

Disinfection of *Salmonella* **in poultry houses**

by

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ABSTRACT

Factors related to cleanability of materials, cleaning and disinfection were tested in a questionnaire-based, retrospective field study of 78 broiler houses that received two *Salmonella* serotypes with day-old chicks. Serotype and combined surface disinfection and fogging vs. any method used separately were significantly associated with house status (infected in one or more crop cycles).

In a worst-case scenario laboratory heating study, *Salmonella* was spiked into organic matter (feed or faeces), and various factors (final heating temperatures, drying or not prior to and during heating) were investigated. A gold standard of 60 °C, 100% RH during a 24-hour period killed all *Salmonella* and naturally occurring *E. coli*, with high correlation between survival of the two species.

This gold standard was tested in field studies performed in naturally *Salmonella* infected layer houses. After application of steam, usually with 30 ppm formaldehyde, no *Salmonella* was detected, there were large reductions in coliforms, and organic samples had no surviving indicator bacteria.

In addition, two identical houses on the same farm were heat treated, one with dry heat, and the other with steam. The bacteriological results were significantly better in the latter.

To determine whether *Salmonella* persistence was related to disinfectant resistance, MICs for 286 *Salmonella* isolates involving five commonly used disinfectants were determined, but no association was found with persistence or the use of certain disinfectant types. Adaptation and de-adaptation studies with these disinfectants did not alter MICs. Selected isolates adapted to triclosan showed increased MICs, but no cross-resistance to the other five disinfectants.

Worst-case scenario surface disinfection tests, using poultry house materials plus feed, egg yolk and oil, were performed at low temperatures. There were significant differences related to the three disinfectants, bacteria (two *Salmonella* and one *E. faecalis* isolate, the latter being more resistant) and some types of organic matter.

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DECLARATION

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original and no part of the dissertation has been submitted for any other academic award. This dissertation represents my own work except:

- Part of the statistical analyses (multivariable analyses in Chapter 7, logistic regression in Chapter 9, Kruskal-wallis and Dunn's tests in Chapter 11, Friedman's two-way ANOVA and Tukey's HSD tests in Chapter 12).
- Part of the technical laboratory work and part of the field sampling (cf. Chapter 9), though I planned, monitored, supervised and participated in the technical work of all the studies.

Any views expressed in the dissertation are those of the author.

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LIST OF ABBREVIATIONS

η	concentration exponent = dilution coefficient
θ, θ^{10}	temperature coefficient (for 1 and 10 °C, respectively)
a_w	water activity
AM	ante mortem
AOAC	Association of Official Analytical Chemists
BA	blood agar
BC	benzalkonium chloride
BPW	buffered peptone water
CEN	European Committee for Standardization
CFU	colony forming units
cm, m, mm	centimetre, metre, millimetre
D, D-value	decimal reduction time
D_x	decimal reduction time at temperature x (°C)
DEFRA	Department for Environment Food and Rural Affairs (UK)
df	degrees of freedom
DGHM	Deutsche Gesellschaft für Hygiene und Mikrobiologie
DNA	deoxyribonucleic acid
DSA	Double Strength ISO-sensitest agar
DT	definitive-type
DVI	Danish Veterinary Institute
DVM	Doctor of Veterinary Medicine
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
EDTA	ethylenediamine tetraacetic acid
g, x g	gram, gravitational force
h, min	hour(s), minute(s)
H	flagellar (in relation to bacterial antigens)
HACCP	Hazard Analysis Critical Control Points
HSP	heat shock protein
ISO	International Organization for Standardization

L, ml, µl	litre, millilitre, microlitre
LB	Luria Bertani
LPS	lipopolysaccharides
LTSF	low temperature steam and formaldehyde
<i>mar</i>	See “MAR”
MAR	multiple antibiotic resistance phenotype associated with <i>mar</i> locus
MDR	multiple drug resistance
MIC	minimum inhibitory concentration
MPN	most probable number
MSRV	Modified Semi-solid Rappaport-Vassiliadis
ND	not done
O	somatic (in relation to bacterial antigens)
Omp	outer membrane protein
PC	personal computer
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
ppm	parts per million
<i>Ps. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PT	phage type
Q ₁₀	temperature coefficient for 10 °C
QAC	quaternary ammonium compound
RH	relative humidity
RNA	ribonucleic acid
RR	relative risk
rRNA	ribosomal RNA
RVS	Rappaport-Vassiliadis soy peptone broth
<i>S.</i>	<i>Salmonella</i>
s.d.	standard deviation
spp.	species
TC 216	Technical Committee 216 Chemical Disinfectants and Antiseptics
v	volume
VBNC	viable but non-culturable
VIB	veal infusion broth

vs.	versus
w	weight
WHO	World Health Organization
WHO water	WHO standard hard water
z, z-value	the increase in temperature required to reduce D to 0.1 D

Chapter 1 – General aspects and epidemiology

of *Salmonella* in the poultry sector

1.1 The genus *Salmonella*

The genus *Salmonella* belongs to the family Enterobacteriaceae, which are Gram-negative, facultatively anaerobic, non-spore-forming rods. Most serotypes have peritrichous flagella and are thus motile.

In the scientific world, there has been much dispute on the taxonomy of *Salmonella*. However, today it is generally agreed that the genus *Salmonella* comprises only two species, *S. enterica* (also named *S. choleraesuis*) and *S. bongori*; *S. enterica* is further divided into six subspecies (Fossum *et al.* 1996; Popoff and Le Minor 1997). The majority of zoonotic important *Salmonella* and about 60% of the more than 2500 known serotypes (also named serovars) belong to subspecies/subgenus I (*S. enterica* subsp. *enterica*) (Fossum *et al.* 1996; Popoff and Le Minor 1997; Popoff *et al.* 2003). According to these principles, the correct notation for a serotype is *Salmonella* [*species*] subsp. [*subspecies*] serovar [*serotype*], e.g. *Salmonella enterica* subsp. *enterica* serovar Enteritidis; however, in most cases this is shortened to *Salmonella* (*S.*) [*serotype*], e.g. *S. Enteritidis*.

Salmonella can be further divided into serotypes, based on somatic (O) and flagellar (H) antigens in the Kaufmann-White system, derived from agglutination with homologous antisera. Most *Salmonella* have two genotypic forms of H-antigens (phases I and II) (D'Aoust 1989). A few serotypes have retained names that show their O- and H-antigens, e.g. *S. 61:k:1,5,7*, which possesses the O-antigen 61, the phase I H-antigen k and the phase II H-antigens 1, 5 and 7. However, for most serotypes trivial names, often derived from diseases (e.g. Typhimurium) or places of first identification (e.g. Dublin), are retained.

Different methods for further differentiation, e.g. antibiograms, biotyping, phage typing, plasmid profile analysis, pulsed field gel electrophoresis (PFGE) and other molecular epidemiological techniques, have been used extensively to characterise *Salmonella*.

Moreover, *Salmonella* may be divided into host-specific, host-adapted and non-host-adapted serotypes. *S. Gallinarum* and *S. Pullorum* are the classical examples of serotypes specific to fowl. Generally, the serotypes of zoonotic importance are non-host-adapted, but exceptions exist, e.g. *S. Dublin* (adapted to cattle and sheep) or *S. Choleraesuis* (adapted to pigs) which can cause severe disease in humans.

As for transmission within the same species, *Salmonella* serotypes are often designated according to their ability to spread vertical or horizontal infections. *S. Enteritidis*, *S. Typhimurium* and *S. Berta* are classical examples of serotypes that are more readily transmitted vertically, whereas most other zoonotic serotypes found commonly in poultry are mainly transmitted horizontally.

1.2 Historical overview

In 1885, the American bacteriologist D.E. Salmon described a bacterium, *Bacterium suipestifer*, which he thought was the cause of hog cholera (Marthedal 1960). At the beginning of the 20th century, hog cholera was found to be a viral disease, and *B. suipestifer*, later named *Salmonella Choleraesuis*, was only a secondary agent (Marthedal 1960). In the years after 1885, there were several reports of disease in animals and humans caused by *Salmonella*. In 1888, 58 persons became ill after having eaten beef from one cow, and *Bacterium enteritidis* was isolated from that outbreak. Three years later, *Bacterium typhimurium* was isolated from dead laboratory mice (Marthedal 1960; Barrow 1993). In 1934, the group of bacteria to which *S. Choleraesuis* belonged was officially named *Salmonella* (Marthedal 1960).

Thus, the zoonotic importance of *Salmonella* has been recognised for more than a century, but the prevalence of *Salmonella* in animals and humans was difficult to assess before the development of current surveillance systems. Most reports of *Salmonella* infections in animals and humans were due to clinical outbreaks (Jepsen 1960; Poppe 1999). Up till the 1960s, eggs from ducks, geese, turkeys and hens were often described as the main food vehicles causing human salmonellosis, and *S. Typhimurium* often caused these outbreaks (McCoy 1975; Poppe 1999). Without a routine surveillance programme, these clinical outbreaks were probably over-represented and did not reflect the true occurrence of salmonellosis in humans (Barrow 1993; Poppe 1999).

Increased routine testing and awareness of the epidemiology of *Salmonella* in the 1950s and 1960s revealed numerous horizontal serotypes in feed and subclinical infections in broilers (Brown *et al.* 1973; Barrow 1993). Moreover, the increased global food trade and the industrialisation of the poultry sector with fewer and bigger enterprises probably also contributed to this increase (McCoy 1975; D'Aoust 1994). As for human salmonellosis, fewer serotypes and larger outbreaks are generally seen in countries with a highly developed commercial poultry sector, whereas a wider range of serotypes and more sporadic and smaller outbreaks occur in countries with a less developed poultry sector (Barrow 1993). Until the 1980s, *S. Typhimurium* was generally the most common serotype in humans (Doyle and Cliver 1990; Le Bacq *et al.* 1993), although many other serotypes were also reported.

In the 1980s and 1990s, a global increase of human salmonellosis due to *S. Enteritidis* was observed (Rodrigue *et al.* 1990). The reason for this global increase is unknown, amongst other things because phage type 8 dominates on the American continent whereas phage type 4 is the most widespread in Europe, so a common source is unlikely (Rodrigue

et al. 1990; Wray 1995). However, the most likely source of a global increase of one serotype that is rarely found in feed is one or more of the few breeding companies that supply most of the world's commercial poultry, although other reasons have been proposed (Rabsch *et al.* 2000; Cogan and Humphrey 2003). It is generally agreed that eggs are the main food vehicle implicated in human salmonellosis due to *S. Enteritidis* (St Louis *et al.* 1988; Bean and Griffin 1990; Rodrigue *et al.* 1990; Petersen and James 1998), though this view has been challenged, e.g. by Duguid and North (1991). An important epidemiological aspect of *S. Enteritidis* is its ability to infect and persist in the ovaries and oviduct (Hopper and Mawer 1988; Shivaprasad *et al.* 1990; Barrow 1993). This means it can more easily pass along the farm-to-fork chain and spread in the hatchery in spite of effective hygiene measures to eliminate eggshell contaminations.

One of the results of the increased *S. Enteritidis* occurrence has been the implementation of systematic surveillance and control programmes in many western countries (Altekruse *et al.* 1993; Edel 1994; Hogue *et al.* 1997; Wegener *et al.* 2003). The combination of these systematic registrations and several typing studies have elucidated many associations between human and animal *Salmonella* occurrence which in the past were suspected but often poorly documented.

1.3 General epidemiological aspects of *Salmonella* in the poultry sector

The following key aspects explain the widespread occurrence of *Salmonella*:

- Most serotypes can infect a wide range of mammals, birds and reptiles. Most of the common food domestic and wild vertebrate animals are birds or mammals, i.e. there is a big potential infection reservoir.
- The natural habitat of *Salmonella* is the gastro-intestinal system. Thus, infected animals and humans excrete the bacteria in their faeces, either continuously or intermittently.

- *Salmonella* can survive for long periods and under dry conditions in the environment, especially in organic matter.

Moreover, the following main characteristics of the commercial poultry sector explain why *Salmonella* infections can become so widespread in poultry:

- There is a hierarchical structure; the number of animal enterprises increases towards the bottom of this hierarchy, so a *Salmonella* infection at the top can easily spread downwards.
- Hatcheries and abattoirs are often “bottle neck” premises with optimal conditions for bacterial growth and cross-contamination, factors that can magnify the number of *Salmonella*-infected flocks or poultry meat products.
- The historical tendency towards bigger and fewer poultry enterprises increases the likelihood of spreading *Salmonella* along the farm-to-fork chain.

Many studies on *Salmonella* occurrence in the poultry sector are based on *Salmonella* sampling results, including either the whole poultry hierarchy or parts of it (Morris *et al.* 1969; Bains and MacKenzie 1974; Bhatia and McNabb 1980; Rigby *et al.* 1982; Lahellec and Colin 1985; Jones *et al.* 1991; Davies *et al.* 1997; Käsbohrer and Blaha 1997; Murase *et al.* 2001), or they focus more on specific aspects, often locally on the premise. Most studies describe the dissemination of *Salmonella* serotypes, but few distinguish between primary infection sources and persistent infections. This distinction may, however, be somewhat artificial, depending amongst other things on which analytical unit is used: *Salmonella*-infected mice may persist on farm level, but may also introduce *Salmonella* into other poultry houses.

Nevertheless, as the main topic here is persistent infections, it is important to distinguish these from primary sources.

1.4 Main sources of *Salmonella* in the poultry sector

Airborne *Salmonella* infections between houses or farms are uncommon (Doyle and Cliver 1990; Wray and Davies 1997), thus they are mainly introduced with any vehicle that comes into the poultry house. These vehicles either have to enter the poultry house (feed, the poultry itself, water, litter and people who attend to the poultry), or their introduction can be avoided (e.g. wild animals or equipment) by bio-security measures.

Though it is difficult to document the infection source in every single case, it is generally agreed that many *Salmonella* infections are introduced either by contaminated feed or the poultry itself (Hinton and Linton 1988; Doyle and Cliver 1990; Wray and Davies 1997).

In feed, many studies have described the presence of various horizontally transmitted serotypes (D'Aoust 1994; Veldman *et al.* 1995; Davies and Wray 1997), whereas vertically transmitted serotypes such as *S. Enteritidis* or *S. Typhimurium* are rarely found (Veldman *et al.* 1995; Davies and Wray 1996a); the reasons for this difference in occurrence are not known. The serotypes found in feed often differ from the serotypes in the poultry that receives this feed (Snoeyenbos *et al.* 1967; Dougherty 1976; Veldman *et al.* 1995). This lack of association might indicate the multifactorial epidemiology of *Salmonella* infections, and/or it reflects differences in infectious doses and susceptibility in chickens. In addition, *Salmonella* can occur in very low numbers in feed and still infect chicks (Milner and Shaffer 1952; Hinton 1988), and the distribution in a solid is often uneven, so when only a minor fraction of the feed is sampled, *Salmonella* will often be undetected (Davies and Wray 1997).

Day-old chicks infected with *Salmonella* from the hatchery are frequently an important *Salmonella* source on the farms (Bhatia and McNabb 1980; Lahellec and Colin 1985;

Cox *et al.* 1990; Bailey *et al.* 1994; Cason *et al.* 1994; Davies and Wray 1994; Christensen *et al.* 1997; Cox *et al.* 2000).

Several studies have described *Salmonella* contamination of water, mainly due to contamination from sewage or sludge (Smith *et al.* 1978; Jones *et al.* 1980; Kinde *et al.* 1996a), but this infection source is not common in poultry, although it has been reported (Kinde *et al.* 1996a).

It is difficult to document the exact role of people in the introduction of *Salmonella* infections, but strict bio-security measures will logically minimise the risk. Poultry attendants who are intestinal carriers have been reported (Savov *et al.* 1976), but their exact role in a possible *Salmonella* introduction has not been elucidated.

The actual contribution of equipment, including litter, has not been investigated in many studies, but logically they can be a hazard, e.g. *Salmonella* contaminated crates from the abattoir or hatchery (Rigby *et al.* 1980). Obviously, the bio-security principles that apply to people also apply to machines and equipment used in other places than the poultry house.

Murray (1991) reviewed *Salmonella* contamination in the environment, amongst other things in wild animals, and its importance for domestic animals. *Salmonella* is found in wild animals, but often in less than 1% of a species (Borg 1985; Murray 1991; Köhler 1993; Cizek *et al.* 1994; Refsum 2003). However, seagulls often have higher *Salmonella* prevalence, and they carry serotypes that reflect those among humans and animals in the region, which is probably due to their foraging on refuse tips, coastal sewage outfalls and manured farmland (Coulson *et al.* 1983; Monaghan *et al.* 1985; Murray 1991). Thus, their role as *Salmonella* vectors over long distances and their proximity to sites of humans and domestic animals can be important. Direct introduction of *Salmonella* from wild animals

into poultry premises is obviously difficult to prove, and it has only been reported anecdotally. However, a primary introduction from wild animals to cattle and sheep on pastures has been described (Reilly *et al.* 1981; Coulson *et al.* 1983; Euden 1990).

In conclusion, exclusion of *Salmonella* from the poultry inserted in the house and from the poultry feed will often reduce *Salmonella* incidence significantly. Also, it seems logical that thousands of animals and tonnes of feed introduced direct into a farm building are more risky than an occasional seagull or migrating rodent that comes into contact with domestic poultry.

1.5 Epidemiology of persistent *Salmonella* infections

Few studies focus on factors that can influence *Salmonella* persistence. This might reflect the situation in most countries where the Scandinavian attitude of zero-tolerance of *Salmonella* in a poultry flock is unusual, but even in the Scandinavian countries there has been little research on persistence of *Salmonella* infections in poultry houses.

Table 1.1 gives an overview of multi-disciplinary studies related to factors that can contribute to persistent *Salmonella* infections on poultry farms; studies on specific aspects (e.g. mice or beetles) are not included. The factors which can contribute to a persistent *Salmonella* infection cannot be compared directly to each other, as the type of study, epidemiological conditions and surveillance systems differed between countries and periods. The studies can be divided into large-scale studies based on questionnaires (Henken *et al.* 1992; Fris and Van den Bos 1995; Rose *et al.* 2000) or compiled from surveillance databases (Angen *et al.* 1996) and field studies where a few poultry enterprises were scrutinised more closely.

Table 1.1: Multi-disciplinary studies investigating risk factors which possibly influence the *Salmonella* persistence on poultry farms.

Country	Type(s) of premise	Factor(s)	Type of study ¹	Source
Canada	Broiler houses	Cleaning and disinfection of ventilation system.	FS	Higgins <i>et al.</i> 1982
France	Broiler flocks	Resident <i>Salmonella</i> spp.	FS	Lahellec <i>et al.</i> 1986
Netherlands	Broiler breeder flocks	Disinfection tubs, hygiene barriers, feed mills.	RCCS(Q)	Henken <i>et al.</i> 1992
USA	Layer flocks	Environment, especially mice, and improperly cleaned and disinfected poultry houses.	FS	Opitz 1992
Netherlands	Egg laying flocks	Improperly cleaned and disinfected poultry houses, vermin.	MS(CIC)	van de Giessen <i>et al.</i> 1994
UK	Broiler breeder and broiler houses	Cleaning, disinfection, disinfectant, cleaning by farmers vs. contractors, mice.	FS	Davies and Wray 1995c
Netherlands	Broiler breeder farms	Several (main groups: hygiene, surroundings, and poultry).	RCCS(Q)	Fris and Van den Bos 1995
Denmark	Broiler flocks	Hatchery, feed mill, numbers of houses on the farm, <i>Salmonella</i> in preceding flocks, season.	RLDS	Angen <i>et al.</i> 1996
UK	Breeder flocks	Cleaning, disinfection, environment.	FS	Davies and Wray 1996a
UK	Broiler breeder houses	Disinfection, mice.	FS	Davies and Wray 1996b
Germany	Broiler houses	Horizontal introduction.	FS	Käsbohrer and Blaha 1997
UK	Commercial turkey farms	Type of disinfectant.	FS	Davies <i>et al.</i> 1998b
Germany	Turkey houses	Cleaning, disinfection.	FS	Ruckaberle <i>et al.</i> 1999
France	Broiler houses	Disinfection, rodents, accessibility of trucks, disease in previous flock.	PS(SQ)	Rose <i>et al.</i> 2000

¹ FS = field study; MS(CIC) = model study (cumulative infection curve); PS(SQ) = prospective study (sampling and questionnaire); RCCS(Q) = retrospective case-control study (questionnaires); RLDS = retrospective, longitudinal database study.

Not surprisingly, each type of study has its pros and cons. In the field studies, the problem was often the low number of analytical units. The large-scale studies often identified more risk factors than the field studies, either because they were more comprehensive or because they had more variables. Many variables increase the chance of finding factors which are statistically, but not necessarily biologically, significant. One way of reducing this pitfall is the use of multivariable analyses (which was done in all the large-scale studies cited). A more specific problem in some studies was the lack of historical data (Henken *et al.* 1992; Rose *et al.* 2000) that could determine if significant factors were related to persistence or introduction of *Salmonella*. Henken *et al.* (1992) found feed mills as one of the main risk factors, but it was not elucidated if this was related to a persistent *Salmonella* infection or successive introductions from the same feed mills. Fris and van den Bos (1995) estimated that during a three-year period, horizontal transmission accounted for about 30% of the *S. Enteritidis* occurrence on the farm. However, these 30% covered both persistent infections and introductions, e.g. from neighbouring farms and by vehicles. Angen *et al.* (1996) conducted a comprehensive database study including many risk factors and serotypes, but rightly emphasised that separate analyses for each serotype could be relevant, as these may have different pathogenesis.

The field studies either investigated persistence as one of several factors that could contribute to the *Salmonella* status of the flock (Higgins *et al.* 1982; Lahellec *et al.* 1986; Davies and Wray 1994; Kinde *et al.* 1996b; Käsbohrer and Blaha 1997; Davies *et al.* 2001) or they focused solely on persistence of *Salmonella* in the poultry enterprise (Davies and Wray 1995c, 1996a, 1996b; Davies *et al.* 1998b; Ruckaberle *et al.* 1999; Fournier *et al.* 2001).

The influence of resident *Salmonella* in the poultry house itself on the *Salmonella* status of the subsequent flock(s) was shown in a few studies (Higgins *et al.* 1982; Lahellec *et al.* 1986; Opitz 1992; Rose *et al.* 1999).

The few detailed field studies on persistent *Salmonella* infections in poultry premises have mainly been performed by one scientific group (Davies and Wray 1995c, 1996a, 1996b; Davies *et al.* 1998b, 2001). Disinfection with formaldehyde or synthetic phenols was generally effective, whereas other disinfectants, e.g. glutaraldehyde or peroxygen compounds, were less efficient. Moreover, a satisfactory *Salmonella* reduction could be achieved with formaldehyde or a synthetic phenolic compound in spite of a relatively poor cleaning standard, whereas an equally effective disinfection with certain other disinfectants could only be achieved if the cleaning standard was very high (Davies and Wray 1995c; Davies *et al.* 1998b). However, an exact assessment of the cleaning standard was not reported. In another study, a standard using pre-determined critical control points in battery cage houses was used, and dirty sites were positively related to the occurrence of *S. Enteritidis* (Fournier *et al.* 2001). The ability of *Salmonella* to survive in poultry houses after depopulation, up to 53 weeks in dust (Davies and Wray 1996a) and up to 26 months in litter, dried faeces and feed (Davies and Breslin 2003b), illustrates its recalcitrance.

The role of animals other than poultry in persistent *Salmonella* infections is beyond the scope of this thesis, so it will only be discussed briefly. The role of mice has been reported in several studies (Krabisch and Dorn 1980; Henzler and Opitz 1992; Opitz 1992; Davies and Wray 1995b, 1996b; Kinde *et al.* 1996b) whereas rats seem to be less important, maybe because these are less prone to establish stationary populations (Hunter *et al.* 1976; Krabisch and Dorn 1980), or they are controlled more effectively because they are more disliked. Though *Salmonella* has been detected in several invertebrates, their exact

role for *Salmonella* persistence is difficult to elucidate. Much focus has been on the lesser mealworm (*Alphitobius diaperinus*) that occurs world-wide in poultry houses, but its importance in persistent *Salmonella* infections remains controversial (De las *et al.* 1968; Krabisch and Dorn 1980; Davies and Wray 1995a).

Chapter 2 – Procedures for cleaning and disinfection of poultry houses

2.1 Recommended cleaning procedures

In the commercial poultry sector, an all-in all-out system is applied, and cleaning is normally performed in depopulated poultry houses.

Cleaning is a mechanical process, where the main objective is the removal of organic matter and – if present – biofilms. It has always to be done prior to disinfection, and these two processes should be performed separately.

Several guidelines on cleaning procedures in poultry houses exist, and the focus on zoonotic *Salmonella* has put much emphasis on biosecurity, cleaning and disinfection of poultry houses. Generally, the following chronological cleaning steps are described (Linton *et al.* 1987; von Löhren 1994; Meroz and Samberg 1995; Sørensen and Pedersen 1998; Anonymous 2002a):

- 1) Manure and feed are removed and disposed of.
- 2) Any necessary repairs to the poultry house and equipment are carried out.
- 3) As much equipment as possible is dismantled and, if possible, soaked in water, preferably with a detergent.
- 4) Some guidelines describe a dry cleaning step (Meroz and Samberg 1995). Removal of dust with an industrial vacuum cleaner will minimise the bacterial load in the aerosols formed during washing, but dry cleaning is probably rarely practised.
- 5) The poultry house is wetted with water, preferably with a detergent.
- 6) High pressure washing of the poultry house, preferably with hot water at 40-50 °C (Sørensen and Pedersen 1998) or 60 °C (Meroz and Samberg 1995), as higher tem-

peratures will denature proteins (Meroz and Samberg 1995). Often, an alkaline detergent is recommended (von Löhren 1994; Sjøberg and Pedersen 1998). The washing is performed from the ceiling towards the floor, and it begins farthest from the drains.

Ventilation systems should be washed from the outside of the house. Water pipes are flushed with the highest possible pressure, and a detergent or acidifier is often recommended. All washing should be continued until there is no visible organic matter.

2.2 Cleaning of Danish poultry houses

Specific procedures and recommendations probably differ between countries, but in order to define the subject Danish procedures will be briefly described. With regard to procedures, Danish chicken farms can generally be categorised into three groups: breeding stock, broilers, and table egg layers (including commercial rearing houses).

The breeding stock farms are either privately owned or they belong to a hatchery. Written guidelines, which follow the principles outlined in official recommendations (cf. Section 2.1), are supplied by the hatcheries (Skaarup 2002). Private breeding stock farmers have to follow these guidelines in order to deliver eggs to the hatchery. Between November 1996 and September 1999, *Salmonella* was detected in 13 breeding stock farms that were visited by the author in the subsequent download period. In general, very little organic matter was seen, and as much equipment as possible had been dismantled. As per December 2003, none of the *Salmonella* types has been re-detected in samples from subsequent flocks, except for one farm which was the only parent stock farm inspected where a substantial amount of organic matter was seen.

Since 1989, routine *Salmonella* samples have been submitted from each broiler flock when the chickens are about three weeks old (Bisgaard 1992; Anonymous 2003), and payments from the abattoirs are reduced in case of *Salmonella* positive flocks. Thus, Dan-

ish broiler farmers have long experience in cleaning and disinfection. On the other hand, most broiler farms are privately owned, and they are not under the same strict control measures as the breeding stock. This means that the cleaning standard varies more, a tendency confirmed by visiting about 100 of the 330 Danish broiler farms after cleaning (pers. obs. and cf. Chapter 7). Generally, removal of manure and washing with a mobile high pressure cleaner is done on the first day of the download period, and disinfection is performed the day after. Equipment is rarely dismantled, even in persistently *Salmonella*-infected broiler houses (Gradel 1998). Repairing in the house is normally done in the period between disinfection and re-stocking of the house. This is not to be recommended, and there are quite a few examples of *Salmonella* re-occurrence in a subsequent flock after dislodging dust when repairing ceilings or air chimneys (pers. obs.).

As regards table egg farms, there are even bigger variations in cleaning standards and procedures. Most table egg farms are privately owned, but contrary to the broiler farms they do not have the same tradition of a routine wet cleaning in the download periods. The reasons for this can only be speculative, but before the present *Salmonella* control programme was implemented in December 1996, *Salmonella* in table egg layers did not cause economic consequences for the farmers. Moreover, many buildings were not designed for wet cleaning, e.g. they lack drains or have ceilings made of materials that are damaged by high pressure cleaning. Finally, the equipment can be very difficult to clean, especially cage systems in battery houses. Thus, many table egg houses have never been wet-cleaned; the procedure in the download periods has often been poor dry cleaning, so only the worst organic matter was removed.

2.3 Documentation for recommended cleaning procedures

Little scientific work has been done on cleaning of animal houses, so most of the experience is empirical (Linton *et al.* 1987). Monitoring of cleaning is difficult to measure in real life situations. Probably, the most important criterion for a successful cleaning is the lack of visible organic matter, but this is based on a subjective estimate. The most widely used parameter is reduction in bacterial counts, a relatively objective parameter, which empirically should be related to the removal of organic matter. However, in two studies from calf and pig houses (Beer *et al.* 1980) and hospital wards (Griffith *et al.* 2000), visual cleanliness was reported to be a poor indicator of the microbial status. Moreover, microbial results have to be interpreted carefully, as the proportions of bacteria adhering to a surface that can be swabbed differ between methods and materials, e.g. related to roughness (also cf. Section 5.2.3). Linton *et al.* (1987) stated as a general rule of thumb that about 99% of the bacteria can be removed by cleaning under experimental conditions, whereas only a 90% reduction can be expected under more realistic conditions. Davies and Wray (1995c) reported increased numbers of *Salmonella* and coliforms after cleaning of poultry houses, amongst other things on beams, which could be due to aerosols from the high pressure washing and/or activation of dormant bacteria in the dry dust when this was moistened.

Recommendations for using hot water and an alkaline detergent are based on the easier dissolution of fats (Gibson *et al.* 1999). However, the few studies in animal houses suggest that the practical relevance of this may be negligible. In two studies, cleaning with cold and hot water and with/without detergents were compared, and the bacterial reductions were the same for all the four possible combinations (Walters 1967; Morgan-Jones 1981). Sundahl (1975) confirmed this for the somewhat subjective term “cleanability” of materials, which were washed after soaking with and without a detergent. The use of a

detergent even made the cleaning of some hard-to-clean materials more difficult, possibly because the detergent dissolved the organic matter which was then sucked into the surface of the materials. In a recent study, Banhazi *et al.* (2003) cleaned concrete hygiene pavers (simulating floors in pig houses) by high pressure cleaning or hosing, the latter with or without pre-soaking with a detergent. High pressure cleaning and the use of a detergent followed by hosing were equally effective, and both were more efficient than hosing without a detergent. The same study showed that drying further decreased the microbial load, but only in a clean area (i.e. the pavers were moved away from the cleaning site), as aerosols re-contaminated the concrete surfaces in dirty areas.

Many other factors also affect the cleaning of premises. In the study by Morgan-Jones (1981), various materials were placed in poultry and pig houses during a two-week period, and big differences in bacterial counts were found, ranging from 5,360 CFU 100 cm⁻² on varnished ply board to 116,000 CFU 100 cm⁻² on block board. A study of floor and wall materials used in the food industry showed that the effectiveness of cleaning, measured by reductions in bacterial counts, could not be predicted from the gross roughness of materials (Taylor and Holah 1996); microscopic irregularities may be more relevant (Holah and Thorpe 1990). In a project on cleaning of commercial kitchens, thousands of samples for total bacterial counts were taken from surfaces, but no general tendencies could be related to cleaning procedures, surfaces or other hygienic parameters (Anonymous 2002d). Gibson *et al.* (1999) described cleaning techniques and bacterial biofilms on floors in the food industry, and found that mechanical high pressure washing was the most effective way to remove biofilms, a result in accordance with several other studies on the removal of biofilms.

2.4 Disinfection procedures in Danish poultry houses

The types of disinfectant used and their mode of application probably vary between countries and poultry sectors, and it is often tradition or effective marketing, rather than a professional judgement, which determines this. In this section, the general situation for Danish poultry houses will be outlined.

Figure 2.1 gives an overview of disinfectants used in the downlead periods after *Salmonella* positive broiler flocks. Disinfectants containing 23% glutaraldehyde and 5% benzalkonium chloride as the active ingredients have gained much popularity in recent years. This is probably due to marketing campaigns that have emphasised the carcinogenic effects of formaldehyde, its supposed low efficacy below 16 °C and its apparent reduced ability to be combined with tensides and alcohols in fogging. Some years ago, oxidising disinfectants based on combinations of peracetic acid and hydrogen peroxide were also introduced to the Danish market, as these are supposedly less detrimental for people and animals.

There are no official records of the mode of application, e.g. surface disinfection or thermal fogging. Thermal fogging has gained much popularity recently because glutaraldehyde/benzalkonium chloride compounds with higher amounts of ancillary substances that promote the hovering effect have been introduced to the market.

The disinfectants in Figure 2.1 are mainly those used for the house itself, as other compounds are normally used for water systems. A variety of disinfectants is used for water systems, such as organic acids, silver salts, chlor compounds or oxidising disinfectants (pers. obs.), though no official records of this exist. When growth promoters were banned from Danish poultry feed in February 1998, broiler farmers were encouraged to use or-

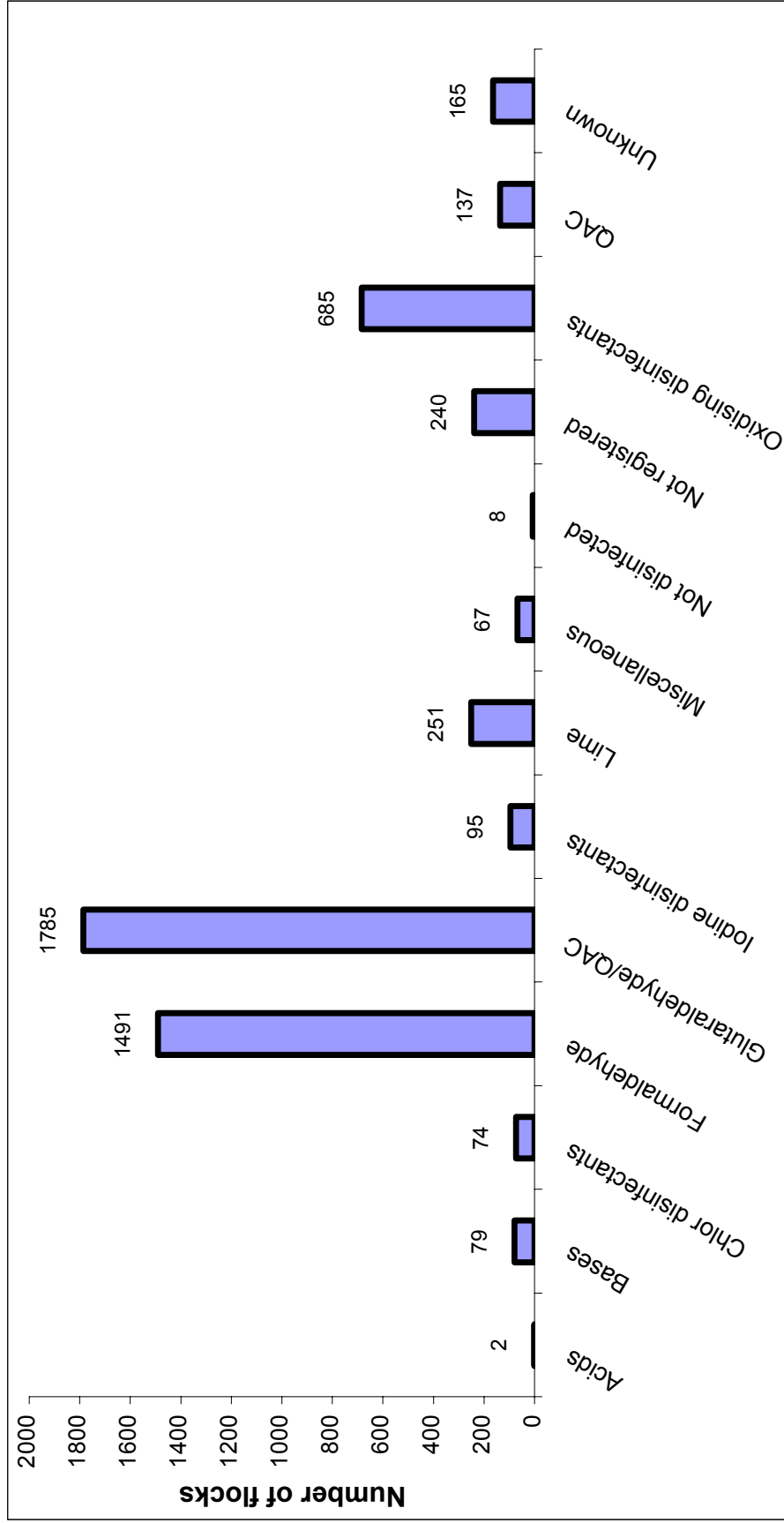


Figure 2.1: Disinfectants used in download periods after 4,629 *Salmonella* positive Danish broiler flocks in the period 3/1/92-3/10/01.
Source: Poultry database under the Danish Poultry Council.
QAC: Quaternary ammonium compound, usually benzalkonium chloride.

ganic acids in the water to minimise the risk of diseases. Another disinfectant type that is probably under-represented in Figure 2.1 is bases, mainly hydrated lime or sodium hydroxide, which are often used on floors and walls after the general disinfection.

One company has specialised in heat-treating poultry houses, using steam at 160-170 °C with formaldehyde (“the Danish method” (Profe and Trenner 1994; von Löhren 1994)). The sealed houses are heated to an air temperature of about 60 °C, which is achieved after 30-60 minutes, and the relative humidity rapidly reaches 100%.

In principle, the above procedures also apply to Danish houses for poultry sectors other than broilers, but as for cleaning procedures, they are applied consistently in breeder houses, but less diligently or not at all in table egg houses. The following is from the quality assurance system run by a broiler hatchery in Denmark (Skaarup 2002): “The house is disinfected with a glutaraldehyde/benzalkonium chloride compound, applied both as surface disinfection and as thermal fogging. In most houses, floors and walls are subsequently surface disinfected with hydrated lime. Water lines are disinfected with an oxidising disinfectant which stays there for at least five hours before rinsing with water. All disinfectants are applied in concentrations according to the manufacturer’s instructions”.

Chapter 3 – Detrimental environment, bacterial stress responses and disinfectant resistance

3.1 General aspects

The concept of stress has been widely and often vaguely used in many contexts to denote something that is not optimal. Storz and Hengge-Aronis (2000) suggested different definitions of bacterial stress. One of these was simply any deviation from optimal growth conditions which results in a reduced growth rate, but there are exceptions, as some bacteria have adapted so well to detrimental conditions that their growth is not impaired. Other definitions include damage to cellular components without a cellular response, concurrent with a stimulation of specific stress-response genes, but these definitions do not necessarily cover the concept because unknown physiological reactions or unidentified genes cannot be ruled out (Storz and Hengge-Aronis 2000). In this context, the most relevant definition is probably any exposure that impairs growth, as this is highly relevant with regard to cleaning and disinfection, where the growth phase of bacteria probably plays an important role. Parallel to this, stress responses in bacteria are thus found in the transitory phase between growth and death.

Many principles which contribute to bacterial stress are general, e.g. bacteria in the stationary phase are generally more resistant to detrimental factors than bacteria in the growth phase (Hansen and Riemann 1963; Gawande and Bhagwat 2002b). Moreover, *E. coli* is probably the bacterium most commonly studied in stress investigations, thus its similarity to *Salmonella* is advantageous (Neidhardt and VanBogelen 1987; Foster and Spector 1995).

Because disinfectant resistance is an inherent part of bacterial responses to detrimental conditions, this will also be considered in this chapter.

The topics in this chapter are extensive; therefore, the focus will be on Gram-negative bacteria which between them have many properties in common, and are likely to be most relevant to *Salmonella* spp.

3.2 Detrimental factors and survival in the extra-animal environment

3.2.1 General aspects

Numerous investigations have dealt with detrimental environment outside of living hosts and stress responses in *Salmonella*. Most studies on detrimental conditions and *Salmonella* are related to food microbiology, i.e. materials that resemble organic matter in poultry enterprises. Other studies, however, have been performed under in-farm conditions (e.g. survival in faeces, dust and soil).

Optimal growth conditions are not necessarily the same as optimal survival conditions, especially not when speaking of long-term survival (more than a few days). Conditions in the natural environment probably only allow growth of bacteria for limited periods; most of the time they experience a stationary/dormant/quiescent phase, sometimes designated a “feast-or-famine lifestyle” (Kolter *et al.* 1993) which, with the definitions in this review, means they are stressed most of the time. The main exception is probably biofilms in which bacteria grow (Costerton *et al.* 1995).

