Evaluation and implication of cleaning and disinfection of broiler houses and pig nursery units

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LIST OF ABBREVATIONS

A

AC   After cleaning

ACC   Available chlorine concentration

ACP   Agar contact plate

AD   After disinfection

AHC   Animal house cleaner

AHS   Animal house stabilizer

ATP   Adenosine triphosphate

B

BC   Before cleaning

BPW   Buffered peptone water

C

C&D   Cleaning and disinfection

CE   Competitive exclusion

CFU   Colony forming units

CMC   Critical micelle concentration

CP   Cleaning product

CRA   Chlorine releasing agents

D

D   Day

DE   Dey engley

DP   Disinfection product

E

EU   European Union

L

LA   Livestock-associated

M

MBC   Minimum bactericidal concentration

MIC   Minimum inhibitory concentration

MRSA Methicillin resistant *Staphylococcus aureus*

N

NA   Nutrient agar

O

OD   Optical density

OM   Organic material

P

PAA   Peracetic acid

PCA   Plate count agar

PCR   Polymerase chain reaction

PIP   Probiotics in process

Q

Q1   First quartile

Q2   Second quartile (*i.e.* median)

Q3   Third quartile

QAC   Quaternary ammonium compound

R

RH   Relative humidity

RLU   Relative light units

S

S&B Slanetz and bartley

SAEW Slightly acidic electrolysed water

SAS Statistical Analysis System Software

T

TAB Total aerobic bacteria

TSB Tryptone soy broth

U

UPGMA Unweighted pair group method with arithmetic averages algorithm

V

VRBGA Violet red bile glucose agar

W

W Week

WHO World Health Organizition

X

XLD Xylose lysine deoxycholate

CHAPTER I
Introduction

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Introduction

1. Livestock meat production

The livestock sector is highly dynamic. According to FAOSTAT, global livestock meat production increased by 35% between 2000 and 2013 (Table I.1). During the same period, the greatest increases were seen in broiler (64%), pig (31%) and cattle (14%) meat production (Table I.1). (FAOSTAT, 2015).

In the European Union (EU), livestock production increased by 2.6% between 2000 and 2013. In 2013, broiler and pig production comprised 74% of the European meat production. (FAOSTAT, 2015). Within Belgium in the same year, broiler and pig production represented 85% of the total meat production (FAOSTAT, 2015).

As the broiler and pig production contribute the most to the global, European and Belgian meat production, the focus in this thesis was on these two sectors. Both sectors are further discussed below.

Table I.1: Livestock, broiler and pig meat production (tons) from 2000 - 2013 globally, in the European Union and Belgium, respectively. Evolution of production between 2000 and 2013 is given in percentage between brackets. Data was obtained from FAOSTAT (2015).

	Global		European Union		Belgium	
	2000	2013	2000	2013	2000	2013
Livestock	2.3×10^8	3.1×10^8	4.3×10^7	4.4×10^7	1.7×10^6	1.8×10^6
		(35%)		(2.6%)		(2%)
Broiler	5.9×10^7	9.6×10^7	8.2×10^6	1.1×10^7	4.0×10^5	3.8×10^5
		(64%)		(28%)		(-5%)
Pig	8.6×10^7	1.1×10^8	2.2×10^7	2.2×10^7	1.0×10^6	1.1×10^6
		(31%)		(3%)		(8%)
Cattle	5.6×10^7	6.4×10^7	8.4×10^6	7.4×10^6	2.8×10^5	2.5×10^5
		(14%)		(-14%)		(-10%)

1.1. Broiler production

The broiler meat industry has had a great contribution to livestock production growth. Several factors have contributed to the success of the increasing broiler production: (i) genetic progress in poultry strains for meat and egg production; (ii) better understanding of nutrition fundamentals; and (iii) disease control (Ravindran, 2013).

In 2013, the five biggest broiler meat producing countries were the United States of America, China, Brazil, Russian Federation and Mexico. Belgium was listed as 40th of the 207 countries in this ranking. Of the 28 EU countries listed, Belgium comes in at 8th (FAOSTAT, 2015). In addition, Belgium is one of the biggest exporters within the EU (VEPEK, 2012).

In 2015, around 23 million broiler chickens were present in Belgium (Table I.2). In that same year, a total of 994 farms with ≥ 1000 broilers were found in Belgium (FOD Economie, 2015a). Of these farms, 75% and 25% were located in the Flemish and Walloon region, respectively. In Flanders, the number of broiler farms decreased while the number of broilers per farm increased between 2004 and 2013 (Figure I.1).

Table I.2: Evolution of broiler chickens in Belgium (FOD Economie, 2015a).

	2000	2009	2010	2011	2012	2013	2014	2015
Number of broilers (x 1000)	24 498	20 659	21 899	23 084	22 705	23 285	21 161	23 838
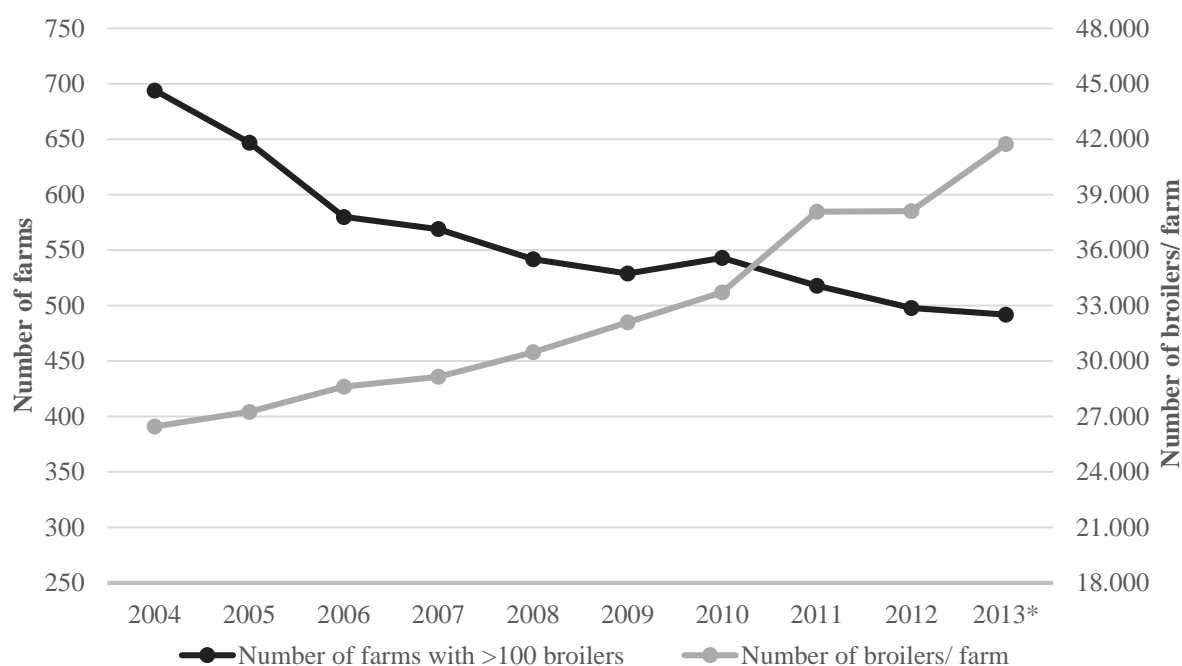
Figure I.1: Evolution of broilers and broiler farms in Flanders (LARA, 2015). **, estimation.*

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1.1.1 <u>Broiler production chain</u>

Figure I.2 represents the position of broiler farms in the broiler production chain. The chain starts with the primary breeding sector comprising large international enterprises (*e.g.* Cobb-Vantress, Aviagen and Hubbard). Primary breeders consist of pureline elite stock, great grandparents and grandparents generations. The progeny of these last flocks are highly efficient breeding lines (*i.e.* parent lines), which are then sold to specialised farms where breeders are housed (parent breeding farms). The parent breeders will produce hatching eggs for the broiler industry. These eggs are transported to the hatchery and subsequently placed in incubators. Eggs are then hatched into day-old chickens. Afterwards, day-old chickens are delivered to broiler farms (VEPEK, 2012). Recently, increasingly more eggs are hatched on-site in the broiler houses. Flocks are thinned around week 5 and remaining broilers are collected, placed into crates and transported to the slaughterhouse at week 6 (age around 38-40 days).
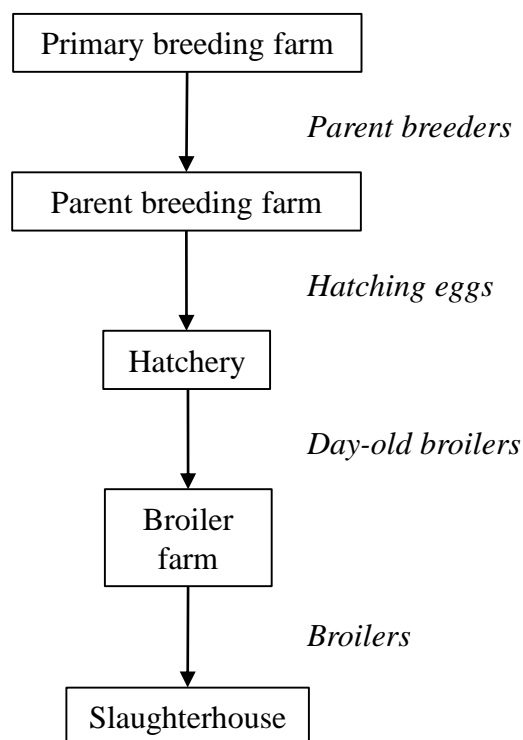
Figure I.2: Overview of the broiler production chain (modified figure from VEPEK, 2012).

1.1.2 <u>Housing of broilers</u>

- <u>Building</u>

In Belgium, broilers are generally reared in floor housing systems with bedding material (*e.g.* wooden shavings), where they can move freely. Standard houses have no windows and are

ventilated with forced air. The walls and the roof are insulated and the floor consists of concrete (Scientific committee on animal health and animal welfare, 2000). Several feed chains and drinker lines are installed, covering the whole length of the house.

Materials used in the broiler houses vary from house to house, but generally consist of concrete (*e.g.* floors and walls), plastic (*e.g.* feed pans and drinking cups), metal (*e.g.* feed chains and heating pipes), synthetic material (*e.g.* ventilation system, walls and roof), wood (*e.g.* ventilation system), etc..

- Stocking density

For farms with more than 500 broilers, stocking density in broilers houses should not exceed 33 kg/m² (*i.e.* 15 broilers/m²). However, upon compliance with additional criteria concerning the ventilation system, heating, low mortality etc., a higher stocking density up to a maximum of 42 kg/m² (*i.e.* 19 broilers/m²) may be authorised (Anonymous, 2007).

- Temperature, relative humidity and ventilation

The temperature in broiler houses is maintained around 33 – 35 °C on arrival of the day-old chicks. From the fourth week until slaughter age, the temperature may decrease up to 3 – 4 °C weekly, resulting in a final temperature around 20 – 22 °C (Table I.3). Relative humidity should be minimum 40% and maximum 70% (Van Gansbeke and Van den Bogaert, 2011). The ventilation system, *i.e.* air inlets and outlets, is designed to provide and spread fresh air throughout the animal house and to decrease relative humidity, toxic gasses (*e.g.* CO_2, NH_3, H_2S) as well as any unpleasant odours (*e.g.* indole). The ventilation system should provide a minimum of 3.6 to 4 m^3 of fresh air per kg body weight and hour (Scientific committee on animal health and animal welfare, 2000).

Table I.3: Recommended ambient temperature and relative humidity (RH) according to the age of broilers (adjusted from Van Gansbeke and Van den Bogaert, 2011).

Age (days)	Recommended ambient temperature	% RH
1	33-35	50-60
3	33-35	50-60
7	30-31	55-65
14	26-28	<70
21	23-26	<70
28	20-24	<70
35	20-33	<75
40	20-22	<75

1.2. Pig production

Globally speaking, 36% of meat production in 2013 was obtained from pigs (FAOSTAT, 2015). Belgium is situated at number 17 of the 189 pig producing countries worldwide. In the EU, Belgium is one of the top ten pig producing countries (FAOSTAT, 2015).

The number of fattening pigs (> 50 kg) in Belgium increased by 174 000 between 2000 and 2014 (Table I.4). In total, 4 727 pig farms were present in Belgium in 2015, of which 87.7%, and 12.3% were situated in the Flemish region and Walloon region, respectively (FOD Economie, 2015b).

Table I.4 Evolution of fattening pigs in Belgium (FOD Economie, 2015a).

	2000	2009	2010	2011	2012	2013	2014
Number of fattening pigs >50 kg (x 1000)	2 749	2 799	2 882	2 955	3 051	3 075	2 923

In the Belgian pig industry, the same trend was observed as in the broiler industry: numbers of pigs per farm are increasing while the number of farms are decreasing (Figure I.3).

In 2014, Belgium was situated in the top 5 exporters of pig meat in the EU and top 10 worldwide (VLAM vzw - Belgian meat office, 2015).
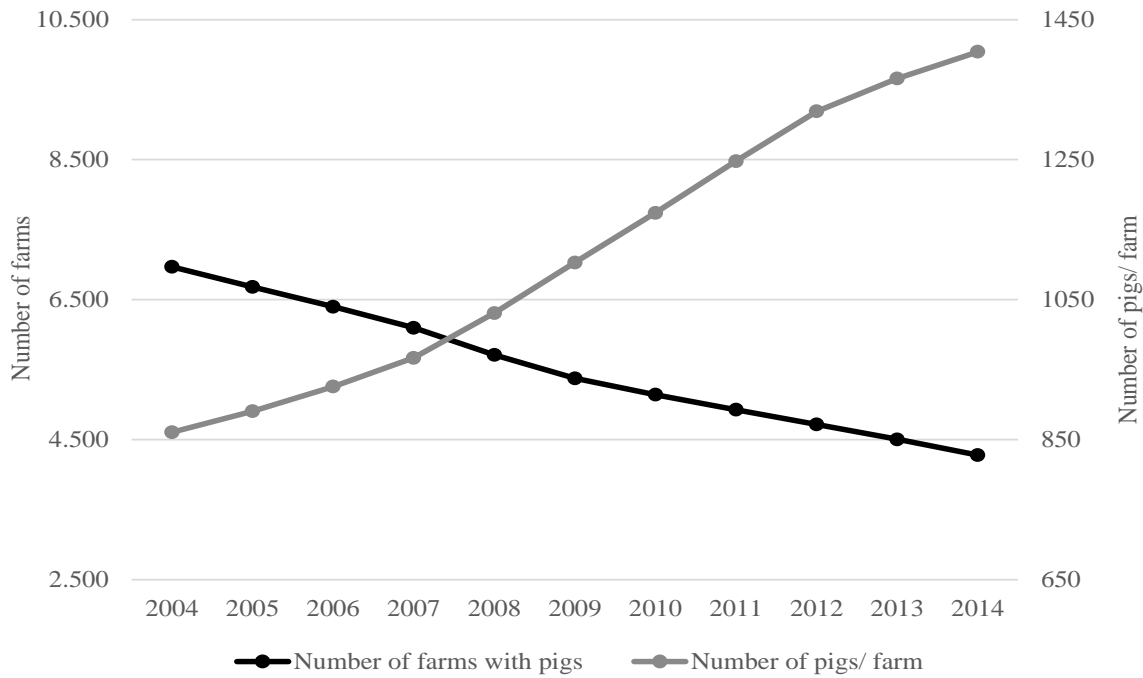
Figure I.3: Evolution of pigs and pig farms in Flanders (LARA, 2015).

1.2.1 Pig production chain

In the pig industry in Europe, three types of farms can be identified: (i) breeding farms, (ii) fattening farms and (iii) farrow-to-finish farms. On breeding farms, sows produce piglets that are subsequently moved to fattening farms after the nursery period. At the fattening farms, piglets are fattened until slaughter age. Farrow-to-finish farms produce piglets, that are fattened on-site (Figure I.4) (FOD Economie, 2015c). In Belgium, fattening pigs are slaughtered at around 115 kg of live weight. Several hybrid systems between the three described systems also exist.

Sows and piglets can be found in different units: breeding, gestation and farrowing units on the one hand and farrowing, nursery and finishing units on the other hand. First, sows are inseminated in the breeding units. During gestation, sows are placed for 15 − 16 weeks in the gestation units (which can be in combination with the breeding unit). From 1 January 2013 onwards, pregnant sows must be group housed during a period from 4 weeks of gestation to 1 week before the expected farrowing date (Anonymous, 2009). One week before farrowing, sows are moved to the farrowing units where they stay until weaning of the piglets (3 − 4 weeks; lactation period). After weaning, piglets are then moved to nursery units where they stay for 5 − 6 weeks. Finally, piglets are relocated to fattening units until slaughter age.
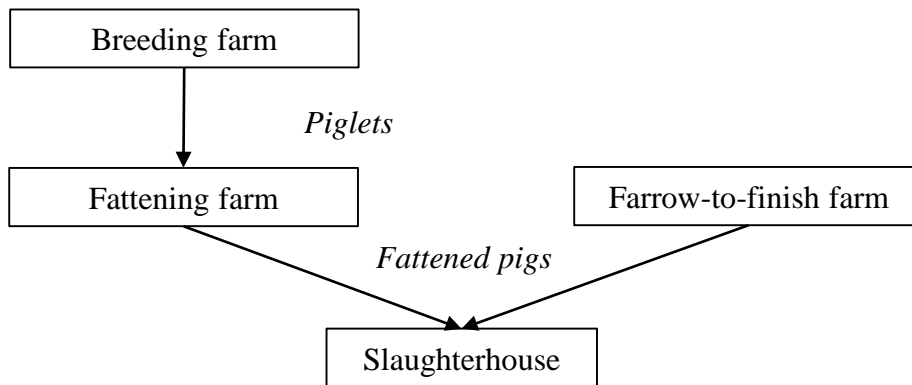
Figure I.4: Overview of the pig production chain.

1.2.2 **Housing of piglets**

As the focus of this thesis is cleaning and disinfection (C&D) of pig nursery units (in addition to broiler houses), the housing of weaner pigs is described.

- Building

The design of pig nursery units is highly variable between pig farms. Fully or partly slatted floors are widely used in pig nursery units throughout the EU. Excreta from pigs can fall through these slatted floors and be stored in a physically separate place from that occupied by the animals (Scientific panel on animal health and welfare, 2005). In case of concrete slatted floors, the maximum width of the openings is 18 mm and the minimum slat width is 50 mm (Anonymous, 2009). Slatted floor systems are also available in plastic (-coated), steel and aluminum. A nursery unit can be divided into several pens by low separation walls (*e.g.* metal, plastic). Each pen contains feed (*e.g.* metal, plastic, wood) and water (*e.g.* metal) dispensers. In addition, enrichment materials (*e.g.* chains) should be available for the piglets.

- Stocking density

In pig nursery units, an unobstructed floor area of 0.20 and 0.30 m² is required per piglet of 10 - 20 kg and 20 - 30 kg, respectively (Table I.5).

Table I.5: Minimal unobstructed floor area that must be available for each weaner or rearing pig kept in a group, excluding gilts after service and sows (Anonymous, 2009).

Live weight (kg)	Unobstructed floor area (m²)
Not more than 10	0.15
More than 10 but not more than 20	0.20
More than 20 but not more than 30	0.30
More than 30 but not more than 50	0.40
More than 50 but not more than 85	0.55
More than 85 but not more than 110	0.65
More than 110	1.00

- <u>Temperature, relative humidity and ventilation</u>

The temperature in pig nursery units is about 28 °C upon arrival of the weaned piglets. Once the piglets reach a weight of 20 kg, the temperature is decreased to 22-23 °C (Varkensloket, 2012). A relative humidity between 50 and 80% in pig houses is advised (Van Gansbeke *et al.*, 2009).

Ventilation in pig nursery units is of great importance to reduce toxic gases as these may cause respiratory diseases in pigs and are harmful for the environment. During the winter, recommendations for ventilation are 0.35 - 0.40 m³ per kg body weight and hour and during the summer 1.60 - 2.10 m³ per kg body weight and hour, as more heat and water vapour (produced by the pigs) must be removed (Madec *et al.*, 2003).

2. Prevention of the introduction and spread of infectious agents on farms (biosecurity)

Biosecurity includes all measures preventing pathogens from entering a herd (*i.e.* external biosecurity) and reducing the spread of pathogens within a herd (*i.e.* internal biosecurity) (Amass and Clarke, 1999). Biosecurity in animal production is key for both farm management (*e.g.* disease prevention) and meeting consumer demands concerning food safety. Good hygiene practices on farms can reduce the risk of introduction and spread of animal diseases and infectious agents that are transmittable from animals to humans (zoonoses). These infectious agents not only lead to disease outbreaks resulting in suboptimal production and flock mortality, but also to an increase of veterinary costs and condemnation rates as well as animal welfare issues. All of this leads to high economic losses for the farmer (Jung and Rautenschlein, 2014) and in case of epidemic diseases, preventive measures such as quarantine or even destruction

of animals (Gelaude *et al*., 2014). It is therefore of great importance to prevent disease outbreaks through biosecurity measures rather than cure them (Gelaude *et al*., 2014; M Laanen *et al*., 2014).

2.1. External biosecurity

External biosecurity can be divided into different categories, such as purchase of animals; removal of manure and dead animals; feed, water and equipment supplies; personnel and visitors; biological vector control; and location of the farm (Gelaude *et al*., 2014; Laanen *et al*., 2014).

2.1.1   Purchase of animals

Direct contact between infected animals (*e.g.* through skin contact) or excretions (saliva, milk, urine, manure, etc.) of infected animals and susceptible animals is an efficient way to introduce diseases (Amass and Baysinger, 2006). To limit the risk of introducing pathogens, it is important that animals are purchased from a farm having the same or higher sanitary status. The same applies for the purchasing of semen. In addition, when animals are purchased, they should preferably be quarantined, during which time they should be observed and tested for possible infectious diseases (Kraeling and Webel, 2015).

2.1.2   Removal of manure and dead animals

Dead animals are often a source of pathogens, as they may have died due to an infection, and should be removed as quickly as possible (Gelaude *et al*., 2014).

Collecting manure and cadavers by transport lorries is a risk for introducing pathogens because these vehicles enter many farms (Doyle and Erickson, 2006; Fritzemeier, 2000; Hege *et al*., 2002). It is therefore recommended that vehicles, or at least their wheels, are disinfected before entering the farm (Casal *et al*., 2007; Gelaude *et al*., 2014; Lister, 2008).

2.1.3   Feed, water and equipment supplies

Feed is generally produced under strict hygienic procedures, but several studies have shown that contaminated feed may be linked to the occurrence of pathogens in animal houses (Davies *et al*., 2004; Dee *et al*., 2014; Fink-Gremmels, 2012; Morgan-Jones, 1981). Feed producers have studied a variety of treatments to decontaminate feed, including chemicals, heat and irradiation (Doyle and Erickson, 2006).

In addition to feed, drinking water can be a source of infectious agents (Herman *et al*., 2003; Heyndrickx *et al*., 2002; Nyachoti and Kiarie, 2010). Water can be contaminated by dust, faeces, wildlife or rodents, hence it is important to store drinking water in closed reservoirs (Lister, 2008). Also, the formation of biofilms in water pipes can lead to contamination of water. Sharing of equipment between farms can be a risk factor for spreading diseases as well (Brennan and Christley, 2012).

2.1.4 **Personnel and visitors**

People have been proven to act as mechanical vectors, *i.e.* vectors that can pick up infectious agents and transmit them through physical contact (EFSA, 2016), of several pathogens (Heyndrickx *et al*., 2002). It is therefore advised to restrict access for visitors, including veterinarians, and limit the number of animal care takers per animal house (Herman *et al*., 2003; Refrégier-Petton *et al*., 2001). Farm workers and visitors should comply with all biosecurity measures regarding washing hands and farm-specific clothing and boots (Amass, 2000). Moreover, basic measures such as a hygiene lock (*e.g.* dressing room) are strongly recommended. Disinfectant footbaths on farms are often highly contaminated with organic material because of improper use. Footbaths, if used inappropriately, may be a risk of pathogen spread rather than a preventive measure (Amass, 2000). Removing all visible manure by scrubbing, followed by soaking the boots in a clean disinfectant bath for a time period according to the disinfectant manufacturer is effective for disinfecting boots (Amass, 2000).

2.1.5 **Biological vector control**

- Vermin

Several studies showed that vermin can be an important vector of pathogens (Dewaele *et al*., 2012b; Hald *et al*., 2004; Meerburg *et al*., 2007).
An important example are flies, which are potential reservoirs and transmitters of several bacteria such as *Salmonella* (Dewaele *et al*., 2012b; Holt *et al*., 2007; Olsen and Hammack, 2000)*, Campylobacter* (Hald *et al*., 2004; Szalanski *et al*., 2004)*, E. coli* O157:H7 (Szalanski *et al*., 2004) and *Staphylococcus aureus* (Owens *et al*., 1998).

Also rodents are recognised as important biological and mechanical vectors for pathogens. House mice (*Mus musculus*) and brown rats (*Rattus norvegicus*) are the most common rodent species on farms (Backhans and Fellström, 2012). Literature showed that wild rodents can carry pathogens such as *Salmonella, Campylobacter*, methicillin resistant *Staphylococcus aureus*

Lawsonia intracellularis, *Brachyspira hyodysenteriae* and *Yersinia*, and can act as transmitters to production animals on farms (Backhans and Fellström, 2012; Dewaele *et al*., 2012b; Pearson *et al*., 2016; Pletinckx *et al*., 2013; van de Giessen *et al*., 2009). In order to limit rodent nesting, feed should be stored in a vermin-free place and buildings should be rodent proof. Other rodent control methods include rodenticides and traps. Using cats for rodent control is not advisable as the cats may transmit diseases as well (Dewaele *et al*., 2012b; Kinde *et al*., 1996).

- Wildlife

Besides vermin, wild birds can play an important role in the spread of diseases on farms, especially on free-range farms. Several studies showed that faeces of wild birds can be contaminated with pathogens such as *Salmonella* Enteritidis (Davies and Breslin, 2001) *Campylobacter jejuni* (Hiett *et al*., 2002; Stern *et al*., 1997) and *Escherichia coli* (Pearson *et al*., 2016).

Wild boars (*Sus scrofa*) can harbour many important infectious agents that are transmissible to domestic pigs and other animal species, such as classical swine fever, Aujeszky disease, brucellosis and trichinellosis (Meng *et al*., 2009). As wild boar populations are growing and spreading in several European countries (Apollonio *et al*., 2010) including Belgium, the risk of disease transmission through direct (contact with other animals) or indirect (air or other vectors) contact with farm animals increases, especially on outdoor and organic farms.

- Pet animals

Pet animals can be infected by pathogens by consuming infected mice, carcasses or by contact with a contaminated environment. Kijlstra *et al*. (2004) showed that cats can be a risk for *Toxoplasma* infection on pig farms. Desrosiers (2011) indicated that *Brachyspira hyodysenteriae* can be transmitted by dogs to pigs. In addition, *Salmonella* has been isolated from dog faeces (Dewaele *et al*., 2012b; Leonard *et al*., 2011). Because of these observations, it is important to prevent contact between pets and farm animals.

2.1.6 **Location of the farm**

Airborne transmission of pathogens is possible through several distance related factors such as: other near farms and backyard animals (Lister, 2008; Van Steenwinkel *et al*., 2011), animal transport on public roads (Graham *et al*.; Vieira *et al*., 2009) and litter spread on nearby arable lands (Lister, 2008). If the farm is located near wild boar populations, preventive measures should be carried out, *e.g.* building fences.

2.2.Internal biosecurity

Internal biosecurity measures aim at prevention or reduction of spread of pathogens within the herd. These measures can be divided into different categories, such as farm management, compartmentalisation, implementing working lines, disease management and cleaning and disinfection (Backhans *et al*., 2015; Gelaude *et al*., 2014; Postma *et al*., 2015). In addition, several categories of external biosecurity (*e.g.* removal of dead animals and measures for pet animals, personnel and visitors) are also part of internal biosecurity measures.

2.2.1 <u>Farm management</u>

On farms, an all-in/all-out system (for each phase/unit) is recommended, whereby premises are emptied, cleaned and disinfected between production cycles to limit the contact between the arriving animals and the dust, manure and debris of the previous round.

Ideally the farm premises are divided into clean and dirty areas (Figure I.5) with clearly identifiable clean-dirty barriers. Allocation of 'clean' and 'dirty' areas is farm dependent, but in general a 'clean' area is the area around and part of the production site with restricted access and a 'dirty' area comprises the cadaver storage facility and the farm entrances for employees, visitors and external transport vehicles. Several clean-dirty locations on farms are discussed below. The location and design of the loading bay should ensure that external vehicles arrive and stay at the dirty entrance of the farm. Another example is the changing room on farms: the dirty zone is where the employees and visitors enter the room, store their personal clothes and shoes and wash/disinfect their hands; and the clean zone is where farm specific overalls and boots are put on. On farms with high hygiene standards, showering is mandatory before entering the clean zone. The two zones should preferable be separated by a physical barrier.
Finally, cross-over between the dirty road/traffic (*e.g.* feed deliveries) and clean road/traffic (employee cars) should be avoided and the number of visitors should be limited to minimise introduction of pathogens.
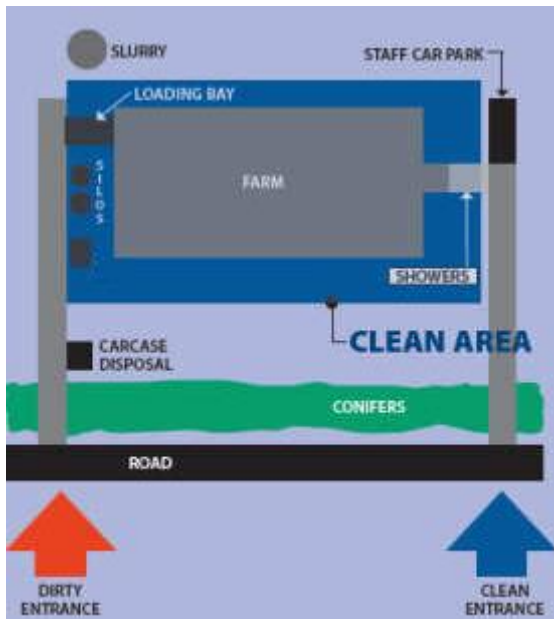
*Figure I.5: An example of clean and dirty areas on a farm
(modified from Blaken (2008)).*

2.2.2   <u>Compartmentalisation</u>

On pig farms, animals should be housed in separate premises according to age (*i.e.* farrowing, nursery and fattening units) in order to minimise disease transmission between the older animals (least susceptible) and young piglets (most susceptible). For each age group, clothing and boots should be provided to prevent contamination with excreta, and equipment such as shovels and brushes should be available. It is advised to wash and disinfect hands between units.

2.2.3   <u>Working lines</u>

Applying working lines or routes on farms, with the youngest age group (most susceptible) at the beginning and the oldest (least susceptible) and diseased at the end, helps to prevent transfer of pathogens to susceptible animals.

2.2.4   <u>Disease management</u>

It is important to separate diseased pigs (possible sources of infectious agents) as soon as possible from healthy pigs and isolate them in closed sickbays.

In addition, it is discouraged to return piglets with retarded growth to a susceptible younger age group, as they are likely to be carriers of pathogens. If these piglets are very weak and considered not to become profitable fattening pigs, euthanasia is a better choice. The same applies for broilers: it is advised to euthanize severe sick broilers, as they may never be a broiler

of good quality, due to the short production cycle, and may be a source of infection for healthy broilers. Therefore it is important to check the broiler houses regularly for sick animals. In addition, a high stocking density may increase the spread of infectious agents rapidly, hence it is advised to rear broilers in lower stocking densities.

Moreover, implementing an effective vaccination programme on pig farms improves the immunity of animals and reduces spread of pathogens within a herd (Amass and Baysinger, 2006).

2.2.5   Cleaning and disinfection

Cleaning and disinfection (C&D) is an important aspect of the internal biosecurity. Not only the interior of the premises should be cleaned and disinfected, but also the hardened environment (*e.g.* concrete) around the premises, as studies showed the presence of pathogens in the vicinity of animal houses (Schulz *et al*., 2012, 2004; Studer *et al*., 1999). In addition, some bacteria can survive for long periods under various conditions in the environment, such as *Salmonella*, *Escherichia coli, Staphylococcus aureus* (including MRSA) and *Enterococcus* spp. (Kramer *et al*., 2006). Effective C&D is therefore a crucial step in reducing the infection pressure in animal houses and preventing both endemic animal diseases and food-borne zoonoses (van de Giessen *et al*., 1998).

Finally, also clothing, boots and equipment should be washed and disinfected, as they could be contaminated with pathogens. Improper disinfection could place the herd at risk of pathogen spread (Amass, 2000).

Cleaning and disinfection of animal houses will be further described below.

3. Cleaning and disinfection of animal houses

3.1   General aspects

A good cleaning and disinfection programme consists of 6 steps. The first 4 take place during cleaning and the last 2 during disinfection. Moreover, 2 additional steps (*i.e.* step 7 and 8) after disinfection could be implemented (Table I.6). After C&D, the hygiene status of animal houses can be evaluated (step 9).

Table I.6: 9 steps of an ideal cleaning and disinfection (C&D) and evaluation programme between production rounds.

Step	Category	Description
1		Dry cleaning
2	Cleaning	Wet cleaning: washing premises with water
3		Wet cleaning: soaking premises with cleaning product
4		Wet cleaning: rinsing premises with water
5		*Drying*
6	Disinfection	Disinfection of premises
7		*Rinsing with water*
8		*Vacancy*
9	Evaluation	Monitoring the hygiene status after C&D

3.1.1 <u>Cleaning</u>

Cleaning refers to physical removal of foreign material from a surface (McDonnell and Russell, 1999). In animal houses, a distinction between dry and wet cleaning is made. Dry cleaning is generally the first step (step 1, Table I.6) that is carried out after removal of animals, whereby manure, dust, feed etc. is mostly removed. Appropriate equipment, such as shovels and brushes (in some cases mounted on agricultural vehicles), are used during this step. After dry cleaning, wet cleaning takes place. Wet cleaning is a process that consists of four factors: time, mechanical action, chemistry and temperature (*i.e.* Sinner's circle) (Friis and Jensen, 2005). If one of the factors is reduced, the other three factors should compensate by increasing them. For example, if a lower concentration of detergent is used, a longer cleaning time is necessary to obtain the same result as cleaning with a higher concentration of detergent.

In the optimal case on farms, premises are first cleaned with water under high pressure to remove loose organic matter (OM) (step 2, table I.6). Subsequently, premises are soaked with a cleaning product (CP) (step 3, table I.6) and afterwards residual dirt and CP are removed with water under high pressure (step 4, table I.6). The soaking with detergent is preferably performed from the floor towards the ceiling, which makes it visually easier to differentiate soaked and non-soaked surfaces, while washing is performed vice versa, to reduce the chance of splashing dirt particles from the floor (heavily soiled) on the walls and ceiling (*i.e.* foaming up, rinsing down principle). When working with high pressure, a pressure of 50 to 200 bar is used with a recommended flow rate of 12 to16 L/min (Cox and Van Meirhaeghe, 2009). Between dry and wet cleaning (*i.e.* before step 2), animal houses can be soaked with water (overnight) in order

to loosen the dirt and facilitate and optimise the C&D process. However, to our knowledge, this hypothesis has not yet been proven in literature. It is also often advised to use warm water and detergent during cleaning as it (theoretically) dissolves fats more easily (Gibson *et al*., 1999), yet there is little recent data underlying its practical relevance in animal houses.

- Working mechanism of detergents

A detergent has an amphipathic structure: a hydrophilic region and a hydrophobic region (hydrocarbon tail) (Field, 2014). First, detergent monomers will reduce the surface and interfacial tension between air/water and soil/surface, which increases contact surface with detergents (mobilization phase). When the detergent monomer concentration exceeds the critical micelle concentration (CMC), molecules will associate to form micelles (Figure I.6). This formation increases the solubility of hydrophobic compounds (solubilisation phase). The insoluble OM and/or bacteria are dispersed as micelle droplets in water (emulsification phase) and will be washed away (Pacwa-Płociniczak *et al*., 2011).
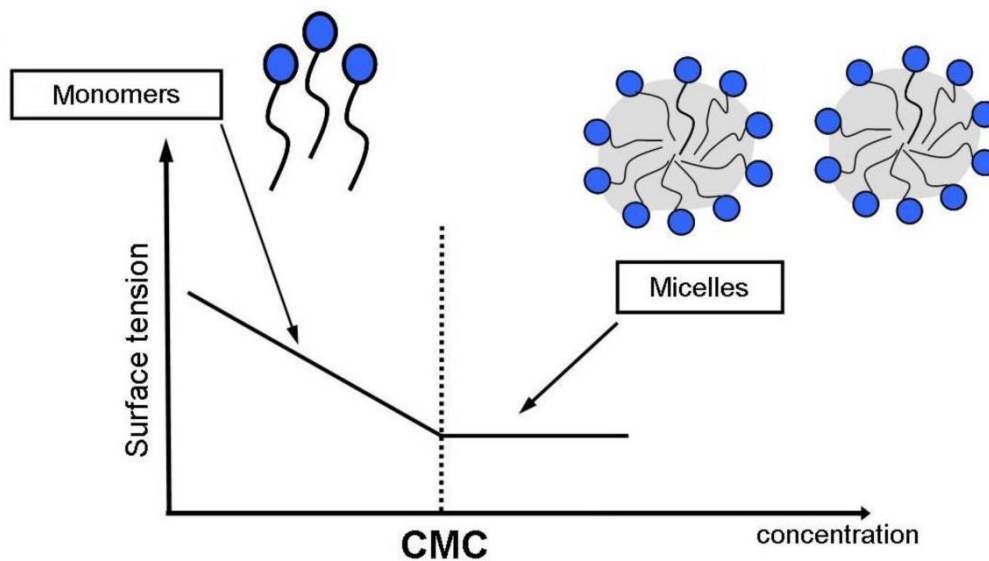
Figure I.6: The relationship between surfactant concentration, surface tension and formation of micelles (adjusted figure from Pacwa-Płociniczak *et al*., 2011).

- Cleaning product compounds

One of the major compounds of cleaning products are detergents. Depending on the type of detergent, the purpose of the cleaning product can differ. Four groups of detergents can be distinguished according to the head group (RIZA, 1998; Salager, 2002): anionic, cationic, non-ionic and amphoteric. Anionic detergents are the most commonly used detergents, and are preferred for cleaning animal houses (Aceto, 2015).

In addition to the type of detergent, pH modifiers also determine the application possibilities of the CP. Alkaline cleaners (pH> 7) are used to remove organic compounds based on carbon (*e.g.* fats, proteins, animals wastes) and acidic cleaners (pH< 7) to remove inorganic compounds (*e.g.* rust, corrosion, scale deposits) (CIMCOOL, 2007). For cleaning animal houses, an alkaline cleaner is chosen (Reus *et al.*, 2008).

The use of tap water with a high mineral content (*i.e.* hard water) during cleaning can diminish the efficacy of anionic detergents, as these detergents have a higher binding affinity to minerals than soil. The content of dissolved minerals in tap water can vary from region. To counteract this problem, chelating agents are added to CP. These agents will bind the minerals and form soluble complexes, whereby the free detergent monomers can actively bind soil (Jennings, 1965).

Moreover, cleaning products are often corrosive. To counteract this problem, inhibitors are added to inhibit corrosion formation on ferrous and non-ferrous metals.

In addition to the above mentioned components, also other components are often added to the CP such as solvents, foam modifiers and antimicrobials (ECOLAB, 2009).

3.1.2   Disinfection

Disinfectants are biocides that are generally used on inanimate objects or surfaces (McDonnell and Russell, 1999). Biocide is a term used for chemical agents that inactivate organisms. Because biocides vary in antimicrobial activity, more specific terms are used, including "-static" (*e.g.* bacteriostatic, fungistatic and sporistatic) and "-cidal" (*e.g.* bactericidal, fungicidal and sporicidal) referring to biocides which inhibit and kill the target organism, respectively.

When animal houses are dried after cleaning (step 5, table I.6), disinfection (step 6, table I.6) can be applied by surface disinfection, thermal fogging or fumigation (Gradel, 2007). Surface disinfection or wet disinfection is often carried out with a pressure cleaner or an orchard sprinkler. In case of thermal fogging (*i.e.* dry disinfection), the biocide, in a higher concentration than in case of surface disinfection, is heated and subsequently converted to a fog by a mobile or fixed fogger. In addition, premises have to be completely sealed off, which is not always easy to do in animal houses. Finally, fumigation can be carried out, whereby the disinfectant is evaporated and spread throughout the premises (Gradel, 2007). On some farms, two disinfection rounds are carried out in which disinfection-methods can be alternated.

