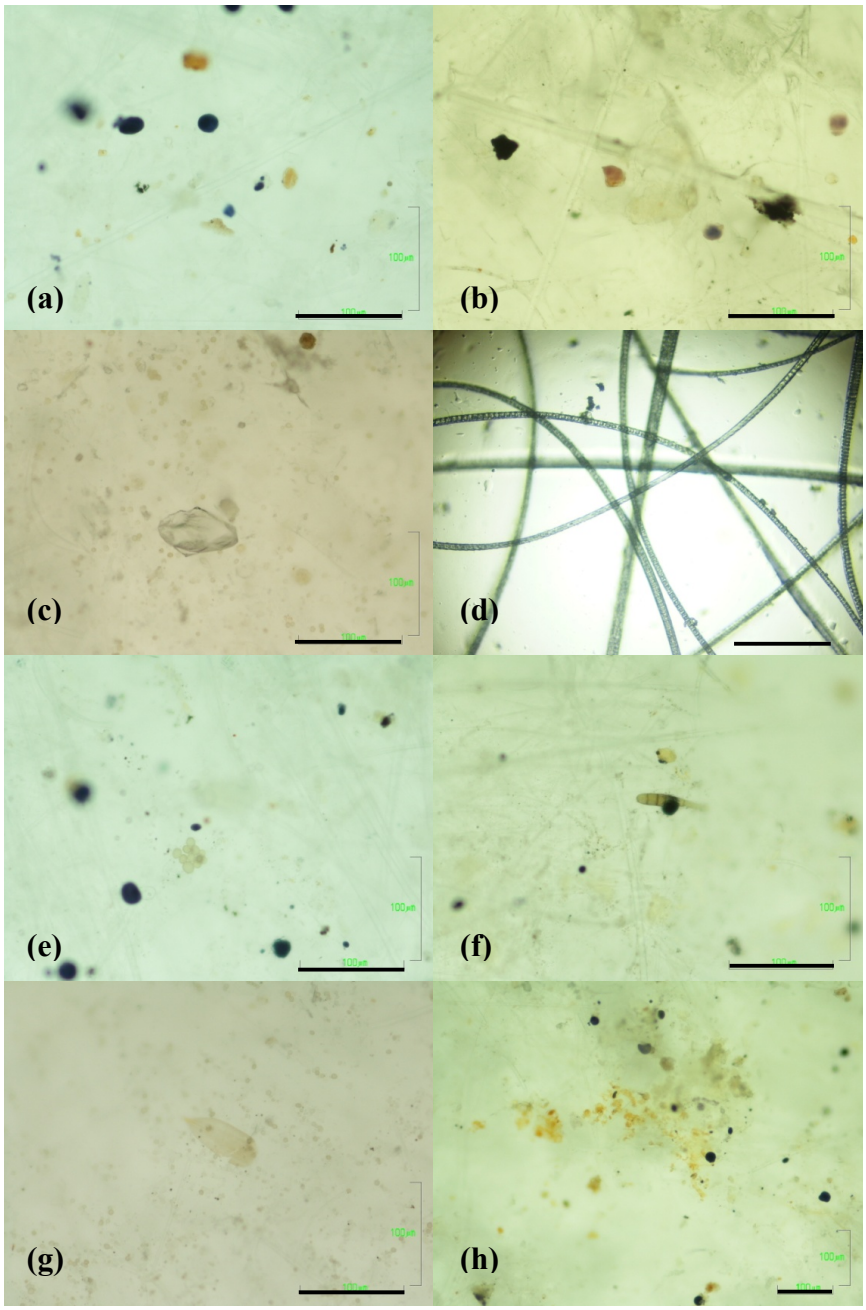

Figure 3.1. Particulate matter components viewed using light microscopy (10x and 20x) on airborne samples collected on glass fibre filters in fattening rabbit and reproductive does. Particles from feed (a), faecal material (b), skin (c), hair (d), fungal spores (e), conidia (f), insect wings (g) and sulphur (h). Scale bar 100 µm.

Besides the qualitative analysis of PM components and their identification through LM, a quantitative analysis of these components was also performed. Results from the quantitative analysis are shown in Table 3.2. This analysis could only be conducted in TSP and PM10 fractions due to the limitations in the magnification of LM in the PM2.5 fraction, together with its small size. Hence, Table 3.2 shows results for just one sample in PM2.5, where the high value corresponding to the "Others" component (62%) reveals the difficulty of such analysis in this fraction. The fraction "Others" represented unclassified particles or fragments of any of the seven identified components not easily distinguished using LM by their shape, colour or size. However, to a certain extent, feed, faecal material and skin particles were identified.

Table 3.2. Number of particles from the different components identified in the collected PM from fattening rabbit and reproductive does, expressed as average relative percentage (%) and standard deviation.

	Components	Fattening rabbits	Reproductive does
PM2.5*	Feed	25.2	N.D.
	Faecal material	11.1	N.D.
	Skin	1.9	N.D.
	Others	61.9	N.D.
	Total counted particles	163	-
PM10	Feed	53.4±6.4	37.5±11.4
	Faecal material	21.7±10.8	23.2±21.5
	Skin	2.1±0.7	13.1±16.1
	Hair	0.1±0.3	0.2±0.3
	Microorganisms	0	3.0±1.9
	Sulphur	0	12.8±18.1
	Others	22.7±15.1	10.1±0.5
	Total counted particles	920	371
TSP	Feed	62.9±7.1	50.8±5.3
	Faecal material	18.4±6.9	17.9±15.5
	Skin	3.0±1.6	12.3±11.5
	Hair	0.3±0.5	1.4±0.2
	Microorganisms	0.1±0.2	1.4±0.4
	Sulphur	0	8.6±12.1
	Others	15.3±8.9	7.7±2.3
	Total counted particles	768	353

N.D.= No data

*Only one sample was observed

Quantitative analysis using LM showed that feed components and faecal material composed the bulk of the collected particles in all fractions, ranging from 25 to 63% for feed and from 11 to 22% for faecal material.

The counted number of particles from feed increased from PM2.5 to TSP. The counted number of particles from faeces remained constant in the three fractions. The high value attributable to the "Others" component in PM2.5 fraction, however, could alter these results. The rest of components were easily counted due to their differential morphologies, and were found in a less extent, with percentages generally below 13% in all cases. Sulphur particles were only present in the reproductive does farm, and ranged from 9 to 13% in PM10 and TSP fractions.

Airborne PM samples collected on polycarbonate filters and examined under SEM are shown in Figure 3.2. This figure illustrates the different PM components and confirms their presence and quantities calculated using LM. In fattening rabbits, Figure 3.2 shows heterogeneous particles, which could be grouped into four particle types: feed, faecal particles, dander, and calcium superphosphate (as explained before, known to be used in fattening rabbit farms as disinfectant). Differences in the abundance of these components between fractions are evident from this figure. In PM2.5, most particles appeared as small bright particles probably from feed, whereas in PM10 and TSP, large skin flakes and irregular layered PM were highly abundant.

Specific individual particles components generated from known sources viewed by SEM are shown in Figure 3.3. Morphological structures ranging from transparent flake-like bent skin cells or rabbit dander (Figure 3.3a), irregular angular and layered faecal particles (Figure 3.3b), round and small particles from feed (Figure 3.3c), aggregates of calcium superphosphate particles (Figure 3.3d), spore-like bioaerosol, presumably conidia from fungus (Figure 3.3e), and long-thin pointed particles from hair (Figure 3.3f) were found.

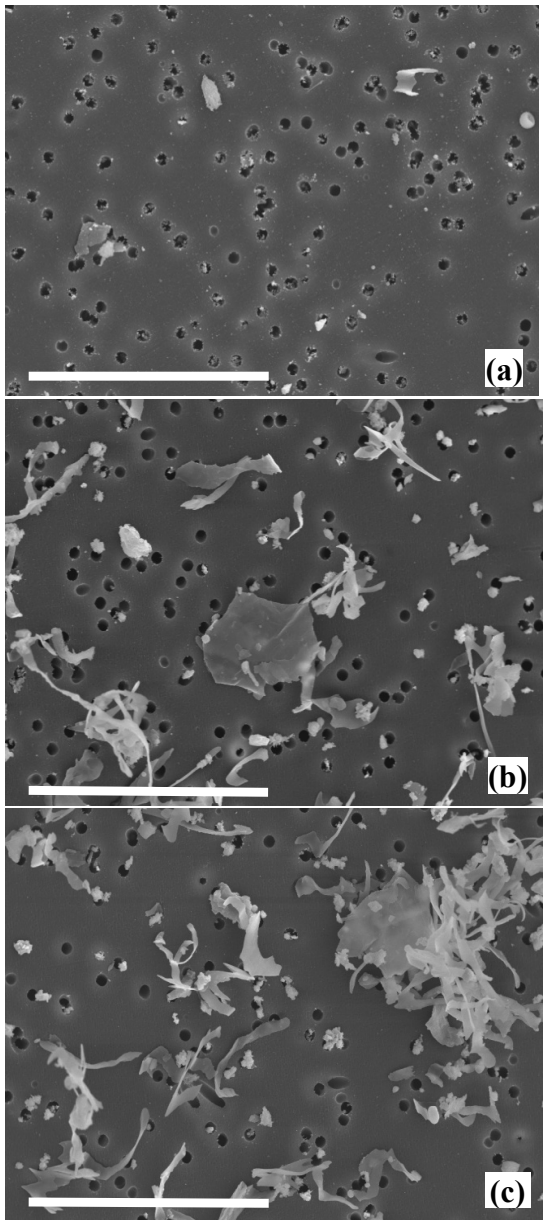

Figure 3.2. Particulate matter airborne samples collected on polycarbonate filters viewed using scanning electron microscopy (600x), from PM2.5 (a), PM10 (b) and TSP (c) samples from fattening rabbit farm. Note: 5 μm diameter filter pores are shown as round dark holes. Scale bar 90 μm.

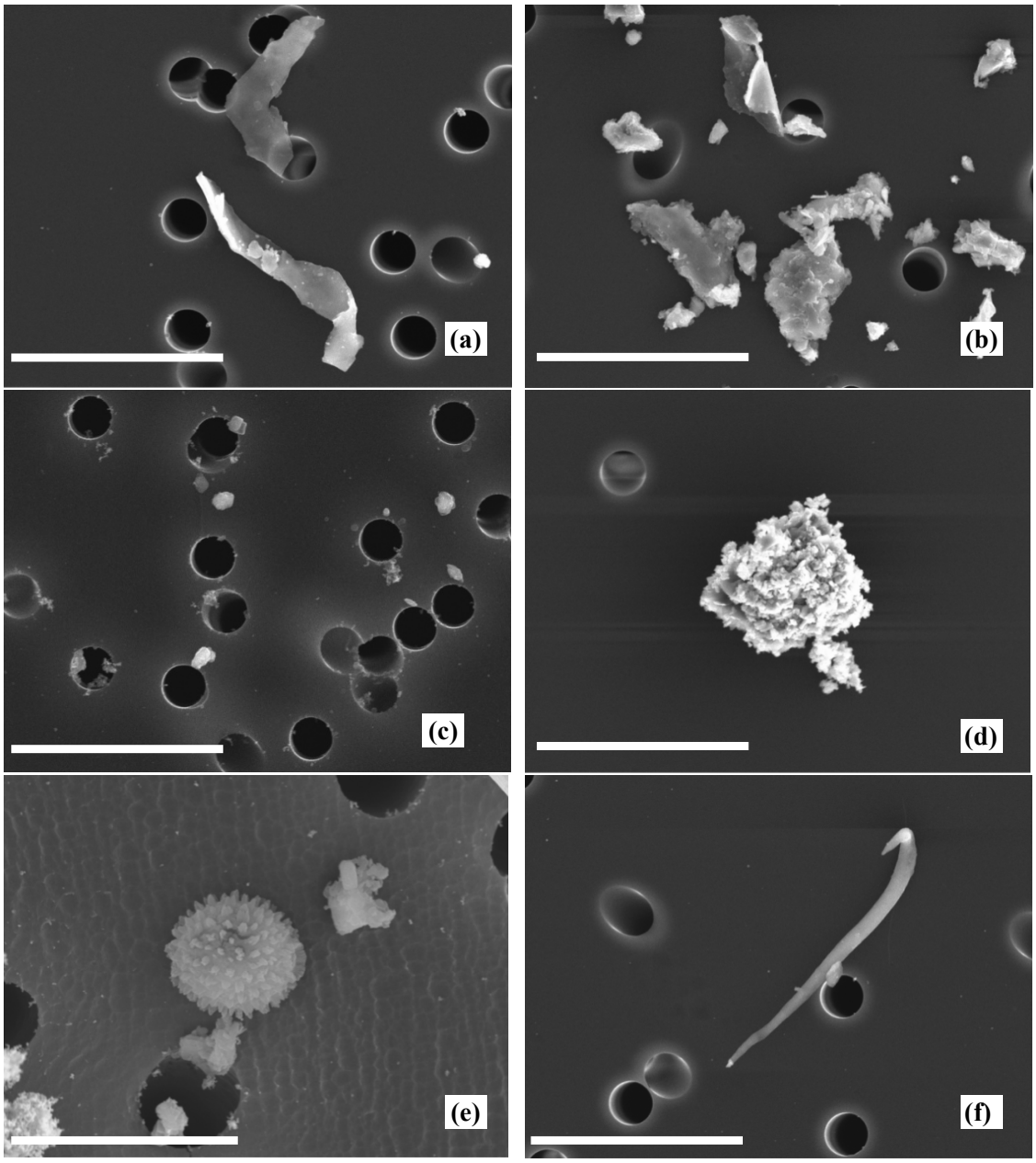

Figure 3.3. Examples of scanning electron microscope photomicrographs from generated PM in the laboratory from know sources collected in fattening rabbits and reproductive does on polycarbonate filters showing rabbit dander (a), layered faecal particles (b), feed (c), calcium superphosphate particles (d), bioaerosol (e), particles from hair (f). Note 5 μm diameter filter pores shown as round dark holes. Scale bar 20 μm, except for fig.e, equal to 10 μm.

3.3.1.2. Chemical composition

Average element chemical composition is presented in Table 3.3, showing differences in element percentages among size fractions.

Glass fibre filters showed presence of sodium (Na), aluminium (Al), silicon (Si), potassium (K), calcium (Ca), zinc (Zn), and barium (Ba). Besides the elements present in the blank filter (glass fibre filter), high contents of sulphur (S) and Ca were identified in all size fractions. Chlorine (Cl) was more abundant in PM10 and TSP fraction compared with PM2.5; whereas other elements such as magnesium (Mg) and phosphorus (P) were the most abundant in TSP, and iron (Fe) was the most abundant in PM2.5. From the ANOVA analysis, it was observed how the differences in the average values of the most abundant elements (Na, Mg, P, S, Cl, K, Ca, Zn and Ba), were significantly different in one or two size fractions. Overall, major elements found in rabbit airborne PM were S, Ca, Mg, Na and Cl.

Table 3.3. Average element composition (%) and standard deviation of the different PM size fractions, including blank filter, and significance level of average values among fractions (n=159).

Element (%)	Blank filter	PM2.5	PM10	TSP	Significance level
Na	6.7±0.4	5.3±2.1^{a}	7.8±4.9^{b}	6.4±3.9^{ab}	p<0.05
Mg	0	0.3±0.5^{a}	0.9±0.8^{b}	1.3±1.3^{c}	p<0.001
Al	4.9±0.3	5.0±2.8	4.4±3.1	4.0±1.8	N.S.
Si	49.6±1.6	39.3±12.8	36.4±12.8	40.8±17.2	N.S.
P	0	0^{a}	0.3±0.9^{a}	1.5±5.1^{b}	p<0.10
S	0.1±0.2	7.7±8.4^{a}	5.0±6.7^{b}	4.2±5.3^{b}	p<0.10
Cl	0	0.1±0.3^{a}	4.3±5.6^{b}	3.1±5.3^{b}	p<0.001
K	6.8±0.1	5.8±1.9^{a}	6.4±1.7^{ab}	7.2±4.6^{b}	p<0.10
Ca	4.5±0.4	14.5±13.3^{a}	10.6±13.3^{ab}	17.8±16.9^{b}	p<0.10
Mn	0	0.0±0.1	0	0.0±0.1	N.S.
Fe	0.2±0.3	2.3±8.3	0.7±1.5	0.4±0.9	N.S.
Ti	0	0.3±2.1	0.1±0.4	0.0±0.1	N.S.
Zn	12.4±1.1	8.2±4.7^{a}	10.5±7.3^{b}	5.4±2.9^{c}	p<0.001
Ba	14.9±1.1	11.1±7.1^{a}	11.8±5.7^{a}	7.8±4.2^{b}	p<0.05
I	0	0.0±0.3	0.9±3.2	0	N.S.
Ce	0	0.1±0.5	0	0	N.S.
Cu	0	0.0±0.2	0	0	N.S.

^{a,b}Averages within a row with different superscripts differ significantly (p<0.05)
N.S.= Not significant differences

3.3.1.3. Bacterial concentration

Table 3.4 shows the results for the average airborne bacteria concentrations in CFU per m^3 of air from the samples collected in fattening rabbit and reproductive doe farms. Average CFU in the air were higher in TSP compared with other fractions, and overall ranged from $1.7x10^4$ to $1.6x10^6$ CFU/m^3. Average CFU in PM10 ranged from $3.6x10^3$ to $3.0x10^4$ CFU/m^3, and from $3.1x10^3$ to $1.6x10^4$ CFU/m^3 in PM2.5.

Table 3.4. Average airborne bacterial concentrations and standard deviation in colony forming units (CFU) per m^3 in fattening rabbit and reproductive does in different PM size fractions and standard deviation (n=2).

	PM2.5	PM10	TSP
Fattening rabbits	$4.2x10^3 \pm 2.3x10^3$	$7.9x10^3 \pm 6.1x10^3$	$4.1x10^5 \pm 5.6x10^5$
Reproductive does	$1.2x10^4 \pm 1.1x10^3$	$1.9x10^4 \pm 1.2x10^4$	$9.4x10^4 \pm 5.1x10^4$

3.4. Discussion

The results presented herein contribute to improving the knowledge on airborne PM in rabbit farms in terms of particle morphology, chemical compositions and bacterial concentrations in different size fractions. Particle characterization revealed high particle diversity in rabbit PM. Although most particles were biological in nature, quantitative analysis using LM showed that feed components and faecal material composed the bulk of the airborne particles in PM2.5, PM10 and TSP size fractions, ranging from 25 to 63% for feed and from 11 to 22% for faecal material.

Previous studies in pigs (Donham et al., 1986; Heber et al., 1988; Feddes et al., 1992) identified feed as predominant components in TSP and in particles larger than 10 μm in diameter. In poultry and pigs, Cambra-López et al. (2011a) found higher contribution of particles from faecal material in PM10 and PM2.5 than in our study. Perhaps, the nature of rabbit's hard faeces, which are highly compressed and contain a mucin cover (Sirotek et al., 2003), could probably explain such differences. Airborne PM in rabbit houses showed a high relative contribution of feed and rabbit's skin and hair, compared with other species. Moreover,

our results showed a high complexity in particle morphology (especially in size, which ranged from a few μm to 90-100 μm) in the examined PM samples. This indicates that source contributions could vary when expressed in particle mass rather than in particle numbers as reported in Cambra-López et al. (2011a). In fact, large particles from skin as shown in Figure 3.2 could gain relative importance when expressed in particle mass.

Both LM and SEM were used in this study to discriminate among particle components and types (i.e. sources). When using LM, iodine was used to stain starch granules and differentiate feed from the rest of sources. Undigested feed components from feed found in faecal material, could also be stained with iodine. Furthermore, faecal particles were difficult to distinguish, especially in PM2.5 fraction, and could be in some cases confused with skin or feed. For this reason, the contribution of feed might have been slightly overestimated in our results using LM. The use of other stain different from iodine to differentiate between feed or faecal material such as undigested feed particles found in faeces could help in the identification of these PM components in the smaller size fractions when using LM. Nile blue sulphate stain has been used before for this purpose (Donham et al., 1986). Nevertheless, to overcome LM limitations, further SEM analysis is encouraged. In fact, SEM analysis in this study was used to examine in more depth PM characteristics and to support and complete LM analysis. The SEM analysis revealed that particles from skin, faeces and feed were abundant in TSP and PM10 fractions, whereas particles from feed and, to a lesser extent, from skin, were the most abundant in PM2.5. These data provide valuable information, especially as regards fine PM2.5, although further examinations using SEM are necessary to acquire additional data on particle characteristics in rabbit farms under different housing and environmental conditions than those in this study.

Our results indicate that most particles were characterized as fragmentation-type particles, with irregular and acute edges (broken skin flakes, disintegrated particles from feed or faecal material from mechanical fracture). These results are in agreement with the results obtained from analysis of the farm activities influencing PM generation (Adell et al., 2012). These authors reported that mechanical activities such as feeding, sweeping and cleaning the cages by burning hair are

major PM-generating activities. Crushing of feed particles during feed distribution could explain the high contribution of feed particles found in airborne PM. Whether the rest of activities would result in the generation of faecal material, rabbit skin and hair would be a matter of discussion, but it could be expected that skin debris would be released through animal manipulation and other farm activities. Besides mechanical fragmentation of particles, a variety of biological structures such as spores were identified, indicating that fungal spores might be abundant in the air in rabbit farms.

Major elements found in rabbit PM were S, Ca, Mg, Na and Cl. These elements were similar to those reported by Aarnink et al. (2004) and Schneider et al. (2001) in airborne PM in pigs, and by Cambra-López et al. (2011b) in poultry, except for certain elements such as Ca (found in a greater extent in this study) and only small amounts of P. The higher content of Ca could be attributable to the use of calcium superphosphate powder in fattening rabbit farm.

Overall, the analytical methods used to characterize PM in this study, based on microscopic techniques can supply valuable but limited data on particle or source chemical composition and morphological characteristics. To further identify and quantify source contributions, the use of source apportionment models is encouraged (Watson et al., 2002). Source apportionment models would allow obtaining quantitative and comparable estimations of source contributions of PM, between and within livestock categories.

As regards the bacterial concentrations in airborne PM, our results were higher than those reported by Navarotto et al. (1995) and Duan et al. (2006) for rabbit farms. The findings of this study are similar to those observed by Seedorf et al. (1998) for cows, pig and poultry houses and Ribikauskas et al. (2010) for rabbit house. Although filtration samplers are not recommended for microbial bioaerosol sampling because of desiccation stresses that occur as air flows through the filters (Crook, 1995), it is a commonly used technique (Thorne et al., 1992) and our findings indicate that airborne bacteria concentration in rabbit farms are comparable with other livestock species. The values observed in airborne bacteria in rabbit farms suggest further research to investigate the presence and levels of infective airborne pathogens would be useful.

3.5. Conclusions

Airborne PM in rabbit farms is highly complex in particle morphology, especially in size, revealing high diversity in particle components and types (i.e. sources). Particle size ranged from a few μm to 90-100 μm and most PM showed fragmentation type particles with irregular and acute edges.

Broken skin flakes, disintegrated particles from feed and faecal material from mechanical fracture are the main sources of airborne PM in rabbit farms. Major elements found in rabbit airborne PM were S, Ca, Mg, Na and Cl. Further research is needed to obtain quantitative and comparable estimations of source contributions of PM, between and within livestock categories using source apportionment models.

Average CFU in the air ranged from $1.7x10^4$ to $1.6x10^6$ CFU/m^3 in TSP; from $3.6x10^3$ to $3.0x10^4$ CFU/m^3 in PM10; and from $3.1x10^3$ to $1.6x10^4$ CFU/m^3 in PM2.5. The existence of infective airborne pathogens in the air in rabbit farms, however, is still unknown.

Our results will improve the knowledge on airborne PM in rabbit farms in terms of morphology, chemical compositions and bacterial concentrations in different size fractions, necessary to understand PM's origin in rabbit farms and to propose adequate source-specific reduction techniques.

3.6. Acknowledgements

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Abstract. The extent of the potential health hazards of particulate matter (PM) inside rabbit farms and the magnitude of emission levels to the outside environment is still unknown, because there are scarce data about PM concentrations and emissions in and from such buildings. The objective of this study was to quantify airborne PM10 and PM2.5 concentrations and emissions in two rabbit farms in Mediterranean conditions, and to identify the main factors related with farm activities influencing PM generation. Concentrations of PM10 and PM2.5 were determined continuously using a tapered element oscillating microbalance (TEOM) in a fattening rabbit and in a reproductive doe farm during autumn. At the same time as PM sampling, the time and type of human farm activity being performed was recorded. Additionally, temperature, relative humidity and ventilation rate were recorded continuously. Emissions were calculated using a mass balance in each farm. Results showed PM concentrations in rabbit farms are low compared with poultry and pig farms. Average PM10 concentrations were 0.082±0.059 mg/m^3 (fattening rabbits), and 0.048 ±0.058 mg/m^3 (reproductive does). Average PM2.5 concentrations were 0.012±0.016 mg/m^3 (fattening rabbits), and 0.012±0.035 mg/m^3 (reproductive does). Particulate matter concentrations were significantly influenced by type of human farm activity performed in the building rather than by animal activity. Major PM-generating activity in fattening rabbit farm was sweeping, and major PM-generating activity in reproductive does was sweeping and burning hair of the cages. Average PM10 emissions were 5.99±6.14 mg/place/day (fattening rabbits), and 14.9±31.5 mg/place/day (reproductive does). Average PM2.5 emissions were 0.20±1.26 mg/place/day (fattening rabbits), and 2.83±19.54 mg/place/day (reproductive does). Emission results indicate that rabbit farms can be considered relevant point sources of PM emissions, comparable to other livestock species. Our results improve the knowledge on factors affecting concentration and emissions of PM in rabbit farms and can contribute to design adequate PM reduction measures to control not only PM inside rabbit houses, but also its emission into the atmosphere.

Keywords. Air quality, animal housing, atmospheric pollution, dust, health.