The external factors which have been described as related to survival of naturally occurring bacteria can be categorised into the following groups:

- Chemical substances
- Humidity
- pH

- Temperature

Other detrimental factors and mechanisms (e.g. osmolarity, oxidative stress, competing bacteria and iron stress) have been extensively studied in the laboratory (Foster and Spector 1995) (also cf. Section 3.3), but generally their role for survival of naturally occurring bacteria has not been elucidated, although they probably also play a significant role for these.

Many detrimental factors influence each other, e.g. water activity and osmolarity are often closely correlated (Ingraham and Marr 1996), and the amounts of different chemical substances (e.g. carbohydrates or fats) influence the water activity (Hansen and Riemann 1963; Senhaji 1977). The development of resistance in bacteria as a result of one stress factor can also influence susceptibility towards other stress factors and general survival. Humphrey *et al.* (1995) showed that *S. Enteritidis* that was tolerant to heat, acid or hydrogen peroxide survived longer on surfaces. In a study by Berchieri and Barrow (1996), certain disinfectants were less effective against *S. Enteritidis* if this had been stressed prior to the disinfection. Such interactions often complicate studies concerning the exact role of specific detrimental factors.

3.2.2 Chemical substances

The role and mechanisms of action of specific substances involved in the survival of bacteria in the natural environment are difficult to assess, and the literature is sparse. In studies involving survival of *Salmonella* in e.g. feed or faeces (Juven *et al.* 1984; Plym-Forshell and Ekesbo 1996), little attention was given to the role of chemical substances. The survival of *Salmonella* for at least three years in fluff (Miura *et al.* 1964) and for more than four years in ward floor dust (Robertson 1972), i.e. materials with low nutrient levels, suggests that nutrients *per se* are not very important for sustaining life. In general, investi-

gations of long term survival of desiccated bacteria on surfaces show that these survive longer if they are protected by various chemical substances (McDade and Hall 1964), but the exact mechanisms have not been elucidated. Fry and Greaves (1949) studied short and long term survival when drying bacteria; much longer survival times – for *S. Typhimurium* up till two years – were reported when 5-10% glucose was added to the nutrient broth. On the other hand, laboratory media for long-term storage of bacteria often have few nutrients to extend their lifetime, but other storage media have more nutrients, and the topic can be debated.

3.2.3 Humidity

The collective term “humidity” covers different aspects of the water content. In food microbiology, water activity (a_w) is often used; in studies related to surfaces, per cent relative humidity in the air (% RH) is often the term; moisture is mainly applied to conditions in various materials. The influence of humidity factors on survival of bacteria in and on various materials has been investigated in many studies. A special aspect is humidity and heating of bacteria (cf. Section 4.1.3).

In general, *Salmonella* is able to survive long periods of desiccation (D'Aoust 1989; Janning *et al.* 1994). Relevant materials include surfaces of farm building materials, dust, fabrics, faeces and feed, which all simulate materials and organic matter found in empty poultry houses. McDade and Hall (1964) investigated several bacterial strains, including *S. Derby*, on surfaces of metal, glass and ceramic tile at 11, 53 and 85% RH. The survival rates were inversely related to increased relative humidities for all bacterial strains and materials. Bale *et al.* (1993) showed that two strains of *E. coli* on glass had the highest death rate at about 80% RH, while it declined both above and below this level. This study differed from the previous one by McDade and Hall in that a wider range of relative hu-

midities (10-100%) was investigated. Lidwell and Lowbury (1950) investigated the effect of atmospheric humidity on the survival in dust of *Staphylococcus aureus* and *Streptococcus pyogenes*, and reported that higher humidities (including % RH close to 100) accelerated death rates. Fourteen years later, Harry and Hemsley (1964) showed the same tendency for coliforms in dust which survived better at 10.1% than at 70.1% RH, but only these two RH were investigated. A study with *S. Typhimurium* on different kinds of fabrics showed persistence for about 24 weeks at 35% RH, whereas this period was significantly shortened at 78% RH (Wilkoff *et al.* 1969). Turnbull and Snoeyenbos (1973) found that death rates for *S. Typhimurium* in poultry litter were significantly increased at higher water activities (including $a_w \sim 1.0$). Tucker (1967) confirmed this tendency for *Salmonella* in poultry litter at 29.8 and 44.0 % moisture. On the other hand, Himathongkham *et al.* (2000) reported that mixing one part of poultry manure with two parts of water decreased the death rate of *S. Typhimurium* and *E. coli*, but this was thought to be due to dilution of the ammonia concentration. Himathongkham *et al.* (1999) investigated *S. Enteritidis* and *S. Typhimurium* at different water activities in chicken manure. The highest death rate was seen at a water activity of 0.89, while it declined both above and below this level. Juven *et al.* (1984) found higher survival rates for both *S. Montevideo* and *S. Heidelberg* in poultry feed, meat and bone meal, dry milk and cocoa powder at water activities of 0.43 and 0.52 than at 0.75. Doesburg *et al.* (1970) distinguished between initial death rates and death rates over longer storage periods. There was no significant difference in the initial death rate when comparing water activities of 0.54 and 0.71, whereas it was reduced significantly at a water activity of 0.34. Long-term death rates were smaller than the initial ones, especially at the two lower water activities (0.34 and 0.54).

In general, it can cautiously be concluded that under conditions simulating those found in poultry houses, lower humidities prolong survival times for *Salmonella*, but this general picture may change and/or become more detailed if studies with a wider range of water activities, especially those approaching 1.0, are performed.

3.2.4 pH

Salmonella are neutrophiles with respect to pH, i.e. they grow in the approximate range 4-9, with optimal growth at 7 (Doyle and Cliver 1990; Ingraham and Marr 1996). Regulation of pH in foods is a well-established way of retarding growth of micro-organisms, but fewer studies on survival exist. In chicken litter, ammonia has mainly been considered to be responsible for a high pH, but as the inactivation mechanisms of bacteria in faeces are largely unknown, the detrimental effect of ammonia is not necessarily related *per se* to the pH (Himathongkham *et al.* 2000). Turnbull and Snoeyenbos (1973) found a relation between an alkaline pH and high ammonia levels, although deviations from this relationship were also noticed. They reported that higher levels of ammonia at water activity above 0.4 increased the death rates for *Salmonella*, whereas there was no difference at water activities below 0.4. Although Tucker (1967) focused on water activity, a lower ammonia level and/or pH could also be involved in the higher survival in new than in old litter, and in pens left unoccupied which prolonged the survival times significantly in both old and new litter.

In general, the few field studies that focus on pH are with faeces, and the dynamics in this material make it difficult to elucidate the exact role of pH in survival. Strong acids and bases are used as chemical disinfectants, but such extreme pHs are probably not found in faeces or other organic materials.

3.2.5 Temperature

Salmonella, like other enteric bacteria, has its optimal growth at about 37 °C; this declines towards the limits of the growth range (ca. 5-45 °C) (Doyle and Cliver 1990; Ingraham and Marr 1996).

Himathongkham *et al.* (2000) compared the survival of *S. Typhimurium* and *E. coli* in manure and manure slurry at 4, 20 and 37 °C, and found that D-values (cf. Section 5.3.1) decreased with increasing temperatures, e.g. 6-22 weeks at 4 °C, but only 1-2 days at 37 °C. The same tendency was reported by Wang *et al.* (1996) in bovine faeces, by Plachá *et al.* (2001) in pig slurry, and about two decades earlier by Williams and Benson (1978), who investigated survival of *S. Typhimurium* in both poultry feed and litter at 11, 25, and 38 °C. The same tendencies have been described for bacteria on various surfaces, e.g. glass, wood or metal (Nakamura 1962; McEldowney and Fletcher 1988).

The exact reasons for the lower survival as the temperature approaches the optimal growth are unknown (Himathongkham *et al.* 2000). However, bacteria in the growth phase are more susceptible to stress factors than in the stationary phase, and this may logically be a main determinant in the higher survival at temperatures below 37 °C.

Salmonella survives freezing, and in this context it does not differ significantly from most other bacteria (D'Aoust 1989).

Heating of *Salmonella* will be dealt with in Section 4.1.

3.3 Stress responses in individual bacteria

3.3.1 General aspects

Research on bacterial stress responses is substantial, and new aspects are continuously being elucidated. Still, much remains to be done to complete the overall understanding, as some of the very basic mechanisms are poorly understood, which is probably partly

related to the fact that the same applies to basic mechanisms of cell injury (cf. Sections 4.1.2 and 4.2.1).

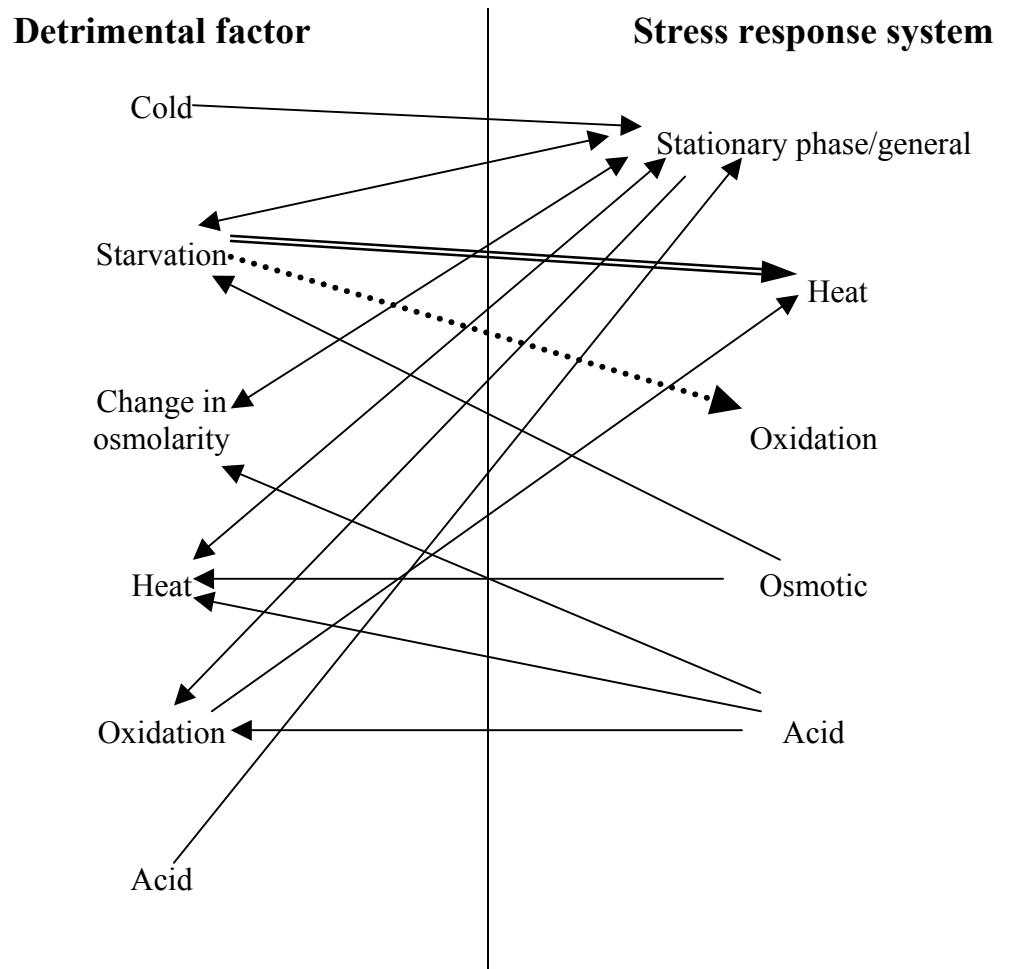
Bacteria in the extra-animal environment probably encounter many different detrimental conditions simultaneously. Therefore, a holistic approach has been attempted, and known cross-protections are highlighted (cf. Figure 3.1).

3.3.2 The general stress response, stationary phase, and starvation

Table 3.1 gives an overview of important features linked to the stationary phase.

The general stress response is closely linked to the stationary phase of non-sporulating Gram-negative bacteria, a phase in which they probably exist most of the time in their natural, extra-animal habitats due to nutrient limitations (Kolter *et al.* 1993). The phase itself is generally triggered by starvation, but the general stress response is also induced by high osmolarity, high or low temperature, low pH (Hengge-Aronis 2000) and competitive microflora (Aldsworth *et al.* 1999).

The general stress response is regulated by the master regulator RpoS (also known as σ^s or σ^{38}). The activity of this gene is hardly detectable in non-stressed rapidly growing bacteria, but as they enter the stationary phase it begins to be increasingly expressed (Lange and Hengge-Aronis 1991; McCann *et al.* 1991; Foster and Spector 1995). However, RpoS is also part of other regulatory networks which are activated by starvation and other stresses, so the complete picture is complicated (Hengge-Aronis 2000). More than 50 genes depend on RpoS, and at least 30 proteins require RpoS for their expression during starvation (McCann *et al.* 1991; Hengge-Aronis 2000). Often, starvation is divided into specific starvation conditions for phosphate (P), carbon (C) or nitrogen (N). Each of these situations triggers proteins that either have functions specific for the condition, or they in-



*Figure 3.1: Known cross-inductions by detrimental factors and cross-protections by stress response systems which are considered to be important for survival of *Salmonella* in the extra-animal environment.*

Arrows to the right show cross-induction, whereas arrows to the left show cross-protection.
====> : only carbon and amino acid starvations are known to induce heat stress proteins.
.....> : only carbon starvation is known to induce the oxidation stress response.

Table 3.1: Putative characteristic features in stationary phase *E. coli* and *Salmonella*. Compiled from Roszak and Colwell (1987); Siegele and Kolter (1992); Kolter et al. (1993); Foster and Spector (1995); Adams and McLean (1999); Hengge-Aronis (2000).

Key area	Brief description	Possible impact on relevant aspects
Morphology	<p>Smaller, almost spherical cells, thus the rod shape of e.g. <i>E. coli</i> is lost.</p> <p>Cytoplasm is condensed and the volume of periplasm is increased. Changes in chromosome topology due to reduced gene expression.</p>	<p>Cell divisions without an increase in cell mass increase the number of bacteria which may improve survival for the clone.</p>
Cell envelope changes	<p>The surface is covered by more hydrophobic molecules which favour adhesion and aggregation.</p> <p><i>E. coli</i> starved at low temperatures produce a fibronectin-binding element (curli) which may be involved in aggregation.</p>	<p>Biofilm formation is favoured.</p>
Metabolism	<p>Fatty acid composition in membranes changes, rendering them less permeable.</p> <p>The overall metabolic rate is reduced, but a low level of metabolism is maintained.</p>	<p>Less permeability to chemicals (e.g. disinfectants and antibiotics) increases the bacterial resistance to these. Protection from autolysis.</p> <p>The low metabolic activity reduces susceptibility to detrimental conditions. Resumption of growth may occur if nutrients become available.</p>
Protein synthesis	<p>Stability of RNA and proteins decreases, whereas DNA stability is maintained.</p> <p>30-50 different proteins are synthesised under the general stress response, the number and kinds of proteins depending on the specific conditions.</p>	<p>DNA stability is important for resumption of growth if conditions improve.</p> <p>Development of various protective mechanisms.</p>
Mutations	<p>Mutation rates increase in starved bacteria.</p>	<p>Possible selection of strains with increased resistance to detrimental conditions.</p>

fluence other conditions such as other starvation conditions, heat, osmotic or oxidative stress (Foster and Spector 1995). Moreover, different genes and proteins are expressed at different stages of the stationary phase, as their induction is generally related either to transcription, translation or proteolysis of RpoS (Lange and Hengge-Aronis 1994; Foster and Spector 1995). This illustrates the ability of Gram-negative bacteria to adapt to both acute and long-term stress conditions.

Among the more than 30 Rpos-dependent proteins, about 12 have been identified. For some of these the functions are known, including Exonuclease III (involved in DNA-repair), a cell-shape determination protein (regulated by the gene *bolA*), trehalose (osmo-protectant, desiccation resistance, thermotolerance), *ecp-htrE* operon (osmotic resistance, thermotolerance) and histone-like protein (DNA protection) (Kolter *et al.* 1993; Hengge-Aronis 2000).

Starvation can also induce RpoS-independent proteins. Carbon-starvation induces about 20 RpoS-independent proteins, three of which are known heat shock proteins (DnaK, GroEL, and HtpG) (Kolter *et al.* 1993).

3.3.3 The heat-shock response

In bacteria, heat-shock proteins (HSPs) are synthesised almost immediately as a result of a modest temperature upshift from 30 to 42 °C, and within 5 minutes they reach their maximum level (10- to 15-fold), followed by a decrease until a steady level after about 20 minutes (2- to 3-fold compared to the pre-shift level) (Lemaux *et al.* 1978; Herendeen *et al.* 1979; Yamamori and Yura 1980; Yura *et al.* 2000). At higher temperatures, the production of HSPs increases and continues as long as the bacteria can produce proteins, i.e. until they die (Yura *et al.* 2000).

Yura *et al.* (2000) described two regulons mainly involved in the heat-stress response, the master regulator σ^{32} (encoded by RpoH) and σ^E (also called σ^{24}) (encoded by RpoE). In general, σ^{32} and σ^E protect against cytoplasmatic and extra-cytoplasmatic stresses, respectively. σ^E is mainly active at higher temperatures (45-50 °C) where it protects the cell by regulation of protein misfoldings (Yura *et al.* 2000).

In 1987, Neidhardt and VanBogelen (1987) listed 17 known HSPs in *E. coli*, whereas Yura *et al.* (2000) 13 years later listed 38 HSPs, among these 34 under the σ^{32} regulon. Many of these have more or less well-defined known functions, such as sigma factors, chaperones, proteases, and peptidyl-prolyl isomerase, i.e. mainly functions related to protein reorganisation and stabilisation (Yura *et al.* 2000). However, many exact functions and mechanisms remain unknown, probably because exact mechanisms involved in heat injury are not known either (cf. Section 4.1.2).

Also for HSPs, there is an overlap to other detrimental factors. The main inducer of HSPs is heat (Neidhardt and VanBogelen 1987), but other external stress factors can induce HSPs, among these ethanol, carbon-starvation, oxidative stress, amino acid starvation, antibiotics and heavy metals, though the mechanisms behind these stress inducers and HSP initiation are generally poorly understood (Neidhardt and VanBogelen 1987; Yura *et al.* 2000).

3.3.4 Other stress systems

3.3.4.1 General aspects

Storz and Hengge-Aronis (2000) described several stress systems relevant for Gram-negative bacteria in extra-animal environments, especially when the role of chemical disinfectants is considered (cf. Sections 3.4 and 4.2). Here, oxidative, acid and osmotic stress will be described briefly.

3.3.4.2 Oxidative stress

Many oxidative stress response genes are under the control of two regulons, *oxyR* and *soxRS*. Some of these genes and *oxyR* itself are controlled by *rpoS* which also controls other oxidative stress genes not regulated by *oxyR* or *soxRS* (Storz and Zheng 2000). Recently, the role of the outer membrane protein Ag43 in biofilm formation was described. Ag43 is also involved in the oxidative stress response, and partially regulated by *oxyR*, so a link between oxidative stress and biofilm formation was elucidated (Danese *et al.* 2000). In *S. Typhimurium*, Christman *et al.* (1985) found a link between the expression of nine oxidative stress proteins (induced by hydrogen peroxide) and three HSPs. A link between the *soxRS* regulon and the *mar* response is also well known (cf. Section 3.4.5).

3.3.4.3 Acid stress

The acid tolerance response was described specifically for *S. Typhimurium* by Foster (2000). Figure 3.2 gives an overview of the inter-dependency between the acid tolerance response and the general stress response, controlled by the general stress response regulon (σ^s / *rpoS*). The acid tolerance response confers cross-protection against heat, oxidative, and osmotic stress (Leyer and Johnson 1993; Foster and Spector 1995; Bearson *et al.* 1997; Foster 2000), whereas other stresses do not induce acid tolerance (Bearson *et al.* 1997). Foster and Spector (1995) emphasised the unique role of the acid tolerance response with regard to its cross-protection to several types of environmental stress, and speculated if this was another way of inducing the general stress response, which previously was only associated with starvation (also cf. Section 4.1.9).

3.3.4.4 Osmotic stress

Osmotic stress probably plays a role when *Salmonella* is desiccated; this was exemplified for trehalose by Welsh and Herbert (1999).

The two outer membrane proteins OmpC and OmpF are crucial in the osmoregulation (Nikaido and Vaara 1987; Foster and Spector 1995; Bremer and Krämer 2000). These two systems are adversely regulated, as OmpC levels increase and OmpF levels decrease in

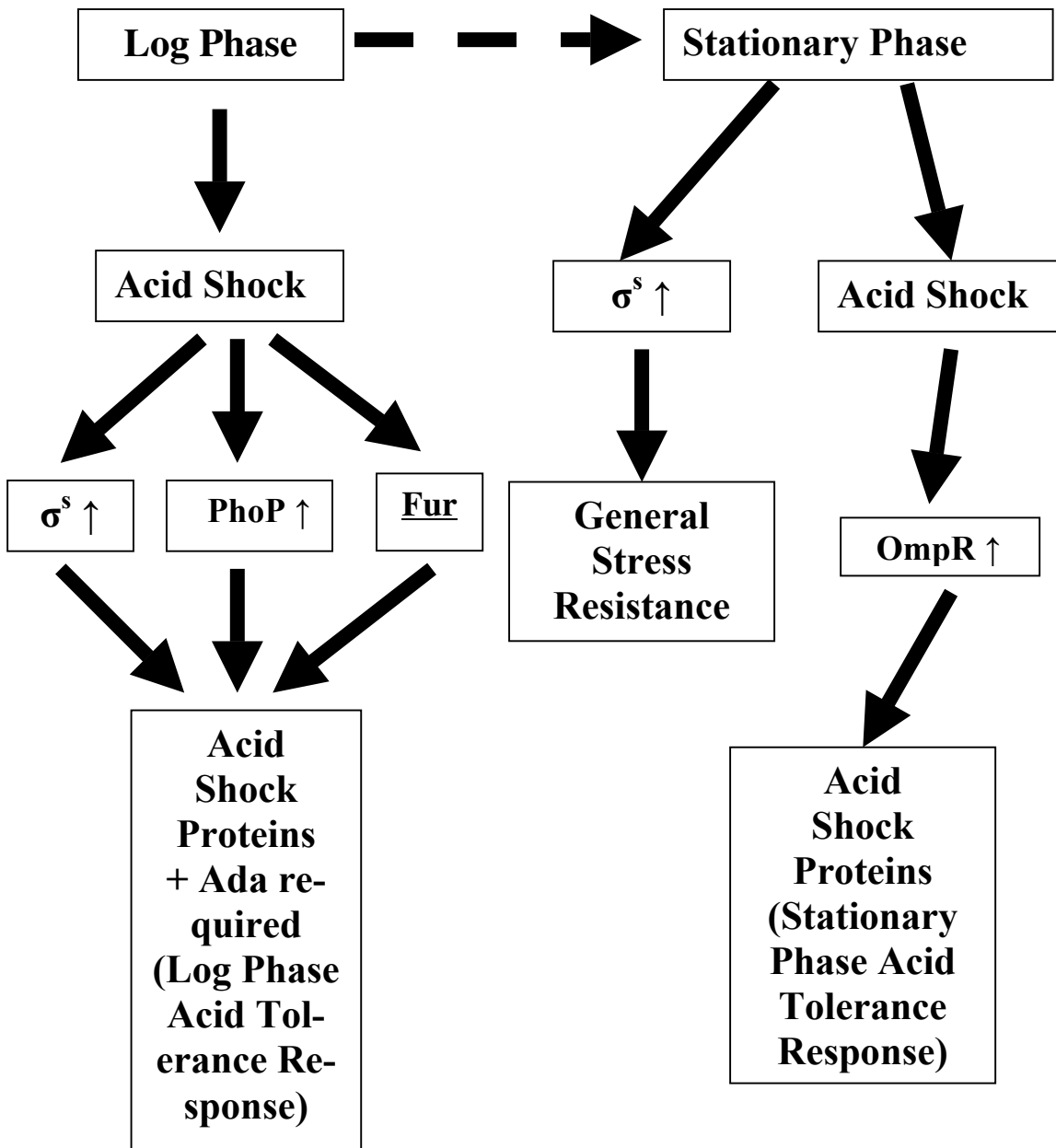


Figure 3.2: Log phase and stationary phase acid tolerance responses of *S. Typhimurium*. Fur is not induced by acid, but it is required for the production of acid shock proteins. The Ada protein is also required for the log phase acid tolerance response, but it does not seem to be induced by acid or to regulate expression of other acid shock proteins. Source: (Foster 2000).

high osmolarity (Nikaido and Vaara 1987). Different compatible solutes, of which trehalose, proline, ectoine, and glycine betaine are central, balance the environmental osmolarity acting as “compatible solutes” in place of water (Bremer and Krämer 2000). Trehalose is also regulated under the general stress response (cf. Section 3.3.2).

3.4 Disinfectant resistance

3.4.1 General aspects

While antibiotic resistance has been thoroughly studied, less is known about resistance to disinfectants. A strain is said to be resistant to a disinfectant if it is either insensitive to a concentration of the disinfectant used in practice or it is not inhibited by a concentration that inhibits the majority of strains of that organism (Russell 1999a). However, these definitions do not necessarily cover the more general aspects of intrinsic resistance, e.g. those related to the outer membrane in Gram-negative bacteria.

Here, the focus will be on disinfectants used commonly in the agricultural sector, hence some better understood but less relevant mechanisms (e.g. plasmid mediated resistance to mercury compounds) will be omitted. Known mechanisms will be outlined, and these will be related to practical studies, if possible.

3.4.2 Mechanisms of resistance to disinfectants in Gram-negative bacteria

Disinfectant resistance is divided into the following categories (McDonnell and Russell 1999):

1. Intrinsic resistance which is due to inherent characteristics of the bacteria, in Gram-negatives mainly related to their outer membrane. An inherent part of this is the membrane changes that occur as a result of different conditions which will also alter the resistance to disinfectants. Also, the phenotypic adaptation seen in biofilms is often classified as intrinsic, but this will be described separately here.

2. Acquired resistance which may arise by mutation or by the acquisition of plasmids or transposons.

3.4.3 Intrinsic resistance

The outer membrane in Gram-negative bacteria functions as the main permeability barrier, which generally makes these more resistant to disinfectants than non-sporulating, non-mycobacterial Gram-positive bacteria (Russell and Gould 1988; McDonnell and Russell 1999; Stickler and King 1999; Denyer and Maillard 2002). A disinfectant must reach its target site in order to be effective, and though these are less specific than for antibiotics, most are found within the cell (Russell 1999a). The following main characteristics of the outer membrane play an essential role in its permeability (Nikaido and Vaara 1987; Russell and Gould 1988; Vaara 1992; Denyer and Maillard 2002):

- Lipopolysaccharides (LPS), which are unique components of the Gram-negative outer membrane. LPS-molecules are strongly linked to each other, among other things by the divalent cations Mg^{2+} and Ca^{2+} . These links form a strong permeability barrier to hydrophobic molecules that do not enter easily in wild-type *E. coli* and *S. Typhimurium* (Nikaido and Vaara 1987). LPS are divided into three parts, a) the hydrophilic O antigen polysaccharide that protrudes into the medium, b) the hydrophobic lipid A region, being closest to the inner membrane, and c) the core polysaccharide region that connects a) and b). In “deep rough” mutants, the part of the core region that is closest to the inner membrane is lost, and as a result the outer membrane permeability to various hydrophobic compounds increases considerably. Deep rough mutants have been used to study the role of LPS in permeability of many different compounds. In other LPS studies, permeabilisers (agents that increase the permeability of the outer membrane) are used, e.g. ethylenediamine tetraacetic acid (EDTA) which chelates divalent

cations from their binding sites in LPS, resulting in LPS release from the outer membrane and thus increased permeability (Vaara 1992).

- The outer membrane proteins (Omp) F, C and D in *Salmonella* (see also Section 3.3.4.4) form porin channels that only allow the passage of small hydrophilic molecules (Nikaido and Vaara 1987; Russell and Gould 1988; Denyer and Maillard 2002). Studies of bacteria with defects in the Omp proteins have provided useful information on the role of the porin channels in the uptake of many compounds.

Based on studies of these structures, two main pathways for antibacterial agents are described, one hydrophobic through the LPS-layer and the other hydrophilic through the porin channels (Russell and Gould 1988; Russell 1999a; Denyer and Maillard 2002). In addition, a third pathway called self-promoted uptake is used by the so-called membrane-active agents, e.g. cationic disinfectants, of which chlorhexidine and quaternary ammonium compounds (QACs) have been much studied (Russell and Gould 1988; Russell 1998; McDonnell and Russell 1999; Maillard 2002). Membrane active agents are thought to damage the outer membrane by displacing divalent cations and thus destabilising LPS-LPS links. The exact mechanisms remain to be elucidated, but the addition of Mg^{2+} ions reverses the uptake of at least some of the active membrane agents (Russell and Gould 1988).

The composition of the outer membrane is closely related to the conditions the bacteria encounter, and several studies have shown that this can have an impact on susceptibility to disinfectants (Brown and Williams 1985; Brown *et al.* 1990; Denyer and Maillard 2002). This is an important aspect of adaptation to disinfectants (cf. Section 3.4.6.1). The growth medium may markedly influence the resistance of bacteria to disinfectants (Russell 1999b). Ten consecutive transfers of *E. coli* were performed in medium containing one

peptone type, except the ninth which was made in another type of peptone medium. Bacteria from the first, eighth and tenth transfer were killed within one minute by benzalkonium chloride, whereas it took more than two minutes after the ninth transfer (Klimek and Bailey 1956). Nutrient limited growth broth generally renders bacteria more resistant to disinfectants (Lisle *et al.* 1998). In one study, *E. coli* developed resistance progressively against chlorine during a 29-day starvation period (Lisle *et al.* 1998). Magnesium limitation in the broth may render *Pseudomonas aeruginosa* more resistant to disinfectants that displace cations, probably because magnesium-bridges in the cell walls are replaced by polyamides (Russell 1999b).

Little is known about resistance to disinfectants related to pH of the growth medium, incubation temperature and oxygen conditions (Russell 1999b). Incubation temperature influences the cell wall lipid composition (Vigh *et al.* 1998), but few studies have specifically related this to a subsequent disinfection susceptibility. In one study by Gawande and Bhagwat (2002b), a lower incubation temperature increased the acid resistance. It was speculated if this was due to decreased fluidity of membrane lipids, but no evidence was given.

Desiccation of bacteria, especially on hard or non-hydroscopic surfaces, render these more resistant to a subsequent disinfection (Russell 1999b). The mechanisms behind this are unknown, but may be similar to what is known from heating studies (cf. Section 4.1.3).

Many studies show that attached bacteria are more resistant to disinfectants than bacteria in suspensions (e.g. LeChevallier *et al.* 1988a; Dhir and Dodd 1995; Foschino *et al.* 1998; Gawande and Bhagwat 2002a), but the impact of specific mechanisms is difficult to elucidate, as other characteristics (e.g. surface material and biofilms) are often involved.

The role of other cell structures in intrinsic resistance in Gram-negative bacteria are at present unclear or not considered important in intrinsic resistance (Russell and Gould 1988; McDonnell and Russell 1999).

3.4.4 Biofilms

3.4.4.1 General aspects

Biofilms are defined as matrix-enclosed bacterial populations that adhere to each other and to surfaces (Costerton *et al.* 1995). The formation of biofilms is influenced by several factors in bacteria and on surfaces, all of which have been thoroughly described in the scientific literature, e.g. hydrophobicity, electric charge, growth conditions, bacterial species, surface materials and nutrients. Because biofilm formation depends on these and several other known and unknown factors, it is difficult to establish a model that will predict a specific biofilm formation. However, the two most important prerequisites are probably the presence of moisture and nutrients (Allison and Gilbert 1995; Kumar and Anand 1998; Barker and Bloomfield 2000), though it has been stated that only low levels of the latter favour biofilm formation (Corry and Allen 2000). Most laboratory experiments have involved only one bacterial species, whereas several bacterial species often form biofilms in the natural environment, often with protozoa, algae, and fungi (James *et al.* 1995; Brown and Barker 1999; Corry and Allen 2000). Little research exists on biofilms in animal houses, but there is every reason to believe that biofilms are formed in water lines (Tuschewitzki *et al.* 1983; Mattila-Sandholm and Wirtanen 1992). Biofilm formation on stainless steel in the food industry has been much studied, but only recently has biofilm formation by *Salmonella* on plastic, cement and steel been described in the laboratory (Joseph *et al.* 2001). However, these materials were immersed in broth, so it is still questionable if *Salmonella* will form biofilms under natural conditions in poultry houses on

similar surfaces, such as feeding systems, walls, and floors. It is noteworthy that classical microbial biofilms have only rarely been reported in food processing plant environments (Gibson *et al.* 1999) in spite of the fact that biofilms on food and food contact surfaces have been thoroughly studied (Zottola and Sasahara 1994).

3.4.4.2 Biofilm structure and function

Costerton *et al.* (1995) reviewed the general structures of biofilms and the different methods for studying these. Generally, bacteria form aggregates/microcolonies in different horizontal and vertical levels of the biofilm, but areas of the highest cell density are species specific, and the structure becomes more complicated in mixed-species biofilms. The glycocalyx, found between the biofilm bacteria, consists of exopolysaccharides produced by these. The structure and composition of this glycocalyx also vary with different factors such as bacterial species and surface conditions, but it is generally highly hydrated and has water channels (Allison and Gilbert 1995; Costerton *et al.* 1995). Another important feature is the detachment and dispersal of planktonic cells that can colonise new niches (Gilbert *et al.* 1993; Costerton *et al.* 1995). The organised structures in biofilms demand cell-to-cell communication, an area much studied in recent years (Swift *et al.* 1994; Salmond *et al.* 1995; Davies *et al.* 1998a; Winans and Zhu 2000).

3.4.4.3 Bacterial resistance in biofilms

Bacteria in biofilms are generally more resistant to antibiotics and disinfectants than their planktonic counterparts (Costerton *et al.* 1987; Allison and Gilbert 1995; Stewart and Costerton 2001). The susceptibility to other detrimental factors has been less studied, but with the many known cross-protections in mind (cf. Table 3.1, Figure 3.1), the same tendencies must logically be expected (Frank and Koffi 1990; Dhir and Dodd 1995). This is supported by the fact that more than 45 genes differ in expression between sessile bacteria

and their planktonic counterparts (Donlan and Costerton 2002). Moreover, as attached bacteria are an inherent part of biofilms, these render a general higher resistance than planktonic cells (cf. Section 3.4.3). This, however, illustrates one of the major obstacles in the investigation of bacterial resistance in biofilms, as we encounter several differences between bacteria in biofilms and their planktonic counterparts. Thus, the relative importance of different mechanisms in biofilm resistance are unknown in spite of the vast scientific literature on this topic (Costerton *et al.* 1995; Gilbert *et al.* 1997). Generally, the biofilm resistance mechanisms to antimicrobials are summarised in three main hypotheses (Gilbert *et al.* 1997; Stewart and Costerton 2001; Gilbert *et al.* 2002):

- Slow or incomplete penetration of the antimicrobial into the glycocalyx, either because of incomplete penetration and/or due to an easy penetration accompanied by deactivation by enzymes in the glycocalyx.
- An altered chemical microenvironment in the biofilm, e.g. levels of nutrients and oxygen, pH and osmolarity which vary within the biofilm. Often, anaerobic conditions, which can minimise the efficacy of some antimicrobials, are found in the deeper biofilm layers. Biofilm niches with low nutrient levels are also often found in the deeper layers, in which the bacteria enter a stationary/dormant phase and become more resistant (Brown *et al.* 1988; Gilbert *et al.* 1990).
- A subpopulation of biofilm bacteria forms a special phenotypic biofilm state which is more resistant. This hypothesis is controversial, but it has been supported by the fact that about 1% of the bacteria often survive antimicrobial treatment, even in thin, newly formed biofilms. Lewis (2001) reviewed this phenomenon in depth.

3.4.4.4 *Salmonella* in biofilms

The literature on *Salmonella* in biofilms is sparse, but several conditions that can influence biofilm formation and resistance to various detrimental factors have been described.

Jones and Bradshaw (1997) reported that the attachment of *S. Enteritidis* to water pipes increased in the presence of *Klebsiella pneumoniae* compared to a situation where only *S. Enteritidis* was present. In another study with *Pseudomonas fluorescens* and *S. Typhimurium* in binary species biofilms, the former was best in adhering to a polycarbonate surface (Leriche and Carpentier 1995). However, this seemed to be advantageous for *S. Typhimurium*, as the restriction of nutrients rendered these more resistant to chlorine. Ronner and Wong (1993) found that iodine, chlorine, a quaternary ammonium compound, and an anionic acid generally caused a 7-8 log reduction of planktonic *S. Typhimurium*, whereas the same bacteria in biofilms on buna-n rubber and stainless steel were reduced by less than 1-2 log and 3-5 log, respectively. Joseph *et al.* (2001) showed that two *Salmonella* serotypes were found in highest density on plastic, followed by cement and steel. Iodine and chlorine caused significantly lower bacterial reductions on all three materials compared to planktonic cells, e.g. 10 ppm Cl₂ generally caused a 1-2 log reduction on the three materials after 25 min, whereas all planktonic cells were killed after 10 min. The increased resistance of biofilm *Salmonella* compared to their planktonic counterparts was confirmed in other studies (Somers *et al.* 1994; Dhir and Dodd 1995). The resistance of biofilm *Salmonella* generally increases with increasing age of the bacteria (Leriche and Carpentier 1995; Korber *et al.* 1997), a phenomenon also reported for other bacteria. The impact of different growth media for *Salmonella* adherence to surfaces has been shown in several studies (Dhir and Dodd 1995; Hood and Zottola 1997a, 1997b; Bonafonte *et al.*

2000). Lee and Falkow (1990) showed for different *Salmonella* serotypes that growth in 0-1% oxygen rendered these 6-70 times more adherent than when they were grown in 20% oxygen. Korber *et al.* (1997) showed an inverse relationship between crevice width and flow velocity of the disinfectant trisodium phosphate on one hand and survival of biofilm *Salmonella* on the other.

3.4.5 Acquired resistance

In Gram-negative bacteria, acquired resistance to disinfectants is generally believed to be less important than intrinsic resistance (Russell 1999a).

The genetic aspects of plasmid-encoded resistance to biocides have only been studied in detail for staphylococci, mainly for the *Qac*-complex (Russell 1997). Plasmid-mediated resistance to mercury, silver and copper is well-characterised in Gram-negative bacteria, but there is probably no cross-resistance to unrelated compounds (Russell 1997), including disinfectants relevant for poultry houses. A few years ago, a formaldehyde dehydrogenase in *E. coli* was reported to be plasmid-mediated (Kummerle *et al.* 1996). The R124 plasmid in *E. coli* alters OmpF, rendering the cells more resistant to various agents (Russell 1998). Other plasmids may be involved in other outer membrane changes, and this has been reported to confer increased resistance to formaldehyde in *E. coli* (Russell 1999a), but more exact mechanisms remain to be elucidated (Russell and Gould 1988).

Mutations causing resistance to disinfectants have not been observed, apart from the putative ones reported from studies of adaptation or efflux pump systems.

In recent years, there has been much research into efflux pumps with regard to disinfectant resistance. Older studies unaware of this mechanism might have interpreted efflux solely as a decreased outer membrane permeability (Russell 1998). Moken *et al.* (1997) found that pine oil could select *E. coli* which over-expressed the *marA* gene, and which

had increased resistance to pine oil and several unrelated antibiotics. Efflux pumps in Gram-negative bacteria are related to multiple drug resistance (MDR) systems. MDR genes are activated by induction or mutation of genes that are part of the normal cell genome. Generally, they are activated by stress factors (McDonnell and Russell 1999; Russell 2000; Randall and Woodward 2002), e.g. the stress inducer salicylate and increasing levels of tetracycline that have been used to induce the *marA* response in *S. Typhimurium* DT104 (Randall and Woodward 2001a) or by oxidative stress (Alekshun and Levy 1999). Most studies of *marA* and other MDR systems have involved *E. coli*, in which at least 29 known or putative MDR systems have been found (Alekshun and Levy 2000), but *Salmonella* has also been studied (Kunonga *et al.* 2000; Randall *et al.* 2001; Randall and Woodward 2001a, 2001b). As regards oxidative stress, it is interesting that the *soxRS* regulon in *E. coli* (cf. Section 3.3.4.2) is part of the *mar* regulon (Alekshun and Levy 1999; Randall and Woodward 2002). *MarA* is known to have the following functions (Alekshun and Levy 1999; Levy 2002; Randall and Woodward 2002): a) activation of the inner membrane protein AcrB that transports drugs out of the cells, possibly through the outer membrane channel protein TolC, b) repression of OmpF synthesis, decreasing the permeability of the outer membrane, c) altering the expression of other membrane proteins and d) increased expression of various protective cytoplasmatic enzymes.