Alternatives for disinfection, such as solutions of competitive exclusion (CE) bacteria are sold for environmental application in animal houses. The efficacy of CE bacteria against pathogens in the gut of animals has been shown in several scientific studies (Doyle and Erickson, 2006; Genovese *et al*., 2003; Mead, 2000; Schneitz and Hakkinen, 2016), however the value of a CE protocol in the environment of animal houses is yet to be determined. Possible mechanisms to explain the working mechanism of CE products are that the introduced bacteria (i) physically obstruct attachment sites preventing attachment of pathogens, (ii) compete for essential nutrients, limiting growth of pathogens, (iii) produce antimicrobial compounds (*e.g.* bacteriocins) and/or (iv) inactivate quorum sensing (Patterson and Burkholder, 2003). Though, the true mode of action of CE bacteria remains unknown.

After disinfection, animal houses or parts (*e.g.* feeding troughs) can be rinsed with water (step 7, table I.6), to remove disinfectant residuals as they can be toxic for animals. Furthermore, a vacancy (8th step, table I.6) may be applied in order to dry the animal houses and further reduce the residual bacteria, however it is not known how long this period should last. In 2009, six member states of the EU required a specific minimum vacancy period after disinfection of broiler houses, in case of *Salmonella* contamination: *i.e.* Austria (14 days), Estonia (21 days), Luxemburg (21 days), Norway (30 days), Denmark (10-14 days), Spain (12 days) (EFSA, 2011). Backhans *et al*. (2015) showed that 92% of the studied Swedish pig farms (n= 60) applied a mean vacancy of 5.3 days. Moreover, few scientific literature is available about the effect of a vacancy on the bacterial load in animal facilities. In addition, a vacancy step is often carried out for practical reasons such as restoration works or a delay of animal delivery.

A disinfection product (DP) contains, besides several compounds common with CP, one or several active components. The type of these components determine against which micro-organisms the DP is active. The ideal animal house DP meets the following criteria (Gradel, 2007): (i) it eliminates micro-organisms rapidly; (ii) it is active against a broad spectrum of micro-organisms; (iii) it is unaffected by low temperatures; (iv) it is unaffected by OM; (v) it retains its activity during storage; (vi) it is non-corrosive; (vii) it is non-hazardous for the farmer; and (viii) it is environment friendly.

The top 5 most commonly components in DP used for the primary sector (SCENIHR and GreenFacts, 2009) and their properties are described below.

- <u>Active components</u>

Alcohols

Alcohols are commonly used for skin and hard-surface disinfection. The most commonly used alcohols are ethanol, isopropanol and n-propanol (especially in Europe). Alcohols exhibit a rapid and broad-spectrum antimicrobial activity against vegetative bacteria (not spores), viruses and fungi. Because of its fast evaporation, other biocides are added in low concentration to DP to increase efficacy (McDonnell and Russell, 1999). The activity of alcohol is slightly affected by OM (Gorman and Scott, 2004).

Aldehydes

The most known aldehydes used as disinfectants, are glutaraldehyde and formaldehyde. Glutaraldehyde has a broad spectrum of activity against bacteria, including spores, fungi and viruses. Formaldehyde is bactericidal, sporicidal and virucidal (McDonnell and Russell, 1999). However, the activity is lower at a temperature beneath 20 °C and relative humidity (RH) should be at least 70% (Reus *et al*., 2008). Moreover, the use of formaldehyde as active substance in biocides is now under review by the European chemicals agency because it has carcinogenic properties (see chapter I – section 3.3) (European Chemicals Agency, 2016). In addition, OM has little influence on the activity of both aldehydes (Reus *et al*., 2008).

Quaternary ammonium compounds

Quaternary ammonium compounds (QAC), are cationic surface-active agents that are used as detergents and disinfectants. Because of their range in chemical structures, the specific activity is quite diverse. Therefore, there is a lot of debate about the activity of QAC against micro-organisms (Walker, 2002). However studies have proved the activity of QAC against vegetative bacteria (mostly Gram positive), yeast, fungi and some viruses (especially enveloped) (Fazlara and Ekhtelat, 2012; McDonnell and Russell, 1999; Reus *et al*., 2008). QAC have a rapid action against micro-organisms, however they are very susceptible to OM and are detrimental to the environment (Gradel, 2007, 2004).

Peroxygens

Hydrogen peroxide and peracetic (peroxyacetic) acid are commonly used peroxygen based disinfectants. Hydrogen peroxide is an environmentally friendly product as it dissolves rapidly in water and oxygen. In addition, it exhibits a broad spectrum and rapid activity against bacteria (especially Gram positive), yeast, viruses and bacterial spores. However, a disadvantage is that these products are corrosive. Peracetic acid (PAA) is considered bactericidal, virucidal and fungicidal at low concentrations. In addition, it remains active in the presence of OM

(McDonnell and Russell, 1999). A combination of both peroxygens is very effective as farm disinfectant (Reus *et al*., 2008).

A more recently developed disinfectant is peroxymonosulfate, with an increasing use as footbath and surface disinfectant. It has a broad microbial spectrum of activity (*i.e.* bactericidal, virucidal and fungicidal) and is effective in the presence of OM (Perry and Caveney, 2012).

Chlorine based compounds
The most important chlorine releasing agents (CRA) are sodium hypochlorite, chlorine dioxide and N-chloro compounds such as sodium dichloroisocyanurate (McDonnell and Russell, 1999). They exhibit a rapid kill against a broad spectrum of micro-organisms. High levels of available chlorine will even eradicate mycobacteria and bacterial spores (Gorman and Scott, 2004). However, these agents are highly susceptible to OM and are very corrosive (Gradel, 2004).

- Antibacterial action

The mechanism of action of disinfectants is poorly understood, as they have multiple bacterial target sites. The overall mechanism may depend on the bacterial structure against which it has its activity. Three levels of interaction of a biocide with the vegetative bacterial cell exist: (i) interaction with the outer cellular components, (ii) interaction with the cytoplasmic membrane and (iii) interaction with cytoplasmic constituents. It is possible that a biocide acts on one or all three levels to produce its antibacterial effect, though the cytoplasmic membrane is considered as major target site (Maillard, 2002).

Examples of these interactions between biocides and the bacterial cell on each level are described below.

First level (outer components)
One effect caused by the interaction between biocides and the bacterial cell, is the change in hydrophobicity of the cell wall. For example, it has been observed that the hydrophobicity of the Gram negative bacterial cell wall can be altered by cationic compounds (*e.g.* QAC), leading to damage and uptake of the biocide so the target sites can be reached (Ferreira *et al*., 2011; Maillard, 2002; Marcotte *et al*., 2005). In addition, cationic disinfectants have a high binding affinity for negatively charged outer components of both Gram positive (*e.g.* teichoic acid and polysaccharide elements) and Gram negative bacteria (lipopolysaccharide) (Fazlara and Ekhtelat, 2012).

Glutaraldehyde is thought to cross link outer membrane components, *e.g.* peptidoglycan (Gram positive bacteria) and lipoproteins (Gram negative bacteria), subsequently leading to hinder of

essential functions (*e.g.* nutrient uptake) and cell death (Russell, 2001). The interaction between alcohols and the cell wall results in coagulation/denaturation of proteins (Ascenzi, 2005).

Second level (cytoplasmic membrane)
The target site of membrane active agents is the cytoplasmic membrane of the bacterial cell. Disruption of the membrane is often demonstrated by the release of intracellular materials, potassium, inorganic phosphates, amino acids, proteins, etc. Alcohol denatures proteins in the cytoplasmic membrane, including enzymes, leading to cell leakage and cell death (Ingram, 1990). Quaternary ammonium compounds bind membrane phospholipids and subsequently induce leakage of intracellular components (Ferreira *et al*., 2011; Ioannou *et al*., 2007). Moreover, it has been shown that hydrogen peroxide can cause membrane damage in bacteria, by oxidising lipids and proteins (Baatout *et al*., 2006; Brandi *et al*., 1991; Peterson *et al*., 1995). Chlorine dioxide exerts a non-specific oxidative attack on membrane proteins, including enzymes involved in transport (Auer, 2009; Jeng and Woodworth, 1990), disrupting the permeability (Oyarzabal, 2005).

Third level (cytoplasmic constituents)
Chlorine dioxide is also associated with oxidative modification and denaturation of constituent proteins, critical to the integrity and functioning of bacteria (Ogata, 2007). Besides, oxidation of DNA and RNA can occur (Auer, 2009). Formaldehyde causes cell death by cross linking proteins and DNA (Schouten, 2002). Hydrogen peroxide causes DNA and protein damage, due to the release of ferryl radicals and hydroxyl radicals, respectively (Linley *et al*., 2012).

Few active components, *i.e.* glutaraldehyde, formaldehyde, PAA and hydrogen peroxide, are actively sporicidal. These components require higher concentrations and longer contact times for this effect than for bactericidal activity (Russell, 1990). The mechanisms of sporicidal activity is poorly understood, probably due to the complex nature of the bacterial spore and the possibility that disinfectants have more than one actual or potential target site.

- Factors influencing the antibacterial effect

The activity of disinfectants on bacteria depends on several factors. It is believed that the antibacterial effect is concentration dependent, whereby at low concentrations more specific interactions might occur, while at higher concentrations non-specific damage likely occurs. The bacteriostatic effects, usually achieved by a lower concentration of a biocide, might correspond to a reversible activity on the bacterial target (Maillard, 2002). The concentration of the disinfectant can be reduced by the presence of diluting water and/or extraneous material (*e.g.*

OM and surface active agents). Moreover, it is known that the cidal activity of disinfectants is strongly impaired under soiled conditions, because of the reaction between OM and the disinfectant. This reduced activity is especially seen with highly reactive compounds (*e.g.* hydrogen peroxide) (Russell, 2004; Smith, 2004). According to kinetic studies, a lower concentration of the disinfectant requires a longer contact time. Theoretically, if the concentration of QAC is halved, it requires a double disinfecting time (Russell, 2004). Several disinfectants based on aldehydes, peroxides, QAC, bis-phenols and iodines were tested in the presence and absence of OM in a study of Ruano *et al*. (2001). In absence of OM, most disinfectant products were effective within 10 minutes of contact time. However, when OM was present, the efficacy decreased and longer contact times and/or higher dosages were necessary. This was also shown by Moustafa Gehan *et al*. (2009) who tested 5 commonly used disinfectants in the poultry industry. This study showed that in presence of OM, a longer contact time than 30 minutes was needed to demonstrate the efficacy.

OM also affects disinfection by adhering to microbial cells and blocking adsorption sites necessary for disinfectant activity (Smith, 2004). As some surfaces in animal houses are difficult to clean and hence possibly still contain OM, these are likely sources for infectious agents. Wooden surfaces are more difficult to clean than plastic or metal, likely due to the porosity of wood (Rathgeber *et al*., 2009). Also, concrete in animal houses is often affected by numerous environmental factors, such as wear caused by animals and vehicles and chemical degradation caused by feeds and manure (Kymalainen *et al*., 2009), making them difficult to clean and disinfect. A study showed that the performance of biocides was reduced on porous or rough surfaces such as wood and concrete compared to smooth surfaces such as metals and plastics (Harding *et al*., 2011). In addition to the type of material, the design of surfaces has an impact on the cleanability.

It has also been shown that surface active agents present in CP can significantly reduce the antibacterial activity of QAC (Russell, 2004). Therefore, it is important that animal houses are thoroughly rinsed with water after applying the CP. Moreover, when high levels of cations (*i.e.* Ca^{2+} and Mg^{2+}) are present in water, the activity of certain disinfectants, *e.g.* chlorhexidine (Rutala and Weber, 2008) and QAC (Bessems, 1998; Fredell, 1994), can be reduced as they interact to form insoluble precipitates. Therefore, hard water should be used in the laboratory to test the efficacy of disinfectants.

Furthermore, the environmental temperature may influence the antibacterial activity of the disinfectant. It is often stated that the activity of a disinfectant increases with an increasing

temperature, but some disinfectants are more temperature dependent than others (Russell, 2004). In addition to temperature, the relative humidity in animal houses can influence gaseous disinfectants, *e.g.* formaldehyde and chlorine dioxide. The relative humidity (RH) for disinfection with formaldehyde is advised to be at least 70% (Reus *et al.*, 2008).

It has also been shown that freshly made disinfectants are more efficient in the presence of OM, than stored disinfectants (Stringfellow *et al.*, 2009). Some disinfectants can lose their antibacterial properties due to several factors, such as pH changes and the temperature during storage (Boucher, 1978; Costa *et al.*, 2015; Kunigk *et al.*, 2001).

Finally, also characteristics of bacteria may influence the activity of disinfectants, *i.e.* vegetative planktonic cells are more susceptible than biofilm cells (Allison and Gilbert, 1995; Costerton *et al.*, 1987; Gradel, 2004; Stewart and Costerton, 2001) or spores (McDonnell and Russell, 1999; Russell, 1999). In case of biofilm cells, the biocide concentration is strongly affected by the reduced diffusion of active components through the biofilm (SCENIHR, 2009). In addition, intrinsic, adaptive or acquired resistance mechanism may lead to survival of bacteria (see chapter I - section 4). Also the number of bacteria can affect the efficacy of disinfectant, as they can provide protection to other bacterial cells. Therefore, high inoculation levels of bacteria are used in laboratory tests (Maillard, 2013). It would be interesting to identify the residual bacterial flora after disinfection of animal houses and test their susceptibility against the disinfectant in order to understand their survival.

3.2   Monitoring hygiene status in animal houses

After cleaning and disinfection, the hygiene status can be monitored (9th step, Table I.6). Several methods to perform hygiene controls, either as a routine control or after a sanitary crisis, have been used and described, *e.g.* agar contact plates (ACP), swab samples, air samples, ATP analysis and visual inspection (Table I.7). In Belgium, ACP for enumeration of total aerobic bacteria are used to assess the efficacy of disinfection of poultry houses in the *Salmonella* control programme. However this is not the case for neighbouring countries: Germany, the Netherlands, France and the United Kingdom (personal communication). Vangroenweghe *et al.* (2009) suggested the use of ACP for enumeration of total aerobic bacteria, to monitor hygiene after C&D in pig facilities. In addition, farmers can join a quality system (e.g. Belplume and IKB) that impose standards including hygiene control with ACP.

It would be useful to select the most suitable sampling methods for monitoring C&D in broiler houses and pig nursery units. In addition, it would be interesting to sample locations that are

difficult to clean and disinfect, as they give a better idea about the hygiene status, *i.e.* presence or absence of pathogens, after C&D. These need to be identified for each type of animal house. Bacteriological monitoring after C&D, often focus on total aerobic bacteria (Corrégé *et al.*, 2003; Hancox *et al.*, 2013; Ward *et al.*, 2006) and/or a specific pathogen analyses (Carrique-Mas *et al.*, 2009; Merialdi *et al.*, 2013; Mueller-Doblies *et al.*, 2010; L J Pletinckx *et al.*, 2013; Rose *et al.*, 1999). Besides total aerobic bacteria also various specific microbiological indicator organisms such as *Escherichia coli, Enterococcus* spp., *Salmonella* and methicillin resistant *Staphylococcus aureus* can be used to evaluate the hygiene of animal houses. *Escherichia coli* has been shown to be a suitable index organism for monitoring the possible presence of *Salmonella* (Dewaele *et al.*, 2011; Gradel *et al.*, 2004a; Winfield and Groisman, 2003).

An advantage of monitoring *E. coli* is that the detection and enumeration method is less time consuming and laborious than analysing *Salmonella*. In addition, the index organism should occur in higher numbers than the pathogen, increasing the chance of detecting/enumerating it (Dewaele et al., 2011). However, a drawback is that the survival rate of the index organism in a given environment should be similar or greater than of the pathogen, which may not always be the case.

Enterococcus spp. is suggested to be an adequate hygiene-indicator organism for faecal contamination of surfaces (Gradel *et al.*, 2004b). In 2008, a high prevalence of LA-MRSA in European pig breeding (n= 1600) and production (n=3473) holdings was found: 37.6% and 43.6%, respectively (EFSA, 2010). Therefore, it seems interesting to monitor MRSA during C&D.

In addition to bacteriological monitoring, also non-bacteriological analyses can be carried out, such as adenosine triphosphate (ATP) monitoring and a visual inspection (Table I.7). Both methods are generally conducted after cleaning. ATP is an energy molecule, present in all eukaryotic and prokaryotic living cells. The principle of the analysis is based on the addition of a solution containing lysis reagent, the substrate luciferin and the enzyme luciferase to the swab sample. The lysis reagent allows the release of ATP from all living cells. Released ATP molecules are used by luciferase to convert the substrate resulting in a bioluminescent reaction. Measurements of the produced light can be immediately carried out with a measurement apparatus.

Table I.7: Advantages and disadvantages given for each monitoring method to assess the bacterial load or hygiene status. Examples of scientific studies, carried out in chicken or pig facilities, are given for each method. Bold and underlined characters represent studies carried out in chicken and pig facilities, respectively.

How to monitor?	What can be monitored?	Advantages	Disadvantages	Studies
ACP [1]	Bacteria	- Ease of use - Fixed sampling area - No need for further processing after sampling - Premade available - Objective	- Limited sampling surface (25 cm²) - Only smooth, firm surfaces - Colony overgrowth - One ACP per specific organism - Need for standardised pressure - Results after incubation period and enumeration	**De Reu *et al*. (2006); Huneau-Salaün *et al*. (2010)**; <u>Kim and Kim (2010)</u>
Swab sampling	Bacteria	- Larger sampling surfaces [2] - Able to sample irregular surfaces - ≥1 analyse/ swab - High upper enumeration limit - Objective	- Laboratory manipulation - No standardised protocol - Results after incubation period and enumeration	<u>Banhazi and Santhanam, (2013)</u>; <u>Beloeil *et al*. (2007)</u>; **Carrique-Mas *et al*. (2009); Davies and Breslin (2003)**; <u>Hancox *et al*. (2013); Mannion *et al*. (2007); Merialdi *et al*. (2013); Oliveira *et al*. (2006)</u>; **Rathgeber *et al*. (2009); Rose *et al*. (2003)**; <u>Schmidt *et al*. (2004)</u>; **Ward *et al*. (2006)**
Air sampling	Bacteria	Dependent on the sampling device and method [3] - Objective	Dependent on the sampling device and method [3] - No standardised protocol - Results after incubation period and enumeration	**De Reu *et al*. (2005)**; <u>Hao *et al*. (2013)</u>; **Kim and Kim (2010); O'Mahony *et al*. (2011);** <u>Oliveira *et al*. (2006)</u>
ATP [4] swab	Eukaryotic and prokaryotic cells	- Results within 1 minute - Able to sample irregular surfaces - No need for further processing after sampling	- Limited sampling surface (100 cm²), depended on manufacturer - Interpretation of results	<u>Corrégé *et al*. (2003); Roelofs and Plagge (1998)</u>

Visual inspection	Dirt	- Immediate results	- Subjective - No standardised protocol	**Huneau-Salaün *et al*. (2010)**

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[1] ACP, agar contact plates; [2] Sponge swabs and environmental swabs may be used for surfaces that are at least 100 cm², however in case of sponge swabs it is recommended to sample larger areas (Lahou and Uyttendaele, 2014); [3] Sampling can be carried out by air plating (*i.e.* sedimentation) or with a mechanical air sampling device (Banhazi *et al*., 2009). Each method has its advantages and disadvantages; [4] ATP, adenosine triphosphate.

3.3   Experimental studies on the efficacy of C&D on bacteria

Several studies have been carried out to test and compare the efficacy of disinfectants and C&D protocols. Generally, two approaches are followed: laboratory studies and/or field studies.

3.3.1   <u>Laboratory studies</u>

- <u>Suspension tests</u>

In suspension tests, a volume of suspension with bacteria is added to the disinfectant, with or without OM. After a predetermined contact time, the aliquot is tested for survival. In order for a farm disinfection product to gain approval for the European market, it must pass a quantitative suspension test with simulation soiling conditions according to the European Standard EN1656. Tested reference bacteria must show a minimum 5 log reduction after exposure to the disinfectant (European Commitee for Standardization, 2000). Various studies conducted suspension test (*i.e.* in vitro tests) to determine the difference in efficacy of disinfectants by standardised laboratory methods. An example of a suspension test with simulating organic soiling is the study of Thomson *et al*. (2007), whereby 7 disinfectant compounds were tested against 10 species of porcine bacterial pathogens (including *Salmonella* Typhimurium, *E. coli*, *Streptococcus suis*, *Brachyspira hyodysenteriae*). They showed that under high OM concentrations (simulating poorly cleaned conditions), the efficacy of the disinfectant compounds was markedly reduced. Also a low temperature and short contact time affected the efficacy of the compounds.

The main disadvantage of suspension tests, is that they are often unrealistic and yield favourable results because bacteria in suspension are more susceptible than when they are attached to surfaces (Gradel, 2004). Therefore, it is not always correct to extrapolate results from suspension tests to field conditions, expecting the same efficacy of the disinfectant (Gradel, 2004).

- <u>Field-like tests</u>

Another approach is to mimic field conditions whereby artificially inoculated samples are used (*i.e.* field-like tests) (Gradel, 2004). The objective of field-like tests is to verify whether the proposed use-dilution of the disinfectant is still adequate in a real-life conditions (Reybrouck, 1998). These tests are performed in the laboratory, so they can be standardised (Reybrouck, 1999). Gradel *et al*. (2004b) tested the efficacy of (i) formaldehyde; (ii) glutaraldehyde/benzalkoniumchloride; (iii) peroxide compound; and (iv) water (*i.e.* control) on commonly found materials (concrete paving stones, steel feed chain links, wooden dowels and jute egg belts) and OM (feed, fats and egg yolk) from poultry houses, artificially inoculated

with two *Salmonella* serotypes or *Enterococcus faecalis*. McLaren *et al.* (2011) used 2 model systems to test the efficacy of 14 commonly-used farm disinfectants against *Salmonella*: a wet (representing boot dips) and dry (representing soiled surfaces and equipment) model. In both models, faecal slurry was used as OM. This study simulated field conditions relevant to poultry and pig units.

3.3.2 Field studies

Although the above mentioned studies mimic farm conditions, it is essential to also conduct experiments in field situations. However, it is difficult to include an identical control in field studies. Gradel *et al.* (2004a) tested the outcome of a field-like test (Gradel *et al.*, 2003), in which a temperature-humidity-time treatment for eliminating *E. coli* and *Salmonella* in OM (*i.e.* poultry faeces and feed) was determined, in a field study. These studies showed that the steam treatment at >60 °C and 100% RH with addition of 30 ppm formaldehyde at the beginning was most effective in eliminating *Salmonella* and the indicator bacteria in OM in layer houses. Similar results regarding the effect of formaldehyde were shown by the study of Carrique-Mas *et al.* (2009), in which the effectiveness of different disinfection protocols were compared in 60 *Salmonella* positive laying houses. They indicated that the use of 10% formalin led to a greater reduction of *Salmonella*. Also Mueller-Doblies *et al.* (2010), demonstrated the importance of disinfection with formaldehyde-based products in reducing *Salmonella* prevalence in 50 turkey houses. However, since 2004, formaldehyde has been proven to be carcinogenic by the World Health Organization (WHO) for the nasopharynx (the throat) and the nasal cavities. The use of formalin (*i.e.* 37% formaldehyde solution) as disinfectant in animal houses is banned in Europe since 2007 under the Biocidal Products Directive. Formaldehyde as substance of a disinfectant for veterinary hygiene, is now also under review by the European commission (European Chemicals Agency, 2016).

A more environmentally friendly disinfectant is slightly acidic electrolysed water (SAEW). SAEW contains primarily hypochlorous acid (HOCl), which is an effective form of chlorine and possesses antimicrobial activity (Hao *et al.*, 2013a; Len *et al.*, 2000). Hao *et al.* (2013) demonstrated that disinfection with SAEW (available chlorine concentration (ACC) of 250 mg/L) significantly reduced bacteria on the equipment and surfaces and decreased survival rates of *Salmonella* and *E. coli* in layer houses. The same researcher group also showed that SAEW disinfection (ACC of 300 mg/L) of pig barns could significantly reduce *Salmonella*, *Staphylococcus aureus* and coliforms on surfaces (Hao *et al.*, 2013b).

In addition to studies that mostly focus on the efficacy of DP, studies evaluating cleaning and disinfection programmes have also been performed. Huneau-Salaün *et al*. (2010) compared common C&D methods, routinely used by the farmers, in 30 layer houses. This study showed that programmes followed by two disinfection rounds (by spraying and/or fogging), were more efficient against streptococci. In addition, surface disinfection (*i.e.* spraying) seemed more efficient than fogging in cage houses. Davies and Breslin (2003) reported that fogging was more efficient on horizontal surfaces, rather than vertical and less accessible surfaces, whereas spraying allowed the direct treatment of all surfaces. Finally, a lower standard of cleaning was observed in cage houses than in on-floor houses. Mannion *et al*. (2007) studied the efficacy of C&D protocols in finisher units on 14 pig farms. They indicated that intensive cleaning and disinfection was effective for reducing the levels of *Enterobacteriaceae* on floors. The study of Merialdi *et al*. (2013) showed that C&D practices, carried out in different units (*i.e.* gestation, farrowing, nursery and fattening units) on 6 pig herds, reduced the MRSA environmental contamination, but were inadequate to eliminate MRSA.

Hancox *et al*. (2013) tested 2 cleaning protocols in pig pens: dry cleaning followed by one hour soaking with cold water or dry cleaning followed by one hour soaking with detergent. Both protocols were followed by a high pressure cleaning with cold water and a disinfection step. This study showed that detergent and disinfectant had varying bactericidal effects depending on different materials (*i.e.* concrete, stock board, metal) and bacterial parameters (total aerobic bacteria and *Enterobacteriaceae*). They could not show a synergetic or additive effect between detergent and disinfectant, but recommended the use of a suitable detergent during cleaning. However, also other parameters such as a soaking step, the applied pressure, water hardness, temperature of the water, etc. during cleaning might affect C&D of animal houses. Therefore, it would be interesting to carry out field studies that look at the effect of these parameters on the bacterial load in order to subsequently optimise the C&D process.

4. **Bacterial resistance to disinfectants**

Disinfectants are one of the several detrimental conditions that micro-organisms encounter in the environment (Gradel, 2007). Table I.8 shows the relative susceptibility of bacteria to disinfectants compared to other groups of micro-organisms.

Table I.8: Relative susceptibility of groups of micro-organisms to disinfectants (Fraise *et al*., 2012).

Range	Group of micro-organisms
Resistant	Prions
	Bacterial endospores
	Protozoal oocysts
	Mycobacteria
	Small non-enveloped viruses
	Protozoal cysts
	Fungal spores
	Gram negative bacteria
	Moulds
	Yeasts
	Protozoa
	Large non-enveloped viruses
	Gram positive bacteria
Susceptible	Enveloped viruses

Antibiotics are mainly selectively toxic, while disinfectants have several bacterial cell targets. Resistance against these compounds can be either a natural property (*i.e.* intrinsic resistance); acquired by one or more target gene mutations or acquisition of genetic elements (*e.g.* plasmids and transposons) (*i.e.* acquired resistance) (McDonnell and Russell, 1999; Russell, 1999). However, a third mechanism of resistance has been described, *i.e.* adaptive resistance, that it is relatively poorly understood. Adaptive resistance is a phenomenon that can be described as an induction of resistance to one or more antibacterial agents in response to a specific signal such as subinhibitory concentrations of an antibacterial agent, an environmental cue (*e.g.* pH) and/or social activities (biofilm formation) (Fernández *et al*., 2011). This resistance involves a temporary increase in the ability of a bacterium to survive an antibacterial agent, mainly as the result of alterations in gene and/or protein expression (Fernandez and Hancock, 2012).

The development of reduced susceptibility increases the probability of further disinfection failure (Chapman, 2003).

4.1 Intrinsic resistance

Intrinsic resistance is due to inherent characteristics of the bacteria. An important intrinsic resistance mechanism is the cell permeability, also referred to as "permeability barrier"

(SCENIHR, 2009). In addition, the phenotypic adaptation in biofilms is also classified as intrinsic (Russell, 1999).

4.1.1 Cell permeability

The outer membrane of bacteria acts as the main permeability barrier, which may reduce uptake of disinfectants. Therefore, spores and mycobacteria are intrinsically more resistant than vegetative non-mycobacterial bacteria (McDonnell and Russell, 1999; Russell, 1999). Spores are formed by spore forming bacteria under stressful conditions. Bacteria in spore form can survive in this state for many years. Because of the presence of a spore-coat, composed of highly cross-linked proteins, spores are intrinsic resistant to antimicrobials (Cole and Robison, 1996; Knapp, 2014).

Mycobacteria possess a complex cell wall structure, *i.e.* a lipid-rich cell envelope composed of mycolic acids, that forms an effective barrier (Knapp, 2014; McDonnell and Russell, 1999; Portevin *et al*., 2004).

Gram negative bacteria are generally more resistant to disinfectants than non-sporulating, non-mycobacterial Gram positive bacteria (McDonnell and Russell, 1999). This is due to the outer membrane, that consists of strong linked lipopolysaccharides, fatty acids and phospholipids and repulses hydrophobic disinfectants away from the cell (Gradel, 2004; Knapp, 2014; Nikaido and Vaaro, 1987). In this outer membrane, also porins that form hydrophilic channels and efflux pump components are embedded. Changes to these components (*e.g.* alteration of porin size, loss of porin proteins, induction of efflux systems) can have an effect on the permeability and consequently on the susceptibility to biocides (Denyer and Maillard, 2002).

4.1.2 Biofilm formation

Biofilms are defined as exopolysaccharide matrix-enclosed bacterial populations that are tightly attached to each other and to surfaces. Bacteria in biofilms are generally more resistant to disinfectants than their planktonic (non-biofilm) counterparts (Allison and Gilbert, 1995; Costerton *et al*., 1987; Gradel, 2004; Stewart and Costerton, 2001). Bacteria in biofilms can be less susceptible to disinfectants as a consequence of multiple reasons such as (i) reduced access of a disinfectant to cells within biofilms; (ii) chemical interaction between the disinfectant and the biofilm; (iii) modulation of the micro-environment; (iv) production of degradative enzymes; and (v) acquired bacterial resistance due to genetic exchange between cells within the biofilm (Gradel, 2007, 2004; McDonnell and Russell, 1999; Rutala and Weber, 2008).

4.2   Acquired resistance

Acquired resistance to disinfectants can occur by either mutation or acquisition of genetic material in the form of plasmids or transposons (Gradel, 2007; McDonnell and Russell, 1999; Russell, 1999). Plasmids and transposons are transferable between bacteria of the same species or bacteria of different species. A gap in scientific knowledge is the effect of biocide exposure on the maintenance and transfer of these extra-chromosomal elements (Knapp, 2014).

When several genes specifying a resistant phenotype are located together on a mobile genetic element such as a plasmid or transposon, co-resistance can occur. Subsequently, the development of resistance to one antibacterial agent can be accompanied by the appearance of resistance to another agent (Chapman, 2003; Condell *et al*., 2012). The same is seen with cross-resistance, when different antimicrobial agents act on the same target, initiate a common pathway to cell death or share a common route of access to their respective targets (Chapman, 2003; Condell *et al*., 2012). There is concern that in case of an impairment of the used disinfectant (due to presence of organic material or diluting water) resulting in exposure to lower active levels of these agents, selection for antibiotic resistant strains could occur. Slifierz *et al*. (2015) showed that the use of quaternary ammonium compound-based disinfectants is a risk for selecting antibiotic resistant MRSA in commercial swine herds. Randall *et al*. (2004) suggested that the use of biocides alone or combined with antibiotic treatment may also increase selective pressure towards antibiotic resistance of *Salmonella enterica*. Furthermore, the induced stress in bacteria by biocides may favour the expression of resistance mechanisms, and their dissemination by horizontal gene transfer (SCENIHR, 2009).

4.3   Resistance against commonly used farm disinfectants

- Resistance against alcohols

Several studies indicated intrinsic resistance of bacteria to alcohol. In a study of Kubota *et al*., (2008), the resistance to ethanol of planktonic and biofilm cells of *Lactobacillus plantarum* was tested. Results showed that biofilm cells were resistant to 30% and 40% ethanol, while no surviving planktonic cells were detected. Woo *et al*. (2002) showed a prolonged survival of four mycobacterial strains in 75% alcohol compared to other skin flora, using a quantitative suspension test. In addition, ineffectiveness against bacterial endospores of genera *Bacillus* and *Clostridium* spp. has been described (Marquis, 2002; Thomas, 2012). Thomas (2012) indicated that alcohol tolerance of spore-forming bacteria is dependent on the stage of spore development

and spore hardiness, which varies between species, strains, age of cultures, growing conditions and other factors.

- <u>Resistance against aldehydes</u>

Intrinsic resistance to aldehydes is developed during spore formation, whereby resistance to formaldehyde is developed in the early stage and glutaraldehyde in the latest stage. Therefore, resistance to formaldehyde may be linked to cortex formation, while resistance to glutaraldehyde may be linked to coat formation (Knott *et al*., 1995; McDonnell and Russell, 1999). Also some mycobacterial strains of different species (*e.g. M. chelonae*, *smegmatis* and *abscessus*), showed resistance to aldehyde-based disinfectants (Carson *et al*., 1978; De Groote *et al*., 2014; Griffiths *et al*., 1997; Nomura *et al*., 2004; Svetlíková *et al*., 2009). Vikram *et al*. (2015) demonstrated that efflux pumps contributed to glutaraldehyde resistance in *Pseudomonas fluorescens* and *P. aeruginosa* biofilms. In addition, known modulators (*e.g.* lipid and polyamine biosynthesis) of biofilms may contribute to this resistance. In addition, several studies showed the presence of aldehyde dehydrogenase, plasmid mediated resistance (Kato et al., 1983; Kümmerle et al., 1996; Zhang et al., 2013) and cell surface alterations (Azachi *et al*., 1996; Kaulfers *et al*., 1987) in several bacterial species.

- <u>Resistance against QAC</u>

QAC are regarded as sporistatic and mycobacteriostatic, however Cortesia *et al*. (2010) showed that QAC can select for non-genetically determined reversible resistant phenotypes of *Mycobacterium abscessus*. In a *Pseudomonas aeruginosa* biofilm, the level of bacterial resistance to benzalkonium chloride increased with the C-chain length of this QAC (C12 to C18). Increase of the chain length is combined with an increase in hydrophobicity of QAC, which could limit the penetration through the hydrophilic matrix (Campanac *et al*., 2002). *Staphylococcus aureus* in biofilm, shows a significant reduction of cell surface hydrophobicity, which makes them highly resistant to QAC (Campanac *et al*., 2002).

In addition, breakdown and inactivation of QAC has been reported. Nishihara *et al*. (2000) isolated a *Pseudomonas fluorescens* from sludge, that was able to degrade QAC via an N-dealkylation process (Knapp, 2014). Moreover, plasmid borne efflux pump genes that confer resistance against QAC has been described among clinical and environmental bacteria, such as *S. aureus*, including MRSA, *Pseudomonas, Enterococcus* spp., *Salmonella* and other *Enterobacteriaceae* (Jaglic and Cervinkova, 2012; Knapp, 2014; Kücken, 2000; Smith *et al*., 2008; White and McDermott, 2001). These genes code for an energy dependent efflux pump,

which confers also resistance to other compounds such as chlorhexidine, intercalating dyes and triclosan (Jaglic and Cervinkova, 2012; Knapp, 2014; Smith *et al.*, 2008).

- Resistance against peroxygens

Some studies isolated *Bacillus* spores that survive treatment with oxidising agents (Casillas-Martinez and Setlow, 1997; Kempf *et al.*, 2005), however a number of oxidising agents have been used to kill spores including chlorine dioxide, hydrogen peroxide and organic hydroperoxides (Cortezzo *et al.*, 2004).

Many bacteria have developed resistance that confer tolerance to peroxide stress (in particular hydrogen peroxide), which includes production of neutralising enzymes (*e.g.* catalases, peroxidases and glutathione reductases) (Baureder *et al.*, 2012; Harris *et al.*, 2002; McDonnell and Russell, 1999; Uhlich, 2009). This was also seen for bacteria in biofilm form (Elkins *et al.*, 1999; Stewart *et al.*, 2000). In addition, Dubois-Brissonnet *et al.* (2011) demonstrated increased tolerance to peracetic acid by a membrane modification of *Salmonella enterica*.

- Resistance against chlorine based compounds

A major factor in spore resistance to hypochlorite and chlorine dioxide appears to be the spore coat (Young and Setlow, 2003). However, resistance against sodium dichloroisocyanurate by *Bacillus subtilus* spores during sporulation happens when the spore coat is not yet fully produced (McDonnell and Russell, 1999). This means that not only the spore coat but also the cortex confers resistance to chlorine releasing agents (Lambert, 2004). Opportunistic environmental mycobacteria, including *M. kansasii*, *M. marinum*, *M. fortuitum*, *M. phlei*, and *M. chelonae*, have been shown to be relatively resistant to chlorine (Carson *et al.*, 1988; Falkinham, 2003; Pelletier *et al.*, 1988; Taylor *et al.*, 2000). In addition, it has been shown that strains of *M. avium* were more than 500 times more resistant to chlorine than *E. coli* (Falkinham, 2003; Taylor *et al.*, 2000).

Also, a 600× higher concentration of hypochlorite was needed to achieve a 4 log killing of *Staphylococcus aureus* in biofilms, than the concentrations needed to achieve this level of killing with the European phase 1 suspension test cells (Luppens *et al.*, 2002). Reactions between strongly oxidizing biocides, such as hypochlorous acid, and biofilm constituents, and the resulting neutralization, have been shown to provide some protection against killing (Chen and Stewart, 1996).

An increased resistance to chlorine has been described for *Vibrio chloreae* by forming cell aggregates (Morris *et al.*, 1996).

CHAPTER II
General aims

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Cleaning and disinfection (C&D) of animal houses is an essential part of a good hygiene management on a farm, which is of great importance to prevent the spread of animal and zoonotic diseases. An on-farm evaluation of different C&D protocols could help farmers in reducing the infection pressure in the animal houses. Therefore, the aim of this thesis was to evaluate commonly used and alternative C&D protocols carried out in broiler houses and pig nursery units.

More specifically, the first aim was to study different sampling methods and microbiological and non-microbiological parameters to evaluate the efficacy of C&D protocols (**chapter III**).

Cleaning of animal houses not only removes organic material and bacteria, but also ensures that the disinfection step has a great impact on the remaining bacteria. Many studies have evaluated the efficacy of disinfectants in practice, however little scientific work has been carried out on cleaning of animal houses. In **chapter IV**, the objective was to determine the effect of a preceding overnight soaking step before high pressure cleaning and to compare the influence of warm or cold water during cleaning on the bacterial load in broiler houses. A second aim was to identify critical locations during C&D.

A prolonged vacancy of animal houses after C&D has been described as a measure to decrease the survival rate of bacteria. The aim in **chapter VI** was to test this theory on several bacteriological parameters in pig nursery units.

Because of the ongoing concern about excessive use of disinfectants and potential resistance development and cross-resistance to clinically important antibiotics, the use of competitive exclusion (CE) agents has often been suggested as an alternative method to antagonise the growth of these pathogens. The purpose in **chapter V**, was to compare the effect of a CE protocol on the bacterial infection pressure in pig nursery units against a classical C&D protocol.

The aim in chapter **VII** was to gain a better understanding of the general and specific dominant bacteria present after cleaning as well as after disinfection. Furthermore the susceptibility against disinfectants of bacteria present after cleaning and surviving disinfection in broilers houses was investigated.

CHAPTER III
Comparison of sampling procedures and microbiological and non-microbiological parameters to evaluate cleaning and disinfection in broiler houses

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CHAPTER III
Comparison of sampling procedures and microbiological and non-microbiological parameters to evaluate cleaning and disinfection in broiler houses

1. Abstract

Cleaning and disinfection (C&D) of the broiler stable environment is an essential part of farm hygiene management. Adequate C&D is essential for prevention and control of animal diseases and zoonoses. The goal of this study was to shed light on the dynamics of microbiological and non-microbiological parameters during the successive steps of C&D and to select the most suitable sampling methods and parameters to evaluate C&D in broiler houses. The effectiveness of C&D protocols was measured in six broiler houses on two farms through visual inspection, adenosine triphosphate (ATP) monitoring and microbiological analyses. Samples were taken at three time points: before cleaning, after cleaning, and after disinfection. Before cleaning and after disinfection, air samples were taken in addition to agar contact plates (ACP) and swab samples taken from various sampling points for enumeration of total aerobic bacteria, *Enterococcus* spp. and *Escherichia coli* and the detection of *E. coli* and *Salmonella*. After cleaning, air samples, swab samples and ATP swabs were taken and a visual score was also assigned for each sampling point. The mean total aerobic bacteria determined by swab samples decreased from 7.7 ± 1.4 to 5.7 ± 1.2 log colony forming units (CFU)/625 cm² after cleaning and to 4.2 ± 1.6 log CFU/625 cm² after disinfection. ACP are used as the standard for evaluating C&D, but in this study they were found to be less suitable than swabs for enumeration. In addition to measuring total aerobic bacteria, *Enterococcus* spp. seemed to be a better hygiene indicator to evaluate C&D protocols than *E. coli*. All stables were *Salmonella* negative, but the detection of its index organism *E. coli* provided additional information for evaluating C&D protocols. ATP analyses gave additional information about the hygiene level of the different sampling points.