4.1. Introduction

Airborne particulate matter (PM) is abundant in the air of livestock houses (Takai et al., 1998; Cambra-López et al., 2010). High indoor concentrations of PM can compromise the respiratory health of animals and humans, causing detrimental effects on animal performance and efficiency (Donham and Leininger, 1984; Donham, 1991; Al Homidan and Robertson, 2003), and on the health and welfare of farmers (Donham et al., 1984; Andersen et al., 2004). Moreover, PM can be emitted to the outside environment through the ventilation exhausts (Phillips et al., 1998), threatening the environment (plants and organisms), causing vegetation stress and ecosystem alteration (Grantz et al., 2003). Although PM concentrations and emissions have been characterised in poultry and pig production systems (Costa and Guarino, 2009; Lacey et al., 2003), little is known about PM concentration and emissions in and from rabbit houses (Navarotto et al., 1995; Ribikauskas et al., 2010). Although PM concentrations in rabbit farms seem to be low compared to other livestock species (Cambra-López et al., 2008), the emission of PM into the atmosphere could be relevant due to the high ventilation rates observed in those European regions where rabbits are reared (typically Mediterranean countries) (Calvet et al., 2011).

Particulate matter in livestock facilities can originate from several sources such as manure, feed, feathers, skin and bedding material (Cambra-López et al., 2011). The size of PM is one of the most relevant properties because it influences its behaviour in the air and in the respiratory tract. Therefore, PM is usually characterised in terms of its size, as regards the occupational health size fractions: inhalable, thoracic, and respirable (CEN, 1993). These fractions, moreover, can be related to the outside air quality cut-off sizes: PM10 and PM2.5 (particulate matter which passes through a size-selective inlet with a 50% efficient cut-off at 10 μm aerodynamic diameter or at 2.5 μm aerodynamic diameter, respectively), regulated in the Council Directive 1999/30/ EC, relating to limit values for sulphur dioxide, nitrogen dioxide and oxides of nitrogen, PM and lead in ambient air. The PM10 fraction can be inhaled and accumulated in the upper respiratory airways. This fraction includes

the smaller PM2.5 fraction, which can penetrate deeper into the respiratory airways and reach the alveoli in the lungs. Since livestock production can emit considerable amounts of PM into the atmosphere (Takai et al., 1998), there is an increasing tendency to monitor PM10 and PM2.5 fractions instead of occupational health size fractions to comply with air quality regulations outside livestock houses (Directive 1999/30/EC and Directive 2008/50/EC).

To assess the extent of the potential health hazards of PM inside rabbit farms and the magnitude of emission levels to the outside environment, further research on PM concentrations and emissions is needed. Hence, enhancing the knowledge of factors affecting concentration and emissions of PM in rabbit farms is necessary to design adequate PM reduction measures to control PM. This would allow an improvement of the air quality inside the animal house, and the development of technically feasible, environmentally acceptable, and economically viable measures to reduce PM emissions into the atmosphere.

The aim of this study was to quantify airborne PM10 and PM2.5 concentrations and emissions in two rabbit farms in Mediterranean conditions and identify the main factors related with farm activities influencing PM generation.

4.2. Material and methods

4.2.1. Housing and animals

Two rabbit farms were surveyed in this study: one rearing fattening rabbits and another rearing reproductive does. Animals were reared in cages in both farms. Manure was accumulated in pits below the cages for three to four weeks.

Both farms were located in the region of Valencia (East of Spain) and were surveyed during 15 consecutive days in each farm during autumn. Table 4.1 describes both farms in terms of housing and animals.

Table 4.1. Description of the surveyed rabbit farms

	Fattening rabbits	Reproductive does
Length x width (m)	30 x 6	30 x 11
Animal places	2100	530
Average animal weight (kg)t	1300	4000 (including litter)
Feed distribution	Manually distributed pellets	Automatically distributed pellets
Ventilation	Tunnel mechanical 2 fans (Q*= 10131 m^3/h)	Transversal mechanical 7 fans (Q*= 1762 m^3/h)

*Q= Fan average airflow rate. All fans were on/off operated.

4.2.2. Environmental parameters

Indoor and outdoor temperature and relative humidity were recorded every minute using data loggers (HOBO H8-007-02, Onset Computer Corporation, Pocasset, MA., U.S.) in both rabbit farms.

Additionally, ventilation rates were continuously monitored. Ventilation rates were calculated considering the operation time of each fan and the corresponding fan extraction rates following Calvet et al. (2010). Direct measurements of fan activity and extraction capacity of each fan were conducted. Fan activity (percentage of time each fan was functioning) was determined by means of a motor on/off sensor (HOBO H06-004-02, Onset Computer Corporation, Pocasset, MA., U.S.). The extraction capacity of each fan (fan's airflow) was registered before and after each measurement period by multiplying the free flow area by the average air speed in each fan. Air speed was measured at 24 points of the cross section of the fan using a hot wire anemometer (Testo® 425, Germany, measurement range 0 to 20 m/s). As a result, the global ventilation rate in each farm during measurements was calculated by multiplying the activity of each fan by its extraction capacity, and summarizing for the total number of fans.

4.2.3. Particulate matter levels: concentration and emissions

Concentrations of PM10 and PM2.5 size fractions were simultaneously determined using a tapered element oscillating microbalance, TEOM (TEOM model 1405-D, Thermo Fisher Scientific, U.S.). This device operated on changes in the resonant frequency of an oscillating element as a function of increases in particle mass collected on a filter. Changes

in the recorded resonant frequency of the element provide continuous and time-averaged measurement of mass accumulation. Filters were exchanged at approximately 50% loading following Heber et al. (2006). The PM concentrations were recorded every minute for both fractions during 15 consecutive days per farm. Average daily PM concentrations (mg/m^3) were calculated from these data.

The TEOM device was located indoors, close to the ventilation exhaust in each farm. Measurements were conducted at a height of 2 m. At the same time as PM concentrations were measured indoors, the time and type of activity being performed by workers in each farm was recorded. Activities varied daily but were repeated weekly, and included routine activities such as: animal handling and supervision, mortality inspection, feed distribution, cleaning cages with pressurized water, cleaning cages by burning hair, application of powdered disinfectant on the floor (calcium superphosphate), and floor sweeping in all farms; as well as the preparation of nests using cotton waste as bedding material, and powdered sulphur as disinfectant, only in reproductive does.

The emission of PM was calculated using a mass balance in the farm, by subtracting the PM concentration measured outdoors (PM_i), from the concentration measured inside the rabbit farms (PM_e), and multiplying it by the ventilation rate (Q_e) (Equation 1). The emission rate according to Equation 1 was calculated from the TEOM data provided at standard conditions (standard temperature, $T_{std} = 298.15$ K and standard pressure, $P_{std} = 1$ atm), correcting for ambient temperature and barometric pressure (T_a and P_a) according to Li et al. (2008):

$$\text{Emission} = Q_e \text{x} \left(PM_e - \frac{\rho_e}{\rho_i} PM_i \right) \text{x} 10^{-6} \text{x} \frac{T_{std}}{T_a} \text{x} \frac{P_a}{P_{std}} \quad \text{Equation 1}$$

where:

Emission: Emission rate (g/h)
Q_e: Ventilation rate (m^3/h)
PM_i: Inlet particulate matter concentration ($\mu g/m^3$)
PM_e: Exhaust particulate matter concentration ($\mu g/m^3$)
ρ_e, ρ_i: Exhaust and inlet air density (kg dry air/m^3 wet air)
T_a: Ambient temperature (K)
P_a: Ambient pressure (atm)

T_{std}: Standard temperature (298.15 K)
P_{std}: Standard pressure (1 atm)

Outdoor PM concentrations were obtained from the nearest air quality sampling station from the "Valencian Community Atmospheric Contamination Surveillance and Control Monitoring Networks" (Red de Vigilancia y Control de la Contaminación Atmosférica de la Comunidad Valenciana) (Generalitat Valenciana, 2009). The sampling station was located at approximately 400 m from the farms. This station recorded hourly PM10 and PM2.5 concentrations.

Finally, hourly emission rates (g/h) were summarized over 24-h periods, and divided by the number of animal places during the sampling period in each farm, to calculate daily emissions per animal (mg/place/day).

4.2.4. Statistical analysis

Effects of type of activity on PM10 and PM2.5 concentrations for each animal type was analyzed with one-way ANOVA using SAS Software (SAS, 2001) with type of activity as the source of variance. Hourly PM10 and PM2.5 concentration values over the sampling period were the experimental unit in this ANOVA analysis. In addition, this analysis was repeated for specific days within the sampling period in fattening rabbits and reproductive does. Differences with p-values less than 0.05 were considered to be statistically significant.

Differences between fattening rabbits and reproductive does for average daily PM concentrations (mg/m^3) and emissions rates (g/h) were determined with a two-tailed t-test for one treatment with two levels (animal type) using SAS Software (SAS, 2001). Differences with p-values less than 0.05 were considered to be statistically significant.

4.3. Results

4.3.1. Environmental parameters

Table 4.2 shows average (± standard deviation, sd) indoor and outdoor temperature and relative humidity during measurements in each farm. Ventilation rates varied from 8.6 to 12.3 m^3/h. Indoor and outdoor

temperature ranged from 18 to 22 ºC, and relative humidity from 48 to 68%.

Table 4.2. Average ventilation, indoor temperature (in T) and relative humidity (in RH), outdoor T (out T) and RH (out RH), and standard deviation, in fattening rabbits and reproductive does.

Animal type	Ventilation rate (m^3/h)	in T (ºC)	in RH (%)	out T (ºC)	Out RH (%)
Fattening rabbits	8.6± 5.0	21.8±1.9	68.0±9.7	20.3±4.2	63.8±15.2
Reproductive does	12.3*	19.4±2.7	54.8±10.6	17.9±3.5	48.1±13.7

*All fans were running constantly

4.3.2. PM concentrations

Average (±sd) concentrations of PM10 and PM2.5 in the air of fattening rabbit and reproductive doe farm are shown in Table 4.3. Average PM10 concentrations were two-fold higher ($p<0.001$) in fattening rabbits compared with concentrations in reproductive does. Average PM2.5 concentrations were similar in both farms. The proportion of PM2.5 in PM10 ranged from 15 to 25% in both farms.

Table 4.3. Average concentration of PM10 y PM2.5 (mg/m^3) and standard deviation, in fattening rabbits and reproductive does.

Animal type	PM10 (mg/m^3)	p-value	PM2.5 (mg/m^3)	p-value
Fattening rabbits	0.082 ± 0.059^a	0.001	0.012±0.016	N.S.
Reproductive does	0.048 ± 0.058^b		0.012±0.035	

[a,b]Averages within a column with different superscripts differ significantly ($p<0.05$)
N.S.= Not significant differences

Figures 4.1 and 4.2 show the daily concentration variation of PM10 and PM2.5 in fattening rabbits and reproductive does, respectively, during the whole sampling period. The evolution of PM concentrations resulted in daily variations, showing isolated spikes followed by periods of low concentrations (below 0.1 mg/m^3). Figure 4.1 shows maximum spikes in PM10 concentration in fattening rabbits reached 0.9 mg/m^3, whereas maximum spikes in PM2.5 concentrations reached 0.3 mg/m^3. Figure 4.2 shows maximum spikes in PM10 concentration in reproductive does reached 0.8 mg/m^3, whereas maximum spikes in PM2.5 concentrations reached 0.6 mg/m^3 for PM2.5.

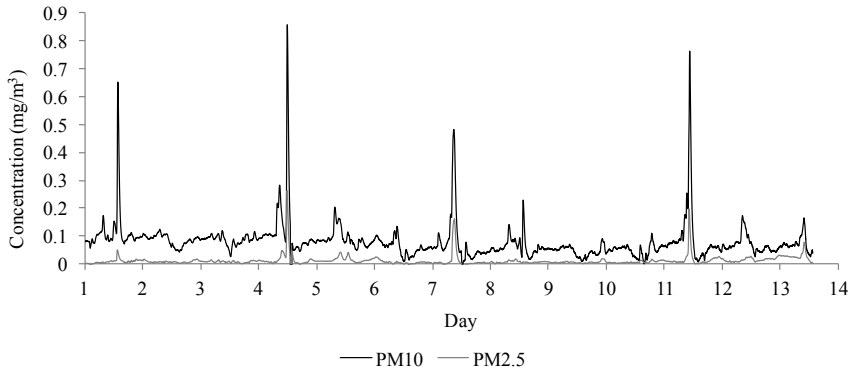

Figure 4.1. Daily PM10 and PM2.5 concentration within sampling period in fattening rabbits.

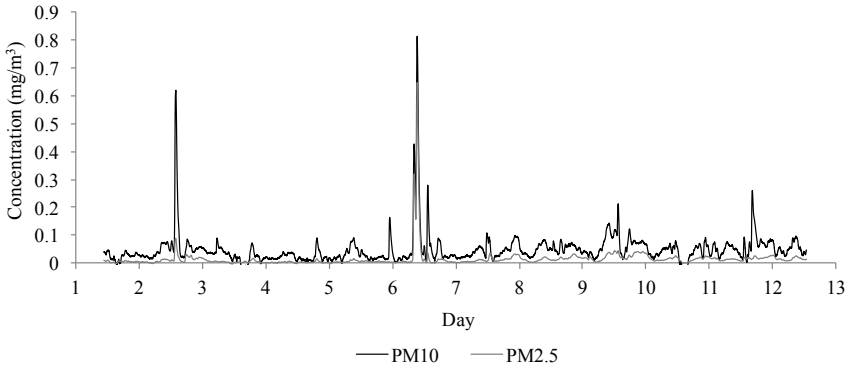

Figure 4.2. Daily PM10 and PM2.5 concentration within sampling period in reproductive does.

The relationship between the type of activity and PM10 and PM2.5 concentrations per farm showed differences between animal type and between PM size-fractions. In fattening rabbits, average PM10 concentrations were three-fold higher ($p<0.0001$) during sweeping compared with when no recorded activity was performed (i.e. no routine activities were conducted because labour hours were over). Average PM10 concentrations were two-fold higher during burning the hair of the cages ($p<0.001$) and during animal handling or cleaning the cages with pressurized water ($p<0.0001$) compared with no recorded activity. Among all activities, sweeping was found to be the activity which generated the highest concentration of PM10. Average PM2.5

concentrations were four to five-fold higher ($p<0.0001$) during sweeping, animal handling or cleaning the cages with pressurized water, than when no recorded activity was performed. However, no statistical significant differences in PM2.5 concentrations among these activities were observed. Average concentrations of PM2.5 during sweeping, animal handling or cleaning the cages with pressurized water were three to four-fold higher ($p<0.001$) than during feeding or burning the hair of the cages.

In reproductive does, average concentrations of PM10 were four to five-fold higher ($p<0.0001$) during sweeping and burning the hair of the cages; and two-fold higher ($p<0.05$) during feeding, than when no recorded activity was performed. Sweeping and burning the hair of the cages did not show statistical significant differences between them, but were statistically significantly different from the rest of activities. Feeding and disinfecting were neither statistically significantly different between them but PM10 concentrations during these activities were lower ($p<0.05$) than during sweeping and burning hair of the cages. Average PM2.5 concentrations were 10 to 12-fold higher ($p<0.0001$) during sweeping and burning the hair of the cages than when no recorded activity was performed. No differences between these two activities were found, but average PM2.5 concentrations during these activities were four to six-fold higher ($p<0.05$) than during feeding and disinfecting.

As an example, Figure 4.3 shows hourly PM10 and PM2.5 concentrations measured in fattening rabbits during two different days (24 h), together with the activities within each day. Variations in PM concentrations coincided with the time when farm activities were performed in the buildings. This figure corresponds to day 1 (Figure 4.3a) and day 11 (Figure 4.3b) of the whole sampling period shown in Figure 4.1. Indoor PM10 and PM2.5 concentrations were below 0.1 mg/m^3 during the whole day, increasing between 07:00 to 15:00 h, coinciding with the hours of higher human activity inside the farm. On day 1 and day 11, PM10 and PM2.5 concentrations were the highest during sweeping ($p<0.0001$). Average PM concentrations were four-fold higher ($p<0.0001$) for PM10, and from five to 20-fold higher ($p<0.0001$) for PM2.5, during sweeping than during feeding or burning hair of the

cages. After 15:00 h, concentrations remained more or less constant until the next day, at about 7:00 h.

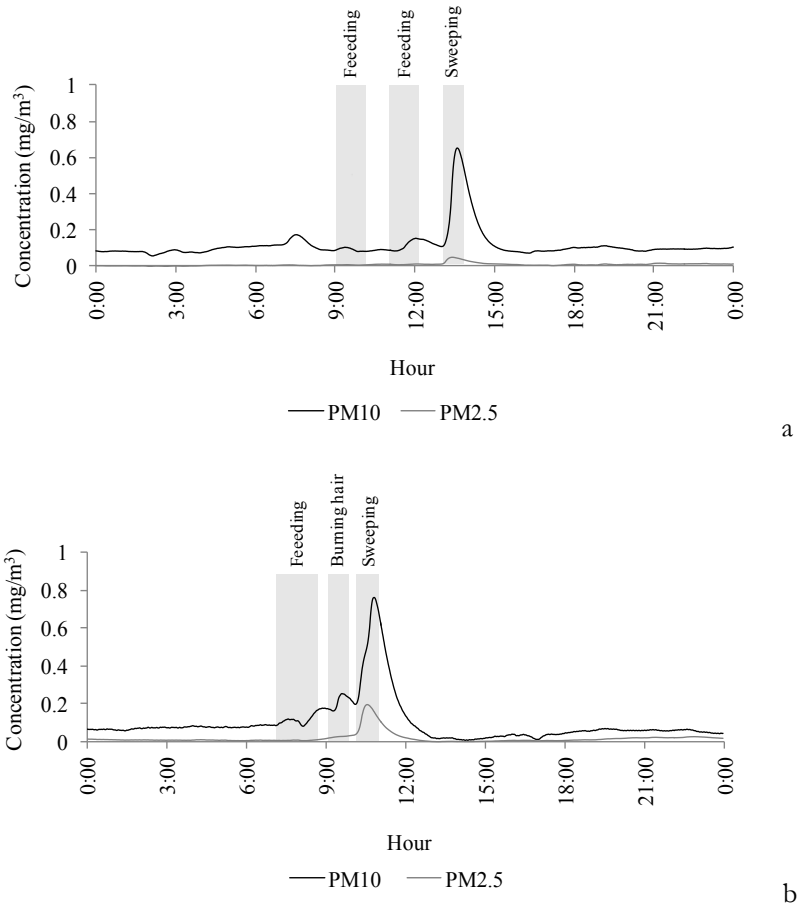

Figure 4.3. Hourly variation in PM10 and PM2.5 concentrations within 24-h periods for day 1 (a) and day 11 (b) of the sampling period, in fattening rabbits with routine farm activities.

Figure 4.4 shows an example of hourly PM10 and PM2.5 concentrations measured in reproductive does during two different days (24 h), together with the activities within each day. This figure corresponds to day 2 (Figure 4.4a) and day 6 (Figure 4.4b) of the whole sampling period shown in Figure 4.2. Indoor PM10 and PM2.5 concentrations also increased between 07:00 to 15:00 h, coinciding with the hours of higher human activity inside the farm, same as for fattening rabbits. On day 2

and day 6, PM10 and PM2.5 concentrations were the highest during sweeping ($p<0.0001$). Average PM concentrations were two to eight-fold higher ($p<0.0001$) for PM10, and from two to 21-fold higher ($p<0.001$) for PM2.5, during sweeping than during feeding, burning hair of the cages or disinfecting. A similar trend as in fattening rabbits was observed in PM concentration evolution in time within a 24-h period.

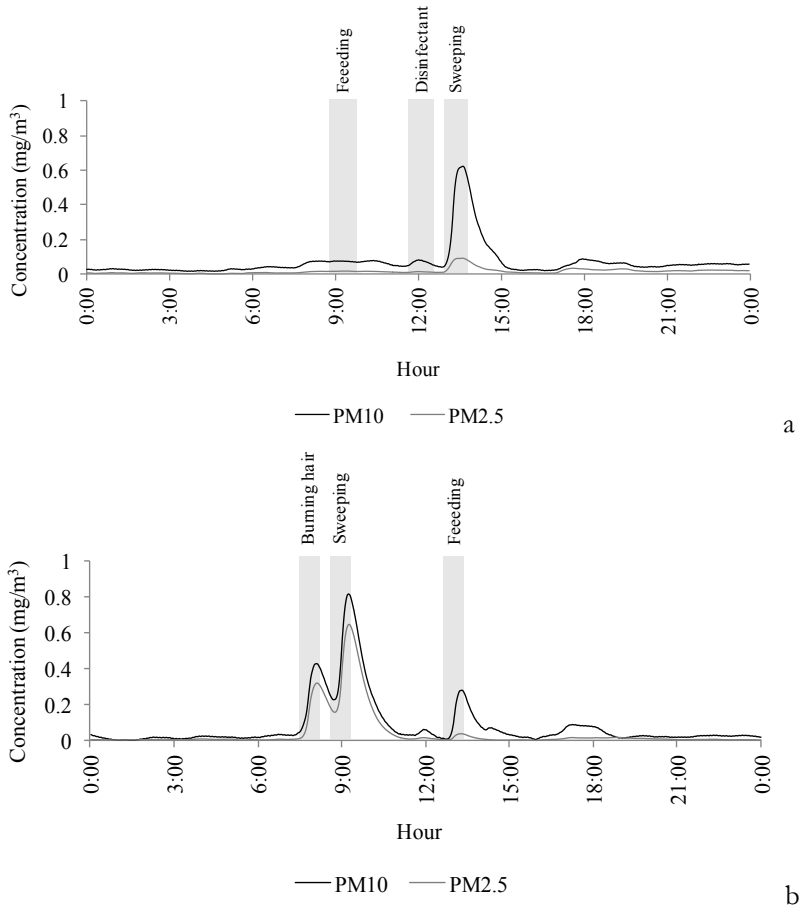

Figure 4.4. Hourly variation in PM10 and PM2.5 concentrations within 24-h periods for day 2 (a) and day 6 (b) of the sampling period, in reproductive does with routine farm activities.

4.3.3. PM emissions

Table 4.4 shows average (±sd) PM emission rates for fattening rabbits and reproductive does. Outdoor PM concentrations during measurements in both farms were low and ranged from 0.002 to 0.113 mg/m^3 for PM10 and from 0.001 to 0.056 mg/m^3 for PM2.5. Average PM emission rates (g/h) were slightly higher in fattening rabbits compared with reproductive does. These differences were more pronounced for PM10 compared with PM2.5, as PM2.5 concentrations shown in Table 4.3 were overall low and similar between farms. Average emission rates per animal place and day showed emission rates were more than two-fold higher in reproductive does, considering animal numbers were higher in fattening rabbits (2100 fattening rabbits) compared with reproductive does (530 does).

Table 4.4. Average PM10 and PM2.5 emissions and standard deviation in fattening rabbits and reproductive does.

Animal type	PM10 (g/h)	p-value	PM10 (mg/place/day)	PM2.5 (g/h)	p-value	PM2.5 (mg/place/day)
Fattening rabbits	0.52±0.54^a	0.008	5.99±6.14	0.02±0.11	0.278	0.20±1.26
Reproductive does	0.329±0.7^b		14.85±31.47	0.06±0.43		2.83±19.54

a,b Averages within a column with different superscripts differ significantly ($p<0.05$)

Figures 4.5 and 4.6 show daily emission variation of both PM10 and PM2.5 emissions in fattening rabbits and reproductive does, respectively. Daily variations in emissions corresponded with variations in indoor concentrations (Figure 4.1 and 4.2), which were furthermore related with human activities inside the farm. Consequently, peaks in PM emissions coincided with peaks in PM concentration. Figure 4.5 shows the emission of PM10 reached maximum values of 5.92 g/h and 1.36 g/h for PM2.5 in fattening rabbits. Figure 4.6 shows the emission of PM10 reached maximum values of 9.80 g/h and 8.04 g/h for PM2.5 in reproductive does.

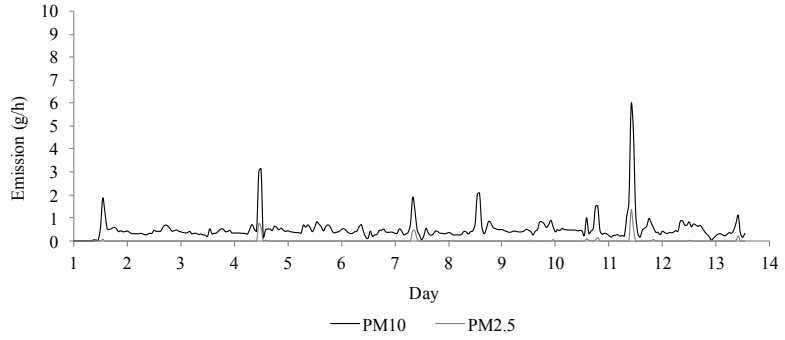

Figure 4.5. Daily PM10 and PM2.5 emission within sampling period in fattening rabbits.

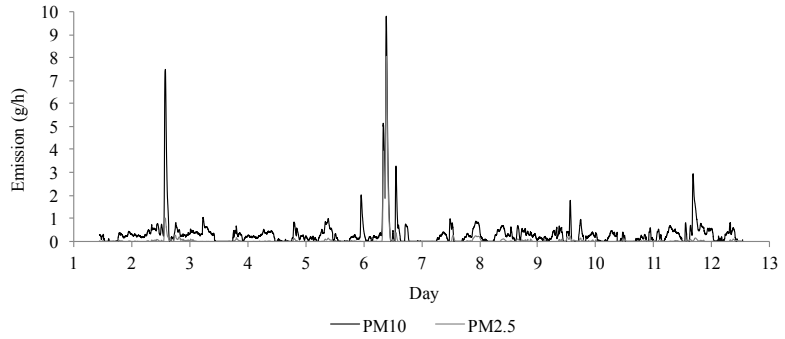

Figure 4.6. Daily PM10 and PM2.5 emission within sampling period in reproductive does.