3.4.6 Studies of disinfectant resistance

Apart from studies on mechanisms, two main categories of studies have dealt with bacterial resistance to disinfectants:

- Studies in which laboratory conditions, such as media composition, temperature and bacterial phase, are regulated and related to bacterial disinfectant resistance. Because

intrinsic resistance is an important feature of such studies, they often expand into other areas (cf. Section 3.4.3); here, the focus will be on adaptation studies.

- Studies involving field strains where the resistance to selected disinfectants has been investigated. Several studies involve testing many isolates, and they give a comprehensive picture of naturally occurring resistance. Moreover, there are a few reports of single cases of natural resistance found in field strains, some of which have been investigated further.

3.4.6.1 Studies where laboratory conditions are regulated

Russell (1999a) reviewed studies where bacteria were exposed to gradually increasing disinfectant concentrations (i.e. adaptation studies), often involving QAC or chlorhexidine. Development of resistance was thought to be due mainly to outer membrane changes. However, most studies related to specific laboratory conditions have been speculative, as they have not investigated specific resistance mechanisms (McDonnell and Russell 1999). Russell (1998) stated that adaptation to QAC and chlorhexidine is normally lost when the bacteria are grown in broth without these disinfectants, but Norwegian studies involving adaptation to benzalkonium chloride (BC) followed by successive growth in BC free broth showed the BC resistance to be stable (Langsrud 1998; Sidhu 2001). Langsrud and Sundheim (1999) reported that just an overnight incubation with a sublethal level of BC in the broth increased the BC resistance of two *Pseudomonas* strains. The same two authors (Langsrud 1998, paper 6) also found that BC resistance in *E. coli* was induced by both sublethal BC concentrations and stress inducers (e.g. salicylate). Guérin-Méchin *et al.* (1999) reported increased QAC resistance in *Ps. aeruginosa* when QAC was added to the medium, and found that QAC resistance significantly altered the outer membrane fatty acid composition. Two studies reported that sublethal levels of formaldehyde in broth increased

the resistance of *E. coli* (Wille 1976) or *Ps. aeruginosa* (Sondossi *et al.* 1985) to this disinfectant. Interestingly, passage of *E. coli* in a medium with a phenolic compound (often involved in efflux pump resistance, cf. Section 3.4.5) did not alter resistance against this disinfectant (Wille 1976).

3.4.6.2 Studies involving field strains

Willinghan *et al.* (1996) tested bacterial isolates from chicken hatcheries in suspension tests using disinfectants containing glutaraldehyde, phenol or QAC as the active compound. From two of the three hatcheries, about 8% were resistant to disinfectant concentrations at or above the recommended in-use concentration. The highest resistance level was seen for glutaraldehyde, and this was related to the common use of this type of disinfectant in the hatcheries. Langsrud and Sundheim (1999) found higher resistance to QAC in *Pseudomonas* spp. isolated from a poultry processing plant where QAC was used than from a plant using chlorine. In a study comprising 1325 Gram-negatives and 500 *Enterococcus* spp. isolated from food, food-processing plants and fish farms, only 16 isolates (15 coliforms and one *E. coli*) were found to be resistant to BC (Sidhu *et al.* 2002). In another study with 802 Gram-negative bacteria isolated from urinary tract infections, *E. coli* (the major species isolated) was equally sensitive to a number of disinfectants and antiseptics, including chlorhexidine, glutaraldehyde, a phenol compound and an antiseptic containing BC (Stickler and Thomas 1980). In a similar study, all *E. coli* isolated from hospitals and standard laboratory strains were equally sensitive to chlorhexidine, BC and other unrelated compounds (Hammond *et al.* 1987). Holah *et al.* (2002) reported that the resistance to a QAC compound and sodium hypochlorite were the same for persistent strains of *Listeria monocytogenes* and *E. coli* isolated from the food industry and laboratory strains of the same two types. Møretrø *et al.* (2003) found that *Salmonella* persisting in fish feed facto-

ries was not more resistant to nine disinfectants used there than *Salmonella* from other sources. In a recent study with *S. Enteritidis*, the resistance against two phenolic, one QAC and one formaldehyde/QAC compound did not differ between isolates from poultry houses regardless of whether this serotype persisted there or not (Davison *et al.* 2003).

3.4.7 Discussion on disinfectant resistance

The words of Russell (1999a) about pine oil and MDR systems (cf. Section 3.4.5 and Moken *et al.* (1997)) illustrate some basic concepts of disinfectant resistance:

“These findings imply the selection by a disinfectant (pine oil) of chromosomal antibiotic resistance with a potential problem in therapy. Their findings have been criticized, in particular that the concentration of pine oil used was only one-tenth of that employed in practice. Furthermore, this type of disinfectant has been used for many years without untoward effects being reported. This highlights the difficulty in translating laboratory findings to the real-life situations.”

Adaptation studies have mainly been performed with disinfectants commonly applied in the food industry, where disinfectants used more commonly in poultry houses (cf. Figure 2.1.) are often not allowed because of their toxicity. Moreover, adaptation may be less important in determining the long-term survival of bacteria in real-life situations, as the adaptation may be reversible (Russell 1998).

Increased resistance to chlorhexidine, QAC and other cationic biocides only results in a 2-8 fold MIC increase (cf. Section 5.4.4.1) which is well below in-use concentrations, and it is therefore difficult to evaluate the practical relevance of such tests (Russell 2000). In addition, huge differences in dilution coefficients between various disinfectant types (cf. Section 4.2.2.1) will further complicate comparisons between these (Russell and McDonnell 2000). One study by Griffiths *et al.* (1997), albeit with mycobacteria (i.e. Gram-positive bacteria), was unusual because it involved both suspension and carrier tests, each

with and without an organic load, and relevant in-use concentrations of disinfectants were applied.

The relevance of specific mechanisms has rarely been evaluated. Despite several papers on the function of *mar*, its natural inducer is unknown (Randall and Woodward 2002). Plasmid-mediated formaldehyde dehydrogenase could be highly relevant if persistent *Salmonella* were resistant to this disinfectant, but nothing is known about the natural occurrence of this enzyme.

Studies with field strains generally indicate that naturally occurring disinfectant resistance in *E. coli* and *Salmonella* is uncommon. It may be significant that resistance is mainly found among *Pseudomonas* spp. which are known for their high intrinsic resistance.

Much research needs to be done on disinfection resistance, and filling in the many gaps and links between laboratory and field situations may alter some of the above interpretations in the future.

Chapter 4 - Factors that influence

the effectiveness of disinfection

4.1 Factors that influence the effectiveness of heat

4.1.1 General aspects

From a time aspect, heat studies can conveniently be divided into conditions before, during and recovery after heating. Recovery of heated bacteria will be discussed in Chapter 5. Many studies deal with sporing bacteria and sterilisation, but the general principles often apply to vegetative bacteria and disinfection. Doyle and Mazzotta (2000) gave an excellent review of factors related to heat resistance of *Salmonella*.

4.1.2 Mechanisms of heat damage and resistance

In vegetative cells, DNA, RNA/ribosomes and the cytoplasmic membrane are the main cell structures which may be damaged by heat (Mossel and Corry 1977; Gould 1989). DNA damage can cause an irreversible loss of cell viability because DNA has the genetic information for re-synthesis of new substances. Gould (1989) did not consider RNA and ribosomes to be key factors in bacterial death, as mild heating accelerates the degradation of rRNA, but this often precedes the loss of viability, whereas Corry (1973) stated that rRNA had a key role in cell death caused by heating. As for membrane leakage, according to Gould (1989) there is generally a poor correlation between cell leakage and death, whereas others have stressed its importance in cell death (Corry 1973; Mossel and Corry 1977). Russell (1999c) described cell damage due to moist heat as heat-catalysed

hydrolytic changes, whereas dry heat initiates an oxidative process. These and other aspects illustrate the words of Gould (1989) which also can be related to sublethal heat injury:

“heat brings about many changes in vegetative microbial cells, and the key event(s) that normally lead to death is not clearly defined. Consequently, rational discussion of ‘resistance’ mechanisms must be limited”.

4.1.3 Water activity

Different aspects of humidity were described in Section 3.2.3. Most food microbiology studies use the parameter water activity, which will also be used in this section.

Generally, the heat resistance of bacteria decreases as higher water activity before and/or during heating increases (Hansen and Riemann 1963; Doyle and Mazzotta 2000). In the *British Pharmacopoeia*, dry sterilisation is 170 °C for at least one hour, whereas wet sterilisation only requires 121 °C for 15 min (Russell 1999c). The same principles apply to heating of vegetative bacteria which have been described both generally (Hansen and Riemann 1963; Corry 1973; Senhaji 1977) and specifically for *Salmonella* (Riemann 1968; Baird-Parker *et al.* 1970; Barrile and Cone 1970; Goepfert *et al.* 1970; Himathongkham *et al.* 1996; Archer *et al.* 1998; Doyle and Mazzotta 2000). No general death kinetic model can be predicted from the water activity, as this depends on several other factors (Baird-Parker *et al.* 1970; Goepfert *et al.* 1970; Corry 1973, 1974; Himathongkham *et al.* 1996). Among these, an increase in solutes will normally lower the water activity, so it may be difficult to know how much of the increased heat resistance is due to the lowered water activity or protection by solutes *per se*. It is, however, generally acknowledged that water activity plays an important role in heat resistance (Hansen and Riemann 1963; Cotterill and Glauert 1969; Barrile and Cone 1970; Senhaji 1977; Kaur *et al.* 1998). In one study, a D_{71} of 20 h for *Salmonella* in milk chocolate (with a high content of sugar and fat and hence a low water activity) was found (Barrile and Cone 1970). By the addition of 2% moisture,

D_{71} was reduced to 4 h; the addition of moisture in the range 2-10% further reduced D_{71} , but without the initial steep decline seen with the first 2%. This study showed a high decline in heat resistance in spite of an insignificant change in relative sugar and fat content, and a moisture threshold beyond which the heat resistance decline was reduced. This illustrated the non-linearity between bacterial death and changing water activities, often encountered in heating studies.

Most studies have dealt with water activity during heating, but equally important for poultry house conditions are studies showing that a decreased water activity before heating also renders *Salmonella* more heat resistant (Kirby and Davies 1990; Mattick *et al.* 2000).

4.1.4 Organic substances

Generally, the presence of fats, proteins and carbohydrates increases the heat resistance of bacteria (Hansen and Riemann 1963; Baird-Parker *et al.* 1970; Corry 1974; Lee and Goepfert 1975; Senhaji 1977; Ahmed *et al.* 1995). Localised low water activity is believed to be the main reason for the protection of bacteria when heated in fats (Hansen and Riemann 1963; Senhaji 1977; Ahmed *et al.* 1995), but the type of fat also influences the heat resistance (Molin 1977; Ababouch *et al.* 1995). The protection by carbohydrates also depends on their type (Goepfert *et al.* 1970; Corry 1974; Molin 1977), whereas little is known about different proteins.

4.1.5 Other substances

The effect of salts on heat resistance is generally very difficult to predict (Hansen and Riemann 1963). In one study, an increased sodium chloride content rendered *Salmonella* more heat resistant, a mechanism that was probably related to the concomitant decreased water activity (Cotterill and Glauert 1969), but another study showed more ambiguous results, as sodium chloride increased the heat resistance of heat sensitive strains

whereas it decreased heat resistance of more heat resistant strains (Baird-Parker *et al.* 1970). Mañas *et al.* (2001) found that minerals in milk, mainly calcium and magnesium, increased the heat resistance of *S. Senftenberg* 775 W (also cf. Section 3.4.3).

In an extensive study, Mitchener *et al.* (1959) investigated the heating of bacterial spores with 650 different substances, many of which are considered to be detrimental for bacteria. The non-uniform results illustrate the complexity of the issue, and emphasise the difficulty in extrapolating to real-life conditions.

4.1.6 Temperature

This section will focus on studies that illustrate general principles for temperatures prior to heating, and heating rates.

There are several studies on application of sublethal temperatures prior to the heating (Mackey and Derrick 1986, 1987b; Bunning *et al.* 1990; Murano and Pierson 1992; Kaur *et al.* 1998). In these studies, sublethal temperatures for *Salmonella* and *E. coli* were in the range 42-52 °C. Mackey and Derrick (1986) found that pre-incubation of *S. Typhimurium* at 48 °C for 30 min prior to heating at 50, 52, 55, 57 or 59 °C increased the heat resistance, a tendency confirmed in other studies (Mackey and Derrick 1987b; Bunning *et al.* 1990; Murano and Pierson 1992). However, these tendencies could not be confirmed by Kaur *et al.* (1998) where *E. coli* was heated at 42, 45, 48 and 50 °C for up till 15 min, i.e. shorter times than used by Mackey and Derrick (1986), but longer than the five minutes reported by Murano and Pierson (1992). The general explanation for the elevated heat resistance after a sublethal heat shock is the formation of heat shock proteins (cf. Section 3.3.3).

Other studies related heat resistance to different rates of heating. Mackey and Derrick (1987a) showed that *S. Typhimurium* was about 1000 times more heat resistant after a heating rate of 0.6 °C min⁻¹ than after rates in the range 5-38 °C min⁻¹. The same tendency

was seen for *S. Typhimurium* in ground beef heated at 6.0, 8.5 and 12.5 °C min⁻¹ (Thompson *et al.* 1979). Again, these tendencies could not be confirmed by Kaur *et al.* (1998) which, among other things, could be due to the use of only one relatively high heating temperature (60 °C). As for sublethal heat shock studies, the likely explanation of a higher heat resistance at lower heating rates is the extra time given to form heat shock proteins.

Numerous studies have dealt with growth temperatures for laboratory bacteria and storage temperatures for bacteria in food. Increased heat resistance of bacteria grown at their optimal temperature compared to those grown below has often been reported (Dega *et al.* 1972; Verrips *et al.* 1980; Katsui *et al.* 1981, 1982; Humphrey 1990; Jackson *et al.* 1996). Heat resistance comparisons between optimal growth temperature and temperatures slightly above this have been more inconsistent. In two studies, an increased heat resistance was seen at higher temperatures (Ng *et al.* 1969; Dega *et al.* 1972), whereas it decreased in another study (Verrips *et al.* 1980). No change in heat resistance was seen for *E. coli* in beef frozen or refrigerated for 48 h (Juneja *et al.* 1997), possibly because there was no growth at these temperatures, and therefore little change in membrane properties. The exact mechanisms for the change in heat resistance as a function of different growth temperatures are not known, but they are probably related to a change in fatty acid composition which alters the membrane permeability (Katsui *et al.* 1981, 1982; Humphrey 1990). This may explain the varying results, as membrane characteristics also depend on several other factors (also cf. Section 3.4.3).

4.1.7 Characteristics of bacteria

Numerous studies show that bacteria in the stationary phase are more heat resistant than in the exponential growth phase (Lemcke and White 1959; Ng *et al.* 1969; Verrips *et*

al. 1980; Humphrey *et al.* 1990, 1995; Jackson *et al.* 1996; Kaur *et al.* 1998). The mechanisms behind this are probably to be found in cross-protections from different stress proteins (cf. Section 3.3).

A few studies reported that *Salmonella* attached to surfaces were more heat resistant than unattached *Salmonella* (Dhir and Dodd 1995; Humphrey *et al.* 1997).

Increasing concentration or density of bacteria may increase their heat resistance (Hansen and Riemann 1963; Humphrey *et al.* 1990; Humpheson *et al.* 1998). This phenomenon is often seen as biphasic death curves (Humpheson *et al.* 1998), but such results can be interpreted in different ways (cf. Section 5.3.2). Some studies have reported that some microbial strains, i.e. a competitive microflora, may increase the resistance in others (Hansen and Riemann 1963; Duffy *et al.* 1995). This may be pertinent in e.g. faeces, but the impact of this under relevant conditions has not been investigated.

Different *Salmonella* types have been compared in many heat resistance studies. Often, D-values change more as a function of some of the previously mentioned factors than because of different serotypes (Thompson *et al.* 1979; Humphrey *et al.* 1990). Studies in eggs have indicated that *S. Enteritidis* is more heat resistant than *S. Typhimurium*, but this tendency is less consistent in culture media (Doyle and Mazzotta 2000). *S. Senftenberg* 775 W has been the “prototype” in many heating studies, and some general tendencies can be given for this type. In some studies, *S. Senftenberg* 775 W was found to have a higher heat resistance than other selected *Salmonella* strains (Read *et al.* 1968; Ng *et al.* 1969), but water activities were not measured or commented. In other studies, the higher heat resistance of *S. Senftenberg* 775W was mainly seen at higher water activities. Goepfert *et al.* (1970) showed that D-values for *S. Senftenberg* 775 W depended much less on water activity than the D-value of seven other *Salmonella* strains. At a water activity of 0.99, *S. Sen-*

ftenberg 775 W had a significantly higher D-value than the other strains, whereas there was no difference at water activities below 0.93. In a study with milk chocolate (with a very low water activity), *S. Typhimurium* had higher D-values than *S. Senftenberg* 775 W at all temperatures (Goepfert and Biggie 1968). Corry (1974) showed that with a high percentage of solutes (and hence a lower water activity), a strain of *S. Typhimurium* had higher D-values than *S. Senftenberg* 775 W, whereas the reverse was seen with less solutes (higher water activities).

4.1.8 Oxygen

Aerobic versus anaerobic conditions play an important role in heating studies with spores, but this aspect has been less studied with vegetative bacteria. George *et al.* (1998) compared growth, heating and recovery of *E. coli*, *S. Enteritidis* and *Listeria monocytogenes* under aerobic and anaerobic conditions. Anaerobic growth and heating did not influence the heat resistance significantly, whereas an anaerobic recovery increased it. Murano and Pierson (1992) found that D_{55} was significantly higher for anaerobic than for aerobic cells of *E. coli*, but they did not distinguish between conditions before and after heating.

4.1.9 pH

The influence of pH on heat resistance varies between different studies. In general, an increase in pH relative to the optimal pH for growth (cf. Section 3.2.4) renders vegetative bacteria more heat resistant (Hansen and Riemann 1963; Doyle and Mazzotta 2000), whereas a pH decrease is reported either to increase (Doyle and Mazzotta 2000) or decrease (Hansen and Riemann 1963) the heat resistance. The former is supported by some studies (Buchanan and Edelson 1999; Ryu and Beuchat 1999; Wilde *et al.* 2000; Bacon *et al.* 2003), the latter by others (Blackburn *et al.* 1997; Casadei *et al.* 2000). Both allegations may be true, as the results probably depend on the extent of pH change and the specific test

conditions. From a mechanistic point of view, cross-resistance between acid and heat resistance may explain an increased heat resistance (cf. Section 3.3.4.3), but a low pH also denatures proteins (Hansen and Riemann 1963; Casadei *et al.* 2000), and the varying results may reflect the borderline zone between sublethal and lethal conditions. The same general arguments probably also apply to an increased pH which may render more varying results if more studies are performed.

4.2 Factors that influence the effectiveness of disinfectants

4.2.1 General aspects

Two systems are involved in the killing of bacteria by disinfectants: a) the bacteria and their suspending medium and b) the disinfectant and its solvent (Klimek and Bailey 1956).

Disinfection is one of several stress factors that bacteria encounter, and their susceptibility to disinfectants should be interpreted as part of this entity (cf. Chapter 3).

Factors related to e.g. osmolarity and oxidation are involved in the effectiveness of disinfection, but as for heat (cf. Section 4.1.2), exact disinfection mechanisms are often obscure as there can be many target sites in the bacteria, and key events that cause bacterial death have not been elucidated (Maris 1995; Russell 1999a). However, disinfectants often attach to proteins and destroy these, either in the cell membranes or in other cell organelles (Russell and Chopra 1996; Anonymous 2002b). This causes leakage of cellular constituents, but also the destruction of various cell organelles, depending amongst other things on the disinfectant and its concentration. Russell (1999b) categorised factors which could influence the effectiveness of disinfectants into pre-treatment, in-treatment and post-treatment phases. Pre-treatment factors are closely associated to intrinsic resistance (cf. Section 3.4.3), whereas post-treatment factors will be discussed in Chapter 5.

4.2.2 Treatment factors

The reactions between disinfectants and other agents follow the general principles for chemical reactions, but the impact of external factors differs according to the type of disinfectant. Here, we will focus on disinfectants used commonly in poultry enterprises (cf. Chapter 2). Table 4.1 shows properties for relevant disinfectants; it only illustrates general principles, as variations occur both within and between different disinfectant types.

Russell (1999b) stated the following general factors that influence the reactions between disinfectants and bacteria, and which are not directly related to characteristics of the bacteria *per se*:

- Concentration of disinfectant.
- Temperature.
- pH.
- Extraneous material.

4.2.2.1 Concentration of disinfectant

The concentration exponent or dilution coefficient (η) is a measure of the effect of change in concentration/dilution on death rate (Bean 1967; Russell 1999b). Reduction in activity, measured as the time to kill a population, is the dilution factor to the power η . For formaldehyde and QACs, $\eta=1$, so the time to kill a bacterial population is increased by 2^1 or 3^1 if the concentration is reduced by a factor 2 or 3, respectively; for phenols, $\eta = 6$, so the parallel time increases are $2^6 (= 64)$ or $3^6 (= 729)$ (Bean 1967; Russell 1999b). Thus,

Table 4.1: General properties for types of disinfectants commonly used in poultry enterprises (compiled from Anonymous (2002b)).

Type of disinfectant	Deter- gent effect	Effective against				Susceptibility to organic matter	Recommended pH/temperature (°C)	Corrosive	Detri- mental for the environ- ment
		Vege- tative bacteria	Spores	Viruses	Rapid action				
Acids	No	+(+) ¹	-	+	++	++	< 2 / > 5	+++	No
Bases (strong)	Yes	++	(+)	+++	+	-	- / -		
Formaldehyde	No	+++	+++	+++	-	-	- / ≥ 16		
Glutaraldehyde	No	+++	+++	+++	+	-	≥ 7 / > 5	-	No
Chlorine compounds ²	No	+++	-	+++	++	+++ / +++ ³	< 7 / < 35 or - ⁴	+++	No
Iodophors	Yes	+++	+	+	+++	++	≤ 6 / < 35	+	?
Oxidising compounds	No	+++	++ ⁵	+++	++	+	< 6 / ≥ 5	+ ⁶	No
Phenols ⁷	Yes	++(+)	-	(+)	++	++	< 7 / < 40	-	Yes
QACs ⁸	Yes	+(+) ⁹	-	-	+++	+++	~8 / -	+	?

¹ -: none, +: low, ++: intermediate, +++: high. Brackets indicate in-betweens.

² Chlorine compounds are divided into hypochlorites and organic chlorine compounds.

³ Hypochlorites: +, ++, +++. Organic chlorine compounds: +, ++.

⁴ Hypochlorites: < 35. Organic chlorine compounds: -.

⁵ ++++ for peracetic acid.

⁶ +++ for peracetic acid.

⁷ Two main groups: a) coal tar agents, b) synthetic phenols. There may be substantial variations in chemical properties between and within these two groups.

⁸ Quaternary ammonium compounds.

⁹ Mainly active against Gram-positive bacteria.

the disinfectant type can have an immense effect on the effectiveness if concentrations are more dilute than recommended.

4.2.2.2 Temperature

Generally, the effectiveness of disinfectants increases with increasing temperature (Russell 1999b) which is a basic concept of a chemical process. The temperature coefficient (θ) is a measure of the increase in bacterial death rate for every 1 °C rise in temperature (Bean 1967; Russell 1999b). Alternatively, θ^{10} (designated Q_{10}), that measures the effect for every 10 °C rise, is used. Q_{10} tends to increase with concentration and the temperature range over which it is measured, but there are generally few references to Q_{10} for disinfectants (Bean 1967; Russell 1999b). Again, some disinfectants are more temperature dependent than others, cf. Table 4.1. It is often stated that glutaraldehyde is active down to about 5 °C, whereas formaldehyde needs at least 16 °C (Anonymous 2002b). The activity of glutaraldehyde increases rapidly above 20 °C, whereas there is little, if any, difference in activity between 40 °C and higher (Russell 1999b).

4.2.2.3 pH

Changes in pH may influence both the cell surface of the bacteria (cf. Section 3.4.3) and the disinfectant (Bean 1967; Russell 1999b).

Phenols, certain acids and agents that release chlorine are mainly active as disinfectants in the unionised form, and the degree of dissociation rises as the pH increases (Bean 1967; Hugo and Russell 1999). For example, chlorine killed spores of *Bacillus metiens* in 2.5 min at pH 6, whereas it took 2 h at pH 10 (Bean 1967). Moreover, the oil-water partition coefficient, and therefore probably also the cell-water coefficient, is reduced for phenols at rising pH, so the concentration near the active site is reduced (Bean 1967). An increase in pH increases the number of negatively charged molecules on the bacterial sur-

face, and these attract cations (e.g. QACs) (Bean 1967; Russell 1999b). QACs and glutaraldehyde are examples of disinfectants that are most effective at alkaline pH (Klimek and Bailey 1956; Gorman *et al.* 1980). The reaction between glutaraldehyde and amino acids occurs most rapidly at $\text{pH} > 7$, though it is unclear if this is the key event that causes bacterial death (Gorman *et al.* 1980; Russell 1999b).

4.2.2.4 Extraneous material

Extraneous material includes organic matter, biofilms (cf. Section 3.4.4), surface-active agents and cations.

As a general rule, organic matter decreases the effectiveness of disinfectants, but again big differences are seen. The decrease in effectiveness is believed to be due partly to a reaction between the disinfectant and the extraneous material, partly to a reduced penetration through the latter (Russell 1999b). The influence of each of these can be difficult to elucidate under real-life conditions (also cf. Section 3.4.4.3). Generally, reduced effectiveness due to reactions with proteins in organic matter is seen with highly reactive disinfectants (such as oxidising, chlorine and iodine compounds), whereas less reactive compounds (e.g. strong bases) are less inactivated (Russell and Chopra 1996; Russell 1999b). One peculiar exception is the aldehydes which are relatively unaffected by organic matter in spite of their high reactivity with the amino groups in proteins (Gorman *et al.* 1980; Russell 1999b).

Some surface-active agents reduce the effect of QACs and phenols significantly (Russell 1999b). Therefore, poultry houses should be rinsed with water before disinfection if certain (e.g. non-ionic) detergents have been used for the cleaning.

The activity of disinfectants may increase, decrease or remain unchanged in the presence of different cations (Russell 1999b). An interesting study showed that *E. coli* prepared

in hard water (i.e. with high levels of Ca^{2+} and Mg^{2+}) became more resistant to benzalkonium chloride, whereas this was not the case when the disinfectant was diluted in hard water (Klimek and Bailey 1956), thus these ions exert their effect on the cell, and not on the disinfectant (also cf. Section 3.4.3). The reduced disinfection effect in hard water compared to distilled water is a well-known parameter incorporated into realistic disinfection tests.

4.3 Combined effects of heat and chemical disinfection

Some important factors impede the efficacy of both heat and disinfectants: desiccation and the presence of substances, both organic and inorganic, have been discussed in depth. Stationary phase bacteria are more heat resistant than their counterparts in the growth phase, and this may also apply to chemical disinfectants, although other conditions, such as attachment to surfaces and biofilm formation, are often involved in disinfectant studies. Temperature is also an important parameter, obviously for heat, but also for chemical disinfectants.

Therefore, there is every reason to believe that a combination of heat and a disinfectant will improve the bacterial killing, and this has also been confirmed in many studies. Glutaraldehyde resistant *Mycobacterium chelonae* survived in a 2% glutaraldehyde solution at 20 °C, but was inactivated at 45-60 °C (van Klingeren and Pullen 1993; Griffiths *et al.* 1997). For spores of two *Bacillus* species, a log reduction of 0.5 and 4-5 after 3 h in 85-90 °C hot water and steam, respectively, was seen, whereas 30-40 min in hot steam at 85-90 °C with formaldehyde resulted in complete inactivation (Alder *et al.* 1966).

One process, designated low temperature steam and formaldehyde (LTSF) sterilisation, has focused on the use of formaldehyde as an alternative to higher temperatures without a chemical disinfectant, hence the “low” temperature. Temperatures around 73 ± 2 °C

have been applied in most cases (Hoxey and Thomas 1999). Evacuation of air followed by pulses of high air pressure are used in steriliser chambers to increase the killing of bacteria (Hoxey and Thomas 1999), and complex technical aspects are described in many studies, but it is often difficult to estimate the impact of these on the results. One of the many problems is the inability to measure formaldehyde concentrations locally (Hoxey and Thomas 1999) which can change considerably as a result of e.g. permeability of materials, local air pockets and organic matter. Thus, the only valid methods for measuring sterility are based on killing of bacterial spores (Wright *et al.* 1996; Hoxey and Thomas 1999). The technical complexities combined with the varying biological parameters can explain many of the inconsistent results, a problem reported by the Committee on Formaldehyde Disinfection of the Public Health Laboratory Service (1958) from their own study performed at seven different laboratories. However, the general principles of the classical work by Nordgren (1939) on LTSF sterilisation have generally not been challenged in subsequent publications (Committee on Formaldehyde Disinfection of the Public Health Laboratory Service 1958; Hoxey and Thomas 1999).

Nordgren (1939) found increased bacterial death with increasing temperatures in the range 10-70 °C. In another study, however, no increased inactivation rates were seen at temperatures between 0 and 30 °C (Committee on Formaldehyde Disinfection of the Public Health Laboratory Service 1958). Hoxey (1984) (quoted by Hoxey and Thomas (1999)) found minor changes in inactivation when the temperature decreased from 80 to 70 °C, but a further decrease to 65 °C caused a substantial decline in lethality.

With regard to humidity, Hoxey and Thomas (1999) reported that little had changed since Nordgren (1939) found an increased bactericidal action up until about 50% RH, whereas there was little further improvement from 50 to 95% RH. However, other studies

showed a more varied picture. Cross and Lach (1990) recommended about 80% RH, as this would minimise condensation, and spores were not killed more rapidly between 80 and 98% RH. Pfeifer and Kessler (1994) found maximum heat resistance at 40% RH whereas it declined above and below, but most when the humidity increased (e.g. $D_{96} = 2.2$ min at 100% RH and $D_{115} = 5.2$ min at 1% RH). Spinner and Hoffmann (1971) investigated spores on cloth and glass at 11, 33, 53, 75 and 100% RH. The killing rates increased with increasing humidity, with the highest increases until 53% RH. Moreover, the spores were killed more rapidly on cotton than on glass at the two lower humidity levels, whereas this was reversed at the three higher ones. This was explained by the increased absorption of water on the impervious material at higher humidity.

As expected, increased formaldehyde concentrations also increase the bacterial lethality, although linear relationships were not always seen throughout the concentration ranges (Hoxey and Thomas 1999). The Committee on Formaldehyde Disinfection of the Public Health Laboratory Service (1958) found a linear relationship in laboratory experiments, whereas the results were more inconsistent in real-life situations because of a high formaldehyde absorption by fabrics.

A combined steam and formaldehyde treatment is performed in many Danish poultry houses (cf. Chapter 2) and for the treatment of *Salmonella* in poultry feed (Anonymous 2001). The above principles probably also apply to these treatments, although air evacuation and high pressure air pulses cannot be performed outside of steriliser chambers. Certain aspects, which may apply more specifically to poultry house conditions, should be considered. Robertshaw (1983) stated that condensation on cold uninsulated surfaces would cause formaldehyde polymerisation (i.e. an inactivation of the active disinfectant), and therefore well-insulated steriliser chambers were advocated. It was also recommended

to apply formaldehyde as early as possible in the process, as this would “saturate” the surfaces before these were “diluted” by steam. In a poultry house, it is difficult to both avoid cold surfaces and apply formaldehyde at the beginning of the process, as the surfaces first have to be warmed by the steam. Another study compared disinfection with gaseous formaldehyde with or without condensation (using 100 and ca. 90% RH, respectively) at 20, 25, 30, 35 and 40 °C, performed at normal pressure and without air evacuation (Casella and Schmidt-Lorenz 1989). It was found that only at 20 °C did condensation reduce the disinfection efficacy. Pfeifer and Kessler (1994) found LTSF to be so effective for spores that there was no increase in heat resistance related to attachment. Spiner and Hoffmann (1971) did not detect any difference in lethality between unwashed and washed spores at 100% RH, whereas unwashed spores were killed even more rapidly than the washed ones at 75% RH. Other studies, however, have reported the “classical” results of decreased bacterial lethality due to organic matter (Nordgren 1939; Gibson 1977). The penetration of steam and formaldehyde into various materials and cavities has been addressed in many studies. Spores in narrow tubes are often not killed by LTSF (Nordgren 1939; Alder *et al.* 1966). The Committee on Formaldehyde Disinfection of the Public Health Laboratory Service (1958) reported low penetration through blankets that covered bacteria-spiked cotton threads, as the formaldehyde was absorbed. In the same study, bacteria from a serum suspension dried on cotton threads were not killed by LTSF either, as the formaldehyde could not penetrate the dried protein layer. This illustrates the importance of humidifying and soaking as much organic matter as possible prior to the LTSF. An interesting study showing formaldehyde residues in wool blankets at least five weeks after the treatment, concomitant with a bacterial killing during this period (Alder *et al.* 1971), is relevant for the disinfection of fabrics, e.g. jute egg belts.

4.4 Practical applications of disinfection in poultry houses

Briefly, the ideal situation can be described as follows: in the poultry house, the equipment is easy to clean, there are no cracks or crevices in the inner building which is made of hard, smooth and impervious surfaces. The cleaning has been performed scrupulously according to the recommendations (cf. Chapter 2). The whole of the inner building is disinfected with a chemical disinfectant that kills *Salmonella* rapidly, even at low temperatures, is unaffected by organic matter, non-corrosive for the equipment and breaks down to harmless components in the environment.

The more realistic situation is less rose-coloured: manufacturers have not spent many resources on the construction of cleanable equipment. Often, this has bends, hidden cavities and other “devices” that impede effective cleaning. No poultry house is completely without cracks or crevices, and many of these have wood, plaster or other surfaces which are porous and difficult to clean. Nobody is perfect, including the farmer or the contractor who has to perform a dirty, tedious and hard job with a high pressure cleaner. Moreover, expectations concerning disinfectant performance have to be reduced, as none of these fulfils all the above criteria (cf. Table 4.1).

Because of some of the above considerations, disinfectants that are relatively unaffected by organic matter and non-corrosive are often chosen. In Denmark, these arguments have been important in the choice of aldehydes. However, their permeability through organic matter is considered to be poor (Anonymous 2002b). Another disadvantage may be their slower action compared to disinfectants that are more susceptible to organic matter. With a reduced contact time between *Salmonella* and the disinfectant (e.g. on vertical surfaces), this could pose a problem. Moreover, maybe *Salmonella* adapts more easily to the disinfectant due to mechanisms described in Chapter 3 if the reaction is slower. The practi-

cal relevance for slow-reacting disinfectants and *Salmonella* in poultry houses is, however, purely speculative, as nothing has been published on these aspects. Drinking systems (water lines) probably contain little organic matter, but biofilms may be present (cf. Section 3.4.4). Disinfectants that damage biofilms would be advantageous, e.g. oxidising agents (Tanner 1989; Takeo *et al.* 1994; Eginton *et al.* 1998) or certain chlorine compounds (LeChevallier *et al.* 1988a, 1988b). Little is known about strong bases as disinfectants (Bennett *et al.* 2003), but their properties make them relevant for floors and walls. Strong bases are also the classical recommendation for outdoor areas (Søeberg and Pedersen 1998). Hydrated lime gives a nice “clean” appearance, it is easy to see which surfaces have been disinfected, and it possibly “seals off” *Salmonella* in cracks and crevices, so the chickens do not get infected when they peck on the floor. However, such speculations are unscientific and difficult to document, though they seem plausible.

Chapter 5 - Investigation principles

for *Salmonella* and disinfectants

5.1 General aspects

Most biomedical laboratory work with bacteria is performed in test tubes with suspensions and involves clinical isolates (Brown *et al.* 1991), and this is not representative of the conditions usually encountered by bacteria in the environment (cf. Section 3.2.1). There are, however, two main aspects of the disparity between field and laboratory situations, namely a) optimisation of the laboratory detection procedures, and b) laboratory models, in this case disinfection tests, ranging from “test tube” (suspension test) types to those that simulate real-life conditions.

In the optimisation of laboratory procedures, suspension tests are often an efficient means of standardising conditions. General principles for the qualitative and quantitative detection of *Salmonella* will be described and related to conditions encountered under stressful conditions in the environment. General concepts of life and death of bacteria will be evaluated and related to common detection procedures. Because efficient sampling is essential for detecting bacteria, principles for sampling from surfaces will be outlined.

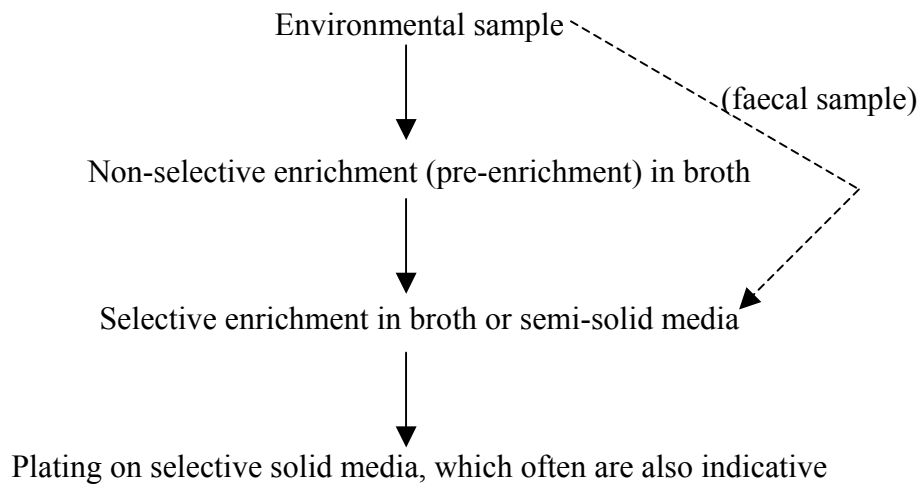
Key aspects of microbial death kinetics and problems related to these will be discussed.

Finally, general principles and pros and cons of the various kinds of disinfection tests will be described.

5.2 *Salmonella* in the extra-animal environment and in the laboratory

5.2.1 Isolation procedures

The classical principles for the qualitative isolation of *Salmonella* from various types of environmental samples are (Fricker 1987; D'Aoust 1989):



The use of different media will usually increase the sensitivity, so two types of selective enrichment broth and two solid media are often incorporated in standard procedures (Anonymous 2002e).

The main role of pre-enrichment is to facilitate repair and growth of *Salmonella* which is often stressed/injured and may occur in low numbers (Fricker 1987; D'Aoust 1989). The incubation period must be long enough to allow for resuscitation of injured *Salmonella*, as these often have prolonged lag phases (Mackey 1999) (cf. Section 5.2.2). An incubation period of 16-24 h is generally applied (Fricker 1987; D'Aoust 1989), as longer time spans may favour competitive flora (Beumer *et al.* 1991). Direct inoculation of samples into selective enrichment broth is sometimes used, but injured *Salmonella* is susceptible to the selective agents, so such methods are generally not recommended for non-

clinical samples (D'Aoust 1989; Mackey 1999), although a few investigations have disputed this for heavily contaminated samples such as faeces (Fricker 1987).

During the selective enrichment, selective agents repress the growth of competitive flora, so resuscitated *Salmonella* can multiply preferentially. Some semi-solid enrichment media also incorporate indicator systems (Goossens *et al.* 1984).

Selective principles are also used in the solid media which also include indicator systems that enable colonies of *Salmonella* to be detected easily, e.g. β -galactosidase in Rambach agar (Rambach 1990).

As each of the above steps takes about 24 hours (sometimes even longer for the selective enrichment (Waltman *et al.* 1993)), it takes a minimum of three days to detect *Salmonella* tentatively, after which serological (cf. Section 1.1), and sometimes biochemical, tests are used for confirmation of suspect colonies. Many alternative rapid methods have been developed in order to shorten the detection time, but these are generally less sensitive and specific than the traditional methods (Mackey 1999), or they do not distinguish between dead and living bacteria (e.g. PCR methods).