2. Introduction

Hygiene in animal production is key for both farm management (*e.g.* disease prevention) and meeting consumer demands concerning food safety. Cleaning and disinfection (C&D) of farm stables form the basis of hygiene management. Good hygiene practice on farms can reduce the risk of introduction and persistence of animal diseases and infectious diseases that are transmittable from animals to humans (zoonoses). A good C&D protocol is based on a thorough cleaning of the stable environment followed by a disinfection step. Cleaning is as crucial as disinfection, because any residual organic material (dirt) can reduce or nullify the efficiency of the disinfectant. In recent years, many C&D guidelines have become available to help farmers to reduce the infection pressure on the farm. Several countries even require official periodic control of the general hygiene status of broiler houses after C&D. In Belgium, this is controlled by determining the total aerobic bacteria with agar contact plates (ACP) taken at different places in the broiler house. However many practical questions regarding optimal temperature of cleaning water, method of cleaning and disinfecting, etc. are not yet thoroughly studied which often results in guidelines that are based on opinions rather than sound scientific data. A prerequisite for the evaluation of the effectiveness of C&D protocols can only be properly evaluated via systems that effectively measure the effectiveness of the different C&D steps.

Many different methods to perform hygiene controls have been used and described. Evaluating C&D of stables can be done as a routine control or after an outbreak of infection. Agar contact plates can be used routinely to assess the efficiency of C&D. Huneau-Salaün *et al*., 2010 used ACP based on enumeration of streptococci to assess the effectiveness of C&D in battery cage and on-floor layer houses. In addition, a visual control inspection was carried out. De Reu *et al*., 2006 used ACP for the enumeration of total aerobic bacteria and *Enterobacteriaceae* to compare the C&D in different housing systems for laying hens. Adenosine triphosphate (ATP) monitoring is capable of providing information about the level of biological residues (eukaryotic cells as part of soil and prokaryotic cells) in less than one minute, to evaluate the quality of C&D. Previous research has shown that ATP analyses can be used to monitor hygiene in pig stables after cleaning (Corrégé *et al*., 2003; Roelofs and Plagge, 1998). With microbiological swabs, larger areas can be sampled to detect for example *Salmonella* persistence after disinfection in contaminated broiler houses. Swab methods have also been used in several studies to evaluate C&D in layer and broiler houses (Carrique-Mas *et al*., 2009; Davies and Breslin, 2003a; Rose *et al*., 2003; Ward *et al*., 2006).

Besides total aerobic bacteria also various specific microbiological indicator-index organisms such as *Enterococcus* spp., *Escherichia coli* and *Salmonella* have been used to evaluate the hygiene of stables. *Salmonella* is an important food-borne pathogen and its presence in the poultry sector plays an important role in the spreading of this pathogen in the food production chain. *Escherichia coli* has been shown to be a suitable index organism for monitoring the possible presence of *Salmonella* (Dewaele *et al.*, 2011; Gradel *et al.*, 2004a; M.D. Winfield and Groisman, 2003). Finally, *Enterococcus* spp. is suggested to be an adequate hygiene-indicator organism for faecal contamination of surfaces (Gradel *et al.*, 2004b).

Although many different methods have been proposed to perform hygienic controls, few studies have compared different C&D methods. To evaluate the effectiveness of different C&D protocols, better understanding of the advantages and limitations of the available methods for evaluating C&D is required, together with observation of the evolution of indicators of bacterial load and cleanliness after performing the different steps of a C&D procedure.

This study was designed to meet the above mentioned requirements for evaluating different C&D procedures. The objectives are thus 1) to compare different sampling methods and parameters best suited to evaluate the effectiveness of C&D in broiler houses and 2) to get more insight into the dynamics of microbiological parameters in the successive steps of C&D in broiler houses. In this study, both easy and exhaustive methods are used and compared.

3. Materials and methods

3.1 Farms, broiler houses and C&D protocols

This study was carried out in six broiler houses on two farms in Belgium. Three C&D rounds, carried out between flocks, were evaluated in four broiler houses on farm A and in two broiler houses on farm B. The various C&D protocols used on both farms consisted of three steps: dry cleaning, wet cleaning and disinfection. During dry cleaning, manure and feed are removed. The wet cleaning protocols were different on the two farms (Table III.1). The cleaning products used were Keno™san (CID LINES, Ieper, Belgium) on farm A and Intra Power Foam (IntraCare, Veghel, the Netherlands) on farm B. Disinfection on farm A and B during the three C&D rounds was carried out by fogging and using an orchard sprinkler, respectively. The disinfection product used on farm A was Cid 20 (CID LINES, Ieper, Belgium); on farm B, Desbest 700 (Frans Veugen, Bedrijfshygiëne, Nederweert, the Netherlands). Both cleaning products consisted of commercial solutions containing sodium hydroxide and both disinfection

products consisted of combination of quaternary ammonium compounds (QAC), aldehydes and alcohol (Table III.1).

Table III.1: Different cleaning protocols carried out repeatedly in six broiler houses on two farms (A and B). The overnight (8 hours) soaking step was carried out with cold water without cleaning product. *Spraying was done using an orchard sprinkler. Warm: 60 °C.

Farm	Stable	Number of C&D rounds	Overnight soaking step?	High pressure cleaning	Cleaning compounds	Disinfection compounds	Disinfection method
A	1	3	Yes	Warm	Sodium hydroxide	QAC [1] + aldehydes + alcohols	Fogging
	2	3	Yes	Cold	Sodium hydroxide	QAC + aldehydes + alcohols	Fogging
	3	3	No	Cold	Sodium hydroxide	QAC + aldehydes + alcohols	Fogging
	4	3	No	Warm	Sodium hydroxide	QAC + aldehydes + alcohols	Fogging
B	1	1	No	Cold	None	QAC + aldehydes + alcohols	Spraying*
	1	2	No	Warm	Sodium hydroxide	QAC + aldehydes + alcohols	Spraying*
	2	1	No	Warm	Sodium hydroxide	QAC + aldehydes + alcohols	Spraying*
	2	2	No	Cold	Sodium hydroxide	QAC + aldehydes + alcohols	Spraying*

[1] QAC, quaternary ammonium compounds
Disinfection compounds on farm A: QAC: alkyldimethylbenzylammoniumchloride (61.5 g/L); Aldehydes: glutaraldehyde (58 g/L), formaldehyde (84 g/L) and glyoxal (19.8 g/L); Alcohols: isopropanol (40 g/L).
Disinfection compounds on farm B: QAC: didecyldimethylammoniumchloride (100 g/L); Aldehydes: formaldehyde (32 g/L) and glutaraldehyde (80 g/L); Alcohols: 2-propanol/methanol and ethanol (10-50 g/L).

3.2 Sampling plan and types of samples

Sampling was performed at the following moments before and during C&D:

- Immediately after depopulation of the broiler house (manure still present), but before the onset of cleaning (BC);

- 24 hours after cleaning but before disinfection (AC) (implemented from the 2nd C&D round);

- 24 hours after disinfection but before chick placement (AD).

Different types of samples (ACP, swab samples and ATP swabs) were taken at 10-12 different sampling points (upon availability) per quarter of a broiler house, resulting in 40-48 sampling points per broiler house (Table III.2).

3.2.1 <u>Before cleaning</u>

Per stable, 10 sampling points were each sampled four times for each type of agar used. This yielded a total of 120 ACP per stable: 40 samples for each type (n=3) of agar used (Table III.2). The agar media used in the ACP were Plate Count Agar (**PCA**, Oxoid, CM0325, Basingstroke, Hampshire, England) for total aerobic bacteria, Slanetz and Bartley (**S&B**, Oxoid, CM0377, Basingstroke, Hampshire, England) for *Enterococcus* spp. and Rapid *E. coli* (Biorad, 356-4024, Marnes-la-Coquettes, France) for *E. coli* counts, respectively. ACP had a surface of 25 cm². Additionally, 40 sponge swab samples (10 sampling points x four samples) premoistened with 10 mL Buffered Peptone Water (**BPW**) (3M, SSL10BPW, St-Paul, USA) were taken per stable. A surface of 625 cm² (*i.e.* A4 format) was swabbed whenever possible. Since the surface of the drinking cups was smaller than 625 cm², five drinking cups in each quarter of the stable were sampled. After dilution, enumeration of the swab samples was also carried out on PCA, S&B and Rapid *E. coli*. The lower limits for enumeration of the aforementioned types of swab samples were 4 log, 4 log and 2 log colony forming units (CFU)/625 cm², respectively. Seven air samples per broiler house were also taken, while walking through the stable, using the Reuter Centrifugal Air Sampler (Hycon® Biotest AG, Dreieich, Germany). This apparatus pulls air over agar filled airstrips containing PCA, S&B or Rapid *E. coli*. Air volumes were sampled in duplicate. The volumes sampled for each type of agar medium were 50 L, 50 L and 100 L, respectively. In addition, 800 L of air was sampled using air strips filled with PCA. These strips were further processed in the lab for the detection of *E. coli* and *Salmonella*.

Table III.2: Analyses performed during the successive C&D steps for each sampling location at each time point. (Number of samples taken before cleaning/number of samples taken after cleaning /number of samples taken after disinfection, -: no samples were taken).

Sampling points	Agar contact plates Enumeration			Swab samples Enumeration			Swab samples Detection		Swab samples ATP	Air samples Enumeration			Air samples Detection		Visual inspection
	Total aerobic bacteria	*Enterococcus* spp.	*E. coli*	Total aerobic bacteria	*Enterococcus* spp.	*E. coli*	*E. coli*	*Salmonella*	Hygiene	Total aerobic bacteria	*Enterococcus* spp.	*E. coli*	*E. coli*	*Salmonella*	Dirt
Floor	4/-/4	4/-/4	4/-/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	-/4/-						-/4/-
Air outlet	4/-/4	4/-/4	4/-/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	-/4/-						-/4/-
Wall	4/-/4	4/-/4	4/-/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	-/4/-						-/4/-
Air inlet	4/-/4	4/-/4	4/-/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	-/4/-						-/4/-
Drinking cup	4/-/4	4/-/4	4/-/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	-/4/-		6/6/6			1/1/1	-/4/-
Feed pan	4/-/4	4/-/4	4/-/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	-/4/-						-/4/-
Feed hopper	4/-/4	4/-/4	4/-/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	-/4/-						-/4/-
Pipes	4/-/4	4/-/4	4/-/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	-/4/-						-/4/-
Drain hole	-/-/4	-/-/4	-/-/4	-/4/4	-/4/4	-/4/4	-/4/4	-/4/4	-/4/-						-/4/-
Loose material	4/-/4	4/-/4	4/-/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	-/4/-						-/4/-
Roof	4/-/4	4/-/4	4/-/4	4/4/4	4/4/4	4/4/4	4/4/4	4/4/4	-/4/-						-/4/-
Floor crack	-/-/4	-/-/4	-/-/4	-/4/4	-/4/4	-/4/4	-/4/4	-/4/4	-/4/-						-/4/-

3.2.2   <u>After cleaning</u>

Forty-eight premoistened swab samples for microbiological analyses were taken at 12 sampling points (Table III.2) in each quarter of the broiler house and enumerated on PCA, S&B and Rapid *E. coli*. The lower limit for enumeration was 1 log CFU/625 cm². The same sampling methods and agar media were used for air sampling. The air volumes were as follows: 100 L (enumeration of total aerobic bacteria) and 800 L (detection of *E. coli* and *Salmonella*), 100 L (enumeration of *Enterococcus* spp.) and 200 L (enumeration of *E. coli*). Moreover, 48 ATP swabs (Hygiena, US2020, Camarillo, CA, USA) were taken at the defined 12 sampling points x four samples. Sampling area of ATP swabs was 100 cm². Analyses were performed immediately after sampling according to the manufacturer's instructions. The principle of the analyses is based on the addition of a solution containing lysis reagent, the substrate luciferin and luciferase. The lysis reagent allows the release of ATP from prokaryotic and eukaryotic cells. Released ATP molecules are used by the enzyme luciferase to convert the substrate resulting in a bioluminescent reaction. Measurements of the produced light were immediately carried out with the Ensure ATP measurement apparatus (Hygiena, Camarillo, CA, USA). In addition, a visual cleaning inspection was performed four times at the 12 sampling points. A visual score to evaluate cleaning was assigned based on Huneau-Salaün *et al*. (2010). A minimum score of 0 was given to the sampling points that were still very dirty and a maximum score of 3 to the completely clean ones, resulting in a possible maximum of 12 (each type of sampling point was evaluated four times).

3.2.3   <u>After disinfection</u>

In total, 144 ACP and 48 swab samples were taken at the same sampling points as AC. The lower limit for enumeration on swab samples was 1 log CFU/625 cm². Air samples and volumes were taken as described at AC. To neutralise the residual action of the disinfectants on the microbiological growth, 10 mL Dey Engley neutralizing broth (Sigma Aldrich, Fluka, D3435, St-Louis, USA) was used to premoisten the sponge swab samples (3M, SSL100, St-Paul, USA). A disinfectant neutralizing solution was also added to the agar media for ACP and airstrips. Three percent (v/v) polysorbate (Merck-Schuchardt, 8.17072.100, Hohenbrunn, Germany) and 0.3% (w/v) L α-lecithin soy bean (Calbiochem, 429415, Darmstadt, Germany) was added to S&B medium (ACP and airstrips) and 3% polysorbate, 0.3% L α-lecithin soy bean, 0.2% (w/v) sodium bisulfite (UCB, Belgium) and 4.2% (w/v) sodium thiosulfate (VWR, 27910260, Leuven, Belgium) was added to PCA (airstrips) and Rapid *E. coli* (ACP and airstrips) media.

The ACP for total aerobic bacteria also contained a neutralizing solution (RODAC, PL-agar, P309.16.0017.025).

3.3 Sample processing

Samples were transported to the lab under refrigeration. Incubation of ACP and airstrips was started on the day of sampling and swab samples were stored at 3 ± 2 °C for 18 h before further processing. One hundred milliliters of BPW was added to the BC swab samples; 10 mL BPW was added to the AC swab samples and to the AD swabs. Prior to plating, swab samples were homogenised by placing them in a Masticator (IUL instruments, S.A., Barcelona, Spain) and diluted in peptone water (Oxoid, TV50I6D, Wesel, Germany) required to produce countable results on the selected agar media. Plating of dilutions was performed by pour plating and on agar plates using a spiral plater (Eddy Jet, IUL instruments, S.A., Barcelona, Spain). ACP, air strips and agar plates were incubated 72 h at 30 °C for PCA, 48 h at 37 °C for S&B and 24 h at 44 °C for Rapid *E. coli*, respectively. The remaining BPW fraction of the BC and the AD swab samples was incubated at 37 °C during 24 h for the detection of *E. coli* and *Salmonella*. One hundred milliliters of BPW was added to PCA air strips (800 L air) and also incubated overnight at 37 °C for the detection methods. Detection of *E. coli* was carried out by plating 10 µl of the enrichment broth on Rapid *E. coli* medium. *Salmonella* detection on the broth was carried out according to ISO 6579:2002 Annex D protocol (Anonymous, 2002). Positive *Salmonella* colonies on Xylose Lysine Deoxycholate agar medium (**XLD**, Oxoid, CM0469, Basingstroke, Hampshire, England) were subcultured on Nutrient Agar (**NA**, Oxoid, CM0003, Basingstroke, Hampshire, England). After incubation, polymerase chain reaction (PCR) confirmation on cel lysates as described by Aabo *et al*. (1993) was performed. Table III.2 provides an overview of the analyses performed at the different time points during C&D.

3.4 Statistical data analysis

Statistical analyses were carried out with Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc., Cary, NC, USA). A histogram and Q-Q plot was made of the obtained data to characterise the distribution of the variables. Mean with standard deviation are given for counts that were normally distributed and median with first and third quartile are given for counts that didn't follow this distribution. In order to detect significant differences in total aerobic bacteria counts (dependent variable) during C&D, a linear regression model was performed with sampling time as categorical independent variable. In case of significant influence of sampling time, a tukey post hoc test was carried out. A Spearman's rank correlation

test was done to evaluate the correlation between the visual scores and ATP values. *P*-values ≤ 0.05 were considered as significant.

4. Results

During three C&D rounds on two farms (six broiler houses), a total of 4508 ACP and 2047 swab samples were taken for microbiological analyses. In addition, 252 air samples and 810 ATP swabs were taken and analysed and 810 visual cleaning scores were assigned to different sampling points.

4.1 Before cleaning

Twenty-two percent of all ACP (n=2102) taken on the two farms were unreadable, mostly caused by trapped dirt particles. Of the ACP, 82%, 70% and 34% for total aerobic bacteria (n= 706), *Enterococcus* spp. (n=698) and *E. coli* (n=698), respectively, were positive for growth (Figure III.1), of which 13%, 36% and 31% were countable ([1-300] CFU/25 cm²) and the remainder were overgrown (> 300 CFU/25 cm²). Descriptive values for ACP of total aerobic bacteria, *Enterococcus* spp. and *E. coli* are given in table III.3. Much higher numbers, *i.e.* 98%, 95% and 82%, of the swab samples (n=705) for total aerobic bacteria, *Enterococcus* spp. and *E. coli* were countable (Figure III.1), respectively. The mean counts of total aerobic bacteria and *Enterococcus* spp. on the countable swab samples were 7.7 ± 1.4 log and 6.6 ± 1.0 log, respectively. The median count for *E. coli* on countable swab samples was 4 log CFU/625 cm² (Table III.3). The results of the air samples showed higher counts for total aerobic bacteria followed by *Enterococcus* spp. and no counts for *E. coli* (Figure III.2). After enrichment, *E. coli* was detected in 92% of the swab samples and 33% or 4 out of 12 air samples. No *Salmonella* was found in any of the samples.

4.2 After cleaning

After cleaning, 97%, 87% and 15% of the swab samples (n= 540) were countable for total aerobic bacteria, *Enterococcus* spp. and *E. coli*, respectively (Figure III.1). The results of the countable swab analyses showed that the mean contamination of the six stables after cleaning was 5.7 ± 1.2 log CFU total aerobic bacteria and 4.0 ± 1.2 log CFU *Enterococcus* spp. per 625 cm². The median count for *E. coli* was 2.7 log CFU *E. coli* per 625 cm² (Table III.3). The average visual score and median ATP values per type of sampling point is shown in Figure III.3. Median ATP values per sampling point ranged from 29 RLU (the roof) to 7671 RLU (drinking cups). Moreover, a large range of ATP values per type of sampling point was found.

No correlation was found between visual scores and ATP values ($\rho s=-0.24$, $P< 0.0001$, n=810). The median bacterial count in 1 m³ of air was 2.36 log CFU for total aerobic bacteria (Figure III.2). In the air samples (n=12), no *E. coli* was enumerated and detected and no *Salmonella* was found.
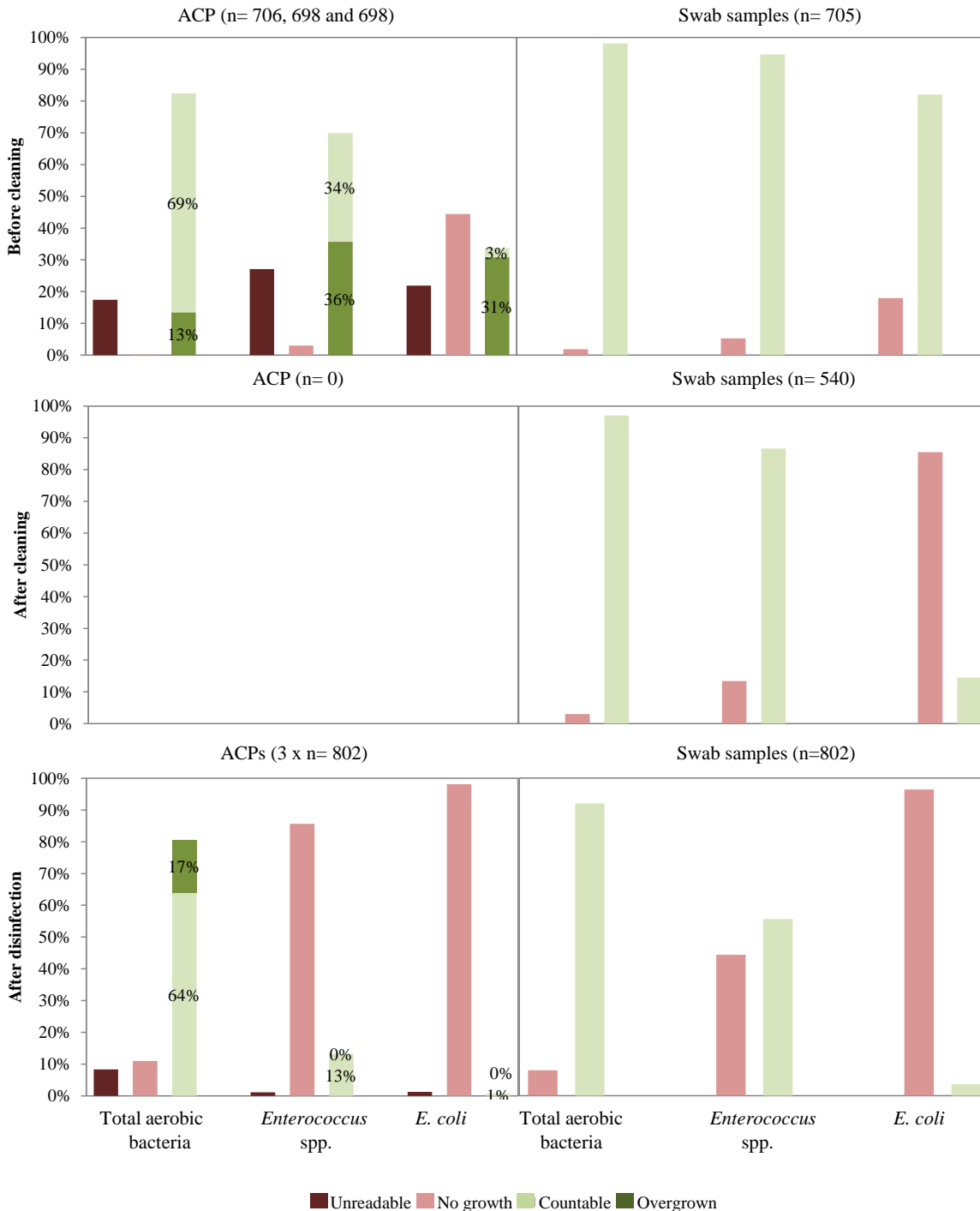
Figure III.1: Categories of microbiological results obtained on agar contact plates (ACP) and swabs for total aerobic bacteria, Enterococcus spp. and E. coli. Samples taken during 3 C&D rounds in six broiler houses. (Overgrown, > 300 colony forming units (CFU)/25 cm²; unreadable, dirt particles trapped in agar; n, number of samples taken).
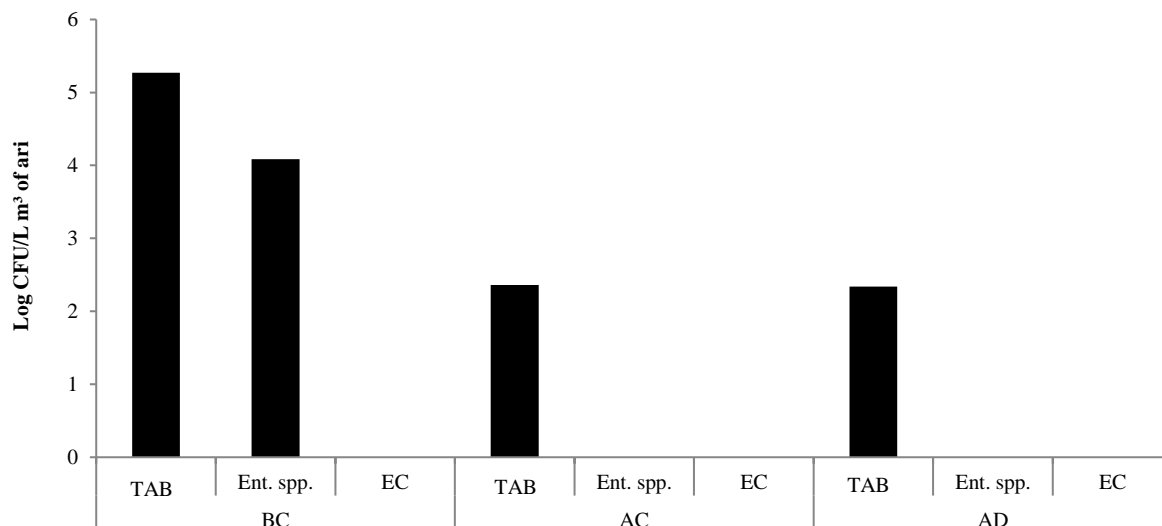
Figure III.2: Median log colony forming units (CFU) counts of total aerobic bacteria (TAB), Enterococcus spp. (Ent. spp.) and E. coli (EC) in CFU per m³ air in six broiler houses. A total of 72 samples of each medium for TAB, Ent. spp. and EC enumeration were taken during C&D, respectively. (BC, before cleaning; AC, after cleaning; AD, after disinfection). Vertical bars denote standard deviation

Table III.3: Contamination of total aerobic bacteria (TAB), Enterococcus spp. (Ent. spp.) and E. coli on countable agar contact plates (ACP) and swab samples. Samples taken during 3 C&D rounds in six broiler houses. Mean log colony forming units (CFU) and standard deviations are given for counts that are normally distributed. First quartile (Q1), median (Q2) and third quartile (Q3) are given for counts that didn't follow this distribution.

Parameter	Time point	ACP			Swab samples				
		Q1	Q2	Q3	Mean ± SD		Q1	Q2	Q3
TAB									
	BC	65	149	228	7.7	1.4			
	AC				5.7	1.2			
	AD	2	7	30	4.2	1.6			
Ent. spp.									
	BC	14	58	115	6.6	1.0			
	AC				4.0	1.2			
	AD	0	0	0			1.9	2.8	3.7
E. coli									
	BC	0	0	3			3.1	4	4.9
	AC						1.9	2.7	3.9
	AD	0	0	0			1.5	2.4	3.7
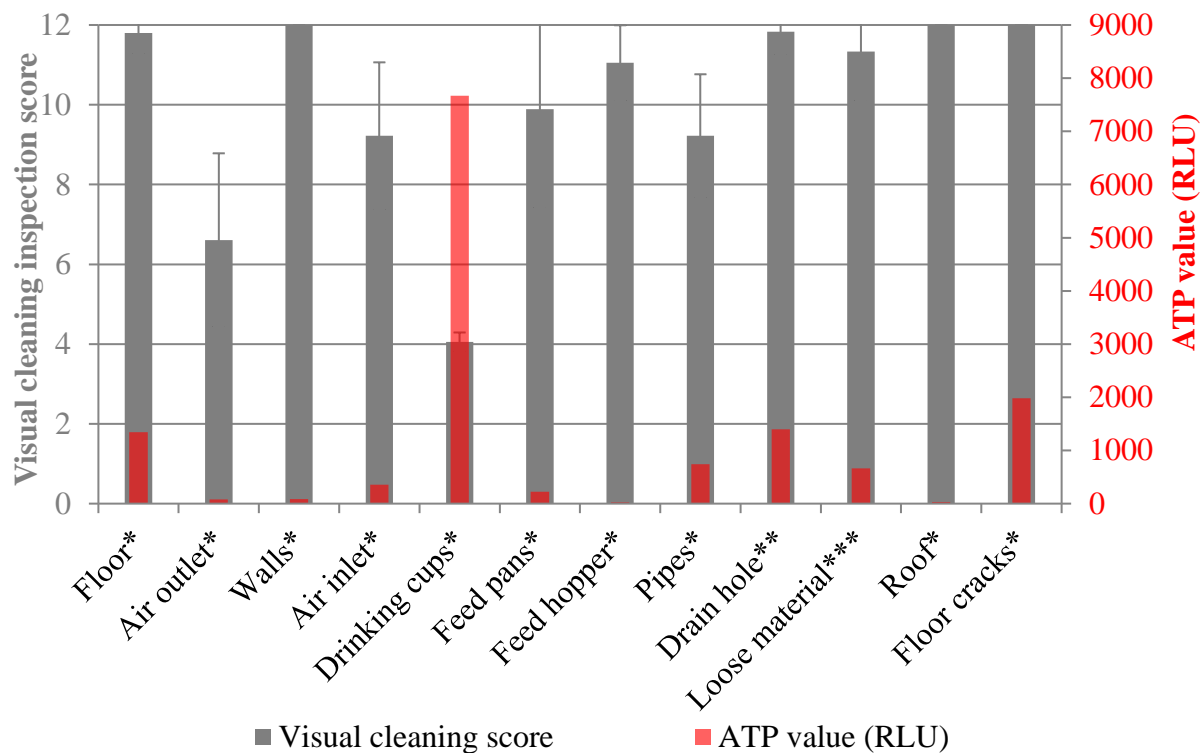
*Figure III.3: Mean visual cleaning inspection scores and median adenosine triphosphate (ATP) values given per sampling point after 3 cleaning rounds in six broiler houses. ATP values are expressed in relative light units (RLU). The higher the visual cleaning score, the cleaner the sampling point visually was and vice versa. *, ** and *** denotes that in total 72, 66 and 24 individual visual scores and 72, 66 and 24 ATP values were obtained, respectively. Vertical bars denote standard deviation.*

4.3   After disinfection

After disinfection, 13% and 1% of the ACP (n= 802) were positive for growth of *Enterococcus* spp. and *E. coli*, respectively (Figure III.1). In contrast, 81% of the total aerobic bacteria ACP (n= 802) were positive for growth, 17% of which were overgrown (> 300 CFU/25 cm²). Besides, 8% of the ACP were unreadable by growth of mold or trapped dirt particles. Forty-eight percent, 12% and 0.6% of the ACP for total aerobic bacteria, *Enterococcus* spp. and *E. coli* had counts between 1-40 CFU/25 cm², respectively (Figure III.4). Descriptive values are given in table III.3. Of the swab samples (n= 802), 92%, 56% and 4% gave countable results for total aerobic bacteria, *Enterococcus* spp. and *E. coli*, respectively (Figure III.1). The results of the countable swab samples showed that the mean total aerobic bacteria contamination of the six stables after disinfection was 4.2 ± 1.6 log CFU per 625 cm². Median counts for *Enterococcus* spp. and *E. coli* were 2.8 log CFU and 2.4 log CFU per 625 cm², respectively (Table III.3). After enrichment, *E. coli* was found in 7% of the swab samples, mostly from drain

holes (Figure III.5). No *Salmonella* was detected in the air or in the swab samples taken at the different sampling points of the stables. The median bacterial count for total aerobic bacteria in the air was 2.34 log CFU per m³ of air (Figure III.2). No *E. coli* was detected in the air samples.
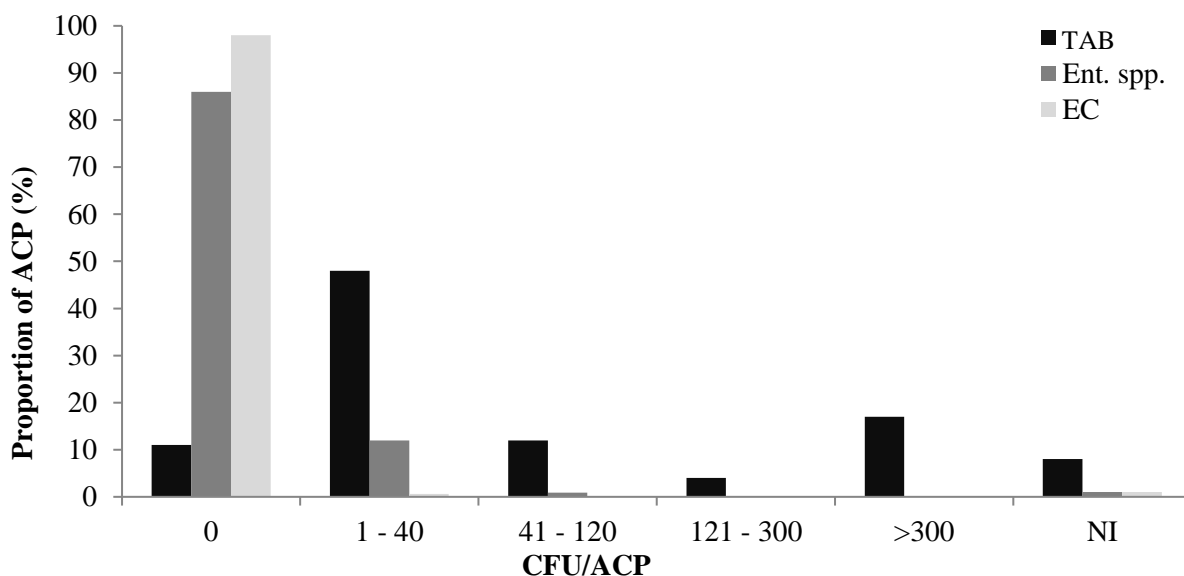
Figure III.4: Distribution of proportion of samples (n= 802) within different categories of enumeration on agar contact plates (ACP) for total aerobic bacteria (TAB), Enterococcus spp. (Ent. spp.) and E. coli (EC). ACP taken after 3 disinfection rounds in six broiler houses. (NI, not interpretable or unreadable).
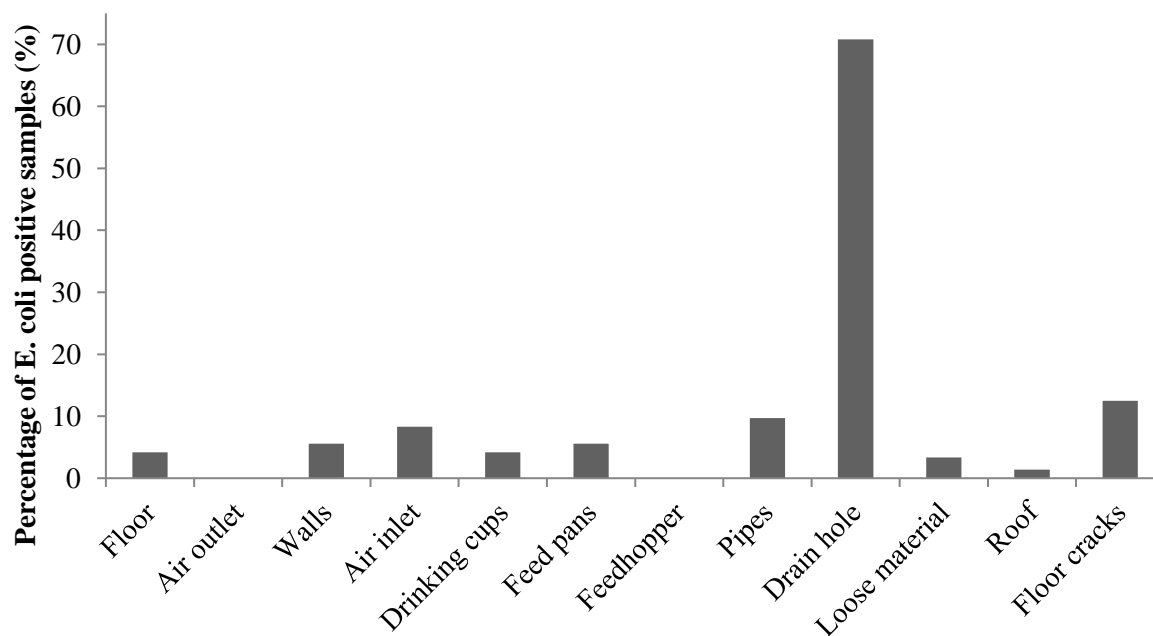
Figure III.5: Percentage of swab samples positive for E. coli within each category of sampling point after 3 disinfection rounds in six broiler houses. Twenty-four and sixty samples of the drain hole and loose material were taken, respectively. All other sampling points had 72 samples each.

5. Discussion

5.1 Sampling methods

ACP are often used to evaluate the hygiene of surfaces. They have the advantage of being fast to apply and easy to process, but can only sample 25 cm². In contrast, swab sampling is better suited for sampling irregular and larger surfaces but swabs needs more handling and laboratory manipulation.

Before cleaning, the ACP were mostly unreadable or overgrown, which gave us little information about the initial bacterial status of the broiler houses. Similar results were found by Huneau-Salaün *et al.*, 2010 at layer farms: 36% of the ACP for *Enterococcus* spp. taken before cleaning were overgrown (> 200 CFU/25 cm²). Moreover, enumeration on ACP selective for *E. coli* obtained fewer countable results compared to enumeration of swab samples. With the swabs, more than 82% were countable for total aerobic bacteria, *Enterococcus* spp. and *E. coli*, thus this type of sampling was more suitable for proper estimation of the initial bacterial status of the stables.

After disinfection, enumeration of *Enterococcus* spp. and *E. coli* on ACP gave few countable results. Huneau-Salaün *et al.*, 2010 showed that 62% of the ACP taken after disinfection in layer houses were negative for *Enterococcus* spp. growth compared to 86% in our study. On the other hand, in our study more than half of the swab samples gave countable results for *Enterococcus* spp.. For total aerobic bacteria only, a considerable number of ACP showed bacterial growth after disinfection. Enumeration of the swab samples revealed only a small fraction of countable sample for *E. coli*. In conclusion, ACP for total aerobic bacteria and enumeration of total aerobic bacteria and *Enterococcus* spp. on swab samples give the most information about the final bacterial status of the stables after disinfection.

Air samples gave us little valuable information about the effectiveness of C&D, as the supply of outside air in the stable differs between BC and AD. Therefore, this parameter not only reflected the influence of C&D on the contamination but also the bacterial load of outside air.

5.2 Hygiene monitoring by ATP analyses and visual cleaning inspection

After cleaning, high ATP values were still found for drinking cups, drain holes and floor cracks, despite the latter two having been visually evaluated as clean. The ATP values indicate that these sampling points still contain a high amount of biological residues (eukaryotic cells as part of soil and prokaryotic cells) after cleaning and that ATP measurements can identify critical

sampling points that are difficult to clean more thoroughly. Results of swab samples also showed that mostly drain holes and floor cracks were still contaminated with *E. coli* after disinfection. On the other hand, air outlets appeared visually to be one of the most soiled points after cleaning, but ATP measurements were low. This indicates that some sampling points look soiled, but that they have actually little biological matter. Tear of materials probably led to this negative visual assessment. After performing Spearman's rank correlation test, results showed that no correlation was found between ATP values and visual cleaning inspection scores. An explanation could be that the cleanliness of some sampling points, such as drain holes and floor cracks, are difficult to assess visually, leading to erroneous visual scoring. Huneau-Salaün *et al*., 2010 showed that a visual inspection can be an unreliable indicator of surface cleanliness. Our observations demonstrate that visual cleaning inspection alone is not reliable to assess the hygiene status of broiler houses.

5.3 Dynamics of microbial counts

The number of swab samples countable for total aerobic bacteria decreased from 98% to 97% AC and to 92% AD. The mean total aerobic bacteria count on these countable swab samples decreased from 7.7 ±1.4 to 5.7 ± 1.2 log CFU/625 cm² AC and to 4.2 ± 1.6 log CFU/625 cm² after disinfection. Surprisingly, total aerobic bacteria was significantly ($P< 0.0001$) reduced by an average of 1.5 log after the disinfection step, which was less than the 2 log reduction obtained by cleaning ($P< 0.0001$). Before a disinfection product gains approval for the European market, it must pass a quantitative suspension test according to the European Standard EN1656. That test simulates soiling conditions. The test results must show a minimum 5 log reduction of some reference bacteria (European Commitee for Standardization, 2000). Our study indicates that in the field, the 5 log reduction is far from achieved during disinfection for total aerobic bacteria. The average decrease of *Enterococcus* spp. after a cleaning step was 2.6 log CFU per 625 cm² (from 6.6 ±1.0 to 4.0 ± 1.2 log CFU). In 44% of the swabs after disinfection, numbers of *Enterococcus* spp. were lower than 1 log. Therefore, the median count after disinfection was even lower than 2.8 log CFU per 625 cm². ACP data yielded insufficient information about the dynamics of the bacterial contamination during C&D. The number of positive samples for *E. coli* detection was reduced from 92% BC to 7% AD. Drain holes (71%), floor cracks (13%) and pipes (10%) were still positive for *E. coli* after disinfection. Drain holes as well as floor cracks were previously identified as critical sampling points for C&D in stables and the most risky places for *Salmonella* contamination (Bolder, 2004; Dewaele *et al*., 2012b; Mueller-Doblies *et*

al., 2010; Rajic *et al*., 2005). Sampling drain holes and floor cracks is advised to evaluate C&D of these locations.