4.4. Discussion

Particulate matter is a highly relevant pollutant found in the air of confined livestock facilities. However, previous studies on air quality in rabbit farms have usually measured airborne pollutants other than PM such as bioaerosols or gases (Duan et al., 2006; Calvet et al., 2011). Furthermore, to our knowledge, data on PM emissions from rabbit farms are limited, thus our findings contribute to fulfil this gap through the characterization of concentrations and emissions of size-fractioned PM2.5 and PM10.

Particulate matter concentrations presented herein resulted in higher PM concentrations in fattening rabbit farms compared with reproductive does, but were overall low compared with other livestock species and regulation thresholds. Airborne PM concentrations in both rabbit farms were below occupational thresholds according to human health (HSE, 2007) and below maximum exposure recommendations for livestock. Although no limits have been established as regards PM concentrations and rabbit health, concentrations did not exceed the recommended limits regarding pig health of 3.7 mg/m^3 for inhalable PM (particles wich can be inhaled through the nose and mouth) and of 0.23 mg/m^3 for respirable PM (particles wich can go beyond the larynx and penetrate into the unciliated respiratory system) (CIGR, 1994).

Measured PM concentrations were below reported values for other livestock housing systems such as poultry or pig in the literature, which range from 0.05 to 15.30 mg/m^3 inhalable PM, and from 0.03 to 1.90 mg/m^3 respirable PM as reviewed in Cambra-López et al. (2010). These differences among species are attributable to the peculiarities of rabbit production systems, where animals are reared in cages with limited movement and use no bedding material, which can be a relevant source of PM. This could result in less generation of PM, less deposition of PM on surfaces, and consequently less PM becoming airborne compared with pig or poultry (especially broiler) production systems. The use of deep pit as manure collection in rabbit houses could also influence such low PM concentrations compared with pig or poultry housings, where animals are directly in contact with dried manure. Contact with dry manure could facilitate dried manure disintegration and its release into the air.

Although few studies have investigated and quantified PM in rabbit farms, work by Kaliste et al. (2002) reported inhalable PM concentrations in laboratory rabbit rooms in the same range as PM10 in this study. Navarotto et al. (1995) reported inhalable PM concentrations to be in the range between 0.10 to 2.55 mg/m^3, and from 0.03 to 0.57 mg/m^3 for respirable PM, in an intensive fattening rabbit farm. Sancilio et al. (1999) reported inhalable PM concentrations to be in the range between 0.75 to 3.62 mg/m^3, and from 0.24 to 0.90 mg/m^3 for the

respirable fraction. Nonetheless, Ribikauskas et al., (2010) reported higher concentrations for inhalable PM for rabbits kept in groups in straw pens, than those reported for size-fractioned PM for rabbits kept in cages in our study. Although direct comparison between occupational health size fractions and PM10 and PM2.5 is not straight forward because it depends on sampling instrument cut-off curves and sampling conventions followed, our results of PM concentrations are found within the lower ranges of the reported values in the literature for occupational health size fractions. This could probably be a consequence of high ventilation rates experienced during the sampling period in our study, which result in a high dilution of indoor air and thus low airborne concentrations.

Among the physical and biological factors affecting PM concentrations in livestock farms, animal species, kind of housing system and environmental factors have been identified as one of the major factors influencing PM concentrations and emissions (Cambra-López et al., 2010). Concentrations of PM in our study were directly affected by hour of the day, increasing between 07:00 to 15:00 h. Rabbits have been reported to be more active during night hours (Estellés et al., 2010) and moreover, Ribikauskas et al. (2010) reported air quality parameters were related to rabbit activity, except for PM concentrations. Our results showed that during 24-h measurements, peaks in PM concentrations were mainly related with human activity rather than with animal activity. Sancilio et al. (1999) reported that burning hair on the cages through "cleaning by flame" was a relevant activity as regards PM generation. Our findings indicated that sweeping in fattening rabbits and both sweeping and burning hair on the cages in reproductive does were related with the highest increases both of PM10 and of PM2.5 concentrations.

Particulate matter emission rates for the rabbit farms were influenced by PM concentration and routine farm activities. These findings can contribute to quantifying PM from livestock production systems. Emission rates expressed per animal place were lower compared with poultry, pig or cattle (Wathes et al., 1997; Takai et al., 1998; Lacey et al., 2003). For instance, average emissions in broiler houses have been

reported to be between 13 and 47 mg/animal/day for PM10 (Lacey et al., 2003; Roumeliotis and Van Heyst, 2007; Calvet et al., 2009), and between 2.8 and 3.8 mg/animal/day for PM2.5 (Roumeliotis and Van Heyst, 2007; Cambra-López et al., 2009). Concentrations in broiler houses in these studies, however, were approximately 10-fold higher than in rabbit farms in our study. The emission rates obtained in this study are thus relatively high compared with other studies, taking into account the low measured concentrations in both rabbit farms. This inbalance can be explained by the high ventilation rates registered during our experiment, in autumn in the Mediterranean area of Spain, compared with other regions. Therefore, the consequences and fate of PM emissions from rabbit farms to the external environment in these conditions must be taken into account and PM emissions from such houses should not be neglected.

The results presented in this study for 15 days of continuous monitoring, are a valuable estimation for PM10 and PM2.5 emission factor for rabbit farms in Mediterranean conditions during autumn. Extrapolation of these results to a different season, however, should be performed with caution, as ventilation rates and indoor and outdoor environmental parameters can remarkably vary within seasons. The ventilation rate during summer is higher than during the autumn and indoor relative humidity can be expected to be lower, as well. On the contrary, in winter, ventilation rates are lower than in summer and indoor relative humidity can also increase. Therefore further research to compare these results with other periods of the year in rabbit farms is necessary.

Overall, the results presented in this study provide necessary data on air quality in rabbit farms, essential to understand and characterize PM concentrations and emissions in such animal facilities. Although PM concentrations inside rabbit farms are clearly under threshold for human health, the effect that PM chemical and biological composition may have on human or animal health and performance are still unknown. Nevertheless, our results improve the knowledge on the levels of PM in rabbit farms, which can be useful to identify factors affecting concentration and emissions and design adequate PM reduction

measures to control PM not only inside rabbit houses, but also emissions into the atmosphere.

4.5. Conclusions

Particulate matter concentrations inside rabbit farms are low compared with poultry and pigs. Average PM10 concentrations measured in this study were 0.08±0.06 mg/m^3 in fattening rabbits and 0.05±0.06 mg/m^3 in reproductive does. Average PM2.5 concentrations were 0.01±0.02 mg/m^3 in fattening rabbits, and 0.01±0.04 mg/m^3 in reproductive does.

Particulate matter concentrations were significantly influenced by type of human farm activity performed in the building rather than by animal activity. Major PM-generating activity in fattening rabbit farm was sweeping and major PM-generating activity in reproductive does was sweeping and burning hair of the cages.

Emissions of PM from rabbit farms are comparable to other livestock species and should not be neglected. Average calculated PM10 emissions in this study were 6.0±6.1 mg/place/d in fattening rabbits and 14.9±31.5 mg/place/d in reproductive does. Average PM2.5 emissions were 0.2±1.3 mg/place/d in fattening rabbits and 2.8±19.5 mg/place/d in reproductive does.

These results improve the knowledge on factors affecting concentration and emissions of PM in rabbit farms and can contribute to designing adequate PM reduction measures to control PM not only inside rabbit houses, but also emissions into the atmosphere.

4.6. Acknowledgements

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4.7. References

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5

Concentración, distribución espacial y por tamaño de bacterias aerobias mesófilas en el aire de granjas de broilers

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Resumen. El material particulado (PM) y los microorganismos en el aire son dos de los principales contaminantes por vía aérea en el ámbito de la ganadería. El PM puede ir asociado a numerosos microorganismos y la inhalación de los mismos constituye un efecto perjudicial para la salud. Los objetivos de este estudio fueron estudiar la distribución espacial de bacterias aerobias mesófilas en el aire de una granja de broilers (granja de cría de pollos para la obtención de carne), cuantificar la concentración de bacterias y de PM en al aire y su evolución a lo largo de un ciclo de producción, evaluar la relación entre el tamaño de partícula y las bacterias aerobias mesófilas, y correlacionar la evolución de las concentraciones de PM con la evolución de las concentraciones de bacterias aerobias mesófilas. Para ello se muestreó el aire semanalmente, utilizando diferentes métodos, en dos salas de una misma nave experimental de broilers durante un ciclo de 42 días. Se observó un gradiente espacial ($p<0,001$) en la concentración de bacterias aerobias mesófilas, entre la altura de los animales y alturas mayores al comienzo del ciclo (día 3). La concentración de bacterias aerobias mesófilas en el aire varió entre 3 y 6,53 logaritmo unidades formadoras de colonia, log UFC/m^3. Entre el 42% y el 96% de las bacterias aerobias mesófilas se encontraron en los rangos de tamaño de partícula entre 3,3 y más de 7 μm. La concentración media de PM en el aire fue de 0,019 mg/m^3 para PM2,5 y 0,189 mg/m^3 para PM10. Las concentraciones de PM y bacterias aerobias mesófilas en el aire evolucionaron de forma similar a lo largo del ciclo de producción (coeficiente de correlación entre 0,78 y 0,89), produciéndose para ambos un máximo en el día 24 del ciclo y disminuyendo a partir de entonces, coincidiendo con un incremento en la tasa de ventilación.

Palabras clave. Bioaerosoles, calidad del aire, material particulado, avicultura.

Abstract. In livestock houses, particulate matter (PM) and airborne microorganism are two of the most relevant air pollutants. Particulate matter may carry microorganisms, the inhalation of which can cause detrimental health effects. The aim of this study was to study the spatial distribution of airborne aerobic mesophilic bacteria in the air of a broiler farm (rearing poultry for meat production), to quantify the concentration of airborne aerobic mesophilic bacteria and PM in the air and to study their evolution in time, as well as to evaluate the relationship between particle size and airborne aerobic mesophilic bacteria, and to correlate the evolution of PM concentrations and aerobic mesophilic bacteria concentrations over time. For this purpose, the air of two broiler rooms in an experimental broiler farm was sampled weekly using different methods during 42 days. There was a spatial gradient ($p<0.001$) in aerobic mesophilic bacteria concentrations between animal height and higher heights at the beginning of the production cycle (day 3). The concentration of aerobic mesophilic bacteria in the air ranged from 3 to 6.53 log colony forming units, log CFU/m^3. Between 42% and 96% of aerobic mesophilic bacteria in the air were found in the particle size ranges between 3.3 to more than 7 μm. The PM concentration in the air was equal to 0.019 mg/m^3 for PM2.5 and equal to 0.189 mg/m^3 for PM10. The PM and bacteria concentrations followed a similar evolution during the production cycle (correlation coefficient between 0.78 y 0.89), showing a maximum concentration on day 24 and decreasing thereafter, coinciding with an increase in the ventilation rate.

Keywords. Bioaerosols, particulate matter, air quality, poultry.

5.1. Introducción

El material particulado (del inglés, Particulate Matter; PM) suspendido en el aire en alojamientos ganaderos se compone principalmente de partículas fecales, pienso, partículas de piel, pelo, plumas y polvo mineral en diferentes proporciones (Cambra-López et al., 2011). Estas partículas pueden ir solas o asociadas a numerosos microorganismos, en ocasiones patógenos, pudiendo facilitar su transmisión entre animales y humanos dentro de las explotaciones y ocasionando problemas sanitarios (Chinivasagam et al., 2009). Se denomina bioaerosol al conjunto de partículas en suspensión con un diámetro que varía generalmente entre 0,5 y 100 μm, de origen o actividad biológica que pueden afectar a seres humanos causándoles algún tipo de patología (Cox and Wathes, 1995). En este sentido, el PM y los bioaerosoles son dos de los principales contaminantes aéreos en el ámbito de la ganadería (Seedorf et al., 1998; Takai et al., 1998).

De entre las partículas biológicas que pueden ir asociadas a los bioaerosoles se encuentran las esporas, hongos, virus, bacterias, toxinas y alérgenos. La principal fuente generadora de bioaerosoles en alojamientos ganaderos son los propios animales a través de sus secreciones y excreciones; además del pienso y la cama, en aquellas explotaciones en las que esté presente (Cox and Wathes, 1995). La concentración de bioaerosoles en el aire varía con el sistema de alojamiento, el animal y los factores ambientales.

La inhalación de todas estas partículas pueden afectar a seres humanos causándoles algún tipo de patología, ya sea de tipo alérgico, tóxico, infeccioso u otros (Cox and Wathes, 1995). Los problemas de salud más frecuentemente asociados a la exposición a bioaerosoles son los problemas respiratorios y las enfermedades infecciosas (Douwes et al., 2003). Además, la exposición aguda y crónica de los animales a los bioaerosoles puede ocasionar una disminución en el rendimiento productivo (Donham, 1991).

La frecuencia y gravedad de los posibles problemas respiratorios y enfermedades infecciosas ocasionados por los bioaerosoles depende del tipo, tamaño, cantidad y supervivencia de los microorganismos. Además,

se ha demostrado que el PM puede actuar como sustrato para los microorganismos ya que proporciona un ambiente adecuado para su supervivencia (Millner, 2009). En este sentido, las partículas pueden proteger a los microorganismos de condiciones climáticas adversas como por ejemplo la temperatura, la humedad relativa y la radiación ultravioleta (Cox and Wathes, 1995), que pueden provocar su inactivación. La forma y el tamaño del PM también determinan el periodo de supervivencia de los microorganismos en el aire. El tamaño de partícula determina el tiempo que la partícula permanecerá suspendida en el aire (Cox and Wathes, 1995) de manera que, a menor tamaño permanecerá más tiempo suspendida.

En general, entre los diferentes alojamientos ganaderos, las granjas de broilers son las que mayores concentraciones de PM y bioaerosoles presentan (Takai et al., 1998). Estas diferencias se deben fundamentalmente a las particularidades de este tipo de animales y del alojamiento: a la presencia de plumas jóvenes en broilers (plumón) que fácilmente pueden ser aerotransportadas, a la excreción de ácido úrico encapsulado en forma de partículas esféricas, así como al tipo de alojamiento (sobre suelo) con material de cama (Cambra-López et al., 2011b). Los microorganismos más comúnmente aislados en los alojamientos ganaderos son las bacterias Gram positivas, siendo las bacterias de los géneros *Staphylococcus* y *Streptococcus* las que predominan (Matkovic et al., 2007). Entre las bacterias Gram negativas, las bacterias de la familia *Enterobacteriaceae* son las más abundantes (Zucker et al., 2000b). Sin embargo, todavía se desconoce cuál es el comportamiento de las bacterias suspendidas en el aire de granjas, su distribución y cómo se relacionan con el PM. Esta información podría ser útil para predecir sus niveles, evaluar los posibles riesgos para la salud y así contribuir a desarrollar medidas para reducir sus efectos perjudiciales en granjas de broilers.

En este contexto, los objetivos de este trabajo son:

> Estudiar la distribución espacial de bacterias aerobias mesófilas en el aire durante un ciclo de producción de broilers.

Cuantificar la concentración de bacterias aerobias mesófilas y de PM en el aire de granjas de broilers y estudiar su evolución a lo largo de un ciclo de producción.

Evaluar la relación entre el tamaño de partícula y las bacterias aerobias mesófilas en el aire de granjas de broilers.

Identificar la correlación entre la evolución de las concentraciones de PM y las concentraciones de bacterias aerobias mesófilas en el aire de granjas de broilers.

5.2. Material y métodos

5.2.1. Instalaciones y animales

La experiencia se llevó a cabo en dos salas piloto (sala 1 y sala 2) de la nave experimental de cebo aviar del Centro de Tecnología Animal perteneciente al Instituto Valenciano de Investigaciones Agrarias (IVIA), situado en Segorbe (Castellón). Cada sala tenía unas dimensiones de 13 x 6 metros. Las salas disponían de un mecanismo de ventilación forzada controlado mediante un sistema copilot (Copilot System®, Barcelona), con dos ventiladores situados en el techo de cada sala. El sistema de refrigeración empleado fue de refrigeración evaporativa, a partir de unos paneles humidificadores por los que se hace pasar el aire procedente del exterior a través de una película de agua que lo enfría.

Para la experiencia se utilizaron un total de 576 pollos macho (línea Hubbard) de un día de vida y 42 ± 3 g de peso. Al comienzo del ciclo, los pollos se dividieron de forma aleatoria en ambas salas. Se utilizó viruta de madera como cama (de 10 cm de espesor). El ciclo de crecimiento se desarrolló en verano, y duró 42 días durante los cuales los animales fueron alimentados con piensos comerciales.

5.2.2. Parámetros productivos

Los pollos se pesaron al inicio y al final del ciclo. El consumo de pienso se valoró al final del ciclo productivo. Con estos datos se calculó el consumo medio diario de pienso (CMD), la ganancia media diaria de

peso (GMD) y el índice de conversión (IC; kg pienso consumido/kg peso) de los animales. La mortalidad fue supervisada diariamente y se tuvo en cuenta para el cálculo de los parámetros productivos.

5.2.3. Parámetros ambientales

Se registró la temperatura y la humedad relativa de forma continua durante todo el ciclo cada 5 minutos en 2 puntos diferentes en el interior de cada sala. Para ello se utilizaron sensores de temperatura y humedad relativa (HOBO U12-O13 Onset Computer Crp, Pocasset, Mass, EEUU). Además el caudal de ventilación se calculó en cada sala a partir de un balance de dióxido de carbono (CO_2) utilizando sondas con sensores de CO_2 (Vaisala GMT-222, 0-1000 ppm) y un datalogger (HOBO U12-O13 Onset Computer Crp, Pocasset, Mass, EEUU) para la recogida de datos en continuo. La emisión de CO_2 de los animales se estimó según valores obtenidos por Calvet et al. (2010). El día 29 del ciclo se incrementó la tasa de ventilación y se puso en marcha el sistema de refrigeración evaporativa.

5.2.4. Muestreo de bacterias aerobias mesófilas en el aire

Para cumplir con los objetivos de este estudio, se utilizaron tres sistemas de muestreo de bacterias en el aire: el sistema de sedimentación en placa (método pasivo); el sistema de borboteador de aire o "impingement" y el sistema de impactación en cascada (los dos últimos, métodos inerciales).

La diferencia entre estos sistemas de muestreo radica en el método de captación de la muestra (sin aire forzado para el método pasivo y con aire forzado para los métodos inerciales), además del diámetro de corte y por lo tanto, el tamaño de partícula muestreado. Mediante la sedimentación en placa se obtienen, principalmente, bioaerosoles adheridos a tamaños de partícula grande o conglomerados de partículas que se depositan sobre la placa con agar por gravedad. Los borboteadotes captan, en un medio líquido mediante aspiración del aire, partículas superiores a 0,3 μm de diámetro. El impactador en cascada recoge la muestra directamente en agar mediante aspiración del aire y separa por tamaños de partículas; permitiendo posteriormente, evaluar la relación entre el tamaño de partícula y los microorganismos en el aire.

Así, el sistema de sedimentación en placa permitió estudiar la distribución espacial de las bacterias aerobias mesófilas cultivables en las salas, el sistema de borboteador de aire permitió cuantificar la evolución de las concentraciones de estas bacterias en el aire a lo largo del ciclo de producción, mientras que el sistema de impactación en cascada permitió relacionar las bacterias aerobias mesófilas cultivables en el aire con el tamaño de partícula. El aire se muestreó semanalmente los días 3, 17, 24 31 y 38 del ciclo.

5.2.5. Distribución espacial de las bacterias aerobias mesófilas cultivables medida mediante sedimentación en placa

Para estudiar la distribución en el aire de bacterias aerobias mesófilas se utilizó la técnica de sedimentación en placa. Para ello se colocaron en cada sala un total de 36 placas con un medio de cultivo para bacterias aerobias mesófilas (PCA, del inglés Plate Count Agar) (Liofolchem, TE, Italia), en tres alturas (12 placas por altura y sala): de 10 a 30 cm del suelo (altura de respiración de los animales, que varió debido al crecimiento de los animales), a 150 cm del suelo (altura de respiración de personas) y a 200 cm del suelo (altura de la salida de la ventilación).

Semanalmente, las placas con PCA se colocaron abiertas durante tiempos variables para obtener un número de colonias entre 30 y 300 para su recuento. Este tiempo varió entre 5 y 30 minutos según la semana del ciclo de engorde de los pollos. Tras la exposición, las placas se incubaron en estufa a 30ºC durante 48 horas. Tras la incubación, las unidades formadoras de colonias (UFC) fueron contadas. Este muestreo se realizó únicamente durante las primeras 3 semanas del ciclo de producción ya que a partir del día 17, el elevado número de microorganismos en el aire imposibilitó la lectura de las placas.

5.2.6. Concentración y evolución de las bacterias aerobias mesófilas cultivables medidas mediante el sistema de borboteadores

Para el recuento de bacterias aerobias mesófilas en el aire, éste se muestreó en un medio líquido utilizando un sistema de frascos borboteadores "impingers" (AGI-30, Ace Glass, Inc., Vineland, NJ, EEUU). Éstos funcionaban recogiendo un volumen de aire conocido en

un medio de captación líquido. El medio de captación utilizado estaba compuesto por 20 mL de agua de peptona tamponada estéril, 0,01% de Tween y 0,005% de antiespumante. El muestreo se hizo por triplicado en cada sala (3 impingers/sala). El caudal de aire muestreado fue 12,5 L/min por cada impinger, que se obtenía mediante una bomba de succión de caudal constante calibrada (75 Mb, Ilmivac, Ilmenau, Alemania). El diámetro de corte de los borboteadores de aire fue de 0,3 μm. Todos los microorganismos por encima de este diámetro fueron recogidos.

El muestreo se llevó a cabo en el centro de cada sala en un punto próximo a la salida del aire, a 1,5 m de altura. Las muestras de aire se recogieron semanalmente en cada sala durante un tiempo de 15 minutos. Las muestras recogidas se mantuvieron refrigeradas (4ºC) durante su transporte hasta el laboratorio.

La muestra se procesó en el laboratorio antes de 2 horas. Se realizaron diluciones seriadas de cada muestra y la solución líquida se sembró en placa con medio de cultivo PCA. Las placas se incubaron en estufa a 30ºC durante 48 horas.

El número de UFC por placa se calculó en relación al volumen de aire muestreado, el tiempo de muestreo y el flujo de aire, tal y como muestra la siguiente ecuación:

$$UFC/m^3 = \frac{UFC \times V.Muestra \times 1000}{V.Sembrado \times FlujoAire \times t}$$

Siendo:

UFC: Unidades formadoras de colonias contadas en la placa
V. Muestra: Volumen de la muestra (20 mL)
V. Sembrado: Cantidad de muestra sembrado en placa (0,1 mL)
Flujo Aire: Flujo de aspiración de la bomba (12,5 L/min)
t: Tiempo de muestreo (15 min)

5.2.7. Distribución según tamaño de partícula de las bacterias aerobias mesófilas cultivables en el aire mediante impactación en cascada

Para muestrear bacterias aerobias mesófilas en el aire en función del tamaño de partícula al que podrían estar adheridas, se utilizó un impactador en cascada en agar (Six Stage Viable, Andersen Cascade Impactor, Thermo Scientific, EEUU). Este impactador separaba las partículas de acuerdo a su diámetro aerodinámico, desde un mínimo de 0,65 μm hasta un máximo de 7 μm, haciendo pasar la muestra por diferentes tamaños de poro (Tabla 5.1). El caudal de aire muestreado fue 28,1 L/min que se obtenía mediante una bomba de succión de caudal constante calibrada.

Tabla 5.1. Rango de tamaños de partículas del impactador en cascada.

	Rango de tamaño de partículas (μm)
Nivel 1	0,65 – 1,1
Nivel 2	1,1 – 2,1
Nivel 3	2,1 – 3,3
Nivel 4	3,3 – 4,7
Nivel 5	4,7 – 7,0
Nivel 6	≥7

Este impactador constaba de seis plataformas consecutivas perforadas con tamaños de poro decrecientes. Sobre cada una de las plataformas se colocó una placa de vidrio (DURAN Group GmbH, Alemania) con 27 mL de medio de cultivo PCA.

El muestreo se realizó semanalmente en cada sala. El tiempo de muestreo varió entre 10 y 90 segundos y fue ajustado para obtener un número de colonias entre 30 y 300 por placa según la semana del ciclo de crecimiento de los pollos, así como para evitar la desecación del agar debido a la corriente de aire forzado que se hace pasar por las placas durante el muestreo.

Entre muestreos, el impactador se desinfectó con una solución de etanol al 70%. Las placas de agar se transportaron en refrigeración (4ºC) al laboratorio antes de haber transcurrido 2 horas desde el muestreo.

Después del muestreo, estas placas se incubaron en estufa a 30ºC durante 48 horas. Tras la incubación, las UFC fueron contadas. El número de UFC en el aire ambiente se calculó en relación al flujo de aire y el tiempo de muestreo para obtener las UFC por metro cúbico de aire muestreado, según la siguiente ecuación:

$$UFC/m^3 = \frac{UFC \times 1000}{Flujo\ aire \times t}$$

Siendo:

UFC: Unidades formadoras de colonias contadas en la placa
Flujo aire: Flujo de aire absorbido (28,1 L/min)
t: Tiempo de muestreo (min)

A partir del día 31 del ciclo, debido al elevado número de microorganismos en el aire en la explotación, las placas fueron lavadas para diluir la concentración de colonias según la metodología propuesta por Zhao et al. (2011a y b). El lavado se realizó añadiendo 2 mL de agua de peptona estéril a cada una de las placas y mediante un asa de siembra se rascó la superficie del agar, sin dañarlo. Los 2 mL de agua de peptona se vertieron en un frasco estéril. Esta operación se repitió tres veces, obteniendo un total de 6 mL por placa. A partir de esta solución, se realizó el recuento en placa.

Para estas placas, el número de UFC en el aire ambiente se calculó en relación al flujo de aire, el tiempo de muestreo y el volumen de agua de peptona añadida, para obtener las unidades formadoras de colonia por metro cúbico de aire muestreado.