A detailed investigation of the impact of one or more detrimental factors often requires a quantitative approach (cf. Chapter 3 and Section 5.4). Because of the enrichment procedures, this cannot be achieved with qualitative *Salmonella* tests.

A semi-quantitative approach is the principle of most probable number (MPN) methods in which traditional procedures are made for replicate volumes of serially diluted samples. Typically, traditional procedures for Y sub-samples weighing X gram are performed, and this is repeated for Y sub-samples weighing 0.1X and 0.01X, respectively (Anonymous 1988). Sub-samples in decimal dilutions of the pre-enrichment broth is another variation of the MPN-method used for *Salmonella* (Davies 2001). Results are inter-

preted by MPN-tables to give an estimated number of CFU g⁻¹ in the sample. MPN-methods are useful but laborious, because the numbers of samples increase immensely (Humbert 2001). Moreover, an idea of the range of CFU g⁻¹ sample beforehand is advantageous in determining X and Y, as results where all samples are *Salmonella* positive or negative cannot be used to determine CFU g⁻¹ within a range.

Traditional tenfold dilutions of samples and direct inoculation onto solid media have been used in many cases, but in natural samples *Salmonella* often occurs in low numbers, is injured, unevenly distributed in solids and there are often high numbers of competitive flora, so such methods are often not suitable. However, they have often been used in laboratory tests with artificially *Salmonella* inoculated material. If these experiments are performed in samples with a competitive flora, the use of antibiotic resistant *Salmonella* strains and the incorporation of the same antibiotic in the media may be advantageous (Humbert 2001). There are, however, many other factors that can prevent satisfactory results by traditional plate count methods. Because laboratory tests often involve the application of detrimental factors that injure *Salmonella*, low numbers may not be detected by direct plating onto selective agar. The use of non-selective media which allow injured bacteria to repair can often - at least partly - compensate for this, and should be used whenever possible (Mackey 1999).

In the traditional “double agar layer methods”, bacteria are first grown on a solid non-selective agar, followed by a layer of selective agar, so the advantages of both types of media are utilised. The application of such a method increased the recovery of injured coliforms from food 2- to 100-fold (Mackey 1999; Blackburn and McCarthy 2000) and heat-injured *Salmonella* 10-fold (Kang and Fung 2000).

5.2.2 Life and death of bacteria

Most laboratory detection procedures are based on culturing of bacteria, and this was formerly the basis for distinguishing between viable/culturable and non-viable/non-culturable bacteria. With the development of new methods, it has become clear that all viable bacteria cannot necessarily be cultured, so the clear-cut distinction between life and death may be more complicated than considered previously. The following definitions are used commonly for bacteria (Oliver 1993; Mackey 1999):

- Viability: normally defined as the ability to multiply, but this implicates the method of measurement which must then be specified. Another definition is simply cellular/morphological integrity (i.e. no lysis has occurred).
- Sublethal injury/stress: is reversible or can be circumvented. The bacteria cannot grow on selective media normally used for their isolation, but they can repair cellular damage and regain all their normal properties under suitable conditions.
- Viable but non-culturable (VBNC) bacteria: cannot grow either on selective or non-selective media that normally support their growth. VBNC bacteria are considered to be viable because they are physically intact, exhibit metabolic activity and sometimes they can infect animals.
- Lethal injury/stress: is irreversible and cannot be circumvented. Lethally injured (i.e. dead) bacteria have irreversibly lost their ability to generate progeny. According to one of the definitions of viability, they have lost their cellular/morphological integrity (i.e. cellular lysis has occurred).

The scientific literature is confusing because the above definitions often overlap or they are used imprecisely. There is especially a lot of confusion between injured and VBNC bacteria (cf. below).

Mackey (1999) described the main characteristics of injured bacteria, known at present (also cf. Sections 4.1.2 and 4.2.1). The outer membrane of Gram-negatives is damaged which is the main explanation for their inability to grow on media containing selective substances such as bile salts. The cytoplasmic membrane is often also injured, and this causes lysis, the degree of which depends on the stress conditions. Hence, the exact distinction between sub-lethal and lethal damage remains to be determined. Similarly, the role of DNA damage in bacterial death is unclear regarding heating, freezing, desiccation or acids, but it is the primary cause of death due to ultraviolet or ionising radiation. RNA damage is probably one of the main reasons for the extended lag phase in injured cells, as time is needed to re-synthesise RNA and proteins.

There is no uniform agreement on the exact definition of the VBNC state, and the concept is controversial (Oliver 1993; Kell *et al.* 1998; Barer and Harwood 1999; Mackey 1999; Edwards 2000; Rozen and Belkin 2001; Winfield and Groisman 2003). VBNC bacteria can usually be cultured after a suitable resuscitation treatment (Oliver 1993; Mackey 1999), and this blurs the distinction between VBNC and injured bacteria. Its relationship to the stationary phase is unclear (Mackey 1999) which makes its relevance for aspects described in Chapter 3 difficult. The VBNC state is a phenomenon of naturally occurring bacteria that has mainly been described in natural water systems, and the relevance for *Salmonella* in poultry houses is difficult to elucidate, because these often involve a small proportion of the bacterial population and have to be studied *in situ*. The few studies dealing with *Salmonella* and VBNC illustrate the controversy of the topic, as these either supported (Roszak *et al.* 1984; Munro *et al.* 1995; Caro *et al.* 1999) or opposed (Bogosian *et al.* 1998; Turner *et al.* 2000) the existence of a VBNC state. The study by Bogosian *et al.* (1998), comprising several enteric bacteria (incl. *S. Choleraesuis*), elucidated one of the

main arguments against the occurrence of a VBNC state. Two easily distinguishable (lactose positive and negative) strains were used to create a mixture containing large numbers of non-culturable cells of both strains, together with a small number of culturable cells of only one strain. For all the bacterial types, only cells of the culturable strains were recovered after various resuscitation procedures. The authors concluded that the non-culturable (“VBNC”) cells were dead, and the apparent resuscitation was due to growth of the few culturable cells.

5.2.3 Sampling procedures

Here, the focus will be on sampling of surfaces, as this is most relevant in empty poultry houses. There are two main aspects of sampling, a statistical and a methodological one.

The statistical aspect is straightforward, as more samples and the covering of bigger areas per sample will increase the likelihood of detecting *Salmonella*. However, only a small proportion of the surface area in empty poultry houses can be sampled. Different studies in poultry flocks have estimated the sensitivity of sample types and numbers of samples necessary for detecting *Salmonella* with 95% confidence, given a certain prevalence and 100% sensitivity of the laboratory method used (as the real sensitivity can never be determined) (Skov *et al.* 1999), but nothing similar has been attempted in empty poultry houses. The same principles could be applied in these, i.e. 60 and 300 samples will detect *Salmonella* with 95% certainty, given a prevalence of at least 5 and 1%, respectively, but the definition of prevalence in an empty poultry house is speculative, and it will depend on the sampling methods and the distribution of *Salmonella* in the house.

Methods for microbiological sampling of surfaces were summarised by Favero *et al.* (1968). Basically, there are four types of methods, each with several variations:

- The swab-rinse technique or swab methods: some form of this method is at present the only one that can be used realistically for sampling *Salmonella* in poultry houses. Subsequently, qualitative traditional *Salmonella* methods are used to resuscitate injured bacteria. Quantitative methods are applied for different types of non-*Salmonella*, but as it is difficult to apply such methods in a uniform way, the reproducibility and repeatability of these methods are poor (Favero *et al.* 1968). Amongst other things, a high proportion of bacteria remains on the surfaces (Moore and Griffith 2002), and different methods may alter the sensitivity significantly (Davies and Wray 1996a; Rolfe *et al.* 2000).
- Rinse test: the contaminated surface is immersed in sterile fluid, which is then agitated. For *Salmonella*, the sterile fluid will typically be the non-selective enrichment broth. These test types are very laborious to apply in the sampling of poultry houses, but in the laboratory they have been used in many surface disinfection and biofilm tests.
- Agar contact methods: a nutrient agar is pressed directly against the test surface. These methods cannot be applied to *Salmonella* in poultry houses, as these will typically be overgrown by other bacteria. Even if a *Salmonella* selective and indicative agar is used, the two enrichment procedures are skipped, so the likelihood of detecting injured *Salmonella* decreases immensely.
- Direct surface agar plating: sterile agar medium is poured on the surface after which it solidifies and is incubated. This can only be used in the laboratory, but its accuracy in quantitative studies was reported to be good (Favero *et al.* 1968).

5.3 Microbial death kinetics models

5.3.1 Definitions

Microbial death is normally illustrated by curves that show numbers of surviving microbes. These are almost always illustrated as curves where the numbers of survivors are plotted on a logarithmic y-axis and the x-axis is an arithmetic time scale, yielding straight lines for first-order reactions (cf. section 5.3.2.). Most often, the decimal logarithm system is used as this complies with D-values. The decimal reduction time (also named the D-value or D) is defined as the time at a particular temperature to reduce a microbial population to a tenth of this population, i.e. a one log-cycle reduction. In heat experiments, D is normally written with the actual temperature in °C, e.g. D_{71} . If D-values are calculated at different temperatures under the same conditions, they can be plotted on a logarithmic y-axis with the corresponding temperatures on an arithmetic x-axis, a plot designated the thermal death time curve (Hansen and Riemann 1963). Here, a first-order reaction yields a straight line, the slope of which is the z-value (or z), expressed as the increase in temperature required to reduce D to 0.1D (Hansen and Riemann 1963). The aforementioned designations may differ according to the context, e.g. the term disinfection rate constant is often used in disinfection studies (Lambert and Johnston 2000), but these linguistic distinctions do not alter the basic definitions.

5.3.2 Factors that influence microbial survival curves

It is generally assumed that the death of microbes in a population follows first order kinetics, regardless of the cause of death (Cerf 1977; Mitscherlich and Marth 1984; Mafart 2000), thus yielding a straight line in a semi-logarithmic plot (cf. Section 5.3.1). Most kinetic studies of microbial death are based on one of the following hypotheses (Cerf 1977; Lambert and Johnston 2000):

- Mechanistic hypothesis: the microbial population is homogeneous, and the reaction between the microbes and the detrimental factor is comparable to a chemical reaction process, i.e. basically following first order kinetics.
- Vitalistic hypothesis: there is a natural distribution of resistance against the detrimental factor in the population. This difference in the degree of resistance is permanent, and it is therefore also named “the theory of variable permanent resistance”.

It has never been determined if one, both or neither of these hypotheses can basically explain miscellaneous death kinetic phenomena (Lambert and Johnston 2000). The reasons for this uncertainty are manifold, but they are probably mainly related to the limited knowledge of exact mechanisms involved in microbial death (Cerf 1977; Lambert and Johnston 2000) (cf. Section 5.2.2) and to many studies showing survivor curves which deviate from first-order reaction kinetics in spite of the above assumptions.

Many designations have been given for semi-logarithmic survivor curves that deviate from straight lines, but the main deviations are either shouldering or tailing (Hansen and Riemann 1963; Cerf 1977; Johnston *et al.* 2000). Shouldering is a slower death rate of the population at the beginning of the detrimental process. Some of the reasons for shouldering are thought to be either clumping of cells or a weak disinfectant concentration which prolongs the time it takes to reach the target cell sites (Hansen and Riemann 1963). Because the ultimate aim is to eliminate all the microbes, and shouldering is a phenomenon at the beginning of the process, tailing is more relevant here. Tailing is characterised by a D-value that increases near the end of the detrimental process compared to the constant D-value of the straight semi-logarithmic line. Death curves with a tail are often described as biphasic, i.e. consisting of two straight lines. When tailing is found, it is assumed that cells of unequal resistance are present (Hansen and Riemann 1963), and this is the main objec-

tion to the mechanistic hypothesis (Lambert and Johnston 2000). On the other hand, tailing is normally seen in only a minor fraction of the whole population, e.g. ca. 10^{-5} in a study with *S. Enteritidis* (Humpheson *et al.* 1998). This is not in accordance with the vitalistic hypothesis of different sub-populations, each with its own intrinsic resistance, because these will give more varied tail fractions (Cerf 1977). Moreover, it has been stated generally that survival curves obtained by re-heating surviving tail populations did not differ from curves obtained by the first heating, i.e. the tail population was not more heat resistant than the parent population (Hansen and Riemann 1963; Moats *et al.* 1971). However, studies in which a surviving tail population was more heat resistant than the parent population have also been reported (Corry and Roberts 1970), so the topic remains controversial. In general, it is not clear if tailing is a naturally occurring phenomenon or an artefact. Cerf (1977) reviewed and discussed possible reasons for tailing. Several studies, in which tailing could be the result of artefacts, were quoted, and it was grouped into the following four categories:

- Two or more homogeneous populations, each with its own level of resistance, are mixed in the investigation. This is different from the vitalistic hypothesis where heterogeneous populations occur naturally, and not as a result of mixing in the laboratory.
- Non-uniform treatment: heating in open vials, so e.g. bacterial spores in small droplets receive lower temperatures. Heat treatment of fatty materials could also render a tail population due to local water activity variation (e.g. in small cavities). The conditions in organic material could change during heat treatment, altering the conditions for the longest surviving microbes compared to those that were killed in the beginning. Several other examples were given on laboratory conditions and procedures that could explain tailing.

- Clumping: this is commonly mentioned in connection with radiation, but it has also been described from heating studies where an uneven distribution of heat among microbes in a clump is a likely explanation (Klijn *et al.* 2001).
- Enumeration techniques: counting of few microbes has a higher variability than when more microbes are counted, thus the calculated number of survivors may be too high at the end of the treatment.

In conclusion, microbial death kinetics are empirically considered to be first-order reactions, but there is no general agreement on reasons for deviations from these.

5.4 Disinfection tests

5.4.1 General aspects

There is no general agreement on what disinfection really means apart from “a reduction of microbes” (Reybrouck 1999), which can be accomplished by several means. Thus, there is no generally agreed success criterion for the efficacy of a disinfectant, although a 5 log reduction in numbers of CFU is demanded in many tests (Cremieux and Fleurette 1991).

Another disadvantage is the lack of harmonisation between countries. Only a few countries (France, Germany, Netherlands, UK, USA) have official disinfectant test methods, and even between these no harmonisation exists (Cremieux and Fleurette 1991; Reybrouck 1999).

In 1990, the European Committee for Standardization (CEN) established Technical Committee 216 Chemical Disinfectants and Antiseptics (TC 216), the scope of which was (Reybrouck 1999):

“the standardisation of the terminology, requirements, test methods including potential efficacy under in-use conditions, recommendations for use and labelling in the whole field of chemical disinfection and antiseptics”.

Today, none of these objectives has been accomplished. Denmark, for example, has no legislation on the efficacy of disinfectants for the agricultural sector. This means that anyone can market a product as a disinfectant without any documentation for its effectiveness. However, results from the European suspension test are reported in many commercial disinfectant brochures, probably because suspension tests often give impressive results for the elimination of bacteria (Bloomfield *et al.* 1991).

5.4.2 The carry-over of disinfectants

All chemical disinfection tests have two phases: 1) contact between the bacteria and the disinfectant, and 2) recovery of the relevant bacteria. Detrimental conditions are an inherent part of the first phase, but these have to be eliminated in the second phase. In other words, the disinfectant should not be carried over in the recovery procedures in which it will inhibit the growth of the bacteria. The procedures for neutralising disinfectants fall into three main categories (Russell 1981; Sutton *et al.* 2002):

- 1) Appropriate dilution in the enrichment broth.
- 2) Treatment with a chemical neutralizer.
- 3) Filtration, whereby the bacteria theoretically stay at the filter while the disinfectants are washed away.

Regardless of the method, it is difficult to estimate the efficacy theoretically, so tests, preferably quantitative ones, are normally necessary (Russell 1981; Sutton *et al.* 2002).

Dilutions are mainly suitable for disinfectants that have high dilution coefficients (cf. Section 4.2.2.1) and do not bind easily to the cell (Sutton *et al.* 2002), but MICs for the actual bacteria should be known.

For neutralizers, four parallel tests are necessary (broth without disinfectant or neutralizer, broth with disinfectant, broth with neutralizer and broth with disinfectant and neu-

tralizer), because neutralizers can also be toxic to bacteria, either alone or in combination with the disinfectant. Ideally, the tests should be repeated with different concentrations of disinfectant and neutralizer (Russell 1981). Moreover, results for one type of bacteria cannot be extrapolated to other types, as each disinfectant-neutralizer-bacteria combination is unique (Sutton *et al.* 2002). This also implies that no neutralizer covers all types of disinfectants, and this poses a problem when different disinfectants have to be compared, as there will invariably be differences in the recovery media. One major disadvantage in the evaluation of neutralizers is that these tests are performed with non-injured bacteria (Russell 1981; Sutton *et al.* 2002). A neutralizer or a disinfectant-neutralizer combination might be harmless for non-injured, but detrimental to injured bacteria (as are often encountered in disinfection tests).

In filtration tests, so little disinfectant has to adhere to the filter or the bacteria that it does not inhibit growth in the subsequent recovery procedures. This is validated by comparisons between known amounts of bacteria on filters that have been treated with disinfectants and bacteria that have not been filtered or disinfectant treated (Russell 1981).

Many chemical disinfectant studies in which no neutralisation procedures were used or considered have been published (Bergan and Lystad 1972; MacKinnon 1974). Results from such studies might have over-estimated the efficacy of chemical disinfectants, because the putative inhibition by disinfectants in recovery media has been neglected.

5.4.3 Types of disinfection tests

Reybrouck (1999) classified disinfection tests into the following categories:

- 1) First stage preliminary tests, also called *in vitro* tests, i.e. all test methods that are not carried out under practical conditions or in the field. These can be divided into bacte-

riostatic tests, i.e. minimum inhibitory concentration (MIC) tests, and bactericidal tests; the latter are often divided into suspension, capacity and carrier tests.

- 2) Practical tests, which are carried out in the laboratory. They mimic natural conditions, but are carried out with artificially inoculated samples (e.g. surfaces).
- 3) In-use or field tests. Covers all tests carried out in the environment with the naturally occurring bacteria where the disinfectants will be used.

5.4.4 In vitro tests

5.4.4.1 MIC-tests

As a disinfectant is normally required to be bactericidal, the bacteriostatic activity of a disinfectant is mainly relevant for the selection of the most resistant bacterial strains. The minimum inhibitory concentration (MIC) can be determined in tests similar to those used for antibiotics (Reybrouck 1999; Randall and Woodward 2001a).

5.4.4.2 Suspension tests

In suspension tests, a volume of the bacterial suspension is added to the disinfectant in the concentration to be tested, and after a predetermined exposure time, an aliquot is examined either qualitatively or quantitatively (standard dilutions and streaking on solid media) (Reybrouck 1999). Quantitative tests are often preferred because these give a more detailed view of the efficacy. Suspension tests are easy to standardise, and various parameters can be compared, e.g. different disinfectants, concentrations, temperatures, bacteria and time schedules. In some tests, an organic load is added (e.g. yeast, serum or albumin). Suspension tests are widely used to document the efficacy of disinfectants, and pass/fail criteria have been determined, e.g. a 5 log reduction in 5 minutes in the European suspension test or the Dutch 5-5-5 suspension test. The main disadvantage of suspension tests is that they are often unrealistic (van Klingeren 1995). Bacteria in suspensions are generally

more susceptible to detrimental factors (including disinfectants) than those attached to surfaces or associated with complex organic matter, matrices and diverse bacterial populations (cf. Section 3.4.3). This means it is hazardous to extrapolate results from suspension tests to real-life conditions and expect the same efficacy of the disinfectant.

Rigorous standardisation is important, even within the same test. Bloomfield and Looney (1992) investigated the repeatability and reproducibility of the European suspension test performed in several laboratories at different time schedules, and they reported significant differences in microbicidal effect, mainly related to different pre-treatments of bacterial strains prior to the investigation. A similar study with the Dutch 5-5-5 test, covering 10 laboratories, 7 disinfectants, 4 bacterial strains and a time span of 5 weeks, reached a similar conclusion (van Klingeren *et al.* 1977) (also cf. Section 3.4.3).

5.4.4.3 Capacity tests

In capacity tests, the ability of a disinfectant to retain activity in the presence of an increasing load of bacteria, and often also of organic matter, is investigated (Reybrouck 1999). Such tests simulate the disinfection of items with bacteria and organic matter that are soaked in a disinfectant suspension. The most widely used capacity test in Europe is the Kelsey-Sykes test (Reybrouck 1999). In this test, bacterial suspensions are added to the same disinfectant solution at predetermined time intervals, thus increasing the bacterial load successively. From each disinfectant/bacterial suspension, serial tenfold dilutions are made and checked for presence/absence of the bacteria (i.e. an MPN-method) (Reybrouck 1999). Croshaw (1981) found the Kelsey-Sykes test to be more realistic than suspension tests, but stated that several factors could still be altered to mimic in-use conditions more closely. Moreover, a major disadvantage was its widespread use without a proper determination of its reproducibility (Croshaw 1981).

5.4.4.4 Carrier tests

In a carrier test, a piece of solid material (e.g. a stainless steel coupon) is soaked in a bacterial suspension, after which it is dried and immersed in a disinfectant solution from which it is transferred to a nutrient broth for cultivation (van Klingereren 1995; Reybrouck 1999). This approaches a practical test, but as carriers are standardised they often represent unrealistic surfaces, so they are characterised as in-vitro tests (Reybrouck 1999). The two most widely used carrier tests are the DGHM and the AOAC use-dilution test (Reybrouck 1999). There are two main factors in a carrier test that are likely to increase the variability compared to a suspension test, namely the more inconsistent recovery of survivors from surfaces (also cf. Section 5.2.3) and the loss of bacterial viability as a result of desiccation on the inoculated surface (Bloomfield *et al.* 1994; van Klingereren 1995). It was therefore surprising that one study reported of less variability in a surface test (Bloomfield *et al.* 1994) than in a suspension test (Bloomfield and Looney 1992). Later, van Klingereren (1995) reviewed the variability of carrier tests performed at different laboratories, and he also confirmed its low variability. The main reason for the variability was thought to be changes in the resistance of the test suspension (i.e. as for suspension tests, cf. Section 5.4.4.2), but this did not explain the smaller variability in surface tests compared to suspension tests.

5.4.5 Practical tests

The main purpose of these tests is to mimic specific real-life conditions as closely as possible. Practical tests are performed in the laboratory, so they can be standardised more easily (Reybrouck 1999). In practical tests, it is difficult or impossible to determine factors that influence the resistance of the test bacteria to disinfectants, and this can render the reproducibility between different laboratories difficult (Reybrouck 1999). Test methods

described by DGHM are probably the most comprehensive practical tests, and several evaluations describe investigations that mimic real-life conditions (Werner 1975; Borneff and Werner 1977; Reybrouck 1999). Generally, the results are very useful for the specific conditions described, but they cannot be extrapolated to other conditions.

5.4.6 In-use tests

The main principle is that the in-use dilution of a disinfectant should not leave surviving naturally occurring target microbes after use, even with an organic load (Reybrouck 1999). Prince and Ayliffe (1972) measured the phenolic concentration and the bacterial contamination in 105 hospital environment samples. The number of samples with contamination was related to the phenolic concentration, so an effective concentration under real-life conditions could be estimated. Published in-use studies on *Salmonella* in the agricultural section have used traditional sampling methods before and after disinfection procedures (cf. Section 1.5). In-use studies have many unknown and uncontrollable factors, and it is often difficult to include identical negative control units in which no disinfection is performed. Probably, the only way of compensating for the many unknown factors is to perform in-use tests under many different conditions in order to deduce general tendencies, e.g. the efficacy of formaldehyde reported from several poultry house studies (cf. Section 1.5).

Chapter 6 – Introduction to studies

6.1 General aspects

This chapter gives a general introduction to the studies described in Chapters 7-12 and Appendix A. The background and aims of the studies are outlined, and general *Salmonella* isolation procedures and non-*Salmonella* methods applied in more than one study are described.

6.2 Background to the studies

In 1988, the WHO recommended nine key *Salmonella* research areas in the farm-to-fork chain, five of which related to the primary agricultural sector (World Health Organization 1988). On the farm level, all topics (such as vaccination and competitive exclusion) dealt merely with a reduction in *Salmonella* occurrence, and not with a complete *Salmonella* elimination. Curiously, cleaning and disinfection of *Salmonella*-infected animal houses were not mentioned. In most countries, it may be more realistic to opt for a *Salmonella* reduction, but in some countries, such as the Scandinavian ones, it was decided politically that *Salmonella* had to be eliminated, at least on the individual farm. Thus, an inherent part of the Danish *Salmonella* Control Programme implemented in 1996 was penalties for farmers who could not eliminate *Salmonella* in the first download period after a *Salmonella*-infected flock. Measures for the elimination of *Salmonella* have to be applied in a download period, so it is advantageous that commercial poultry enterprises in the Western hemisphere run an all-in all-out system. With the implementation of the Danish *Salmonella* Control Programme, two DVMs (Susanne Kabell and the author of this dissertation) were employed at the DVI, Århus. One of their main tasks was to advise farmers with persistently *Salmonella*-infected poultry houses on how to eliminate *Salmonella*

on their farm. This required a holistic approach, e.g. one should not focus solely on cleaning and disinfection without implementing effective rodent control and strict bio-security measures. However, it was soon discovered that the scientific community had adhered to the WHO recommendations, as there was very little sound scientific knowledge in areas related to the elimination of *Salmonella* on farm level. This was especially the case for cleaning and disinfection of animal houses. It is beyond any doubt that thorough cleaning which removes all visible organic matter will improve the efficacy of disinfection, but persistent *Salmonella* infections were also encountered in many poultry houses in which very thorough cleaning had been performed. In addition, some types of poultry house are difficult to clean effectively, so disinfection methods that could still be effective in spite of the presence of organic matter were also needed. This required applied research into borderline conditions so results could be used directly by farmers and contractors for disinfection. Due to these needs, money was allocated under the Danish *Salmonella* Control Programme for the so-called cleaning and disinfection projects. There were two main pillars of these projects, one focusing on heat, the other on chemical disinfection. The studies reported here are based on most of the results from these projects, except those in Chapters 7 and 10, which were performed in the author's "consultancy period" prior to the cleaning and disinfection projects.

6.3 Aims

The main aim was the development of methods, which could be realistically applied in order to eliminate *Salmonella* in poultry houses.

More specifically, the studies had the following aims:

- To find factors related to cleanability of materials, cleaning and disinfection procedures in poultry houses that could influence *Salmonella* persistence (Chapter 7).
- To pinpoint factors related to heating that were important for the elimination of *Salmonella* (Chapters 8-10).
- To find a temperature-humidity-time treatment that could eliminate all *Salmonella* in worst-case laboratory and field heating studies (Chapters 8-9).
- To investigate whether there was an association between *Salmonella* persistence and resistance to commonly used disinfectants (Chapter 11).
- To test a putative role of *mar* in resistance to commonly used disinfectants (Chapter 11) – whether there is a need to vary disinfectants applied, in order to avoid selection of disinfectant resistant strains of *Salmonella*.
- To test the efficacy of commonly used disinfectants against *Salmonella* in worst-case scenario surface disinfection tests (Chapter 13).
- To evaluate the use of putative indicator bacteria in the monitoring methods (Chapters 8-10, 12).

6.4 Media, disinfectants and chemicals

An overview of media, disinfectants and chemicals is seen in Table 6.1.

Table 6.1: Media, disinfectants and chemicals used in the laboratory tests.

Name	Description (source)
Bacto agar	(Difco 0140)
Bio Komplet Plus	(Korn- og Foderstofkompagniet, 8260 Viby J, Denmark)
Blood agar	Blood agar base (Oxoid CM271), 5% calf blood
Buffered peptone water	(Merck 1.07228.)
Cyclohexane	(Bie & Berntsen A/S, 2610 Rødovre, Denmark, no. 40006)
Deionised water	ELGA LabWater (Violia Water Systems, HP14 3JH Bucks, UK)
DIASALM	(Merck 1.09803.)
Dorset's egg slopes	(Med-Ox Diagnostics Inc., Ontario K2S 1E7, Canada, EM300)
Enterococcus broth	ad modum Enterococcosel broth (Becton Dickinson, 211213)
Ethanol, 96%	969.6 ml 99.9% ethanol (Bie & Berntsen A/S, 2610 Rødovre, Denmark, no. BBB14050) and 30.4 ml deionised water
FAM 30	(Evans Vanodine International, Lancashire PR5 8AH, UK)
Farm Fluid S	(Antec International, Suffolk CO10 2XD, UK)
Formalin	24.5% v/v formaldehyde (Bie & Berntsen A/S, 2610 Rødovre, Denmark, no. 4552)
ISO-sensitest agar, double strength	(Oxoid CM 471 B double strength)
Luria Bertani broth	Bacto LB broth, Miller (Difco 0446)
MacConkey agar	(Difco 0075)
Mueller Hinton agar	(Becton Dickinson, 211438)
Modified Semi-solid	(Oxoid CM910)
Rappaport-Vassiliadis n-hexane	(Bie & Berntsen A/S, 2610 Rødovre, Denmark, no. 40008)
Nutrient broth	(Difco 0003)
Physiological saline	0.85% sodium chloride in deionised water
Rambach agar	(Merck 1.07500.)
Rappaport-Vassiliadis soy peptone broth	(Oxoid CM866)
Rifampicin	(Sigma R-3501)
Slanetz agar	Membrane-filter Enterococcus Selective Agar acc. to Slanetz and Bartley (Merck 1.05289), 1% 2.3.5 triphenyltetrazoliumchloride (Fluka 71379)
Sodium chloride	(Bie & Berntsen A/S, 2610 Rødovre, Denmark, no. LAB00333)
Sodium hydroxide	(Merck 1.01621)
Standard Count Agar	(Ciba Specialty Chemicals, 8000 Århus C, Denmark, Irgasan DP300)
Triclosan	(Difco 0344)
Veal infusion broth	(Antec International, Suffolk CO10 2XD, UK)
Virkon S	0.404 g CaCl ₂ ·2H ₂ O (Merck 2382) and 0.139 g MgCl ₂ ·6H ₂ O (Merck 5833) per L deionised water
WHO standard hard water	

6.5 Bacterial field isolates

Table 6.2 shows the bacterial field isolates used in all studies, except Chapter 11 (see details in Tables 11.1 and 11.5).

Table 6.2: Bacterial field isolates used in all studies, except Chapter 11.

Type	Isolate no.	Source, all from poultry	Used in Chapter(s)	Used in Figure(s) in Appendix A
<i>S. Enteritidis</i> , PT8	7278230-01	Faecal sample	8,12	A3,A8,A10,A11,A12
<i>S. Typhimurium</i> , DT110	9974037-01	Faecal sample	8	A7
<i>S. Infantis</i>	9879483-01	Faecal sample	8	A4
<i>E. coli</i>	7330455	Cloacal swab sample	9	A10,A11,A12
<i>E. faecalis</i>	7330481	Cloacal swab sample	9,12	A10,A11,A12
<i>S. Senftenberg</i>	S 8827 97	UK (cf. Tables 11.1 and 11.5)	12	
<i>S. Enteritidis</i> , PT8	9981190-21	House floor sample		A1,A9
<i>S. Enteritidis</i> , PT8	7260281-02	Chicken		A2
<i>S. Infantis</i>	9888954-07	House sample		A5
<i>S. Typhimurium</i> , DT110	9976240-04	House sample		A6
<i>S. Enteritidis</i> , PT4	7278447-01	Faecal sample		A8
<i>S. Enteritidis</i> , PT6	9979724-01	Faecal sample		A8
<i>S. Enteritidis</i> , PT4	9969695-01	House sample		A9
<i>S. Enteritidis</i> , PT6	7260731-16	House sample		A9

6.6 Storing of bacterial isolates

Unless otherwise stated, all isolates were kept on BA plates, storing at 5 °C. At least once a month, one colony was subcultured in VIB, incubated at 37 °C aerobically for 18-24 hours and streaked onto a new BA plate.

6.7 *Salmonella* isolation procedures

Evaluation of *Salmonella* isolation procedures, including comparisons between different media, was not part of this project. Therefore, the general procedures applied at DVI, Århus, for environmental *Salmonella* samples submitted under the Danish *Salmonella* Control Programme were used in this study. A modified ISO 6579 method (Anonymous 2002e) was used at DVI, Århus. In October 1996, DVI, Århus, performed an

evaluation of Rambach agar vs. Brilliant Green Agar, modified by Lutensit A-LBA (see Petersen (1997) for details) for environmental samples, and found that omitting the latter did not decrease the sensitivity (DVI, Århus, internal evaluation report (J.C. Jørgensen, pers. comm.)). Therefore, traditional *Salmonella* isolation procedures involving BPW → RVS → Rambach agar were used for the heating studies (cf. Section 6.7.1 and Chapters 8-10). In May-July 2002, RVS vs. MSRV were evaluated at DVI, Århus, and because MSRV did not decrease the sensitivity (J.C. Jørgensen, pers. comm.) this medium replaced RVS in the standard procedures, and it was therefore used for the chemical disinfection studies (cf. Section 6.7.2 and Chapter 12).

6.7.1 *Salmonella* procedures with RVS

The sample was diluted 1:9 in BPW, incubating 16-20 h at 37 °C, after which 0.1 ml BPW was enriched in 10 ml RVS, incubating 18-24 h at 42 °C, and plated (10 µl) on Rambach agar which was incubated 18-24 h at 37 °C. Results from Rambach agar were recorded for both *Salmonella* like and non-*Salmonella* like colonies. Most of the latter were blue or bluish colonies, representing coliform bacteria (cf. Tables 8.3 and 9.3). Presumptive *Salmonella* colonies on Rambach agar were serotyped by agglutination with relevant O-antisera (Popoff and Le Minor 1997).

6.7.2 *Salmonella* procedures with MSRV

The sample was diluted in BPW (different sample:BPW proportions, cf. Section 12.2.8), incubating 16-20 h at 37 °C, after which MSRV was inoculated from BPW and incubated for both 18-24 h and 42-48 h. Plates with swarming after 18-24 or 42-48 h were plated (10 µl) on Rambach agar incubating 18-24 h at 37 °C. Presumptive *Salmonella* colonies on Rambach agar were agglutinated with O-antisera relevant for either *S. Enteritidis* or *S. Senftenberg* (Popoff and Le Minor 1997).

6.8 *E. coli* isolation procedures

Unless otherwise stated, the sample was diluted 1:9 in BPW, incubating 16-20 h at 37 °C and plated (10 µl) on MacConkey agar which was incubated 18-24 h at 37 °C. Red/reddish colonies with precipitate zones were classified as presumptive *E. coli*. Atypical colonies were checked by routine tests (motility, growth on Drigalski agar, indole test, citrate utilisation on Simmons citrate agar, methyl red test, Voges Proskauer test) for identity as *E. coli*.

6.9 Enterococci isolation procedures

Unless otherwise stated, the sample was diluted 1:9 in Enterococcus broth, incubating 18-24 h at 42 °C and plated on Slanetz agar which was incubated 42-48 h at 37 °C. Purplish colonies on Slanetz agar were characterised as presumptive enterococci.

6.10 Rifampicin resistant isolates

Rifampicin-resistant isolates were used in the investigations to facilitate isolation and check for unintentional cross-contamination. The chromosomal mutation of rifampicin-resistance is unusual in naturally occurring bacteria, and it is not easily transferable (Compeau *et al.* 1988). The gradient plate technique described by Eisenstadt *et al.* (1994) was used, however, with some modifications in the media (Nutrient agar: 8.5 g sodium chloride, 13.0 g Bacto agar, 20.0 g Nutrient broth, 1000 ml deionised water; Rifampicin solution: 5.0 g rifampicin, 1000 ml 96% ethanol; Nutrient agar with rifampicin (50 µg ml⁻¹): 500 ml Nutrient agar, 5 ml Rifampicin solution). Isolates with a susceptibility zone of 0 mm, using Neo-sensitabs Rifampicin (Anonymous 1998) were used in the relevant investigations.

6.11 Quantitative tests

Isolates were inoculated in broth and incubated 12-24 hours at 37 °C, i.e. until the stationary phase. Numbers of CFU ml⁻¹ were determined by means of tenfold dilutions and spreading of 0.10 ml aliquots on agar plates which were incubated for 18-24 hours at 37 °C. Numbers of CFU in the range 20-300 were counted and used for calculating numbers of CFU in the material. In tests where all plates had <20 colonies, plates with these were used for counting. Various media were used in different test series (described in the relevant sections and in Appendix A). In all quantitative heat experiments (cf. Appendix A), VIB and BA were used for the initial inoculation and plating.

Chapter 7 - Impact of cleanability, cleaning and disinfection on persistence of *S. Enteritidis* and *S. Typhimurium* in Danish broiler houses

7.1 Introduction

Since 1989, samples for *Salmonella* examination (ante mortem samples, i.e. “AM-samples”) have been submitted from all Danish broiler flocks delivering to Danish abattoirs (Bisgaard 1992; Anonymous 2003). All *Salmonella* results have been registered continuously in a database (Angen *et al.* 1996).

In January-April 1997, only about 5% of the AM-samples were *Salmonella* positive. Most of these came from broiler houses which had been persistently infected for long periods, often with *S. Infantis* or *S. 4.12:b:-*. At the end of April 1997, *Salmonella* was detected in two parent-stock enterprises which delivered to the same hatchery. *S. Enteritidis*, phage type 8 (SE8), was detected in one and *S. Typhimurium*, definitive-type 66 (ST66), in the other. Several broiler flocks became infected even though the two parent flocks were culled immediately after *Salmonella* detection.

The pattern of a few broiler houses having persistent infections with horizontal *Salmonella* types and intermittent waves of *S. Enteritidis* and/or *S. Typhimurium* is common in Denmark. It is often difficult to trace the infection sources of the persistently infected broiler houses, but the effective surveillance programs facilitate this task for *S. Enteritidis* and *S. Typhimurium* because these are most often detected in the parent stock and/or in the hatchery before they reach the broiler house. In spring 1997, so many broiler houses were infected with SE8 and/or ST66 that it was possible to conduct a retrospective investigation

into factors associated with the number of subsequent crops infected with these two *Salmonella* types. Moreover, no other serotypes were introduced into the broiler houses during the study period.

In this study, various conditions on the farms and in the broiler houses were investigated in a questionnaire-based, retrospective field study. Conditions related to house materials, equipment, cleaning and disinfection will be described in more detail, and they will be associated with the number of crops in which *S. Enteritidis* or *S. Typhimurium* were detected in the houses.

7.2 Materials and methods

7.2.1 Samples

Broiler houses with *Salmonella* positive AM-samples, taken by the farmer when the chickens were about three weeks old (Skov *et al.* 1999), were used to identify the broiler houses of this study. Positive broiler houses had one or more samples with *S. Enteritidis*, phage type 8 (SE8), and/or *S. Typhimurium*, definitive-type 66 (ST66).

7.2.2 Criteria for inclusion in the study

All broiler farms where SE8 and/or ST66 was found in AM-samples received at the DVI in a given period, and which received day-old chicks from the aforementioned hatchery, were invited to participate in the study. Before this time, SE8 or ST66 had never been detected in Danish broilers, and the two implicated broiler parent flocks were also the first ones where SE8 and ST66 had been found.

The given period for SE8 was 29/4/97-1/7/97, and 30 farms (50 broiler houses) fulfilled the above criteria. Twenty-seven of these farms (44 houses) were included in the study, representing 90% and 88% of the farms and broiler houses, respectively. The own-

ers of two farms (five broiler houses) did not wish to participate, and one farm with one broiler house had ceased producing broilers when the owner was contacted.

For ST66, 1/5/97-15/7/97 was the given period; 26 farms (45 broiler houses) met the criteria. Among these, 24 farms (40 broiler houses) were included in the study (92% of the farms and 89% of the broiler houses). One farm with three broiler houses had ceased broiler production, and another farm had two broiler houses in which *S. Typhimurium* DT193 was detected in subsequent crops. This definitive-type is thought to be a laboratory artefact, so we do not know if DT193 represented a persistent ST66 infection or the introduction of a new definitive type; therefore, these two houses were excluded.

Altogether, 42 farms (78 broiler houses) were included in the study. These numbers differ from 51 farms and 84 broiler houses (which are obtained by adding farms and broiler houses for SE8 and ST66) because some farms and houses had both SE8 and ST66.

7.2.3 Data collection procedures

In the period 2/6/97-11/3/99, the author visited the 78 broiler houses in a download period after cleaning.