5.4 Suitable measurement system

Besides enumeration of bacteria, one important aim of our study was to generate sufficient information for selecting sampling methods and identifying analytical parameters for later study of differences between C&D protocols. After disinfection, ACP of *Enterococcus* spp. and *E. coli* are not suitable enough to make comparisons between C&D protocols compared to swab analysis of the same parameters. On the other hand, ACP of total aerobic bacteria after disinfection resulted in sufficient numbers (64%) of countable results and ACP are easy to use. Enumeration of *E. coli* after cleaning and after disinfection yielded very few countable results, allowing only to evaluate the presence or absence of *E. coli* in our evaluation system.

In conclusion, enumeration of swab samples showed that the mean total aerobic bacteria in the broiler houses decreased from 7.7 ± 1.4 to 4.2 ± 1.6 log CFU/625 cm² due to C&D. ACP, the standard used for evaluating the effectiveness of C&D, were shown to be less suitable compared to swab sampling. ATP analyses gave us more objective information about the level of hygiene compared to visual evaluations. The measurements system that provide valuable information for evaluating C&D protocols consists of: ACP for total aerobic bacteria counts AD; swab enumeration for total aerobic bacteria and *Enterococcus* spp. BC, AC and AD; and the detection of *E. coli* on those swab samples. After cleaning, ATP analyses could also be carried out.

6. Acknowledgements

CHAPTER IV
On-farm comparisons of different cleaning protocols in broiler houses

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CHAPTER IV
On-farm comparisons of different cleaning protocols in broiler houses

1. Abstract

The present study evaluated the effectiveness of four cleaning protocols in order to reduce the bacteriological infection pressure on broiler farms and prevent food-borne zoonoses. Additionally, locations that are difficult to clean and possible sources of infection were identified. Cleaning and disinfection rounds were evaluated in 12 broiler houses on five farms through microbiological analyses and adenosine triphosphate hygiene monitoring. Samples were taken at three time points: before cleaning, after cleaning, and after disinfection. At all time points, swab samples were taken from various sampling locations for enumeration of total aerobic bacteria and *Enterococcus* spp. In addition, before cleaning and after disinfection, also detection of *Escherichia coli* and *Salmonella* was carried out. Finally, adenosine triphosphate swabs and agar contact plates for total aerobic bacteria counts were taken after cleaning and after disinfection, respectively. Total aerobic bacteria and *Enterococcus* spp. counts on swab samples showed that cleaning protocols preceded by an overnight soaking step with water, caused a higher bacterial reduction compared to protocols without a preceding soaking step. Moreover, soaking of broiler houses leads to less water consumption and working time during high pressure cleaning. No differences were found between protocols using cold or warm water during cleaning. Drinking cups, drain holes and floor cracks were identified as critical locations for cleaning and disinfection in broiler houses.

2. Introduction

In 2011, most reported food-borne outbreaks (69553 human cases) in the European Union were associated with food originating from animals. *Salmonella* was the most frequently detected causative agent (26.6% of outbreaks) (European Food Safety Authority (EFSA), 2014). *Salmonella* is present in the intestinal tract of a wide range of animals such as birds, making commercial poultry flocks a potential reservoir for *Salmonella*. This pathogen can contaminate carcasses and equipment during processing of poultry meat (Tadesse and Cízek, 1994). To decrease the contamination level on poultry carcasses, it is important to control *Salmonella* infection at farm level (Rose *et al.*, 2000). Other organisms such as *Enterococcus cecorum* (Borst *et al.*, 2012; Chadfield *et al.*, 2004; Jung and Rautenschlein, 2014), *Enterococcus faecalis* (Tankson *et al.*, 2001) and *Escherichia coli* (Dho-Moulin and Fairbrother, 1999) have been associated with clinical diseases in broiler chickens. These infectious agents not only lead to disease outbreaks and flock mortality, but also to an increase of veterinary costs and condemnation rates at slaughterhouses. This all leads to high economic losses for the farmer (Jung and Rautenschlein, 2014).

An effective cleaning and disinfection (C&D) of broiler houses at the end of a production round is a crucial step in reducing the infection pressure on broiler farms and preventing both food-borne zoonoses (van de Giessen *et al.*, 1998) and endemic animal diseases. One of the important risk factors for contamination of flocks is the *Salmonella* status of the broiler house after C&D (Marin *et al.*, 2011). Also, residual organic debris (faeces, feathers, etc.) has to be removed properly before disinfection because it has an adverse effect on disinfectants (Hoff and Akin, 1986). Furthermore, organic material still present after cleaning can form a physical barrier that protects bacteria from disinfectants (Stringfellow *et al.*, 2009).

Little research has been published on the effectiveness of cleaning methods in animal houses. An on-farm evaluation and comparison of different cleaning protocols could help farmers in selecting the most appropriate cleaning method and in reducing or even eliminating zoonotic and pathogenic infectious organisms. Also, insight in working time, consumption of water, electricity and heating oil could have an impact on selecting a cleaning protocol.

The identification of locations that are difficult to clean could help in improving C&D protocols to better prevent infections through these residual sources of infectious material. Mueller-

Doblies *et al*. (2010) showed that areas that are difficult to clean, such as floor cracks, had a higher *Salmonella* prevalence than an intact floor, which is easier to clean.

Costs associated with the analyses needed for evaluating cleaning protocols should also be considered. A reduction of the number of samples to be analysed results in a lower work load and costs for the lab.

The first objective of this on-farm study was to compare the effectiveness of different cleaning protocols. The difference between whether or not applying an overnight soaking step after dry cleaning and/or the use of warm (60 °C) or cold water during cleaning was studied. Additionally, the number of samples needed for the evaluation of C&D was determined. Finally, critical locations that are difficult to clean in broiler houses were searched for.

3. Materials and methods

3.1 Cleaning and disinfection

Four different cleaning protocols were carried out and compared in 12 broiler houses on five farms, including one pilot farm, in Northern Belgium. Selection of farms was based on the willingness of the farmers to participate and the presence of at least two comparable (age, size, construction of the building, use of building materials, etc.) broiler houses on each farm. Three C&D rounds were evaluated in 4 broiler houses on the pilot farm (farm A) and two C&D rounds in 2 broiler houses on each of the four other farms (farm B-E). The C&D protocols consisted of different steps: dry cleaning (manure and feed removal), whether or not overnight soaking of the stable with water, wet cleaning (either with warm or cold water) and disinfection. Wet cleaning was further divided into three steps: 1) removal of organic material by high pressure cleaning with warm or cold water, 2) soaping and 3) removal of soap and any remaining dirt by high pressure cleaning with warm or cold water. On each farm, different cleaning protocols were carried out (Table IV.1): Protocol 1: overnight (8 hours) soaking with cold water followed by cleaning with warm water (60 °C) and cleaning product (CP), protocol 2: overnight (8 hours) soaking with cold water followed by cleaning with cold water and CP, protocol 3: no overnight soaking and cleaning with warm water (60 °C) using CP and protocol 4: no overnight soaking and cleaning with cold water using CP. All CP consisted of sodium hydroxide (and potassium hydroxide) and all disinfection products consisted of a combination of quaternary ammonium compounds (QAC), aldehydes and alcohols.

Table IV.1: Four cleaning protocols carried out repeatedly in 12 broiler houses on five different farms.

Farm	Cleaning protocols				CP [1]	Active components CP	Conc. [2] CP	D [3]	Disinfection method	Active components D: QAC [7] + aldehydes + alcohols	Conc. D
	1	2	3	4							
A	3x [4]	3x	3x	3x	Keno™San [8]	Sodium hydroxide	1.0%	Cid 20 [8]	Fogging	Alkyldimethylbenzylammoniumchloride (61.5 g/L) + glutaraldehyde (58 g/L); formaldehyde (84 g/L); glyoxal 19.8 g/L + isopropanol (40 g/L)	2.0%
B			2x [5]	2x	Intra Power Foam [9]	Sodium hydroxide	3.0%	Desbest 700 [12]	Spraying [6]	Didecyldimethylammoniumchloride (100 g/L) + glutaraldehyde (80 g/L); formaldehyde (32 g/L) + isopropanol; methanol; ethanol (conc.: 10-50 g/L)	1.0%
C		2x	2x		Keno™San [8]	Sodium hydroxide	1.0%	ViroCid [8]	Spraying	Alkyldimethylbenzylammoniumchloride (170.6 g/L); didecyldimethylammoniumchloride (78 g/L) + glutaraldehyde (107.25 g/L) + isopropanol (146.25 g/L)	1.8%
D			2x	2x	Sodium Hydroxide 50% [10]	Sodium hydroxide	1.0%	Cid 20 [8]	Spraying	See above	2.0%
E	2x			2x	Ino Net [11]	Sodium hydroxide + potassium hydroxide	3.0%	Hyprelva SL[11]	Fogging	Benzylalklyldimethylchloride (80 g/L); didecylmethylammoniumchloride (15 g/L) + glutaraldehyde (130 g/L) + isopropanol; methanol (conc. <10 g/L)	2.0%

[1] CP, cleaning product; [2] Conc., concentration; [3] D, disinfectant; [4] 3x, cleaning protocol conducted during three C&D rounds; [5] 2x, cleaning protocol conducted during two C&D rounds; [6] Spraying was done by using an orchard sprinkler; [7] QAC, quaternary ammonium compounds; [8], CID LINES, Ieper, Belgium; [9], IntraCare, Veghel, Netherlands; [10], Brenntag NV, Deerlijk, Belgium; [11], Distrifarm, Deerlijk, Belgium; [12], Frans Veugen, Bedrijfshygiene, Nederweert, Netherlands

3.2   Sampling and sampling processing

Sampling was performed on following moments during C&D:

- Immediately after depopulation of the broiler house (manure still present) (before cleaning, BC);
- 24 hours after cleaning but before disinfection (after cleaning, AC);
- 24 hours after disinfection but before chick loading (after disinfection, AD).

The method of sampling (number of samples per house, sampling points, surface…), sample processing and microbiological analyses was based on Luyckx *et al*. (2015) (*i.e.* chapter III). Briefly, at each time point 10-12 locations were sampled in quadruplicate (625 cm² area was sampled). Drain holes were usually present in smaller amounts than four per broiler house. Swab samples (3M, St-Paul, USA) were used at each sampling point (BC, AC and AD) and adenosine triphosphate (ATP) swabs (Hygiena, US2020, Camarillo, CA, USA) and agar contact plates (ACP) were taken AC and AD, respectively. On the swab samples, enumeration of total aerobic bacteria and *Enterococcus* spp. was carried out. In addition, the BC and AD swab samples were enriched in Buffered Peptone Water (BPW, Oxoid, CM0509, Basingstroke, Hampshire, England) during 24 h at 37 °C for the detection of *E. coli* and *Salmonella*. Detection of *E. coli* was followed by plating 10 µl of the enrichment broth on Rapid *E. coli* medium (Biorad, 356-4024, Marnes-la-Coquettes, France). *Salmonella* isolation was also attempted according to ISO 6579:2002 Annex D protocol (Anonymous, 2002). Agar contact plates were used for the enumeration of total aerobic bacteria (RODAC, PL-agar, P309.16.0017.025).

3.3   Monitoring power consumption and working time

Consumption of water, electricity, heating oil, cleaning product and working time was monitored for each protocol during four successive C&D rounds on farm A.

3.4   Statistical processing of the results

Statistical analyses were carried out with Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc., Cary, NC, USA). A histogram and Q-Q plot was made of the obtained data to characterise the distribution of the variables. The log transformed counts of total aerobic bacteria on swab samples and the log transformed ATP values followed a normal distribution. Log transformed counts of *Enterococcus* spp. on swab samples, detection results of *E. coli* on swab samples and counts of total aerobic bacteria on ACP did not follow this distribution.

For ACP with counts higher than 300 colony forming units (CFU) or with non-countable CFU counts (completely overgrown), counts were altered to 350 CFU and 450 CFU, respectively. For all swab samples, 1 CFU was added up to the absolute counts before a log transformation was performed, in that way counts of zero CFU were first transformed to the value one and then log transformed which turned them back to the value zero.

To assess the effect of each individual cleaning measure and their combinations (independent variables: soaking; temperature; time; interaction soaking*temperature and interaction time*soaking) on the total aerobic bacteria counts on swab samples (AC and AD) and ATP values (AC) (dependent variables), a linear mixed regression model was used. The not normally distributed data was transformed to a binomial dataset (dependent variables) with the group with zero-values containing counts that were lower than the detection limit and the group with one-values counts higher than the detection limit. On this dataset a logistic regression test was carried out. Counts on swab samples (BC) were added as continuous independent variables and the variable farm was included as a random effect in the model to correct for measurements within one farm. Post-hoc comparison was performed with a Bonferroni test. *P*-values≤ 0.05 were considered as significant.

In order to determine the number of samples needed to evaluate C&D protocols a two way-ANOVA was carried out on the normal distributed data and a Friedman's two way nonparametric ANOVA test was performed when data was not normally distributed (independent variables: sampling time and section, dependent variable: counts on swab samples). These tests were conducted on results obtained for total aerobic bacteria and *Enterococcus* spp. counts on swab samples, respectively. In both tests, a contrast statement was carried out, whereby samples taken in 1, 2 and/or 3 sections (*e.g.* 14 different combinations) were compared to samples taken in all 4 sections.

A linear discriminant analysis was conducted using location as grouping variable. The stepwise variable selection algorithm selected specific variables (ATP values from AC, counts of total aerobic bacteria and *Enterococcus* spp. AD on swab samples, *E. coli* detection AD on swab samples and the decrease of total aerobic bacteria counts on swab samples during C&D) that were capable of classifying a sample to a specific location (1 to 13).

4. Results

From October 2012 until February 2014, C&D rounds were studied in 12 broiler houses on five different farms. The four different cleaning protocols were compared. A total of 3473 swab samples for microbiological analyses were taken, whereof 1107 BC, 1091 AC and 1275 AD. In addition, 1274 ATP samples and 1275 ACP for total aerobic bacteria counts were taken AC and AD, respectively.

4.1 Comparison of Cleaning Protocols

4.1.1 <u>Comparisons between total aerobic bacteria counts</u>

Of all the swab samples, 98%, 98% and 95% were countable BC (lower limit: 4 log CFU/625 cm²), AC (lower limit: 1 log CFU/625 cm²) and AD (lower limit: 1 log CFU/625 cm²), respectively. After disinfection, 11% of ACP were negative for growth of total aerobic bacteria (< 1 CFU/ACP). In addition, 6% of ACP were unreadable, which was caused by trapped dirt particles, and 83% were positive for growth of which 15% were overgrown (> 300 CFU/25 cm²). Descriptive values for total aerobic bacteria counts on swab samples and ACP are given for each protocol in table IV.2. After cleaning, little differences in counts on swab samples were found for the four cleaning protocols (maximum difference of 0.2 log CFU/625 cm²). Swab samples showed that mean total aerobic bacteria contamination after disinfection was the lowest for protocols with soaking step (protocols 1 and 2 – variable 1 in table IV.2) and the highest for protocols without soaking step (protocols 3 and 4 – variable 2 in table IV.2). In addition, the number of countable swab samples was the lowest for protocols with soaking step (10% of the swab samples showed no growth after disinfection). Linear regression analysis on results of swab samples showed a significant lower amount of total aerobic bacteria ($P< 0.01$) after disinfection for protocols using a soaking step (0.5 log CFU/625 cm² difference in least square means). No significant differences were found when broiler houses were cleaned with warm or cold water. Also, no interaction was found between the variables soaking and temperature of water during cleaning (soaking*temperature). Median total aerobic bacteria counts on ACP after disinfection were the lowest for protocols with soaking step (protocols 1 and 2). Also, 14% of ACP showed no growth for protocols with soaking step compared to 10% for protocols without a soaking step (protocols 3 and 4). No differences in ACP counts between cleaning protocols were found after logistic regression analysis.

Table IV.2: Descriptive values for total aerobic bacteria (TAB) and Enterococus spp. (Ent spp) counts on swab samples and ACP during C&D. Samples taken during C&D in 12 broiler houses on 5 farms. Variable 1: soaking (protocol 1 and 2), 2: not soaking (protocol 3 and 4), 3: warm (protocol 1 and 4) and 4: cold (protocol 2 and 3). Mean and standard deviation are given for results that are normally distributed. First quartile (Q1), median (Q2) and third quartile (Q3) are given for results that didn't follow this distribution.

		Swab samples (log CFU/625 cm²)								
		BC [4]			AC [5]			AD [6]		
V [1]	PM [2]	N [3]	Countable [7] (%)	Count	N	Countable [8] (%)	Count	N	Countable** (%)	Count
1		393	391 (98)	**7.6** ± 1.7	371	363 (98)	**5.8** ± 1.5	463	417 (90)	**3.7** ± 1.8
2	TAB	709	621 (97)	**7.4** ± 1.8	720	711 (99)	**6.0** ± 1.5	812	790 (97)	**4.3** ± 1.7
3		552	542 (98)	**7.6** ± 1.7	540	531 (98)	**5.8** ± 1.5	634	607 (96)	**4.0** ± 1.7
4		555	540 (97)	**7.3** ± 2.0	551	543 (99)	**6.0** ± 1.5	641	600 (94)	**4.2** ± 1.8
1		393	363 (92)	**6.1** ± 2.0	369	310 (84)	**3.5** ± 1.8	432	171 (37)	0.0 – **0.0** – 2.3
2	Ent	705	659 (93)	**6.0** ± 1.9	720	668 (93)	**4.1** ± 1.7	811	442 (55)	0.0 – **1.3** – 3.2
3	spp	547	578 (95)	**6.2** ± 1.8	538	485 (90)	**3.9** ± 1.7	632	292 (46)	0.0 – **0.0** – 2.8
4		551	504 (91)	**5.8** ± 2.0	551	493 (89)	**3.9** ± 1.8	641	321 (50)	0.0 – **1.3** – 3.0

		ACP (CFU/25 cm²)		
		AD		
V	PM	N	Positive for growth (%)	Count
1		429	369 (86)	2 – **7** – 31
2	TAB	764	686 (90)	4 – **21** – 132
3		601	537 (89)	3 – **13** – 62
4		592	518 (88)	3 – **17** – 90

[1] V, variable; [2] PM, parameter; [3] N, number; [4] BC, before cleaning; [5] AC, after cleaning; [6] AD, after disinfection; [7] lower limit: 4 log CFU/625 cm²; [8] lower limit: 1 log CFU/625 cm².

4.1.2 Comparisons between *Enterococcus* spp. counts

More than half of the swab samples AD were negative (< 1 log CFU/625 cm²) for enumeration of *Enterococcus* spp.. When comparing the proportion of countable samples after C&D, protocols with soaking step showed the smallest proportion: 37% (Table IV. 2). Logistic regression analysis showed a stronger decrease of *Enterococcus* spp. after disinfection for C&D protocols with a soaking step ($P < 0.05$). These results confirmed the observations with total aerobic bacteria counts. No differences were found between protocols using warm and cold water.

4.1.3   Detection of *E. coli*

Before cleaning, 93% (ranging from 92% to 94% per variable) of the swab samples were positive for *E. coli*, while after disinfection only 7% were positive. Of swab samples taken after disinfection, 8% (35 out of 463), 7% (59 out of 810), 6% (37 out of 634) and 9% (57 out of 639) were positive for *E. coli* for protocols with soaking step, without soaking step, using warm water and using cold water, respectively. Logistic regression analyses on results of *E. coli* detection showed no significant differences between the four protocols.

4.1.4   Comparisons between ATP values

Mean ATP values were 2.5 ± 0.9, 2.4 ± 1.0, 2.4 ± 1.0 and 2.5 ± 0.9 log RLU (relative light units) for protocols with soaking step, without soaking step, using warm water and using cold water, respectively. Linear regression analysis showed that protocols without a soaking step, had lower ATP values after cleaning than protocols with a soaking step ($P < 0.05$), with a least square means difference of 0.1 log RLU. No differences were found between protocols using warm or cold water.

4.1.5   Comparisons between power consumption and working time

Results on working time during cleaning, consumption of water, electricity and cleaning product used during the different protocols are listed in table IV.3. Because consumption of heating oil is strongly dependent on type of high pressure cleaners, data is not shown.

Table IV.3: Comparison of power consumption and working time between cleaning protocols. Variable 1: soaking (protocol 1 and 2), 2: not soaking (protocol 3 and 4), 3: warm (protocol 1 and 4) and 4: cold (protocol 2 and 3).

Parameters	1	2	3	4
Working time during high pressure cleaning (min/m²)	2.02 [1]	2.20	1.94	2.27
Water needed for soaking step (m³/m²)	0.0010			
Water during high pressure cleaning (m³/m²)	0.016 [1]	0.018	0.016	0.018
Electricity (Wh/m²)	0.0023			
Cleaning product (L/m²)	0.0066	0.0057	0.0064	0.0059

[1] Soaking step was not taken into consideration for calculation of working time and water consumption during cleaning.

4.2 Sampling locations.

4.2.1 <u>Bacterial analyses</u>

Means (with standard deviations) and medians (with first and third quartiles) are shown for each parameter, time point and location in table IV.4. Before cleaning, drinking cups had total aerobic bacteria counts higher than 9 log CFU/625 cm² and floors (manure still present), pipes and loose materials (heating devices) had counts higher than 8 log CFU/625 cm³. *Enterococcus* spp. counts were also found in high amounts in the same 4 locations (> 6.5 log CFU/625 cm²). After cleaning, drinking cups and drain holes had the highest counts (total aerobic bacteria: > 7.5 log CFU/625 cm² and *Enterococcus* spp.: $\geq$ 4.9 log CFU/625 cm²) and feed hoppers and roofs had the lowest counts (total aerobic bacteria: < 5 log CFU/625 cm² and *Enterococcus* spp.: 2.4 log CFU/625 cm²). Results of swab samples taken after disinfection confirmed these results. Also, total aerobic bacteria enumerations of air outlets were below 3 log CFU/625 cm² after disinfection. Mean total aerobic bacteria counts for roofs were higher after cleaning (4.9 ± 1.3 log CFU/625 cm²) than before cleaning (4.5 ± 2.5 log CFU/625 cm²). After disinfection, *E. coli* was mostly still found at drain holes (53% of the samples), floor cracks (24%) and drinking cups (10%). Other locations had a prevalence of less than 10%. At farm C, *Salmonella* was detected in 11 swab samples BC (taken from floor, air outlet, drinking cups and loose material) and 2 samples AD (taken from drinking cups and floor cracks). No *Salmonella* was found on the other farms.

Results of ACP ([0-450] CFU/ACP) after disinfection showed that floors, drain holes and floor cracks had highest total aerobic bacteria counts. More than 30% of ACP taken at drain holes, floor cracks and floors were overgrown (> 300 CFU).

Table IV.4: Microbiological and ATP values given for each sampling location. Samples taken during C&D in 12 broiler houses on 5 farms. Number of samples ranged BC from 108 to 112, AC from 42 (drain hole) to 96 and AD from 51 (drain hole) to 112 per location. Results in log CFU/625 cm² for swab samples, CFU/25 cm² for ACP and RLU/100 cm² for ATP values. Mean and standard deviation are given for results that are normally distributed. First quartile (Q1), median (Q2) and third quartile (Q3) are given for results that didn't follow this distribution.

| Sampling location | TAB [1] | | | | | *Enterococcus* spp. | | | EC [2] detection | | ATP [3] |
	BC	AC	Swab samples	AD ACP [4] Count [0, 450]	Overgrown	BC	AC	AD	BC	AD	AC
Floor	8.6 ± 1.0	6.1 ± 0.9	4.5 ± 1.1	32 – **94** – 350	35%	7.0 ± 0.8	4.2 ± 1.0	0.0 – **1.3** – 2.5	100%	9%	2.8 ± 0.7
Air outlet	7.2 ± 0.9	5.9 ± 0.9	3.0 ± 1.8	1 – **4** – 14	7%	5.6 ± 1.8	3.9 ± 1.6	0.0 – **0.0** – 1.3	100%	0%	2.0 ± 0.6
Wall	7.1 ± 0.6	5.7 ± 1.2	4.6 ± 1.0	15 – **38** – 74	9%	6.0 ± 1.2	3.5 ± 1.7	0.0 – **2.6** – 3.5	100%	3%	1.8 ± 0.6
Air inlet	7.5 ± 1.8	6.0 ± 1.4	4.8 ± 1.4	3 – **24** – 144	18%	6.2 ± 2.0	3.8 ± 1.9	0.0 – **2.3** – 3.7	97%	5%	2.3 ± 0.8
Drinking cup	9.5 ± 0.8	7.7 ± 0.9	5.5 ± 1.7	2 – **15** – 199	25%	7.1 ± 0.7	4.9 ± 1.3	0.0 – **2.0** – 3.9	100%	10%	3.7 ± 0.4
Feed pan	7.0 ± 1.1	5.8 ± 1.3	3.5 ± 1.8	1 – **5** – 14	6%	5.5 ± 1.7	4.6 ± 1.0	0.0 – **0.0** – 2.8	100%	2%	2.3 ± 0.7
Feed hopper	6.7 ± 0.8	4.3 ± 1.9	2.6 ± 1.5	1 – **4** – 20	8%	5.4 ± 1.7	2.4 ± 2.0	0.0 – **0.0** – 0.0	99%	0%	1.4 ± 0.7
Pipe	8.5 ± 0.9	6.2 ± 1.1	4.5 ± 1.4	7 – **19** – 64	19%	7.4 ± 0.9	4.3 ± 1.7	0.0 – **1.3** – 3.3	99%	4%	2.6 ± 0.7
Drain hole	n.a. [5]	7.6 ± 0.8	6.0 ± 1.2	221 – **400** – 450	75%	n.a.	5.2 ± 0.7	1.3 – **3.4** – 4.8	n.a.	53%	3.4 ± 0.5
Loose material	8.1 ± 1.0	5.4 ± 1.5	3.5 ± 1.5	3 – **9** – 29	12%	6.8 ± 0.8	3.5 ± 1.8	0.0 – **0.0** – 2.0	100%	2%	2.5 ± 0.6
Roof	4.5 ± 2.5	4.9 ± 1.3	3.2 ± 1.7	1 – **5** – 19	11%	3.1 ± 2.7	2.4 ± 1.9	0.0 – **0.0** – 1.8	99%	2%	1.5 ± 0.6
Floor crack	n.a.	6.5 ± 1.0	4.5 ± 1.5	17 – **60** – 350	34%	n.a.	4.6 ± 1.0	0.0 – **1.3** – 3.0	n.a.	24%	3.4 ± 0.5

[1] TAB, total aerobic bacteria; [2] EC, *E. coli*; [3] ATP, adenosine triphosphate; [4] ACP, agar contact plates; [5] n.a., not accessible.

4.2.2 Number of samples

No differences in mean and median total aerobic bacteria and *Enterococcus* spp. contamination respectively, was seen when samples were taken in quadruplicate (n=3473) or when samples were taken in one fold (n=893), duplicate (n=1775) or threefold (n=2627) ($P > 0.05$).

4.2.3 ATP analyses

ATP values were the highest for drinking cups, drain holes and floor cracks (≥ 3.4 log RLU/100 cm²). Lowest ATP values (≤ 1.5 log RLU/100 cm²) were found for feed hoppers and roofs.

4.2.4 Locations

A linear discriminant analysis showed a separation of drinking cups, drain holes and floor cracks. ATP values and enumeration of total aerobic bacteria AC and AD on swab samples contributed the most to this observation. A new analysis was conducted with only these parameters and comparable results were obtained. Detection of *E. coli* and *Salmonella* at these sampling points after disinfection confirmed these results.

5. Discussion

5.1   Cleaning of broiler houses

When broiler houses were soaked during C&D, a greater reduction of total aerobic bacteria and *Enterococcus* spp. counts on swab samples was found, whether or not warm or cold water was used during cleaning. Although counts on ACP after disinfection showed the same trend, logistic regression analysis on these counts couldn't confirm this observation.

Considering ATP values, only a small difference of 0.1 log RLU/100 cm² was found between protocols without a soaking step and protocols preceded by a soaking step. ATP-metry measures the amount of eukaryotic (as part of soil) and prokaryotic (bacteria, molds…) cells. These ATP measurements gave contradictory results, since lower bacterial counts were found after disinfection for protocols with a soaking step. Green *et al*. (1999) showed that commercial sanitisers and cleaning products may quench or increase the light signal during ATP measurements, which could lead to false positives and negatives. This was not taken into account in this study. Also, a poor repeatability and reproducibility for commercially available

rapid ATP monitoring systems has been reported (Shama and Malik, 2013). Therefore, the found differences in ATP values between protocols seemed negligible.

Recommendations for using warm water are based on the easier dissolution of fats (Gibson *et al.*, 1999), improved action of the cleaning product and quicker drying of the house. In practice however, no differences were found between cleaning protocols using warm water and cold water concerning total aerobic bacteria and *Enterococcus* spp. contamination (whether or not a soaking step was applied). Other studies in animal houses also showed that the use of warm water in practice is negligible (Morgan-Jones, 1981; Walters, 1967). One explanation could be that the actual cleaning products in combination with cold water are sufficiently able to dissolve fats. However, when broiler houses were cleaned with warm water, less water and working time were spend in comparison with protocols using cold water. It should also be taken into consideration that the use of warm water contributes to the comfort of farmers during cleaning. Water consumption was higher for protocols without a soaking step. Even though broiler houses were soaked with water overnight (mean water consumption during soaking: 0.0010 m³/m²), the water consumption was still lower. This means that a preceding soaking step reduced the amount of water needed to clean broiler houses afterwards. Soaking can loosen organic material, making removing it is easier during high pressure cleaning. In addition, working time spent on cleaning after soaking were less than cleaning without a preceding soaking step. However, it should be taken into account that soaking of broiler houses can be time consuming by postponing the high pressure cleaning. Automatic sprinkler systems, mostly present in the broiler house for cooling broilers during summer, can be used overnight for soaking the stable.

5.2   Sampling

Statistical analyses showed that sampling 12 locations in one fold per broiler house was sufficient to evaluate C&D. This means that costs and working time can be reduced for future research on evaluating C&D methods.

5.3   Identification of critical locations

High counts on swab samples showed that drinking cups, drain holes and floor cracks are critical locations during C&D in broiler houses. Next to the high bacterial load BC and AD, these locations also contained a lot of water after cleaning, causing dilution of the used disinfection products. High bacterial counts found after disinfection confirmed this observation. Also, ATP values were the highest for these three locations, which could give an indication that there was still a high amount of organic material present after cleaning. Drain holes as well as

floor cracks were previously identified as critical locations and possible sources for pathogens (Dewaele *et al*., 2012b; Mueller-Doblies *et al*., 2010; Rajic *et al*., 2005). Because these locations are covered with pellet before chick loading, there is no direct contact with the animals, but they still remain a risk. To reduce this risk, floor cracks can be regularly repaired by filling and more attention can be given to C&D of drain holes. On the other hand, drinking cups are capable of immediately contaminating a new flock. Because of their fragile and angular construction, drinking cups are difficult to clean and are therefore critical locations. In addition, broiler chickens can contaminate these drinking cups by defecating in them or by (particularly when they are young) stepping and walking in it. Heyndrickx *et al*. (2002) showed that drinking water in broiler houses is one of the risk factors significantly related to the *Salmonella* flock status. Renwick *et al*. (1992) also showed that there was a greater risk of contamination of drinking water with *Salmonella* from trough drinkers and plastic bell drinkers than from nipple drinkers.

Feed hoppers, roofs and air outlets seemed the cleanest locations (low bacterial counts and ATP values) in broiler houses after C&D. A logical explanation is that these locations do not come into direct contact with any manure or chickens because they are (one of) the highest locations in broiler houses. Another explanation would be that these locations have a smooth surface and are therefore also easy to clean. Remarkably, roofs were more contaminated with total aerobic bacteria after cleaning than before cleaning. This could be explained by the fact that when cleaning floors, dirt (manure) can be splashed on the roof.

Results showed that ATP-metry could be capable of providing additional information to identify critical locations in broiler houses, although results should be interpreted cautiously. *E. coli* detection can also be used to quickly detect the less sanitised and critical locations.

6. Conclusion

Total aerobic bacteria and *Enterococcus* spp. counts on swab samples showed that C&D protocols using a soaking step caused a higher bacterial reduction compared to protocols without a soaking step. Although total aerobic bacteria counts on ACP showed the same trend, statistical analyses could not confirm this. Furthermore, a preceding soaking step leads to less water consumption and working time during high pressure cleaning. No differences were found between protocols using cold or warm (60 °C) water. The number of samples needed for the evaluation of broiler houses can be reduced from samples taken in fourfold (*i.e.* 48 samples) to

samples taken in one fold (*i.e.* 12 samples). Drinking cups, drain holes and floor cracks are critical locations during C&D in broiler houses and therefore possible sources of pathogens.

7. Acknowledgements

CHAPTER V
Comparison of competitive exclusion with classical cleaning and disinfection on bacterial load in pig nursery units

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CHAPTER V
Comparison of competitive exclusion with classical cleaning and disinfection on bacterial load in pig nursery units

1. Abstract

Colonisation of the environment of nursery units by pathogenic bacteria is an important factor in the persistence and spread of endemic diseases in pigs and zoonotic pathogens. These pathogens are generally controlled by the use of antibiotics and disinfectants. Since an increasing resistance against these measures has been reported in recent years, methods such as competitive exclusion (CE) are promoted as promising alternatives.

In this study the effect of a CE protocol on the bacterial infection pressure in nursery units was compared to a classical cleaning and disinfection (C&D) protocol (control). Tests were performed during 3 successive production rounds using multiple identical nursery units. CE protocol consisted of microbial cleaning (*Bacillus* spp. spores) and spraying the *Bacillus* spp. spores during down-time and production. Sampling was performed: immediately after pig removal; 24 h after cleaning (CE units) or disinfection (control units) and after 1 and 5 weeks of production (piglets present). On these samples, analyses of bacterial spores, *Enterococcus* spp., *Escherichia coli*, faecal coliforms, methicillin resistant *Staphylococcus aureus* and *Salmonella* were performed. In addition to the bacterial analyses, feed conversion, faecal consistency and antibiotic use were monitored.

This study showed that the infection pressure in CE units after microbial cleaning was not reduced to the same degree as in control units. Despite sufficient administration of probiotic-type spores, the analysed bacteria did not decrease in number after 3 production rounds in CE units, indicating no competitive exclusion. These results indicate that the CE protocol is not a valuable alternative for classical C&D.

2. Introduction

Colonisation of the environment in nursery units by pathogenic bacteria is an important factor in the persistence and spread of endemic diseases in pigs and of zoonotic pathogens. These infections are often controlled by the use of antibiotics and disinfectants. However, an increasing level of resistance against these substances has been observed in recent years (Callens *et al*., 2013; Mateu and Martin, 2001; Russell, 1998; Soumet *et al*., 2012). Since 2005, methicillin resistant *Staphylococcus aureus* sequence type 398 (MRSA ST398) has been found on farms and farm animals, especially pigs (Smith and Pearson, 2011; Vanderhaeghen *et al*., 2010; Weese, 2010). MRSA ST398 has a multiresistant phenotype (Kehrenberg *et al*., 2009), a zoonotic character (Catry *et al*., 2010) and can also pick up new resistance genes (Pletinckx *et al*., 2013). Wong *et al*., 2013 described the presence of disinfectant resistance genes in porcine MRSA. Although the minimum inhibitory and bactericidal concentrations (MIC and MBC) of resistant strains remain lower than the recommended working concentrations of disinfectants, there is concern that an impairment of the used disinfectant (due to presence of organic material) resulting in exposure to lower active levels of these agents, selection for more resistant strains harbouring these genes may occur (Wong *et al*., 2013). Slifierz *et al*. (2015) showed that the use of quaternary ammonium compound-based (QAC) disinfectants is a risk for selecting (antibiotic resistant) MRSA in commercial swine herds. Antibiotic multiresistant *Salmonella* strains on pig farms have been described in several countries (Chuanchuen and Padungtod, 2009; Rajic *et al*., 2004; Sisak *et al*., 2008). Randall *et al*. (2004) suggested that the use of biocides alone or combined with antibiotic treatment may also increase selective pressure towards antibiotic resistance of *Salmonella enterica*. Beier *et al*. (2008) showed that β-haemolytic enterotoxigenic *Escherichia coli* (*E. coli*) strains isolated from neonatal pigs, were resistant to chlorhexidine and QAC. Some of these resistant strains had also multiple antibiotic resistance.

Because of the ongoing concern about excessive use of biocides and potential resistance development and cross-resistance to clinically important antibiotics, the use of bacterial biocontrol agents has often been suggested as an alternative method to antagonise the growth of these pathogens. The working mechanism of these biocontrol agents is based on the concept of bacteria that should compete with pathogens in the environment by competitive exclusion, influencing quorum sensing, producing antimicrobial compounds (*e.g.* bacteriocins) and/or competition for attachment sites (Patterson and Burkholder, 2003). However, only very few reports describing the use and the effectiveness of microbial biocontrol agents on farms are

available in literature. The aim of this study was to compare the effectiveness of a commercial competitive exclusion (CE) protocol with a classical cleaning and disinfection (C&D) protocol in decreasing *Salmonella*; (haemolytic) *E. coli*, faecal coliforms, *Enterococcus* spp. and MRSA contamination of nursery units during 3 successive rounds.

3. Materials and methods

3.1   Management in control and CE units

This study was carried out in 6 identical nursery units at the experimental pig farm of the Institute for Agricultural and Fisheries Research (ILVO) during 3 successive production rounds. Piglets were moved to these units immediately after weaning (4 weeks of age) and stayed there for 6 weeks. Three units were assigned to the control group (classical C&D protocol) and 3 to the treatment group (CE protocol). Each unit consists of eight identical pens of 1.8 m² (Figure V.1). Piglets were raised per six in one pen. After 6 weeks, piglets were transported to fattening units and pens were cleaned (and disinfected) according to the tested protocols.
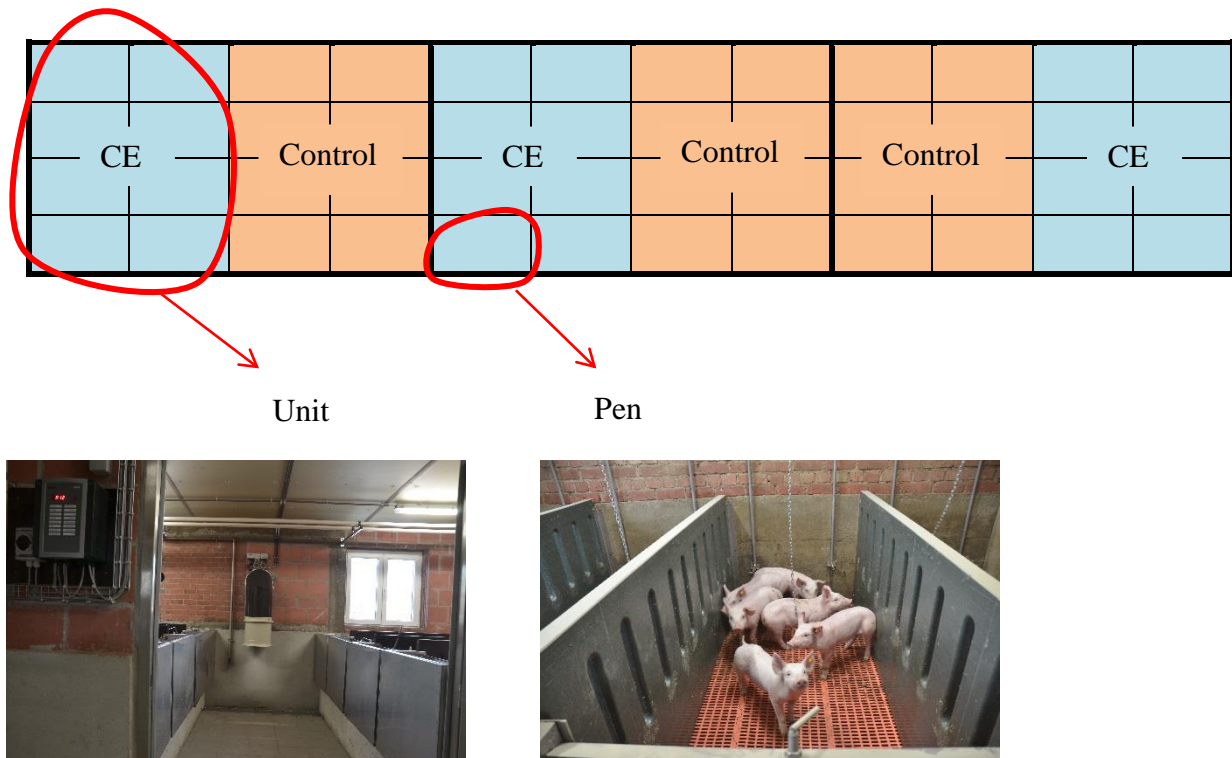
Figure V.1: Overview of the experimental set up in the pig nursery units at the experimental pig farm. Three units were assigned to the competitive exclusion (CE) group and three to the control group.