5.2.8. Concentración y evolución de material particulado en el aire

Semanalmente, se registraron en continuo las concentraciones de dos fracciones de partículas en el aire en cada sala: PM10 (material particulado de 10 μm de diámetro o inferior) y PM2,5 (material particulado de 2,5 μm de diámetro o inferior) con el sistema "Tapered element oscillating microbalance", TEOM (TEOM®, modelo 1405-D Thermo Fisher Scientific, EEUU). De esta forma nos fue posible relacionar la evolución del material particulado a lo largo del ciclo con la

evolución de las bacterias aerobias mesófilas. El TEOM medía la masa de PM mediante determinación de la frecuencia propia de vibración de un dispositivo de vidrio de forma tubular anclado en uno de sus extremos.

Las muestras de PM se tomaron a 2 m de altura. El muestreo fue semanal en ambas salas. El tiempo de muestreo fue de 24 horas por sala y semana. El equipo se programó para registrar concentraciones cada minuto.

5.2.9. Análisis estadístico

Los resultados obtenidos a partir de la sedimentación en placa se analizaron mediante un análisis de varianza (ANOVA) utilizando el paquete estadístico SAS System Software (Version 9.1, SAS Institute Inc., Cary, Carolina del Norte, EEUU) para determinar la variación en el número de colonias por altura en cada sala. Los valores medios de recuento por alturas, salas y semanas fueron comparados mediante el test de comparación de medias tukey para un nivel de significación estadística del 5% (0,05).

Los resultados obtenidos semanalmente mediante cada uno de los métodos de muestreo se presentan como medias por sala y semana.

Para identificar la correlación entre la evolución de las concentraciones de PM y las concentraciones de bacterias aerobias mesófilas en el aire, se calculó el coeficiente de correlación entre la concentración de PM2,5 y PM10 y la de bacterias aerobias mesófilas, utilizando los recuentos de bacterias aerobias mesófilas medidos con los borboteadores y utilizando el paquete estadístico SAS System Software (Version 9.1, SAS Institute Inc., Cary, Carolina del Norte, EEUU).

5.3. Resultados y discusión

5.3.1. Parámetros productivos

Los parámetros productivos de los animales se encontraron dentro de los rangos habituales en broilers. Los valores medios de consumo medio

diario de pienso (CMD), ganancia media diaria (GMD) e índice de conversión (IC) durante todo el ciclo se presentan en la Tabla 5.2 para cada sala.

Tabla 5.2. Valores medios de ganancia media diaria (GMD), consumo medio diario (CMD), e índice de conversión (IC) durante todo el ciclo de producción de broilers en cada sala.

	GMD (g/día)	CMD (g/día)	IC (g pienso/g peso)
Sala 1	63,2	103,7	1,64
Sala 2	67,6	110,5	1,63

5.3.2. Parámetros ambientales

La Tabla 5.3 resume la temperatura y la humedad relativa en las dos salas para todo el ciclo de producción. Éstas fueron similares a lo largo del ciclo en ambas salas y se encontraron dentro de los rangos normales para el adecuado crecimiento de los animales.

Tabla 5.3. Media de temperatura (T), humedad relativa (HR) y desviación estándar (Desvest) a lo largo del ciclo de producción de broilers en cada sala.

	T (ºC)	Desvest T	HR (%)	Desvest HR
Sala 1	28,0	2,1	44,8	15,6
Sala 2	27,6	1,5	46,5	13,4

En la Figura 5.1 se presenta la evolución de la tasa de ventilación a lo largo del ciclo para cada sala. La ventilación fue similar durante la primera mitad del ciclo en ambas salas, sin embargo, la tasa de ventilación de la sala 1 fue superior a la de la sala 2 en la segunda mitad del ciclo, debido al control de la climatización. No obstante, la tasa de ventilación media en la sala 1 para todo el ciclo (0,7±0,5 m^3/h/animal) fue comparable con la tasa de ventilación media en la sala 2 (0,5±0,3 m^3/h/animal).

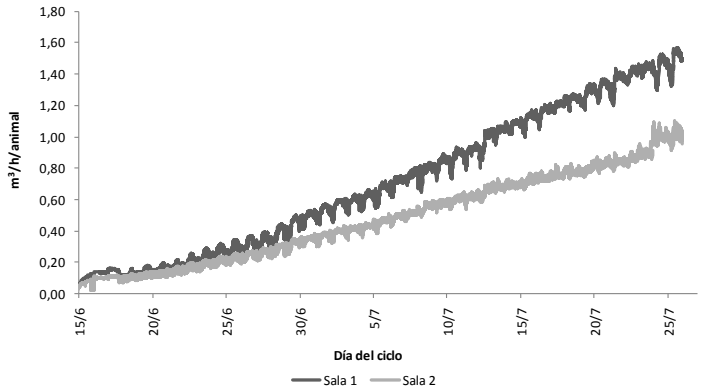

Figura 5.1. Tasa de ventilación (m³/h/animal) durante el ciclo de producción de broilers en cada sala.

5.3.3. Distribución espacial de las bacterias aerobias mesófilas en el aire

En la Tabla 5.4 se presenta el recuento medio de los 12 puntos en cada una de las alturas muestreadas mediante la técnica de sedimentación pasiva, por día de muestreo y sala. En general, se observó un incremento de la concentración bacteriana del día 3 al día 17 de ciclo en ambas salas.

Tabla 5.4. Media del logaritmo de unidades formadoras de colonia (log UFC), error estándar de la media (EEM) y p-valor del recuento de bacterias aerobias mesófilas a diferentes alturas por día de del ciclo, sala y altura.

	Día del ciclo	Altura	N muestras	log UFC/placa	EEM	p-valor
Sala 1	3	30 cm	12	3,02[a]	0,038	<0,001
		150 cm	12	2,56[b]		
		200 cm	12	2,17[c]		
	17	30 cm	12	3,18	0,040	0,179
		150 cm	12	3,28		
		200 cm	12	3,24		
Sala 2	3	30 cm	12	2,54[a]	0,029	<0,001
		150 cm	12	2,21[b]		
		200 cm	12	2,29[b]		
	17	30 cm	12	3,33	0,037	0,252
		150 cm	12	3,27		
		200 cm	12	3,25		

a,b Medias con diferente superíndice difieren significativamente (p<0,05)

En cuanto a la distribución de las bacterias, en el muestreo realizado el día 3 del ciclo se observó un gradiente significativo de bacterias entre las tres alturas ($p<0,001$), encontrándose un mayor número de bacterias aerobias mesófilas a la altura de los animales (30 cm) en ambas salas, tal y como describieron Saleh et al. (2005). Esto es debido a que los animales y sus deyecciones son la principal fuente de microorganismos al aire (Seedorf et al., 1998). Sin embargo, dos semanas después (día 17 del ciclo), se observó un incremento en el número de bacterias respecto al muestreo anterior, aunque no se observaron diferencias significativas en el recuento de bacterias aerobias mesófilas entre las tres alturas. Estos resultados indican una distribución más homogénea de las bacterias aerobias mesófilas en el aire a partir de este momento, probablemente debido a un incremento de la ventilación que favoreció la homogeneización del aire en las salas.

5.3.4. Concentración y evolución de las bacterias aerobias mesófilas en el aire

La Figura 5.2 muestra el recuento de bacterias aerobias mesófilas obtenido utilizando la técnica de borboteadores de aire a lo largo del ciclo para cada sala. El promedio de concentración de UFC en el aire teniendo en cuenta las dos salas fue 6,28±1,14 log UFC/m^3, muy similar al recuento obtenido en otros estudios. Por ejemplo, Seedorf et al. (1998) obtuvieron un recuento de 6,43 log UFC/m^3, y Bakutis et al. (2004), obtuvieron 6,67 log UFC/m^3 en broilers.

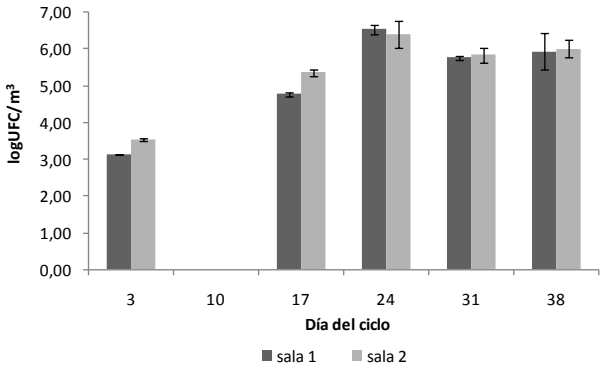

Figura 5.2. Recuento de bacterias aerobias mesófilas de la muestra obtenida mediante los borboteadores de aire a lo largo del ciclo de producción de broilers en cada sala.

En ambas salas, la evolución en el tiempo de la concentración de bacterias aerobias mesófilas fue similar. Se mostró un incremento semanal en el número de UFC desde el comienzo de la experiencia hasta alcanzar el máximo de concentración el día 24 del ciclo, en el que se obtuvo el recuento de 6,53 log UFC/m^3 en la sala 1 y de 6,41 log UFC/m^3 en la sala 2 (ambos equivalen a 10^6 UFC/m^3). A partir de entonces, los recuentos disminuyeron aunque los valores se mantuvieron elevados respecto a los valores iniciales, entre 5,7 y 6 log UFC/m^3.

En la bibliografía se describe que el recuento de bacterias en el aire incrementa con la edad de los pollos (Saleh et al., 2005; Oppliger, 2008), mostrando su concentración máxima alrededor de la cuarta semana del ciclo (Cox and Wathes, 1995), tal y como sucede en nuestro estudio. Después, puede descender ligeramente debido al aumento en la tasa de ventilación, considerando los valores elevados de ventilación que normalmente se alcanzan a finales del ciclo de producción de broilers.

5.3.5. Relación entre el tamaño de partícula y las bacterias aerobias mesófilas

En la Tabla 5.5 se observa el recuento de bacterias aerobias mesófilas a lo largo del ciclo, recogidas mediante la técnica de impactación en cascada. Se presenta para cada sala el logaritmo de UFC medio obtenido en cada uno de los 6 niveles del equipo de muestreo, correspondientes a los distintos tamaños de partículas. Esta tabla muestra un incremento semanal de microorganismos durante el ciclo para todos los tamaños de partícula en ambas salas.

El promedio del logaritmo de la concentración de UFC en el aire teniendo en cuenta las dos salas fue 7,11±1,60 log UFC/m^3, siendo ligeramente superior en la sala 2.

Tabla 5.5. Recuento de bacterias aerobias mesófilas (log UFC/m³) obtenidos mediante impactación en cascada según rango de tamaño de partículas a lo largo del ciclo de producción de broilers en cada sala.

	Día del ciclo	Rango de tamaño de partículas (μm)					
		0,65–1,1	1,1–2,1	2,1–3,3	3,3–4,7	4,7–7	>7
Sala 1	3	1,67	2,37	3,11	3,27	3,11	3,07
	10	–	–	–	–	–	–
	17	3,39	3,97	4,40	4,50	4,66	4,44
	24	4,79	4,95	5,07	5,15	5,37	5,33
	31	6,88	6,88	6,92	5,80	6,71	8,76
	38	6,60	6,89	7,22	6,71	6,41	7,41
Sala 2	3	1,37	2,58	3,18	3,37	3,40	3,41
	10	–	–	–	–	–	–
	17	4,47	4,30	4,40	4,54	4,52	4,50
	24	5,10	5,45	5,45	5,71	5,34	5,46
	31	5,80	5,80	6,28	7,03	6,10	6,58
	38	7,74	6,27	6,53	6,62	6,64	7,22

La Figura 5.3 y la Figura 5.4 muestran los resultados de la Tabla 5.5 expresados en porcentaje de bacterias para cada uno de los 6 niveles del equipo de muestreo y en cada sala, a lo largo del ciclo de producción. Se observa que el recuento máximo de bacterias aerobias mesófilas en general se encontró en los rangos de partículas gruesas. La Figura 5.3 muestra que entre el 42 y el 96% de las bacterias aerobias mesófilas se encontraron en los rangos de tamaño de partícula entre 4,7 y 7 μm en la sala 1. La Figura 5.4 muestra que entre el 57 y el 83% de las bacterias aerobias mesófilas se encontraron en los rangos de tamaño de partícula entre 3,3 y 7 μm en la sala 2, excepto el día 38 en el que se obtuvo un 64% de bacterias aerobias mesófilas en los rangos de tamaño de partícula más pequeños (entre 0,65 y 1,1 μm).

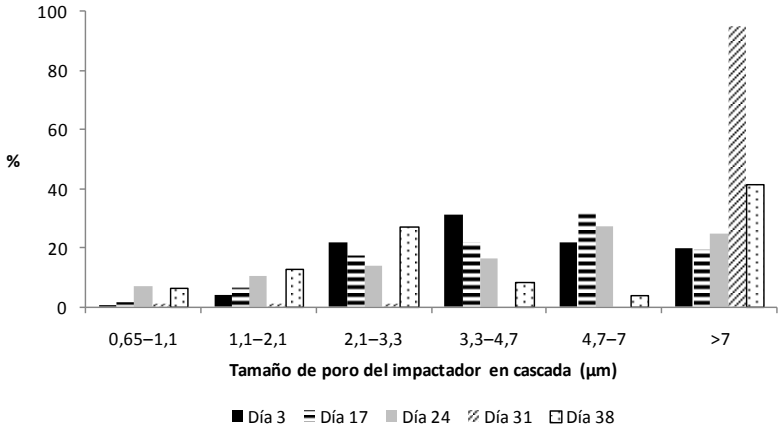

Figura 5.3. Porcentaje de microorganismos según el tamaño de poro del impactador en cascada durante el ciclo de producción de broilers en la sala 1.

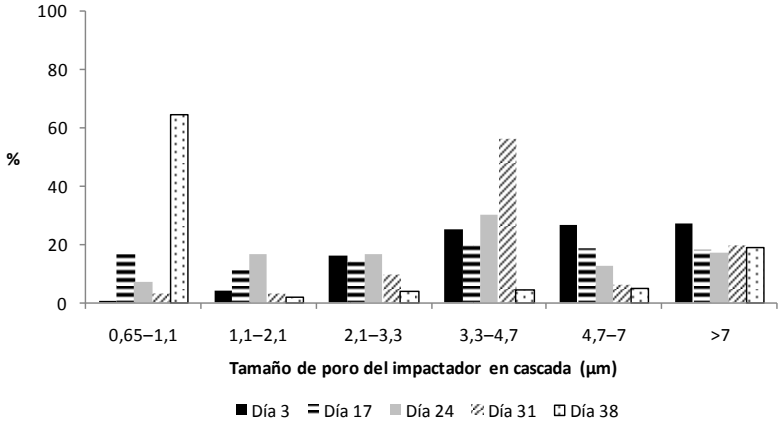

Figura 5.4. Porcentaje de microorganismos en cada uno de los niveles del impactador en cascada durante el ciclo de producción de broilers en la sala 2.

Estos resultados indican una mayor asociación de las bacterias aerobias mesófilas a las partículas de tamaños entre 3,3 y 7 µm o superior, comparado con las más pequeñas (entre 0,65 y 3,3 µm). Lee et al. (2006) también encontraron una tendencia a la asociación de microorganismos con partículas de tamaños grandes.

5.3.6. Concentración y evolución del material particulado en el aire

Como se observa en la Figura 5.5 y Figura 5.6, las concentraciones de PM medidas con el TEOM tanto de PM2,5 como de PM10 variaron en el tiempo en ambas salas. La concentración media para ambas salas fue de 0,019 mg/m^3 para PM2,5 y 0,189 mg/m^3 para PM10, en todo el ciclo. En todos los casos, la concentración del PM10 fue superior a la concentración del PM2,5, indicando una mayor concentración de partículas gruesas (entre 2,5 y 10 μm de diámetro) que finas (menores de 2,5 μm de diámetro).

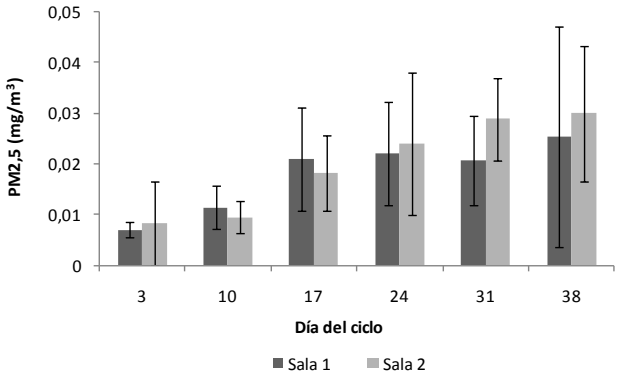

Figura 5.5. Concentración de PM2,5 medido con el equipo de muestreo en continuo de material particulado (TEOM) a lo largo del ciclo de producción de broilers en cada sala. Barras de error indican desviación estándar.

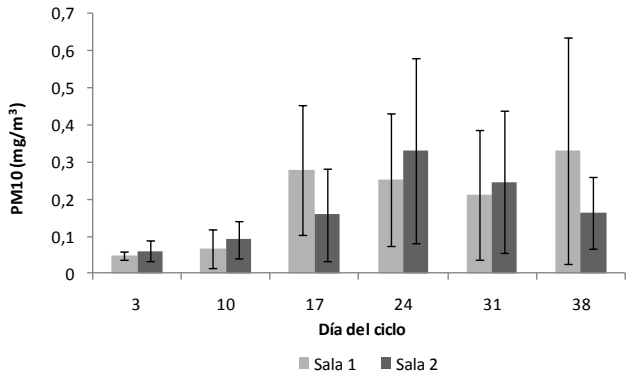

Figura 5.6. Concentración de PM10 medido con el equipo de muestreo en continuo de material particulado (TEOM) a lo largo del ciclo de producción de broilers en cada sala. Barras de error indican desviación estándar.

Los niveles de PM fueron bajos durante toda la experiencia, aunque se observó una elevada variación diaria en las concentraciones que se refleja en una amplia desviación estándar de la media (Figura 5.5 y Figura 5.6). Esta variación es habitual y se debe a la fluctuación horaria de las concentraciones de PM a lo largo de un día. Los niveles de PM fueron muy inferiores a los valores máximos propuestos para protección de la salud de los trabajadores y de los animales: 3,4 mg/m^3 de PM10 y 1,7 mg/m^3 de PM2,5 (CIGR, 1992). Igualmente, no se superaron los valores máximos de concentración según criterios de salud humana en el ámbito laboral, que están cifrados en 10 mg/m^3 de PM10 y 4 mg/m^3 de PM2,5, (HSE, 2007).

Además, la concentración de PM total obtenida en este estudio es inferior a la mayoría de resultados aportados por otros autores para el mismo tipo de alojamiento. La PM10 indicada en los diferentes estudios es de 0,69 mg/m^3 (Roumeliotis and Van Heyst, 2007) y de 1,21 mg/m^3 (Cambra-López et al., 2009), y la PM2,5 es de 0,19 mg/m^3 (Roumeliotis and Van Heyst, 2007) y de 0,069 mg/m^3 (Cambra-López et al., 2009). Esta diferencia en las concentraciones puede ser debido a la baja densidad de animales de nuestro estudio en condiciones experimentales, comparado con otros estudios.

La concentración de PM aumentó a lo largo del ciclo. A partir del día 29 del ciclo, aumentó la tasa de ventilación (especialmente en la sala 1) y se puso en marcha la refrigeración evaporativa, disminuyendo la concentración de partículas, sobre todo la fracción más gruesa (PM10). Esta evolución coincide con el comportamiento de los microorganismos aerobios mesófilos obtenido con los borboteadores de aire (Figura 5.2). Se obtuvo una correlación positiva (Tabla 5.6) entre la concentración de PM y la concentración de bacterias aerobias mesófilas en el aire medida con los borboteadotes. Los coeficientes de correlación variaron entre 0,78 y 0,89, para PM2,5 y PM10, siendo superiores para PM2,5. Estos resultados confirma la relación positiva entre estas variables descrita por otros autores (Bakutis et al., 2004).

Tabla 5.6. Coeficiente de correlación entre la concentración de material particulado (PM2,5 y PM10) y el recuento de bacterias aerobias mesófilas medido con los borboteadotes.

	Coeficiente de correlación	
	Sala 1	Sala 2
PM2,5	0,89	0,89
PM10	0,78	0,86

5.4. Conclusiones

La distribución espacial de las bacterias aerobias mesófilas en el aire varió a lo largo del ciclo. Al comienzo del ciclo (día 3), se observó un mayor número de bacterias ($p<0,001$) a la altura de los animales (30 cm del suelo) respecto a alturas mayores (entre 150 y 200 cm del suelo) indicando un gradiente en su concentración. Sin embargo, estas diferencias desaparecieron a partir del día 17 del ciclo, probablemente debido a un aumento de las concentraciones de bacterias aerobias mesófilas totales y a una distribución más homogénea de éstas en el aire.

Las concentraciones de bacterias aerobias mesófilas medidas en el aire de una granja de broilers variaron entre 3 log UFC/m^3 (equivalente a 10^3 UFC/m^3) y 6,53 log UFC/m^3 (equivalente a 10^6 UFC/m^3). Las concentraciones aumentaron semanalmente, hasta alcanzar el máximo el día 24 del ciclo. A partir de entonces, y probablemente debido al aumento de la tasa de ventilación, los recuentos disminuyeron aunque se mantuvieron con valores elevados.

Entre el 42% y el 96% de las bacterias aerobias mesófilas se encontraron en los rangos de tamaño de partícula entre 3,3 y más de 7 μm, comparado con las más pequeñas (entre 0,65 y 3,3 μm).

La concentración media de PM en el aire fue de 0,019 mg/m^3 para PM2,5 y 0,189 mg/m^3 para PM10. La evolución de las concentraciones de PM a lo largo del ciclo fue similar a la de las bacterias aerobias mesófilas en el aire. A partir del día 24, y probablemente debido al aumento de la tasa de ventilación, las concentraciones, especialmente de la fracción más gruesa (PM10) disminuyeron. Existe una correlación

positiva (coeficiente de correlación entre 0,78 y 0,89) entre las concentraciones de PM2,5 y PM10 y las de bacterias aerobias mesófilas.

Los resultados de este estudio son útiles para el desarrollo de medidas de reducción del PM así como de bioaerosoles en granjas de broilers, y contribuyen a entender su comportamiento en el aire.

5.5. Agradecimientos

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6

Comparative performance of three sampling techniques to detect airborne *Salmonella* species in poultry farms

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Abstract. Sampling techniques to detect airborne *Salmonella* species (spp.) in two pilot scale broiler houses were compared. Broilers were inoculated at seven days of age with a marked strain of *Salmonella* Enteritidis. The rearing cycle lasted 42 days over the summer. Airborne *Salmonella* spp. were sampled weekly using impaction, gravitational settling, and impingement techniques. Additionally, *Salmonella* spp. were sampled on feeders, drinkers, walls, and in the litter. Environmental conditions (temperature, relative humidity, and airborne particulate matter (PM) concentration) were monitored during the rearing cycle. The presence of *Salmonella* spp. was determined by culture-dependent and molecular methods. No cultivable *Salmonella* spp. were recovered from the poultry houses' surfaces, the litter, or the air before inoculation. After inoculation, cultivable *Salmonella* spp. were recovered from the surfaces and in the litter. Airborne cultivable *Salmonella* spp. were detected using impaction and gravitational settling one or two weeks after the detection of *Salmonella* spp. in the litter. No cultivable *Salmonella* spp. were recovered using impingement based on culture-dependent techniques. At low airborne concentrations, the use of impingement for the quantification or detection of cultivable airborne *Salmonella* spp. is not recommended. In these cases, a combination of culture-dependent and culture-independent methods is recommended. These data are valuable to improve current measures to control the transmission of pathogens in livestock environments and for optimising the sampling and detection of airborne *Salmonella* spp. in practical conditions.

Keywords. Airborne transmission, air quality, bioaerosol, broiler housing, particulate matter.

6.1. Introduction

Airborne microorganisms are abundant in the air of livestock houses (Dungan, 2010). They can cause detrimental effects to the health of farmers and animals and can be responsible for infectious and non-infectious diseases (Bonlokke et al., 2009; Donham et al., 2000). Although most airborne microorganisms in livestock houses are non-pathogenic, airborne pathogens can be found in minor concentrations (Zucker et al., 2000). Seedorf et al. (1998) reported concentrations of total airborne bacteria of 6 log colony forming units (CFU) per m^3 in broiler houses, whereas the levels of *Enterobacteriaceae* (family which includes pathogenic species) were 3 log CFU/m^3. When pathogens are zoonotic and airborne transmittable, long-distance transmission to nearby farms can occur, and the health of not only farmers but also people living near the livestock houses may be threatened (Radon et al., 2007).

In the environments in livestock houses, the biological survival of airborne microorganisms is affected by environmental conditions such as temperature, relative humidity, and ultraviolet radiation (Cox and Wathes, 1995). Moreover, the survival of microorganisms in air can be influenced to a large extent by airborne particulate matter (PM) (Adell et al., 2011; Cambra-López et al., 2010; Nimmermark et al., 2009) because their physical deposition is affected by particle characteristics, mainly the size of particle they attach to (Just et al., 2009). Although many bacteria and fungi have been recovered from airborne PM (Andersson et al., 1999; Curtis et al., 1975; Martin et al., 1996), the role of PM in the airborne transmission of specific pathogens is not fully understood.

Poultry production is a source of human pathogens such as *Salmonella* species (spp.), which are a major cause of foodborne illness throughout the world (WHO, 2011). These bacteria are generally transmitted to humans through the consumption of contaminated food of animal origin, mainly meat, eggs, and milk. *Salmonella* spp. can cause adverse health effects such as fever, diarrhoea, abdominal cramps, and headache (Blaser and Newman, 1982).

In poultry houses, *Salmonella* spp. can survive and remain viable in different reservoirs even after cleaning and disinfection (Davies and Breslin, 2003; Marin et al., 2009). Several authors have isolated *Salmonella* spp. from surfaces or litter in poultry farms (Chinivasagam et al., 2009; Davies and Breslin, 2003; Mallinson et al., 2000; Wales et al., 2006). Marin et al. (2009) reported that farm surfaces, faeces, and settled dust were the most relevant sources of *Salmonella* spp. contamination in poultry flocks. Furthermore, *Salmonella* spp. can become airborne and remain viable in the air. Their presence in the air has been confirmed inside poultry farms (Chinivasagam et al., 2009; Davis and Morishita, 2005). Additionally, it has been recognised that the airborne transmission of *Salmonella* spp. among animals over short distances can occur (Davis and Morishita, 2005; Oliveira et al., 2006). David and Morishita (2005) also recovered airborne *Salmonella* spp. 12 meters from a layer farm, thus indicating that the spread of *Salmonella* spp. to the outside environment may also occur through ventilation exhausts.