Personal observations focused on factors related to cleanliness of the broiler house. Cleanliness was assessed at critical control points (i.e. sites that were difficult to clean and/or that the persons in charge of cleaning might overlook). These sites included ledges, air inlets, heating pipes, feed-chain links and the inner “corner” of feed-chain corner wheels. It was generally easy to characterise these sites objectively as dirty or clean, thus minimising subjective judgement. But the drawback was that the critical control points were determined in equipment which was not found in all broiler houses. On the other hand, it was difficult to use critical control points for sites found in all broiler houses, be-

cause these represented big areas (floors, walls, ceilings), i.e. sites where only a more subjective estimate of the cleanliness and no dichotomous measures could be made.

The visit was completed by filling in a questionnaire based both on personal observations and on information from the person in charge of attending to the broilers. Questions which ought to have been included in the original questionnaire were noted during the visits. “New” questions which did not require another visit to the farm were answered by the person in charge of the broilers in a telephone interview (which took place in May 1999).

7.2.4 Statistical procedures

The broiler house was the epidemiological/analytical unit, regardless of the number of broiler houses (*Salmonella* infected or not) on the farm. The farms were only considered in the statistical analyses if we observed that the same outcomes were recorded for “all” houses on the same farm. Table 7.1 gives an overview of numbers of single-infection and multiple-infection houses (see later) dispersed on the farms.

Table 7.1: Numbers of single-infection and multiple-infection houses (cf. Tables 7.2 and 7.3), distributed on numbers of farms (General/Salmonella Enteritidis, phage type 8/Salmonella Typhimurium, definitive-type 66).

		Number of multiple-infection houses			
		0	1	2	3
Number of single- infection houses	0	-	6/6/5	5/6/1	1/0/0
	1	14/9/10	6/2/3	2/2/0	1/1/0
	2	5/1/3	-	-	-
	3	1/0/0	-	-	-
	4	0/0/1	-	-	-
	5	-	-	-	-
	6	-	-	-	-
	7	1/0/1	-	-	-

House IV, V and VI (cf. Table 7.3) were each on one farm having 2, 3 and 4 houses, respectively, that were part of this study. Because no general house status could be determined for these houses, they were subtracted from the number of houses (general), yielding 1, 2 and 3 houses, respectively, that were part of this study.

One farm with seven ST66 single-infection houses was an outlier. Though no cluster analysis was made, we believe there was generally an even distribution between single-infection and multiple-infection houses on the other farms, and the minor variations seen

between these were mainly due to the uneven distribution of the two serotypes between single-infection and multiple-infection houses. A flock/crop within a broiler house was defined as all of the day-old chicks received on the same day from the hatchery, regardless of whether the broilers were slaughtered in one or more batches.

All 78 broiler houses were divided into multiple-infection and single-infection houses. First, these categories were defined separately for SE8 and ST66, and then the general house status was defined (Tables 7.2 and 7.3).

Table 7.2: Salmonella status of broiler houses in the study which had only Salmonella Enteritidis, phage type 8 (SE8) or Salmonella Typhimurium, definitive-type 66 (ST66).

Number of crops with that Sal- monella type	Number of houses that had only		House status (n=72)		
	SE8 (n=38)	ST66 (n=34)	General	SE8	ST66
1	15	27	Single-infection	Single-infection	Single-infection
2	10	3	Multiple-infection	Multiple-infection	Multiple-infection
3	9	2	Multiple-infection	Multiple-infection	Multiple-infection
4	2	2	Multiple-infection	Multiple-infection	Multiple-infection
5	1	0	Multiple-infection	Multiple-infection	Multiple-infection
6	1	0	Multiple-infection	Multiple-infection	Multiple-infection

Table 7.3: Salmonella status of broiler houses in the study which had both Salmonella Enteritidis, phage type 8 (SE8) and Salmonella Typhimurium, definitive-type 66 (ST66).

House code	Number of crops with		House status		
	SE8	ST66	General	SE8	ST66
I	1	1	Single-infection	Single-infection	Single-infection
II	3	7	Multiple-infection	Multiple-infection	Multiple-infection
III	4	8	Multiple-infection	Multiple-infection	Multiple-infection
IV	2	1	Omitted	Multiple-infection	Single-infection
V	1	2	Omitted	Single-infection	Multiple-infection
VI	3	1	Omitted	Multiple-infection	Single-infection

As of January 2002, neither SE8 nor ST66 had been found in the single-infection houses after the study period. On average, six to seven broiler flocks are raised annually in each Danish broiler house; thus, about 11 broiler flocks passed through each broiler house in the study period covering ~ 22 months.

In the definition of multiple-infection houses, we had to rule out that a so-called “persistence” was actually from re-infection from the parent flock(s) and/or the hatchery. Cross-infections between day-old chicks from different parent flocks in the hatchery were seen soon after the detection in the two infected parent flocks; thus, many broiler farms were infected with SE8 and/or ST66 by day-old chicks which came from several parent flocks. However, all dates for the delivery of day-old chicks to the second crop were compared with the *Salmonella* status at the hatchery and associated breeding stock. By the earliest of these dates, the two *Salmonella* infected parent flocks had been culled, eggs from these flocks had been destroyed and neither SE8 nor ST66 was detected in any samples related to the hatchery (e.g. fluff or day-old chicks from each parent flock). Moreover, neither SE8 nor ST66 was detected in any of the hundreds of broiler houses supplied by the hatchery but which were not part of this study. Thus, it is very unlikely that the hatchery was delivering SE8/ST66-infected day-old chicks at the time of delivery to the second crop in the broiler houses of this study.

All univariable analyses were done in Epi Info, (Anonymous 1996), ANALYSIS. Alpha was 5% (2-tailed) throughout the study. Dichotomous variables were analysed by chi-square or 2-tailed Fisher exact tests (for expected values < 5) (PROC TABLES). Continuous variables were analysed in PROC MEANS where t-test was used if Bartlett’s test for homogeneity of variance had $p > 0.05$ and data were normally distributed; if not, the rank-sum test was used. Because we used all infected houses on the farms, valid relative risks (RR) could be recorded for dichotomous variables. All factors were analysed separately related to the houses’ general status or status concerning SE8 and ST66, because the serotypes *per se* seemed to be associated with the house status; we therefore wanted to see if general tendencies were confirmed separately within serotypes.

Multivariable data analyses were done for factors which as a group had a logical biological interdependency. These analyses were done with a point system (which added points for each factor we believed *a priori* to be preventive). Moreover, all data were transferred to SAS (Anonymous 1999), where PROC GENMOD was used in multivariable analyses for interaction with and confounding from the serotype. This procedure was run for all factors having $p < 0.10$ for univariable RRs and where the result did not depend on the results of other factors (which might have given too many missing values). Interaction with serotype was tested for significance ($\alpha = 0.05$, 2-tailed), and the effect of confounding was estimated by comparing odds ratios with and without the serotype in the model.

7.3 Results

Table 7.4 shows results for dichotomous variables related to house materials, equipment and cleaning and disinfection procedures. In addition, two continuous variables were connected to cleaning and disinfection procedures, i.e. times per year that dead animal containers were cleaned and disinfected. These had no association to house status, either generally or specifically for any of the serotypes (data not shown).

Use, cleaning and disinfection of vehicles and trailers used for cleaning out were investigated in depth. These were most often used for all broiler houses on the farms, but no cross-infection was observed between houses on any farms (data not shown), so their role for re-infecting houses seemed insignificant, regardless of procedures used. There was a higher risk of becoming a multiple-infection house if the vehicle was used for grain, both generally and for the two serotypes, whereas the risk was lower if the trailer was used for new straw. In order to investigate an association between house status and procedures for

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vehicles and/or trailers, a point system was made (see Figure 7.1). There was no significant association with house status ($p=0.67$).

Table 7.4: Definitions of dichotomous variables and relative risks of multiple-infection houses (General, Salmonella Enteritidis, phage type 8 (SE8), Salmonella Typhimurium, definitive-type 66 (ST66)).

Category (in bold) and definition of dichotomous variable	Relative risk of multiple-infection house if "Yes"		
	General	SE8	ST66
Indoor factors, inside the broiler house, materials that enter			
Vehicles/trailers used for cleaning out are used for other purposes	0.89 ¹	0.73	1.16
(Vehicle used for new straw) ²	1.18	1.96	0.50
(Vehicle used for grain)	2.29	2.00	2.60
(Vehicle used for other purposes than new straw or grain)	0.97	0.84	0.98
(Trailer used for new straw)	0.41	0.33	0.56
(Trailer used for grain)	1.41	1.35	1.67
(Trailer used for other purposes than new straw or grain)	0.92	0.86	1.00
Vehicles/trailers are cleaned after cleaning out	- ³	-	-
(Only vehicles are cleaned after cleaning out)	0.70	1.19	0.62
(Only trailers are cleaned after cleaning out)	None ⁴	None	None
(Both vehicles and trailers are cleaned after cleaning out)	1.43	0.84	1.62
Vehicles/trailers are disinfected after cleaning out	1.20	1.22	0.92
(Only vehicles are disinfected after cleaning out)	0.57	1.30	0.43
(Only trailers are disinfected after cleaning out)	1.52	1.11	None
(Both vehicles and trailers are disinfected after cleaning out)	1.20	0.79	2.33
Indoor factors, inside the broiler house, materials of the house			
Floor cracks ever repaired ⁵	3.04	4.05	1.50
(Floor cracks repaired in the preceding 12 months)	0.95	1.21	1.50
Drain present	1.29	0.60	-
Washing water collected in tank	0.74	0.56	1.59
Concrete walls	0.98	0.83	0.86
Plastered walls	0.86	1.14	0.54
Wall materials other than concrete or plastered	0.85	0.88	1.48
Visible beams	0.52	0.91	0.17
(Wooden beams)	0.97	0.95	0.52
(Steel beams)	0.83	0.93	1.21
(Clean ledges observed at visit)	1.00	1.11	1.74
Wooden gates	0.74	0.73	0.90
Metal gates	1.03	1.22	0.71
Hoisting gates	0.99	1.09	0.89
Swing gates	1.01	0.92	1.12
Ceiling covered by a cleanable material	1.05	1.18	1.22
Ceilings covered by armoured plastic	0.63	0.93	0.34

Continued

*Chapter 7- Impact of cleanability, cleaning and disinfection on
persistence of S. Enteritidis and S. Typhimurium in Danish broiler houses*

Table 7.4 (continued)

Category (in bold) and definition of dichotomous variable	Relative risk of multiple-infection house if "Yes"		
	General	SE8	ST66
Indoor factors, inside the broiler house, equipment			
Clean heating pipes observed at visit	0.42	1.83	0
Outlet chimneys washed in all download periods	0.92	0.90	1.35
Outlet chimneys washed in some download periods	0.90	1.03	0.58
Outlet chimneys never washed in download periods	1.34	1.11	1.42
Clean outlet chimneys observed at visit	1.59	1.18	2.89
Hole in the bottom of ventilators	0.64	1.38	0.12
Low-pressure ventilation system	4.77	-	3.00
("Plug holes" in air inlets)	1.49	1.55	1.09
(Clean air inlets observed at visit) ⁵	2.2	1.17	5.63
Equal-pressure ventilation system	0.21	0	0.33
Chain feeding system	1.42	1.01	2.25
(Clean chain feeding system observed at visit)	1.33	1.07	2.75
Pan feeding system	0.70	0.99	0.44
Nipple drinkers	1.20	1.26	1.29
(With water cups under the nipples)	1.40	1.20	0.92
(Acids ever used for decalcification of water cups)	1.22	1.64	2.10
Bell drinkers	0.93	0.98	0.78
Indoor factors, inside the broiler house, cleaning and disinfection			
Contractor performs cleaning of the broiler house	0.88	1.51	0
Only cold water used for cleaning	0.81	0.79	1.13
Detergent used for cleaning, in some or all download periods	1.20	1.07	1.23
Outer feed systems cleaned, in some or all download periods	1.93	1.74	3.78
Outer feed systems disinfected, in some or all download periods	1.67	None	1.50
The feed system or part of it is dismantled before cleansing	1.19	1.25	0
Disinfectants used in water systems in all download periods	0.91	1.51	0.41
(Acids used in download periods)	1.16	1.14	1.75
(Disinfectant other than acids used in download periods)	1.01	1.14	0.83
(Acids and other disinfectants are used in each download period)	1.39	1.35	2.08
Outer platforms disinfected	1.80	2.33	1.21
Formaldehyde disinfection, in some or all download periods	0.49	0.66	0
Glutaraldehyde disinfection, in some or all download periods	1.17	1.33	0.89
Lime disinfection, in some or all download periods	1.23	1.16	0.89
Oxidising disinfection, in some or all download periods	1.50	1.44	1.42
More than one disinfectant, in some or all download periods	0.94	1.14	0.49
Only surface disinfection used regularly	1.72	1.67	1.00
Only fogging used regularly	1.07	0.80	3.14
Both surface disinfection and fogging used regularly ⁵	0.17	0.82	0
Special measures taken after <i>Salmonella</i> in the preceding crop(s)	0.79	0.93	1.05

¹ In bold if $p < 0.05$, not in bold if $p \geq 0.05$. ² Variables in brackets were only answered in case of "yes" to the immediately preceding variable that was not in brackets. ³ Could not be calculated (0 in the denominator).

⁴ Only one answer given. ⁵ Included in PROC GENMOD (see Table 7.5).

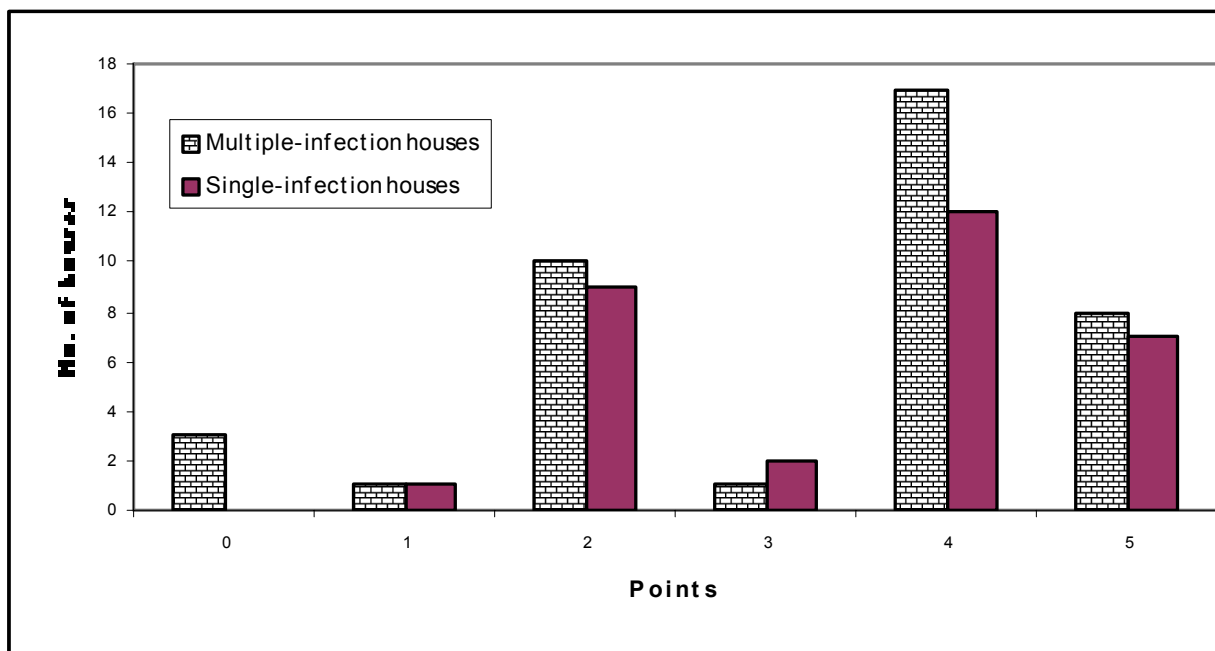


Figure 7.1: Points given for use, cleaning, and disinfection of vehicles and trailers used for cleaning out, and association to numbers of multiple-infection and single-infection houses.

Legends:

Use, cleaning, and disinfection of vehicle/trailer	Points
• Vehicle and trailer are only used for cleaning out	5
• Vehicle and/or trailer are/is used for other purposes than cleaning out, and if this is the case the vehicle/trailer is/are cleaned and disinfected	4
• Vehicle and trailer are used for other purposes than cleaning out. Both are cleaned, but only one of them is disinfected	3
• Vehicle and/or trailer are/is used for other purposes than cleaning out, and if this is the case the vehicle/trailer is/are cleaned, but not disinfected	2
• Vehicle and trailer are used for other purposes than cleaning out. Only vehicle or trailer is cleaned, and neither of them is disinfected.	1
• Vehicle and/or trailer are/is used for other purposes than cleaning out, and if this is the case the vehicle/trailer is/are neither cleaned nor disinfected	0

Very few factors related to house materials or equipment seemed to influence the house status. The repair of floor cracks increased the likelihood of becoming a multiple-infection house, but it is important here to distinguish between cause and effect, and a more important parameter was the repair of floor cracks within the last year, which did not influence house status. Apparently, the type of ventilation system seemed to influence the house status, but here a clustering effect could not be ruled out, as the seven single-

infection ST66-houses on the outlier farm (cf. Table 7.1) all had equal-pressure ventilation system (which only three other broiler houses had). Clean air inlets were associated with increased risk of general multiple-infection houses. Because clean conditions illogically were related to increased risk of multiple-infection status and the cleanliness of other critical control points was not associated with house status, this result was probably less important.

Cleaning procedures did not seem to influence the house status. In 59/75 broiler houses, only cold water was used for washing, whereas 43/74 used detergent, either for all the washing or only for the equipment.

A combined surface and fogging disinfection decreased the risk of becoming a multiple-infection house. However, the aforementioned outlier farm used both these disinfection methods in all their broiler houses, and this was only done in five other houses, so a clustering effect could not be ruled out.

The serotype *per se* was significantly associated with house status (data not shown), so interaction with and confounding from the serotype was investigated (cf. Table 7.5).

Table 7.5: Results from PROC GENMOD model investigating interactions with serotype and confounding from the serotype.

Definition of dichotomous variable	p for interaction parameter	Odds ratio without interaction parameter ¹	Odds ratio without interaction parameter and serotype ¹
Floor cracks ever repaired	1.00	0.39	0.21
Clean air inlets observed at visit	0.29	0.37	0.27
Both surface disinfection and fogging	- ²	6.82	9.03

¹ Odds ratios calculated for "yes=1" versus "no=0".

² Not calculated due to zero in one interaction cell.

There was no interaction ($p \geq 0.05$), and the change in odds ratio when the serotype was removed from the model was not alarming.

7.4 Discussion

In this study, the same person was mainly responsible for dealing with the data and he visited all farms, and this should make the data collection and interpretation more uniform. However, it might have been a disadvantage that the visitor knew the *Salmonella* status before the visits; this could bias the data collection (especially the subjective ones). Many factors which could be associated with each other were recorded - we could not know if the factor itself or another “hidden” factor was associated with the outcome. The serotype could be an important confounder or interaction factor, so this was investigated in depth. All interaction p were > 0.05 , and the changes in odds ratios were generally not alarming - but because there are no exact limits, this is open for discussion. Moreover, the inclusion of many factors (also such that were not linked to the topic of this chapter) increased the chance of obtaining coincidental statistical results, even if these were not biologically relevant. Nevertheless, many factors which were not considered very relevant biologically were chosen for investigation, because if many “non-relevant” factors were non-significant this would confirm our “comfort” with statistical highlighting of “more biological” factors. We were reassured to see that most significant factors were related to the environment closest to the *Salmonella* - whereas virtually no significant factors were found for the outdoor conditions (data not shown). The numbers of farms and broiler houses were relatively low (especially when the tests were performed separately for the serotypes); this means that statistical non-significance should be interpreted with caution as relevant factors might have been missed.

Surprisingly, whether or not vehicles used were used for cleaning out, and whether or not these vehicles were subsequently cleaned and disinfected, had no effect on house status. This, combined with the fact that no cross-infection was observed in other broiler

houses on the farms, strengthens the impression that persistence is a house-related phenomenon, a tendency confirmed in other studies (Higgins *et al.* 1982; Lahellec *et al.* 1986; Opitz 1992). Moreover, it is apparently difficult to transfer *Salmonella* by vehicles, at least in a concentration so it is detected in other broiler houses. There may be many reasons for this, but studies have shown that the *Salmonella* level in manure declines towards the end of the rearing period (Turnbull and Snoeyenbos 1973; Gradel *et al.* 2002), and this will minimise the risk of transfer by vehicles used for removing manure.

Cleanliness (measured at various critical control points) was not associated with house status. This could be for several reasons, including that cleaning is less important for the elimination of *Salmonella* than is claimed in various guidelines. Another reason could be that the chosen critical control points were not representative of the general cleanliness. However, we speculate that the cleanliness in most Danish broiler houses (both those in this study and others) is so good, that the little variation seen had no influence on the ability to eliminate *Salmonella* infections. Other studies have related *Salmonella* persistence to poor cleaning of the broiler houses (Higgins *et al.* 1982; Davies and Wray 1995c) or other types of poultry houses (Opitz 1992; Davies and Wray 1995c, 1996b; Ruckaberle *et al.* 1999). Two studies found that contractors were better at cleaning poultry houses than the farmers themselves (Davies and Wray 1995c; Rose *et al.* 2000); this tendency was not confirmed in our study.

No disinfectants were superior to others, in contrast to observations made in other studies where formaldehyde was superior to glutaraldehyde (Davies and Wray 1995c; Davies *et al.* 1998b). Rose *et al.* (2000) found that *Salmonella* persistence increased as the number of disinfections (2, 1 or 0) decreased - a result comparable to ours.

Chapter 7- Impact of cleanability, cleaning and disinfection on persistence of *S. Enteritidis* and *S. Typhimurium* in Danish broiler houses

In conclusion, this study suggests that the type of house materials, equipment or cleaning procedures do not seem to influence the persistence of *Salmonella* in broiler houses. The type of disinfectant, or its application either for surface disinfection or fogging did not seem to influence the house status either, whereas a combined surface and fogging disinfection reduced the risk of becoming a multiple-infection house.

Chapter 8 - Worst-case scenario

laboratory heating studies with

Salmonella* spp. and *Escherichia coli

8.1 Introduction

Guidelines on cleaning and disinfection are based on the assumption that all organic matter can be removed by cleaning, and that the subsequent chemical disinfection will cover all surfaces. This ideal situation is rarely achieved in poultry houses (cf. Section 4.4). Most of the present *Salmonella* occurrence in the Danish table egg sector is due to persistently infected layer houses, mostly with *S. Enteritidis*, phage type 8 (SE8). This *Salmonella* type often came with chickens delivered by one hatchery that hatches most of the Leghorn chickens for the Danish farmers. As this breed is best suited to be housed in battery cages, the occurrence of SE8 is over-represented in these systems, which are also the most difficult to clean and disinfect properly due to their complex inaccessible equipment.

Thus, there is an urgent need to investigate disinfection methods that will be effective in animal houses with organic matter, and which can be evenly distributed by an automatic method. Heat rather than chemical disinfection of contaminated items is recommended whenever possible (Russell 1999c). If heat can be evenly distributed in poultry houses, and if it can penetrate organic matter without damaging materials, it may be useful for dealing with persistent *Salmonella* infections. Therefore, a heat research project was implemented under the Danish *Salmonella* Control Programme. The project was divided into phase I laboratory tests and phase II field tests.

This chapter describes the laboratory heating tests in which worst-case scenarios found in badly cleaned poultry houses with high amounts of stressed *Salmonella* were used to determine a heating scheme which could be realistically applied in field tests. *E. coli* was used as a possible indicator bacterium that occurs in higher numbers than *Salmonella* in poultry houses, and might be used to verify the effectiveness of the treatment against *Salmonella*.

The corresponding field tests are described in Chapter 9.

8.2 Materials and methods

8.2.1 *Salmonella* test isolates

All test isolates (see Tables 6.2 and 8.1) were field isolates from persistently infected Danish poultry houses. They were stored as described in Section 6.6.

Rifampicin-resistant isolates were used, cf. Section 6.10.

8.2.2 Heating in broth and enumeration using plate counts

These tests were performed to select the most heat resistant among the three most common *Salmonella* (*S.*) Enteritidis phage types (4, 6 and 8) in Denmark, and to see if rifampicin-resistance induced in the laboratory altered the heat resistance of three *Salmonella* serotypes. A description is given in Section 6.11 and Appendix A.

8.2.3 Heat survival investigations that mimicked worst case scenarios

8.2.3.1 Principle

A full factorial design with different factors was used (Table 8.1):

Table 8.1: Factors investigated in worst-case scenario heating tests.

Factor	Reason(s) for inclusion in study
Test material (Feed – Faeces)	It is difficult to mimic the composition of organic matter found in poultry houses, but feed represents the input and the highest amounts of fats, carbohydrates and proteins (that protect bacteria against detrimental conditions), whereas faeces represent the output of these components. On the other hand, faeces will theoretically have the highest numbers of bacteria found in the poultry house.
<i>Salmonella</i> type (<i>S. Enteritidis</i> , phage type 8 – <i>S. Typhimurium</i> , definitive- type 110 – <i>S. Infantis</i>)	<i>S. Enteritidis</i> , PT8 (SE8), is the most common <i>Salmonella</i> type in persistently <i>Salmonella</i> -infected Danish layer houses. The two other types were included to see the impact of <i>Salmonella</i> type on heat resistance.
Drying prior to heating (Yes – No)	Theoretically, drying reduces the number of bacteria, but also renders the surviving bacteria more heat resistant. These factors illustrate dry vs. wet cleaning of the poultry house prior to the heat treatment.
Heating rate (1 °C h ⁻¹)	A slow heating rate gives the bacteria ample opportunities to adapt to the higher temperatures, amongst other things by producing heat stress proteins.
Relative air humidity during heating (Low (16-30%) – High (100%))	Moist heat is considered to be more detrimental to bacteria than dry heat.
Final heating temperature (50 – 55 – 60 – 65 – 70 °C)	These temperatures can realistically be achieved in poultry houses, while minimising the risk of damaging equipment.

The tests were performed in series, each with one relative air humidity during heating and one final temperature.

Supplementary tests were carried out with one isolate of *Salmonella* using egg yolks and finely ground feed as well as faeces and pelleted feed (cf. Section 8.2.3.4).

8.2.3.2 Test materials

Fresh faeces were taken at least once a month from the same battery cage house with droppings belts, and from where *Salmonella* had never been detected culturally or serologically. The faeces were stored at 5 °C in tightly closed plastic bags. Pelleted full feed for egg layers (code no. 3103, free from antibiotics and coccidiostatics) was supplied by Korn-

og Foderstofkompagniet A/S (Havnen 2, 6440 Augustenborg, Denmark). During the replicate tests (cf. Section 8.2.3.4) some additional materials were examined. Pelleted feed crumbled in a sterile mortar was used to see if feed in a more compact form would alter the results. Egg yolks, two per beaker, were used as a fourth substrate. The replicate test was repeated four times for each type of material.

8.2.3.3 Test protocol

Thirty (± 1) g of test material were put in 5.0 cm diameter beakers, yielding a layer 2-2.5 cm thick. Presence of naturally occurring *E. coli* in each beaker of faeces was checked by streaking onto MacConkey agar (cf. Section 8.2.3.4), and all the beakers were inoculated with one of the three isolates of rifampicin-resistant *Salmonella* (cf. Table 8.1).

The beakers containing faeces and feed were put in a climate chamber set to 20 (± 0.5) °C and 30 (± 3)% relative humidity (RH) for 10 days. Lids were put on half the beakers, and the other half were left open to equilibrate to the 30% RH, which caused them to dry. All series were run at 30 (± 3)% RH, but additional series were run with deviating RH due to a defect in the climate chamber (cf. Table 8.2).

After exactly 10 days, some samples (“10-day samples”) were weighed and examined for numbers and presence/absence of *Salmonella*. Moreover, faecal samples were examined for presence/absence of *E. coli*. The remaining beakers were transferred to a cooled incubator for the heat tests after removing the lids from the non-dried samples (see Figure 8.1). The PC-software programme Apt-Com, version 1.0 (Binder GmbH, 78532 Tuttlingen, Germany) was used to increase the temperature from the initial 20 °C to the final heating temperature at 1 °C h⁻¹. The RH and temperature inside the incubator were monitored continuously at 5 min intervals. In order to achieve an atmosphere of 100% RH,

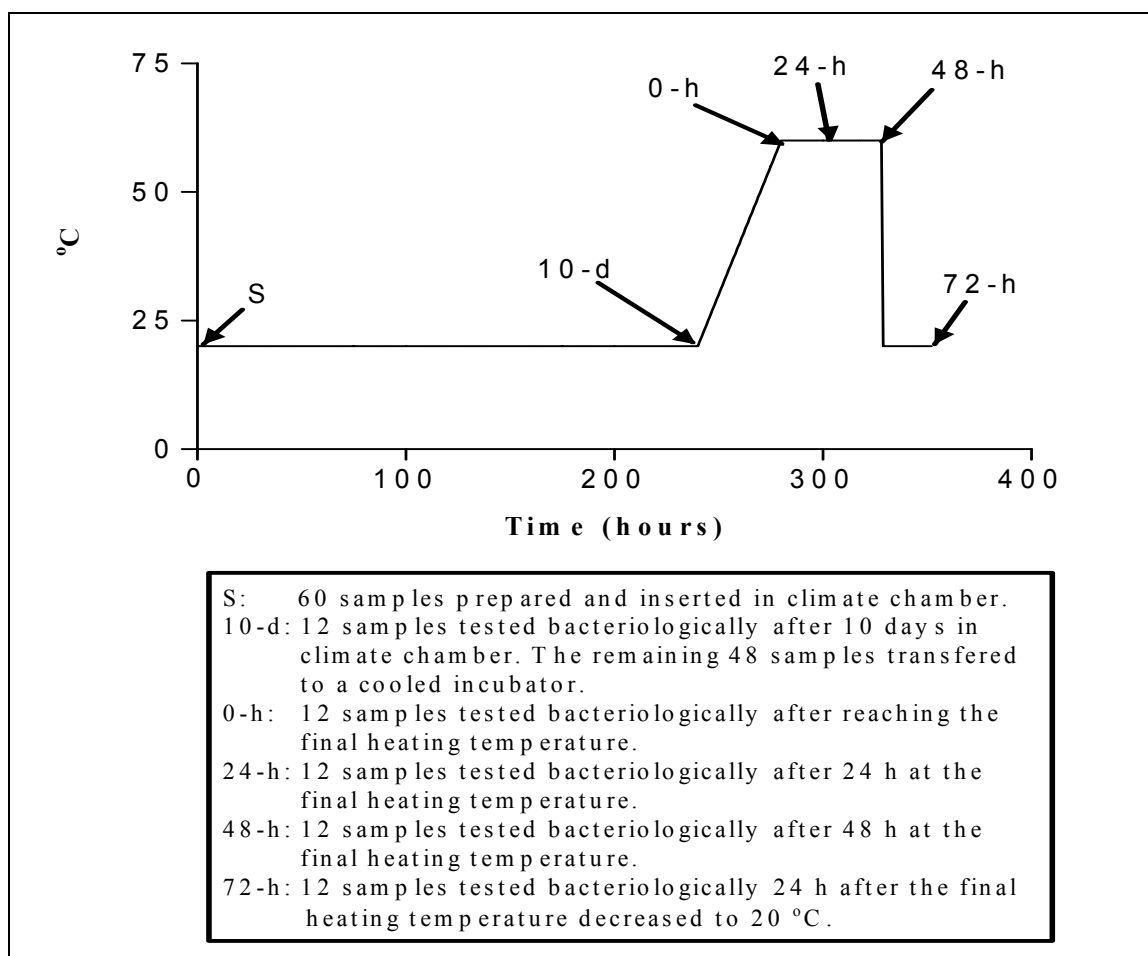


Figure 8.1: Time schedule and samples for a heating series, here with a final heating temperature of 60 °C.

some samples were sealed individually in plastic boxes with water in the bottom. The RH in these boxes reached 100% within about half an hour. Heating was continued for 48 h, after which 20 °C was maintained for a further 24 h. Samples were weighed and examined as the “10-day samples” (see above) as soon as they reached the final heating temperature, after 24 and 48 h of heating, and 24 h after heating had ceased.

Replications of the above procedures were not performed, so when a satisfactory temperature-humidity-time combination was found the experiment was repeated, but with some modifications: only dried samples were used (no samples were lidded during the initial 10-day equilibration period), quantitative tests were omitted, and only one serotype

(SE8) was used, as the results were very similar for the three isolates. Checks for surviving *Salmonella* were more rigorous after replicate testing (cf. Section 8.2.3.4). Tests were carried out four times in each of the test materials (pelleted and ground feed, faeces and egg yolk).

8.2.3.4 Microbiological procedures

An overview of the microbiological procedures is given in Figure 8.2.

Cultures from BA plates were incubated in VIB at 37° C until stationary phase. Numbers of CFU were determined as described in Section 6.11. Three ml of the 10⁻² dilution were added to the test material in each beaker, mixing immediately with a sterile spoon, after which the samples were incubated as described above.

After incubation, 0.3-0.4 g (faecal samples) or ca. 1.0 g (feed samples) were taken from the middle of each sample and examined for numbers of CFU of *Salmonella* as described in Section 6.11, except that the Rambach agar contained 50 µg ml⁻¹ rifampicin. The remaining test materials were examined using a routine presence/absence test, cf. Section 6.7.1. Results from Rambach agar plates were recorded for both *Salmonella* and non-*Salmonella* colonies, i.e. if Rambach plates were sterile or not. In addition, randomly selected *Salmonella*-positive colonies were checked for rifampicin-resistance using Neo-Sensitab rifampicin tests as described in Section 6.10. To check for presence/absence of *E. coli* in faecal samples, the BPW pre-enrichment used to inoculate RVS was streaked on MacConkey agar, cf. Section 6.8. MacConkey agar was also used to check that faecal

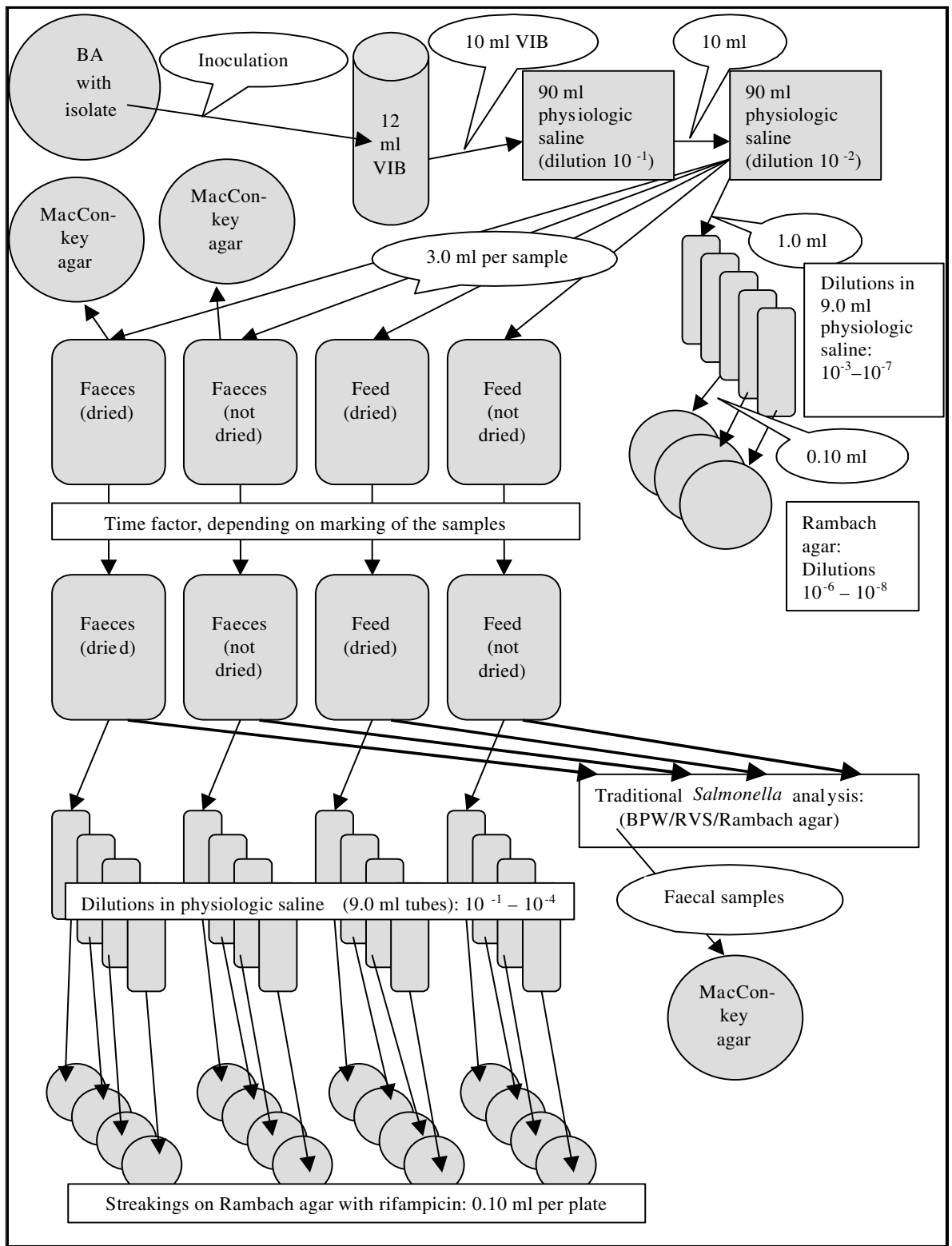


Figure 8.2: Overview of microbiological procedures (cf. text).

samples contained *E. coli* before the experiment started.

Extra *Salmonella* checks in replicate tests: DIASALM and MSRV agar were all inoculated from the original BPW pre-enrichment, as well as RVS, plating onto Rambach agar. RVS was streaked onto Rambach agar after both 18-24 and 42-48 h of incubation.

8.2.3.5 Statistical analysis

All data were subjected to an Access database (Anonymous 1997a) and an Excel spreadsheet for calculations (Anonymous 1997b). Confidence intervals (95%) were calculated for heating in VIB, but not for solids, as no replicate tests were made, and a Poisson distribution was not expected in these. Student's t-test with different variances ($\alpha=0.05$, two-sided p) was used to compare log-numbers of CFU between dried and non-dried samples and between the isolates. Association between occurrence of *Salmonella* and *E. coli* was assessed by Cohen's Kappa (Sackett 1992), McNemar chi-square test (5% significance levels) (Martin *et al.* 1987) and negative predictive value (Toma *et al.* 1999).

8.3 Results

8.3.1 Heating in broth and enumeration using plate counts

Appendix A shows that rifampicin resistance did not alter the heat resistance of the isolates (Figures A1-A7) and that *S. Enteritidis*, phage type 8, was no less heat resistant than phage types 4 and 6 (Figures A8-A9); hence, SE8 and rifampicin resistant isolates could be used in the proper heating studies.

8.3.2 Heat survival investigations that mimicked worst case scenarios

8.3.2.1 Weight loss of samples

Faeces and feed were 20.0-34.2% and 83.5-87.9%, respectively, of their original weight after equilibration (drying) for 10 days. Faeces and feed that were lidded for 10 days were 88.6-98.7% and 94.9-99.5%, respectively, of their original weight indicating that the lids on the beakers were not completely tight.

8.3.2.2 Initial inoculum of *Salmonella*

Numbers of CFU of *Salmonella* ml⁻¹ VIB used for inoculation were in the range 1.9x10⁸ -3.1x10⁹, thus initially there was 2x10⁵ - 3x10⁶ CFU g⁻¹ organic matter.

8.3.2.3 Survival during heating

Results are shown in Table 8.2.

In faeces, very few or no *Salmonella* colonies were detected by direct plating of faeces (data not shown), although enrichment frequently gave positive results. At low humidity, survivors were found in dried faeces at every sampling time and every final heating temperature. In the undried faeces exposed to low humidity and in both the dried and undried faeces exposed to 100% RH, *Salmonella* was not detected after 24 h at 55°C and above.

In feed, *Salmonella* survived in both dried and undried feed at low humidity, regardless of the final heating temperature. Heating at 100% RH resulted in all *Salmonella* samples testing negative after 24 h at 60 °C and above; at all temperatures up to 65 °C, *Salmonella* died more rapidly in undried than in dried feed, whereas no difference was seen at 70 °C. *Salmonella* was frequently detected by direct plating. At low humidity, *Salmonella* was often detected by direct plating at all stages, albeit in low numbers after 24 h and beyond. There were big quantitative variations between series and samples, but some general trends

Table 8.2: Survival of Salmonella in experiments at five final heating temperatures (50, 55, 60, 65 or 70 °C) (TEMP) using faeces or feed as organic matter (ORG), three serotypes (S. Enteritidis, phage type 8 (SE8), S. Typhimurium, definitive-type 110 (ST110), S. Infantis (Inf)) (TYPE), with samples dried (RH sometimes deviated from 30%) or not dried in a 10-day period prior to the final heat treatment (DRD).