Classical C&D protocol was carried out after pigs were removed. Manure was removed by cleaning with cold water. Twenty-four hours later, pens were soaked with 2% MS Topfoam (sodium hydroxide) (Schippers, Bladel, The Netherlands) for 30 min. The cleaning product and any remaining dirt was removed under high pressure with cold water (150 bar) and pens were disinfected with 1% (v/v) MS Megades (glutaraldehyde and quaternary ammonium compounds) (Schippers). Finally, the pens were kept empty during two weeks of down-time.

The CE units pens were first hosed down with cold water to remove manure; 24 h later they were soaked with 1.5% (v/v) PIP AHC (Probiotics In Progress Animal House Cleaner, Chrisal, Lommel, Belgium) at 40 °C for 10 min and rinsed with warm water (40 °C). PIP AHC consists of cleaning compounds, *Bacillus* spp. spores and enzymes. In CE units, no disinfection was carried out. In addition, during the 2-week down-time period as well as during production, CE units were sprayed 2 – 3 times per week with pure PIP AHS (Animal Housing Stabilizer, Chrisal) to bring and retain biocontrol agents into the stall environment. In the first week of production during the third round, CE units were sprayed every day of the week with PIP AHS. The AHC and AHS PIP products contained *Bacillus* spp. spores of five different species in a concentration of 8.5 and 7.5 log colony forming units (CFU)/ mL, respectively.

Both protocols were carried out according to the manufacturers guidelines. For each protocol an individual and identical high pressure jet (Kärcher, HDS 6/14-4CX, Temse, Belgium) was used.

3.2 Sampling scheme

Sampling was performed at different time points (" sampling moments"): (1) immediately after pig loading (before cleaning, BC); (2) 24 h after cleaning (CE units) (AC) or 24 h after disinfection (control units) (AD); (3) after 1 week (W1) and (4) after 5 weeks of production (W5) (piglets present). Three pens per unit were sampled at each sampling moment. Premoistened sponge swab samples with 10 mL Buffered Peptone Water (BPW) (3M, SSL10BPW, St-Paul, USA) were taken at five locations per pen: synthetic grid floor, concrete wall, synthetic wall, drinking nipples and feeding trough. Samples were taken in triplicate per type of location resulting in 15 swab samples per nursery unit at each sampling moment. After disinfection, 10 mL Dey Engley neutralising broth (Sigma Aldrich, Fluka, D3435, St-Louis, USA) was used to premoisten the sponge swab samples (SSL100, 3M) used. When possible, a surface of 625 cm² was swabbed.

3.3   Sample processing

Samples were transported to the lab under refrigeration and stored at 3 ± 2 °C for 18 h before further processing. Samples were first diluted with 30 mL of BPW (Oxoid, CM0509, Basingstroke, Hampshire, England) and then homogenised by placing them in a Masticator (IUL instruments, S.A., Barcelona, Spain). Prior to plating, swab samples were further diluted in peptone physiological salt water (Bio Trading, K110B009AA, Mijdrecht, The Netherlands) to produce countable results on the selected agar media: Slanetz-and-Bartley (Oxoid, CM0377) for *Enterococcus* spp., Rapid *E. coli* (Biorad, 356-4024, Marnes-la-Coquettes, France) for *E. coli* and faecal coliforms and chromID® MRSA-SMART (MRSM, bioMérieux, 413050,Marcy l'Etoile, France) for MRSA enumerations. A 3 mL BPW-fraction was heated for 10 minutes at 80 °C, diluted in peptone water and plated on Plate Count Agar (Oxoid, CM0325) for spore enumerations in order to determine the CFU count in both PIP products and to test if *Bacillus* spp. spores were well distributed and sufficiently present in pens. Also, a 10 mL BPW-fraction was mixed with 10 mL double concentrated Mueller Hinton Broth (Oxoid, CM0405) and 13% (w/v) sodium chloride (Merck, 1.06404.500, Darmstadt, Germany). After overnight incubation at 37 °C, 100 µl was plated on MRSM for detection of MRSA. The remaining BPW fraction (original sample) was also overnight incubated at 37 °C for detection methods. Detection of *E. coli* and faecal coliforms was carried out by plating 10 µl of the enrichment broth on Rapid *E. coli* medium. *Salmonella* detection on the broth was carried out according to ISO 6579:2002 Annex D protocol (Anonymous, 2002).

3.4   Confirmation of, MRSA, *Salmonella* and haemolytic *E. coli*

Five positive MRSA colonies (if present) were subcultured on Tryptone Soy Agar (Oxoid, CM0131) and DNA was extracted according to the method of Stranden *et al*. (2003). A multiplex PCR, as described by Maes *et al*. (2002), was performed for MRSA and a CC398 specific PCR, as described by Stegger *et al*. (2011), for MRSA ST398 confirmation.

Positive *Salmonella* colonies on Xylose Lysine Deoxycholate agar medium (Oxoid, CM0469) were subcultured on Nutrient Agar (Oxoid, CM0003). After incubation, PCR confirmation on cel lysates was performed as described by Aabo *et al*. (1993).

From the third down-time and production round, five positive *E. coli* colonies (when possible) were subcultured on Columbia base Blood Agar (Oxoid, CM0331) with 5 % sheep blood and incubated for 24 hours at 37 °C for enumeration of hemolytic *E. coli*. If a plate was negative after 24 hours, it was incubated for a further 24 hours. To calculate the enumerations of

haemolytic *E. coli*, the ratio of the number of positive haemolytic *E. coli* colonies on the 5 selected colonies was multiplied by the mean *E. coli* enumeration of that sample.

3.5   Other analyses

Piglets were weighed individually at the age of 4, 6 and 9 weeks. Also feed intake was monitored per pen on the same moments allowing to calculate feed conversion ratio of every pen.

In addition, faecal consistency was evaluated according to Pedersen and Toft (2011): a score from 1 (no diarrhea) to 4 (serious diarrhea) was assigned per pen.

Finally, clinical manifestations and treatment with antibiotics were registered. Treatments days per 100 days at risk (TD100) was calculated per pen for each protocol. This was done by calculating the ratio of treatments days (number of days that piglets received antibiotics) and the number of days at risk (time that pigs could be exposed to antibiotics), taking the number of dead piglets into account. This ratio was then multiplied by 100.

3.6   Statistical analysis

The distribution of the variables was characterised with a histogram and Q-Q plot. Log transformed enumerations of spores and *Enterococcus* spp. and results of average daily gain, daily feed intake, feed conversion ratio and TD100 ratio followed a normal distribution. Log transformed enumerations of *E. coli*, haemolytic *E. coli*, faecal coliforms and MRSA did not follow this distribution.

The 4 point scale faecal consistency score was reduced to a binary scale: 0 = pens with score 1 and 1 = pens with score > 1.

The effect of the predictor variables on the normal distributed data (dependent variables) was assessed using multivariate linear regression. The effect of predictor variables on the non-normally distributed outcome variables describing the enumeration and detection of the different bacteria (absence or below the detection limit =0, presence =1) was tested by means of multivariate logistic regression analysis.

A backward stepwise elimination was performed to determine the final statistical model for each bacteriological parameter, starting with the global model (predictor variables: protocol used, sampling moment, production round and location) and subsequently removing all non-significant terms. Only biologically relevant interaction effects were considered. In each model, the variables unit and pen were included as a random effect to correct for measurements within

one pen and unit. The predictor variable sampling moment was included as a repeated measure. Post-hoc comparison was performed with a Tukey-Kramer test. Throughout the analyses, *P*-values ≤ 0.05 were considered as significant.

All statistical analyses were carried out with Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc.).

4. Results

In total 1074 swab samples were taken during 3 successive rounds. At each sampling moment approximately 90 samples were taken: *i.e.* 45 in CE units ($n = 3$) and 45 in control units ($n = 3$).

4.1 Spore enumerations

At every sampling moment and in each production round, higher spore enumerations were found for CE units compared to control units ($P < 0.01$) (Figures V.2a and V.2b), with a minimal difference of 0.70 log (BC) and 1.15 log (first round) CFU (colony forming units)/sampling surface. Further, spore enumerations increased after every round in CE units ($P < 0.01$) (Figure V.2b). Mean spore enumerations ranged from 2.88 log CFU/sampling surface AC to 4.89 log CFU/sampling surface at W5 during production piglets present and from 1.25 log CFU/sampling surface AD to 2.61 log CFU/sampling surface at W5 for CE and control units, respectively.

Spore enumerations

Figure V.2: Mean spore enumerations in log colony forming units/sampling area for CE and control units. At each sampling moment per round (b), 135 and 180 samples were taken per unit type, respectively. Significant differences between sampling moments or rounds within one type of unit are indicated by different letters above bars. Significant differences between protocols within one sampling moment or round are indicated by a star (*) on the horizontal axis. Vertical bars denote standard errors. BC, before cleaning; AC/ AD, after cleaning (CE unit) or after disinfection (control unit); W1, after 1 week of production; W5: after 5 weeks of production.

4.2   *Enterococcus* **spp. enumerations**

When considering the overall contamination level in both units, higher *Enterococcus* spp. enumerations, with a mean difference of 0.80 log CFU/sampling surface, were found in CE units ($P< 0.01$). After disinfection of control units, lower *Enterococcus* spp. enumerations were observed compared to cleaned CE units ($P< 0.01$) (Figure V.3a). The mean difference was 2.88 log CFU/sampling surface. Cleaning of CE units caused a reduction of 0.42 log CFU/sampling surface, while in disinfected control units a reduction of 3.54 log CFU/sampling surface was noticed. Before cleaning and after 1 week of production, no differences in *Enterococcus* spp. enumerations were found between units. However, at W5, higher *Enterococcus* spp. enumerations were found in CE units ($P= 0.05$). In addition, *Enterococcus* spp. enumerations were higher in every production round for CE units ($P< 0.01$) (Figure V.3b).
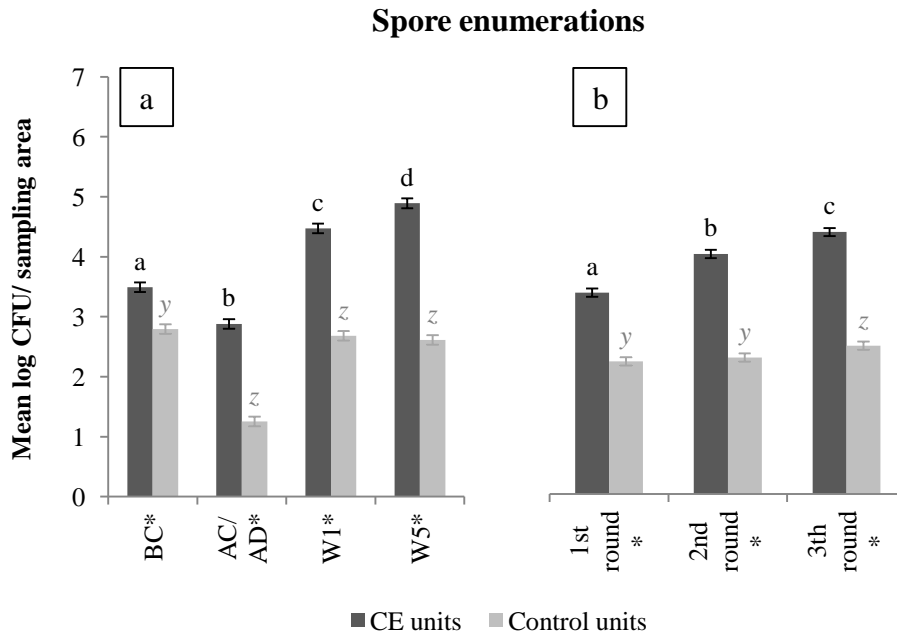
Enterococcus spp. enumerations
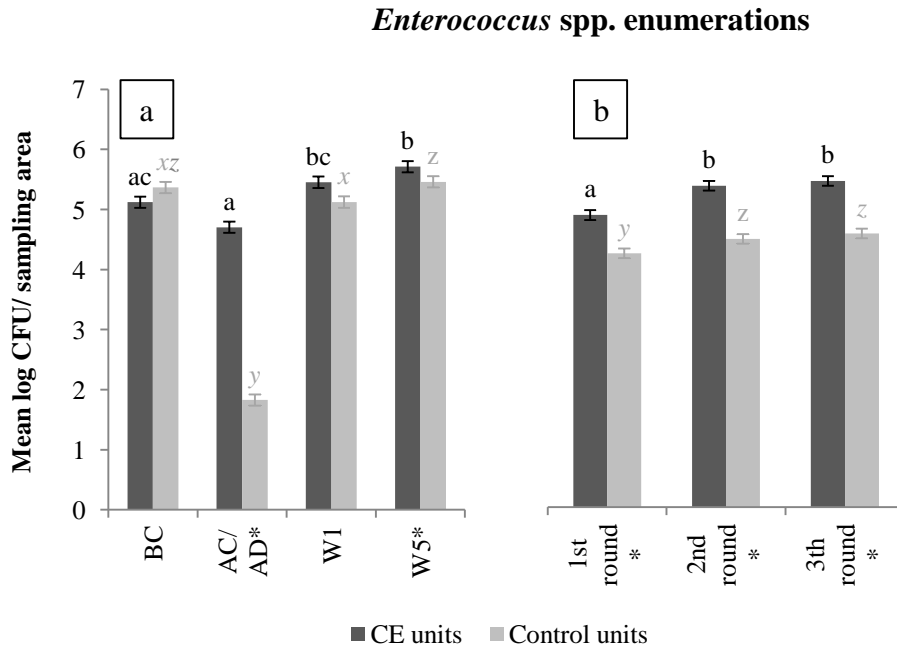
Figure V.3:Mean Enterococcus spp. enumerations in log colony forming units/sampling area for CE and control units. At each sampling moment (a) and per round (b), 135 and 180 samples were taken per unit type, respectively. Significant differences between sampling moments or rounds within one type of unit are indicated by different letters above bars. Significant differences between protocols within one sampling moment or round are indicated by a star (*) on the horizontal axis. Vertical bars denote standard errors. BC; before cleaning, AC/ AD, after cleaning (CE unit) or after disinfection (control unit); W1, after 1 week of production and W5: after 5 weeks of production.

4.3   *E. coli* enumerations

More *E. coli* countable samples were found for CE units after cleaning compared to control units after disinfection (*P*< 0.01) (Figure V.4a). Proportion of countable samples was reduced by 9% AC of CE units, while a reduction of 41% was obtained after disinfecting control units. During production and before cleaning, no differences were found in amount of countable *E. coli* samples between both types of units.

In control units, lower amounts of countable samples were found AD compared to amounts found BC and W1 (*P*< 0.01) while this was not seen AC of CE units (Figure V.4a).

Descriptive values of *E. coli* enumeration at each sampling moment are given in Table V.1.

Table V.1:Descriptive values for Escherichia coli (E. coli), faecal coliforms and methicillin resistant Staphylococcus aureus (MRSA) enumerations (log colony forming units/sampling area) given for each sampling moment for CE units and control units. Mean and standard deviation are given for enumerations that are normally distributed. First quartile (Q1), median (Q2) and third quartile (Q3) are given for enumerations that did not follow this distribution.

Sampling moment	*E. coli*	Faecal coliforms	MRSA
CE units			
BC [1]	0.0 – **1.6** – 2.8	2.7 ± 1.5	2.9 ± 1.4
AC/ AD [2]	0.0 – **0.0** – 2.8	0.0 – **1.9** – 3.8	0.0 – **0.0** – 0.0
W1 [3]	0.0 – **0.0** – 2.8	0.0 – **2.7** – 3.8	3.3 ± 1.1
W5 [4]	2.5 ± 1.6	3.1 ± 1.5	3.2 ± 1.1
Control units			
BC	0.0 – **0.0** – 3.0	2.6 ± 1.7	2.9 ± 1.4
AC/AD	0.0 – **0.0** – 0.0	0.0 – **0.0** – 0.0	0.0 – **0.0** – 0.0
W1	0.0 – **0.0** – 3.0	0.0 – **2.0** – 3.6	3.2 ± 1.3
W5	2.5 ± 1.8	3.1 ± 1.6	2.9 ± 1.3

[1] BC, before cleaning; [2] AC/ AD, after cleaning/ after disinfection; [3] W1, after 1 week of production; [4] W5, after 5 weeks of production.

4.4 Haemolytic *E. coli* enumerations

Of all samples taken in CE units ($n = 180$) and control units ($n = 180$) during the 3rd round, 24% and 23% were positive for haemolytic *E. coli*, respectively. Of these positive samples, 16% were obtained AC (CE units) and 0% were obtained AD (control units), respectively. Mean enumerations were 3.0 log CFU/sampling surface for both types of units. No significant differences were noticed between units.

4.5 Faecal coliform enumerations

When comparing CE and control units, results of faecal coliform enumeration confirmed the observations obtained with *E. coli* analyses (Figure V.4c). A reduction of 26% and 51% of faecal coliform countable samples was obtained AC and AD of CE and control units, respectively.

After cleaning as well as AD, a significant reduction of faecal coliform countable samples was obtained ($P< 0.01$).

Faecal coliform enumerations at each sampling moment for both types of units are given in Table V.1.

4.6   *E. coli* **and faecal coliform detection**

Detection results of *E. coli* (Figure V.4b) and faecal coliforms (Figure V.4d) confirmed the enumeration results of both parameters.

4.7   **MRSA enumerations**

After cleaning, countable samples were reduced 61% for CE units, 20% less than the observed reduction in disinfected control units ($P<0.01$) (Figure V.4e). When pens were soiled (BC, W1 and W5), no differences in MRSA contamination were found between both types of units. Mean and median enumerations for MRSA are given for each sampling moment in Table V.1.

4.8   **MRSA detection**

Detection results showed that the number of MRSA positive samples was the highest (90%) for CE units compared to the control units (81%) ($P<0.01$) (Figure V.4f).

Enumerations   **Detection**
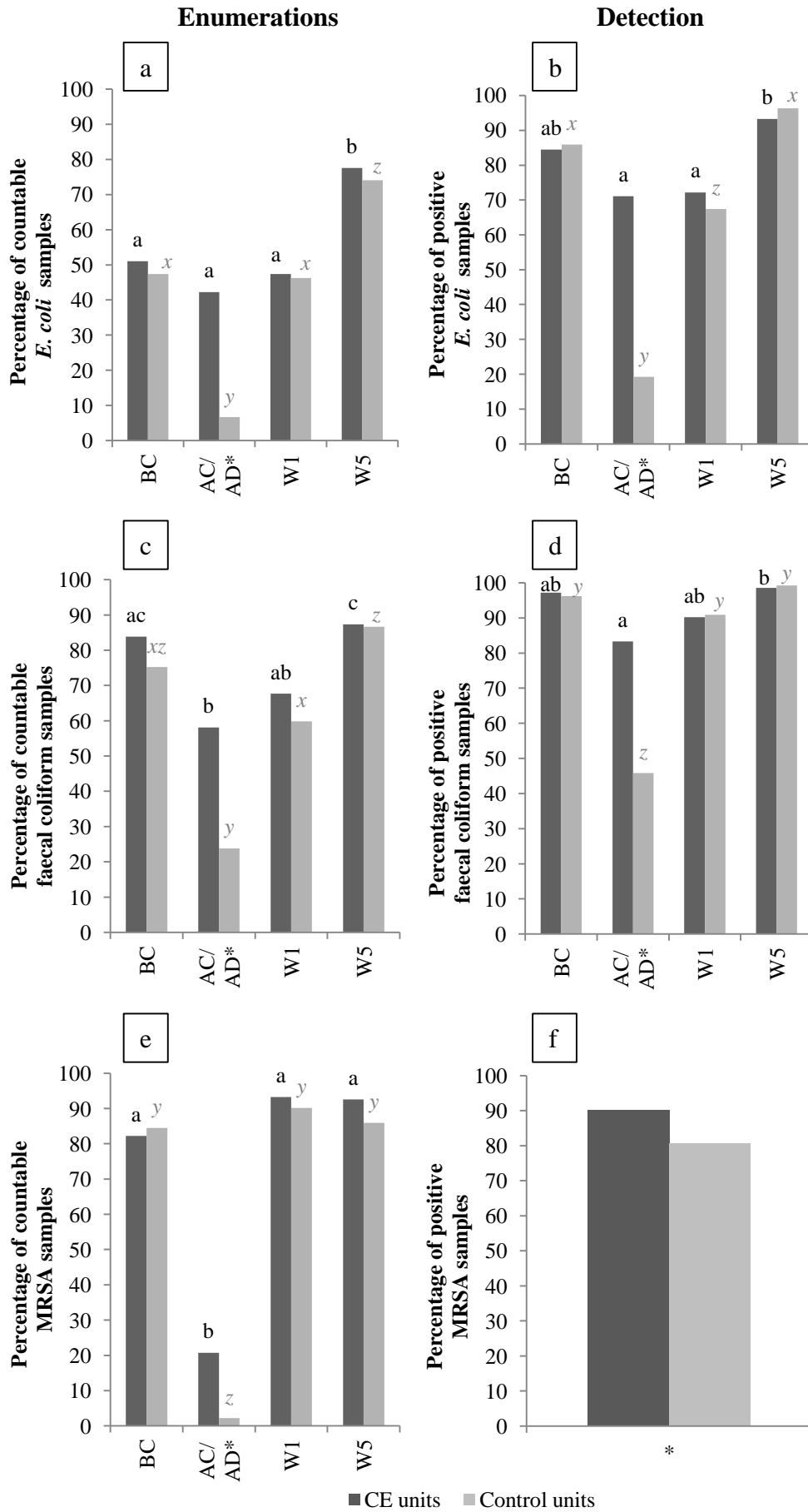
Figure V.4: Percentage of positive samples before (enumerations) and after enrichment (detection) for E. coli, faecal coliforms and MRSA given for CE and control units. At each sampling moment and in total 135 and 540 samples were taken per unit type, respectively. Significant differences between sampling moments within one type of unit are indicated by letters above bars. Significant differences between protocols within one sampling moment are indicated by a star (*) on the horizontal axis. BC, before cleaning; AC/ AD, after cleaning or after disinfection; W1, after 1 week of production and W5: after 5 weeks of production.

4.9 *Salmonella* detection

No *Salmonella* was found in this study.

4.10 Sampling locations

Mean enumerations (with standard deviation) and median enumerations (with first and third quartile) of *Enterococcus* spp., *E. coli*, faecal coliforms and MRSA after cleaning (CE units) and disinfection (control units) are given per type of sampling location in table V.2. In addition, the percentage of countable swab samples (enumerations) and positive samples after enrichment (detection) is shown for both types of units. Also, mean spore and *Enterococcus* spp. counts on all samples taken in CE and control units are given for each type of location in figures V.5 and V.6, respectively.

After cleaning of CE units, enumerations of *Enterococcus* spp. were the highest for floors, concrete walls and drinking nipples. In addition, highest percentage of countable *E. coli* samples and median enumerations were found for floors and concrete walls. Moreover, after enrichment also drinking nipples were still often contaminated with *E. coli*. Results of faecal coliforms and MRSA confirmed these observations.

In control units, high numbers of *Enterococcus* spp. were found on floors and drinking nipples. Most *E. coli* positive samples after enrichment were found for floors, drinking nipples and feeding troughs. In addition, highest enumerations for faecal coliforms were also found at these locations. Finally, for MRSA, drinking nipples were the most contaminated after disinfection.

More spore enumerations were found at every location for CE units (Figure V.5), with a minimal difference of 1.2 log CFU/sampling surface.

In addition, when considering the overall *Enterococcus* spp. contamination level, higher levels were found for each location in CE units (Figure V.6).

Table V.2: Descriptive values for Escherichia coli (E. coli), faecal coliforms and methicillin resistant Staphylococcus aureus (MRSA) enumerations (log colony forming units/sampling area) and detection after cleaning (CE units) and disinfection (control units) for each type of sampling location. Detection method was carried out after an overnight enrichment of samples. Mean and standard deviation are given for enumerations that are normally distributed. First quartile (Q1), median (Q2) and third quartile (Q3) are given for enumerations that did not follow this distribution.

Location	*Enterococcus* spp.		*E. coli*			Faecal coliforms			MRSA		
	CS (%) [6]	Enumerations	CS (%)	Enumerations	D (%) [7]	CS (%)	Enumerations	D (%)	CS (%)	Enumerations	D (%)
					CE units						
1 [1]	100	5.0 ± 0.8	59	0.0 - **1.6** - 3.0	85	67	0.0 - **3.2** - 3.7	96	44	0.0 - **0.0** - 1.9	81
2 [2]	100	4.8 ± 1.0	67	0.0 - **1.6** - 4.1	78	90	2.6 - **3.9** - 4.9	92	22	0.0 - **0.0** - 0.0	74
3 [3]	100	4.4 ± 0.9	4	0.0 - **0.0** - 0.0	48	19	0.0 - **0.0** - 0.0	50	11	0.0 - **0.0** - 0.0	56
4 [4]	100	4.9 ± 0.4	41	0.0 - **0.0** - 3.0	85	52	0.0 - **2.5** - 3.7	96	19	0.0 - **0.0** - 0.0	63
5 [5]	96	4.4 ± 1.3	41	0.0 - **0.0** - 2.2	59	62	0.0 - **2.5** - 3.6	83	7	0.0 - **0.0** - 0.0	44
					Control units						
1	70	2.1 ± 1.6	11	0.0 - **0.0** - 0.0	26	33	0.0 - **0.0** - 2.5	58	0	0.0 - **0.0** - 0.0	26
2	48	$0.0 – **0.0** – 3.0$	0	0.0 - **0.0** - 0.0	4	10	0.0 - **0.0** - 0.0	46	0	0.0 - **0.0** - 0.0	19
3	33	$0.0 – **0.0** - 1.7$	0	0.0 - **0.0** - 0.0	7	5	0.0 - **0.0** - 0.0	17	4	0.0 - **0.0** - 0.0	19
4	89	3.3 ± 1.5	19	0.0 - **0.0** - 0.0	30	43	0.0 - **0.0** - 3.2	67	4	0.0 - **0.0** - 0.0	37
5	48	$0.0 – **0.0** - 2.9$	4	0.0 - **0.0** - 0.0	30	29	0.0 - **0.0** - 2.8	42	4	0.0 - **0.0** - 0.0	15

[1] 1, floors; [2] 2, concrete walls; [3] 3, synthetic walls; [4] 4, drinking nipples; [5] 5, feeding trough; [6] CS (%), proportion of countable samples given in percentage; [7] D (%), proportion of positive samples after detection given in percentage.
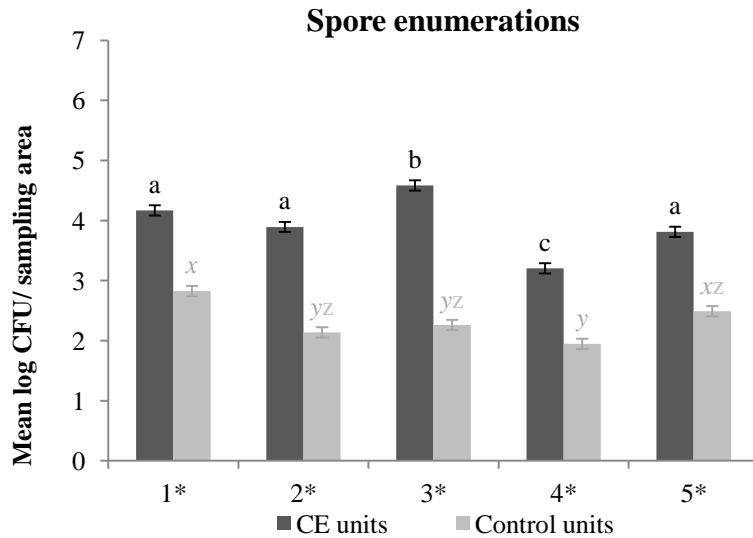
Figure V.5: Mean spore enumerations in log colony forming units/sampling area for CE and control units for each location. At each location, 108 samples were taken per type of unit. Significant differences between sampling moments within one type of unit are indicated by different letters above bars. Significant differences between protocols within one sampling moment are indicated by a star (*) on the horizontal axis. Vertical bars denote standard errors. 1, grid floor; 2, concrete wall; 3, synthetic wall; 4, drinking nipples; 5, feeding trough.
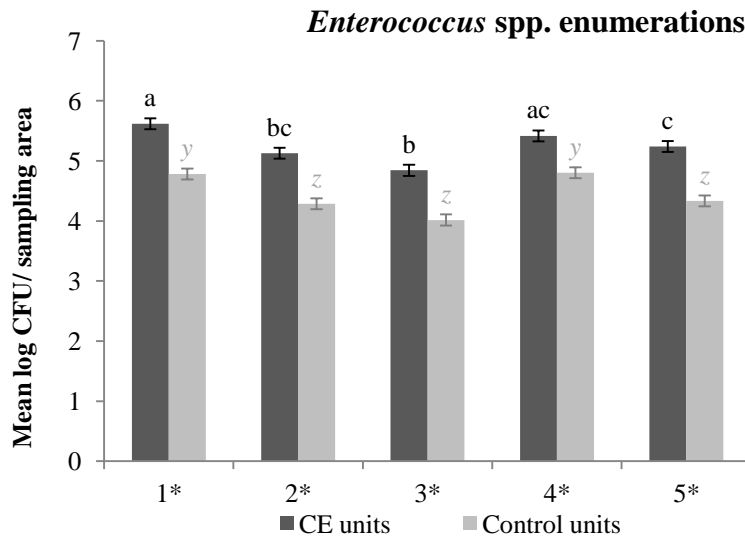
Figure V.6: Mean Enterococcus spp. enumerations in log colony forming units/sampling area for CE and control units for each location. At each location, 108 samples were taken per type of unit. Significant differences between sampling moments within one type of unit are indicated by different letters above bars. Significant differences between protocols within one sampling moment are indicated by a star (*) on the horizontal axis. Vertical bars denote standard errors. 1, grid floor; 2, concrete wall; 3, synthetic wall; 4, drinking nipples; 5, feeding trough.

4.11 Performance results

Mean starting weight of piglets in CE and control pens was 7.4 ± 1.5 and 7.1 ± 1.5 kg, respectively. A mean feed intake of 0.539 ± 0.078 and 0.521 ± 0.065 kg/ day was observed for CE and control units, respectively. No significant differences were found between feed intake of piglets raised in CE and control pens. When considering results of daily gain, no significant differences were found. Average daily gain was 0.407 ± 0.056 and 0.395 ± 0.053 kg for piglets in CE and control pens, respectively. In addition, no significant differences in mean feed conversion were found: 1.327 ± 0.072 and 1.324 ± 0.085 for pigs in CE and control units, respectively.

4.12 Faecal consistency

No significant differences in scores of faecal consistency between protocols were noticed (data not shown).

4.13 Antibiotic treatment

The mean TD100 for CE and control units was 27.9 ± 0.9 % and 28.3 ± 2.1 %, respectively. No significant differences were found between protocols.

5. Discussion

The emergence of multiresistant (pathogenic) bacteria is of great concern for animal and human health. Excessive use of antibiotics (Gullberg et al., 2014; Nikaido, 2009) and disinfectants (Karatzas et al., 2007; Randall et al., 2005; Whitehead et al., 2011) in for example the animal primary production, could possibly contribute to this phenomenon. Therefore, alternative methods such as competitive exclusion (CE) are promoted as promising. In this study a commercial CE protocol (by probiotic-type bacteria) was compared with a classical C&D protocol in nursery units.

According to the manufacturer of the PIP products, a reduction of pathogenic bacteria and improvement in hygiene after CE during 3 successive production rounds should be obtained. The first statement could not be confirmed by this study: *E. coli* (*Salmonella*-indicator), haemolytic *E. coli* and MRSA analyses showed that the infection pressure after CE cleaning was not reduced to the same extent as implementing a disinfection step. Furthermore, during production no differences were noticed. Also no improvement in hygiene was seen compared

to the control units: during the 2nd and 3rd production round higher *Enterococcus* spp. enumerations (hygiene indicator) were observed compared to the 1st round and no differences in faecal coliforms (faecal indicator) contamination between the two types of units were found. Because, higher contamination levels of MRSA and pathogen-indicator organisms (*E. coli*) were found in CE units after cleaning, there may be a greater chance of infecting young piglets arriving in those nurseries.

Several hypotheses have been proposed to explain the mechanisms of CE cultures. One is that CE bacteria should compete with other bacteria for adhesion sites, nutrients and energy, which results in preventing growth and proliferation of pathogenic bacteria in the environment (Cummings and Macfarlane, 1997). Another hypothesis is that these bacteria influence the quorum sensing communication and therefore inhibit expression of virulence and colonisation genes of pathogens (Vilà *et al.*, 2010). Besides CE bacteria, also enzymes were administered during cleaning, with the aim of helping to eliminate biofilms. In this study, no reduction of the analysed bacteria after 3 production rounds in CE units was seen. Several explanations were found to clarify this observation: (i) adhesion sites are abundantly present in animal houses, hence there is no need for competition; (ii) removal of organic debris is only carried out when piglets are removed from pens, therefore CE-, pathogenic and other bacteria have an abundance of nutrients during production, eliminating the need for competition between bacteria; (iii) however, in order to compete for nutrients, spores need to germinate, which may not be the case for all spores.

Moreover, Luyckx, *et al.* (2015a) (*i.e.* chapter III) showed that a cleaning step in broiler houses caused a reduction of total aerobic bacteria with 2 log CFU/sampling surface and that a disinfection step caused a further reduction of 1.5 log CFU. Although, cleaning caused a greater reduction of total aerobic bacteria, both the above study and this one showed that a disinfection step is still an important step for further reducing the bacterial infection pressure in barns with naturally high levels of environmental bacteria.

Improvement of feed conversion efficiency by probiotic-type bacteria could be obtained by a shift in intestinal flora, stimulating growth of nonpathogenic facultative anaerobic bacteria, inhibiting growth of pathogens, and enhancing digestion and utilisation of nutrients (Lutful Kabir, 2009). However, no differences were found between piglets raised in CE and control units in our study. Also, no differences in faecal consistency was noticed. A possible

explanation could be that not enough CE bacteria could be administered directly to the animals through the environmental spray application.

Finally, the contamination levels of the different sampling locations were analysed after cleaning of CE units and disinfection of control units. In CE units, grid floors, concrete walls and drinking nipples were still mostly contaminated by *Enterococcus* spp., *E. coli*, faecal coliforms and MRSA after cleaning. Although spore counts showed that high numbers of CE bacteria were present at these locations, the contamination level of different bacteria was still much higher compared to the microbial load after disinfection of control units. In addition, the overall *Enterococcus* spp. contamination of all locations during the experiment was higher in CE units. In control units, grid floors and drinking nipples seemed critical locations after disinfection. Luyckx, *et al*. (2015b) also showed that drinking cups are critical locations for C&D in broiler houses.

6. Conclusions

Very few studies about the impact of microbial cleaning and administration during production on the environment in animal houses are available. Our results showed that competitive exclusion by probiotic-type bacteria could not meet the claims provided by the manufacturer. Moreover, this study showed that a good C&D protocol during down-time is still very important for reducing infection pressure in nursery units. However, more research should be carried out for a valuable alternative, because disinfectant resistance might be an upcoming problem.

7. Acknowledgements

CHAPTER VI
A 10-day vacancy period after cleaning and disinfection has no effect on the bacterial load in pig nursery units

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CHAPTER VI
A 10-day vacancy period after cleaning and disinfection has no effect on the bacterial load in pig nursery units

1. Abstract

Biosecurity measures such as cleaning, disinfection and a vacancy period between production cycles on pig farms are essential to prevent disease outbreaks. No studies have tested the effect of a longer vacancy period on bacterial load in nursery units.

The present study evaluated the effect of a 10-day vacancy period in pig nursery units on total aerobic flora, *Enterococcus* spp., *Escherichia coli*, faecal coliforms and methicillin resistant *Staphylococcus aureus* (MRSA). Three vacancy periods of 10 days were monitored, each time applied in 3 units. The microbiological load was measured before disinfection and at 1, 4, 7 and 10 days after disinfection.

No significant decrease or increase in *E. coli*, faecal coliforms, MRSA and *Enterococcus* spp. was noticed. Total aerobic flora counts were the lowest on day 4 after disinfection (i.e. 4.07 log CFU/625 cm²) ($P<0.05$), but the difference with other sampling moments was limited (i.e. 0.6 log CFU/625 cm²) and therefore negligible. Furthermore, this observation on day 4 was not confirmed for the other microbiological parameters. After disinfection, drinking nipples were still mostly contaminated with total aerobic flora (i.e. 5.32 log CFU/625 cm²) and *Enterococcus* spp. (i.e. 95% of the samples were positive) ($P<0.01$); the feeding troughs were the cleanest location (total aerobic flora: 3.53 log CFU/625 cm² and *Enterococcus* spp.: 50% positive samples) ($P<0.01$).

This study indicates that prolonging the vacancy period in nursery units to 10 days after disinfection with no extra biosecurity measures has no impact on the environmental load of total aerobic flora, *E. coli*, faecal coliforms, MRSA and *Enterococcus* spp..

2. Introduction

Weaned piglets are subjected to many environmental, behavioural and dietary stresses. Moreover, the intestinal gut flora is still precarious, which makes them highly susceptible to enteric diseases (Hopwood and Hampson, 2003). Disease outbreaks in animal houses can lead to animal mortality and higher condemnation rates at slaughterhouses. The resulting economic damage can be severe (Jung and Rautenschlein, 2014) together with preventive measures (e.g. quarantine in case of epidemics) and even destruction of farm animals (Gelaude et al., 2014). In addition, foodborne zoonotic diseases are a significant and widespread global public health threat.

In nursery units, diarrhoea is one of the most important causes of economic losses in the pig industry. Post-weaning diarrhoea is multifactorial but the proliferation of pathogenic *E. coli* strains throughout the intestinal tract of piglets after weaning has been shown to play a significant role (Hampson, 1994; Richards and Fraser, 1961). Another important pathogen for the pig industry is *Salmonella*. In 2011, most of the reported food-borne outbreaks (69 553 human cases) in the European Union were associated with food originating from animals. *Salmonella* was the most frequently detected causative agent (26.6% of outbreaks) (European Food Safety Authority (EFSA), 2014).

Methicillin resistant *Staphylococcus aureus* sequence type 398 (MRSA ST398) is an emerging opportunistic pathogen among farm animals, especially pigs (Smith and Pearson, 2011; Vanderhaeghen et al., 2010; Weese, 2010). Epidemiological studies have shown that they not only colonise pigs, but can also be transmitted to persons with direct livestock exposure. Moreover, it is indicated that MRSA ST6398 represents an increasing cause of infections in humans (Köck et al., 2013).

It is of great importance to prevent disease outbreaks through biosecurity measures rather than cure them (Gelaude et al., 2014). Biosecurity includes all measures that prevent pathogens from entering a herd (external biosecurity) as well as reducing the spread of pathogens within the herd (internal biosecurity) (Sarrazin et al., 2014). Between production cycles, internal biosecurity measures such as cleaning, disinfection and a vacancy period are applied. Every biosecurity measure can influence the degree of infection pressure before new animals arrive. Luyckx et al. (2015a) showed that a cleaning step in broiler houses caused a reduction of total aerobic flora by 2 log CFU/625 cm² and that a disinfection step caused a further reduction of 1.5 log CFU. In piglet nursery units, the importance of a prolonged vacancy period is unknown. The aim of the present study was to assess the evolution of the bacterial load of total aerobic

flora, *Enterococcus* spp., *Escherichia coli* (*E. coli*), faecal coliforms and MRSA during a 10-day vacancy period in piglet nursery units.