Nevertheless, to determine whether a certain airborne pathogen can furthermore cause infection, not only its presence, but also its concentration in the air is necessary (Blackall et al., 2010). Although research has dealt with the detection and quantification of *Salmonella* spp. in different reservoirs in poultry environments including the air, and using several sampling techniques and culture-dependent as well as culture-independent methods such as polymerase chain reaction (PCR) (Chinivasagam et al., 2009; Davies and Breslin, 2003; Eriksson and Aspan, 2007; Gradel et al., 2003); literature shows that these results can vary considerably depending on the sampling techniques and method for analysis. Moreover, although practical measures to control airborne transmission of *Salmonella* spp. in poultry environments are necessary, the behaviour of *Salmonella* spp. in the air still remains unpredictable. Furthermore, there is currently a lack of standardised techniques to detect and quantify airborne pathogens, specifically airborne *Salmonella* spp.

The problem resides in the control of airborne pathogens being complicated because sampling and analytical techniques have been developed and validated in other matrices, such as water (Peccia and

Hernandez, 2006), which differ from air, where airborne pathogens are found in low concentrations. At present, only limited efforts have been made to compare the different techniques and to apply them to livestock-derived pathogens in the air. Therefore, to improve current measures to control the transmission of pathogens in livestock environments, the performance of sampling techniques and analytical methods under different housing and environmental conditions needs to be assessed.

The objective of this study was to compare the performance of techniques to sample and detect airborne *Salmonella* spp. in broiler (poultry for meat production) farms. The study was conducted in two pilot scale broiler houses over a summer rearing cycle in experimentally inoculated birds. Air sampling techniques based on impaction, gravitational settling, and impingement followed by culture-dependent and molecular methods were tested. The comparison between techniques will provide insight into the advantages and disadvantages of the sampling techniques and analytical methods used to detect pathogens found at low concentrations in the air. Additionally, the relationship between airborne *Salmonella* spp. and airborne PM characteristics and the processes leading to *Salmonella* spp. becoming airborne were examined. These data will be useful to improve current control measures for pathogenic and non-pathogenic airborne bacteria inside and outside livestock houses.

6.2. Materials and methods

6.2.1. Experimental poultry houses and broilers

The study was conducted in two identical poultry houses in the pilot scale broiler farm at the Animal Technology Centre (CITA-IVIA) located at Segorbe (Castellón, Spain). Each poultry house measured 13 x 6 meters. The houses were heated by a central heating system and mechanically ventilated with two ventilators suspended from the ceiling.

At the start of the rearing cycle, 288 one-day-old male broiler chicks (Hubbard) were introduced in each house. The birds were placed

randomly into 24 floor group pens with an area of 1.3 m^2 for a pen in each house (12 pens per house and 12 animals per pen). Each pen contained wood shavings as litter to a depth of 10 centimetres. The rearing cycle lasted 42 days over the summer. The broilers had free access to feed and drinking water. *Salmonella* spp. was confirmed in some one-day birds.

6.2.2. Animal inoculation with *Salmonella* spp.

On day 7 of the rearing cycle, the broilers were orally inoculated with 1 mL of a bacterial solution containing 10^8 CFU *Salmonella* Enteritidis with kanamycin resistance (clinical isolate from faeces, wild-type mutant strain 3934 yhjL-km from the Instituto Universitario de Agrobiología y Recursos Naturales and Departamento de Producción Agraria, Universidad Pública de Navarra-CSIC, Spain; deposited in the Spanish Type Culture Collection (CECT) with the accession number CECT 7236). It was previously confirmed that this mutant behaved like a standard *S*. Enteritidis strain and that the resistance was stable. This protocol was revised and accepted by the Animal Welfare Committee of the Instituto Valenciano de Investigaciones Agrarias.

6.2.3. Animal productive parameters

Bird weight was recorded at the beginning and at the end of the rearing cycle by weighing the animals in each house. Feed consumption was recorded at the end of the rearing cycle in each house. With these data, total average daily gain, average daily feed intake, and feed conversion in each house were calculated. Mortality was supervised daily and was used in the calculation of the productive parameters.

6.2.4. Environmental parameters and airborne particulate matter

The temperature and relative humidity were recorded in each house using data loggers (HOBO U12-O13, Onset Computer Corp, Pocasset, MA, U.S.). Two data loggers were placed inside each house, and two were placed outside. Data were recorded at five-min intervals. The ventilation rate in each house was calculated using a carbon dioxide (CO_2) balance (Calvet et al., 2011). The CO_2 concentration was

measured every five minute inside each house, at a representative sample point near one of the two exhaust ventilators in each house using a CO_2 sensor with a measurement range from 0 to 10000 ppm (Vaisala GMT-222, Vaisala Oyj., Helsinki, Finland) coupled with a data logger (HOBO U12-O13, Onset Computer Corp, Pocasset, MA, U.S.). The CO_2 concentration of the inlet air was considered constant and equal to 350 ppm (clean air) as a result of previous measurements conducted at our installations.

Additionally, concentrations of PM10 (particles smaller than 10 μm) and PM2.5 (particles smaller than 2.5 μm) were simultaneously determined using a "tapered element oscillating microbalance" (TEOM model 1405-D, Thermo Fisher Scientific, Franklin, MA, U.S.). This device operated on changes in the resonant frequency of an oscillating element as a function of increases in the particle mass collected on a filter. Changes in the recorded resonant frequency of the element provide continuous and time-averaged measurements of mass accumulation. The TEOM device was located indoors, close to the ventilation exhaust in each poultry house. Measurements were conducted at a height of 2 meters. Particulate matter concentrations were measured weekly in each house. The sampling duration was 24 hour, and recordings were stored every minute. Average one-minute records were summarised to calculate the 24-hour PM concentrations.

6.2.5. Sampling and microbiological analysis of *Salmonella* spp. on surfaces

Prior to the arrival of the chicks, the absence of *Salmonella* spp. on the farm surfaces (floor and wall), feed, and litter was confirmed following the ISO 6579:2002 method (ISO 6579:2002).

During the rearing cycle, settled dust on surfaces was collected by means of sterile wet gauze pads (AES Chemunex, Bruz Cedex, France). Samples were collected on two days of the rearing cycle (days 23 and 37) at eight different points distributed randomly across the feeders, drinkers, and walls in each poultry house. The presence of *Salmonella* spp. in these samples was tested following the ISO 6579:2002 method (ISO 6579:2002). Isolated colonies were further confirmed for *Salmonella* spp.

using biochemical confirmation (API-20E, bioMérieux, Madrid, Spain). The same biochemical confirmation of *Salmonella* spp. was performed for all the samples that were analysed with a culture-based method in this study.

6.2.6. Sampling and microbiological analysis of *Salmonella* spp. in the litter

The litter was sampled weekly in each poultry house, starting on day 3 pre-infection. The litter samples were collected in each house by randomly sampling 24 spots per house to a depth of 1 to 4 cm. The samples were pooled per house, homogenised to achieve a uniform sample, stored in sterile bags and refrigerated between 4ºC and 8ºC until transport to the laboratory.

A 25-g aliquot of each litter sample was prepared in 225 mL of buffered peptone water. Each sample was manually shaken, and 1 mL of appropriate serial dilutions was inoculated into 9 mL of buffered peptone water. *Salmonella* spp. colonies were determined by culturing 1 mL of the continuous dilutions in duplicate brilliant green agar (BGA) (Liofichen, Roseto degli Abruzzi, Italy) plates with 50 μg/mL of kanamycin (kanamycin sulphate, Sigma-Aldrich, Steinhem, Germany). Plates were incubated at 37ºC for 24 h, and then CFUs were counted on plates containing between 30 and 300 colonies. The colonies were further confirmed to be *Salmonella* spp. using biochemical confirmation.

Additionally, the dry matter content of the litter was determined. A sample of 80 to 100 g of litter was dried in the oven at 104ºC for 24 hour according to AOAC International (AOAC International, 2003). Dry matter analyses were conducted in triplicate per house.

6.2.7. Sampling and microbiological analysis of airborne *Salmonella* spp.

The air in each poultry house was sampled weekly, on the same day, using three techniques: impaction, gravitational settling, and impingement. Impaction and impingement samplings were conducted within a 20 minutes interval between houses. Gravitational settling was conducted simultaneously in both houses.

Air sampling by impaction was conducted with a six-stage viable Andersen Impactor (Thermo Scientific, Franklin, MA, U.S.). The Andersen sampler had six stages, each of which consisted of a plate with agar placed under a screen with 400 holes. The diameter of the holes decreases in each successive stage. Airborne microorganisms were retained on the agar plates in different stages according to their size. From the first stage to the sixth stage, bacterial particles larger than 7 μm, from 4.7 to 7.1 μm, from 3.3 to 4.7 μm, from 2.1 to 3.3 μm, from 1.1 to 2.1 μm, and from 0.65 to 1.1 μm in size, were collected. Plates containing BGA (Liofichen, Roseto degli Abruzzi, Italy) with 50 μg/mL of kanamycin (kanamycin sulphate, Sigma-Aldrich, Steinhem, Germany) were used in the Andersen sampler. The sampling airflow rate was 28.3 L/min. Three repetitions were conducted in the centre of each house at different heights: 10-30 cm (animal breathing height), 150 cm (human breathing height) and 200 cm (exhaust fan height). Sampling duration was 90 seconds per repetition and height. Plates were directly incubated at 37ºC for 24 hour and then CFUs were counted and divided by the volume of the sampled air. Colonies were further confirmed to be *Salmonella* spp. using biochemical confirmation. Plates positively confirmed for *Salmonella* spp. were considered positive plates.

The gravitational settling technique was used to sample airborne *Salmonella* spp. across the whole house space. Gravitational settling sampled microorganisms adhered to coarse particles or particle aggregates, which settled by gravitational forces, without using forced air. Therefore, it allowed sampling for airborne *Salmonella* spp. without size discrimination (as for impaction or impingement) and for longer sampling durations, overcoming the short sampling times required for impaction and impingement. Thirty-six Petri plates with BGA (Liofichen, Roseto degli Abruzzi, Italy) and 50 μg/mL of kanamycin (kanamycin sulphate, Sigma-Aldrich, Steinhem, Germany) were placed open at three in each poultry house, 12 plates per height: 10-30 cm (animal breathing height), 150 cm (human breathing height) and 200 cm (exhaust fan height). The sampling time was 24 hour. The plates were directly incubated at 37ºC for 24 h, and then CFUs were counted. Colonies were further confirmed to be *Salmonella* spp. using biochemical

confirmation. Plates positively confirmed for *Salmonella* spp. were considered positive plates.

Air sampling using liquid impingement was conducted with AGI-30 samplers (Ace Glass Co., Vineland, NJ, U.S.). The AGI-30 sampler worked by accelerating airborne particles through a narrow orifice placed at a fixed distance from the bottom of a flask containing a liquid. A pressure drop is created in the flask and forces the air to enter through the inlet of the impinger. The AGI-30 sampler worked with a cut-off diameter of 0.31 μm. Each sampler contained 20 mL of buffered peptone water, 0.01% of Tween, and 0.005% of anti-foam and was operated at a flow rate of 12.5 L/min for 15 minute. Sampling was performed in triplicate at a height of 1.5 meters in the centre of each house, near the exhaust air. The three samples were then pooled and refrigerated between 4ºC and 8ºC until transport to the laboratory (within 2 hour). The final volume was measured and corrected for evaporation before using culture-dependent and molecular methods.

A schematic diagram of one poultry house showing the sampling locations is provided in Figure 6.1. The sampling locations were the same in each house.

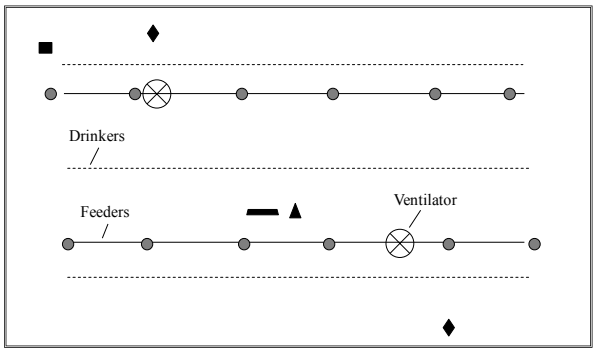

Figure 6.1. Schematic diagram of a poultry house showing sampling locations (■ Particulate Matter sampler (TEOM); ▬ Andersen cascade impactor; ▲ Impingers; ● Gravitational plates; ♦ Temperature and relative humidity sensors).

6.2.8. Culture-dependent analysis of airborne *Salmonella* spp.

For the selective detection of *Salmonella* spp. in air samples collected using liquid impingement, three methods were used: serial dilutions and plating, most probable number (MPN), and the ISO 6579:2002 method. First, the sampled liquid was serially diluted 10-fold in 0.1% buffered peptone water, and then 0.1-mL samples were plated onto duplicate BGA plates (Liofichen, Roseto degli Abruzzi, Italy) with 50 μg/mL of kanamycin. Plates were incubated at 37ºC for 24 h, and then the CFUs were counted. Colony forming units were counted on plates containing between 30 and 300 colonies (Thorne et al., 1992). The concentrations of *Salmonella* spp. in the liquid samples were determined by multiplying the CFU by the dilution volume and dividing by the volume plated (0.1 mL). The concentrations of *Salmonella* spp. in the air were then calculated by introducing the volume of sampled air. Colonies were further confirmed to be *Salmonella* spp. using biochemical confirmation. Plates positively confirmed for *Salmonella* spp. were considered positive plates.

Second, a five-tube MPN analysis was performed for *Salmonella* spp. One-mL of liquid impingement was used to make decimal dilutions (10^0 to 10^{-4}) in buffered peptone water and incubated at 37ºC for 24 hour. Aliquots of 0.1 mL from each incubated broth were inoculated onto 10 mL of Rappaport Vassiliadis (RV), followed by incubation at 42ºC for 24 hour. Positive tubes were cultured onto duplicate BGA with 50 μg/mL of kanamycin and xylose lysine deoxycholate agar (XLD, Difco, Le Pont de Claix, France) plates and incubated at 37ºC for 24 hour, and colonies were further confirmed using biochemical confirmation (API-20E, bioMérieux, Madrid, Spain).

Third, the samples from the liquid impingement were also analysed following the ISO 6579:2002 method to confirm the presence of *Salmonella* spp. Finally, the remaining liquid was stored under refrigeration at 4ºC and then processed for DNA extraction.

6.2.9. Molecular methods

Approximately 25 mL of the liquid impingement was centrifuged at 4200 rpm in a microcentrifuge tube for 20 minute at 4ºC. The supernatant was discarded and the pellet was suspended in 1 mL of PBS 1X (phosphate-buffered saline, 10 mM Tris HCl, 1 mM EDTA, pH 8) and stored at -20ºC prior to DNA extraction. The DNA was extracted with Real Pure Genomic DNA Extraction (Durviz, Valencia, Spain). The total extracted DNA was suspended in a final volume of 100 µL and stored at -20ºC.

Salmonella species-specific PCR primers, ST11 (5'-AGCCAACCATTGCTAAATTGGCGCA-3') and ST15 (5'-GGTAGAAATTCCAGCGGGTACTG-3'), purchased at Roche Diagnostics (Roche Diagnostics, Mannheim, Germany) and published previously by Aabo et al. (1993), were used to amplify a 429-bp fragment. The PCRs were performed in a PTC®-100 thermocycler (BioRad, Hercules, CA, U.S.). A 25-µL PCR mixture contained the following concentrations of the reagents: 0.4 µM of each primer, 200 µM of each dNTP (Bioline, London, U.K.), 1X PCR buffer (20 mM Tris-HCL[pH 8,4], 50 mM KCl), 1.5 mM $MgCl_2$, 0.75 U BIOTAQ™ polymerase (Bioline, London, U.K.), and 5 µL of sample DNA. The incubation conditions were 95ºC for 1 min, followed by 35 cycles of 95ºC for 30 s, 60ºC for 30 seconds and 72ºC for 30 seconds. A final extension of 72ºC for 4 minutes was used. The PCR products were visualised by agarose gel electrophoresis. Four repetitions of the PCR analyses of the extracted DNA were conducted. The sample was considered positive as long as one repetition was positive.

A summary of the sampling techniques used and the analytical method to detect *Salmonella* spp. during the rearing cycle is presented in Table 6.1.

Table 6.1. Summary of evaluated parameters, sampling techniques, description of sampling, and analytical methods used in this study to detect Salmonella spp. per sampling event. Sampling frequency was weekly for all parameters.

Parameter	Sampling technique (sampling points)	Number of samples per house and sampling event	Sampling duration	Airflow (L/min)	Analytical method
Salmonella spp. on surfaces	Sterile wet gauze pads (8 points)	1	-	-	ISO 6579:2002
Salmonella spp. in litter	Litter sampling (24 points)	3	-	-	Direct count (dilutions)
Airborne *Salmonella* spp.	Impaction (3 points)	18	90 s	28.3	Direct count
	Gravitational settling (36 points)	36	24 h	-	Direct count
	Impingement (1 point)	3	15 min	12.5	Direct count (dilutions) Most Probable Number ISO 6579:2002 Polymerase Chain Reaction

6.2.10. Data analyses

All data were summarised and analysed per house and week. The airborne distribution of *Salmonella* spp. obtained with gravitational plates was compared among the different heights using analysis of variance with SAS software (SAS, 2001), comparing the average *Salmonella* spp. counts per height, house, and week using the Tukey test with a significance level of 5%.

The detection limits for each culture-dependent sampling technique were calculated assuming a single CFU in the agar plate considering the sampler's airflow and the sampling duration (Buttner and Stetzenbach, 1991). For impingement, the detection limit was calculated from the total volume of the liquid plated.

The relationship between *Salmonella* spp. concentration in the litter and in the air (using gravitational settling) with the productive and

environmental parameters was investigated using Pearson's correlation coefficient for the whole sampling period with SAS software (SAS, 2001).

6.3. Results

6.3.1. Productive and environmental parameters

The animals performed similarly in each poultry house. The average productive parameters are shown in Table 6.2.

Table 6.2. Average daily gain (ADG), average daily feed intake (ADFI), and feed conversion in each poultry house during the broiler rearing cycle.

House	ADG (g/day)	ADFI (g/day)	Feed conversion (g feed/g weight)
1	63.2±3.4	103.7±4.5	1.64±0.03
2	67.6±2.2	110.5±3.0	1.63±0.04

Ventilation increased throughout the rearing cycle and varied from 0.04 to 1.56 m^3/h/animal (house 1) and from 0.04 to 1.09 m^3/h/animal (house 2). The outdoor temperature ranged from 16.2ºC to 27.1ºC and the outdoor relative humidity ranged from 38.3% to 72.5%. The average indoor temperature varied from 24.5ºC to 31.2ºC in house 1 and from 24.5ºC to 30.7ºC in house 2. The average indoor relative humidity varied from 24.3% to 72.6% in house 1 and from 27.3% to 71.1% in house 2. The average concentration of PM during the whole cycle for both houses was 0.019±0.008 mg/m^3 for PM2.5 and 0.189±0.104 mg/m^3 for PM10. Both PM2.5 and PM10 concentrations generally increased during the rearing cycle in both houses. The maximum PM2.5 concentrations registered during the whole cycle were 0.082 mg/m^3 for house 1 and 0.079 mg/m^3 for house 2. The maximum PM10 concentrations were 1.14 mg/m^3 for house 1 and 1.79 mg/m^3 for house 2 (data not shown). The weekly averages for indoor and outdoor temperature and relative humidity and the indoor PM2.5 and PM10 concentrations during the rearing cycle in both houses are shown in Table 6.3.

Table 6.3. Average outdoor temperature (out T) and relative humidity (out RH), indoor T (in T) and RH (in RH), PM2.5 and PM10 concentration, and standard deviation in each poultry house during the broiler rearing cycle.

Day	House	out T (ºC)	out RH (ºC)	in T (ºC)	in RH (%)	PM2.5 (mg/m³)	PM10 (mg/m³)
3	1	18.7±4.2	56.5±9.0	33.0±0.9	33.6±3.2	0.007±0.002	0.045±0.014
	2			30.8±3.2	45.8±8.7	0.009±0.009	0.051±0.030
10	1	21.0±4.9	65.9±11.9	32.5±0.6	41.6±3.1	0.013±0.010	0.091±0.123
	2			29.6±0.1	46.0±3.2	0.011±0.005	0.107±0.064
17	1	26.4±5.4	45.8±10.7	29.1±1.4	45.6±3.4	0.019±0.011	0.270±0.208
	2			28.4±1.0	47.6±4.3	0.019±0.007	0.179±0.123
24	1	26.2±5.2	54.1±12.2	27.7±1.9	60.2±4.4	0.022±0.010	0.263±0.169
	2			28.4±2.4	54.6±4.7	0.024±0.014	0.337±0.250
31	1	25.5±4.0	63.5±8.8	25.3±1.4	77.0±5.8	0.021±0.008	0.178±0.152
	2			26.2±1.6	77.0±3.9	0.030±0.011	0.300±0.287
38	1	25.5±3.7	64.3±11.5	24.6±0.9	64.0±6.1	0.023±0.021	0.304±0.287
	2			25.6±0.7	80.0±2.0	0.028±0.012	0.153±0.090

6.3.2. *Salmonella* spp. on surfaces

Before the arrival of the animals, analyses of the farm facilities (floor and wall), feed, and litter resulted in the absence of *Salmonella* spp. in such facilities.

The settled dust that was collected on surfaces (feeders, drinkers, and walls) using sterile wet gauze pads on days 23 and 37 of the rearing cycle was positive for *Salmonella* spp. in both days and houses.

6.3.3. *Salmonella* spp. in the litter

Salmonella spp. in the litter was not detected on day 3 of the rearing cycle, previous to the experimental infection. After the experimental infection, *Salmonella* spp. was detected and quantified in both houses, showing no clear pattern along the rearing cycle. Ten days post-infection (day 17 of the rearing cycle), the levels of *Salmonella* spp. in the litter were equal to 4.4 log CFU/g (house 1) and 3.2 log CFU/g (house 2). Overall, the concentrations of *Salmonella* spp. in the litter ranged from 3 log CFU/g to 4.6 log CFU/g. Figure 6.2 shows the evolution of log CFU of *Salmonella* spp. per g of litter throughout the rearing cycle in each poultry house.

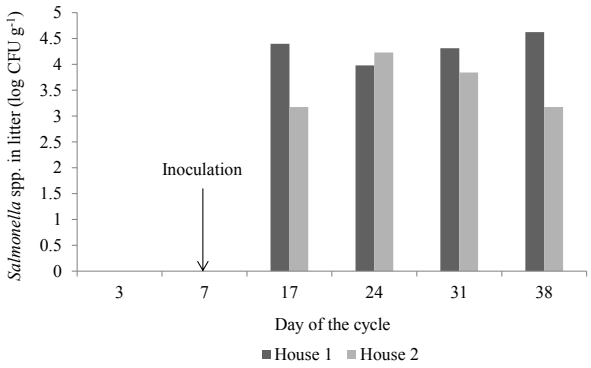

Figure 6.2. *Salmonella* spp. counts in the litter (log CFU/g litter) during the cycle in both poultry houses.

The dry matter content of the litter decreased during the rearing cycle in both houses. The dry matter percentage varied from 86% (day 3 of the rearing cycle) to 69% (day 31 of the rearing cycle) in house 1, and from 85% to 61% in house 2. Dry matter values were similar between houses (data not shown).

6.3.4. Airborne *Salmonella* spp.

Differences in the detection of airborne *Salmonella* spp. were recorded using the impaction, gravitational settling, and impingement sampling techniques.

By means of impaction using the Andersen cascade impactor, positive samples for *Salmonella* spp. were only randomly detected at the end of the cycle (days 24 and 38) in house 2, in size ranges between 0.65-1 μm (1.97 log CFU/m^3 at 200 cm), 3.3-4.7 μm (1.15 log CFU/m^3 at 150 cm), and 7 μm or higher (1.15 log CFU/m^3 at 150 cm). No positive samples for *Salmonella* spp. were observed in house 1 using impaction. The calculated detection limit of the Andersen cascade impactor (90-seconds sampling duration) was 1.38 log CFU/m^3.

Using gravitational plates collocated at different heights, *Salmonella* spp. were detected towards the end of the rearing cycle only on day 38 (house 1) and on days 24 and 38 (house 2). Nevertheless, the analysis and counting of the gravitational plates was complicated due to the

accumulation of dirt during the 24 hour of exposition, and the overgrowth of other Gram-negative bacteria (e.g., coliforms) that differed from *Salmonella* spp. in the culture plates. For these reasons, only those results corresponding to days 3, 24, and 38 of the rearing cycle are shown (Table 6.4). The results are shown as a percentage of *Salmonella* spp.-positive plates out of 12 plates used in each of the sampled heights, per sampling day and house. In the first three weeks of the rearing cycle, *Salmonella* spp. were not detected in any house. *Salmonella*'s prevalence increased at the end of the cycle, and house 2 presented a higher percentage (64%) of positive plates than house 1 (11%). No statistically significant differences among heights were observed per house.

Table 6.4. Percentage of positive plates of *Salmonella* spp. using gravitational settling, and p-values at different heights per day in the cycle, by poultry house and height.