TEMP (°C)	ORG	TYPE	DRD	RH(SD) ¹	Heating at 16-30% RH					Heating at 100% RH				
					10d ²	0 ²	24 ²	48 ²	72 ²	10d	0	24	48	72
50	Faeces	SE8	Yes	30 (0.2)	- ³	+ ⁴	+	+	+	+	-	-	-	-
50	Faeces	SE8	Yes	39 (3.12)	ND ⁵	ND	ND	ND	ND	-	+	+	-	-
50	Faeces	SE8	Yes	50 (27.8)	ND	ND	ND	ND	ND	+	-	-	-	-
50	Faeces	SE8	No		+	-	-	-	-	+	-	-	-	-
50	Faeces	ST110	Yes	30 (0.2)	+	+	+	+	+	+	+	-	-	-
50	Faeces	ST110	Yes	39 (3.12)	ND	ND	ND	ND	ND	+	+	+	-	-
50	Faeces	ST110	Yes	50 (27.8)	ND	ND	ND	ND	ND	+	+	-	-	-
50	Faeces	ST110	No		+	-	-	-	-	+	-	-	-	-
50	Faeces	Inf	Yes	30 (0.2)	+	+	+	+	+	+	+	-	-	-
50	Faeces	Inf	Yes	39 (3.12)	ND	ND	ND	ND	ND	+	+	-	-	-
50	Faeces	Inf	Yes	50 (27.8)	ND	ND	ND	ND	ND	+	+	-	-	-
50	Faeces	Inf	No		+	-	-	-	-	+	-	-	-	-
50	Feed	SE8	Yes	30 (0.2)	2.8 ⁶	2.3	0.8	0.6	+	11.0	3.0	1.3	+	+
50	Feed	SE8	Yes	39 (3.12)	ND	ND	ND	ND	ND	2.0	0.2	+	+	+
50	Feed	SE8	Yes	50 (27.8)	ND	ND	ND	ND	ND	0.8	+	+	+	-
50	Feed	SE8	No		5.8	0.8	0.6	0.6	+	0.9	+	+	-	-
50	Feed	ST110	Yes	30 (0.2)	5.3	1.6	0.9	0.5	0.6	8.7	2.1	1.0	1.0	+
50	Feed	ST110	Yes	39 (3.12)	ND	ND	ND	ND	ND	1.5	0.4	+	+	+
50	Feed	ST110	Yes	50 (27.8)	ND	ND	ND	ND	ND	+ ⁷	+ ⁷	+	+	-
50	Feed	ST110	No		10.5	1.0	0.8	+	+	+	+	+	-	-
50	Feed	Inf	Yes	30 (0.2)	19.7	13.4	3.3	2.9	3.0	6.0	1.3	0.8	+	+
50	Feed	Inf	Yes	39 (3.12)	ND	ND	ND	ND	ND	1.4	0.3	+	+	+
50	Feed	Inf	Yes	50 (27.8)	ND	ND	ND	ND	ND	0.5	0.1	+	+	-
50	Feed	Inf	No		17.4	2.7	2.3	2.5	2.6	4.2	0.6	+	-	-
55	Faeces	SE8	Yes	30 (0.2)	+	+	+	+	+	+	-	-	-	-
55	Faeces	SE8	Yes	33 (2.7)	ND	ND	ND	ND	ND	+	+	-	-	-
55	Faeces	SE8	Yes	41 (20.9)	ND	ND	ND	ND	ND	+	+	-	-	-
55	Faeces	SE8	No		+	-	-	-	-	+	-	-	-	-
55	Faeces	ST110	Yes	30 (0.2)	+	+	+	+	+	+	-	-	-	-
55	Faeces	ST110	Yes	33 (2.7)	ND	ND	ND	ND	ND	+	+	-	-	-
55	Faeces	ST110	Yes	41 (20.9)	ND	ND	ND	ND	ND	+	+	-	-	-
55	Faeces	ST110	No		+	-	-	-	-	+	-	-	-	-
55	Faeces	Inf	Yes	30 (0.2)	+	+	+	+	+	+	-	-	-	-
55	Faeces	Inf	Yes	33 (2.7)	ND	ND	ND	ND	ND	+	+	-	-	-
55	Faeces	Inf	Yes	41 (20.9)	ND	ND	ND	ND	ND	+	+	-	-	-
55	Faeces	Inf	No		+	-	-	-	-	+	-	-	-	-
55	Feed	SE8	Yes	30 (0.2)	2.4	0.5	0.7	+	0.3	1.7	+	+	-	-
55	Feed	SE8	Yes	33 (2.7)	ND	ND	ND	ND	ND	1.7	+	+	-	-
55	Feed	SE8	Yes	41 (20.9)	ND	ND	ND	ND	ND	19.5	3.0	+	+	+
55	Feed	SE8	No		5.9	0.3	0.5	0.2	0.2	28.3	+	-	-	-
55	Feed	ST110	Yes	30 (0.2)	3.0	2.1	1.1	0.5	0.3	1.8	0.4	+	-	-
55	Feed	ST110	Yes	33 (2.7)	ND	ND	ND	ND	ND	1.7	0.2	+	-	-
55	Feed	ST110	Yes	41 (20.9)	ND	ND	ND	ND	ND	8.1	1.2	+	+	-
55	Feed	ST110	No		10.3	1.8	1.4	1.3	1.2	19.1	+	-	-	-
55	Feed	Inf	Yes	30 (0.2)	6.2	6.1	1.7	1.2	1.7	2.7	0.2	+	-	-
55	Feed	Inf	Yes	33 (2.7)	ND	ND	ND	ND	ND	3.2	0.7	+	-	-
55	Feed	Inf	Yes	41 (20.9)	ND	ND	ND	ND	ND	6.6	0.9	+	+	-
55	Feed	Inf	No		29.1	3.0	2.7	2.6	2.4	31.2	+	-	-	-

Continued

*Chapter 8 – Worst-case scenario laboratory heating
studies with Salmonella spp. and Escherichia coli*

Table 8.2 (continued)

TEMP (°C)	ORG	TYPE	DRD	RH(SD) ¹	Heating at 16-30% RH					Heating at 100% RH				
					10d ²	0 ²	24 ²	48 ²	72 ²	10d	0	24	48	72
60	Faeces	SE8	Yes	30 (0.2)	+	+	+	+	+	+	-	-	-	-
60	Faeces	SE8	Yes	33 (2.9)	-	-	+	+	+	+	-	-	-	-
60	Faeces	SE8	No		+	-	-	-	-	+	-	-	-	-
60	Faeces	ST110	Yes	30 (0.2)	+	+	+	+	+	+	-	-	-	-
60	Faeces	ST110	Yes	33 (2.9)	+	+	+	+	+	+	-	-	-	-
60	Faeces	ST110	No		+	-	-	-	-	+	-	-	-	-
60	Faeces	Inf	Yes	30 (0.2)	+	+	+	+	+	+	-	-	-	-
60	Faeces	Inf	Yes	33 (2.9)	+	+	+	-	+	+	-	-	-	-
60	Faeces	Inf	No		+	-	-	-	-	+	-	-	-	-
60	Feed	SE8	Yes	30 (0.2)	2.2	1.1	0.2	0.3	0.3	1.1	+	-	-	-
60	Feed	SE8	Yes	33 (2.9)	0.1	0.1	0.1	0.1	+	0.2	+	-	-	-
60	Feed	SE8	No		1.2	+	+	+	+	3.0	-	-	-	-
60	Feed	ST110	Yes	30 (0.2)	2.6	1.1	0.7	0.7	0.8	1.7	+	-	-	-
60	Feed	ST110	Yes	33 (2.9)	0.1	0.1	+	0.1	+	0.1	+	-	-	-
60	Feed	ST110	No		0.6	0.1	+	0.1	0.1	2.5	+	-	-	-
60	Feed	Inf	Yes	30 (0.2)	6.2	1.6	1.2	1.1	1.1	6.4	1.0	-	-	-
60	Feed	Inf	Yes	33 (2.9)	0.2	0.2	0.1	0.1	+	0.4	+	-	-	-
60	Feed	Inf	No		3.3	0.6	+	+	+	4.4	-	-	-	-
65	Faeces	SE8	Yes	30 (0.2)	+	-	+	+	+	+	-	-	-	-
65	Faeces	SE8	No		-	-	-	-	-	+	-	-	-	-
65	Faeces	ST110	Yes	30 (0.2)	+	+	+	+	+	+	-	-	-	-
65	Faeces	ST110	No		+	-	-	-	-	+	-	-	-	-
65	Faeces	Inf	Yes	30 (0.2)	+	+	+	+	+	+	-	-	-	-
65	Faeces	Inf	No		+	-	-	-	-	+	-	-	-	-
65	Feed	SE8	Yes	30 (0.2)	3.0	0.3	0.2	+	+	0.5	+	-	-	-
65	Feed	SE8	No		9.1	1.0	0.9	+	+	3.6	-	-	-	-
65	Feed	ST110	Yes	30 (0.2)	3.0	1.1	0.7	0.7	+	1.7	+	-	-	-
65	Feed	ST110	No		5.9	1.1	0.9	+	+	5.7	-	-	-	-
65	Feed	Inf	Yes	30 (0.2)	3.0	2.9	0.7	0.6	0.6	2.4	+	-	-	-
65	Feed	Inf	No		16.0	2.2	1.5	1.4	1.3	8.5	-	-	-	-
70	Faeces	SE8	Yes	30 (0.2)	+	+	+	+	+	+	-	-	-	-
70	Faeces	SE8	Yes	31 (1.9)	ND	ND	ND	ND	ND	+	-	-	-	-
70	Faeces	SE8	No		-	-	-	-	-	-	-	-	-	-
70	Faeces	ST110	Yes	30 (0.2)	+	+	+	+	+	+	-	-	-	-
70	Faeces	ST110	Yes	31 (1.9)	ND	ND	ND	ND	ND	+	-	-	-	-
70	Faeces	ST110	No		+	-	-	-	-	+	-	-	-	-
70	Faeces	Inf	Yes	30 (0.2)	+	+	+	+	-	+	-	-	-	-
70	Faeces	Inf	Yes	31 (1.9)	ND	ND	ND	ND	ND	+	-	-	-	-
70	Faeces	Inf	No		+	-	-	-	-	+	-	-	-	-
70	Feed	SE8	Yes	30 (0.2)	1.4	0.3	+	+	+	4.4	-	-	-	-
70	Feed	SE8	Yes	31 (1.9)	ND	ND	ND	ND	ND	0.2	-	-	-	-
70	Feed	SE8	No		4.3	0.8	0.8	+	+	0.2	-	-	-	-
70	Feed	ST110	Yes	30 (0.2)	1.6	0.5	+	+	+	4.9	-	-	-	-
70	Feed	ST110	Yes	31 (1.9)	ND	ND	ND	ND	ND	0.8	-	-	-	-
70	Feed	ST110	No		5.2	0.7	0.7	+	+	1.0	-	-	-	-
70	Feed	Inf	Yes	30 (0.2)	4.6	0.5	0.4	0.3	+	5.8	-	-	-	-
70	Feed	Inf	Yes	31 (1.9)	ND	ND	ND	ND	ND	0.9	-	-	-	-
70	Feed	Inf	No		5.5	0.7	0.4	0.4	+	1.2	-	-	-	-

¹ Mean % relative humidity (standard deviation). ² 10d = 10-day samples, 0, 24, 48, 72 = 0-, 24-, 48-, and 72-hour samples, respectively (cf. Figure 8.1). ³ Salmonella not detected in qualitative or quantitative tests.

⁴ Salmonella detected in qualitative but not in quantitative tests. ⁵ Not done. ⁶ Percent surviving Salmonella in quantitative tests; all detected in qualitative tests. ⁷ Quantitative results not available, as the initial CFU g⁻¹ organic matter could not be calculated.

were observed. In the initial 10-day period, there was a significantly higher survival in undried than in dried samples ($p=0.024$), a tendency observed with each of the serotypes, though differences for each individually were not significant. Between 10-day and 0-hour samples (see Figure 8.1), there was often a bigger relative reduction in numbers of CFU in undried than in dried feed in spite of the higher 10-day survival in the former. Moreover, *S. Infantis* apparently survived better than the two other serotypes, though this difference was not significant. The quantitative results confirmed the qualitative ones, i.e. humidity during heating was an important factor in the elimination of *Salmonella*. In no case was *Salmonella* detected in quantitative tests without being found in the parallel qualitative ones. Numbers of *Salmonella* detected after the 10-day pre-treatment and beyond were too low to compare the heat resistance of the three serotypes.

In replicate tests (SE8 in four substrates, with four replicates, using predried samples and more rigorous tests for *Salmonella*), *Salmonella* was not detected in any 24-, 48- or 72-h sample in this experiment, regardless of the method of detection or type of test material (pelleted or ground feed, egg yolk or faeces). Thus, 60 °C and 100% RH during a 24-hour period was identified as a suitable standard to be applied in field studies.

E. coli was detected in all faecal samples prior to the testing. Table 8.3 shows the association between results for *Salmonella*, including Rambach agar plate readings, and *E. coli*. Kappa analysis showed a “substantial agreement” between *Salmonella* and *E. coli* results, whereas the agreement became “almost perfect” between non-*Salmonella* colonies and *E. coli* results. For the latter, we had McNemar $\chi^2 < 3.84$ (df=1), i.e. the two tests could statistically replace each other with 95% certainty. Moreover, for the *Salmonella* negative

samples McNemar χ^2 was still significant and there was a high negative predictive value (0.99), which indicated that *E. coli* results could statistically predict the *Salmonella* status.

Table 8.3: Results from all faecal samples for Escherichia (E.) coli readings on MacConkey agar and qualitative Salmonella experiments, including readings on Rambach agar.

<i>E. coli</i> detected on MacConkey agar?	<i>Salmonella</i> spp. detected on Rambach agar?				SUM
	Yes		No		
	Pure cul- ture of <i>Sal- monella</i>	<i>Salmonella</i> and non- <i>Salmonella</i>	Growth of non- <i>Salmonella</i>	Sterile	
Yes	8	90	10	8	116
No	40	8	3	222	273
SUM	48	98	13	230	389

General:

Kappa/McNemar values, respectively, for *E. coli* versus:

Salmonella: 0.62/12.74

Non-*Salmonella* colonies: 0.83/1.33

Sterile Rambach agar plates: -0.58/38.69

Negative predictive value (*E. coli* vs. *Salmonella*): 225/273 = 0.82

Only for *Salmonella* positive samples:

Kappa/McNemar values for *E. coli* versus only *Salmonella* colonies: - 0.59/20.0

Only for *Salmonella* negative samples:

Kappa/McNemar values for *E. coli* versus non-*Salmonella* colonies: 0.62/3.27

Negative predictive value: 222/225 = 0.99

Kappa values (Sackett 1992):

0.0-0.2, "slight"; 0.2-0.4, "fair"; 0.4-0.6, "moderate"; 0.6-0.8, "substantial"; 0.8-1.0, "almost perfect".

Positive values: "Agreement"; Negative values: "Disagreement".

8.4 Discussion

This study aimed at finding a temperature-humidity-time treatment that would be effective in eliminating *Salmonella* from the organic matter remaining in poultry houses after cleaning, but without damaging the fabric and equipment in the houses. The organic matter in a poultry house is difficult to characterise, but as feed and faeces represent the input and output, respectively, of fats, carbohydrates and proteins in the poultry house (i.e. components that protect bacteria from detrimental conditions such as heat), these test materials were chosen to represent organic matter. Previous studies have shown that organic

matter and low water content protect bacteria from heating, but it was not possible to predict from the literature exactly what treatment would be effective.

In order to test a "worst case scenario" stationary phase inocula (cf. Section 4.1.7) were allowed to adapt to the environment for 10 days before the heat treatment was applied, as various stress factors, e.g. drying, might induce regulatory stress protein systems that protect both against the inducing factor and other stresses, e.g. heat (cf. Section 3.3 and Figure 3.1). In addition, a very slow heating rate was applied (cf. Section 4.1.6).

Overall, the results show the important effect of humidity on heat resistance, both before and during the heat treatment (cf. Section 4.1.3).

Salmonella was more heat resistant in dried feed than dried faeces, which could indicate the protective effect of fats, carbohydrates and/or proteins in the former (cf. Section 4.1.4). Other studies have shown that increasing numbers and densities of bacteria might increase their heat resistance (cf. Section 4.1.7), and this would favour survival in faeces compared to feed, but in this study the presence of the above compounds seemed more important. There were no differences in heat resistance among the three serotypes, and this is in accordance with other studies where the heat resistance was influenced more by external factors than by the serotype *per se* (cf. Section 4.1.7).

In the quantitative studies, injured bacteria were streaked directly on a selective solid medium, which is not the most sensitive method (cf. Section 5.2.1), and no replicates were performed. However, the main purpose of the quantitative studies was to see if there were differences between serotypes, as no *Salmonella* was detected from quantitative tests without being detected in the qualitative ones. The better survival in non-dried than dried samples during the 10-day preheating period could be real, but it could also be due to higher numbers of injured bacteria from dried samples that could not grow on selective agar (cf.

Section 5.2.1). On the other hand, the reverse was seen during the heating where more bacteria were recovered from low humidity than from 100% RH series, and this is more likely to be a real difference. *S. Infantis* tended to have a higher survival during the 10-day pre-heating period than the two other serotypes both in dried and in non-dried samples. This is likely to be a real difference, as the proportion of injured cells probably does not differ between *Salmonella* serotypes. One explanation could be a better survival of *S. Infantis* in the extra-animal environment compared to the other two more invasive serotypes that are better adapted to the animal environment, but more research is needed on this topic.

The most likely explanation for the nearly complete failure to detect *Salmonella* in faecal samples in the quantitative tests is the high numbers of competitive flora. Faecal bacteria with faster generation times apparently outnumbered *Salmonella*, even with Ram-bach agar made selective with rifampicin.

Monitoring presence/absence of naturally-occurring *E. coli* showed that it could be used as a reliable and convenient indicator for the presence or absence of *Salmonella* after heat treatment, and that differences in susceptibility between naturally-occurring bacteria and laboratory isolates are probably negligible in situations that mimic field conditions. Enterobacteriaceae are used as indicator bacteria for identification of conditions which would allow survival of *Salmonella* in e.g. feed mills (Anonymous 2001), but few scientific publications have given statistical evidence for the use of these or related bacteria.

In conclusion, investigations that mimicked worst case scenarios encountered in persistently *Salmonella* infected empty poultry houses with a bad cleaning standard showed that 60 °C and 100% RH for 24 hours could eliminate high numbers of *Salmonella* and naturally-occurring *E. coli*.

Chapter 9 - Monitoring the efficacy of

steam and formaldehyde treatment of

naturally *Salmonella*-infected layer houses

9.1 Introduction

Results from the laboratory heating studies (cf. Chapter 8) showed that 60 °C and 100% RH for a 24-h period was effective under worst-case scenarios, and this was applied as the gold standard for the heating field studies reported in this chapter. In the laboratory, heat could not be combined with a chemical disinfectant because of working environmental problems. However, the inclusion of a chemical disinfectant will theoretically increase the efficacy of heat (cf. Section 4.3). Therefore, formaldehyde was added to the steam in some of the houses.

As there are no gold standards for disinfecting animal houses satisfactorily, and this was a field study without control houses and with many uncontrollable parameters, several monitoring methods were included.

Thus, the aim of this study was to monitor the effectiveness of steam heating of table egg layer houses naturally infected with *Salmonella*, aiming to maintain ≥ 60 °C and 100% RH during a 24-h period. Different methods were compared, such as steam with and without formaldehyde, shorter heating times, and chemical disinfection (applied both as surface disinfection and thermal fogging). In addition, various monitoring methods were applied to enhance the validation of the treatment procedures.

9.2 Materials and methods

9.2.1 Farms and houses

Danish commercial table egg layer premises with one or more naturally *Salmonella*-infected houses were invited to participate in the study. Altogether, four battery cage and two barn houses, distributed on five farms, were heat-treated (Table 9.1). Farm B had three *Salmonella*-infected barn houses, of which the first was heat-treated, the second surface disinfected, and the third thermally fogged. All houses in the study were treated in a down-draw period after cleaning but before any chemical disinfection. However, sodium hypochlorite was used in the washing water for the house on Farm D.

9.2.2 Treatment procedures

One company that has steam treated poultry houses for years was used for heating all the houses (cf. Section 2.4). A hose attached to a steam generator was inserted through an opening at one end of the house, e.g. through the door opening, which was then sealed tightly with plastic. All other openings, such as outlet chimneys and air inlets, were also sealed tightly to prevent outside air from being sucked into the house, as negative pressure was created during the steam treatment. Ancillary rooms were sometimes included in the heat treatment (cf. Section 9.2.4). The company estimated that ca. 1000-1500 L water h⁻¹ was used, but this probably varies with different external factors such as house size and season.

Different procedures were used between farms and houses (Table 9.1). Because of the large size of House D1, three steam hoses were used, one at each end of the house and one in the manure pit. In House C1, an extra hose was used for the ancillary rooms.

Table 9.1: Layer farms and houses studied, with summary of the disinfection treatments and microbiological checks.

Farm	House	House type	Treat- ment ¹	No. of Salmonella samples		Organic indicator samples ² used	Comments (treatment month and year)
				Before treatment	After treatment		
A	A1	Barn	Model 1	287	288	Yes ³	Pre-treatment samples taken ca. 1 day after cold water cleaning without detergent was finished (Aug. 2001).
	A2	Battery cage with droppings belts	Model 1	302	303	Yes ³	As for House A1.
	B1	Barn	Model 2	100	102	Yes	Pre-treatment samples taken ca. 7 days after hot water cleaning with detergent was finished. Slat, bell drinkers and nest mats were dismantled and soaked 24 h in 5% NaOH (Nov. 2001).
B	B2	Barn	Model 3	100	96 ⁴	No	As for House B1.
	B3	Barn	Model 4	100	100	No	As for House B1, except that detergent was not used.
	C1	Battery cage with droppings belts	Model 2	298	308	Yes	Pre-treatment samples taken ca. 1 day after hot water cleaning without detergent was finished. Droppings and egg belts were dismantled and soaked in 85 °C water (March 2002).
D	D1	Battery cage with droppings belts	Model 2	289	290	Yes	Pre-treatment samples taken ca. 5 days after cold water cleaning with detergent and sodium hypochlorite was finished. Egg belts were dismantled (April 2002).
	E1	Battery cage with droppings belts	Model 2	308	308	Yes	Pre-treatment samples taken ca. 3 days after cold water cleaning with detergent was finished (Nov. 2002).

¹ Model 1: 60 °C and 100% RH for 24 h without formaldehyde (Period I), followed by a short heating to ca. 60 °C with formaldehyde, 13 and 22 ppm in House A1 and A2, respectively (Period II). Model 2: 60 °C and 100% RH for 24 h with 30 ppm formaldehyde at the beginning of the process. Model 3: Thermal fogging (2.75 L Bio Komplet Plus (cf. Section 11.2.3), 0.28 L pH-regulator (KOH and H₃PO₄) and 5.5 L tap water was run for ca. 25 min. Model 4: Surface disinfection (using 15 L Bio Komplet Plus, 1.5 L pH-regulator, dissolved in 500 L tap water) that took ca. 3 h.

² Samples of feed inoculated with Enterococcus faecalis or Escherichia (E.) coli or faeces with naturally occurring enterococci and E. coli, placed next to temperature probes (cf. text).

³ Organic indicator samples placed both during Period I, Period II and Period I+II.

⁴ By a mistake, 4/100 samples were taken from the outside of air inlets (which were closed during the treatment).

Formaldehyde at 23.4% (v/v) was used for all relevant houses; a dose to yield 30 ppm formaldehyde was calculated from the volume to be heat-treated, i.e. it was not measured.

9.2.3 *Salmonella* sampling procedures

Generally, 300 samples were taken both before and after the treatment. However, due to limited resources, the 300 samples were dispersed around the three houses studied on Farm B (Table 9.1).

Sample sites were selected beforehand to provide an even “geographical” distribution in the house and on different types of equipment (Table 9.2). All sample sites were marked (including cages labelled with tape or clothes pegs) as precisely as possible before treatment, and sampling after treatment was made from the same sites. A few samples were taken from additional sites after treatment, which is the main reason for differing numbers of samples before and after treatment (Table 9.1). All battery cage houses had either five or six tiers, and the first, third and fifth/sixth tiers were used consistently for sampling; each tier was evenly divided into seven sample sites, including one at each end.

For all samples, beakers containing a 10x10 cm sterile gauze swab (Simonsen & Weel, 2630 Tåstrup, Denmark, catalogue no. 487-003) immersed in 100 ml BPW were used. Each sampling site was swabbed vigorously with the swab, which was then returned immediately to BPW in the beaker. Disposable gloves were used and changed between each sample. All samples were returned to the laboratory on the sampling day where they were either incubated directly or stored at 5 °C until incubation the next day. Thereafter, the procedures were as described in Section 6.7.1.

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Table 9.2: Before treatment: number of Salmonella-positive samples/total samples (%), distributed according to categories across houses.

Main category	Subcategory	Salmonella/total (%)	
Equipment, faeces	Cage wire floors	3/73	(4.1)
	Cage partitions	1/81	(1.2)
	Droppings belts/rollers	5/48	(10.4)
	Manure dryers	5/65	(7.7)
	Slats	0/6	(0)
Equipment, water	Nipple drinkers	3/68	(4.4)
	Drip channels	12/51	(23.5)
Equipment, feed	Feed chains	2/29	(6.9)
	Feed chain “corner” wheels	6/33	(18.2)
	Feed systems, miscellaneous	0/26	(0)
	Feed troughs, bends	1/48	(2.1)
	Feed troughs, surfaces	4/108	(3.7)
Equipment, eggs	Egg belt grills	0/12	(0)
	Egg belts	6/49	(12.2)
	Egg brushes	3/10	(30.0)
	Egg elevators	5/34	(14.7)
Equipment, various	Nests	4/69	(5.8)
	Wooden structures	0/7	(0)
	Miscellaneous	0/6	(0)
Surroundings, related to floors	Drains	6/23	(26.1)
	Floor, barn houses	23/168	(13.7)
	Floor, under cages	14/68	(20.6)
	Floor, walking area	15/62	(24.2)
	Manure pits	7/14	(50.0)
	Wall floor junctions	38/172	(22.1)
Surroundings, not related to floors	Air inlets	1/73	(1.4)
	Beams/ledges	9/74	(12.2)
	Ceilings	0/7	(0)
	Miscellaneous	2/10	(20.0)
	Outlet chimneys	1/32	(3.1)
	Wall fabric junctions	4/131	(3.1)
	Walls	3/87	(3.4)
Surroundings, miscellaneous	Ancillary rooms	5/16	(31.3)
	In poultry houses	1/19	(5.3)
Unknown sites		1/5	(20.0)
SUM		190/1784	(10.7)

9.2.4 Monitoring of temperature and humidity

The company monitored the temperature continuously using four temperature loggers, but as these were not calibrated the results from these will not be reported here.

In all houses that were steam treated, temperature or temperature/relative humidity (RH) were logged at 5-min intervals, using Testo 175 or Testo 171 loggers (Testo, 79853 Lenzkirch, Germany). After the treatments, all loggers were read in the corresponding PC-programmes.

Temperature loggers were placed at six sites per house, one in each corner and two evenly distributed in the middle. In houses where ancillary rooms were included in the heat treatment (Houses A2, C1), one “corner” measurement was taken from the farthest corner of the ancillary room. A temperature logger was also placed in the farthest corner of the egg storeroom attached to House D1, but no heat treatment was performed here because steam entering the electric cabinets caused a power cut. At each of the six sites, there was a “low” measurement, normally logging 2.5 cm above floor level, and a “high” measurement that in most cases logged 2-2.5 m above floor level, but in some cases also at lower (1-2 m) or higher (4-5 m) levels due to different house constructions and practicalities of placing probes. Thus, each heat-treated house had 12 standard sites where the temperature was always measured, and these were also the sites where organic indicator samples were placed (cf. Section 9.2.5).

RH was measured in some houses not less than 2 m above floor level. Temperatures were measured at different heights above the floor to see where 60 °C was achieved. As heating of Houses A1 and A2 showed that 60 °C was not achieved 2.5 cm above floor level, measurements at different heights (22, 48, 66, 98, 116, 148 and 196 cm above floor level) were made in House B1. Here, 61.9 °C was recorded 22 cm above the floor, and as

the temperature did not decrease above this level, the height range was narrowed in the subsequent houses (10/12.5/50, 8.3 and 4.5/10/22 cm above floor level in Houses C1, D1 and E1, respectively). In Houses B1, C1, D1 and E1, additional temperature and humidity measurements were performed in holes drilled in the concrete floor to monitor temperatures achieved in cracks and the concrete itself. The diameters of holes drilled in the concrete floor were ca. 4, 12 or 16 mm, using probes with diameters of ca. 4, 12 and 12 mm, respectively, to measure both closely to the concrete and with a few mm space for the steam. In addition, sealant was used around the probes in some holes in two houses.

9.2.5 Organic indicator samples

As the *Salmonella* checks were in-use tests with the uncertainties connected with these (cf. Section 5.4.6), they were supplemented with organic indicator samples, i.e. samples with high amounts of organic matter placed beside the standard site temperature probes (cf. Section 9.2.4) containing indicator bacteria (as it was too hazardous to place *Salmonella*-inoculated samples in the layer houses).

Bacteria found in the same habitats as *Salmonella* spp., but often in higher numbers, which are at least as heat resistant as *Salmonella* spp., would be suitable indicator bacteria. A field isolate of *E. coli* and *Enterococcus (E.) faecalis* (cf. Table 6.2), both isolated from poultry samples submitted to our laboratory, were investigated for use in organic indicator samples.

Initially, both isolates were made rifampicin resistant (cf. Section 6.10).

The two isolates were compared to one SE8 isolate in three series of traditional quantitative heating studies (cf. Section 6.11 and Appendix A, Figures A10-A12). As both *E. coli* and *E. faecalis* were at least as heat resistant as SE8 they were found to be suitable as indicator bacteria.

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Organic matter was either feed or fresh poultry faeces. The feed was pelleted full feed for egg layers (code no. 3103, free from antibiotics and coccidiostats) supplied by Korn- og Foderstofkompagniet A/S (Havnen 2, 6440 Augustenborg, Denmark). The fresh faeces were taken at least once a month from a battery cage house with droppings belts, from which *Salmonella* had never been detected culturally or serologically in any sample submitted under the Danish *Salmonella* Control Programme. Feed was stored at room temperature and faeces at 5°C in tightly closed plastic bags.

The *E. coli* or *E. faecalis* isolate was incubated for 12-24 h at 37 °C in VIB until stationary phase. Three ml of a 10⁻² dilution in physiological saline was used to inoculate each feed sample of 30 (± 1) g. In faecal samples (each weighing 30 (± 1) g), the naturally occurring *E. coli* and enterococci were used. Initially, fresh faeces were streaked directly onto MacConkey agar and Slanetz agar to check for *E. coli* and enterococci, respectively (cf. Sections 6.8 and 6.9).

Organic indicator samples were weighed into beakers and put in a climate chamber set to 20 (± 0.5) °C and 30 (± 3)% RH to dry. However, due to a temporary malfunction of the climate chamber, organic indicator samples for Farms A and B were kept at room temperature. Organic indicator samples for Farms A, B/C/E and D were dried for 4, 10 and 15 days, respectively. For Farm B samples, a mean temperature of 19.4 (s.d. 0.9) °C and %RH of 40.1 (s.d. 8.5) were measured for the 10-day period. Control samples (three for each combination of bacterial type and organic matter, i.e. 12 per house) remained in the climate chamber or under ambient conditions without lids.

In the animal house, lids were removed and samples placed at the same height as and not more than 20 cm from a temperature probe placed at one of the 12 standard sites (cf.

Section 9.2.4). After treatment, new lids were put on beakers before taking them back to the laboratory.

In the laboratory, *E. coli* or *E. faecalis*/enterococci samples were processed as described in Sections 6.8 and 6.9, respectively. In addition, randomly selected colonies from feed samples were checked for rifampicin resistance (Anonymous 1998).

9.2.6 Statistical analyses

All *Salmonella* sample data were entered in an Access database (Anonymous 1997a). Five percent significance levels were used in all tests.

Chi-square or two-tailed Fisher exact tests (for expected values < 5) were used for categorical data.

Percentages were calculated from categorical data and used for regression tests. Percentages (*Salmonella*-positive samples vs. Rambach agar plates with growth) were compared by linear regression using the function “Regression” in Excel (Anonymous 1997b).

Comparisons between houses related to reductions in Rambach agar plates with growth, both generally and specifically for coliforms, were investigated by fitting a generalized linear model with binomial family and logit link (Anonymous 2002f). Two explanatory variables were included (“Treatment” and interaction between “House” and “Treatment”). Differences between houses in the reductions due to treatment were found by testing the significance of the interaction term. To keep the overall risk of type I error equal to 0.05, an alpha of 0.0073 ($= 1.00 - 0.95^{1/N}$, where $N=7$ (number of comparisons per houses)) was used.

9.3 Results

9.3.1 *Salmonella* samples before treatments

All 190 *Salmonella*-positive samples were serotyped as *S. Enteritidis*, which was also the serotype persisting in all eight houses. In the study period (1/8/01-31/12/02), *S. Enteritidis* was not detected in any samples from hatcheries or parent stock premises delivering to Danish commercial layer premises. Moreover, *S. Enteritidis* was rarely detected in samples from Danish layer premises in which it had not occurred before the study period.

There were big variations in numbers of *Salmonella*-positive samples between houses, ranging from 0/100 in House B1 to 65/302 (21.5%) in House A2 (Table 9.3). Cleaning standards were high in all the houses, except House A2 where more organic matter was seen, but other factors might also influence the percentage of *Salmonella*-positive samples (cf. Table 9.1). Generally, fewer equipment and surroundings samples, not related directly to floors, were *Salmonella*-positive than surroundings samples related to floors ($p < 10^{-7}$), but there were several exceptions, as samples from droppings belts, drip channels, feed chain “corner” wheels and egg equipment often yielded *Salmonella* (cf. Table 9.2). There were no conspicuous differences between barn and battery cage houses, and the distribution in all houses with high *Salmonella*-percentages (A1, A2, C1, E1) was “geographically” even, both longitudinally, transversely and at different heights, including *Salmonella*-positive samples from the whole length of all three tiers sampled in battery cage houses (data not shown).

When the houses were compared, linear regression analyses showed significant associations between % *Salmonella*-positive samples and % Rambach agar plates with growth ($R^2=0.7018$, $p=0.0094$, cf. Table 9.3).

Table 9.3: Results for *Salmonella* samples, including readings on Rambach agar plates (numbers of samples/sum (%))

House	Before treatment						After treatment				R ²	Signi- fiance ³	
	Salmonella detected			Salmonella not detected			Salmonella not detected						
	Sum ¹	Only S ¹	S + blu ¹	S + oth ¹	No growth	Blu ¹	Oth ¹	Salm. detected	No growth	Blu			Oth
A1	36/287 (12.5)	2/36 (5.6)	33/36 (91.7)	1/36 (2.8)	9/251 (3.6)	240/251 (95.6)	2/251 (0.8)	0/288 (0)	194/288 (67.4)	90/288 (31.3)	4/288 (1.4)	3.0/3.0	a
A2	65/302 (21.5)	4/65 (6.2)	61/65 (93.8)	0/65 (0)	18/237 (7.6)	217/237 (91.6)	2/237 (0.4)	6/303 ⁴ (2.0)	189/297 (63.6)	108/297 (36.4)	0/297 (0)	2.5/2.5	a
B1	0/100 (0)	-	-	-	59/100 (59.0)	41/100 (41.0)	0/100 (0)	0/102 (0)	99/102 (97.1)	3/102 (2.9)	0/102 (0)	14.1/14.0	bc
B2	6/100 (6.0)	0/6 (0)	6/6 (100)	0/6 (0)	42/94 (44.7)	48/94 (51.1)	4/94 (4.3)	0/96 (0)	59/96 (61.5)	35/96 (36.5)	2/96 (2.1)	1.7/1.5	a
B3	5/100 (5.0)	2/5 (40.0)	3/5 (60.0)	0/5 (0)	30/95 (31.6)	62/95 (65.3)	3/95 (3.2)	0/100 (0)	92/100 (92.0)	8/100 (8.0)	0/100 (0)	8.5/8.1	b
C1	37/298 (12.4)	9/37 (24.3)	21/37 (56.8)	7/37 (18.9)	118/261 (45.2)	134/261 (51.3)	9/261 (3.4)	0/308 (0)	306/308 (99.4)	2/308 (0.6)	0/308 (0)	92.9/80.1	c
D1	1/289 (0.3)	0/1 (0)	1/1 (100)	0/1 (0)	211/288 (73.3)	76/288 (26.4)	1/288 (0.3)	0/290 (0)	289/290 (99.7)	1/290 (0.3)	0/290 (0)	79.4/77.3	c
E1	40/308 (13.0)	8/40 (20.0)	32/40 (80.0)	0/40 (0)	84/268 (31.3)	178/268 (66.4)	6/268 (2.2)	0/308 (0)	299/308 (97.1)	8/308 (2.6)	1/308 (0.3)	25.1/26.3	bc
SUM	190/1784 (10.7)	21/190 (11.1)	157/190 (82.6)	8/190 (4.2)	571/1594 (35.8)	996/1594 (62.5)	27/1594 (1.7)	6/1795 (0.3)	1527/1795 (85.1)	255/1795 (14.2)	7/1795 (0.4)		

¹ For "Before treatment"/"Salmonella detected": Sum = all *Salmonella*-positive samples; Only S = only red/reddish ("Salmonella-like") colonies; S + blu = red/reddish ("Salmonella-like") and blue/bluish ("coliform") colonies; S + oth = red/reddish ("Salmonella-like") and colonies that were not blue/bluish. For "Before/After treatment"/"Salmonella not detected": Blu = blue/bluish colonies; Oth = colonies that were not blue/bluish. All Rambach agar plates that had both blue/bluish colonies and colonies that were not blue/bluish were categorised as "S + blu" or "Blu" for *Salmonella* positive and negative samples, respectively.

² Reduction factor, i.e. the proportion between % Rambach agar plates with growth before and after treatment (all non-*Salmonella*/coliforms).

³ Results from logistic regression, both for all non-*Salmonella* and coliforms. Houses with different letters had significantly different reduction factors.

⁴ All six samples also had blue/bluish colonies.

9.3.2 *Salmonella* samples after treatments

No *Salmonella* was found in any house except for six samples with *S. Enteritidis* in House A2 (Table 9.3), all of which were related to floors. Comparisons of the numbers of *Salmonella*-positive samples before and after treatment were highly significant for Houses A1, A2, C1 and E1 ($p < 10^{-7}$). For Houses B2 and B3, the reductions were around significance limits ($p = 0.03$ and 0.06 , respectively), but because of the low numbers of *Salmonella*-positive samples before treatment this should be interpreted with caution. For the remaining two houses (B1 and D1), the *Salmonella* results *per se* cannot be used for evaluating the efficacy of the treatments.

Logistic regression models for reductions in percent Rambach agar plates with growth showed the same tendencies both for all non-*Salmonella* and coliforms, as these were very similar (cf. Table 9.3). Houses A1, A2 and B2 did not differ from each other, but all were significantly worse than all the other houses ($p \leq 5.57 \times 10^{-7}$). The four houses treated with steam and formaldehyde (Houses B1, C1, D1 and E1) were not different from each other. Among these, Houses B1 and E1 were not different from House B3 that was surface disinfected. The highest reduction factors were seen for the steam and formaldehyde treated houses, regardless of significance.

9.3.3 Temperature and humidity

In the air, 100% RH was accomplished 10-15 min after the steam treatment commenced, and it was maintained throughout the heat treatment period (data not shown).

In tightly sealed houses, the temperature that was maintained during the 24-h period was achieved within the first hour, and this temperature was generally evenly dispersed both longitudinally and transversely in the house (data not shown). The main difficulty was to achieve the required 60 °C near the floor (data not shown), but measurements at differ-

ent heights near the floor indicated that the first ca. 10 cm were the most critical, as 60 °C was achieved at this height, and the temperature changes were minor at higher levels (data not shown). In House E1, 60 °C was not achieved, probably because the ridge roof was not tightly sealed, so cold air from the outside was sucked into the house. In the same house, it also took 4-6 h before the 60 °C was achieved (data not shown).