3. Materials and methods

3.1 Sampling plan

This study was carried out in 6 identical nursery units (A1 to A3 and B1 to B3) on the experimental pig farm at the Institute for Agricultural and Fisheries Research (ILVO, Merelbeke, Belgium) (Figure VI.1) . Each unit consists of 8 pens of 1.8 m². Piglets were moved to these units immediately after weaning (4 weeks of age) and stayed there for 6 weeks. Each pen housed 6 piglets. Pen flooring was a synthetic grid, under which a board slopes towards a centrally-located slurry pit. Units A1 to A3 were monitored during 2 successive vacancy periods in February and April 2015 and units B1 to B3 during 1 vacancy period in March 2015. After pig removal, the units were cleaned with warm water, then disinfected with 1% (v/v) MS Megades (Schippers, Bladel, The Netherlands) on the same day. The disinfection product consists of glutaraldehyde and quaternary ammonium compounds. After cleaning and disinfection, the pen remained vacant for 10 days. During this vacancy period, the temperature and relative humidity (RH) were monitored hourly using thermo-hygrometers (Ilog EI-HS-D-32-L, ESCORT data logging systems). Three random pens per unit were sampled before disinfection and at 1, 4, 7 and 10 days after disinfection. Per sampling moment, 135 samples were taken, for a total of 675 samples.
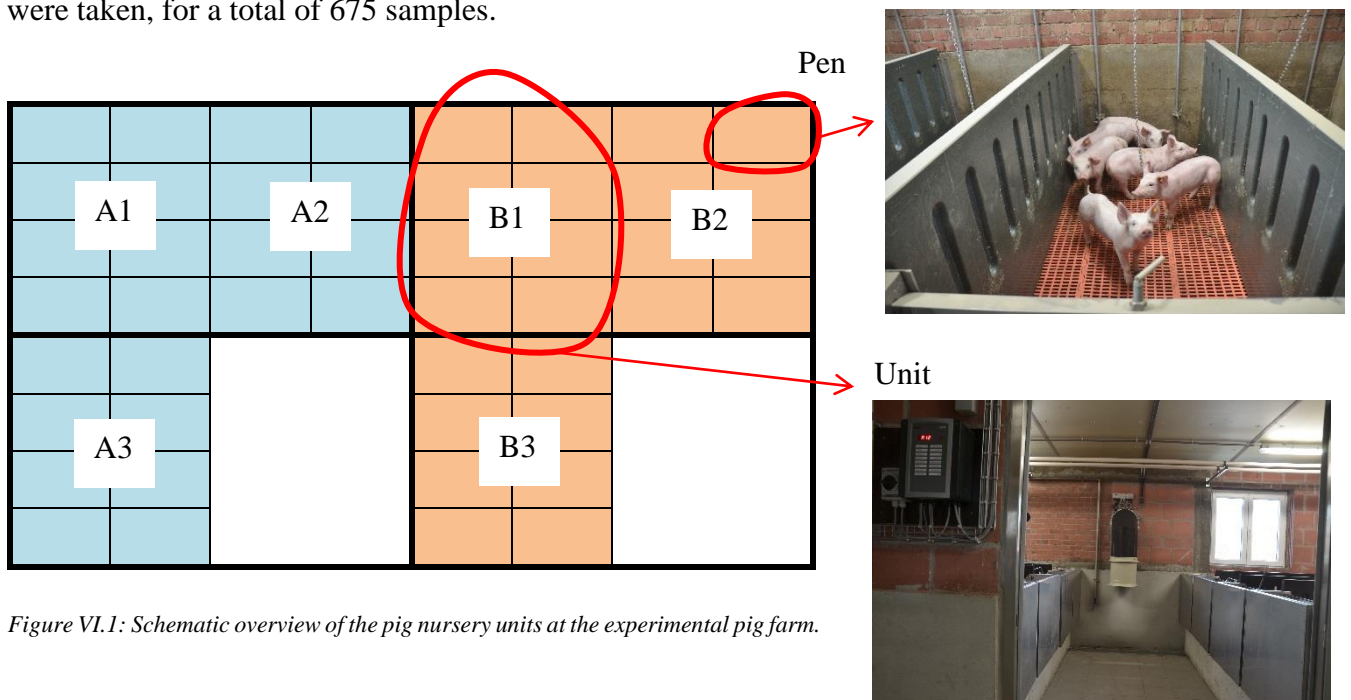
Figure VI.1: Schematic overview of the pig nursery units at the experimental pig farm.

3.2 Sample processing

Sponge swab samples (3M, SSL100, St. Paul, MN, USA), pre-moistened with 10 mL Ringers solution (Oxoid, BR0052G, Basingstroke, Hampshire, England), were taken at 5 locations per pen: floor, concrete wall, synthetic wall, drinking nipples and feeding trough. Sampling of 3 pens per unit resulted in triplicates per type of location or 15 swab samples per unit at each time point. To neutralise the residual action of the disinfectants on the microbiological growth, 10 mL Dey Engley neutralising broth (Sigma Aldrich, Fluka, D3435, St-Louis, MO, USA) was used to pre-moisten the sponge swab samples that were used on day 1 after disinfection. A surface of 625 cm² (A4 paper format) was sampled whenever possible. Because the surface of the drinking nipples was smaller than 625 cm², 2 drinking nipples per pen were sampled. Samples were transported to the lab under refrigeration and were processed immediately. For all measured pathogens, selected relevant bacteriological parameters and enumeration or detection analyses were based on Luyckx et al. (2015a). Swab samples were first diluted with 30 mL of Buffered Peptone Water (BPW, Oxoid, CM0509) and then homogenised by placing them in a Masticator (IUL instruments, S.A., Barcelona, Spain). Prior to plating, swab samples were further diluted in dilution series in saline peptone water (Bio Trading, K110B009AA, Mijdrecht, The Netherlands) to produce countable results on the selected agar media: Plate Count Agar (Oxoid, CM0325) for total aerobic bacteria and Slanetz and Bartley (Oxoid, CM0377) for *Enterococcus* spp. (lower enumeration limit 30 CFU/ 625 cm²). Plate Count Agar and Slanetz and Bartley plates were incubated at 30 °C and 37 °C during 72 h and 48 h, respectively. A 10 mL BPW fraction was also transferred to a Stomacher® bag and mixed with 10 mL double concentrated Mueller Hinton Broth (Oxoid, CM0405) and 13% (w/v) sodium chloride (Merck, 1.06404.500, Darmstadt, Germany). After overnight incubation of this solution at 37 °C, 100 µl was plated on chromID® MRSA SMART (MRSM, bioMérieux, Marcy l'Etoile, France) for the detection of MRSA. ChromID® MRSA SMART were incubated at 37 °C for 24 h – 48 h. The remaining BPW fraction (original sample) was also incubated overnight at 37 °C for additional analyses: for detection of *E. coli* and faecal coliforms, 10 µl of the enrichment broth was plated onto Rapid *E. coli* medium (Biorad, 356-4024, Marnes-la-Coquettes, France) and incubated for 24 h at 44 °C.

3.3   Statistical analysis

The distribution of the log-transformed enumerations of total aerobic bacteria and *Enterococcus* spp. was analysed via graphs (Q-Q plot and histogram). The log-transformed enumerations of total aerobic bacteria followed a normal distribution. A linear regression model was conducted to evaluate the effect of a vacancy period and location on the log-transformed total aerobic bacteria enumerations (dependent variable). To assess the effect of predictor variables (vacancy period and location) on the non-normally distributed outcome variables, variables describing the enumeration and detection of the different bacteria (*Enterococcus* spp., *E.* coli, faecal coliforms and MRSA) were transformed into binary variables (absent or below the detection limit = 0, present = 1). Subsequently a logistic regression analysis was carried out. Temperature and RH were added as covariates in both models. Variable "unit" was included as a random effect in both models to correct for measurements within one unit.

Post-hoc comparison was performed with a Tukey-Kramer test. *P*-values ≤ 0.05 were considered as significant. All statistical analyses were carried out using Statistical Analysis System software (SAS$^{®}$, version 9.4, SAS Institute Inc., Cary, NC, USA).

4. Results

Before disinfection, the mean enumeration of total aerobic flora was 5.64 log CFU/625 cm² (Figure VI.2a). The proportion of positive samples for *E. coli*, faecal coliforms and MRSA (after enrichment) and *Enterococcus* spp. was 49%, 65% and 16% (Figure VI.3a) and 95% (Figure VI.4a), respectively.

On day 1 after disinfection, mean enumeration of total aerobic bacteria was significantly reduced to 4.44 log CFU/625 cm² (*P*<0.01) (Figure VI.2a). Of the 135 samples taken on day 1, 13%, 23% and 7% were positive for *E. coli*, faecal coliforms and MRSA detection, respectively (Figure VI.3a). In addition, 70% of the samples gave countable results for *Enterococcus* spp. (Figure VI.4a). The proportion of positive samples for *E. coli*, faecal coliforms and *Enterococcus* spp. was significantly lower compared to the proportions found before disinfection (*P*< 0.01).
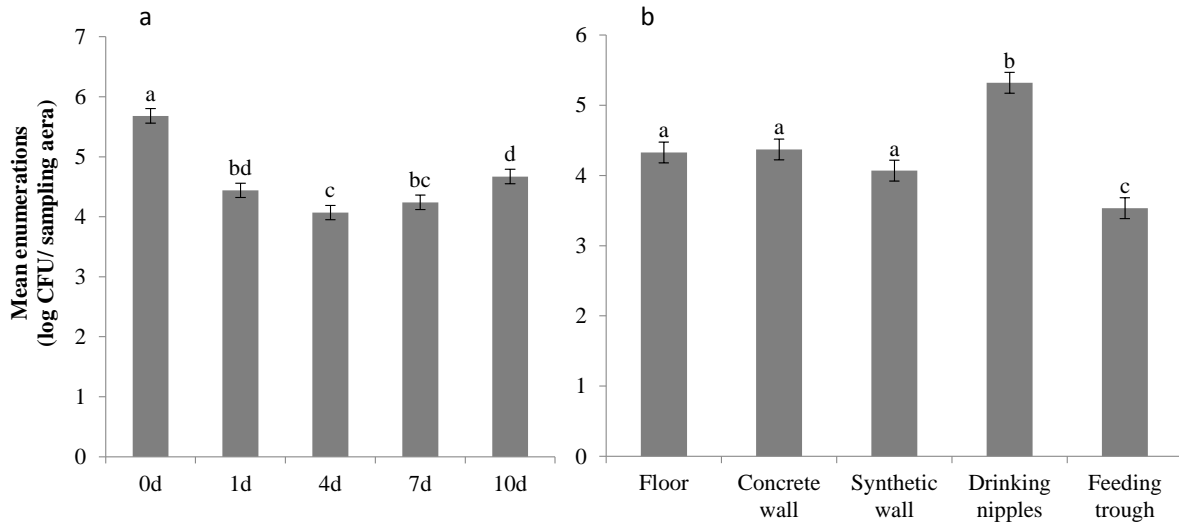
Figure VI.2: Mean enumeration of total aerobic bacteria with standard errors. Mean enumerations or given for each sampling moment (a) and location after disinfection (b). Samples (n = 135) were taken before disinfection (0d) and 1 day (1d), 4 days (4d), 7 days (7d) and 10 days (10d) after disinfection. Samples (n=108) were taken from each location. Significant differences between sampling moments/ locations are indicated by different letters above bars.
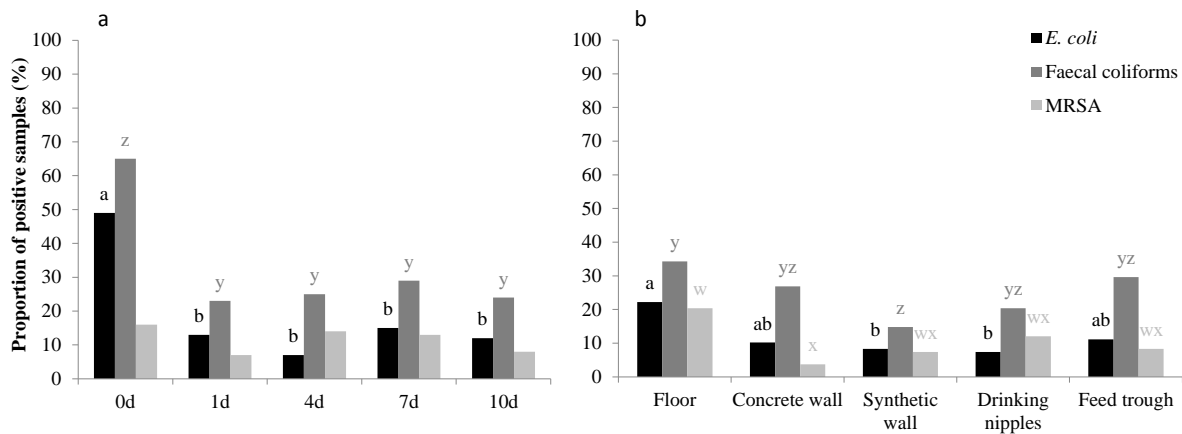
Figure VI.3:Proportion of positive samples given for detection of E. coli, faecal coliforms and MRSA, respectively. Proportions are given for each sampling moment (a) and location after disinfection (b), in percentage. Samples (n = 135) were taken before disinfection (0d) and 1 day (1d), 4 days (4d), 7 days (7d) and 10 days (10d) after disinfection. Samples (n = 108) were taken from each location. Significant differences between sampling moments/ locations per bacteriological parameter are indicated by different letters above bars.
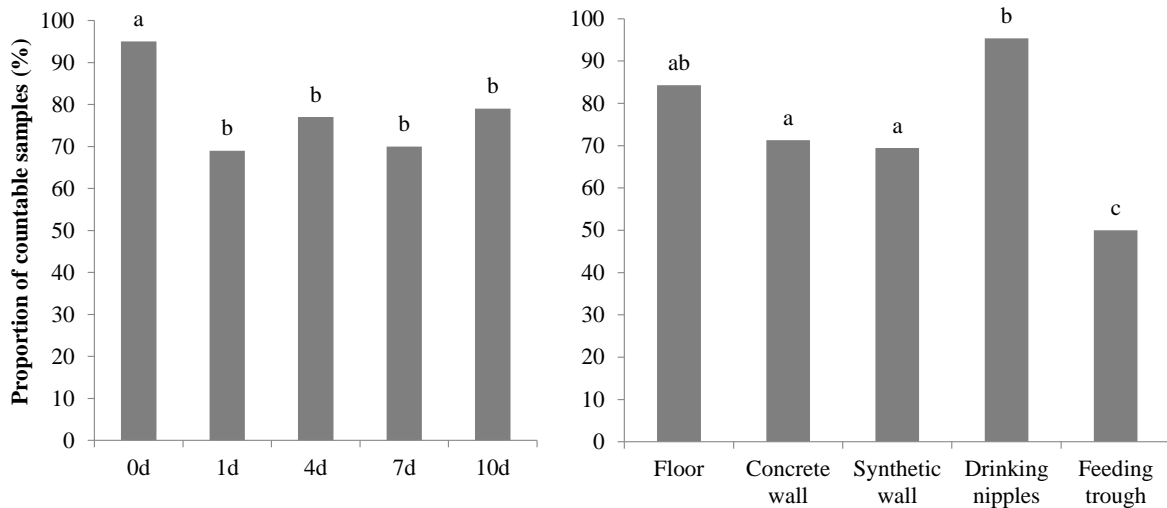
Figure VI.4:Proportion of countable samples given in percentage for Enterococcus spp.. Proportions are given for each sampling moment (a) and location after disinfection (b). Samples (n = 135) were taken before disinfection (0d) and 1 day (1d), 4 days (4d), 7 days (7d) and 10 days (10d) after disinfection. Samples (n =108) were taken from each location. Significant differences between sampling moments are indicated by different letters above bars.

Three days later (day 4), total aerobic bacteria were significantly reduced to 4.07 log CFU/sampling area ($P< 0.05$). Only 7% of the samples were positive for *E. coli*, but the number of positive samples found for faecal coliforms and MRSA were higher (25% and 14%, respectively). Countable results for *Enterococcus* spp. also increased to 77%.

On day 7 after disinfection, mean enumeration of total aerobic bacteria was 4.24 log CFU/sampling area. Of all samples, 15%, 29% and 13% were positive for *E. coli*, faecal coliforms and MRSA detection, respectively and comparable to day 1, 70% of the samples gave countable results for *Enterococcus* spp..

On day 10, total aerobic bacteria increased further to 4.64 log CFU/ sampling area, which was 0.6 log CFU more than 4 days after disinfection ($P< 0.01$), but not significantly different from day 1. Proportion of positive samples for *E. coli*, faecal coliforms and MRSA were 12%, 24% and 8%, respectively. In addition, 79% of the samples were countable for *Enterococcus* spp..

Overall, no significant differences were noticed between sampling moments after disinfection for *E. coli*, faecal coliforms, MRSA and *Enterococcus* spp..

During the entire 10-day vacancy period, the overall contamination level (total aerobic bacteria) was the highest for drinking nipples (*i.e.* 5.32 log CFU/625 cm²) ($P<0.01$) and the lowest for feeding troughs (*i.e.* 3.53 log CFU/625 cm²) ($P<0.01$) (Figure VI.2b). Results of *Enterococcus* spp. confirmed these observations ($P<0.01$) and also showed that the floors were still highly contaminated (*i.e.* still 84% of the samples were positive) ($P<0.01$) (Figure VI.4b). Results for *E. coli*, faecal coliforms and MRSA did not indicate the most critical locations after cleaning and disinfection (C&D) (Figure VI.3b).

During the vacancy period, the mean temperature ranged from 15 °C to 16 °C and RH from 57% to 67% (Figure VI.5).
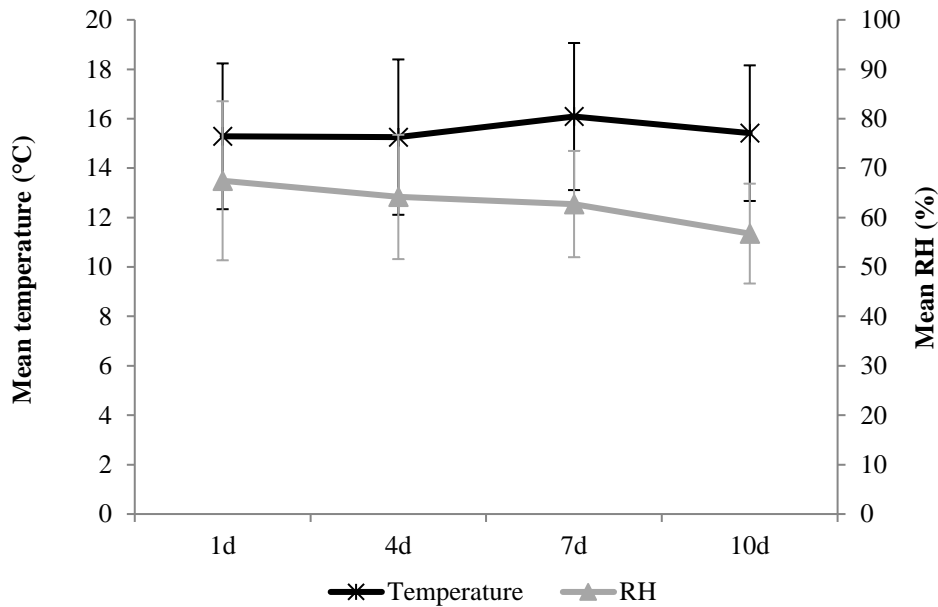
Figure VI.5: Mean temperature (°C) and relative humidity (RH, %) with standard deviations given per sampling moment. Sampling moments: day 1 (1d), 4 (4d), 7 (7d), 10 (10d) after disinfection.

5. Discussion

Biosecurity measures, such as cleaning and disinfection (C&D) and a prolonged vacancy period of the animal houses are an essential part of the hygiene management on the farm to prevent disease outbreaks. The effect of a vacancy period of 10 days after disinfection on several bacteriological parameters was examined during this study.

Disinfection reduced the total aerobic flora by 1.2 log CFU/ sampling surface. During the following 10 day vacancy, only a small reduction was observed on day 4, though this seemed microbiological negligible (maximum difference of 0.6 log CFU/625 cm²). One possible explanation for the observed small fluctuations and the decline of total aerobic flora on day 4 is that some bacteria can survive stressful conditions by entering a viable but nonculturable state (M. D. Winfield and Groisman, 2003). These nonculturable bacteria were not enumerated nor detected by the methods used in this study. Another possible explanation is that residual flora could proliferate again after disinfection, due to lack of niche and nutrient competition with other bacteria. These residual bacteria could have survived the disinfection step by the presence of a resistance mechanism (Callens et al., 2013; Mateu and Martin, 2001; Russell,

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1998; Soumet et al., 2012) or by detrimental factors present during disinfection, such as residual organic material.

Moreover, a longer vacancy period can even have a negative effect, not only financially because of a lower number of production cycles (i.e. lower income) but also bacteriologically. For example, recontamination could occur by vectors such as vermin and rodents in case of biosecurity breaches (Dewaele et al., 2012b; Hald et al., 2004; Meerburg et al., 2007), especially when other compartments in the same building are still occupied with animals or if residual organic material (*e.g.* faeces and feed) is present after C&D. Flies may be reservoirs and vectors of several bacteria such as *Salmonella* (Dewaele et al., 2012b; Holt et al., 2007; Olsen and Hammack, 2000)*, E. coli* O157:H7 (Szalanski et al., 2004), *Staphylococcus aureus* (Owens et al., 1998) and *Streptococcus suis* type 2 (Marois et al., 2007). Wild rodents can also carry pathogens such as *Salmonella, Campylobacter, Yersinia* and MRSA ST398 (Backhans and Fellström, 2012; Dewaele et al., 2012a; L J Pletinckx et al., 2013; van de Giessen et al., 2009). As biosecurity measures are very well implemented on the pilot farm, it can be assumed that on other farms, the bacteriological load and infection pressure may even increase during vacancy.

Some bacteria can survive for long periods under various conditions in the environment, such as *Salmonella*, *Staphylococcus aureus* (including MRSA) and *Enterococcus* spp. (Kramer et al., 2006). The results from the present study indicate that a prolonged vacancy period without extra biosecurity measures creates no reduction in these bacteria. Extra biosecurity measures such as specific pathogen control programs and pest control during the vacancy period could therefore be beneficial.

Finally, the contamination levels of several locations were analysed during the vacancy period. Drinking nipples were still mostly contaminated with total aerobic flora and *Enterococcus* spp.. Luyckx et al. (2015b) showed that drinking cups are critical locations for C&D in broiler houses. Drinking water from these contaminated sources could be a possible cause for disease in animals. Therefore extra attention should be given to these locations during C&D and during the vacancy period. In addition, also disinfection of drinking lines is recommended as they can be contaminated with biofilms, including pathogenic bacteria (Gannon et al., 2012). As this study is carried out on an experimental farm, also other locations can be identified as critical locations for C&D, due to their different specific structural design or composition compared to the studied farm.

6. Conclusion

This study indicates that a vacancy period up to 10 days after cleaning and disinfection with no extra biosecurity measures has no beneficial effect on the bacterial load of total aerobic bacteria, *E. coli*, faecal coliforms, MRSA and *Enterococcus* spp. in piglet nursery units.

7. Acknowledgements

CHAPTER VII
Identification and biocide susceptibility of dominant bacteria after cleaning and after disinfection of broiler houses

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CHAPTER VII
Identification and biocide susceptibility of dominant bacteria after cleaning and disinfection of broiler houses

1. Abstract

Hygiene in animal production is key for both farm management and food safety. Cleaning and disinfection (C&D) of broiler houses is essential to manage farm hygiene. Still high levels of total aerobic flora after C&D in broiler houses are reported. However, little is known about the microbial composition after cleaning (AC) and after disinfection (AD). In addition, the question why some bacterial species/isolates are still present AD whereas other are killed remains.

The study was carried out in 4 broiler houses. Sampling was performed AC and AD. The disinfectant was based on hydrogen peroxide and peracetic acid. Enumerations were carried out for total aerobic flora, *Enterococcus* spp. and *Enterobacteriaceae*. The dominant bacteria present was assessed by (GTG)$_5$ analysis and 16S rRNA gene sequence analysis. In addition, minimum bactericidal concentration (MBC) tests were carried out on 18 selected isolates belonging to the *Enterobacteriaceae* family and 10 *Enterococcus faecium* isolates.

A wide variety of bacteria were detected AC and AD. In total, 363 and 255 isolates were identified AC and AD, respectively, resulting in a total of 109 identified species. The most dominant bacteria belonged to *Brevibacterium*, *Brachybacterium* and *Staphylococcus* AC and *Bacillus*, *Brevibacterium* and *Staphylococcus* AD. In addition, at both sampling moments, *Enterococcus faecium* was dominant amongst the *Enterococcus* spp. isolates. On the selective medium for *Enterobacteriaceae*, the genera *Enterobacter* and *Pantoea* and *Aeromonas* (non *Enterobacteriaceae*) were dominant AC while *Escherichia*, *Lelliottia* and *Pantoea* were dominant AD. In addition, species pathogenic to poultry and humans were identified not only AC but also AD. MBC results showed no trend in selection of less susceptible isolates for the used disinfectant AD compared to AC. In addition, the recommended concentration of the disinfectant (i.e. 0.5% commercial solution of hydrogen peroxide and peracetic acid) seemed too low to kill *Enterobacteriaceae*.

2. Introduction

Hygiene in animal production is key to good farm management (e.g. disease prevention) as well as meeting legal and consumer demands concerning food safety. Good hygiene practices on farms can reduce the risk of introduction and persistence of animal and zoonotic diseases. Cleaning and disinfection (C&D) of animal houses form the basis of hygiene management. Luyckx *et al.* (2015) show that the mean total aerobic flora count after cleaning and after disinfection of broiler houses was still high at 5.7 ± 1.2 log CFU/ 625 cm² and 4.2 ± 1.6 log CFU/ 625 cm², respectively. It is important to assess the risk associated with this observation for both human and broiler health. However, little is known about these residual bacteria after cleaning and disinfection (C&D). In addition, the question remains why some bacterial isolates are still present after disinfection whereas others are eliminated. One hypothesis is that isolates could have become resistant against the used disinfection compounds (Russell 1998; Soumet *et al.*, 2012). In addition, some bacterial species are intrinsically resistant to certain disinfectant compounds, often caused by cell impermeability (Russell, 1998). Further, biofilm formation by bacteria is not only a protection against disinfectants but can also induce tolerance against disinfectants (Bridier *et al.*, 2011). Organic debris (faeces, feathers, etc.) remaining after improper cleaning may also form a physical barrier that protects bacteria from disinfectants (Stringfellow *et al.*, 2009) and may have an adverse effect on disinfectants (Hoff and Akin, 1986).

The aim of this study was to better understand which general and specific dominant bacteria remain present after cleaning and after disinfection. A selection of bacteria remaining after cleaning and disinfection were investigated for their susceptibility against disinfectants.

3. Materials and methods

3.1 Cleaning and disinfection of broiler houses

The study was carried out in 4 identical broiler houses, of 5400 broilers, all located on a pilot farm (Experimental Poultry Centre, EPC) in Geel, Belgium. Broilers were raised in floor housing systems with wooden shavings as bedding material ("deep litter system"). After approximately 6 weeks of broiler production, cleaning and disinfection (**C&D**) took place. The C&D protocol consisted of 4 steps: dry cleaning, soaking with water, wet cleaning and disinfection. Immediately after removal of broilers, manure and feed were removed ("dry cleaning"). After dry cleaning, broiler houses were soaked with water overnight. On the

following day, the houses were washed and soaked for 30 minutes with a foaming cleaning product containing sodium hydroxide as active component (1% Keno™san, CID LINES, Ieper, Belgium) and warm water. Twenty-four hours later, disinfection was carried by fogging with a solution of hydrogen peroxide (220 g/L) and peroxyacetic acid (55 g/L) (D50, CID LINES, Ieper, Belgium). Three litres of the disinfectant and 6 litres of water were used per broiler house (1005 m³). According to the manufacturer, a minimum of 1 litre of the disinfectant in 4 litres of water is recommended per 1000 m³ for thermal fogging.

3.2 Sampling

Sampling was performed at the following moments during C&D:

- 24 hours after cleaning but before disinfection (**AC**)
- 24 hours after disinfection (**AD**)

Pre-moistened sponge swab samples (3M, SSL100, St-Paul, USA) with 10 mL Ringers solution (Oxoid, BR0052G, Basingstroke, Hampshire, England) were taken AC at seven predefined locations per broiler house: floor, wall, air inlet, drinking cups, pipe, drain hole and floor crack. The study of Luyckx *et al.* (2015b) showed that these locations were still mostly contaminated AC and AD with total aerobic flora, *Enterococcus* spp. and/ or *E. coli*. To neutralise the residual action of the disinfectants on the microbiological growth, 10 mL Dey Engley neutralising broth (DE broth, Sigma Aldrich, Fluka, D3435, St-Louis, MO, USA) was used to pre-moisten the sponge swab samples used AD.

A surface of 625 cm² (i.e. A4 format) was sampled whenever possible. Because the surface of a drinking cup was smaller than 625 cm², 5 drinking cups per broiler house were sampled and pooled as one sample.

3.3 Sample processing

Swab samples were first diluted with 10 mL of Buffered Peptone Water (**BPW**, Oxoid, CM0509, Basingstroke, Hampshire, England) and then homogenised by placing them in a masticator (IUL instruments, S.A., Barcelona, Spain). Prior to plating, swab samples were further diluted in 10 fold dilution series in peptone physiological salt water (Bio Trading, K110B009AA, Mijdrecht, The Netherlands) to produce countable results on the selected agar media: Plate Count Agar (**PCA**, Oxoid, CM0325) for total aerobic flora, Slanetz and Bartley (**S&B**, Oxoid, CM0377) for *Enterococcus* spp. and Violet Red Bile Glucose Agar (**VRBGA**, Oxoid, CM1082) for *Enterobacteriaceae*. PCA, S&B and VRBGA plates were incubated at 30 °C, 37 °C and 37 °C for 72 h, 48 h and 24 h, respectively.

3.4 Isolate collection

Isolates were collected from agar plates with the highest serial 10 fold dilution, representing the most dominant flora. Depending on the number of colonies on these agar plates, plates were divided into 4 (when [100-200] colonies/agar plate) or 8 areas (when >200 colonies/agar plate). Five colonies from S&B and VRBGA and 10 colonies from PCA were randomly collected from one area. In this way, colonies were randomly selected without taking their morphology into account. Colonies were streaked onto new agar plates to obtain single colonies. This process was repeated three times to obtain pure isolates. Isolates were stored as glycerol stocks at -80 °C. In total, 800 isolates were collected.

3.5 Isolate identification

From each isolate, DNA was extracted according to Stranden *et al.* (2003). On the same day, a repetitive-element PCR, i.e. polytrinucleotide $(GTG)_5$ PCR, was carried out on each DNA extract based on Calliauw *et al.* (2015). PCR products were analysed using the QIAxcel Advanced System (QIAGEN GmbH, Hilden, Germany) and QIAxcel DNA High Resolution Kit (QIAGEN) (method OM1200 with an additional 120-second separation time). For each PCR product, a QX Alignment Marker (15 bp/3 kb, QIAGEN) was included in the run. The obtained fingerprints were then clustered in BioNumerics version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) based on their similarity using UPGMA (unweighted pair group method with arithmetic averages algorithm) with 1% curve smoothing. For isolates where no $(GTG)_5$ fingerprint was obtained with DNA prepared according to Stranden *et al.* (2003), DNA was extracted additionally with GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, NA2100, Diegem, Belgium). For 182 of 800 isolates, the $(GTG)_5$ fingerprint of DNA extracted using both methods contained weak or no bands. These isolates were excluded from the study. Out of the 618 isolates included in the $(GTG)_5$ fingerprint clusters, 355 were selected for partial 16S rRNA gene analysis. They were chosen based on the occurrence of their pattern and as representatives for visually defined clusters. A minimum of 2 isolates per cluster was selected to identify each complete cluster. For identification, the 16S rRNA gene was partially amplified using universal bacterial primers 16F72 and 16R1522 according to Brosius *et al.* (1978). PCR products were analysed using the QIAxcel Advanced System and QIAxcel DNA High Resolution Kit (method OM500). QX Alignment Marker (15 bp/3 kb) was included in the run. PCR products were sequenced with both primers (Macrogen Europe, Amsterdam, the Netherlands). Sequence reads of at least 500 bp were used for further analysis in EZtaxon (Kim

et al., 2012). The species with the highest similarity ($\geq 98.5\%$) and completeness was used to identify the isolates to the putative species level. When similarity and completeness percentage was the same for different species found for one isolate, the first match of the list was used.

3.6 Minimal Bactericidal Concentrations (MBC)

The minimal bactericidal concentration (MBC) method used during this study was based on Knapp *et al.* (2015) and described below.

3.6.1 Isolate selection for MBC study

Collection of isolates on genus/species level was based on their abundance AC and AD within the *Enterobacteriaceae* and *Enterococcus* group. Moreover, isolates were chosen based on their (GTG)₅ fingerprint: when possible, isolates obtained AC and AD were selected from the same (GTG)₅ cluster. A total of 18 isolates (9 AC and 9 AD) of the *Enterobacteriaceae* group were selected for MBC tests: 3 *Pantoea agglomerans* (AC), 2 *Escherichia vulneris* (AC), 5 *Lelliottia amnigena* (2 AC and 3 AD), 4 *Enterobacter soli* (2 AC and 2 AD), 3 *Escherichia albertii* (AD) and 1 *Pantoea rodasii* (AD). Isolates were obtained from drinking cups, pipes and drain holes in the 4 investigated broiler houses. In addition, 10 *Enterococcus faecium* isolates (5 AC and 5 AD, i.e. the most dominant species of the *Enterococcus* spp. group AC and AD) from the same (GTG)₅ cluster ($> 90\%$ related) were selected. The isolates were isolated from 3 of the broiler houses at the following locations: floor, air inlets, drinking cups, pipes and floor cracks.

3.6.2 Optical density versus enumeration

An optical density (OD_{600}) range was calculated for each species, to determine at which OD_{600} $1 - 5 \times 10^8$ CFU bacteria/ mL were present, according to (Knapp, 2014).

3.6.3 Neutralisation efficacy

The neutralising efficacy of DE broth was tested against disinfectant D50. One millilitre liquid bacterial culture ($1 - 5 \times 10^8$ CFU/ mL) was added to a solution of one mL 0.5 % (v/v) D50 and 8 mL DE broth and left in contact for 5 min (Knapp, 2014). As positive and negative control, disinfectant was replaced by 1 mL Ringers solution and DE broth by 8 mL Ringers solution, respectively. Because ≤ 1 log difference in CFU/mL was observed between initial counts of liquid bacterial culture and counts taken after bacterial exposure to biocide treated with neutraliser, DE broth was considered effective to neutralise the disinfectant. No growth was observed when DE broth was replaced by Ringers solution.

3.6.4 Test inocula

The selected isolates were grown on PCA (i.e. *Enterobacteriaceae*) or S&B (*Enterococcus faecium*) and incubated 24 h and 48 h at 37°C, respectively. Three different colonies per agar plate were each grown in 10 mL Tryptone Soya Broth (TSB, Oxoid, CM1108) at 37 °C during 16 h to obtain fresh liquid cultures. Subsequently, cultures were centrifuged at $5,000 \times$ g for 10 min and resuspended in Ringers solution to an OD_{600} corresponding to a viable count of $1 - 5 \times 10^8$ CFU bacteria/ mL. As control, enumerations on PCA or S&B were carried out.

3.6.5 MBC

Tests were carried out in 96 microtiter plates with U-shaped bottoms (Novolab, KIM650111). To test the reproducibility of the assay, one isolate was tested on 3 different occasions in triplicate. The other isolates were tested in triplicate. Microtiter plates contained dilutions of D50 (end concentration: 1.0 % - 0.03125 % (v/v); 0.5% is the recommended concentration according to the manufacturer for killing bacteria) in TSB. Fifty microlitres of test inocula ($1 - 5 \times 10^8$ CFU bacteria/ mL) were added resulting in a total volume of 100 µL per well. Plates were incubated at 37 °C during 24 h. After incubation, 20 µL of each suspension was transferred into 180 µL DE broth and left in contact for 5 min. Subsequently, 12.5 µL of each suspension was spotted in duplicate on agar plates and incubated at 37°C. The MBC was defined as the lowest concentration of D50 at which no bacterial growth was observed on the agar plate. When triplicates of one isolate showed different MBC, the highest MBC result was reported.

Statistical analysis

Statistical analyses were carried out with Statistical Analysis System software (SAS®, version 9.4, SAS Institute Inc., Cary, NC, USA). The proportion of isolates belonging to a certain genus versus the total number of isolates collected AC or AD was compared between both sampling moments using Fisher's exact test (in case of a frequency <5) or a chi-square test (in case of all frequencies >5). In addition, the proportion of *Enterobacteriaceae* isolates surviving the 0.5% disinfectant solution was compared between sampling moments using Fisher's exact test.

4. Results

4.1 Bacteriological analysis

Of all samples taken AC, 100 %, 100% and 25% were countable for total aerobic bacteria, *Enterococcus* spp. and *Enterobacteriaceae*, respectively. Of these countable samples, the mean

enumeration was 5.87 ± 0.75 log, 4.09 ± 0.52 log and 3.04 ± 1.98 log CFU/sampling surface, respectively. In addition, 280, 140 and 26 colonies per medium were isolated, respectively. After disinfection, 93%, 64% and 18% of the samples gave countable results for total aerobic bacteria, *Enterococcus* spp. and *Enterobacteriaceae*, respectively. The mean countable enumeration was 4.47 ± 1.43 log, 2.78 ± 0.94 log and 3.11 ± 1.15 log CFU/sampling surface, respectively. In total 354 colonies were isolated AD: 249, 82 and 23 colonies per medium, respectively.

4.2 Identification results

Identification results (family, genera, species) of isolates dominantly present on VRBGA, S&B and PCA are given in Tables VII.1, VII.2 and VII.3, respectively. In addition, the mean log CFU enumeration of agar plates from which isolates were collected was calculated and subsequently classified into one of the 3 abundance classes. Finally, the obtained *P*-values, using Fisher's exact or a chi-square test, are given.

4.2.1 <u>Isolates from VRBGA</u>

Genera *Enterobacter* and *Pantoea* (both *Enterobacteriaceae*); and *Aeromonas* (non *Enterobacteriaecea*) were most abundant AC and *Escherichia*, *Lelliottia* and *Pantoea* (all *Enterobacteriaceae*) were most abundant AD. In addition, *Curtobacterium* (not belonging to the *Enterobacteriaecea* family) grew on the selective medium VRBGA.

No significant changes were observed between proportions of isolates identified as *Enterobacter*, *Escherichia, Leclercia, Lelliottia* and *Pantoea* AC and AD.

Most isolates were isolated from samples originating from drain holes (58% AC and 85% AD). Other than drain holes, samples (and thus isolates) also originated from floors (4% AC and 15% AD), drinking cups (21% AC), air inlets (4% AC) and pipes (13% AC).

Table VII.1: Family, genera and species isolated from Violet Red Bile Glucose Agar (VRBGA, Enterobacteriaceae selective medium) after cleaning (AC) and after disinfection (AD). Species pathogenic for poultry and/or humans are indicated by bold and/or underlined text, respectively. In addition, the magnitude of mean enumeration of samples whereof bacteria were isolated, is indicated by an X in one of the 3 abundance classes.

Family	Organism	AC				AD				P-value[4]
		%[1]	Classes (log)[3]			%[2]	Classes (log)[3]			
			<1.5	[1.5 - 3[	>3		<1.5	[1.5 - 3[	>3	
Gram negative										
Enterobacteriaecae	*Citrobacter gillenii*	4.17			X	n.i.				
	Enterobacter cancerogenes*, kobei, soli*	16.67			X	15.00			X	P=1.00
	Erwinia persicina	4.17			X	n.i.				
	*Escherichia **albertii** and vulneris*	12.5	X			25.00		X		P=0.43
	Leclercia adecarboxylata	4.17	X			10.00			X	P=0.58
	Lelliottia amnigena	8.33		X		30.00			X	P=0.11
	*Pantoea **agglomerans** and rodasii*	16.67		X		20.00		X		P=1.00
	Providencia rettgeri	4.17		X		n.i.				
	Siccibacter turicensis	4.17			X	n.i.				
Aeromonadaceae	*Aeromonas **hydrophila** subsp. hydrophila and media*	20.83			X	n.i.				
Gram positive										
Microbacteriaceae	*Curtobacterium plantarum*	4.17		X		n.i.				

[1] Ratio between number of isolates within one genus and total number of identified isolates AC (n= 24) given in percentage; [2] Ratio between number of isolates within one genus and total number of identified isolates AD (n= 20) given in percentage; [3] Classes are given in log CFU/ sampling surface; [4] Fisher's exact or a chi-square test was carried out for the genera that were identified both AC and AD. The obtained P-values are given. Significant values are indicated with bold characters; n.i., not identified; *, One isolate had a match with other species with same similarity/completeness percentage.

4.2.2 Isolates from S&B

Enterococcus faecium was the most dominant species belonging to the genera *Enterococcus* on S&B both AC and AD, with mean enumerations between 2 and 4 log CFU/sampling surface. A significant decrease was observed between the proportion of isolates identified as *Enterococcus faecium* AC and AD. Nonspecific genera *Aerococcus*, *Desemzia* and *Staphylococcus* (representing the majority within the nonspecific genera) were also found on S&B. In addition, a significant increase in proportion of isolates identified as *Staphylococcus* was observed AD. *Staphylococcus* spp. isolates from AD originated from all locations, while *Enterococcus* spp. isolates were mostly isolated from drain holes. An exception was *Enterococcus faecium* isolates, which also originated from floors, air inlets and pipes.