House	Days of the cycle		Height	Number of samples	Number of *Salmonella*-positive plates (%)	p-value
1	Before inoculation	3	30 cm	12	0	-
			150 cm	12	0	
			200 cm	12	0	
	Post-inoculation	24	30 cm	12	0	-
			150 cm	12	0	
			200 cm	12	0	
		38	30 cm	12	1 (8.3%)	0.140
			150 cm	12	0	
			200 cm	12	3 (25%)	
2	Before inoculation	3	30 cm	12	0	-
			150 cm	12	0	
			200 cm	12	0	
	Post-inoculation	24	30 cm	12	0	0.140
			150 cm	12	3 (25%)	
			200 cm	12	1 (8.3%)	
		38	30 cm	12	7 (58.3%)	0.887
			150 cm	12	8 (66.7 %)	
			200 cm	12	8 (66.7%)	

No *Salmonella* spp. were detected by liquid impingement during the whole cycle in any house by culture-dependent methods using dilutions and plating, MPN, or the ISO 6579:2002 method. Using impingement, the calculated detection limit (15-min sampling duration) was 3.48 log CFU/m^3.

Positive results for *Salmonella* spp., however, were obtained using PCR. Table 6.5 presents the results of samples from the AGI-30 analysed by PCR in both poultry houses during the cycle, showing a positive detection of *Salmonella* spp. in all samples, except for day 24 of the rearing cycle in house 1. Samples were positive on day 3 (pre-infection). Figure 6.3 shows the expected PCR products visualised by agarose gel electrophoresis.

Table 6.5. Positive (+) and negative (-) results for *Salmonella* spp. detection using PCR for 4 repetitions from impingement samples.

Days of the cycle		House	*Salmonella* spp. detection
Before inoculation	3	1	+
		2	+
Post-inoculation	17	1	+
		2	+
	24	1	-
		2	+
	31	1	+
		2	+
	38	1	+
		2	+

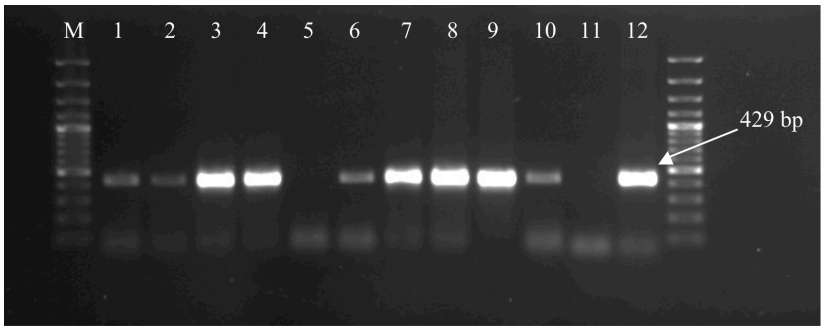

Figure 6.3. PCR amplification profiles. Lane 1-10: houses 1 and 2 on different days of the rearing cycle; Lane 11: negative control; Lane 12; positive control 3934 yhjL-km, kanamycin-resistant strain; M lane contains the 100-bp molecular size ladder marker.

Correlation coefficients between *Salmonella* spp. concentration in the litter and in the air (using gravitational settling) and productive and environmental parameters were low (data not shown) except for ambient relative humidity and airborne *Salmonella* spp. which showed the

strongest correlation (correlation coefficient equal to 0.80, p=0.06). Airborne *Salmonella* spp. also correlated fairly well with the dry matter content of the litter (correlation coefficient equal to 0.72, p=0.11), with PM2.5 (correlation coefficient equal to 0.64, p=0.17), and with animal weight (correlation coefficient equal to 0.69, p=0.12). We found a negative correlation between ambient temperature and concentration of airborne *Salmonella* spp. (correlation coefficient equal to -0.55, p=0.25). The concentration of *Salmonella* spp. in the litter showed no strong correlation with any of the measured environmental or productive parameters (correlation coefficients below 0.42). The correlation between *Salmonella* spp. concentration in the litter and *Salmonella* spp. concentration in the air was -0.82 (p=0.18).

6.4. Discussion

Our results revealed differences using three sampling techniques and two analytical methods to detect airborne *Salmonella* spp. in a broiler farm. Experimentally inoculated birds released *Salmonella* spp. in variable amounts during the rearing cycle, which could be detected along the experimental period mainly in litter and dust reservoirs. *Salmonella* spp. have been reported to survive desiccation better than other *Enterobacteriaceae* (Carrique-Mas and Davies, 2008). It can survive in old fan dust up to 30 weeks after depopulation in poultry houses (Davies and Wray, 1996), and in litter, dry faeces, and feed, it can survive up to 26 months after depopulation (Davies and Breslin, 2003). Its behaviour in the air, however, remains unpredictable. Its survival in the air is probably different from other more appropriate substrates and may lead to nutrient stress and shock.

During the broiler rearing cycle, with regards to the animal productive parameters, these were generally found within the upper ranges of other studies (Al Homidan et al., 1998; Feddes et al., 2002). This could be due to the controlled environmental conditions in this study conducted in a pilot scale broiler house. Environmental parameters, such as ventilation rates and outdoor temperature and relative humidity, were typical of summer conditions in the study area.

During the experimental period, the detection of airborne cultivable *Salmonella* spp. occurred towards the end of the rearing cycle, coinciding with the highest ventilation rates, the highest airborne PM2.5 and PM10 concentrations, and the highest indoor relative humidity, along with the lowest indoor temperatures. We found a strong correlation between airborne *Salmonella* spp. and ambient relative humidity. Research has reported that primarily temperature and relative humidity affect the survival of airborne microorganisms and that temperatures above 24ºC can decrease airborne bacterial survival (Tang, 2009). Additionally, Zhao et al. (2011a) reported that airborne microorganisms were associated with the PM. A higher PM concentrations could have enhanced bacteria's growth, although we only found a small correlation between airborne *Salmonella* spp. and PM2.5. Adell et al. (2011) reported that airborne mesophilic bacteria were generally attached to particles from 3.3 to >7 μm. These authors reported increasing PM and airborne mesophilic bacteria concentrations in the air in a broiler farm as a function of time, showing a high correlation coefficient (0.78-0.89) between both variables. In our study, however, airborne *Salmonella* spp. were randomly found attached to particles from 0.65 to > 7 μm in diameter.

Before the infection of the chicks, no cultivable *Salmonella* spp. were recovered from the poultry houses' surfaces or in the litter, indicating that the experimental inoculation was probably the only source of cultivable *Salmonella* spp. Recovery of cultivable *Salmonella* spp. on settled dust collected using sterile wet gauze pads from the houses' surfaces, was positive in all samples taken post-infection. These results are also in accordance with Marin et al. (2009) who identified settled dust collected on surfaces as a relevant risk of *Salmonella* spp. contamination among poultry flocks.

Positive samples for *Salmonella* spp. were first obtained in the litter ten days post-infection (day 17 of the rearing cycle). Chinivasagam et al. (2009) obtained similar concentrations of *Salmonella* spp. in broiler litter in a commercial broiler house. *Salmonella* spp. in the litter was probably a result of bacterial shedding in faeces by inoculated birds. Animal faeces found in the litter have been reported to be one of the main sources of

pathogens in the air in livestock houses (Chinivasagam et al., 2009; Mallinson et al., 2000). Furthermore, particles from broiler excreta have been identified as one of the major sources of airborne fine and coarse PM in broiler houses (Cambra-López et al., 2011).

There was a delay in the detection of *Salmonella* spp. in the air compared with the litter of one or two weeks, depending on the poultry house. This could possibly be explained by the time needed for the faeces in the litter to dry, disintegrate, and become airborne. Although we found no correlation between dry matter content of the litter and *Salmonella* spp. concentration in the litter as described by Hayes et al. (2000); when litter is dry it is more prone to becoming airborne as a consequence of increased ventilation rate or birds movement (Cambra-López et al., 2010). In fact, we found strong correlations between airborne *Salmonella* spp. and dry matter litter content as well as with *Salmonella* spp. in the litter. Therefore, our results suggest that the airborne process may take some time and that the excretion of *Salmonella* spp. from inoculated chicks occurs earlier than its presence in the air. Adell et al. (2011) reported a higher bacterial concentration of airborne mesophilic bacteria near the litter than at higher levels at the beginning of a broiler rearing cycle. Our results, however, showed no differences in *Salmonella* spp. distribution in the poultry house space. In practical conditions, the detection of infected animals, as well as the detection of *Salmonella* spp. in the litter above certain thresholds, as shown in our results, can be considered a surrogate indicator of possible air contamination and a useful preventive measure of airborne transmission. Nevertheless, further research is necessary to better understand the processes leading to airborne *Salmonella* spp. under practical conditions in non-inoculated, *Salmonella* spp.-free birds.

The different air sampling techniques used in this study to detect airborne *Salmonella* spp. were impaction, gravitational settling, and impingement. The differences in the results among techniques could be explained by differences related to the sampling technique used: (i) the use of forced air and direct impaction on agar or not; (ii) the cut-off diameter of each sampling device (i.e., the size of sampled particles); and (iii) their detection limits.

The Andersen impactor and impingers used forced air, whereas gravitational settling plates worked without forced air. Moreover, using impaction and gravitational settling, air impacted directly onto the agar, reducing problems associated with sample processing in the laboratory compared with impingement, where air was sampled into liquid media and then transferred onto agar. The Andersen impactor could discriminate between particle sizes of 0.65 μm in diameter up to a maximum of 7 μm in diameter. Gravitational settling plates, however, sampled all airborne microorganisms adhered to coarse particles that can settle by gravity and probably large particle aggregates as well. Impingement had a cut-off diameter of 0.31 μm.

These differences related to the sampling techniques used resulted in cultivable *Salmonella* spp. being positive using impaction and gravitational settling in some cases and negative using impingement. Nevertheless, using impaction, *Salmonella* spp. were only recovered in a few samples at the end of the rearing cycle. Using impingement, no cultivable *Salmonella* spp. were recovered by the different culture-dependent methods during the experimental period. The results show that the performance of the sampling techniques can be improved when the sampling devices sample directly onto agar (i.e., impaction or gravitational settling). The sampling performance and collection efficiency using the impingement technique could be affected by the sampling stress caused when cells are accelerated in the nozzle at high velocities (equal to 313 m/s) (Lin et al., 2000) and particles bounce and re-aerosolise from the liquid (Grinshpun et al., 1997). This could result in a loss of culturability and reduced collection efficiency. Additionally, the survival of *Salmonella* spp. in the impingement liquid and the competition between other bacteria (in peptone water), together with the nutrient stress and shock caused by *Salmonella* spp. inhabiting the air, could also explain these unexpected results. The manipulation and processing of the liquid impingement in the laboratory may also influence the detection of cultivable *Salmonella* spp. Using impingement, Brooks et al. (2010) reported the difficulties in isolating *Salmonella* spp. from air samples. These authors could only isolate *Salmonella* spp. once from 38 impinger samples in a commercial broiler house, although *Salmonella* spp. were quantified in the litter.

Although most common airborne microorganism sampling techniques involve filtration, impaction and/or impingement (Cox and Wathes, 1995), our results show that no sampling approach can be considered universally suitable for *Salmonella* spp. Therefore, although air sampling by impingement has been recognised as an appropriate sampler for assessing other airborne microorganisms, it has not been fully validated for airborne *Salmonella* spp. According to our results, and in agreement with Brooks et al. (2010), the use of impingement for quantification or detection of cultivable airborne *Salmonella* spp. is not recommended.

With regards to the sampler detection limits, these could also partly explain the controversial results among sampling techniques. In our study, the detection limit calculated for impaction showed better sensitivity (1.38 log CFU/m^3) than using impingement (3.48 log CFU/m^3). Therefore, when reporting negative results, the lowest sampler detection limit should be considered.

In addition to intrinsic sampling characteristics, differences in results among techniques could also be explained by intrinsic microbiological factors, such as shifts between modes of survival and competition with other microorganisms. The concentration of airborne microorganisms could be underestimated with culture-dependent methods because airborne bacteria may utilise survival strategies, such as the formation of biofilms, resistance to low water activity, rugose formation, and entry into a viable but non-culturable (VBNC) state (Gupte et al., 2003), in which viable bacteria have lost their ability to form colonies in a reversible process. Additionally, interferences and competition with other microorganisms can occur, especially when airborne pathogen concentrations are low (Qasem et al., 2005). In inoculated animals, Lever and Williams (1996) reported airborne cultivable *Salmonella* spp. concentrations to be relatively low at 1 log CFU/m^3. Therefore, such low concentrations could favour the growth of competitors of *Salmonella* spp. Consequently, culture methods can greatly underestimate the real populations of pathogenic bacteria and their health threat to workers and animals (Chi and Li, 2006).

For these reasons, most authors have used more sensitive laboratory methodologies to detect pathogens in the air, such as the ISO 6579:2002

presence and absence technique or the semi-quantitative technique of MPN (Davis and Morishita, 2005; Gast et al., 2004). These techniques, however, are qualitative and are not valid for quantifying microorganisms. In our study, using the ISO 6579:2002 presence and absence technique and the semiquantitative technique of MPN, *Salmonella* spp. were not detected in impingement samples. Eriksson and Aspan (2007) affirmed that the qualitative ISO 6579:2002 presence and absence technique was the most sensitive and specific method among presence/absence, PCR or ELISA to detect *Salmonella* spp. in faeces. The fact that the prevalence of *Salmonella* spp. in the litter is higher than in the air (Chinivasagam et al., 2009; Kwon et al., 2000) could explain such differences. In practice, other authors also reported difficulties in detecting airborne *Salmonella* spp. using culture-dependent methods in poultry farms when other airborne pathogens, such as *Escherichia coli*, were detected in concentrations ranging from 2 to 5 log CFU/m^3 (Chinivasagam et al., 2009). Nevertheless, the use of standardized ISO technique in this study, provides the possibility to further compare culture quantification between institutions and researchers.

When attempting to detect pathogens in the air, such as *Salmonella* spp., the use of PCR can provide rapid and sensitive results (Alvarez et al., 1995). The detection limit of PCR is lower than that of culture-dependent techniques because it can detect a single cell in the sample aliquot (Alvarez et al., 1995). Although our results were obtained using conventional PCR and real-time PCR is considered the gold standard nowadays, conventional PCR proved to be sufficiently sensitive in this study at the tested concentrations. The results using PCR analyses of the liquid impingement in this study demonstrated the presence of *Salmonella* spp. in the air, contrary to the results obtained with culture-dependent methods. Zhao et al. (2011b) obtained similar results with airborne *Campylobacter*. These authors did not detect airborne *Campylobacter* by culture-dependent methods, but they obtained positive results using PCR. Furthermore, Hospodsky et al. (2010) reported that the accuracy, precision, and method detection limits of real-time PCR for airborne microorganisms are influenced by several factors during the sampling, DNA extraction, and analytical phases.

In addition to the advantages of molecular methods, analytical methods to detect airborne pathogens based on PCR can have drawbacks related to their limited ability to provide information on pathogen viability and ability to cause infection. When monitoring airborne pathogens, an assessment of viability to investigate whether they pose a threat to human or animal health is necessary (Keer and Birch, 2003). Bacterial pathogens are able to infect animals and humans, but molecular methods cannot easily differentiate between viable and dead pathogens (Keer and Birch, 2003), and in our study, some samples were positive for *Salmonella* spp. prior to bird inoculation. Stojek et al (2012) in a study to detect *Legionella* spp. in water conclude that PCR cannot be a substitute for the culture methods, nonetheless it could be regarded as an useful complementary method. Although the analyses of *Salmonella* spp. in farm facilities, feed, bedding, and animals before the arrival of the animals were all negative for cultivable *Salmonella* spp., *Salmonella* spp. from a previous flock could have remained in VBNC form, or bacterial DNA from dead cells could also be detected.

Moreover, the presence of a certain pathogen does not necessarily mean infection will occur. For infection to occur, a human must be exposed to a pathogen's infective dose (the amount that will cause 50% of exposed individuals to suffer illness) (Blackall et al., 2010). The infective dose for *Salmonella* spp. has been reported to range between 10^3 and 10^5 organisms, being dependent upon the strains used, and the age and physical condition of the individuals (Blaser and Newman, 1982; Kothary and Babu, 2001).

Overall, Table 6.6 summarises the detection results using the different sampling techniques and analytical methods in this study, and it presents recommendations for optimising the sampling and detection of airborne *Salmonella* spp. in practical conditions. Although the use of gravitational settling was complicated in this study, it can still be recommended for viability assessment in combination with other culture-dependent method (i.e. impaction) because they are simple and easy to use and can sample during long periods (hours). From our results, overall recommendations include a combination of culture-dependent and

culture-independent methods to overcome the limitations of a single method.

Table 6.6. Detection results (positive, + or negative, -) and recommendations for sampling and detecting airborne *Salmonella* spp. according to the techniques and analytical sampling methods used in this study.

Parameter	Technique	Sampling duration	Airflow (L/min)	Collectium medium	Analytical method	*Salmonella* spp.detection	Recommendation
Before arrival of chicks							
Salmonella spp. on water, feed and litter	1 mL sample for water 25 g sample for feed and litter	-	-	-	Culture- dependent ISO 6579:2002	-	Viability assessment
After arrival of chicks, weekly							
Salmonella spp. on surfaces	Sterile gauze pads	-	-	-	Culture- dependent ISO 6579:2002	+	Viability assessment
Salmonella spp. in litter	25 g sample	-	-	-	Culture- dependent Direct count (dilutions)	+	Viability assessment. Could serve as indicator of presence in the air.
Airborne *Salmonella* spp.	Impaction	90s	28.3	Agar	Culture- dependent Direct count	+	Viability assessment
	Gravitational settling	24 h	-	Agar	Culture- dependent Direct count	+	Viability assessment
	Impingement	15 m	12.5	Liquid	Culture- dependent Direct count (dilutions)	-	Not recommended
					Most Probable Number	-	Not recommended
					Culture- dependent ISO 6579:2002	-	Not recommended
					Culture-independent Polymerase Chain Reaction	+	Detection combined with culture-dependent method for viability assessment

6.5. Conclusions

We evaluated the performance of air sampling techniques based on impaction, gravitational settling, and impingement, followed by culture-dependent and molecular methods to detect airborne *Salmonella* spp. in experimentally inoculated birds in two pilot scale broiler houses. Our results revealed differences using three sampling techniques and two analytical methods and that no sampling approach is universally suitable for airborne *Salmonella* spp. These data are valuable to improve current measures to control the transmission of pathogens in livestock environments. From our results, we can conclude the following:

During the experimental period, the detection of airborne *Salmonella* spp. occurred towards the end of the rearing cycle (from day 24 onwards). The environmental conditions at the end of the rearing cycle could have positively influenced bacteria survival and growth, especially ambient relative humidity, litter dry matter content, and PM2.5 concentration. Airborne *Salmonella* spp. were randomly found attached to particles ranging from 0.65 to > 7 μm in diameter.

There was a delay of one or two weeks in the detection of *Salmonella* spp. in the air compared with in faeces (litter). Further research, however, is necessary to better understand the processes leading to *Salmonella* spp. becoming airborne under practical conditions in non-inoculated, *Salmonella* spp.-free birds.

Positive samples for airborne cultivable *Salmonella* spp. were obtained by sampling directly onto agar (i.e., impaction or gravitational settling), while samples were negative using impingement. At low airborne concentrations, the use of impingement for the quantification or detection of cultivable airborne *Salmonella* spp. is not recommended.

A combination of culture-dependent and culture-independent methods is recommended to prevent undetected pathogen concentrations; however, when monitoring airborne pathogens, an assessment of viability to investigate whether they pose a threat to human or animal health is necessary.

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7

Air disinfection in laying hen houses: Effect on airborne microorganisms with focus on *Mycoplasma gallisepticum*

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Abstract. Poultry production is a source of airborne microorganisms and particulate matter (PM). In laying hen houses, infection by the respiratory pathogen *Mycoplasma gallisepticum* can cause a decrease in laying eggs and their quality. We evaluated the application of thermonebulized disinfectant in the air to reduce airborne microorganisms, with emphasis on its effect on *M. gallisepticum*. The study was conducted in a commercial laying hen farm in Toledo (Spain). Two air disinfectant tests were conducted in two identical laying hen houses. One of them was used as treatment house, whereas the other was used as control. Airborne microorganisms were sampled before, 1 h and 6 h after disinfection. Additionally, the environmental hygiene and air quality status of the laying hen houses prior disinfection tests was evaluated. Ambient temperature (T), relative humidity (RH), wind speed, wind direction, and solar radiation were continuously recorded outdoors. T, RH, concentrations of particulate matter (PM10 and PM2.5), and airborne microorganisms (mesophilic aerobic bacteria, *Enterobacteriaceae* and *M. gallisepticum*) were measured indoors. Spatial distribution of airborne mesophilic bacteria was investigated. The morphology of PM was examined by scanning electron microscopy. Prior disinfection tests, average PM concentration was 0.024±0.025 mg/m^3 for PM2.5 and 0.546±0.377 mg/m^3 for PM10; being two (PM10) and three (PM2.5) times higher ($p<0.01$) during light than during dark periods. The concentration of airborne mesophilic aerobic bacteria ranged from 4.1 to 5.7 log colony forming units, log CFU/m^3. There were no differences among heights and corridors. In our conditions, air disinfection using wide spectrum thermonebulized disinfectant was not effective in reducing the concentration of mesophilic aerobic bacteria, *Enterobacteriaceae* and *M. gallisepticum* in the air. It would be desirable to evaluate different air disinfectant doses, products and application methods. *Mycoplasma* spp. was confirmed by qPCR on cage surfaces and chicken's feathers before and after disinfection. The presence of outdoor *Mycoplasma* spp. suggests that the inlet air could be a source of entry of this pathogen. Further information on the relationship between PM and airborne microorganisms and their behavior in the air are necessary to design adequate techniques to reduce them in livestock houses.

Keywords. Poultry, Bioaerosol, Air Quality, Reduction

7.1. Introduction

Good environmental hygiene in poultry farms is critical to the welfare and productivity of the animals and for the health of workers. Poultry production is a source of air pollutants such as microorganisms (bacteria, viruses, and fungi) or portions of them (endotoxins and lipopolysaccharides), particulate matter (PM), and gases (Wathes et al., 1997). As regards airborne microorganisms, a large number of bacterial species have been isolated in laying hen farms. Some of them can be pathogenic for laying hens and humans such as *Pseudomonas, Bacillus, Corynebacterium, Pasteurella, Vibrio, Enterobacter, Salmonella, Brucella, Leptospira, Hamophilus, Mycoplasma, Yersinia, Staphyloccocus, Streptococcus, Micrococcus, Pantoea* and *Sarcina* species (Sauter et al., 1981; Lonc and Plewa, 2010; Zucker et al., 2000).

There is a close relationship between airborne microorganisms and particulate matter (PM) in the air of poultry farms (Nimmermark et al., 2009). Particles can act as a substrate for microorganism because they provide a suitable environment for their survival (Just et al., 2009). Inhalation of PM and its components can aggravate health effects, both for animals and for workers (Wathes et al., 2002; Bonlokke et al., 2009). In addition, the emission of airborne pathogens outside the animal houses may threat the health of nearby farms or even neighboring population (Heber et al., 2001; Otake et al., 2010).

In laying hen houses, infection by the respiratory pathogen *Mycoplasma gallisepticum* is very common. According to Sagardia (2008), 85% of laying hen farms in Spain are infected with *M. gallisepticum*. This pathogen can cause a decrease in laying eggs and their quality without showing any clinical signs (Peebles et al., 2010). *M. gallisepticum* can survive in different reservoirs within a poultry farm. Among these reservoirs, food, drinking water, feathers, droppings or dust are the most common (Marois et al., 2002). Although *Mycoplasma* spp. has been reported to be airborne transmittable (Landman et al., 2004; Feberwee et al., 2005), the factors affecting *M. gallisepticum* aerosolization from its reservoirs, its dispersion and transmission remain unknown.

Exposure of hens to unfavorable environmental conditions such as inadequate ventilation, temperature and humidity, high concentrations of gases (like ammonia) or high concentrations of PM and microorganisms, could aggravate respiratory problems caused by *Mycoplasma* spp. (Kleven, 1998; Wathes, 1998). Thus, an exhaustive understanding of a farm's hygienic status by assessing environmental conditions can provide useful information on the potential ways of improving farm's air quality to reduce risk of respiratory problems and colonization by respiratory pathogens such as *M. gallisepticum*. Moreover, there is need for evaluating techniques which can favor air quality inside poultry farms and which can reduce airborne microorganisms. Until now, not many studies had tackled reducing airborne microorganisms by using air disinfection in laying hen houses.

The aim of this study was to evaluate the application of an air disinfectant to reduce airborne microorganism in a commercial laying hen house, with focus on its effect on *M. gallisepticum*. Moreover, the environmental hygiene and air quality status in terms of concentrations of PM and airborne microorganisms (mesophilic aerobic bacteria, *Enterobacteriaceae* and *M. gallispeticum*) prior disinfection tests was evaluated.

7.2. Material and Methods

7.2.1. Facilities and animals

The study was conducted in a commercial laying hen farm in Toledo (Spain). The laying hen farm consisted of ten identical houses of 100,000 places each. Each house was 140 m long x 23 m wide x 4 m at its lowest height. Hens were reared in enriched battery cages. Each house had eight lines of batteries, with six levels each. Houses were mechanically ventilated, with 42 exhaust fans in a forced tunnel ventilation system. Lighting system consisted of 16 hours light and 8 hours dark. Measurements were conducted during spring-summer months. Hens had free access to food and drink. They received all necessary vaccinations and were positive for M. *gallisepticum* determined by serology.

7.2.2. Outdoor environmental conditions

Outdoor temperature, relative humidity, wind direction and solar radiation were recorded continuously outside the laying hen houses using a weather station (Hobo Weather Station, Onset Computer Corp., USA). The weather station was installed at a high and open point free from obstacles and from the influence of the buildings. Data were recorded every 5 minutes during the experimental period.

7.2.3. Indoor environmental hygiene and air quality prior disinfection

Concentrations of PM were recorded indoors prior disinfection tests in one house. Moreover, PM was characterized morphologically in the same house. Additionally, spatial and temporal distribution of airborne microorganism concentration was studied in three different houses. These results were necessary to design the disinfection test.