Not all measurements in concrete holes were reliable, as implausible temperatures were often seen when 100% RH was achieved (temperature increase of 60 °C within 10 min). Generally, 100% RH was achieved in all the concrete holes within 1 h (data not shown). In holes with little space for the steam (i.e. where the probes filled the holes as much as possible), the temperature was generally ca. 3-5 °C lower than in corresponding measurements 2.5 cm above floor level, whereas similar temperatures were achieved where more room was left for the steam to enter (data not shown). Moreover, it normally took 9-10 h to achieve a stable temperature in the holes, regardless of their type (data not shown). Sealing of concrete floor holes generally lowered the mean temperature by ca. 10 °C (data not shown). However, there were more measurement deviations in concrete holes than in the air, which could be due to the unreliable measurements (see above), but probably the climate also influenced temperatures in and around concrete floors more than in the air, so seasonal variations may be more important.

9.3.4 Organic indicator samples

The relevant bacteria survived in all the control samples. All colonies selected from organic indicator feed samples after treatment were fully rifampicin-resistant.

Nearly all *E. coli* in faecal samples were eliminated regardless of procedure and mean temperature, whereas the three other organic indicator sample types showed almost uniform reduction tendencies (Table 9.4).

Generally, few bacteria survived above 60 °C, a tendency seen especially when 30 ppm formaldehyde was used. The use of 30 ppm formaldehyde seemed to lower the lethal temperature by 2-5 °C. The combination of a 24-h steam treatment without formaldehyde followed by a short heating with formaldehyde added to the steam seemed to be as effective as adding 30 ppm formaldehyde to the steam at the beginning of the 24-h treatment. A short heating to ca. 60 °C with formaldehyde in the steam (Period II) was generally ineffective, as there were surviving organic indicator bacteria at all temperatures.

9.4 Discussion

In a population with more than 500 individuals, 300 or 60 samples will with 95% certainty detect down to 1% or 5% of infected individuals in the flock, respectively, given that laboratory sensitivity is 100%. Similar principles are difficult to extrapolate to sampling in empty poultry houses where sampling units are uncountable, and, as far as is known, recommendations based on specific prevalences have not been reported in the literature, but nevertheless 300 samples in five of the six houses that were heat-treated were considered adequate. This number of samples also enabled us to detect sites and/or equipment where the likelihood of detecting *Salmonella* was higher or lower. The *Salmonella* results *per se* after treatment generally showed a positive effect of the treatments, as only six samples, all from House A2, had *Salmonella*. Other studies have generally found that *Salmonella* was not eliminated by chemical disinfection, though formaldehyde was one of the most effective disinfectants (Davies and Wray 1995c; Davies *et al.* 1998b, 2001; Valancony *et al.* 2001; Davies and Breslin 2003a), and its effectiveness

Table 9.4: Results (positive/all samples, •1 positive sample illustrated by shaded areas) for organic indicator samples related to mean temperatures.

Mean temperature (°C) ¹	Feed										Faeces							
	<i>Enterococcus faecalis</i>					<i>Escherichia coli</i>					Enterococci				<i>Escherichia coli</i>			
	PI ²	PII ³	PI/II ⁴	WF ⁵	PI	PII	PI/II	WF	PI	PII	PII	PI/II	WF	PI	PII	PI/II	WF	
> 62.5	0/4	2/4	0/4	0/11	0/4	2/4	0/4	0/11	0/4	1/4	1/4	0/4	0/11	0/4	0/4	0/4	0/11	
]60.0-62.5]	0/8	6/8	0/8	0/8	1/8	3/8	0/8	0/8	2/8	3/8	3/8	0/8	1/7	0/8	1/8	0/8	0/8	
]57.5-60.0]	1/3	2/3	0/3	0/9	0/3	2/3	0/3	0/9	1/3	2/3	2/3	0/3	2/9	0/3	0/3	0/3	0/9	
]55.0-57.5]	2/4	4/4	1/4	1/2	1/4	2/4	1/4	1/2	2/4	3/4	3/4	0/4	1/2	0/4	0/4	0/4	0/2	
]52.5-55.0]				2/5				0/5					1/5				0/5	
]50.0-52.5]	0/1	1/1	1/1	0/3	0/1	1/1	0/1	0/3	0/1	1/1	1/1	0/1	0/3	0/1	0/1	0/1	0/3	
]47.5-50.0]	1/1	1/1	1/1	0/1	1/1	1/1	0/1	0/1	0/1	1/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1	
]45.0-47.5]	2/2	2/2	2/2	0/2	2/2	2/2	1/2	1/2	2/2	2/2	2/2	2/2	1/2	0/2	0/2	1/2	0/2	
]42.5-45.0]																		
]40.0-42.5]				1/1				1/1					1/1				0/1	
]37.5-40.0]				1/1				1/1					1/1				1/1	
]35.0-37.5]																		
]32.5-35.0]																		
< 32.5	1/1	1/1	1/1	3/3	1/1	1/1	1/1	1/3	1/1	1/1	1/1	1/1	1/3	1/1	0/1	0/1	0/3	

¹ Mean temperature during 24-h period, using the 288 highest temperatures (logged at 5-min intervals).

² Period I (cf. Table 9.1) for Houses A1 and A2.

³ Period II (cf. Table 9.1) for Houses A1 and A2. The mean temperatures in the left column are invalid for Period II.

⁴ Period I and II (cf. Table 9.1) merged.

⁵ Heat treatment with 30 ppm formaldehyde for Houses B1, C1, D1 and E1.

seemed to depend less on the cleaning standard if this was reasonable (Davies and Wray 1995c).

Like *Salmonella*, the natural habitat of coliforms is the intestine, and they are similar in resistance to extrinsic conditions, e.g. heat and chemical disinfection, but they normally occur in much higher numbers, all characteristics that make them suitable as indicator bacteria. Heating tests in the laboratory showed a high association between survival of *E. coli* and the occurrence of Rambach agar plates with growth (cf. Chapter 8), and it was appropriate to see if the same high association was seen under field conditions. With the actual distribution between numbers of samples from different categories (cf. Table 9.2), an association was seen when houses were compared. Due to this association, the reduction in numbers of Rambach agar plates with growth, among which most non-*Salmonella* were coliforms, could be used for comparing different treatments. Dosing with 30 ppm formaldehyde at the beginning of the 24-h steam heating seemed to be the most effective, whereas a 24-h treatment without formaldehyde did not differ much from thermal fogging. However, the relatively high number of floor-related samples could bias results from House B2, because thermal fogging might be more efficient at higher levels due to the hovering effect. Moreover, it was reported that the thermal fogging machine did not work properly, as ca. 30 min passed before the fog was released. As a result of this, some disinfectant might have decomposed before it was released in the fog. On the other hand, House B1 was the most difficult to clean due to numerous cracks, crevices and inaccessible equipment, B3 was a much newer house with more accessible equipment and relatively smooth surfaces, whereas House B2 was intermediate. Thus, the proportion of Rambach agar plates with growth before treatment illogically increased with the “cleanliness” of the house. Possible explanations for this could be that it is easier to sample bacteria from

smooth than from rough surfaces (also cf. Section 5.2.3), or that different types of bacteria which are not equally inhibited by culture media, especially the selective ones, occur in different houses, and this can also bias results when comparing other houses.

Few other studies have included other bacteria than *Salmonella* in monitoring effectiveness of disinfecting these. Davies and Wray (1995c) used an MPN-method for counting coliforms in samples taken from a trial poultry house with three pens infected with *S. Enteritidis*. The pen with the highest number of coliforms before cleaning and disinfection had the lowest number of *Salmonella*. After chemical disinfection of the three pens, using different disinfectants in each, there was a reduction in coliforms in only one (of only 1-2 log).

Valancony *et al.* (2001) took ca. 100 samples from each of 14 battery cage houses both before and after cleaning and disinfection with formaldehyde. They reported that cages and air circulation systems were not reliable sites for sampling as these were disinfected most effectively. In our study, samples from cages and air circulation systems also generally yielded few *Salmonella*-positive samples after cleaning. On the contrary, *Salmonella* was often found in samples taken from floor-related sites before disinfection, and all six *Salmonella*-positive samples after disinfection were from the floor, a tendency reported from other studies after cleaning and disinfection (Davies and Wray 1995c, 1996a, 1996b, 1996c; Davies *et al.* 1998b, 2001; Valancony *et al.* 2001). In addition, concrete is considered to be one of the materials most difficult to heat, and floors were at the level where the lowest temperatures were achieved. All these traits make floors suitable “worst case” areas for the monitoring of the efficacy of treatment, and it is recommended to focus on these in future heating studies, especially if fewer samples are taken. However, samples from equipment should still be included, but sampling from certain sites (droppings belts, drip

channels, feed chain “corner wheels” and egg-equipment) increases the likelihood of obtaining *Salmonella*-positive samples before the heating. These sites also yielded relatively high numbers of *Salmonella*-positive samples in other studies (Davies and Wray 1995c, 1996a; Valancony *et al.* 2001).

The results of organic indicator samples showed that 60 °C and 100% RH during a 24-h period generally could eliminate putative indicator bacteria in organic matter, represented by desiccated feed (each sample yielding a 2.5-3 cm thick layer) and faeces. Naturally occurring *E. coli* in faecal samples were eliminated too easily to be suitable indicator bacteria for the heat treatment, but these results also indicate that naturally occurring *Salmonella* in faeces is eliminated relatively easily. Unfortunately, the different pre-treatments of organic indicator samples for some houses could bias the comparisons between them, but nevertheless the results showed clear tendencies related to the mean temperature. Enterococci are thought to be relatively resistant to various detrimental conditions (Russell 1999a), a tendency confirmed in quantitative heating tests in this study. Their habitat is also the intestines, and they often occur in higher numbers than *Salmonella*. In addition, the organic indicator samples could be placed next to the temperature probes so more exact effects of temperature could be evaluated.

Most other disinfection studies are quantitative as they report on log-reductions in CFU, but the criteria applied in this study were more restrictive. No bacteria should be detected, even after a non-selective enrichment step, which is normally recommended (cf. Section 5.2.1). Formaldehyde neutralisers were not used in any enrichment procedures (cf. Section 5.4.2). We believe that most formaldehyde had broken down to carbon dioxide and water by the time of post-treatment sampling, as none of the samplers experienced irritation of mucous membranes. Moreover, one way of compensating for the lack of neutralis-

ers was probably the dilution of samples (and remaining formaldehyde) in BPW on the spot. Davies and Wray (1996a) (who also brought BPW to the poultry houses for sampling) reported that the use of disinfectant neutralisers reduced the *Salmonella* isolation rates from field samples.

It was surprising to see how quickly the required temperature was achieved and distributed evenly longitudinally and transversely in the poultry houses, which is definitely an asset due to the even “geographical” distribution of *Salmonella* found in 29/35 subcategories (cf. Table 9.2). However, a tightly sealed house appeared to be a very important precondition for this, illustrated by the slow heating of House E1 where a mean temperature of 60 °C during 24 h was not achieved. Another weak point was the lower temperature at floor level in all houses, but measurements showed that the required temperature could be accomplished ca. 10 cm above floor level, a height above which are the battery cages, i.e. equipment difficult to clean and disinfect properly. All farmers were advised to disinfect their floors after the heat treatment, either chemically or by flame burning. Alternatively, the temperature could be increased to e.g. 70 °C in the hope that the temperature at floor level would then reach 60 °C, but this might damage some equipment. It was difficult to achieve the required temperature in concrete holes, but it was facilitated in holes where the steam could enter more easily. This is likely to represent the areas in cracks and crevices where *Salmonella* is found.

As per December 2003, no *Salmonella* has been detected in any samples submitted under the Danish *Salmonella* Control Programme from the houses of this study.

In conclusion, most results indicated that a steam treatment yielding ≥ 60 °C and 100% RH during minimum 24 h, with 30 ppm formaldehyde added at the beginning of this treatment, was effective in eliminating naturally occurring *Salmonella* in layer houses.

Chapter 10 - Dry and moist heat treatment

of two identical battery cage houses

10.1 Introduction

Chapter 9 described field studies where heat treatments, measurements and sampling were controlled rigorously. However, all houses and the equipment in these differed, and as they were distributed on several farms, various management procedures were used. These differences could bias results when these were compared between houses and farms.

In this chapter, the farmer measured temperatures and relative humidities using un-calibrated equipment. Moreover, the heat treatment procedures were developed and performed by the farmer, and were only applied on the actual farm, so it is difficult to know if the procedures could be applied on other farms. Nevertheless, the results are interesting for the following reasons:

- The comparison of two identical poultry houses minimised the bias from house-related factors. In addition, the houses were situated on the same farm (even attached to each other), so the same management procedures were probably applied.
- The two houses were the biggest battery cage houses in Denmark, i.e. the heat distribution was performed under worst-case conditions from a volume point of view.
- Although the temperature loggers were un-calibrated, the same equipment was used in both houses, and temperature probes were placed at the “same” sites, so reasonably valid comparisons between these could be made.

- As dry heat was applied in one house and moist heat in the other, the impact of humidity could be investigated.

Thus, this chapter describes a field study of heating procedures, measurements and bacteriological sampling from two identical battery cage houses, where bias from different types of houses, farms and management was minimised.

10.2 Materials and methods

10.2.1 Description of the farm and houses

The farm was the biggest layer farm in Denmark, having three battery cage houses (ca. 220,000 hen sites), three barn houses (ca. 29,600 hen sites) and three commercial rearing houses.

The two identical houses in this study (A and B) were attached end to end, but had separate entrances. Each house measured 110x20 m, the height of walls was 4.3 m, and the roof angle was 16.7°, yielding 7.3 m from floor to roof ridge.

The two houses also had identical equipment (battery cages with droppings belts). Each house had eight tiers, each tier with six stacks. A metal gangway on both sides of all tiers was placed between the third and the fourth stack. Each house could accommodate ca. 72,000 layers.

10.2.2 *Salmonella* history

Since March 1994, *Salmonella* Enteritidis had been detected in many samples submitted from the farm, until December 1996 under the voluntary *Salmonella* programme for layer farms, afterwards under the official Danish *Salmonella* Control Programme. All *S. Enteritidis* isolates, except the first from March 1994, were phage typed, most as PT6 or PT21. Thus, *S. Enteritidis* had persisted on the farm for years, but it is not clear whether

the persistence was mainly farm- or house-related, amongst other things because of the many houses and poor rodent control.

In Houses A and B, *S. Enteritidis* was detected for the first time in hens in December 1999 and December 1998, respectively. However, because house registration was less rigorous under the voluntary *Salmonella* programme for layer farms (January 1992-December 1996), some of the *Salmonella*-positive samples from this period might have come from House A and/or B.

10.2.3 Chain of events and procedures for House A

In November 1999, the flock in House A was put under suspicion because of high *Salmonella* antibody levels in eggs (Feld *et al.* 2000), and in December 1999, *S. Enteritidis* was detected in hens submitted from the flock. The flock was culled immediately thereafter, and the farmer opted for dry cleaning of the equipment and wet cleaning of floor and walls, followed by a dry heat treatment at the turn of the year. Two space heaters were used for heating the house, and 11 probes measured the temperature (Figure 10.1). A digital screen showed all temperatures as integers.

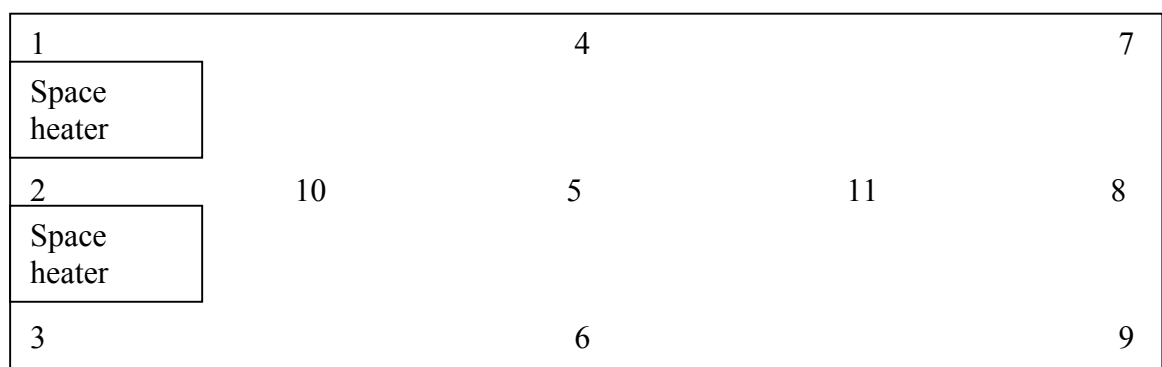


Figure 10.1: House A; outline of heating and measurements. Probes 1-9 and 10-11 were placed 5-10 cm and ca. 3 m above floor level, respectively.

The farmer had not recorded the temperatures, but said that all 11 probes had shown 58-62 °C during a 7-day period. After this heat treatment, the author visited the farm in January

2000. Faeces were visible in many battery cages, on and under droppings belts, and on floors at the end of the tiers. The faeces were dry, had a “chewing gum” consistency, and a handful could be taken from many cages. Moreover, a 1-2 cm thick feed layer was seen at the inner side of the feed troughs. Much less organic matter was seen on floors, walls and the ceiling. The whole of the inside of the house and the equipment were covered by hydrated lime. During this visit, 31 bacteriological samples were taken (cf. Section 10.2.5), and because *Salmonella* was found in these, the owner repeated the dry heating in the period 27/1-6/2-00, albeit at higher temperatures. Temperatures were measured at the same 11 sites as shown in Figure 10.1, but this time the farmer kept a written record. After this second heat treatment, the farm was revisited. Conditions were as described above, except that the faeces were now so dry that they had a powder-like consistency. During this visit, 31 bacteriological samples were again taken (cf. Section 10.2.5).

10.2.4 Chain of events and procedures for House B

In April 2000, the flock in House B was put under suspicion because of high *Salmonella* antibody levels in eggs (Feld *et al.* 2000), and in May 2000, *S. Enteritidis* was detected in hens submitted from the flock. The flock was culled in December 2000, after which the house was cleaned with hot water at 40-50 °C without detergent. Sixty-eight samples were taken immediately after this cleaning and examined for *Salmonella* (cf. Section 10.2.6). The main principles in the heat treatment were the use of steam to achieve a relative humidity in the air as high as possible, combined with formaldehyde added to the steam at the beginning of the process. The “steam trailer”, constructed by the farmer, had a steam generator able to supply 600 L water h⁻¹ and a heat fan that could atomise up till 400 L water h⁻¹. Steam was inserted at one end of the house and dispersed in it by various devices (cf. Figure 10.2).

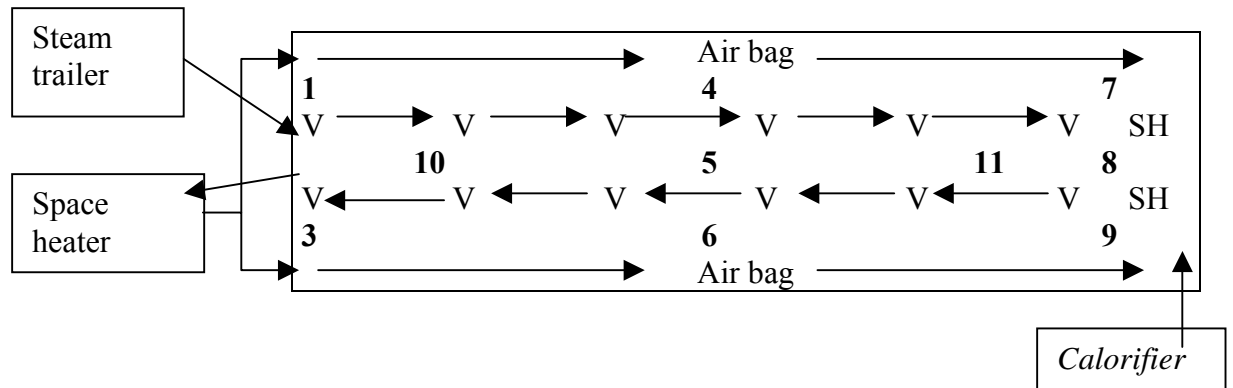


Figure 10.2: House B; outline of heating, air currents and measurements. Numbers show temperature probes (probe 2 omitted, as it broke down during the heat treatment), all placed in the lower battery cage floor. Moreover, relative humidity was measured at probe no. 5 and at the outgoing air (where the arrow to the outdoor space heater begins). Arrows to and from the house show heat supply and removal, respectively. The arrows in the house show the active heat dispersion by air bags and ventilators (V). SH = space heaters in the house.

Probes 1 and 3-9 were placed exactly as in House A, related both to equipment and heat sources (compare with Figure 10.1). Formaldehyde was added to the steam in the heating-up phase, covering a temperature span from ca. 40 to 55 °C over a 15-h period. Seventeen hundred litres of 23.4% formaldehyde were used, yielding ca. 31 ppm in the atmosphere. After the heat treatment, 68 samples were taken again for testing for *Salmonella* (cf. Section 10.2.6).

10.2.5 Sampling and microbiological procedures in House A

Thirty-one samples were taken after both the first and the second heat treatment, as closely as possible from the same sites. Among these, 16 were faecal samples, 14 were feed scraped from the feed troughs, and one sample was from an egg belt. Samples were taken from tiers 1, 3, 5, 6 and 8, from the middle and each end of every tier. All samples were taken from the middle or the lower stack, as the lowest temperatures were expected there. Beakers with 100 ml BPW and sterile gauze swabs (cf. Section 9.2.3) as well as OB[®]

tampons were brought to the house. Gauze swabs were used for all samples, except the egg belt sample for which one OB[®] tampon was used. Swabs/tampons were immersed in BPW, and then used to swab a surface vigorously. Disposable gloves were used and changed between each sample. Faecal material, however, was so abundant that they could be gathered in handfuls, and so were also sampled. All samples were incubated on the sampling day, and *Salmonella* procedures were as described in Section 6.7.1. In addition, selected *Salmonella*-positive isolates were phage typed (Ward *et al.* 1987). The BPW pre-enrichment used to inoculate RVS was streaked on MacConkey agar and on BA to check for presence/absence of *E. coli* and enterococci, respectively. Procedures for *E. coli* were as described in Section 6.8, whereas small (< 1 mm), white-brownish colonies surrounded by an irregular greenish/brownish zone on BA (α haemolysis) were recorded as enterococci.

10.2.6 Sampling and microbiological procedures in House B

Sixty-eight samples were taken from pre-determined sites before the heat and formaldehyde treatment. After treatment, 68 samples were taken 1-2 m from the same sites, as most visible organic matter had been scraped off when sampling before treatment. Samples were taken from the middle and each end of all tiers (cf. Figure 10.3). In addition, after the treatment eight samples were taken from sites in air inlets and outlet chimneys that had not been treated (as these were closed during the heating). Sampling and microbiological *Salmonella* procedures were as for House A (cf. Section 10.2.5), except that disposable dish cloths or OB[®] tampons were used for surfaces, whereas sterile cotton swabs (six per sample) were used for sampling crevices at the underside of the feed trough (cf. Table 10.2); the same materials were used for the same sites before and after heat treatment. No procedures for bacteria other than *Salmonella* were used in the laboratory, but the occurrence of

non-*Salmonella* colonies (mostly blue/bluish colonies, i.e. coliforms) on the Rambach agar plates was recorded.

10.2.7 Statistical analyses

Differences before and after treatments were compared by chi-square or two-tailed Fisher exact tests (for expected values < 5). Associations between occurrence of *E. coli* and “blue/bluish” colonies on Rambach agar in samples from House A were tested by McNemar chi-square test (Martin *et al.* 1987) and Cohen’s kappa (Sackett 1992). Linear regression analysis was used to calculate correlations between mean and maximum temperatures (Anonymous 2002c). Five per cent significance levels were used in all relevant tests.

10.3 Results

10.3.1 Bacteriological results

Results from House A are shown in Table 10.1.

Table 10.1: Positive/total samples for House A.

Sample type	Heat treatment	<i>Salmonella</i>	<i>E. coli</i>	Enterococci
Faeces	First	8/16	15/16	11/16
	Second	3/16	13/16	9/16
Residue from feed troughs	First	0/14	3/14	7/14
	Second	1/14	1/14	7/14
Residue from egg belts	First	0/1	0/1	1/1
	Second	0/1	0/1	1/1
SUM	First	8/31	18/31	19/31
	Second	4/31	14/31	17/31

After the first heat treatment, *S. Enteritidis* was found in 8/31 samples, all of which were faeces. All phage typed isolates were PT6 (also found in the layers before depopulation of the house). Moreover, there were *Salmonella*-positive samples from both ends and the middle of all tiers sampled (data not shown). Seventeen samples also had “blue/bluish” colonies on the Rambach agar plates (data not shown), and the occurrence of these and *E. coli* was associated (McNemar $\chi^2=0.25$, i.e. $p<0.05$, and Cohen’s kappa=0.74, i.e. “sub-

stantial agreement”). The only faecal sample without *E. coli* was also *Salmonella*-negative, whereas enterococci were detected more commonly in all three sample types (6/8 and 13/25 among *Salmonella*-positive and *Salmonella*-negative samples, respectively ($p=0.42$)) (data not shown). After the second heat treatment, *S. Enteritidis* was found in 4/31 samples, this time also in a sample from a feed trough; the reduction from 8/31 to 4/31 *Salmonella*-positive samples was not significant ($p=0.33$). The three *Salmonella*-positive faecal samples were from sites that were also *Salmonella*-positive after the first heat treatment (data not shown). Eighteen samples also had “blue/bluish” colonies on the Rambach agar plates, and the occurrence of these and *E. coli* was associated, albeit less than after the first heat treatment (McNemar $\chi^2=1.13$, i.e. $p<0.05$, and Cohen’s kappa=0.49, i.e. “moderate agreement”). The small reductions in numbers of samples positive for *E. coli* and enterococci after the second heat treatment were not significant ($p=0.31$ and $p=0.61$, respectively).

Table 10.2 and Figure 10.3 show results from House B before the heat treatment.

Table 10.2: *Salmonella*-positive/total samples for House B before the heat treatment.

Sample category	Stack ¹					Total per sample category
	Lower	Middle	Upper	> 1	Unknown	
Manure system ²	2/2	2/8	4/7		1/3	9/20
Feed system ³		4/8	1/1	1/3	1/7	7/19
Crevice ⁴		1/7	0/1	1/4	0/4	2/16
Egg belts ⁵		2/2		1/1	0/2	3/5
Miscellaneous ⁶	0/1	1/2	0/2		1/3	2/8
SUM	2/3	10/27	5/11	3/8	3/19	23/68

¹ All samples were taken from the three lowest stacks.

² From edge of droppings belts and/or from stiffeners transversing these, i.e. the sample material was mainly faeces.

³ From the surface of feed troughs, i.e. the sample material was mainly feed.

⁴ From the crevice on the underside of feed troughs, using cotton swabs. This was a favourite site for the red poultry mite (*Dermanyssus gallinae*), and the sample material was blackened, probably by blood from the hens.

⁵ From egg belts; the sample material was probably mainly feed, dust and eggs.

⁶ Could not be placed in any of the above sample categories.

N1/M3/N	M2/M1	F3/N2/M2
M2/C2	M2/C2	M2/E123/C23
M3/C23/F2	M3/C2	F2/M2/C12
M3/C2/F23	M3/M2/E2	N3/M2/M1
C2/M3/M3	F2/C2/F2	F2/N2/C2
F2/F2/C23	F12/C3/E2	N3/C/F12
N/F/M	F/E/M	F/C/F2
F/C/E	F/C/M	N/F/E

Figure 10.3: House B; outline of *Salmonella* samples taken before heat treatment, each grey bar representing a tier. Letters designate sample category (M=manure system; F=feed system; C=crevice; E=egg belts; N=miscellaneous, see definitions in Table 10.2). Numbers designate stack (1,2,3=lower, middle, upper stack, respectively, see definitions in Table 10.2). Samples taken from > 1 stack or with unknown stack category (cf. Table 10.2) are not numbered. *Salmonella*-positive samples are bold and underlined, *Salmonella*-negative samples are not.

S. Enteritidis was found in 23/68 samples, and all isolates phage typed were PT21, which was also the type found in layers before depopulation of the house. *Salmonella* was distributed evenly both between sample categories (Table 10.2) and geographically (Figure 10.3). Thirty-one Rambach agar plates also had “blue/bluish” colonies (i.e. coliforms) (7/23 and 24/45 among *Salmonella*-positive and *Salmonella*-negative samples, respectively ($p=0.07$)) (data not shown).

After the heat treatment, no *Salmonella* was found in the 68 samples, and all the 68 Rambach agar plates were sterile (i.e. no non-*Salmonella* colonies grew either); reductions in numbers of samples positive for *Salmonella* and coliforms were highly significant ($p=5 \times 10^{-7}$ and $p < 10^{-7}$, respectively). The eight samples taken from air inlets and outlet chimneys after the heat treatment were also *Salmonella*-negative, but all these had blue/bluish (i.e. coliform) colonies on the Rambach agar plates (data not shown).

10.3.2 Temperature and humidity

In House A, mean temperatures at the 11 sites during treatment (191.5 h, i.e. ca. 8 days) were in the range 61-70 °C. However, it took more than 72 h before temperatures were stabilised (Figure 10.4). Maximum temperatures recorded were in the range 68-78 °C, and these were highly correlated to the mean temperatures ($R^2=0.8840$, $p < 0.0001$) with differences in the range 6-10 °C.

In House B, mean temperatures at the ten sites (as probe 2 broke down during the heat treatment, cf. Figure 10.2) in the registration period (ca. 142 h) were in the range 50-59 °C, and it took ca. 30 h before temperatures were stabilised (Figure 10.5). Maximum

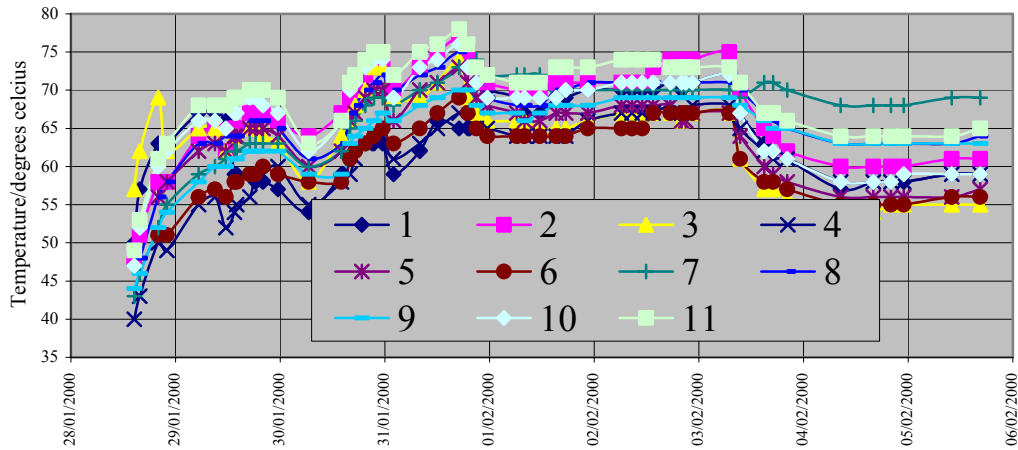


Figure 10.4: House A; temperatures recorded by the 11 probes (cf. Figure 10.1) during the second treatment. For each probe, measurements (marked by symbols) are connected by lines.

temperatures recorded were in the range 61-68 °C, and these were highly correlated to the mean temperatures ($R^2=0.9737$, $p<0.0001$) with differences in the range 8-10 °C.

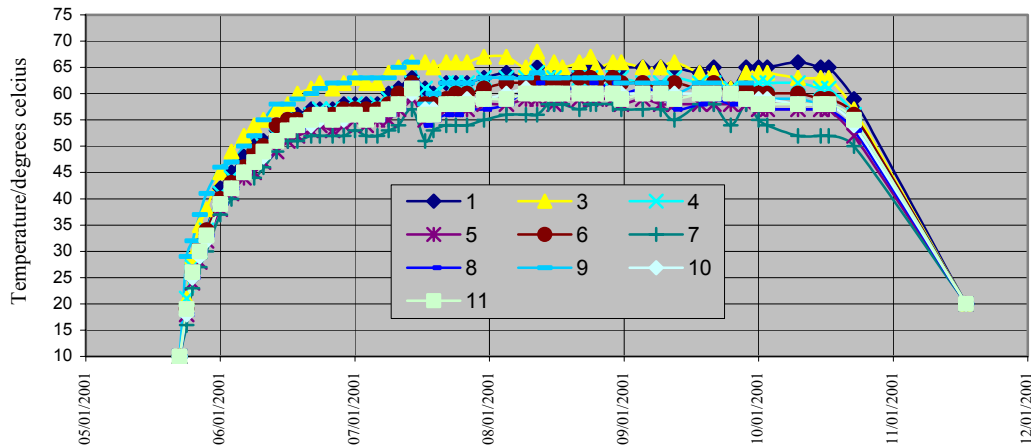


Figure 10.5: House B; temperatures recorded by the ten probes (cf. Figure 10.2) during the treatment. For each probe, measurements (marked by symbols) are connected by lines. The last temperature for all curves (20 °C) was not recorded, but based on information from the farmer.

For both houses, an exact determination of the time before a stable temperature was achieved is difficult, so it is more pertinent to compare temperatures between the houses graphically. The time of commencing the heating was recorded for both houses, so all tem-

perature measurements could be related to this. Moreover, probes 1 and 3-9 were placed at similar sites in the two houses, both related to height, equipment and heating source, so temperatures for these could be compared fairly accurately (Figure 10.6). In House A, the temperatures before stabilisation were more variable than in House B. After stabilisation, the temperatures in House A were generally ca. 10 °C higher than in House B, and the heating was also continued for a longer period in the former.

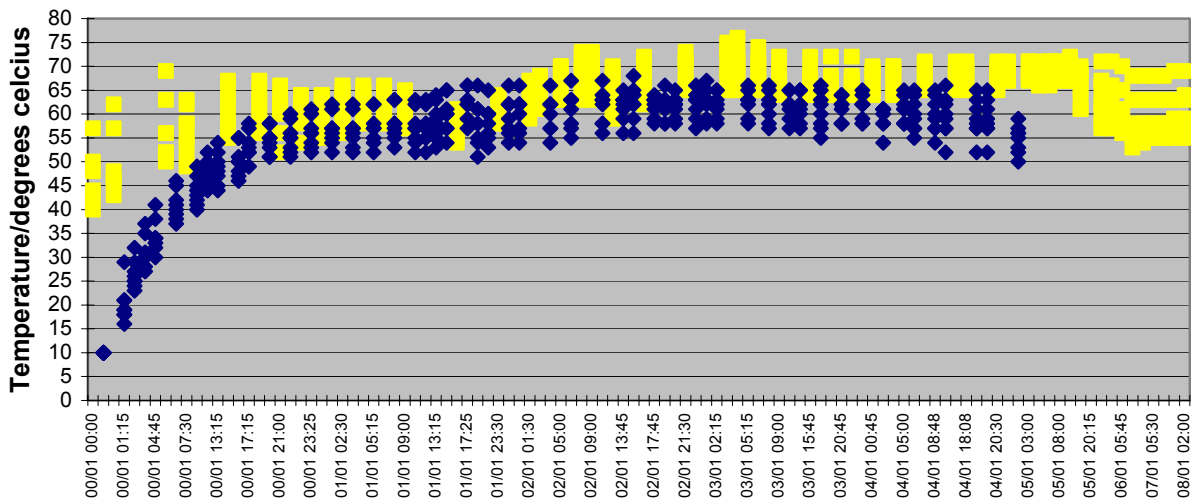


Figure 10.6: Temperatures recorded by probes 1 and 3-9 in Houses A (light squares) and B (dark diamonds). The time scale begins when the first temperatures were recorded in House B, but as the time of commencing the heating was recorded, measurements for the two houses can be plotted in the same figure.

Relative humidity in House B was in the range 55-100, albeit with some fluctuations (Figure 10.7).

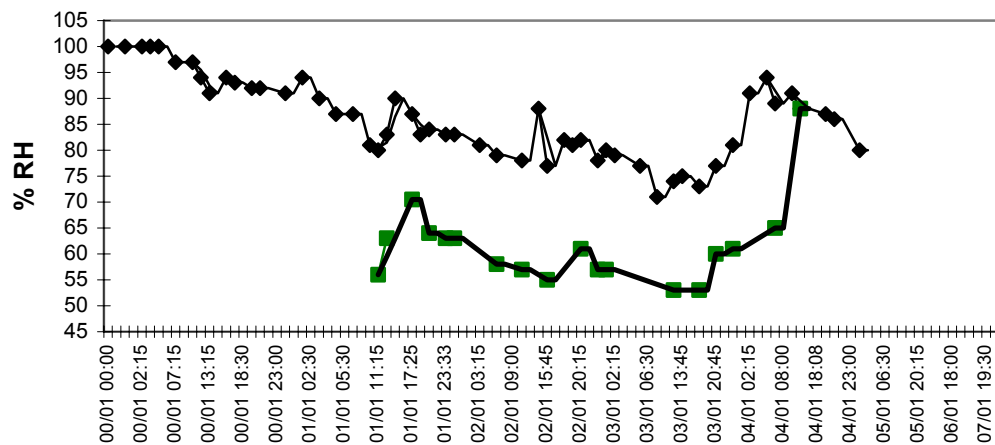


Figure 10.7: House B; relative humidity in the outgoing air (diamonds, connected by a thin line) and at probe 5 (squares, connected by a thick line) (cf. Figure 10.2).

10.4 Discussion

In this field study, different heating procedures were compared between two identical battery cage houses, thus minimising biases related to house structure, equipment and management. However, there were other differences in procedures applied in the two houses, especially related to cleaning:

- House A was dry-cleaned, whereas hot water at 40-50 °C was used in House B.
- After cleaning, more organic matter was found in House A than in House B.
- House A was heated two times, whereas House B was only heated one.
- Formaldehyde was used only in House B.

Thus, although bacteriological results after heating were significantly better in House B than in House A, we can only conclude that the combination of all procedures applied in House B seemed more effective in the elimination of *Salmonella* and coliforms than all procedures applied in House A, but the impact of each of these cannot be quantified. However, humidity was an important difference between the two houses, both in the cleaning

and the disinfection procedures. The effect of humidity has been described both in the literature (cf. Section 4.1.3) and in our laboratory (cf. Chapter 8). The role of formaldehyde was not investigated in this study, but the use of 30 ppm formaldehyde in the steam appears to lower the lethal temperature by 2-5 °C (cf. Chapter 9). This study did not indicate either if the treatment in House B would be equally effective if there had been the same amount of organic matter as in House A, but our laboratory trials showed that moist heat apparently eliminates *Salmonella* and *E. coli* in substantial amounts of organic matter (cf. Chapter 8).

There were slight differences in bacteriological procedures between the two houses, but within each house, the sampling and bacteriological procedures were the same before and after treatment (for which a comparison is most important). This also applied to the use of un-calibrated equipment for measuring temperature and relative humidity, i.e. differences were more important than the numbers *per se*.

The occurrence of “blue/bluish” colonies on Rambach agar plates (i.e. coliforms) also indicates the efficacy of the heat treatment, although the number of samples was too low to evaluate this statistically (also cf. Chapter 9). Moreover, the occurrence of “blue/bluish” colonies on Rambach agar plates and *E. coli* detected in separate procedures from the same samples was associated (also cf. Chapter 8), but more research is needed to evaluate if this is still valid with higher numbers and other types of samples.

In spite of the above reservations, it is clear that moist heat treatment, where mean temperatures were ca. 10 °C lower during a shorter period compared to dry heat treatment, was significantly more lethal to naturally occurring *Salmonella* and coliforms. Thus, this study indicates the importance of high humidity in killing *Salmonella* and coliforms during heat treatment.

Chapter 11 - *Salmonella* persistence in poultry houses and resistance to disinfectants

11.1 Introduction

The epidemiological background of this study is outlined in Section 7.1.

Repeated use of the same types of antibiotics is known to favour the development of antibiotic resistance, but less has been investigated on disinfectants. A small range of disinfectant types are used commonly in the Danish poultry sector, and on many farms, the same disinfectant brand is often used for years in all down periods in all broiler houses. Theoretically, this could induce resistance against the disinfectant used, and this could, at least partly, explain the persistence of certain *Salmonella* serotypes.

Little has been published on the role of efflux mechanisms (cf. Section 3.4.5) with regard to resistance to disinfectants commonly used in the agricultural sector.