Table VII.2: Family, genera and species of bacteria isolated from Slanetz and Bartley (Enterococcus spp. selective medium) after cleaning (AC) and after disinfection (AD). Species pathogenic for poultry and/or humans are indicated by bold and/or underlined text, respectively. In addition, the magnitude of mean enumeration of samples whereof bacteria were isolated, is indicated by an X in one of the 3 abundance classes.

Family	Organism	AC %[1]	Classes (log)[3] <2	[2 - 4[	>4	AD %[2]	Classes (log)[3] <2	[2 - 4[	>4	P-value [4]
Gram positive										
Enterococcaceae	*Enterococcus alcedinis*	n.i.				1.25			X	
	Enterococcus avium	0.72			X	n.i.				
	Enterococcus casseliflavus*	5.80		X		1.25			X	P=0.16
	*Enterococcus **durans***	0.72		X		n.i.				
	*Enterococcus **faecalis***	7.25			X	1.25			X	P=0.06
	*Enterococcus **faecium****	23.91		X		6.25		X		**P<0.01**
Aerococcaceae	*Aerococcus viridans/ urinaeequi* [e]	12.32			X	15.00			X	P=0.68
Carnobacteriaceae	*Desemzia incerta*	n.i.				1.25		X		
Staphylococcaceae	*Staphylococcus saprophyticus* subsp. *bovis/ **arlettae*** [e]	49.28		X		73.75		X		**P<0.01**

[1] Ratio between number of isolates within one genus and total number of identified isolates AC (n= 138) given in percentage; [2] Ratio between number of isolates within one genus and total number of identified isolates AD (n= 80) given in percentage; [3] Classes are given in log CFU/ sampling surface; [4] There is no difference between 16S rRNA gene sequences of these species; [4] Fisher's exact or a chi-square test was carried out for the genera that were identified both AC and AD. The obtained P-values are given. Significant values are indicated with bold characters; n.i., not identified; *, One isolate had a match with other species with same similarity/completeness percentage.

4.2.3 <u>Isolates from PCA</u>

Among the Gram positive isolates (n=259) isolated from PCA, 14 families were found representing 19 genera. Gram negative bacteria (n=97) belonged to 13 families representing 16 genera. The most dominant genera found on PCA were *Brevibacterium*, *Brachybacterium* and *Staphylococcus* AC and *Brevibacterium*, *Microbacterium* and *Staphylococcus* AD.

The proportion of isolates identified as *Bacillus*, *Brevibacterium* and *Microbacterium* significantly increased AD. In contrast, the proportion of isolates identified as *Staphylococcus* and *Comamonas* significantly decreased AD.

The obtained isolates originated from floors (14% and 18%), walls (11% and 10%), air inlets (15% and 15%), drinking cups (13% and 19%), pipes (15 % and 11%), drain holes (16% and 17%) and floor cracks (15% and 9%) AC and AD, respectively. Per sampling point, 4 to 9 genera were found AC, and 6 to 12 genera AD.

Table VII.3: Family, genera and species of bacteria isolated from Plate Count Agar (total aerobic flora) after cleaning (AC) and after disinfection (AD). Species pathogenic for poultry and/or humans are indicated by bold and/or underlined text, respectively. In addition, the magnitude of mean enumeration of samples whereof bacteria were isolated, is indicated by an X in one of the 3 abundance classes.

Family	Organism	AC				AD				P-value[4]
		%[1]	Classes (log)[3]			%[2]	Classes (log)[3]			
			<3	[3- 6[	>6		<3	[3- 6[	>6	
Gram positive										
Bacillaceae	Bacillus endophyticus and galactosidilyticus	1.00		X		7.74		X		**P<0.01**
	Psychrobacillus psychrodurans	n.i.				0.65	X			
Brevibacteriaceae	Brevibacterium oceani*, casei, **avium**, epidermidis, iodinum and permense	10.45		X		18.06		X		**P=0.04**
Corynebacteriaceae	Corynebacterium stationis	n.i.				1.29	X			
Deinococcaceae	Deinococcus ficus	n.i.				2.58		X		
Dermabacteraceae	Brachybacterium nesterenkovii and paraconglomeratum	7.96		X		6.45		X		P=0.59
Dietziaceae	Dietzia aurantiaca	n.i.				0.65		X		
Intrasporangiaceae	Janibacter melonis	1.49	X							
Leuconostocaceae	Weissella thailandensis	n.i.				0.65		X		
Microbacteriaceae	Microbacterium esteraromaticum, lactis, mitrae, paraoxydans, phyllosphaerae and testaceum	2.99		X		12.26		X		**P<0.01**
Micrococcaceae	Aerococcus viridans and urinaeequi	1.00			X	2.58		X		P=0.41
	Arthrobacter bergerei, creatinolyticus and oryzae	3.48			X	0.65		X		P=0.14
	Kocuria gwangalliensis and palustris	4.48		X		3.87		X		P=0.78
	Micrococcus endophyticus	0.50		X		n.i.				
Nocardioidaceae	Nocardioides daedukensis	0.50		X		n.i.				
Promicromonosporaceae	Cellulosimicrobium cellulans	n.i.				1.29		X		
Staphylococcaceae	Macrococcus caseolyticus	0.50		X		n.i.				
	Staphylococcus **arlettae**, caprae, cohnii subsp cohnii, equorum subsp. equorum, lentus, saprophyticus subsp. saprophyticus and simulans	39.80		X		9.68		X		**P<0.01**
Streptococcaceae	Streptococcus australis*, pseudopneumoniae and sanguinis	n.i.				2.58	X			
Gram negative										
Aeromonadaceae	Aeromonas hydrophila subsp hydrophila*	0.50			X	n.i.			X	
Alcaligenaceae	Alcaligenes **faecalis** subsp faecalis	n.i.				2.58				

Caulobacteriaceae	*Brevundimonas diminuta, intermedia*, naejangsanensis*, nasdae**, terrae, vancanneytii* and *vesicularis*	3.98	X		3.23	X	X	*P*=0.78
Comamonadaceae	*Comamonas jiangduensis* and *koreensis*	5.97		X	1.29	X		**P=0.03**
	variovorax paradoxus	n.i.			2.58	X		
Flavobacteriaecae	*Chryseobacterium arthrosphaerae*	1.00		X	n.i.			
Moraxellaceae	*Acinetobacter beijerinckii*, bouvetti, indicus, **lwoffii** and oryzae*	3.98		X	5.16			*P*=0.59
	Enhydrobacter aerosaccus	1.00	X		1.94	X		*P*=0.66
Neisseriaceae	*Prolinoborus fasciculus*	1.00		X	0.65		X	*P*=1.00
Pseudomonadaceae	*Pseudomonas japonica, libanensis, montelli*, putida* and *rhizosphaerae*	2.49		X	1.29		X	*P*=0.70
Rhizobiaceae	*Rhizobium massilae* and *radiobacter*	1.00	X		0.65	X	X	*P*=1.00
Rhodobacteraceae	*Paracoccus huijuniae, sediminis, siganidrum* and *yeei*	1.49	X		4.52	X		*P*=0.11
Sphingobacteriaceae	*Sphingobacterium faecium hotanense, kyonggiense, lactis* and *multivorum*	2.49		X	3.23	X		*P*=0.75
Sphingomonadaceae	*Novosphingobium anipatense*	n.i.			0.65	X		
	Sphingomonas hankookensis and panni				1.29	X		

[1] Ratio between number of isolates within one genus and total number of identified isolates AC (n= 201) given in percentage; [2] Ratio between number of isolates within one genus and total number of identified isolates AD (n= 155) given in percentage; [3] Classes are given in log CFU/ sampling surface; [4] Fisher's exact or a chi-square test was carried out for the genera that were identified both AC and AD. The obtained P-values are given. Significant values are indicated with bold characters; n.i., not identified; *, One isolate had a match with other species with same similarity/completeness percentage; **, Three isolates had a match with other species with same similarity/completeness percentage

4.3   MBC of *Enterobacteriaceae* isolates

MBC results for *Enterobacteriaceae* isolates are given in Table VII.4. The MBC of the disinfectant for all *Enterobacter* and *Escherichia* isolates, independent of being isolated AC or AD, was 1%. More diversity in MBC within *Pantoea* and *Lelliottia* species was noticed. MBC method was highly reproducible for the *Lelliottia* isolate tested in triplicate on 3 different occasions. Of the tested *Enterobacteriaceae* isolated AC and AD, 62.5% and 70% survived exposure to 0.5% disinfectant, respectively (*P*>0.05).

Table VII.4: Minimum bactericidal concentration (MBC) results given for each Enterobacteriaceae isolate tested in triplicate.

Organism	Survival at different concentrations (% solution of hydrogen peroxide and peracetic acid)					
	0.03125	0.0625	0.125	0.25	0.5	1
Isolated AC						
Pantoe agglomerans 1	3 [1]	**0** [3]	0	0	0	0
Pantoe agglomerans 2	3	**0**	0	0	0	0
Pantoe agglomerans 3	3	3	3	3	**0**	0
*Lelliottia amnigena*1	3	3	3	2	1	**0**
*Lelliottia amnigena*2	3	3	**0**	0	0	0
Enterobacter soli 1	3	3	3	3	3	**0**
Enterobacter soli 2	3	3	3	3	3	**0**
Escherichia vulneris 1	3	3	3	3	3	**0**
Escherichia vulneris 2	3	3	3	3	3	**0**
Isolated AD						
Pantoea rodasii 1	3	3	3	3	3	**0**
Lelliottia amnigena 3	3	3	3	3	**0**	0
Lelliottia amnigena 4	3x3 [2]	3x3	3x3	3x3	**0**	0
Lelliottia amnigena 5	3	3	3	**0**	0	0
Enterobacter soli 3	3	3	3	3	3	**0**
Enterobacter soli 4	3	3	3	3	3	**0**
Escherichia albertii 1	3	3	3	3	3	**0**
Escherichia albertii 2	3	3	3	3	3	**0**
Escherichia albertii 3	3	3	3	3	3	**0**

[1] 3, Three of the three tested replicates of a single isolate gave same survival results; [2] 3x3, Three of the three tested replicates of a single isolate gave same survival results on three different occasions (*i.e.* reproducibility test); [3] Digits in bold correspond to concentration with no survival of any of the three replicates, corresponding with the MBC

4.4 MBC of *Enterococcus faecium* isolates

MBC results for *Enterococcus* spp. isolates are given in Table VII.5. MBC of the disinfectant for all tested *Enterococcus faecium* isolates was either 0.0625 or 0.125%. None of the tested isolates AC and AD survived exposure to 0.5% disinfectant.

Table VII.5: minimum bactericidal concentrations (MBC) results given for each Enterococcus faecium isolate tested in triplicate

Organism	Survival at different concentrations (% solution of hydrogen peroxide and peracetic acid)					
	0.03125	0.0625	0.125	0.25	0.5	1
Isolated AC						
Enterococcus faecium 1	3 [1]	2	**0** [2]	0	0	0
Enterococcus faecium 2	3	3	**0**	0	0	0
Enterococcus faecium 3	3	3	**0**	0	0	0
Enterococcus faecium 4	3	**0**	0	0	0	0
Enterococcus faecium 5	3	**0**	0	0	0	0
Isolated AD						
Enterococcus faecium 6	3	**0**	0	0	0	0
Enterococcus faecium 7	3	**0**	0	0	0	0
Enterococcus faecium 8	3	2	**0**	0	0	0
Enterococcus faecium 9	3	**0**	0	0	0	0
Enterococcus faecium 10	3	**0**	0	0	0	0

[1] 3, Three of the three tested replicates of a single isolate gave same survival results; [2] Digits in bold correspond to concentration with no survival of any of the three replicates, corresponding with the MBC

5. Discussion

The identification of bacteria in broiler houses is key to better understanding the dynamics of bacteria during C&D and knowing the impact of the residual bacteria on the health of both animals and humans. Enumerations of total aerobic flora, *Enterococcus* spp. and *Enterobacteriaceae* were carried out after cleaning and after disinfection. Similar results as in the study of Luyckx *et al*. (2015a) were obtained for total aerobic flora and *Enterococcus* spp.. Although the number of countable samples for *Enterobacteriaceae* was reduced by disinfection, mean enumerations on the countable samples were not decreased, also shown by Ward *et al*. (2006). In addition, the dominant species of the families *Enterobacteriaceae* and *Enterococcaceae* and total aerobic flora were identified after cleaning and after disinfection.

The genera *Pantoea* (AC and AD), *Lelliottia* (AD)*, Enterobacter* (AC) and *Escherichia* (AD) were the most dominant *Enterobacteriaceae* isolated from VRBGA. No significant increase or decrease in the proportion of isolates belonging to these genera, was observed between the two sampling moments. Several studies show the presence of these genera in the poultry industry

(Morgan-Jones 1981; Kilonzo-Nthenge *et al.*, 2008; Oaks *et al.*, 2010; Bródka *et al.*, 2012; Gole *et al.*, 2013). Within the genus *Pantoea*, *Pantoea agglomerans* (previously known as *Enterobacter agglomerans*) is the most commonly isolated species in humans, originating from soft tissue or bone/joint infections (Cruz *et al.*, 2007). In addition, *Pantoea agglomerans* has also been isolated from cellulitis lesions in chickens, but these are not believed to be significant (Derakhshanfar and Ghanbarpour, 2002; Vaillancourt and Barnes, 2009). *Lelliottia amnigena* (previously known as *Enterobacter amnigenus*) has been recently associated with raw broiler products (Olobatoke *et al.*, 2015) and has also been found at egg processing plants (Jones and Musgrove, 2008; Musgrove *et al.*, 2009). *L. amnigena* has also been described as a rare pathogen for humans (Bollet *et al.* 1991; Capdevila *et al.* 1998), and a causative agent of limb infections (Corti *et al.*, 2009). *Escherichia albertii* (AD) was found in moderate numbers during this study. *E. albertii* has been reported to be a potential pathogen for humans and animals (Oaks *et al.*, 2010; Oh *et al.*, 2011). Oaks *et al.* (2010) findings indicate that *E. albertii* is likely pathogenic to birds including chickens, and can be associated with epornithics and sporadic disease. *Escherichia vulneris* has been isolated from animals, humans, the environment, and potable water. *E. vulneris* can colonise the respiratory tract, female genital tract, urinary tract, and stool in humans (Shobrak and Abo-Amer, 2014). After cleaning, the genus *Aeromonas* (non *Enterobacteriaceae*) was also isolated in high numbers from VRBGA. *Aeromonas hydrophila* can occasionally cause diarrhoea in broilers. This species has significance for public health, usually through contaminated poultry meat, because it causes gastroenteritis in humans (Barnes, 2003). On VRBGA a larger variety of species was found AC compared to AD.

In conclusion, the 4 dominant genera belonging to *Enterobacteriaceae* identified in this study have been previously linked to the poultry industry. Several species belonging to this family are pathogenic for both poultry and humans. This confirms the importance of reducing *Enterobacteriaceae* as much as possible during C&D.

The most dominant species of *Enterococcus* were *E. faecium* (AC and AD), *E. faecalis* (AC) and *E. casseliflavus* (AC). The proportion of isolates identified as *E. faecium* was significantly reduced AD. All three species have previously been isolated from broilers. *Enterococcus* spp. are generally considered commensal bacteria but do have the potential to cause infections in humans, especially *Enterococcus faecium* and *E. faecalis.* In addition, both species are reported as potential pathogens for poultry (Cauwerts *et al.*, 2007). The third dominant *Enterococcus* species, *E. casseliflavus*, has been isolated from human patients with bacteremia (Reid *et al.*, 2001). Although *Staphylococcus arlettae* and *S. saprophyticus* do not belong to the genus

Enterococcus, they were highly abundant on the *Enterococcus* specific medium, especially AD. *S. arlettae* was previously isolated from skin and nares of poultry (Schleifer *et al.*, 1984). Both species have been found in the indoor air of broiler houses (Chinivasagam et al., 2010; Devriese et al., 1985; Schulz et al., 2004). *S. saprophyticus* has also been isolated from food and food environments (Marino *et al.*, 2011). Hedman and Ringertz (1991) found that urinary tract infections caused by *S. saprophyticus* were common among professionals handling meat products. The genus *Aerococcus* has also been found in high amounts on S&B. The genus *Aerococcus* has been found in the air of poultry houses in different studies (Bródka et al., 2012; Fallschissel et al., 2010; Nielsen and Breum, 1995). *A. viridans* has also been associated with several human infections (Facklam and Elliott, 1995). On S&B, the species isolated AC, were also mostly isolated AD.

In conclusion, the 3 dominant *Enterococcus* species found in this study are generally commensal bacteria for broilers and humans. Besides these species, bacteria belonging to the two genera *Staphylococcus* and *Aerococcus* also grew abundantly on the *Enterococcus* specific medium, resulting in colonies with the same morphology as enterococci. For this reason, enumerations perfomed on S&B could result in an overestimation of *Enterococcus* spp..

Finally, the most dominant genera (i.e. >5% present AC or AD) isolated from PCA were *Bacillus* (AD), *Brevibacterium* (AC and AD), *Brachybacterium* (AC and AD), *Microbacterium* (AD), *Staphylococcus* (AC and AD), *Comamonas* (AC) and *Acinetobacter* (AD). One hypothesis that could explain the increase in the proportion of isolates belonging to *Bacillus*, *Brevibacterium* and *Microbacterium* AD, is that disinfection created an opportunity for otherwise transient species to gain dominance. In addition, the proportion of *Staphylococcus* and *Comamonas* isolates was significantly reduced by disinfection.

Spores of *Bacillus* species are found in soil, dust, and water as well as in the air (Tam *et al.*, 2006). Furthermore, studies of Bródka *et al.* (2012) and Nasrin *et al.* (2007) isolated *Bacillus* species from the air of poultry houses. Previous studies described the genus *Brevibacterium* as one of the abundant taxonomic groups in poultry litter (Dumas et al., 2011; Lu et al., 2003). In general, *Brevibacterium species* are not pathogenic for poultry, but there are known pathogenic species such as *B. avium* (Dumas et al., 2011; Pascual and Collins, 1999). In addition, *B. casei* and *B. epidermidis*, both of which were isolated AC as well as AD, have been described as a cause of bacteremia and central venous line infection in humans, respectively (Brazzola et al., 2000; Gruner et al., 1994; McCaughey and Damani, 1991). Members of the *Brachybacterium* genus have also been isolated from poultry deep litter (Lu *et al.*, 2003; Dumas *et al.*, 2011). No

reports on pathogenicity of *Brachybacterium* have been published. *Microbacterium* species have been found on freshly killed chickens (Cunningham, 1987). Another study isolated a *Microbacterium* species from poultry waste and characterised it as a feather-degrading bacterium (Sangali and Brandelli, 2000; Thys *et al.*, 2004). *M. paraoxydans*, which was isolated both AC and AD, is one of the most frequently isolated microbacteria in human clinical specimens (Gneiding et al., 2008; Laffineur et al., 2003). Besides the 2 abovementioned *Staphylococcus* species, also *S. caprae, S. cohnii, S. lentus and S. simulans* were isolated in this study from PCA and are described as potential pathogens for humans (Mallet et al., 2011; Mazal and Sieger, 2010; Seng et al., 2014; Soldera et al., 2013). The 4 latter species have been isolated from the air originating from broiler houses (Chinivasagam et al., 2010; Devriese et al., 1985). De Reu *et al.* (2006, 2008) also found *Staphylococcus* spp. to be the dominant bacterial flora in the air of laying hen houses and on eggshells. The members of the genus *Comamonas* frequently occur in diverse habitats, such as animal and plant tissues (Ma *et al.*, 2009). To our knowledge no studies have yet revealed the presence of *Comamonas* species in poultry houses. Members of the genus *Acinetobacter* are usually commensal organisms, but can cause infections in susceptible human patients (Dahiru and Enabulele, 2015). Schefferle (1965) found *Acinetobacter* on feathers of poultry and suggested they may originate from deep litter. In addition, *Acinetobacter* species (psychrotrophic spoilage bacteria) are often found on chicken carcasses (Russel, 2010). *A. lwoffii* can cause bacteremia in immunocompromised individuals (Ku *et al.*, 2000). This species has also been involved in several infections in animals, e.g. severe respiratory symptoms in lovebirds (Robino *et al.*, 2005) and septicaemia in hens (Kaya *et al.*, 1989). Other species found in this study with clinical significance for animals are *Enterococcus durans* and *Alcaligenes faecalis*. *Enterococcus durans* can cause bacteremia and encephalomalacia in young chickens (Cardona *et al.*, 1993) and septicaemia and endocarditis in mature birds (Chadfield *et al.*, 2004). *Alcaligenes faecalis* can cause respiratory disease in chickens (Berkhoff et al., 1984, 1983; Simmons et al., 1981).

In conclusion, most of the dominant genera found on PCA, have been previously isolated from (the environment of) poultry. Several pathogens for poultry and humans were isolated AC and even AD. Surprisingly, the genus *Comamonas* was found to be dominant in this study, while to our knowledge, no studies have reported the occurrence of these bacteria in poultry houses.

Because the samples originated from only one pilot farm, conclusions should be drawn with caution. Other factors such as sampling method, meteorological conditions, type of broiler

house and topographic features could also affect the bacterial composition. Additional, studies are needed to verify these results.

The presence of several pathogenic species for poultry and humans not only AC but also AD, indicates that the disinfection step was not able to kill these organisms. Luyckx *et al.* (2015a) also reported the limited reduction of bacterial flora by disinfection in broiler houses. Possible reasons are interference with residual organic matter (Hoff and Akin, 1986), reduced effect of the disinfection step in practice or resistance against the disinfectant (Russell 1998; Soumet *et al.*, 2012). To test this last hypothesis, MBC was determined for several *Enterobacteriaceae* isolates and *Enterococcus* spp. isolates obtained AC and AD. The MBC results did not suggest a selection towards less susceptible isolates AD compared to AC at a concentration of 0.5%. Gram negative bacteria such as *Enterobacteriaceae* are generally more resistant to disinfectants than Gram positive bacteria because they have an outer membrane (Knapp, 2014; McDonnell and Russell, 1999; Nikaido and Vaaro, 1987). However, it has been shown that enterococci can be more resistant than Gram negative bacteria to disinfectants (Bradley and Fraise, 1996; Eginton et al., 1998; Gradel, 2007, 2004). In this study, more than 77.8% of the tested *Enterobacteriaceae* isolates showed a MBC of $\geq 0.5\%$, while all *Enterococcus faecium* isolates showed a MBC of ≤ 0.125. These results indicate that *Enterobacteriaceae* isolates are more resistant to the used disinfectant than *Enterococcus* spp.. This finding is in agreement with Dewaele *et al.* (2011), who showed that *E. coli* was more resistant than *Enterococcus faecalis*, although other disinfectants were tested in that study. As the *Enterococcus faecium* isolates were susceptible to the recommended concentration, the presence of *Enterococcus* spp. and other bacteria AD could be due to the presence of either extraneous material (e.g. organic material), which has a detrimental effect on the disinfectant, or residual water, resulting in dilution of the disinfectant. Moreover, the recommended concentration of the disinfectant (i.e. 0.5%) seemed too low to kill *Enterobacteriaceae*, including pathogenic species for poultry and humans found in this study such as *Escherichia albertii* and *Pantoea agglomerans*. As the recommended concentration of the disinfectant was not able to kill the field isolates in the MBC test, it can be assumed that the recommended concentration of 0.5% of the disinfectant is too low for farm conditions.

Furthermore, literature reports that many bacteria have developed resistance that confer tolerance to peroxide stress (in particular hydrogen peroxide), which includes production of neutralizing enzymes (e.g. catalases, peroxidases and glutathione reductases) (Baureder et al., 2012; Harris et al., 2002; McDonnell and Russell, 1999; Uhlich, 2009). In addition, Dubois-

Brissonnet *et al.* (2011) have demonstrated increased tolerance to peracetic acid by a membrane modification of *Salmonella enterica*. The survival of the *Enterobacteriaceae* isolates might also be the result of such a resistance mechanism among the present bacteria. This needs to be determined in future research.

6. Acknowledgements

CHAPTER VIII
General discussion

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General discussion

According to World Health Organization (WHO), hygiene refers to conditions and practices that help to maintain health and prevent the spread of diseases (WHO, 2016). Hygiene in animal production is key for both farm management (*e.g.* disease prevention) and meeting legal and consumer demands concerning food safety. Biosecurity practices on farms include external and internal measures that minimise horizontal transmission of infectious agents. Among internal biosecurity measures, cleaning and disinfection (C&D) between production rounds and after replacement or transport of animals is a crucial measure and has been shown to be of high importance for the prevention of diseases (Gelaude *et al.*, 2014; Postma *et al.*, 2015). Therefore, in this thesis, the focus was on C&D in broiler and pig facilities, as the broiler and pig production contribute the most to the global, European and Belgian meat production.

Aspects to consider when monitoring the efficacy of C&D on farms

Before comparing different C&D protocols, it is necessary to accurately measure the efficacy of these interventions. To do so, the optimal locations and sampling methods need to be determined and proper bacteriological parameters should be used.

1. Locations and surfaces

Inadequately cleaned and disinfected locations in animal houses and equipment may act as a source of infection for new arriving animals. To break the cycle of infection, it is important to identify locations in broiler and pig houses that are difficult to clean and disinfect in order to improve the C&D protocol as well as to identify the locations for evaluation of the efficacy of the C&D protocol.

In this thesis, several locations were identified as critical locations for C&D due to their structure (chapter IV, V and VI). Drain holes as well as floor cracks were identified as critical locations in broiler houses in chapter IV and by previous studies in other types of animal houses (Dewaele *et al.*, 2012b; Mueller-Doblies *et al.*, 2010; Rajic *et al.*, 2005). These locations can remain soiled because of the difficult access for cleaning and they are often still filled with water when disinfected. The residual organic material protects the bacteria from contact with the disinfectants, affects the action of disinfectants and is a source of nutrients for surviving

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bacteria. Therefore, floor cracks should be regularly repaired by filling, whereas drain holes should be adequately rinsed after cleaning to flush residual organic material. Moreover, it is advisable to disinfect these locations twice as several studies showed that two disinfection rounds, rather than a single treatment, are more efficient in eliminating pathogens such as *Salmonella* (Gradel and Rattenborg, 2003; Huneau-Salaün *et al*., 2010; Rose *et al*., 2000). This thesis also showed that drinking cups are critical locations for C&D of broiler houses (chapter IV). Because of their fragile and angular construction, drinking cups are difficult to clean (Figure VIII.1). Moreover, these cups are often filled with water after cleaning, which subsequently dilutes the applied disinfectant. Therefore, farmers should empty drinking cups by turning the drinking lines before disinfection. In pig nursery units, slatted floors and drinking nipples were found to be critical locations (chapter V and VI). Slatted floors and drinking nipples are difficult to clean due to their specific design including many edges. Moreover, since the quality of drinking water is crucial for profitable production of animals, both the drinking water as well as the dispensers should to be pathogen-free. Besides the identified critical locations in the tested nursery units, probably others exist as there is a great variety in housing designs in pig production. To identify and list all of these, more similar studies, such as done in this thesis, should be performed in different housing systems.

Figure VIII.1: Soiled drinking cups in broiler houses after disinfection. Source: Kaat Luyckx – CLEANDESOPT project

In contrast, as ATP values (after cleaning) and bacterial counts (after cleaning and after disinfection) were the lowest for feed hoppers, roofs and air outlets in broiler houses, they seemed to be the most hygienic locations (chapter IV). These locations are however not in direct contact with broilers during the production and consists of smooth surfaces which makes them easy to clean. After disinfection of pig nursery units, the bacterial load (*i.e.* total aerobic bacteria

and *Enterococcus* spp.) was the lowest for feeding troughs, possibly due to their metal, easily cleanable surface (chapter VI). However, this should be interpreted with caution, as only one farm was sampled. In addition, during sampling the feeding troughs were in some cases still filled with feed (during production), therefore a sampling surface was chosen which was not in direct contact with feed but could be in contact with piglets. It could be possible that the surfaces normally covered by feed, and not sampled in our studies, are critical locations for C&D.

It is known that the composition and structure of materials and design of animal houses can be quite diverse and that their cleanability has an impact on the C&D efficacy. For example, there is a difference in the efficacy of C&D of battery-cage houses and on-floor houses, as battery-cage houses are more difficult to clean (Davies and Breslin, 2003b; Gradel *et al*., 2003; Huneau-Salaün *et al*., 2010). Besides, wooden surfaces may be more difficult to clean than metal or plastic surfaces, likely because of the porous nature of wood (Rathgeber *et al*., 2009). Also, concrete is often affected by numerous environmental factors, such as wear caused by animals and vehicles and chemical degradation caused by feeds and manure (Kymalainen *et al*., 2009), making them difficult to clean and disinfect.

In order to ascertain if a C&D protocol is capable of eliminating infectious agents in animal houses, it is recommended to include the critical locations in the sampling scheme. As the most hygienic locations had mean total aerobic bacteria enumerations below 3.5 log CFU/625 cm² after disinfection, this indicates that one should strive to achieve mean enumerations of ± 3.5 log CFU/625 cm² after C&D throughout the animal house. On the other hand, the cleanest locations may be omitted from the sampling scheme for broiler houses, which will reduce the working load and costs. However, it would be interesting to include locations that are in direct contact with animals (*e.g.* walls and feed pans) as they are of great importance in the spread of pathogens when still contaminated after C&D.

2. **Methods to assess the hygiene status**

Most studies concentrate on finding a suitable sampling method for the recovery of a specific pathogen in a specific environment, *e.g. Salmonella* in poultry houses (Carrique-Mas and Davies, 2008), *Listeria monocytogenes* in food industry (Lahou and Uyttendaele, 2014), *Legionella* (Ta *et al*., 1995) and methicillin resistant *Staphylococcus aureus* (MRSA; (Dolan *et al*., 2011)) in human hospitals, etc.. However, little research is performed to compare different sampling methods to assess the overall hygiene status during C&D of an environment.

Therefore, the aim of the study described in chapter III was to select suitable sampling methods to evaluate C&D in broiler houses (chapter IV) and pig nursery units (chapter V and VI).

- Before cleaning

In chapter III, we showed that swab samples of a defined surface (625 cm²) provided better insight into the initial bacterial load than agar contact plates (ACP), as these were often overgrown by bacteria or unreadable due to macroscopic particles.

- After cleaning

In the past, the most frequently used criterion to assess the efficacy of cleaning was the lack of visible organic material. However, as shown in chapter III, visual inspection is often unreliable as the cleanliness of some locations is difficult to assess with the naked eye.

Other methods we used to assess the hygiene status of surfaces after cleaning, were ATP monitoring and bacteriological analyses on swab samples. ATP analysis is used to provide information on the level of biological residues (both eukaryotic cells and prokaryotic cells), whereas bacteriological analyses of swab samples is used to enumerate the amount of residual bacteria or detect the presence of a defined species. In contrast to the visual inspection, both ATP swabs and bacteriological analyses on swab samples turned out to be good methods to objectively determine the hygiene status of the locations after cleaning (chapter III and IV).

- After disinfection

After disinfection, both ACP as swab samples were efficient in estimating the final bacterial load. However, ACP have several disadvantages, such as the disability of sampling irregular surfaces, (Introduction, section 3.2), that does not apply when using swab samples.

To compare different C&D protocols, it is advised to use bacteriological analyses on swab samples as this method is more able to analyse the reduction of the bacterial load during the successive C&D steps (*i.e.* from before cleaning, to after cleaning, to after disinfection).

3. **Quantitative and qualitative parameters**

- Bacteriological parameter

An important parameter to assess the efficacy of C&D, is the determination of the reduction in bacterial load. In most of the C&D studies, total aerobic bacteria (Corrégé *et al*., 2003; Hancox *et al*., 2013; Ward *et al*., 2006) and/or a specific pathogen is/are monitored (Carrique-Mas *et al*., 2009; Merialdi *et al*., 2013; Mueller-Doblies *et al*., 2010; L J Pletinckx *et al*., 2013; Rose

et al., 1999). Also in chapter III-VI, total aerobic bacteria (*i.e.* quantitative parameter) and the (opportunistic) pathogens methicillin resistant *Staphylococcus aureus* (in pig nursery units) (*i.e.* quantitative parameter when enumerated, qualitative parameter when detected after enrichment) and *Salmonella* (i.e. qualitative parameter) (in broiler houses and pig nursery units) were analysed during C&D. In addition, *E. coli* was evaluated as index organism for *Salmonella* and as hygiene indicator organism for faecal contamination as quantitative (*i.e.* when enumerated) and qualitative (*i.e.* when detected after enrichment) parameter. Moreover, it is known that some *E. coli* types can be pathogenic for chickens (Dho-Moulin and Fairbrother; Mellata, 2013) and piglets (Rossi *et al*., 2012). Other faecal indicator organisms monitored during this thesis, were *Enterococcus* spp. (*i.e.* quantitative parameter). Enterococci can also be involved in infections in poultry (Cardona *et al*., 1993; Chadfield *et al*., 2004) and pigs (Cheon and Chae, 1996). Finally, faecal coliforms were analysed as hygiene indicator organisms (*i.e.* quantitative parameter when enumerated, qualitative parameter when detected after enrichment), as this group of organisms is present in higher numbers compared to *E. coli*, which is a member of this group. Based on the outcome of the different studies (chapter III - VI), it appeared that *Enterococcus* spp. were the most interesting hygiene indicators for C&D studies, as the probability of recovering these organisms was higher and because *Enterococcus* spp. enumerations could show differences between C&D protocols. During the course of the studies, we also learned that the results of *Enterococcus* spp. enumerations during C&D did not follow a normal distribution and often fell below the lower enumeration limit. This led to a simplification of the data into a binomial dataset with a group of zero-values containing counts that were below the enumeration limit and a group of one-values with counts above the lower enumeration limit. Due to this observation, the workload in future studies can be decreased by replacing enumerations of *Enterococcus* spp. with determining only their presence or absence in the sample. In addition to *Enterococcus* spp. analyses, enumerations of total aerobic bacteria on swab samples were also capable of showing differences between C&D protocols (chapter IV and V).

Moreover, the results presented in this thesis also showed that detection of *E. coli* after disinfection, allows to identify critical locations for C&D (chapter IV). Recently, several methods have been developed to rapidly test for the presence of *E. coli* (*e.g.* MicroSnap™ *E. coli*, Hygiena). In the future, these new methods, after validation, could possibly be used by farmers to monitor the hygiene status, and thus the efficacy of the C&D procedure at different locations by themselves.

Although, a limited number of bacterial species was analysed in this thesis, these species could be index-organisms for a wide range of other vegetative and non-mycobacterial Gram positive and Gram negative pathogens. For example, *E. coli* could be used as index organism for pathogens belonging to the *Enterobacteriaceae* family. Consequently, results of *E. coli* analyses described in this thesis, may be extrapolated to obtain conclusions for specific pathogens, that are of importance for the primary sector. However, future research is necessary as index-organisms should comply to several criteria. First, they should originate from the same source (*e.g.* faeces or skin) (Dewaele *et al*., 2011; Ghafir *et al*., 2008). Secondly, they should be present in higher numbers than the pathogen and the detection and enumeration method should be easy, quick and cheap (Dewaele *et al*., 2011; Ghafir *et al*., 2008). Finally, the index organism should have a survival rate equally or higher than the pathogen and should respond in the same manner to disinfection treatments (Dewaele *et al*., 2011; Gradel *et al*., 2004a; Winfield and Groisman, 2003).

Moreover, as Gram negative bacteria are intrinsically more resistant to disinfectants than enveloped viruses (Table I.8), it is assumed that if the Gram negative bacterium *E. coli* is eliminated by disinfection, these viruses, if present in equal or lower amounts, are likely also eliminated. In contrast, if *E. coli* survives the disinfection step, then small non-enveloped viruses (intrinsically more resistant than Gram negative bacteria) will probably also survive the disinfection step.

Table I.8: Relative susceptibility of groups of micro-organisms to disinfectants (Fraise *et al*., 2012)

Range	Group of micro-organisms
Resistant	Prions
	Bacterial endospores
	Protozoal oocysts
	Mycobacteria
	Small non-enveloped viruses
	Protozoal cysts
	Fungal spores
	Gram negative bacteria
	Moulds
	Yeasts
	Protozoa
	Large non-enveloped viruses
	Gram positive
Susceptible	Enveloped viruses

- ATP

Another quantitative method used for hygiene monitoring after cleaning is ATP analysis, which has been widely adopted in the food industry (Betts and Chroleywood food research assocation, 2000) but is until now rarely used in the evaluation of cleanliness in animal housing.

As demonstrated in chapter IV, ATP measurements were able to identify critical locations for cleaning. However, for other locations, large variations in ATP values were found. These variations could be explained by several reasons. It has been demonstrated that the detection limit of ATP tests for the Gram negative bacterium *E. coli* is higher (10^4 colony forming units (CFU)/100 cm²) than for the Gram positive bacterium *Staphylococcus aureus* (10^2 CFU/100 cm²) (Turner *et al*., 2010). In addition, Turner *et al*. (2010) demonstrated that sonication of *E. coli* improved detection indicating incomplete bacterial lysis in the detection system. Therefore, the ratio Gram positive/Gram negative bacteria on surfaces may influence the outcome of ATP measurements. When measuring ATP, not only prokaryotic, but also eukaryotic cells are analysed. Moreover, it is known that eukaryotic cells contain approximately 100-fold more ATP than prokaryotic cells (Aldsworth *et al*., 2009). Therefore, it can be assumed that the number of eukaryotic cells on a specific surface will influence the ATP signal the most. It should also

be noted that commercial sanitisers and cleaning products may quench or enhance the light signal during ATP measurements, which could lead to false positives and negatives (Green *et al*., 1999). As a consequence of these large variations, the results described in this thesis showed that ATP measurements are interesting to identify critical locations but are of little use to compare the efficacy of different cleaning protocols throughout animal houses (chapter IV). The fact that ATP measurements does work to identify critical locations is likely due to the abundance of bacteria (exceeding the detection limit of both Gram negative and positive bacteria) and eukaryotic cells at these locations. A future perspective is to set a cut-off value for farmers indicating the need of extra cleaning. Our data in chapter IV suggest a cutoff value of 3 log relative light units (RLU) as a warning level.

Field studies, an important step towards optimisation of cleaning and disinfection

Many studies have evaluated the efficacy of disinfectants on farms (Carrique-Mas *et al*., 2009; Espinosa-Gongora *et al*., 2013; Gradel *et al*., 2004a; Mueller-Doblies *et al*., 2010), however little scientific work has been carried out on cleaning of animal houses, therefore most of the guidelines for farms are based on assumptions and extrapolations. To be able to assess to what extent these are valid, it is crucial to perform field studies to gain insight in the efficacy of commonly-used cleaning and alternative cleaning protocols in animal houses for maintaining good hygiene and safeguarding animal health.

In the studies described in this thesis, it was found that the mean total aerobic bacteria enumerations on swab samples, taken in broiler houses, decreased with 2 log colony forming units (CFU)/625 cm² after cleaning and with 1.5 log CFU/625 cm² after disinfection (chapter III). In pig nursery units, mean total aerobic bacteria enumerations were only reduced by 1.2 log CFU/625 cm² after disinfection (chapter VI). This was a surprising result, as farm disinfectants must show a minimum 5 log reduction of several reference bacteria, starting at a concentration of $1 - 5 \times 10^8$ CFU/mL in standardised challenge trials, according to the European Standard EN1656 (European Commitee for Standardization, 2000). This thesis showed that in the field, a 5 log reduction on the level of total aerobic bacteria is far from achieved during disinfection.

Moreover, the results showed that the cleaning step was able to reduce the bacterial load more than the disinfection step. A good cleaning step not only strongly reduces/removes bacteria and

organic material but also ensures that the subsequent disinfection step has a greater impact on the remaining bacteria.

Hence, it is of paramount importance to optimize the cleaning step in animal houses. Theoretically, cleaning with warm water and an alkaline detergent is preferred because of their properties to dissolve fats (Gibson *et al*., 1999).