7.2.3.1. Particulate matter: PM10 and PM2.5

Concentrations of PM in two size fractions: PM2.5 (particles smaller than 2.5 μm) and PM10 (particles smaller than 10 μm) inside the sampled house were simultaneously determined using a tapered element oscillating microbalance, TEOM (TEOM model 1405-D, Thermo Fisher Scientific, USA). The TEOM device was located indoors, in the center of the house. Measurements were conducted at a height of 2 m. The PM concentrations were recorded every 5 minutes during 18 consecutive days. Additionally, indoor temperature and relative humidity were recorded continuously every 5 minutes with a sensor coupled to the TEOM.

Differences in hourly PM concentrations between light and dark periods were examined with an analysis of variance (ANOVA), where the light/dark periods were used as a source of variance using SAS System Software (Version 9.1, SAS Institute Inc., Cary, Carolina del Norte, USA).

To characterize PM, two virtual cascade impactors (RespiCon® model 8522, Helmunt Hund GmbH, Wetzlar, Germany) were used in the

sampled house. The PM2.5 and PM10-2.5 size fractions were simultaneously sampled in the air. Two portable pumps (Genie VSS5, Buck Inc, USA) were used to draw air through each virtual cascade impactor at a constant flow of 3.11 L/min. Each PM size fraction was collected onto separate polycarbonate filters (37 mm Ø, 5 μm pore size, Dräger Safety Ag & Co., Luebeck, Germany). Sampling duration ranged between 24 and 45 minutes to obtain a mass particle ranging from 5 to 20 μg per cm^2 of filter (Willis et al., 2002). Samples of PM2.5 and PM10-2.5 were morphological examined using a high-resolution scanning electron microscope (SEM) (JSM-5410, JEOL Ltd., Tokio, Japan), following the methodology described in Cambra-López et al. (2011b).

Photomicrographs of particle components were compared with published photographs of known particles from different sources in laying hen houses in Cambra-López et al. (2011b).

7.2.3.2. Airborne microorganisms: Mesophilic aerobic bacteria, Enterobacteriaceae and Mycoplasma gallispeticum

Airborne microorganisms were sampled in sterile liquid media using AGI-30 impingers (Ace Glass Co., Vineland, NJ, USA). Impingers worked by accelerating airborne particles through a narrow orifice placed at a fixed distance from the bottom of a flask containing a liquid. A minimum pressure drop of (0.5 atm) was created by a constant flow vacuum pump (Model ZA60S, DVP Vacuum Technology, Italy) which forced the air to enter through the inlet of the impinger at a flow rate of 12.5 L/min. Impingers worked with a cut-off diameter of 0.31 μm. Each sampler contained 20 mL of Phosphate Buffered Saline (PBS), 0.01% of Tween, and 0.005% of anti-foam. Sampling duration was 20 min.

Sampling was performed during three consecutive weeks in three houses. Each sampling was conducted in three corridors, at two lengths (section A: 46 m, and section B: 92 m from the front of the house) and at two heights (1,5 m and 3,5 m) (Figure 7.1). Samplings were conducted between 9 a.m. and 14 p.m. (light period). In addition, two samples were collected outdoor in duplicate, at a height of 4 m, representative of the inlet air. Overall, a total of 12 indoor samples and four outdoor samples were sampled weekly.

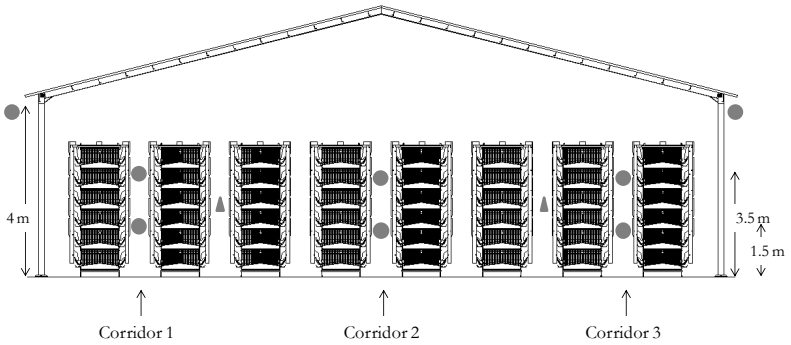

Figure 7.1. Schematic diagram of the hen house showing sampling locations of AGI-30 impingers during indoor airborne microorganism sampling prior disinfection (●) and during disinfetion test (▲).

Samples were refrigerated between 4ºC and 8ºC until being processed. In the laboratory, the sampled liquid was serially 10-fold diluted in sterile distilled water, and then 1 mL samples were plated onto duplicate Plate Count Agar (PCA) for mesophilic aerobic bacteria, and 0.1 mL samples were plated onto duplicate McConkey Agar for *Enterobacteriaceae*.

Plates of PCA were incubated at 30ºC for 48 h and plates of McConkey at 35ºC for 24 h. Colony forming units (CFU) were then counted. CFU were counted on plates containing between 30 and 300 colonies (Thorne et al., 1992). The concentration of airborne bacteria in liquid samples were determined by multiplying the CFU by the dilution volume and dividing by the volume plated. The concentrations of bacteria in the air were then calculated by dividing by the sampled air volume.

Additionally, impingement liquid was used to detect *M. gallisepticum* by real time quantitative polymerase chain reaction (qPCR). The remaining impingement liquid after culture methods was pooled by section (independent from height and corridor). A total of two indoor, plus one outdoor pooled samples were analysed per week. Each sample was filtered using a cellulose nitrate filter with a pore size of 0.45 μm. Particles retained on the filter were washed by placing the filter in a 30 mL falcon tube with 4 mL of identical solution as that used in as impinger liquid (PBS + 0.01% Tween and 0.005% anti-foam) and vortexed for 1 minute. The filter elution solution was stored at -80ºC until qPCR analysis. The qPCR for *M. gallisepticum* was conducted using

an in-house protocol (VISAVET, Centro de Vigilancia Sanitaria Veterinaria, Madrid, Spain).

The spatial distribution of the concentration of airborne mesophilic bacteria were analysed using ANOVA to determine the influence of the section, corridor and height. Section, corridor and height were used as a source of variance at each sampling time, using the SAS System Software (Version 9.1, SAS Institute Inc., Cary, Carolina del Norte, USA).

7.2.4. Air disinfection tests

The evaluation of air disinfectant to reduce airborne microoganisms was conducted in two identical laying hen houses. In one of them, the disinfection test was carried out (treatment house), whereas the other house was used as control. The test was performed twice in two consecutive weeks. Air disinfection was performed using a thermonebulizer (Model Swinfog SW-81, Tectraplant, Valencia, Spain). A mixture of 200 mL of Despadac® disinfectant (Calier, Barcelona, Spain), 3 L of monoethylene glycol, and 6 L of water was thermonebulized in each disinfection test.

During each disinfection test, airborne mesophilic aerobic bacteria and *Enterobacteriaceae* were sampled using AGI-30 impingers (Ace Glass Co., Vineland, NJ, USA) before and after disinfection in the treatment and control houses. Airborne microorganisms were sampled 1 hour before disinfection, 1 hour after disinfection, and 6 hours after disinfection. According to previous results on baseline microorganism concentrations and spatial distribution (section 7.2.3.2.) sampling was conducted in duplicate, in the center of two corridors at a height of 2 m (Figure 7.1). Sampling duration was 20 min. Samples were refrigerated between 4ºC and 8ºC until being processed at the laboratory. In the laboratory, the sampled liquid was serially 10-fold diluted in sterile distilled water, and then 1 mL samples were plated onto duplicate PCA for mesophilic aerobic bacteria, and 0.1 mL samples were plated onto duplicate McConkey Agar, for *Enterobacteriaceae*. These samples were processed in the same way as for baseline airborne microorganism concentrations measurements.

Additionally, the air and other surfaces (cages, feeders and hen's feathers) were sampled for *M. gallisepticum* using sterile swabs and gravitational plates, before and after disinfection (treatment house) and once in the control house. Before disinfection and in the control house, one sterile swab was used for sampling cages and feeders. Approximately, 10 cm^2 of cages and feeders were sampled with a sterile swab in the center of the house, in two corridors at a height of 2 m, in an area close to airborne microorganism sampling position. Two swabs were used for sampling hen's feathers by swiping the feathers of four hens. After disinfection, the samplings were repeated in the treatment house using two sterile swabs in cages, feeders and hen's feathers. The content of sterile swabs was directly plated in Mycoplasma Experience (ME) agar (Reigate, UK). Additionally, swabs were introduced in a test tube containing ME broth. Both agar plates and broth were incubated at 37ºC for 14 to 21 days. For gravitational plates, two ME agar plates were placed open in the center of each house, in two corridors at a height of 2 m, in an area close to airborne microorganism sampling position. A plastic mesh was used to prevent large particles from settling onto the plates. Sampling time was from 3 to 6 h depending on disinfection. After the exposure time, plates were sealed with parafilm and transported to the laboratory at ambient temperature. Plates were incubated at 37ºC for 14 to 21 days. Suspicious colonies from surfaces and gravitational settling technique were isolated in ME agar and cryopreserved at -80ºC for further confirmation by qPCR, as previously described.

Data were analysed using an ANOVA to determine the effect of the application of thermonebulized disinfectant on the concentration of airborne mesophilic aerobic bacteria. Disinfection was used as a source of variance at each sampling time (before disinfection, 1 hour after disinfection, and 6 hours after disinfection), using the SAS System Software (Version 9.1, SAS Institute Inc., Cary, Carolina del Norte, USA).

7.3. Results

7.3.1. Outdoor environmental conditions

Average outdoor temperature, relative humidity and solar radiation was 14.1 ºC, 58.7 % and 217.3 W/m², respectively. Predominant wind direction was west-north-west (WNW) and to a lesser extent north-west (NW).

7.3.2. Indoor environmental hygiene and air quality prior disinfection

7.3.2.1. Particulate matter: PM10 and PM2.5

Average PM concentration was 0.024±0.025 mg/m³ for PM2.5 and 0.546±0.377 mg/m³ for PM10. Maximum levels were 0.222 mg/m³ for PM2.5 and 3.281 mg/m³ for PM10. Average indoor temperature and relative humidity was 23.9 ºC and 45.2%, respectively. Figure 7.2 shows the temporal variation of indoor PM2.5 and PM10 concentrations recorded using TEOM. There was a clear and repeated pattern in daily PM concentrations. There was an increase in PM concentration coinciding with the start of the light period (6 a.m.). The PM concentration remained high, with variations, throughout the light period until 10 p.m. (start of the dark period). From 10 p.m., there was a decrease in PM concentration until the following light period (6 a.m. the next day)

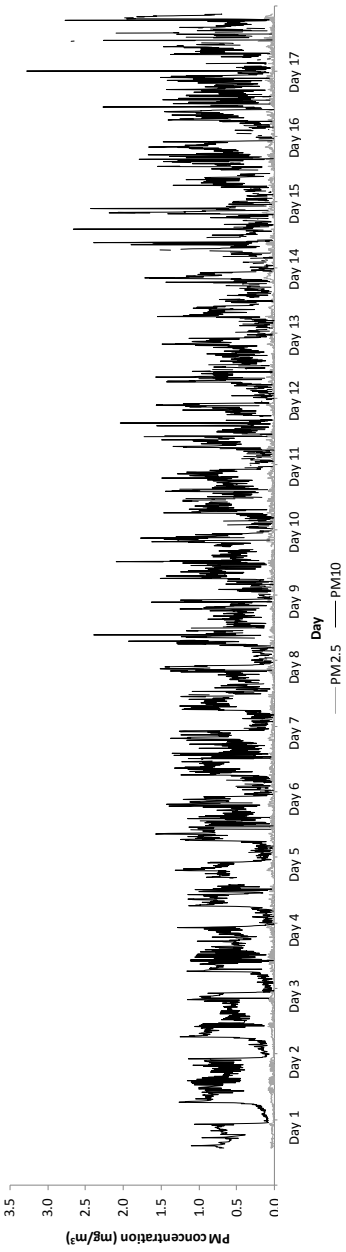

Figure 7.2. Temporal variation in PM2.5 and PM10 concentration (mg/m³) during the sampling period.

Table 7.1 shows average concentration for PM2.5 and PM10 during light and dark periods. The concentration of PM2.5 and PM10 during the light period was, on average, between two ($p<0.0001$) for PM10 and three times higher ($p<0.01$) for PM2.5 than during dark periods.

Table 7.1. Average PM2.5 and PM10 concentration and standard deviation during light and dark period in the sampled house.

	PM2.5 (mg/m^3)	PM10 (mg/m^3)
Light period	$0.028^{a}\pm0.011$	$0.679^{a}\pm0.148$
Dark period	$0.015^{b}\pm0.007$	$0.228^{b}\pm0.103$

[a,b] Averages within a column with different superscripts differ significantly ($p<0.05$)

Photomicrographs of the PM samples collected using the virtual cascade impactors and examined by SEM are shown in Figure 7.3 (PM2.5) and Figure 7.4 (PM10-2.5). Individual irregular particles of various sizes from 1 μm to 100 μm in diameter were observed. Observed particles were mainly from feathers, skin dander, manure, and encapsulated uric acid crystals.

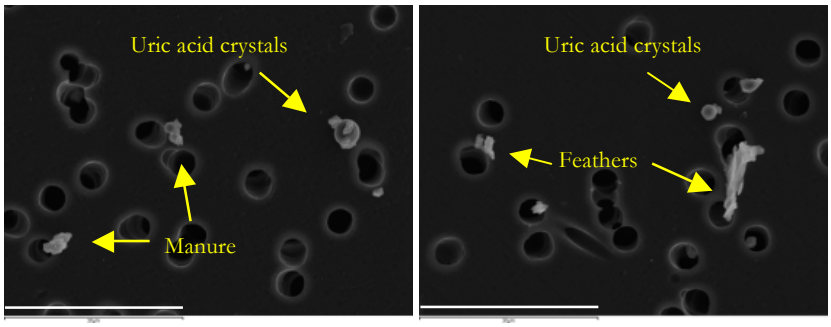

Figure 7.3. Representative PM2.5 airborne samples collected on polycarbonate filters viewed using SEM (1800x) from the sampled laying hen house. Note: 5 μm diameter filter pores are shown as round dark holes. Scale bar 30 μm.

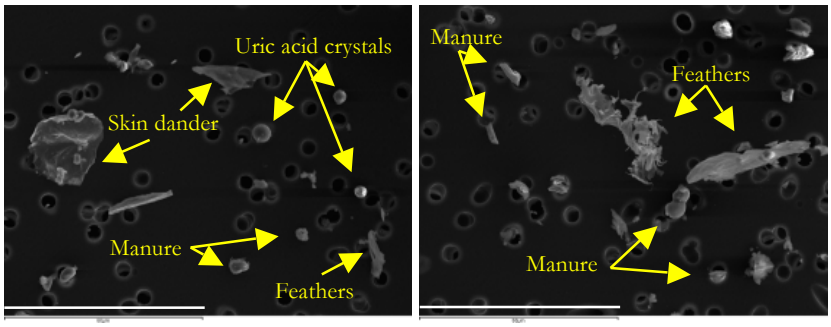

Figure 7.4. Representative PM10-2.5 airborne samples collected on polycarbonate filters viewed using SEM (1000x) from the sampled laying hen house. Note: 5 µm diameter filter pores are shown as round dark holes. Scale bar 60 µm

7.3.2.2. Airborne microorganisms: Mesophilic aerobic bacteria, Enterobacteriaceae and Mycoplasma gallispeticum

The concentration of airborne mesophilic aerobic bacteria ranged from 4.1 to 5.7 log CFU/m^3. Average concentration of airborne mesophilic aerobic bacteria in the different measurement points during the three sampling weeks are shown in Table 7.2.

Table 7.2. Average concentration of airborne mesophilic aerobic bacteria (log CFU/m^3) in different measurement points in laying hen house (n=3), in two sections (Section A: 46 m and Section B: 92 m from the head of the house), three corridors, and two heights (1.5 and 4 m).

		Height	log CFU m^{-3}
Section A	Corridor 1	1.5 m	4.93±0.23
		4 m	4.53±0.18
	Corridor 2	1.5 m	5.01±0.19
		4 m	5.09±0.19
	Corridor 3	1.5 m	4.99±0.19
		4 m	4.86±0.23
Section B	Corridor 1	1.5 m	5.02±0.19
		4 m	5.11±0.19
	Corridor 2	1.5 m	5.39±0.19
		4 m	5.26±0.19
	Corridor 3	1.5 m	5.25±0.19
		4 m	5.06±0.19

Concentration of airborne mesophilic aerobic bacteria in section B were higher than concentration in section A ($p<0.05$). No differences were obtained between heights and corridors. The presence of

Enterobacteriaceae indoors was intermittent throughout the sampling period. Only four positive samples were determined, ranging from 1.6 to 3.8 log CFU/m^3. Airborne *Mycoplasma* spp. was detected by qPCR in 5 out of 6 total indoor samples.

Average outside concentration of airborne mesophilic aerobic bacteria was 5.24±1.17 log CFU/m^3. Average concentration of *Enterobacteriaceae* varied from 0 to 3.20 log CFU/m^3. Airborne *Mycoplasma* spp. was detected by qPCR in all outdoor samples.

7.3.3. Air disinfection tests

Average concentrations of mesophilic aerobic bacteria are shown in Table 7.3. Overall, the concentrations of airborne mesophilic aerobic bacteria during disinfection tests in the treatment and control house were similar ($p > 0.05$), ranging from 4.1 to 4.9 log CFU/m^3. There were no differences between the treatment house and the control house at different sampling times (before disinfection, 1 hour after disinfection, and 6 hours after disinfection).

Table 7.3. Average concentration of airborne mesophilic aerobic bacteria (log CFU/m^3) and standard deviation for two disinfection trials (n=8) in treatment and control house, before disinfection, 1 h after disinfection, and 6 h after disinfection.

	Before	After (1 h)	After (6 h)
Treatment	4.49±0.37	4.46±0.33	4.37±0.51
Control	4.58±0.22	4.44±0.39	4.62±0.36

Enterobacteriaceae were detected in isolated cases during the two disinfection tests: 1.20 log CFU/m^3 in the treatment house before disinfection (disinfection 1) and 1.90 log CFU/m^3 in the control house, one hour after disinfection (disinfection 2).

Suspicious colonies of *M. gallisepticum* recovered using gravitational settling were confirmed to be *M. gallisepticum* by qPCR, both before and after disinfection. Additionally, *M. gallisepticum* were detected in three isolated samples collected using swabs on cage surfaces and hen's feathers in the treatment house, both before and after disinfection.

7.4. Discussion

Measures to reduce airborne pollution in livestock houses help to maintain adequate environmental hygiene both inside and outside. These measures can contribute to prevent health problems in humans and animals and atmospheric emissions, as well. Reduction techniques have been developed in recent years to decrease the concentration of airborne PM and microorganisms, but the effectiveness and applicability of wide spectrum thermonebulized chemical disinfectant to reduce airborne microorganisms had not been tested in battery caged laying hen houses. Therefore, this study contributes to increasing the knowledge on application of disinfectant to reduce airborne microorganisms in the air of laying hen houses and discusses its limitations to further improve its practical use and effectiveness.

To reduce PM and airborne microorganisms at source, firstly, the air quality status must be examined in terms of levels (concentrations) and temporal and spatial distribution. Therefore, this study provides an insight into air pollutant's concentrations and their temporal and spatial distributions inside a laying hen house. This information is a necessary first step in evaluating an air disinfection technique.

Measured PM and mesophilic aerobic bacteria concentrations in this study were in accordance with the literature for caged laying hen houses which ranges from 0.04 to 0.27 mg/m^3 for PM2.5, from 0.39 to 0.57 mg/m^3 for PM10 (Li et al., 2011; Lim, 2007; Wathes et al., 1997) and from $1.6x10^4$ to $1.7x10^5$ CFU/m^3 for mesophilic aerobic bacteria (Saleh et al., 2007; Vucemilo, 2007). The PM concentrations did not exceed the concentration limit recommended by CIGR (1992) and CIGR (1994) of 3.7 mg/m^3 (total dust) and 1.7 mg/m^3 (respirable particles, comparable to PM2.5) to protect animal health. The concentrations of airborne mesophilic aerobic bacteria did not exceed the air quality threshold recommended by Donham (1991) for pigs and humans, which stands at 5 log CFU/m^3.

The daily evolution and variation in the concentration of PM was probably affected by the activity of the hens, mainly due to flutter. During light periods, PM concentration was higher than during darks

periods; Li et al. (2011) observed a similar pattern of PM in a hen farm in the U.S.A. However, the effect light/dark and hens activity was more evident in PM10 concentration than in PM2.5. This is probably because the PM2.5 fraction contains small particles finer than PM10, which may be suspended in the air for a longer time due to their lower settlement velocity. Different authors have observed a close relationship between PM concentrations and airborne microorganism (Adell et al., 2011; Nimmermark et al., 2009), therefore a similar daily evolution and variation of airborne microorganism could be expected, although it was not measured in this study. Beside animal activity, other factors could affect the release of PM and microorganism such as inside temperature (Haeussermann et al., 2008), relative humidity (Yao et al., 2010) or air distribution and ventilation (Costa et al., 2009).

Our results from particle characterization (both PM10-2.5 and PM2.5) with SEM showed that the main sources of PM were feathers, dander and manure, including encapsulated uric acid. Cambra-López et al (2011a) found higher contribution of particles from faecal material and to lesser extent particles from feathers in aviary and floor systems for laying hens. According to our results, airborne microorganisms exhaled and excreted by hens could be probably associated with particles from feathers and manure and microorganisms could be thus expected to be carried by them. Zheng et al. (2013) reported the majority (>95%) of airborne bacteria were carried by particles >3.3 μm in diameter in an experimental aviary laying hen chamber. Aerosolization of these type of particles promoted by hen's activity could enhance airborne microorganisms. Understanding the variations of PM during the day and its main sources is valuable information to design techniques and protocols to reduce airborne microorganisms, as well.

Indoor environmental hygiene and air quality prior disinfection revealed similar levels of mesophilic aerobic bacteria among sampling points inside the house indicating a homogeneous bacterial load in both height and length (sections) along the entire house. These data provide valuable information on the airborne spatial distribution of microorganisms in an approximately 12880 m^3 battery caged laying hen house. These results

could help to identify an appropriate airborne microorganisms sampling scheme for such large buildings.

Our results indicate that air disinfection using wide spectrum thermonebulized chemical disinfectant was not effective in reducing the concentration of mesophilic aerobic bacteria and *M. gallisepticum* in the air. Disinfection consisted of a mixture of a wide spectrum chemical disinfectant Despadac®, plus oil and water. Oil spraying mixed with different substances has been described as an effective technique to reduce PM and airborne microorganism. The main effect of spraying oil is that it promotes particle aggregation and sedimentation, preventing PM from becoming airborne again. Aarnink et al. (2011) obtained a decrease of approximately 80% of PM2.5 and PM10 concentration by spraying rapeseed oil in broilers. Kim (2006) reported an average reduction of 30% of TSP (Total Suspended Particles) and 53% of airborne bacteria spraying different oils in a swine house. Rule et al. (2005) showed a reduction in the concentration of total bacteria spraying a mixture of acid, oil and alcohol, but could only reduce them one order of magnitude. Zheng et al. (2014) showed a slight reduction in the concentration of airborne bacteria using slightly electrolyzed water in an experimental laying hen house. Reductions were predominantly observed in the size range of particles >2.1 μm in diameter, but no reduction was observed for PM. They associated the bacterial decreased with the antimicrobial power of electrolyzed water rather than the effect of water on particles aggregation (Zheng et al., 2014).

According to the manufacturer, the disinfectant used in this study is typically recommended for disinfection of surfaces, but there is no information on its performance when thermonebulized in the air to reduce airborne microorganisms. The air disinfection technique used in our study was not successful in reducing airborne microorganisms. Similar results were obtained by Costa et al. (2014) using a chemical disinfectant fogging system procedure in a farrowing-weaning pig house. They did not obtain a reduction in airborne bacteria with the exception of *Micrococcacceae*. Thus it would be desirable to evaluate different doses and products, as well as application methods in future research. As regards the method of application, the thermonebulization of a liquid

produces very fine droplets which could be too small to promote particle aggregation and sedimentation by gravity. Therefore, other techniques which can generate larger drops must be evaluated. Moreover, evaluation of air disinfection during different period within the day could be investigated. We applied disinfectant during the light period, when hen's activity was the highest. Sampling during dark periods, when hen's activity is low, could increase the effectiveness of the technique.

Detection of *Mycoplasma* spp. in the air of commercial farms has not been previously described in the literature. Results regarding *Mycoplasma* spp. indicated presence of this pathogen both indoor and outdoor prior the air disinfection test. The presence of outdoor *Mycoplasma* spp. suggests that the inlet air could be a source of entry of this pathogen. Thus, to implement a reduction technique, inlet air should be considered as an entry of possible pathogens. Dee et al. (2010) described a mechanical filter as an adequate technique to avoid the infection with *Mycoplasma* spp. from other infected farms. Additionally, transmission between houses of the same farm could occur. Detection of *Mycoplasma* spp. in the air of commercial farms, however, has not been previously described in the literature. Therefore, the results presented herein contribute to increasing the knowledge of airborne *Mycoplasma* spp. dynamics and risks of transmission in commercial facilities. Nevertheless, quantifying airborne microorganisms, especially pathogens found in low concentrations, is complicated and no standardized techniques are available. With impingement, dehydration of bacteria when exposed to air speeds may occur (Landman et al., 2004). Furthermore, culture of *Mycoplasma* spp. is laborious (Feberwee et al., 2005) and complicated, probably due to their lack of cell wall. In our study, no *Mycoplasma* spp. was detected using culture methods, but it was confirmed by PCR. Adell et al (2014) obtained similar results with airborne *Salmonella* spp. These authors did not detect airborne *Salmonella* spp. by culture-dependent methods, but they did obtain positive results using PCR. In the same way, Zhao et al. (2011) only detected airborne *Campylobacter* using PCR, not by using culture dependent methods. A combination of culture-dependent and culture-independent methods, as those used in our study, are always preferred to prevent undetected pathogens in the air of livestock houses.

7.5. Conclusions

We evaluated the application of thermonebulized disinfectant in the air to reduce airborne microorganisms in a commercial laying hen house, with emphasis on its effect on *M. gallisepticum*. Additionally, an evaluation of the environmental and hygienic air quality baseline status prior disinfection test was performed. From our study we can conclude the following:

Prior disinfection tests, average PM2.5 were 0.024±0.025 mg/m^3 and 0.546±0.377 mg/m^3 for PM10; being two (PM10) and three (PM2.5) times higher ($p<0.01$) during light than during dark periods. The concentration of airborne mesophilic aerobic bacteria ranged from 4.1 to 5.7 log CFU/m^3. No differences were obtained between heights and corridors.

Particle characterization with SEM showed high proportion of feathers and manure in airborne PM10-2.5 and PM2.5. To reduce PM and airborne microorganism these sources must be considered.

Air disinfection by applying thermonebulized wide spectrum disinfectant in a commercial laying hen farm was not effective in reducing the concentration of mesophilic aerobic bacteria, *Enterobacteriaceae* and *Mycoplasma* spp. in the air.

It would be desirable to evaluate different air disinfectant doses, products and application methods. Additionally, it is necessary to acquire additional data on the relationship between PM and airborne microorganisms and their behavior in the air to design adequate techniques to reduce them in livestock houses.

The presence of outdoor *Mycoplasma* spp. suggests that the inlet air could be a source of entry of this pathogen. Therefore, the results presented herein contribute to increasing the knowledge of airborne *Mycoplasma* spp. dynamics and risks of transmission in commercial facilities.

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8

Discusión general

La ganadería ha sufrido una gran intensificación productiva en los últimos años para cubrir las necesidades de una población creciente y optimizar los costes de producción. Como resultado de esta intensificación, la tradicional producción ganadera caracterizada por explotaciones pequeñas y dispersas se ha sustituido por pocas y grandes explotaciones concentradas en determinadas áreas geográficas. Los sistemas de producción ganaderos son una fuente de contaminantes atmosféricos reconocida, por lo que la producción ganadera en determinadas zonas puede provocar una alteración en el medio ambiente, además supone un riesgo para la salud de los animales y de las poblaciones vecinas, sobre todo en áreas con gran densidad ganadera (Groot Koerkamp et al., 1998; Seedorf et al., 1998; Takai et al., 1998).

Los alojamientos ganaderos son fuentes inevitables de PM y bioaerosoles (Cambra-López et al., 2010). Actualmente se conocen los niveles habituales de PM y bioaerosoles de algunos de los alojamientos ganaderos, especialmente de aves y porcino. No obstante, los factores que afectan la generación de PM, los posibles efectos sobre otros compuestos como gases o la relación entre el PM aerotransportado y los bioaerosoles patógenos todavía se desconoce. Además, las diferencias entre especies ganaderas en cuanto a tipo de alojamientos, alimentación, condiciones ambientales, etc, exige un estudio diferenciado para cada una de ellas y para cada sistema de alojamiento.

Para desarrollar o implementar una medida de reducción de PM y/o bioaerosoles que sea efectiva en alojamientos ganaderos, deben considerarse los siguientes aspectos:

i. El origen (fuentes)
ii. Los niveles (concentración)
iii. El tamaño, morfología, composición química y propiedades biológicas del PM
iv. La viabilidad y patogeneicidad de los bioaerosoles
v. Los factores que contribuyen a la generación y suspensión en el aire del PM y bioaerosoles

En este sentido, esta Tesis Doctoral aborda aspectos relacionados con el origen, la concentración y la caracterización (propiedades físicas, químicas y biológicas) del PM suspendido en el aire de granjas de conejos y aves, su relación con los bioaerosoles patógenos y técnicas para reducirlos.

Estos cinco aspectos son los aspectos más importantes a considerar no sólo a la hora de diseñar medidas de reducción sino para evaluar los posibles efectos tanto para la salud de animales y personas como para el rendimiento productivo de los animales. Así, los resultados presentados en esta Tesis Doctoral pueden ayudar a la toma de decisión sobre el PM y bioaerosoles en alojamientos ganaderos y proponer la técnica de reducción más adecuada a cada caso, así como a mejorar la calidad del aire tanto en el interior como en el exterior de los alojamientos ganaderos.

8.1. Origen, concentración y caracterización del PM y los bioaerosoles. Factores de los que dependen.

Como primer paso para diseñar una técnica de reducción adecuada de PM y bioaerosoles procedentes de alojamientos ganaderos es necesario mejorar el conocimiento sobre el origen de los mismos y los factores que afectan a su generación y suspensión en el aire. Conocer las fuentes generadoras es fundamental para actuar sobre ellas y evitar que se generen y/o suspendan al aire en la medida de lo posible. Para identificar las fuentes generadoras, es necesario caracterizar las partículas según su tamaño, morfología, composición química y biológica. Esta Tesis Doctoral se ha centrado en el estudio de estos aspectos para el PM y los bioaerosoles. En el capítulo 3 y capítulo 4 se investigó las fuentes generadoras del PM y su concentración y emisión en alojamientos cunícolas. En el capítulo 5 se estudió los factores relacionados con la generación de bioaerosoles y su relación con el PM en alojamientos para broilers. Este mismo enfoque se utilizó en el capítulo 7 previo a la evaluación de una medida de reducción de bioaerosoles en un alojamiento de gallinas ponedoras.

Las fuentes generadoras de PM en alojamientos cunícolas no se habían estudiado hasta el momento. Los resultados de esta Tesis Doctoral indican que las concentraciones en alojamientos cunícolas son bajas comparadas con aves o porcino (por debajo de 0,9 mg/m^3 y 0,3 mg/m^3 para PM10 y PM2,5, respectivamente) (capítulo 4), sin embargo las emisiones medias de PM10 (entre 5,99±6,14 y 14,9±31,5 mg/plaza/día) y PM2,5 (entre 0,2±1,26 y 2,83±19,54 mg/plaza/día) son comparables a las emisiones en alojamientos avícolas o porcinos. Las fuentes más abundantes del PM en el aire en alojamientos cunícolas fueron las partículas procedentes de restos de pienso y material fecal (capítulo 3). Estos resultados se confirman con el análisis físico, químico y microbiológico de las partículas en el que la mayoría del PM en alojamientos cunícolas se mostró como partículas irregulares con aspecto fragmentado, ricas en azufre, calcio, magnesio, sodio y cloro. Éstas además transportaban microorganismos (capítulo 3). Estos datos son útiles para diseñar medidas de reducción en origen, enfocadas a minimizar la generación de estas fuentes y proteger la salud humana y animal. Sobre todo, la recomendación del uso de materiales de protección laboral como mascarillas durante las actividades diarias relacionadas con estas fuentes.

En alojamientos de gallinas (capítulo 7) las partículas más abundantes fueron partículas procedentes de plumas, descamaciones de piel, material fecal y cristales de ácido úrico. Cambra-López et al. (2011), en un exhaustivo estudio sobre las fuentes generadores de PM en gallinas en aviario y suelo obtuvo que las fuentes más abundantes fueron plumas y cristales de ácido úrico. En este caso, la retirada frecuente de la gallinaza de las instalaciones contribuiría a reducir la fuente generadora de PM en los alojamientos de gallinas ponedoras.

En cuanto a los bioaerosoles, los resultados obtenidos en el capítulo 5 en el que se estudió la relación en el tiempo y en el espacio entre el PM y las bacterias suspendidas en el aire, demostraron una mayor asociación de bacterias aerotransportadas con partículas en un rango entre 3,3 y más de 7 μm de diámetro, en un alojamiento de broilers. Del mismo modo, Zheng et al (2013a) obtuvieron en una granja experimental de gallinas ponedoras un mayor recuento de bacterias en partículas mayores de 3

μm que en tamaños inferiores. Por tanto, técnicas de reducción enfocadas a reducir los niveles de partículas de un tamaño superior a 3 μm, podrían ser efectivas para reducir al mismo tiempo los niveles de bacterias en el aire de explotaciones ganaderas.

En esta Tesis se apreció una variación de la distribución espacial de las bacterias aerobias mesófilas en un alojamiento de broilers a lo largo del ciclo (capítulo 5), de forma que durante el inicio del ciclo se observó un mayor número de bacterias a la altura de los animales respecto a alturas mayores. Sin embargo, a partir del día 17 del ciclo, estas diferencias desaparecieron. Con estos resultados, medidas encaminadas a reducir los niveles de bioaerosoles en el aire, como la desinfección del aire, deben ser aplicadas a partir de la segunda semana de ciclo cuando los bioaerosoles ocupan todo el volumen de aire de la sala, de forma que nos aseguramos que la medida de reducción pueda ser eficaz y evitamos mermas económicas por un uso a un tiempo indebido.

8.2. Retos en el muestreo de bioaerosoles patógenos y su cuantificación

El punto de partida para reducir los niveles de bioaerosoles y PM en el aire de los alojamientos ganaderos es utilizar métodos de muestreo precisos y fiables que recojan una muestra representativa del ambiente. Actualmente, el muestreo, la cuantificación de bioaerosoles y la detección de patógenos en el aire representan un reto.

Por una parte, en relación al muestreo, es necesario evaluar en primera instancia el rendimiento de las diferentes técnicas de muestreo. A diferencia de los muestreadores de PM donde la gravimetría es el método de muestreo de referencia según norma UNE-EN 12341:1999 para PM10 y UNE-EN 14907:2006 para PM2.5, no existe una técnica estandarizada para el muestreo de bioaerosoles. Esto es especialmente grave cuando se trata de bioaerosoles patógenos donde es clave realizar una detección fiable y a ser posible temprana en las explotaciones ganaderas y fuera de ellas.

En esta Tesis Doctoral se han evaluado tres técnicas de muestreo de bioaerosoles (impactación, borboteo y sedimentación) para detectar *Salmonella* spp. (capítulo 6). Nuestros resultados mostraron grandes diferencias según la técnica utilizada, que indican que los resultados obtenidos con distintas técnicas de muestreo deben de compararse con cautela. La falta de una técnica estandarizada dificulta el primer paso para diseñar una medida de reducción, es decir, establecer las concentraciones habituales de bioaerosoles, y en particular bioaerosoles patógenos. En este sentido, existe una gran necesidad de evaluar las técnicas disponibles de muestreo en función del tipo de microorganismo a muestrear y seleccionar la más adecuada para alojamientos ganaderos.

Por otra parte, en relación a la cuantificación de bioaerosoles, una vez realizado el muestreo existe la dificultad añadida de detectar o cuantificar al microorganismo en cuestión en el laboratorio ya que los microorganismos tienen diferentes requerimientos de supervivencia y algunos patógenos son muy susceptibles a los cambios (Hubad and Lapanje, 2013). En esta Tesis Doctoral se ha trabajado con dos patógenos: *Salmonella* Enteritidis y *Mycoplasma gallisepticum*. Este último es especialmente sensible fuera de sus reservorios habituales y complicado de cultivar ya que no presenta pared celular.

Tanto en el caso de la detección de *Salmonella* Enteritidis como de *Mycoplasma gallisepticum* (capítulo 6 y capítulo 7) el uso de la PCR fue necesaria para detectar estos patógenos que no fueron detectados mediante sistemas de cultivo tradicional. Por tanto, es necesaria la combinación de técnicas moleculares y técnicas de cultivo para detectar los patógenos presentes en el aire de explotaciones ganaderas. Esto resulta de gran importancia ya que las técnicas moleculares no aportan información sobre la viabilidad de los microorganismos. Conocer la viabilidad de estos patógenos para conocer si podrían poner en peligro la salud humana y animal es fundamental para establecer medidas de prevención y reducción de los mismos. Existen métodos moleculares para detectar la viabilidad de células (e.g. Live/dead cell viability assays o viable real time PCR) aunque no han sido probados en esta Tesis Doctoral. El uso de estas técnicas en combinación con técnicas

moleculares y de cultivo será recomendable en cualquier esquema de detección de bioaerosoles patógenos.

8.3. Técnicas de reducción de bioaerosoles y PM

A la vista de los resultados obtenidos en esta Tesis Doctoral, no resulta fácil proponer una única medida eficaz para reducir los niveles de PM y bioaerosoles en el aire en los alojamientos ganaderos. Las medidas de reducción se pueden clasificar en dos grandes grupos. Por un lado aquellas que tratan de minimizar la generación de bioaerosoles y la suspensión en el aire, es decir, medidas de reducción en origen, entre las que se encuentran las medidas relacionadas con técnicas de manejo de las instalaciones y los animales y la desinfección del aire. Y por otro lado, medidas que tratan de evitar que el PM y los bioaerosoles salgan al exterior de las naves, esto es, medidas de limpieza y purificación del aire.

Hasta el momento se han realizado diferentes estudios sobre posibles técnicas de reducción de PM y bioaerosoles en diferentes alojamientos ganaderos, sin embargo, no se ha desarrollado todavía una técnica 100 % eficaz para reducir tanto su generación como su emisión.

8.3.1. Manejo de las instalaciones y los animales

Se ha demostrado que una forma sencilla de reducir los niveles de bioaerosoles y PM se basa en técnicas de manejo de los animales o de las condiciones ambientales (McCubbin et al., 2002) de modo que se controlan las fuentes generadoras. Este tipo de técnicas basadas en el control de las fuentes generadoras son soluciones atractivas ya que a la vez que se reduce la concentración interior de PM y bioaerosoles, mejoran las condiciones de bienestar para animales y trabajadores.

Los resultados de esta Tesis (capitulo 4) indican que las prácticas de manejo y limpieza pueden afectar a la suspensión del PM y por tanto los microorganismos asociados a él, incrementando los niveles de estas sustancias en el aire.

Modificar las actividades de limpieza y manejo animal podrían contribuir de una forma sencilla y eficaz a reducir la suspensión del PM y

bioaerosoles y por tanto sus niveles en el aire de forma satisfactoria en granjas de conejos, sobre todo. En el capítulo 4 demostramos que la actividad que más eleva las concentraciones de PM en el aire de alojamientos de conejos, sobre todo del PM10, es barrer. Las actividades que elevaron las concentraciones de PM2,5 fueron barrer, el manejo animal y la limpieza de las jaulas. Sancilio et al. (1999) detectó que la actividad de quema de pelo de las jaulas mediante "limpieza en llama" fue la actividad que más PM generó. En un alojamiento de gallinas ponedoras, Li et al (2011) obtuvieron que las actividades de alimentación, sacar basura y limpiar alojamiento produjeron picos de incremento del PM en gallinas ponedoras. Por tanto, una modificación de estas actividades podría mejorar la calidad del aire. La limpieza con agua del suelo (en vez de barrer) y tratar de evitar realizar estas actividades simultáneamente o realizarlas durante las horas del día de mayor renovación del aire podrían ser alternativas de manejo sencillas y eficaces en conejos.

En gallinas ponedoras, de acuerdo con otros autores (Li et al., 2011) se obtuvo una clara relación entre las horas de luz/oscuridad y los niveles de PM (capítulo 7). Esto se debe a la mayor actividad de las gallinas durante las horas de luz, lo cual propicia la suspensión de partículas de plumas así como de partículas sedimentadas como puede ser el pienso o el polvo mineral. Además, en el momento de encendido y apagado de la luz se produce un mayor incremento de la actividad, por lo que medidas encaminadas a disminuir el incremento repentino en la actividad de las gallinas contribuiría a reducir el PM y bioaerosoles suspendidos en el aire, por ejemplo mediante un encendido gradual de las luces.

Las variables ambientales (temperatura, humedad relativa (HR) y ventilación) también influyen en la suspensión en el aire y supervivencia de microorganismos (Haeussermann et al., 2008; Yao et al., 2010). Se sabe que el incremento de la humedad del aire reduce los niveles de PM aerotransportado y una mayor tasa de ventilación favorece la suspensión del PM y bioaerosoles (Lin et al., 2012). Yao et al. (2010) obtuvieron en condiciones de verano (32ºC, 63% HR) unos niveles de PM10 de 0.03 mg/m^3 y 10^4 UFC/m^3 de bacterias totales aerotransportadas frente a 0.01 mg/m^3 de PM10 y 10^2 UFC/m^3 bacterias totales en condiciones de

invierno (23ºC, 74% HR) en un alojamiento porcino. Los resultados de esta Tesis (capítulo 5) revelaron mayor presencia de bacterias aerobias mesófilas en el aire al final del ciclo productivo de pollos coincidiendo con las mayores tasas de ventilación (0.7-1.07 m^3/h/animal), la mayor HR (53.4-56.5%), la menor temperatura (26.6-26.9 ºC) y las mayores concentraciones del PM2.5 y PM10 en el aire (0.025, 0.255 mg/m^3, respectivamente).

No obstante, las modificaciones de manejo así como de las variables ambientales deben ser compatibles con la producción y el bienestar animal, por tanto estas modificaciones deberán realizarse con precaución. Por ejemplo, un incremento de la HR, además de favorecer la sedimentación de partículas, puede tener efectos perjudiciales sobre la proliferación de microorganismos en la cama y la humedad de ésta. Es necesario considerar estos efectos secundarios a la hora de implementar cambios en el manejo de las instalaciones o de los animales.

8.3.2. Desinfección del aire

La desinfección del aire se presenta como una alternativa novedosa para reducir la carga microbiana del aire de los alojamientos ganaderos. La pulverización y nebulización de diferentes sustancias desinfectantes es una técnica actualmente en estudio y evolución. En esta Tesis Doctoral se evaluó la termonebulización de un desinfectante químico de amplio espectro en el aire (usado habitualmente para desinfectar superficies) con una mezcla de agua y aceite (capitulo 7). Los resultados esperados eran una reducción de la carga microbiana debido a dos factores: por un lado por las características bactericidas del desinfectante y por otro lado una mayor sedimentación del PM y bioaerosoles por efecto del aceite, que encapsula las partículas incrementando su peso y por tanto, favoreciendo su sedimentación. No obstante, no observamos ningún efecto sobre el nivel de bacterias aerostransportadas tras la termonebulización del desinfectante. Costa et al. (2014) estudió el efecto de la nebulización de un desinfectante químico en un alojamiento porcino obteniendo una reducción del PM en partículas mayores a 2,1 μm y una reducción de esporas fúngicas con respecto a una nave control, sin embargo no

obtuvieron tampoco reducción de bacterias suspendidas (a excepción de *Micrococcaceae*).

Actualmente, no existe un diseño optimizado para reducir la carga microbiana del aire de alojamientos ganaderos, por tanto existe la necesidad de evaluar diferentes productos, dosis, así como el método y frecuencia de aplicación. Además de los desinfectantes químicos, los productos habitualmente utilizados en los diferentes estudios son, aceites, alcoholes o mezclas de éstos y agua electrolizada. Estos productos, al estar libres de productos químicos, reducen el impacto ambiental asociado a los productos químicos. La pulverización de aceites esenciales como tomillo, orégano, menta o vapor de aceite de hierba de limón se presenta como una nueva vía de estudio ya que pudiera tener un elevado potencial para reducir los microorganismos dado su poder desinfectante (Bolashikov and Melikov, 2009; Tyagi and Malik, 2012). Rule et al. (2005) evaluó la eficacia de la pulverización de una mezcla a base de ácido, aceite y alcohol en una alojamiento porcino mostrando una reducción de PM2.5 y PM10 de entre el 75 y 90%, y una reducción de bacterias totales de un orden de magnitud.

La pulverización de agua electrolizada en alojamientos ganaderos se ha descrito como una alternativa eficiente para reducir los niveles de bacterias aerotransportadas por su poder bactericida. Zheng. et al. (2014), obtuvieron en un alojamiento de gallinas ponedoras una ligera reducción en la concentración de bacterias aerotransportadas, predominantemente en el rango de tamaño de partículas >2,1 μm, aunque no observaron ninguna reducción del PM, por lo que asociaron la reducción del nivel bacteriano al poder antimicrobiano del agua electrolizada y no por el efecto del agua sobre la sedimentación de las partículas. Trabajos recientes comparan la eficacia de la pulverización de un desinfectante químico y de agua electrolizada sobre los niveles de bacterias aerotransportadas. Hao et al. (2013) obtuvo una reducción del 59% (con agua electrolizada) y entre un 26 y 49% (con desinfectantes químicos) en el nivel de bacterias aerotransportadas en un alojamiento porcino, inmediatamente tras la pulverización de éstos. Del mismo modo, Zheng et al. (2013b) en una sala experimental de gallinas ponedoras demostraron una reducción de un orden de magnitud

($p<0,05$) en bacterias totales en el aire mediante la pulverización de agua electrolizada, mientras que no observaron reducción mediante la pulverización de un desinfectante químico. Es necesario evaluar estas técnicas en condiciones comerciales.

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9

Conclusiones y líneas de investigación futuras

9.1. Conclusiones

En los alojamientos avícolas y cunícolas se generan y emiten cantidades importantes de PM y bioaerosoles, por encima de los valores límite de exposición que marca la Directiva 2008/50/CE relativa a la calidad del aire ambiente y a una atmósfera más limpia en Europa, sobre todo en granjas de aves. Estas sustancias deben ser controladas y reducidas para proteger el medio ambiente, la salud y bienestar de las personas y animales.

El PM en alojamientos cunícolas mostró una morfología y composición química compleja. El tamaño de partícula varió entre 90-100 μm, siendo el S, Ca, Mg, Na y Cl los elementos químicos más abundantes. La concentración de bacterias en el aire varió entre $3{,}1x10^3$ y $1{,}6x10^6$ unidades formadoras de colonia, UFC/m^3.

Las principales fuentes generadoras de PM en alojamientos cunícolas fueron la piel, el pienso y las heces provenientes de las actividades de limpieza de la nave y de los propios animales. Una modificación de estas actividades podría mejorar la calidad del aire.

La concentración media de PM10 en alojamientos cunícolas fue $0{,}08\pm0{,}06$ mg/m^3 para conejos de cebo y $0{,}05\pm0{,}06$ mg/m^3 para conejas. La concentración media de PM2,5 fue $0{,}01\pm0{,}02$ mg/m^3 para conejos de cebo y $0{,}01\pm0{,}04$ mg/m^3 para conejas. Las emisiones variaron entre 6 y 15 mg/plaza/día para PM10 y entre 0,2 y 3,0 mg/plaza/día para PM2,5.

Las concentraciones de bacterias aerobias mesófilas medidas en el aire de una granja de broilers variaron entre 3,0 y 6,5 log UFC/m^3. Se observó un mayor número de bacterias a la altura de los animales durante los primeros 17 días del ciclo. La mayoría de bacterias se asociaron con partículas entre 3,3 y más de 7,0 μm de diámetro. Se obtuvo una correlación positiva (coeficiente de correlación entre 0,78 y 0,89) entre las concentraciones de PM2,5 y PM10 y las de bacterias aerobias mesófilas.

Utilizando borboteadores, no se detectó *Salmonella* spp. mediante cultivo en alojamientos de broilers, aunque sí se detecto mediante PCR. No se

recomienda el uso de borboteadores para la detección y/o cuantificación de *Salmonella* spp. cultivable en el aire.

En alojamientos de gallinas, la concentración media de PM10 fue 0,546±0,377 y 0,024±0,025 mg/m^3 para PM2,5; siendo entre dos y tres veces mayores durante el periodo con luces encendidas que durante el periodo de oscuridad. La concentración de bacterias aerobias mesófilas varió entre 4,1 y 5,7 log UFC/m^3. No hubo diferencias entre alturas y pasillos.

La termonebulización de un desinfectante químico de amplio espectro en un alojamiento de gallinas ponedoras no fue efectiva para reducir los niveles de bacterias aerobias mesófilas en el aire ni de *Mycoplasma gallisepticum*. Es necesario estudiar diferentes productos, dosis o técnicas de aplicación.

Los resultados presentados en esta Tesis Doctoral proporcionan una información útil sobre el PM y los bioaerosoles en el aire de alojamientos ganaderos, que permitirá diseñar e implementar medidas de reducción prácticas y eficaces que mejoren la calidad del aire en los alojamientos ganaderos y reduzcan su emisión al exterior.

9.2. Líneas de investigación para futuros trabajos

En esta Tesis Doctoral se han abordado los distintos objetivos, respondiendo a las preguntas de investigación planteadas, y han surgido otras preguntas sin responder. Éstas, marcan el camino que queda por recorrer en cuanto a la mejora de la calidad ambiental de los alojamientos ganaderos en general y en cuanto a la reducción de PM y bioaerosoles en particular. En este sentido, las líneas de investigación recomendadas para trabajos futuros son:

a. Caracterizar los bioaerosoles, sobre todo patógenos, presentes en sistemas de alojamientos ganaderos concretos, en relación a: niveles habituales, relación con el PM y factores que contribuyen a su generación y suspensión en el aire. Por ejemplo, campylobacteriosis, colibacilosis o virus de la enfermedad de bursitis infecciosa en broilers, el virus de la influenza aviar en

broilers y gallinas y la fiebre Q o virus boca-mano-pie en pequeños rumiantes, entre otros.

b. Diseñar técnicas de muestreo y protocolos estandarizados de muestreo, detección y cuantificación de bioaerosoles (patógenos y no patógenos) en alojamientos ganaderos.

c. Estudiar la eficacia de las medidas relacionadas con el manejo animal e instalaciones para la reducción de PM y bioaerosoles. Algunas de estas medidas propuestas son la modificación de variables ambientales (temperatura, humedad relativa y tasa de ventilación), sistemas de limpieza en granjas de conejos alternativas a barrer o a la quema de pelo o modificar el encendido de las luces en gallinas para evitar el incremento repentino de la actividad animal.

d. Evaluar medidas de reducción de bioaerosoles mediante la aplicación de desinfectantes químicos y no químicos en el aire, estudiando distintas variables como productos, dosis o frecuencia de aplicación y en distintas condiciones. Evaluar el uso de aceites esenciales y agua electrolizada en condiciones comerciales.

e. Investigar el efecto combinado de las técnicas de reducción de las concentraciones de PM y bioaerosoles sobre gases contaminantes (amoniaco y gases de efecto invernadero).

f. Evaluar el efecto de las técnicas de reducción de las concentraciones de PM y bioaerosoles sobre las emisiones de estos compuestos al exterior, para evitar poner en peligro la salud de las poblaciones vecinas o incluso otras granjas cercanas.

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