It is advised to use water at a temperature higher than the melt temperature of fats during cleaning. As high melting fats have a melt temperature around 40 °C-55 °C (Koyano and Sato, 2002), cleaning with 60 °C should be able to melt most fats. In addition, Parkar et al. (2004) showed that dissolution and removal of polysaccharide from *Bacillus* spp. biofilms by cleaning with a cleaning product at a temperature 60 °C was successful in cleaning biofilm from test coupons in laboratory trials. However, the composition and quantity of the polysaccharides varies between types of bacteria within biofilms, age of the biofilms and the different environmental conditions under which the biofilms exist (Mayer et al., 1999), therefore the effect of temperature on biofilm removal during cleaning may differ. Moreover, the temperature may not be too high as proteins denature at temperatures above 75 °C, forming a film on substrates that is hard to remove (Rovira, 2016). Finally, it is important to consult the manufacturer concerning the thermal stability of the used cleaning product. In chapter IV, it was shown that there was no significant difference in reducing total aerobic bacteria and *Enterococcus* spp. contamination level after cleaning broiler houses with warm (60 °C) or cold (non-heated) water. Also other older studies in animal houses showed that the relevance of using warm water during cleaning of animal premises is negligible (Morgan-Jones, 1981; Walters, 1967). One explanation could be that the actual cleaning products in combination with cold water are sufficiently able to dissolve fats. On the other hand, we also demonstrated that when broiler houses were cleaned with warm water, less water and working time were spent in comparison with protocols using cold water. Therefore, there are some benefits related to working with warm water as it reduces the workload and contributes to the comfort of the farmers, especially in the winter.

It is difficult to speculate to what extent this result can be extrapolated to other animal species, as for example more fats are excreted by pigs than chickens (*i.e.* the crude fat content of dried poultry and pig manure is 2.3% (Arfan-ul-Haq *et al*., 2015) and 16.4% (Dong, 2009), respectively). Therefore, it is expected that the fat dissolving characteristics are more important for C&D protocols in pig production. Consequently, the effect of warm water versus cold water during cleaning of pig pens needs to be further assessed.

Besides the water temperature, there are other factors that may improve the efficacy of cleaning animal premises, such as an overnight soaking step before the high pressure cleaning. This thesis showed that an overnight soaking step (*e.g.* by automatic sprinkler systems, normally used for cooling broilers during summer) before high pressure cleaning caused a greater reduction of total aerobic bacteria and *Enterococcus* spp. than cleaning without a soaking step (chapter IV). A preceding soaking step also reduced working time and the amount of water needed to clean. This is likely the result of the fact that soaking will loosen organic material, which makes removal easier during high pressure cleaning. It is therefore also recommended to apply in pig barns. A downside of soaking is that the high pressure cleaning needs to be postponed, however the soaking step could be implemented overnight. Based on the results obtained in this thesis it is advised to implement a soaking step, when it is possible to postpone the following C&D protocol with one day.

In addition to cleaning, a prolonged vacancy of animal houses has been described as a measure to decrease the survival rate of bacteria. Natural desiccation is thought to be the main cause of this decrease (Hancox *et al*., 2013). In broiler houses, it is recommended to apply a vacancy period of at least two weeks (Lacy, 2002; Prabakaran, 2003) as this was associated with fewer *Campylobacter* positive flocks (Hald *et al*., 2000). However, such long vacancy periods are generally not carried out in practice, because this results in a lower number of production cycles and thus a lower income. In addition, during a long vacancy period recontamination could occur through the introduction of pathogens by farmers or other vectors such as vermin and rodents (Dewaele *et al*., 2012b; Hald *et al*., 2004; Meerburg *et al*., 2007). Backhans *et al*. (2015) showed that the mean vacancy period in Swedish pig farms was 5.3 days, but no literature was found on the mean vacancy period on Belgian farms. The effect of a vacancy period of 10 days, was tested in pig nursery units (chapter VI). We found no significant effect of a prolonged vacancy of 10 days on several bacteriological parameters. A possible explanation is that some bacteria, such as *Salmonella*, *Staphylococcus aureus* (including MRSA) and *Enterococcus* spp., can survive for long periods under various conditions in the environment (Kramer *et al*., 2006). Also surface characteristics can affect the survival of bacteria during vacancy: concrete is often rough and porous and has the ability to adsorb liquids; whereas steel is smooth and less porous allowing easier evaporation, more drying, and hence, more desiccation and possible microbial death (Hancox *et al*., 2013). A number of studies investigated the effect of relative humidity of the air on the survival of bacteria on surfaces, in dust, on fabrics, etc. (Bale *et al*., 1993; Habimana *et al*., 2014; Harry and Hemsley, 1964; Lidwell and Lowbury, 1950; McDade and

Hall, 1964; Turner and Salmonsen, 1973; Wilkinson, 1966; Wilkoff *et al.*, 1969). Most of these studies showed a higher survival rate of bacteria at low RH than at high RH conditions (Table VIII.1). Moreover, it has been even shown that a variety of airborne bacteria tends to be most susceptible at intermediate-high RH levels ($50 - 70\%$) (Dunklin and Puck, 1948; Sainsbury, 1992; Simensen, 1994; Webb, 1959; Won and Ross, 1966). Therefore, it can be assumed that during vacancy, a mid-high range RH should be obtained, as carried out in chapter VI.

In addition, the temperature during vacancy may have an influence on the growth of bacteria. The growth range of bacteria is typically 25-40 °C. For *E. coli*, the optimum temperature is approximately 39 °C, with a maximum and minimum growth temperature of 48 °C and 8 °C, respectively (Madigan et al., 2009). However, to increase or decrease the temperature beyond these temperatures to assure that no growth could occur during vacancy, would be difficult if not possible in animal houses

In conclusion: our study suggests that implementing a vacancy period of 10 days does not reduce the infection pressure in pig nursery units.

Table VIII.1: Literature review concerning the effect of relative humidity of the air on the survival of bacteria on surfaces, in dust and on fabrics.

Type of substrate	Bacterial species	RH - lowest survival rate	RH - greatest survival rate	Study
Dust	Total aerobic bacteria	66%	44%/ 93%	Lidwell and Lowbury (1950)
Dust	Coliforms	70.1%	10.1%	Harry and Hemsley (1964)
Glass, steel and ceramic tiles	*Escherichia coli*, *Morganella morganii*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Derby	53%/ 85%	11%	McDade and Hall (1964)
Metal surfaces	*Pasteurella tularensis*	65%	10%	Wilkinson (1966)
Fabric	*Salmonella* Typhimurium	78%	35%	Wilkoff, Westrbook and Dixon (1969)
Glass	*Klebsiella*	53%/ 85%	11%/ 33%	Turner and Salmonsen (1973)
Glass	*Pseudomonas* spp., *Acinetobacter calcoaceticus, Staphylococcus* spp., and *Staphylococcus aureus*			
Glass	*Escherichia coli*	80%	< 80% and > 80%	Bale *et al.* (1993)
Steel coupon	*Salmonella* Agona	85%	35%	Habimana *et al.* (2014)

What is the goal that needs to be reached?

As previously mentioned, hygiene refers to conditions and practices that help to maintain health and prevent the spread of diseases. This definition shows that the goal of a good C&D is to obtain farms with a low pathogen infection pressure and consequently healthy animals as a source for safe food. The results of this thesis showed that the mean bacterial contamination level after disinfection was still 4.2 log CFU/625 cm² and 4.4 log CFU/625 cm² in broiler and piglet facilities, respectively (chapter III and VI). Based upon this observation, the question arises whether this is an allowable contamination level or whether a lower number of total aerobic bacteria in animal houses is preferable.

1. **The importance of the in-house microbiota after C&D**

Broilers for commercial production are hatched in a clean environment, and unlike other farm animals such as pigs, broilers will never come into contact with adult birds to become colonised by the healthy microbiota of adults (Crhanova *et al*., 2011). It has been shown that the composition of the litter/house microbiota acts as the seed stock for the gut microbiota of the incoming broilers (Collett, 2007). Therefore, it could be advocated that a minimal contamination level in the cleaned and disinfected broiler house is needed. The formed gut microbiota is thought to prevent colonization by pathogens via mechanisms such as competition for nutrients or for epithelial attachment sites (Canny and McCormick, 2008; Lu and Walker, 2001). However, if pathogens are present in the environment of the newly arriving broilers, they can colonise and replicate in the intestinal tract.

Weaned piglets are immediately subjected to many environmental, behavioural and dietary stresses. Moreover, the intestinal gut flora is still precarious, which makes them highly susceptible to enteric diseases (Hopwood and Hampson, 2003). Therefore, exposure to pathogens upon arrival in pig nursery units, needs to be avoided to allow for a healthy intestinal microbiota to be established.

2. **Goal**

In chapter VI, more insight in the bacterial composition AC and AD in broiler houses, was obtained. As a great amount of the residual bacteria were identified as non-pathogenic organisms, which can compete with remaining and incoming pathogens, this confirms that it is not the intention to remove all bacteria. However, the results of our study also indicate that when the level of total aerobic bacteria is high, there is a great chance that pathogens are still present. Therefore, as previously mentioned, one should strive for a mean total aerobic bacteria level of $\pm$ 3.5 log CFU/625 cm² after C&D. In addition to monitoring total aerobic bacteria, also index organisms (*e.g. E. coli*) should be analysed to determine the hygiene status, *i.e.* pathogen status, of animal houses after disinfection.

3. **Future perspectives**

Of each bacteriological group (*i.e. Enterobacteriaceae*, *Enterococcus* spp. and total aerobic bacteria) that was investigated, several pathogens for poultry and humans, were identified after cleaning and even after disinfection. It would be interesting to select index organisms, belonging to each group, to check for the presence of these and other pathogens in future studies. Another future perspective would be to identify the residual dominant flora and

pathogens after C&D in pig pens. It can be speculated that also pathogens for pigs and humans may still be present, as the residual bacterial load after disinfection in pig nursery units was comparable to the level in broiler houses.

Why do bacteria survive?

As the disinfection step was not able to kill the identified organisms, the subsequent question "why do bacteria survive" raises. In order to answer this question, the minimum bactericidal concentration (MBC) of the disinfectant used in the study of chapter VII (*i.e.* hydrogen peroxide and peracetic acid based product), on several isolates collected after cleaning and after disinfection in broiler houses, was determined. It was shown that Gram negative *Enterobacteriaceae* isolates were less susceptible to the disinfectant than Gram positive *Enterococcus faecium* isolates. This was expected, as Gram negative bacteria are intrinsically less susceptible to disinfectants (McDonnell and Russell, 1999). Moreover, none of the *Enterococcus faecium* isolates could survive the recommended concentration of 0.5%. Therefore we can conclude that the survival of different bacterial species after disinfection, including *Enterococcus faecium*, is probably because of the presence of residual organic matter or diluting water, resulting in a reduction of the disinfection efficacy. This again demonstrates the need for improvement of cleaning. Besides, it was shown that the manufacturer's recommended concentration of the disinfectant (*i.e.* 0.5%) was too low to kill the strains belonging to the *Enterobacteriaceae* sampled in the farm, including pathogenic species for humans and animals. This proposed working concentration is determined by a suspension test carried out by the manufacturer. One difference between this test and the MBC test used in our study is the incubation time of the suspension of isolates with the disinfectant: 5-60 min versus overnight incubation, respectively. As the contact time in our study was longer and similar to field conditions, it should even increase the efficacy of the disinfectant. It was therefore a surprising result that the *Enterobacteriaceae* field isolates survived exposure to 0.5% disinfectant, and thus no 5 log reduction was obtained. As the recommended concentration of the disinfectant of 0.5% was not able to kill the field isolates in the MBC test, it can be concluded that the MBC test gave more accurate results compared to the suspension test and therefore better predicts the field efficacy. Based on this observation, it can also be assumed that the concentration of the disinfectant is too low for farm conditions.

Hence, the survival of the *Enterobacteriaceae* isolates might either be the result of a too low concentration or of the presence of a resistance mechanism among the present bacteria. This needs to be determined in future research.

Another future perspective is to test the susceptibility of other isolates, especially pathogens. Moreover, it would be interesting to test the efficacy of other commonly used disinfectants at their recommended concentration against field isolates.

Competitive exclusion, a good alternative for conventional biocides?

It has been suggested that the use of biocides, especially at sub inhibitory concentrations, may also increase selective pressure towards antibiotic resistance (Beier *et al*., 2008; Knapp *et al*., 2015; Randall *et al*., 2007, 2004). Because of the ongoing concern on this potential resistance development and cross-resistance to clinically important antibiotics, the use of bacterial biocontrol agents has been suggested as an alternative method to antagonise the growth of pathogens. In chapter V, a commercial competitive exclusion (CE) protocol based on Probiotics In Progress (PIP) products (Chrisal, Lommel, Belgium) was tested in pig nursery units during three successive production and C&D rounds. This study showed that the infection pressure in CE units after this microbial cleaning was not reduced to the same degree as in control units (classical C&D). Despite sufficient administration of the probiotic type spores, the analysed bacteria did not decrease after 3 production rounds in CE units, indicating no competitive exclusion effect. Also other claims of the producer regarding antimicrobial use and feed conversion could not be demonstrated.

An explanation for the fact that this concept did not work in our study is likely related to the fact that organic debris are only removed when pig nursery units are emptied. Therefore, nutrients are abundantly available during production, eliminating the opportunity for competition between bacteria, which is the hall mark of competitive exclusion.

According to Vandini *et al*. (2014), a similar CE protocol was able to lower the number of Healthcare-Associated Infections related micro-organisms on surfaces in hospitals (Vandini *et al*., 2014). Therefore, we can conclude that the applied CE protocol is not a valuable alternative in heavily soiled conditions such as animal houses but it may work in other, less heavily soiled circumstances. In addition, a limitation of our study was that the CE protocol was only carried out in pig nursery units, and not in farrowing units. Therefore, the piglets gut microbiota was already formed, which could contain pathogens and contaminate pig nursery units on arrival.

Conversely, this is also a drawback of the CE protocol. A future perspective could be to determine the efficacy of a CE protocol applied on the whole farm, however this approach would substantially increase the work load and associated costs for the farmer.

Another issue concerning the use of CE bacteria, is that they could also acquire and pass on antibiotic resistance genes to pathogens. It has been shown that typical probiotic bacteria are often carriers of specific antibiotic resistance determinants carried on mobile genetic elements (*e.g.* tetracycline resistance genes) (Sharma *et al*., 2014). For the assessment of the safety of probiotic micro-organisms and products, FAO/WHO has formulated guidelines, recommending that probiotic strains for food uses, should be evaluated for a number of parameters, including antibiotic susceptibility patterns, toxin production, etc. (FAO/WHO, 2002). However, for the use of probiotic (type) bacteria for microbial cleaning, no specific guidelines were found.

To conclude…

This thesis showed the importance of a good cleaning step in the reduction of bacteria during C&D as the cleaning step was able to reduce the overall contamination level even more than the disinfection step. Therefore, it is important to continue to evaluate commonly used as well as alternative cleaning protocols in order to lower the infection pressure and optimise hygiene on farms. In this manuscript we showed that:

- Implementing an overnight soaking step before high pressure cleaning is advised as it contributes to the efficacy of cleaning.
- There is no difference between cleaning with cold and warm water of broiler houses, however this still needs to be assessed in pig pens.
- A competitive exclusion (CE) method is not a valuable alternative in animal houses
- A vacancy period of 10 days or less after C&D in pig nursery units, without any extra biosecurity measures, does not further decrease bacteria.
- Critical locations during C&D are
    - Drinking nipples, floor cracks and drain holes in broiler houses
    - Slatted floors and drinking nipples in pig nursery units.
- The survival of bacteria after disinfection may be due to inadequate cleaning or the use of insufficient concentrations.

Several manuals with guidelines for implementing a good and complete hygiene management on farms are already available for the poultry and pig sector. It would be beneficial to replenish

these information sources with the results obtained in the present thesis. Briefly, a good cleaning and disinfection protocol should consist of

(i)   Dry cleaning

(ii)   Overnight soaking step

(iii)   Washing with water

(iv)   Soaking with a detergent

(v)   Rinsing with water

(vi)   Drying step

(vii) Disinfection

(viii) Monitor the hygiene status of several locations, including the critical locations.

SUMMARY

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Good hygiene practices on farms can reduce the risk of introduction and persistence of animal diseases and diseases that are transmittable from animals to humans (zoonoses). These infectious agents can not only lead to disease outbreaks resulting in sub optimal production and flock mortality, but also to an increase of veterinary costs and condemnation rates at slaughterhouses as well as animal welfare issues. This all leads to high economic losses for the farmer (Jung and Rautenschlein, 2014) and in case of epidemic diseases, preventive measures such as quarantine or even destruction of animals (Gelaude *et al*., 2014). It is therefore of great importance to prevent disease outbreaks through biosecurity measures rather than cure them (Gelaude *et al*., 2014; Laanen *et al*., 2014). Biosecurity includes all measures preventing pathogens from entering a herd (*i.e.* external biosecurity) and reducing the spread of pathogens within one herd (*i.e.* internal biosecurity) (Sarrazin *et al*., 2014). In this thesis the focus was on internal biosecurity and more specifically on cleaning and disinfection (C&D) on broiler and pig farms as their production contribute the most to the global, European and Belgian meat production.

In order to evaluate C&D in animal houses, an evaluation tool was designed in **chapter III**. Sampling methods such as surface sampling with swabs and agar contact plates (ACP) and air sampling were tested during the successive C&D steps, *i.e.* before cleaning (BC); after cleaning (AC) and after disinfection (AD), in six broiler houses on two farms. During surface sampling, ten to twelve defined locations were sampled in quadruplicate. The effectiveness of cleaning was investigated by bacteriological analyses on swabs, ACP and air samples; adenosine triphosphate (ATP) monitoring and a visual inspection. The effectiveness of disinfection was examined by bacteriological analyses on swabs, ACP and air samples. In addition, surface and air samples were taken before cleaning to determine the initial bacteriological status of the broiler houses. On swab and air samples and on ACP, enumerations of total aerobic bacteria, *Enterococcus* spp. (hygiene indicator) and *Escherichia* coli (hygiene indicator and index organism for *Salmonella*) was carried out. In addition, an enrichment of swab and air samples was carried out for the detection of *E. coli* and *Salmonella*.

The results of the study showed that ACP were found to be less suitable than swabs for enumeration. In addition to measuring total aerobic bacteria, *Enterococcus* spp. seemed to be a better hygiene indicator to evaluate C&D protocols than *E. coli*. All broiler houses were *Salmonella* negative, but the detection of its index organism *E. coli* provided additional

information for evaluating C&D protocols. ATP analyses gave additional information about the hygiene level of the different sampling points.

In conclusion, the evaluation tool that provides valuable information for evaluating C&D protocols consists of: ACP for total aerobic bacteria counts AD; swab enumeration for total aerobic bacteria and *Enterococcus* spp. BC, AC and AD; and the detection of *E. coli* on those swab samples. After cleaning, ATP analyses could also be carried out for additional information about the hygiene status of the different locations.

In addition to the evaluation tool, the dynamics of the different bacteriological parameters was examined. It was shown that the mean total aerobic bacteria determined by swab samples decreased from 7.7 ± 1.4 to 5.7 ± 1.2 log colony forming units (CFU)/625 cm² after cleaning and to 4.2 ± 1.6 log CFU/625 cm² after disinfection. Surprisingly, total aerobic bacteria was significantly reduced by an average of 1.5 log after the disinfection step, which was less than the 2 log reduction obtained by cleaning ($P< 0.01$) which indicates that in practice, a 5 log reduction, a European Standard (EN1656) that needs to be fulfilled by disinfectants, is far from achieved during disinfection for total aerobic bacteria.

The final evaluation tool was used to evaluate the effectiveness of four cleaning protocols: the difference between whether or not applying an overnight soaking step after dry cleaning and/or the use of warm (60 °C) or cold water during cleaning was studied (**chapter IV**). Two to three C&D rounds were evaluated in 12 broiler houses on five farms. Total aerobic bacteria and *Enterococcus* spp. enumerations on swab samples showed that cleaning protocols preceded by an overnight soaking step with water, caused a greater bacterial reduction compared to protocols without a preceding soaking step. No differences were found between protocols using cold or warm water during cleaning. When analysing ACP for total aerobic bacteria counts, taken AD, no differences were found between protocols.

Additionally, statistical analyses showed that sampling 10-12 locations in one fold per broiler house was sufficient to evaluate C&D. This means that costs and working time can be reduced for future research on evaluating C&D methods.

Furthermore, a comparison between power consumption and working time of the four protocols was carried out. When broiler houses were cleaned with warm water, less water and working time were spent in comparison with protocols using cold water. Although broiler houses were soaked with water overnight, water consumption was still lower than when houses were cleaned without a preceding soaking step. This means that a preceding soaking step reduced the amount of water needed to clean broiler houses afterwards. In addition, working time spent on cleaning

after soaking was less than cleaning without a preceding soaking step. However, it should be taken into account that soaking of broiler houses can be time consuming by postponing the high pressure cleaning.

Finally, locations that are difficult to clean and possible sources of infection were identified. Drinking cups, drain holes and floor cracks were identified as critical locations for C&D in broiler houses, while feed hoppers and roofs were identified as the cleanest.

The same evaluation tool, although slightly adjusted, was used to compare the efficacy of a competitive exclusion (CE) protocol against a classical C&D protocol (control) in **chapter V**. As recently weaned pigs are generally more susceptible to infectious diseases compared to mature or suckling pigs (Blecha *et al*., 1983; Genovese *et al*., 1998), tests were carried out in pig nursery units. The study was performed during 3 successive production rounds using 6 identical nursery units on a pilot farm. CE protocol consisted of microbial cleaning (*Bacillus* spp. spores, enzymes and detergent) and spraying the *Bacillus* spp. spores during down-time (after cleaning) and production. Sampling was performed: immediately after pig removal; 24 h after cleaning (CE units) or disinfection (control units) and after 1 week and 5 weeks of production (piglets present). On these samples, analyses of bacterial spores, *Enterococcus* spp., (haemolytic) *E. coli*, faecal coliforms, methicillin resistant *Staphylococcus aureus* (MRSA) and *Salmonella* were performed. In addition to the bacterial analyses, feed conversion, faecal consistency and antibiotic use were monitored. Analyses of haemolytic *E. coli*, *E. coli* (index organism for *Salmonella*) and MRSA showed that the infection pressure after CE cleaning was not reduced to the same extent after classical C&D during down-time. Therefore, we can assume that no improvement of pathogen elimination is noticed. In contrast, young piglets have a greater chance of being infected when arriving in these CE units. In addition, no improvement in hygiene was found: during the 2nd and 3rd production round, higher *Enterococcus* spp. (hygiene indicator) enumerations were found than after the 1st production round and no differences in faecal coliforms contamination between the two types of units were found.

In addition, no difference in feed conversion nor faecal consistency (indicator for gut infections) of piglets raised in CE and control units was seen. Finally, also no differences in treatments with antibiotics was found.

As it is also important to identify critical locations, contamination levels of locations after cleaning or disinfection were analysed in CE and control units. In CE units, grid floors, concrete walls and drinking nipples seemed still highly contaminated by *Enterococcus* spp., *E. coli*,

faecal coliforms and MRSA after microbial cleaning while in control units these were grid floors and drinking nipples.

Another objective in this study was to test the effect of a 10-day vacancy period in pig nursery units on the following bacteriological parameters: total aerobic bacteria, *Enterococcus* spp., *E. coli*, faecal coliforms and MRSA (**chapter VI**). Three vacancy periods of 10 days were monitored, each time applied in 3 units. The microbiological load was measured before disinfection and at 1, 4, 7 and 10 days after disinfection.

No significant decrease or increase in *E. coli*, faecal coliforms, MRSA and *Enterococcus* spp. was noticed. Total aerobic flora counts were the lowest on day 4 after disinfection (i.e. 4.07 log CFU/625 cm²) (*P*<0.05), but the difference with other sampling moments was limited (i.e. 0.6 log CFU/625 cm²) and therefore negligible. Furthermore, this observation on day 4 was not confirmed for the other microbiological parameters. After disinfection, drinking nipples were still mostly contaminated with total aerobic flora (i.e. 5.32 log CFU/625 cm²) and *Enterococcus* spp. (i.e. 95% of the samples were positive) (*P*<0.01); the feeding troughs were the cleanest location (total aerobic flora: 3.53 log CFU/625 cm² and *Enterococcus* spp.: 50% positive samples) (*P*<0.01).

This study indicates that prolonging the vacancy period in nursery units to 10 days after disinfection with no extra biosecurity measures has no impact on the environmental load of total aerobic flora, *E. coli*, faecal coliforms, MRSA and *Enterococcus* spp..

Finally, in **chapter VII** the residual dominant bacteria after C&D was identified in broiler houses. Therefore, sampling was carried out in 4 broiler houses on a pilot farm AC and AD. The used disinfectant was based on hydrogen peroxide and peracetic acid. Enumerations were carried out for total aerobic bacteria, *Enterococcus* spp. and *Enterobacteriaceae* on Plate Count Agar (PCA), Slanetz and Bartley (S&B) and Violet Red Bile Glucose Agar (VRBGA), respectively. The dominant bacteria was assessed by (GTG)$_5$ analysis and 16S rRNA gene sequence analysis. In addition, minimum bactericidal concentration (MBC) tests were carried out on 18 selected isolates belonging to the *Enterobacteriaceae* family and 10 *Enterococcus faecium* isolates, to determine the susceptibility of these isolates against the used disinfectant. A great variety of bacteria was detected. In total, 363 and 255 isolates were identified AC and AD, respectively. The most dominant bacteria belonged to *Brevibacterium*, *Brachybacterium* and *Staphylococcus* AC and *Bacillus*, *Brevibacterium* and *Staphylococcus* AD. In addition, on both sampling moments, *Enterococcus faecium* was dominant amongst the *Enterococcus* spp. isolates. On the selective medium for *Enterobacteriaceae*, genera *Enterobacter* and *Pantoea*

and *Aeromonas* (non *Enterobacteriaceae*) were dominant AC and *Escherichia*, *Lelliottia* and *Pantoea* AD. In addition, pathogenic species for poultry and humans were identified not only AC but also AD. MBC results showed no obvious trend in selection of less susceptible isolates for the used disinfectant AD compared to AC. In addition, the results showed that *Enterobacteriaceae* isolates are less susceptible to the used disinfectant than *Enterococcus faecium* isolates. In addition, the recommended concentration of the used disinfectant (*i.e.* 0.5%) seemed too low to kill *Enterobacteriaceae*.

SAMENVATTING

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Een goede hygiëne op veebedrijven kan de kans op introductie en persistentie van dierziekten en overdraagbare ziekten van dier op mens (zoönose) verminderen. Deze infectieuze kiemen kunnen niet enkel leiden tot ziekte-uitbraken en sterfte en in geval van epidemische ziekten tot preventieve maatregelen zoals quarantaine of zelfs het opzettelijk doden van dieren (Gelaude *et al*., 2014), maar ook tot een stijging van dierenartskosten en afkeuringsprijzen van het slachthuis, en dus economische schade voor de veehouder (Jung and Rautenschlein, 2014). Het is dus zeer belangrijk om ziekte-uitbraken te vermijden door bioveiligheidsmaatregelen toe te passen (Gelaude *et al*., 2014; Laanen *et al*., 2014). Bioveiligheid omvat alle maatregelen om het introduceren van ziekteverwekkende kiemen (pathogenen) te voorkomen (externe bioveiligheid) en het verspreiden van deze kiemen binnen het bedrijf tegen te gaan (interne bioveiligheid) (Sarrazin *et al*., 2014). In deze PhD thesis lag de focus op de interne bioveiligheid, en dan meer specifiek de reiniging en ontsmetting (R&O) van braadkippen en varkensstallen, omdat hun productie het sterkste bijdraagt tot de globale, Europese en Belgische vleesproductie.

Om de R&O van stallen te evalueren, werd een evaluatie-systeem ontwikkeld in **hoofdstuk III**. Er werden stalen genomen in 6 braadkippenstallen op 2 bedrijven aan de hand van swabs, agar contact plaatjes (ACP) en een luchtbemonsteringstoestel tijdens de verschillende stappen van het R&O proces: voor reiniging (VR); na reiniging (NR) en na ontsmetting (NO). Er werden 10 tot 12 verschillende locaties bemonsterd in viervoud. De efficiëntie van de reinigingsstap werd nagegaan door bacteriologische analyses op swabs, ACP en luchtstalen; adenosine trifosfaat (ATP) analyses en een visuele reinheidsinspectie. De effectiviteit van de ontsmetting werd geanalyseerd door bacteriologische analyses op swabs, ACP en luchtstalen. Bovendien werden ook stalen genomen voor reiniging, om de initiële bacteriële status van de stallen te bepalen. Op de stalen werden tellingen van totaal aeroob kiemgetal, *Enterococcus* spp. (hygiëne indicator) en *Escherichia coli* (hygiëne indicator en indexorganisme voor *Salmonella*) uitgevoerd.

De resultaten van deze studie toonden aan dat ACP minder geschikt waren dan swabs om tellingen van de bacteriologische parameters uit te voeren. Naast het bepalen van het totaal aeroob kiemgetal, leek *Enterococcus* spp. een betere hygiëne indicator dan *E. coli* om R&O te evalueren. Alle stallen waren *Salmonella* negatief, maar de detectie van het indexorganisme *E. coli* gaf bijkomende informatie om de R&O te evalueren. ATP analyses gaven eveneens aanvullende informatie over de hygiënestatus van de verschillende locaties.

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Uit de resultaten volgde dat het evaluatie-systeem dat de meeste waardevolle informatie om R&O protocollen te evalueren bestaat uit: ACP voor tellingen van totaal aeroob kiemgetal NO, swab tellingen voor totaal aeroob kiemgetal en *Enterococcus* spp. VR, NR en NO; en detectie van *E. coli* op deze stalen. Na reiniging, kunnen ATP analyses uitgevoerd worden om extra informatie te voorzien over de hygiëne status van de verschillende locaties.

Daarnaast werd het verloop van de verschillende bacteriologische parameters onderzocht. Er werd aangetoond dat de gemiddelde tellingen voor totaal aeroob kiemgetal op swabs daalde van 7.7 ± 1.4 naar 5.7 ± 1.2 kolonie vormende eenheden (kve)/625cm² na reiniging en naar 4.2 ± 1.6 log kve/625 cm² na ontsmetting. Verrassend genoeg daalde het gemiddelde totaal kiemgetal slechts met 1.5 log kve na ontsmetting, wat minder was dan de 2 log reductie verkregen na reiniging ($P< 0.01$). Dit toont aan dat de 5 log reductie, een norm die behaald moet worden volgens Europese Standaard EN1656 voor ontsmettingsmiddelen, ver van volbracht was na ontsmetting.

Het finale evaluatie-systeem werd vervolgens gebruikt om het verschil in efficiëntie van vier reinigingsprotocollen na te gaan: het al dan niet toepassen van een overnacht inweekstap na de droge reiniging en/of het gebruik van warm (60 °C) of koud water tijdens de natte reiniging (**hoofdstuk IV**). Twee tot drie R&O ronden werden geëvalueerd in 12 braadkippenstallen op 5 bedrijven. Totaal aeroob kiemgetal en *Enterococcus* spp. tellingen op swabs toonden aan dat reinigingsprotocollen met een voorafgaande inweekstap zorgde voor een sterkere bacteriële daling dan reinigingsprotocollen zonder inweekstap. Er werd geen bacteriologisch verschil tussen reinigen met warm of koud water waargenomen. Tellingen op ACP, genomen na ontsmetting, konden geen verschillen tussen de protocollen aanduiden.

Daarnaast, toonden statistische analyses aan dat het bemonsteren van 10-12 locaties in eenvoud per stal, reeds voldoende was om de R&O van braadkippenstallen te evalueren. Dit betekent dat de kosten en werklast sterk verminderd kunnen worden in toekomstige onderzoeken.

Bijkomend werd het energieverbruik en de werktijd nodig voor het uitvoeren van de 4 protocollen vergeleken. Wanneer stallen gereinigd werden met warm water, was er minder water en tijd nodig dan wanneer er gereinigd werd met koud water. Bovendien toonde deze studie aan dat wanneer stallen overnacht ingeweekt werden met water, het waterverbruik nog steeds lager lag dan wanneer stallen niet vooraf ingeweekt werden. Dit betekent dat een voorafgaande inweekstap, het waterverbruik dat nodig is om stallen nadien te reinigen doet dalen. Daarnaast werd ook nog aangetoond dat er minder tijd nodig was om de stallen te

reinigen na inweken. Er moet echter wel rekening gehouden worden met het feit dat het inweken eveneens tijdrovend kan zijn omdat de reiniging moet worden uitgesteld.

Ten slotte werden locaties geïdentificeerd die moeilijk te reinigen en ontsmetten zijn, en dus mogelijke bronnen van pathogenen zijn. Drink-cupjes (lekbakjes), afvoerputjes en vloerspleten werden geïdentificeerd als kritische locaties voor R&O in braadkippenstallen, terwijl voerhoppers en daken als meest propere werden geïdentificeerd.

Het evaluatie-systeem werd vervolgens aangepast en gebruikt om de efficiëntie van een competitief exclusie (CE) protocol tegenover een klassiek R&O protocol (controle) te vergelijken in **hoofdstuk V**. Omdat recent gespeende biggen vaak gevoeliger zijn voor infectieuze ziekten vergeleken met volwassen varkens en speenbiggen, werden de proeven uitgevoerd in biggenbatterijen. De studie werd gedurende 3 opeenvolgende ronden (productie en leegstand) uitgevoerd in 6 identieke biggenbatterijen (of units) op een proefbedrijf. Het CE protocol bestond uit een microbiële reiniging (*Bacillus* spp. sporen, enzymen en een detergent) en het vernevelen van *Bacillus* spp. sporen tijdens de leegstand (na reiniging) en tijdens de productie (biggen aanwezig). Stalen werden genomen: direct na het weghalen van de biggen, 24 u na reiniging (CE units) of ontsmetting (controle units) en na de eerste en vijfde week van de productieronde. Op deze stalen werden analyses van bacteriële sporen, *Enterococcus* spp., (haemolytische) *E. coli*, faecale coliformen, methicilline resistente *Staphylococcus aureus* (MRSA) en *Salmonella* uitgevoerd. Daarnaast werd ook de voederconversie, de faecale consistentie en het antibioticumgebruik van de biggen gemonitord.

Haemolytische *E. coli*, *E. coli* (index organisme voor *Salmonella*) en MRSA analyses toonden aan dat tijdens de leegstand, de infectiedruk na CE reiniging niet zo sterk gereduceerd was als na een klassieke R&O. Er kon dus geen verbetering in pathogeen-reductie aangetoond worden. In tegenstelling, jonge biggen hebben een grotere kans op infecties wanneer ze geplaatst worden in deze CE units. Bovendien werd er ook geen verbetering in hygiëne waargenomen: tijdens de 2de en 3de productieronde werden zelfs hogere *Enterococcus* spp. (hygiëne indicator) tellingen teruggevonden dan tijdens de eerste productieronde en werd er geen verschil in faecale coliform belasting tussen de twee soorten units waargenomen.

Daarnaast werd er geen verschil in voederconversie noch in faecale consistentie (indicator voor darminfecties) tussen biggen uit de CE en controle units teruggevonden. Tenslotte, werd eveneens geen verschil in antibioticumgebruik gevonden.

Daar het ook belangrijk is om kritische locaties te identificeren, werd de bacteriële belasting van de verschillende locaties geanalyseerd na reiniging of ontsmetting. In de CE units waren

de vloerroosters, betonnen muren en drinknippels nog het meest besmet met *Enterococcus* spp., *E. coli*, faecale coliformen en MRSA na reiniging terwijl na het ontsmetten van de controle units voornamelijk de vloerroosters en drinknippels nog het sterkst besmet waren.

In een tweede studie in biggenbatterijen werd het effect van een leegstand van 10 dagen na R&O op volgende bacteriologische parameters onderzocht: totaal aeroob kiemgetal, *Enterococcus* spp., *E. coli*, faecale coliformen en MRSA (**hoofdstuk VI**). Er werden drie leegstanden gemonitord in telkens 3 identieke biggenbatterijen (of units). De bacteriologische status werd geanalyseerd op dag 1, 4, 7 en 10 na ontsmetting.

Er werd geen significante daling of stijging van *E. coli*, faecale coliformen, MRSA en *Enterococcus* spp. gevonden, hoewel er toch kleine schommelingen, zonder duidelijke trend, in de tijd werden geobserveerd. Het totaal aeroob kiemgetal was het laagste op dag 4 na ontsmetting (4.07 log kve/625 cm²) (*P*<0.05), maar het verschil met de andere staalname momenten was zo klein (max 0.6 log kve/staalname oppervlak), dat dit verschil verwaarloosbaar lijkt. Bovendien werd deze observatie op dag 4 niet bevestigd door de andere parameters. De resultaten van deze studie tonen aan dat een leegstand van 10 dagen na ontsmetting zonder extra bioveiligheidsmaatregelen, geen voordeel qua hygiëne in biggenbatterijen oplevert. Ten slotte werd ook de bacteriële belasting van de verschillende locaties geanalyseerd gedurende de leegstand. Hieruit bleek dat de drinknippels nog het sterkst besmet waren met aerobe kiemen (5.32 log kve/625 cm²) en *Enterococcus* spp. (95% van de stalen waren positief) (*P*<0.01). Voerhoppers werden de properste locaties geïdentificeerd (totaal aeroob kiemgetal: 3.53 log kve/625 cm² eb *Enterococcus* spp.: 50% positieve stalen) (*P*<0.01).

Deze studie toont aan dat een verlengde leegstand tot 10 dagen na ontsmetting in biggenbatterijen zonder extra bioveiligheidsmaatregelen geen impact heft op de bacteriële belasting van totaal aerobe kiemen, *E. coli*, faecale coliformen, MRSA en *Enterococcus* spp..

Ten slotte, werden in **hoofdstuk VII** de residuele dominante bacteriën na R&O geïdentificeerd in braadkippenstallen. Hiervoor werden stalen genomen NR en NO in 4 braadkippenstallen op een proefbedrijf. Het gebruikte ontsmettingsmiddel bevat waterstof peroxide en perazijnzuur. Tellingen van totaal aeroob kiemgetal, *Enterococcus* spp. en *Enterobacteriaceae* werden respectievelijk uitgevoerd op Plate Count Agar (PCA), Slanetz and Bartley (S&B) en Violet Red Bile Glucose Agar (VRBGA). De dominante bacteriën werden bepaald door een (GTG)$_5$ en 16 rRNA gen sequentie analyse. Bovendien werden minimale bactericide concentratie

(MBC) testen uitgevoerd op 18 geselecteerde *Enterobacteriaceae* isolaten en 10 *Enterococcus faecium* isolaten, om de gevoeligheid tegenover het gebruikte ontsmettingsmiddel te bepalen. Een brede waaier aan verschillende species werden geïdentificeerd. In totaal werden respectievelijk 363 en 255 isolaten NR en NO geïdentificeerd. De meest dominante bacteriën behoorden tot de genera *Brevibacterium*, *Brachybacterium* en *Staphylococcus* NR en *Bacillus*, *Brevibacterium*, en *Staphylococcus* NO. Daarnaast werd op beide staalname momenten *Enterococcus faecium* als meest dominante species van het genus *Enterococcus* geïdentificeerd.

Na reiniging en na ontsmetting waren respectievelijk de genera *Enterobacter* en *Pantoea* en A*eromonas* (niet *Enterobacteriaceae*) en genera *Escherichia*, *Lelliottia* en *Pantoea* dominant aanwezig op het medium, selectief voor *Enterobacteriaceae*. Daarnaast werden pathogene species voor pluimvee en mensen geïdentificeerd, en dit ni*et al*leen NR maar ook NO. De MBC resultaten toonden geen duidelijke trend in selectie van minder gevoelige species tegenover het ontsmettingsmiddel NO vergeleken met NR. Daarnaast, toonden de resultaten aan dat de *Enterobacteriaceae* isolaten minder gevoelig zijn tegenover het ontsmettingsmiddel dan de *Enterococcus faecium* isolaten. Bovendien bleek de aangeraden concentratie van het ontsmettingsmiddel (0.5%) te laag om de *Enterobacteriaceae* isolaten af te doden.

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CURRICULUM VITAE

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Personalia

Kaat Luyckx werd geboren op 11 januari 1989 te Sint-Niklaas. Na haar middelbare studies Wetenschappen-Wiskunde 8 u, behaalde ze in 2012 haar diploma master in de Biochemie en biotechnologie aan de Universiteit Gent (UGent) met onderscheiding. Datzelfde jaar startte ze haar doctoraatsonderzoek aan het Instituut voor Landbouw en Visserij Onderzoek (ILVO), eenheid Technology en Voeding (T&V). Gedurende vier jaar werkte zij in samenwerking met de vakgroep Verloskunde, Voortplanting en Bedrijfsdiergeneeskunde van de Faculteit Diergeneeskunde (UGent) en Proefbedrijf Pluimveehouderij aan het onderzoeksproject "Optimalisatie van reiniging en ontsmetting in de veehouderij", dat gefinancierd werd door de Federale Overheidsdienst (FOD) Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu.

Kaat Luyckx is auteur van meerdere wetenschappelijke publicaties in nationale en internationale tijdschriften en nam actief deel aan verschillende nationale en internationale congressen aan de hand van posters en mondelinge presentaties.

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