Thus, there were several aims in this study. Minimum inhibitory concentration (MIC) studies involving five disinfectants commonly used in the poultry sector were performed for six *Salmonella* serotypes to see if MICs could be related to *Salmonella* persistence or use of disinfectants in Danish broiler houses. *Salmonella* isolates from other sources were included in the MIC-studies to obtain a broader epidemiological perspective. In addition, six isolates, three with high and three with low MICs, were used in adaptation and de-adaptation studies involving the same five disinfectants to see if resistance could be induced or maintained in the laboratory. Finally, triclosan resistant mutants were selected from some isolates, and these mutants were tested for cyclohexane resistance and resistance to the five disinfectants and triclosan to determine if there was some shared resistance mechanism such as possible up-regulation of the *acrAB* efflux pump.

11.2 Materials and methods

11.2.1 Bacterial isolates

Table 11.1 shows the bacterial isolates used in this study.

Table 11.1: Sources of bacterial isolates.

C ¹	Type	n ²	Source and description
DK	<i>Salmonella</i> (<i>S.</i>) Enteritidis	34	Danish broiler houses, “non-persistent type”
DK	<i>S.</i> Typhimurium	39	Danish broiler houses, “non-persistent type”
DK	<i>S.</i> Tennessee	24	Danish broiler houses, “non-persistent type”
DK	<i>S.</i> 4.12:b:-	81	Danish broiler houses, “persistent type”
DK	<i>S.</i> Infantis	61	Danish broiler houses, “persistent type”
DK	<i>S.</i> Indiana	17	Danish broiler houses, “persistent type”
DK	<i>S.</i> Senftenberg	13	Poultry sector
UK	<i>S.</i> Choleraesuis NCTC 10653	1	Strain used in UK disinfection tests
UK	<i>S.</i> Typhimurium, DT104	8	Pig and broiler farms, before and after disinfection with phenol, formaldehyde or peroxygen
UK	<i>S.</i> 4.12:d:-	4	Feed mill and hatchery, before and after disinfection with formaldehyde
UK	<i>S.</i> Senftenberg	4	Hatchery, before and after disinfection with formaldehyde, glutaraldehyde or QAC
UK	<i>E. coli</i> NCTC 10418	1	Control strain
UK	<i>E. coli</i> AG100	1	Control strain
UK	<i>E. coli</i> AG102	1	Control strain, <i>mar</i> mutant of <i>E. coli</i> AG100

¹ Country (DK=Denmark; UK=United Kingdom); ² Number of isolates.

Danish *Salmonella* isolates were stored in Standard Count Agar, whereas UK isolates were stored on Dorset’s egg slopes, all at room temperature. Three strains of *Escherichia coli* (NCTC 10418, AG100, AG102) were used as controls in all MIC-test batches. All UK *Salmonella* isolates, except *S. Choleraesuis* NCTC 10653, were from samples taken both before and after disinfection. Because the UK *S. Senftenberg* isolates had high MICs, Danish *S. Senftenberg* isolates were included to see if this applied generally to this serotype.

11.2.2 Epidemiology of *Salmonella* from Danish broiler houses

The somewhat arbitrary designations “persistent” and “non-persistent” serotypes (cf. Table 11.1) could not be used for statistical evaluations, so more strict definitions were needed. Both “non-persistent” and “persistent” serotypes that had persisted in many or in a few crops, respectively, were included in the study. From most of the houses shown in Table 11.2, two or more isolates were selected, representing both the beginning and the end of the persistence period; a total of 67 and 21 broiler houses were represented with two or more than two isolates of the same serotype, respectively.

Table 11.2: Salmonella serotypes from Danish broiler houses used in this study: numbers of houses and crops with the serotype (period 3/1/92-3/10/01).

<i>Salmonella</i> serotype	Number of crops with the same <i>Salmonella</i> type								
	1	2	3	4	5	6-10	11-20	21-30	> 30
Enteritidis		5 ¹	6	4		2			
Typhimurium	2	7	4	3	1	2	2		
Tennessee		4	4	1	1	2			
4.12:b:-	1	3	2	2	4	6	4	5	1
Infantis		9	2	3	3	6	4		
Indiana	1	1	2	1	1	3			

¹ numbers of broiler houses.

11.2.3 Disinfectants used in the MIC-tests

There were 4,629 *Salmonella*-positive Danish broiler flocks in the period from 3/1/92 to 2/10/01. During this time, a glutaraldehyde/benzalkonium chloride compound, formaldehyde and an oxidising compound were used most commonly for disinfection of broiler houses (38.8, 32.4 and 14.9%, respectively, cf. Figure 2.1), whereas phenols and iodophors were used rarely. In the UK, phenols were used commonly in poultry houses, whereas iodophors were used mainly for water systems, foot dips and general disinfection (R.H. Davies, pers. comm.). Therefore, the following three “Danish” and two “UK” disin-

fectants were chosen for this study: a glutaraldehyde (23% v/v) and benzalkonium chloride (5% v/v) compound (Bio Komplet[®] Plus and the corresponding pH-regulator (KOH and H₃PO₄)), formalin (24.5% v/v formaldehyde), an oxidising compound (Virkon[®] S), a high boiling point tar acid phenol compound (Farm Fluid S[®]) and an iodophor (FAM 30[®]) (see Table 6.1 for details).

11.2.4 MIC-tests

MIC-tests were performed as previously described (Randall *et al.* 2001). On the day of performing the MIC-tests, pH-regulator was added to Bio Komplet Plus (1:11), and disinfectant solutions in sterile deionised water were prepared and used for double dilutions. Ranges of 0.002-0.25 ml, 0.008-1.00 g and 0.008-1.00 ml 100 ml⁻¹ were made for formaldehyde, Virkon S and the remaining disinfectants, respectively. For all *Salmonella* isolates, the tests were performed at least in duplicate on different days. The *E. coli* control strains were included in each batch to check for deviations between batches.

11.2.5 Disinfectant adaptation and de-adaptation tests

Six isolates, three with high and three with low MICs (cf. Table 11.5; parent isolates that did not grow in cyclohexane), were used for the adaptation and de-adaptation tests which were performed in duplicate, each involving one of the five disinfectants. Initially, isolates were grown overnight in 3.0 ml LB broth at 37 °C. A 0.1 ml inoculum was passaged to 3.0 ml LB broth with a disinfectant concentration of half the lowest recorded MIC, incubating overnight at 37 °C. Each consecutive day, the disinfectant concentration in LB broth was increased by a factor of 1.5, and a 0.1 ml inoculum from the LB broth grown the previous day was inoculated into this. Turbidity was checked visually, and cultures were plated on BA plates to check for growth and purity. The passages ceased when no turbidity and no growth on BA were observed. LB broth (1.5 ml) with growth at the

highest disinfectant concentration was transferred to an Eppendorf tube and centrifuged for 5 min at 15,890 x g. The pellet was suspended in physiological saline to McFarland 0.5 and used for MIC-tests as described above. For de-adaptation, 0.1 ml LB broth was passed to 3.0 ml LB broth without disinfectant for six consecutive days, after which the MIC-tests were repeated.

11.2.6 Triclosan studies

Strains up-regulated for efflux (cf. Section 3.4.5) are likely to show reduced susceptibility to the biocide triclosan (Levy 2002), but little is known about a putative association between efflux type resistance and resistance to the five disinfectants of this study.

An amount of 32 mg triclosan was weighed in 5.0 ml sterile ELGA water, and 1 N NaOH was added drop by drop until all triclosan was dissolved; then sterile ELGA water was added up to 4000 ml, yielding 8 µg ml⁻¹ triclosan.

The six isolates from the adaptation and de-adaptation studies (cf. Section 11.2.5) and the one isolate that grew in cyclohexane (cf. Section 11.2.7) were used to select isolates for growth at high triclosan concentrations. Agar plates were made with DSA and the 8 µl ml⁻¹ triclosan solution (1:1), thus yielding 4.0 µg ml⁻¹ triclosan. Isolates were grown overnight in 500 ml LB broth at 37 °C. Forty-five ml LB broth culture was centrifuged five times for 20 min at 4,388 x g, each time discarding the supernatant and adding more broth culture. Then, the final pellet was resuspended in 2.5 ml physiological saline, and tenfold dilutions were plated on BA to determine numbers of CFU ml⁻¹. For each isolate, 0.1 ml of the undiluted suspension was spread on each of five agar plates containing 4.0 µg ml⁻¹ triclosan, incubating 42-48 h at 37 °C after which CFU were counted. For each isolate, one resistant mutant colony was isolated and used for MIC-tests. The parent isolates, their tri-

closan resistant counterparts and the *E. coli* control strains were used for MIC-tests with the five disinfectants and triclosan, performed in duplicate on different days.

For the same seven isolates, adaptation and de-adaptation studies with triclosan were made in duplicate as for the other five disinfectants (cf. Section 11.2.5), beginning with $0.13 \mu\text{g ml}^{-1}$ triclosan (dissolved in NaOH as described above). The adaptation studies were discontinued on day 20, as so much NaOH had to be used for dissolving triclosan for day 21 that pH of the agar became too alkaline. After adaptation, MIC-tests were performed in duplicate for the parent and the adapted isolates and the *E. coli* control strains against both the five disinfectants and triclosan (double dilutions in the range 0.03-128.8 mg L⁻¹ (pH of the latter was 6.8)). After de-adaptation, only triclosan was used in the MIC-tests, as no changes were seen for the other five disinfectants after adaptation, and the parent isolates were omitted in these studies.

11.2.7 Cyclohexane resistance tests

Strains with up-regulation of efflux have been shown to be cyclohexane resistant (White *et al.* 1997). As such, cyclohexane resistance is a useful marker for strains with up-regulated efflux (Randall *et al.* 2001), and such strains may show resistance to unrelated antibiotics and disinfectants. For this reason, 60 isolates, representing high MICs for different disinfectants, were selected for cyclohexane resistance tests as described by Asako *et al.* (1997). In addition, cyclohexane tests were performed for all the triclosan mutant isolates. All tests were performed in duplicate.

11.2.8 Statistical analysis

All data were entered in an Access database (Anonymous 1997a).

For each disinfectant and for *Salmonella* and *E. coli* separately, Kruskal-Wallis one-way analysis of variance of the MICs was performed (Anonymous 2002g). For significant

bacteria/disinfectant combinations, Dunn's method was used for multiple comparisons between the bacterial types (Anonymous 2002g), as this method sets an error level for the whole set of comparisons, thus avoiding too many 'significant' differences due to the many comparisons.

In order to obtain 2x2 Tables for pair wise comparisons and increases/decreases in MICs during the persistence period, MICs were merged (formaldehyde: 0.004/0.008 vs. 0.015/0.030; Bio Komplet Plus: 0.060 vs. 0.125/0.250; Virkon S: 0.060/0.125 vs. 0.250; Farm Fluid S: 0.015/0.030 vs. 0.060/0.125; FAM 30: 0.060/0.125 vs. 0.250/0.50). Putative associations were tested by McNemar chi-square tests (Martin *et al.* 1987) and by Cohen's kappa (Sackett 1992). Increases and decreases in MICs during the persistence period were tested by chi-square tests. A significance level of 5% was used in all relevant tests.

11.3 Results

11.3.1 MIC-tests

MIC-test and statistical results are shown in Tables 11.3 and 11.4, respectively.

Table 11.3: Minimum inhibitory concentrations (MICs) of isolates.

Country/ isolate ¹	Formaldehyde				Bio Komplet Plus					Virkon S				Farm Fluid S				FAM 30																																												
	MIC (x10 ⁻³) ²				MIC (x10 ⁻³)					MIC (x10 ⁻³)				MIC (x10 ⁻³)				MIC (x10 ⁻³)																																												
	4	8	15	30	15	30	60	125	250	30	60	125	250	15	30	60	125	60	125	250	500																																									
DK/Ent	34 ³				14					20					6				22				6				1				12				21				4				9				21															
DK/Typ	39				20					17					2					12				24				3				19				20				7				15				17														
DK/Ten	9				8				7				6					16					2				1				7				16				4				20				2				22											
DK/4.12:b:-	66				15				12					68					1					1				58				22				17				64				1				13				66				1						
DK/Inf	1	60				18					43					6				37				18				29				31				1				17				37				7														
DK/Ind	16				1				11					6					7				9				1				16				1				14				3																			
DK/Sen					12				1				5					8									10				3				1				12				10				3															
UK/Chol	1									1					1									1									1																													
UK/DT104	8									7					1									8									8									2				6																
UK/4.12:d:-	4									4										4									4													4																				
UK/Sen					4									4										4									4													4																
UK/NCTC	1				30				12					17									1				19				11				2				26				4								6				25				1			
UK/AG100	8				23				5					24					1					3				13				14				6				25				1				12				17				1						
UK/AG102	11				20									26					4					1				13				16				2				28				1				6				23				1						

¹ Cf. Table 11.1. ² ml 100 ml⁻¹, except g 100 ml⁻¹ for Virkon S. ³ No. of isolates/batches for *Salmonella* and *E. coli*, respectively.

Table 11.4: Statistical results for MICs of isolates.

<i>Salmonella</i>		
Disinfectant	Kruskal-Wallis overall p	Individual differences (p<0.05)
Formaldehyde	<0.001	DK/Ten > DK/Ent, DK/Typ, DK/4.12:b:-, DK/Inf, DK/Ind, UK/DT104 ¹ DK/Sen > DK/Ent, DK/Typ, DK/4.12:b:-, DK/Inf, DK/Ind, UK/DT104, UK/4.12:d:- UK/Sen > DK/Ent, DK/Typ, DK/4.12:b:-, DK/Inf, DK/Ind, UK/DT104, UK/4.12:d:-
Bio Komplet Plus	<0.001	DK/4.12:b:- > DK/Typ, DK/Ind UK/DT104 > DK/Ind
Virkon S	<0.001	DK/Ten > DK/Ent, DK/Typ, DK/Ind DK/4.12:b:- > DK/Typ, DK/Ind DK/Inf > DK/Typ UK/Sen > DK/Typ, DK/Ind
Farm Fluid S	<0.001	DK/Ind < DK/Ent, DK/Ten, DK/4.12:b:-, DK/Inf, DK/Sen, UK/DT104, UK/4.12:d:-, UK/Sen
FAM 30	<0.001	DK/Ten > DK/Typ, DK/Ind, DK/Sen DK/4.12:b:- > DK/Typ, DK/Ind, DK/Sen DK/Inf > DK/Typ, DK/Ind, DK/Sen UK/Sen > DK/Ent, DK/Typ, DK/Inf, DK/Ind, DK/Sen
<i>E. coli</i>		
Disinfectant	Kruskal-Wallis overall p	Individual differences (p<0.05)
Formaldehyde	0.007	NCTC > AG102
Bio Komplet Plus	<0.001	AG100, AG102 > NCTC
Virkon S	<0.001	NCTC > AG100, AG102
Farm Fluid S	0.102	-
FAM 30	<0.001	NCTC > AG100, AG102

¹ x>y/x<y: x has significantly higher/lower MICs than y. Designations for isolates. cf. Table 11.3.

For both *Salmonella* and *E. coli* and for all disinfectants, the Kruskal-Wallis analysis was significant, except for *E. coli* and Farm Fluid Super (cf. Table 11.4).

Among the isolates from Danish broiler houses, multiple comparisons generally showed that significantly higher MICs were observed for *S. Tennessee* to formaldehyde, Virkon S and FAM 30, for *S. 4.12:b:-* to Bio Komplet Plus, Virkon S and FAM 30, and for

S. Infantis to Virkon S and FAM 30. Thus, higher MICs were found for both “non-persistent” and “persistent” serotypes, and included “UK” disinfectants.

Among isolates not coming from Danish broiler houses, *S. Senftenberg*, both from DK and the UK, had high MICs to formaldehyde, and the UK *S. Senftenberg* had high MICs to Virkon S and FAM 30. Among the *S. Typhimurium* DT104 isolates, there were generally few deviations from the general distribution although five isolates were resistant to at least six types of antibiotics (data not shown). The MICs of all the UK isolates were the same before and after disinfection. MICs for *S. Choleraesuis* NCTC 10653 did not deviate significantly for any of the five disinfectants, indicating it is representative for *Salmonella* in the official UK DEFRA disinfection tests.

For formaldehyde, most isolates differed from the isolates with high MICs, whereas fewer isolates differed from the high MIC isolates for the other four disinfectants (range 1-3, except for the UK *S. Senftenberg* and FAM 30).

Among the *E. coli* control strains, AG100 and AG102 generally had similar MICs, so *mar* did not seem to play a significant role. Compared to these two isolates, *E. coli* NCTC 10418 had significantly higher (formaldehyde, Virkon S, FAM 30) or lower (Bio Komplet Plus) MICs.

Disinfectants were compared pair wise (MICs merged, cf. Section 11.2.8) to deduce putative associations for isolates. Only Bio Komplet Plus vs. FAM 30 ($\chi^2 = 1.96$, Cohen’s kappa = 0.59) and Farm Fluid S vs. FAM 30 ($\chi^2 = 0.31$, Cohen’s kappa = 0.60) showed significant associations (data not shown).

For each of the 67 and 21 broiler houses with two or more than two isolates, respectively, increases and decreases in MICs during the persistence period were recorded. For all five disinfectants merged, there were 93 increases and 82 decreases, but none of the five

disinfectants deviated significantly from this (Virkon S had the lowest p (0.10) with 32 increases and 19 decreases) (data not shown).

For the three “Danish” disinfectants (formaldehyde, Bio Komplet Plus, Virkon S), cross-tabulations between their use in the preceding download period and merged MICs (cf. Section 11.2.8) were made, but no significant differences in MICs were seen between crops using or not using the actual disinfectant (Virkon S had the lowest p (0.18), but the RR of high MICs was 1.16 if Virkon S was not used in the preceding download period) (data not shown).

11.3.2 Disinfectant adaptation and de-adaptation tests

In LB broth, growth ceased at concentrations up to ca. 13xMIC (highest range for formaldehyde (5.5-12.5) and Virkon S (3-12.5), lowest range (0.7-4.2) for Bio Komplet Plus) (data not shown). This was, however, not reflected in similar high MICs after adaptation, where all isolates except one were within one double dilution compared to the MICs of the parent isolates (data not shown). Four of the six isolates adapted to Virkon S grew weakly in the last LB broth before growth ceased, so a pellet big enough to obtain McFarland 0.5 could not be obtained, and growth could not be re-established in the six de-adaptation passages. Moreover, four isolates after adaptation did not grow in the MIC-tests with formaldehyde, regardless of which disinfectant they had been adapted to, but all of these could be tested after de-adaptation. After de-adaptation, no changes beyond one double dilution compared to the MICs of the parent isolates were seen (data not shown).

11.3.3 Selection of triclosan resistant mutants

Table 11.5 (which also refers to Sections 11.3.4 and 11.3.5) shows results for the seven isolates used in the triclosan resistance studies.

Table 11.5: Data for the seven isolates used in mutant, adaptation and de-adaptation studies with triclosan.

Isolate	Type	GIC ¹	Original MIC (F/B/V/S/M) ²	Mutation rate ³	LDG ⁴	MIC ($\mu\text{g ml}^{-1}$) to triclosan		
						Parent isolate	Mutant isolate	After adaptation de-adaptation
7266444	<i>S. Enteritidis</i>	No	(8/60/60/30/125)	4.9×10^{-10}	20/20 ⁵	0.25/0.25/0.125 ⁶	32.2/32.2 ⁷	<u>>128.8/</u> <u>>128.8</u> ⁵
9675922	<i>S. Infantis</i>	No	(8/60/60/30/125)	1.4×10^{-10}	20/20	0.25/0.25/0.125	32.2/16.1	>128.8/>>128.8
9578243	<i>S. Tennessee</i>	No	(8/60/60/30/125)	5.2×10^{-10}	1/8	0.50/0.25/0.25	32.2/32.2	0.50/0.50
S 4880 98	<i>S. Typh.</i> , DT104	No	(8/125/125/60/250)	1.3×10^{-10}	20/20	0.50/0.25/0.25	32.2/32.2	>128.8/>>128.8
9572762	<i>S. Tennessee</i>	No	(30/125/250/60/250)	3.7×10^{-9}	20/20	0.50/0.25/0.25	32.2/32.2	<u>32.2/</u> <u>>128.8</u>
S 8827 97	<i>S. Senftenberg</i>	No	(30/125/250/60/500)	2.0×10^{-9}	8/20	0.50/0.25/0.25	>128.8/32.2	<u>2.0/</u> <u>>128.8</u>
9577210	<i>S. Tennessee</i>	Yes	(30/250/250/60/250)	2.0×10^{-8}	20/20	2.0/1.0/0.50	<u>64.4/</u> <u>128.8</u>	<u>>128.8/</u> <u>>128.8</u>

¹ Growth in cyclohexane (parent isolates).

² Units, cf. Table 11.3. F = formaldehyde; B = Bio Komplet Plus; V = Virkon S; S = Farm Fluid Super; M = FAM 30.

³ Proportion of cells growing on agar containing $4.0 \mu\text{g ml}^{-1}$ triclosan, cf. text.

⁴ Last day of growth during adaptation to triclosan.

⁵ Replicate 1/replicate 2 (applies to the entire column).

⁶ All results shown in triplicate (applies to the entire column).

⁷ Day 1/day 2 (same isolate used both days) (applies to the entire column).

MICs for mutant, adapted and de-adapted isolates: growth in cyclohexane in both duplicate tests;
growth in cyclohexane in one duplicate test; no growth in cyclohexane in either of the duplicate tests.

After centrifugation, the concentration range for the seven isolates was $1.4\text{--}2.0 \times 10^{10}$ CFU ml⁻¹. On the five agar plates, 1/1/4/4/15/37/143 CFU grew per isolate, thus yielding mutation rates in the range from 5.2×10^{-10} to 2.0×10^{-8} (highest for the isolate that grew in cyclohexane).

For the five disinfectants, virtually all changes in MICs for the mutants and adapted isolates were within one double dilution compared to the seven parent isolates (data not shown), whereas there were big increases in MICs for triclosan.

11.3.4 Triclosan adaptation and de-adaptation tests

Most isolates grew until day 20 of the adaptation, as only three replicates ceased their growth before (one on day 1 and two on day 8). In general, the MICs to triclosan after adaptation and de-adaptation correlated well with the number of days they grew, except that the second replicate of isolate 9578243 illogically had a low MIC after adaptation whereas it was high after de-adaptation.

11.3.5 Cyclohexane resistance

All isolates used in cyclohexane resistance studies grew on agar without organic solvent. In nearly all batches, *E. coli* AG102 grew in both cyclohexane and n-hexane, *E. coli* AG100 grew in only n-hexane, whereas *E. coli* NCTC 10418 did not grow in either of these.

All 60 isolates were resistant to n-hexane, but only one isolate was resistant to cyclohexane.

Among the seven isolates used for the triclosan studies, only the 1/60 isolate growing in cyclohexane was cyclohexane resistant after selection on agar plates with $4.0 \mu\text{g ml}^{-1}$ triclosan. However, most of the isolates that grew until day 20 in the triclosan adaptation studies became cyclohexane resistant, and this was maintained after de-adaptation. All

cyclohexane resistant isolates also grew in n-hexane, whereas a few of the cyclohexane sensitive isolates did not grow in n-hexane either (data not shown).

11.4 Discussion

Theoretical aspects related to this study are mainly described in Sections 3.4 (except Section 3.4.4 that deals with biofilms), 4.2.1 and 5.4.4.1.

The disinfectants probably reacted with compounds in the agar, e.g. glutaraldehydes binding to peptones. Therefore, it is more pertinent to validate the MIC variations than the MICs *per se*. In general, there were few variations in MICs to the five disinfectants used commonly in the Danish or the UK poultry sector. There were no obvious associations between MICs on one side and tendencies to persist or the use of relevant disinfectants on the other. A few serotypes (*S. Tennessee*, *S. 4.12:b:-* and *S. Senftenberg*) tended to have higher MICs to some disinfectants, but not necessarily the ones they had previously encountered. In general, these results confirm other studies (cf. Section 3.4.6.2), i.e. resistance could not be linked epidemiologically to persistence or the frequent use of a few types of disinfectants. Thus, the higher MICs of some serotypes seem to be intrinsic.

This study differed from most of the others (cf. Section 3.4.6.2) by focusing on the primary agricultural sector and disinfectants normally considered more potent (hence normally not allowed in e.g. food premises or hospitals). As many disinfectants were combination products, and ancillary substances (e.g. methanol in the formaldehyde solution) are detrimental *per se*, we do not know if the same mechanisms are involved for related disinfectants. Formaldehyde and glutaraldehyde are both aldehydes, but *S. 4.12:b:-* only had higher MICs to Bio Komplet Plus, whereas *E. coli* NCTC 10418 had higher MICs to formaldehyde but lower MICs to Bio Komplet Plus, maybe because BC in the latter disinfect-

tant also influences the MICs. These and other differences in MICs illustrate the different uptake and resistance mechanisms that may be involved (Maillard 2002).

No increase in MICs was seen after growth in increasing concentrations of the five disinfectants, for which few previously published adaptation and de-adaptation studies exist to our knowledge (cf. Section 3.4.6.1). These results confirm the MIC-tests in which no clear development of resistance under real-life conditions was seen either.

The *mar*-locus has been shown to be involved in resistance to some disinfectants (pine oil, triclosan, certain oxidative stress agents) as well as the organic solvent cyclohexane (Asako *et al.* 1997; Randall and Woodward 2002) (cf. Section 3.4.5), but its role for disinfectants more relevant in the agricultural sector has not been elucidated. In this study, results suggested that MAR type resistance was not involved in resistance to the five disinfectants: MICs were very similar between *E. coli* AG100 and AG102 (the *mar*-mutant of AG100). Cyclohexane resistance was only observed in 1/60 isolates selected for their high MICs. Several isolates that became resistant to triclosan also developed resistance to cyclohexane. This latter resistance pattern is known as MAR type resistance, suggesting up-regulated efflux, but only genotypic studies can elucidate which genes (e.g. *acrAB*, *marRAB* or *soxRS*) were up-regulated. Finally, it was apparent that isolates that developed the MAR phenotype did not show increased resistance to the five disinfectants.

In conclusion, the small variations in MICs to disinfectants used commonly in the poultry sector could not be related clearly to persistence of *Salmonella* or the use of disinfectants. Adaptations to these disinfectants did not alter MICs, and efflux type resistance mechanisms as indicated by organic solvent resistance did not seem to be involved in their higher MICs.

Chapter 12 – Surface disinfection tests mimicking

worst-case scenarios in poultry houses

12.1 Introduction

The scientific literature on disinfection of animal houses is sparse. Amongst other things, information on specific disinfectants can often only be obtained from the companies that produce or market these. Most scientific disinfection studies are from the food industry or hospitals where other conditions prevail and different disinfectants are used, making it difficult to extrapolate results to the agricultural sector.

Poultry houses with the most inaccessible equipment belong to the table egg sector, especially battery cage houses that are the most difficult to clean properly. In general, the efficacy of disinfectants increases with higher temperatures, but lower temperatures are often encountered in the down periods. These and other factors often experienced in real-life situations (cf. Section 4.4) advocate an investigation into borderline conditions, not in order to encourage poor disinfection procedures, but to obtain information on safety margins when cleaning and disinfecting poultry houses. In addition, it is pertinent to know more about poultry house materials and types of organic matter in which *Salmonella* is most difficult to eliminate by disinfection, so more attention can be put on critical control points.

Due to the above considerations, this surface disinfection study was set up to simulate disinfection procedures and worst-case scenarios in empty poultry houses, i.e. considerable amounts of organic matter with high numbers of bacteria and high contents of protective compounds, low temperatures, and materials found in poultry houses that are difficult to clean properly.

12.2 Materials and methods

12.2.1 General principles

Bacteria (*S. Enteritidis*, *S. Senftenberg* or *E. faecalis*) were grown to stationary phase, and high numbers were spiked in organic matter (feed, fat or egg yolks), which was either smeared on the surface of the materials (feed on concrete flags) or the materials were immersed in the organic matter with bacteria (feed chain links, wooden dowels, jute egg belts). Materials with spiked organic matter were then dried for 24 h at a set temperature. Thereafter, they were immersed in a 1% disinfectant solution (formaldehyde, glutaraldehyde/benzalkonium chloride or oxidizing compound) or water (control) for exact time periods after which they were re-dried 25 h at a set temperature. For detection of survivors, all materials with organic matter were immersed in enrichment broth (10^0 dilution) which was also used for 10-fold dilutions until 10^{-4} . During the incubation period, all materials were left in the 10^0 dilutions. Traditional bacteriological procedures were used for all dilutions, i.e. a most probable number (MPN) method was used.

12.2.2 Bacterial isolates

S. Enteritidis, PT8, was the most common type in persistently *Salmonella* infected Danish table egg houses. Moreover, this isolate had relatively low MICs against five disinfectants, including the three of this study; on the other hand, *S. Senftenberg* was chosen because this serotype had relatively high MICs (cf. Chapter 11). *E. faecalis* was used in some series with the worst conditions to test its use as an indicator organism for future field studies. All isolates were made rifampicin resistant (cf. Section 6.10). They were stored as described in Section 6.6. Isolate numbers are shown in Table 6.2.

12.2.3 Poultry house materials and organic matter

Four types of materials (concrete flags, feed chain links, wooden dowels, jute egg belts) and three types of organic matter (feed for layers, rape seed oil, egg yolk) were used (Table 12.1).

Table 12.1: Poultry house materials and organic matter used in the study.

Main category	Description	Comments, incl. company
Poultry house materials	Concrete flags	The mason company Henriksen og Madsen A/S, 9670 Løgstør, Denmark, specializes in making concrete floors for poultry houses. Concrete flags (25x25 cm) with a surface as in poultry houses were made for this study. The flags were ca. two years old before they were used.
	Feed chain links	Second-hand feed chain link pairs (i.e. two links per unit) made of steel (not stainless), donated by M.H. Jensen Maskinfabrik, 8850 Bjerringbro, Denmark.
	Wooden dowels	Ribbed beech wood dowels, 40x10 mm, Bosch, cat. no. 2609250449-710.
	Jute egg belts	America A/S, 7700 Thisted, Denmark, cat. no. 973, width 9 cm. Pieces of 6 cm were cut for use.
Organic matter	Feed for layers	Korn- og Foderstofkompagniet A/S, 6440 Augustenborg, Denmark, code no. 3103. Free from antibiotics and coccidiostatics.
	Rape seed oil	Colzawar TM 32 (cat. no. 26173200-D) (melting point 32 °C), donated by Aarhus Olie, 8100 Aarhus C, Denmark.
	Egg yolk	Grade A battery cage eggs from Hedegaard Foods, 9560 Hadsund, Denmark, bought in the local supermarket, not more than five days prior to each test. Pure yolk could not be obtained from egg packing stations, as these automatically add preservatives to the yolk immediately after breaking the eggs.

These materials are often found in poultry houses, and they have rough surfaces that are difficult to clean and disinfect properly. Only some combinations of materials and organic matter (concrete flags/feed, feed chain links/fat, feed chain links/feed, wooden dowels/fat, wooden dowels/feed, jute egg belts/egg yolk) were included in the study, as it was most pertinent to simulate conditions likely to be found in poultry houses, and resources did not allow the design of a full factorial study.

12.2.4 Preparation of spiked organic matter

Isolates were grown in LB broth 18-24 h at 37 °C, i.e. until stationary phase. LB broth was diluted 1:10 in BPW, which was further diluted in sterile deionised water, 1:10 or 1:100 to obtain high or low numbers of CFU, respectively, in the organic matter. Feed

for layers (cf. Table 12.1) was added to the LB/BPW/deionised water mixture in the proportion 1:2 (w/v). After 1 h, this mixture was shaken vigorously until it had a porridge-like consistency. The same LB/BPW/deionised water mixture was used for spiking egg yolks. For each sample, two eggs (cf. Table 12.1) were broken, the whites were discarded, the yolks were poured into a measuring glass, and the volume was recorded. The same volume of LB/BPW/deionised water mixture was added, and the whole mixture was poured into a jar, lidded and shaken vigorously. The rapeseed oil (cf. Table 12.1) was melted at 42 °C, after which 600 ml was mixed with 2.0 ml stationary phase culture LB broth and shaken vigorously.

12.2.5 Inoculation of materials with spiked organic matter

Concrete flags and feed chain links were re-cycled and washed between the tests, whereas the other materials were discarded after each test. All materials were sterilized before each test series. For concrete flags, an amount of 10.25 (± 0.25) or 20.15 (± 0.17) g spiked feed was distributed evenly with sterile scrapers on an area of 20x20 cm, after which each flag was put in a tub with the inoculated surface up. All other materials were immersed in the relevant spiked organic matter, shaken and left for varying time periods (feed, 15 min; fats, 5 min; egg yolk, 1 h, shaken with 20 min intervals). Thereafter, they were placed on stainless steel trays.

12.2.6 Incubation before and after disinfection

Before and after disinfection, all materials with spiked organic matter were placed in a cooling incubator (Binder KB 115, Binder GmbH, 78532 Tuttlingen, Germany) or a climatic cabinet (Termaks KBP 6395, Termaks, 5057 Bergen, Norway) set at pre-determined temperatures, using the same cabinet type for all samples in a series. Temperature and relative humidity were logged at 10-min intervals (Testo 175 loggers, Testo, 79853 Lenzkirch,

Germany). All materials remained in the cabinets exactly 24 and 25 h before and after the disinfection procedures, respectively.

12.2.7 Disinfection procedures

There is reliable information on the use of disinfectants in Danish broiler houses (cf. Sections 2.4 and 11.2.3 and Figure 2.1). The use of disinfectants in Danish layer houses has not been recorded systematically, but it is our experience that the same three disinfectant types are also used most commonly in these. Therefore, the following three disinfectants were chosen for this study: Bio Komplet® Plus, formalin and Virkon® S (see Table 6.1 for details). WHO water was used both for controls and for disinfectant solutions, all of which were 1% (v/v for Bio Komplet Plus and formaldehyde, w/v for Virkon S). At least 12 h before the disinfection procedures, all WHO water was placed at the temperature used for incubating materials prior to disinfection. All disinfectant solutions were made on the day of use. Bio Komplet Plus was mixed 1:11 with its pH-regulator before further dissolving in WHO water. Two hundred ml disinfectant solutions were used for one concrete flag, three wooden dowels or one jute egg belt piece, in a tub for the concrete flag, in jars for the other materials, whereas a 250 ml solution was used for two feed chain link pairs, except in one series (cf. Table 12.3). All materials with spiked organic matter were immersed in disinfection solutions for exact time periods (cf. Tables 12.2-12.5) before they were placed in new tubs (concrete flags) or on new stainless steel trays (other materials).

12.2.8 Microbiological procedures

All materials with spiked organic matter were immersed individually in pre-enrichment broth, *Salmonella* in BPW and *E. faecalis* in Enterococcus broth, wooden dowels in 20.0 ml vials, concrete flags with the surface down in 200 ml in tubs, the others in 200 ml jars. Materials were left in the broth for 30 (concrete flags/feed, wooden dow-

els/feed), 60 (feed chain links/feed, jute egg belts/egg yolk) or 120 min (feed chain links/fat, wooden dowels/fat) before dilution so organic matter could be dispersed in the broth. Vessels containing materials with fat were placed at 37 °C to let the fat become fluid. After these time periods, vessels were shaken vigorously and feed was scraped from the surface of concrete flags with a sterile Drigalski spatula before dilution. Tenfold dilutions were made until 10^{-4} , using BPW and Enterococcus broth for *Salmonella* and *E. faecalis*, respectively. All vessels were lidded and tubs with concrete flags sealed with plastic bags during incubation with all materials remaining in the 10^0 dilutions. Further procedures for *Salmonella* and *E. faecalis* were as described in Sections 6.7.2 and 6.9, respectively. From the lowest of all dilutions with suspect colonies, rifampicin resistance was checked with Neo-sensitabs Rifampicin (Anonymous 1998).

12.2.9 Statistical analysis

All data were entered in an Access database (Anonymous 1997a). Though the number of CFU could be calculated, this gave no meaning for materials other than the concrete flags, as the amount of spiked organic matter was unknown for these. It was therefore more relevant to derive a point system related to growth in the dilutions: if no growth was seen in any dilutions, 0 points were given, growth in only 10^0 scored 1 point, growth in only 10^0 and 10^{-1} scored 2 points and so on, i.e. a scale from 0 to 5 points was derived. A few series had some illogical growth patterns, as there was no growth in a dilution in between dilutions with growth. In these series, x.5 points were given, where x was the dilution above the dilution without growth (e.g. growth in 10^0 , 10^{-1} , 10^{-2} and 10^{-4} , but not in 10^{-3} , would render 4.5 points). This point system was first used for a Friedman's two-way ANOVA to see if there were overall differences, after which individual differences were compared by Tukey's HSD test (Anonymous 2002g). In addition, in order to test for statistical signifi-

cance in a traditional *Salmonella* presence/absence test, 2x2 tables were made by merging 1-5 point samples, comparing 0 vs. >0 points by chi-square or two-tailed Fisher exact tests (for expected values <5). For each isolate, disinfectants were compared pair wise, and isolates were compared pair wise for each disinfectant in series where both isolates were included (cf. Tables 2-5). Statistical tests were not made between types of poultry house materials or organic matter, as other conditions (e.g. different temperatures before and after disinfection) also differed between these. All tests were made with the individual replicate points using a significance level of 5% ($p=0.05$).

12.3 Results

Tables 12.2-12.5 show the results. All positive samples had agar plates with pure cultures of suspicious colonies. All tested colonies were fully rifampicin resistant and selected suspect *Salmonella* colonies reacted with relevant O-antisera. Only significant differences will be commented here.

As all of the F-tests for the Friedman's ANOVA test were significant ($p<0.001$), individual comparisons using the Tukey's HSD test could be made. For *S. Enteritidis*, both formaldehyde (F) and Bio Komplet Plus (B) were more effective than Virkon S (V) ($p<0.001$ and $p=0.002$, respectively) and WHO water (W) (both $p<0.001$). For *S. Senftenberg*, the three disinfectants were more effective than W ($p<0.011$ for F and B; $p=0.013$ for V) and F was more effective than V ($p=0.005$). For *E. faecalis*, all three disinfectants were more effective than W (F: $p=0.001$; B: $p<0.001$; V: $p=0.007$). Pair wise comparisons between the bacteria only showed significance when *S. Enteritidis* and *S. Senftenberg* were compared for Virkon S, as the former was more resistant ($p=0.023$). Though no significant differences were detected, *E. faecalis* generally had higher mean points than either *Salmonella* isolates.

Table 12.2: Results for concrete flags inoculated with feed for layers (disinfection conditions deteriorating towards the bottom of the table).

CFU ¹	GPF ²	TB ³	TA ⁴	DT ⁵	S. Enteritidis			S. Senftenberg				
					F ⁶	B ⁶	V ⁶	WHO ⁶	F	B	V	WHO
Low	10.0-10.5	20.2 (±0.2)	10.9 (±0.5)	30	0 ⁷	0	0	1	0	0	0	2
Low	20.0-20.3	20.2 (±0.2)	10.9 (±0.5)	30	0	0	4	ND ⁸	0	0	0	2
Low	20.0-20.3	10.9 (±0.5)	10.9 (±0.5)	30	0	0	3	3	0	0	2	2
Low	20.0-20.3	5.9 (±0.5)	5.9 (±0.5)	30	0	1	3	5	0	0	1	1
Low	20.0-20.3	5.9 (±0.5)	5.9 (±0.5)	15	0	0	2	4	0	0	2	1
High	20.0-20.3	5.9 (±0.5)	5.9 (±0.5)	15	0/0	0/1	4.5 ⁹ /4	5/5	0	0	2	2
High	20.0-20.3	5.9 (±0.5)	5.9 (±0.5)	5	0	2	4	5	0	1	2	5

¹ Low = ca. 4×10^3 - 6×10^6 g⁻¹ organic matter; High = ca. 4×10^6 - 6×10^7 g⁻¹ organic matter (cf. Section 8.3.2.2).

² G organic matter per flag.

³ Mean temperature (± deviations merged for all series) (°C) during 24-h period before disinfection.

⁴ Mean temperature (± deviations merged for all series) (°C) during 25-h period after disinfection.

⁵ Disinfection time (minutes).

⁶ F = formaldehyde; B = Bio Komplet[®] Plus, V = Virkon[®] S, WHO = WHO water (control).

⁷ 0 = no growth in any dilution (in the range 10^0 - 10^4); 1 = only growth in 10^0 ; 2 = only growth in 10^0 and in 10^{-1} , etc.

Replicate results written in the same row, separated by /.

⁸ Not done because MSRV plates crystallized during incubation; no growth was seen either on Rambach agar plates when streaking from MSRV plates.

⁹ x.5: no growth in dilution x-1, but in dilution x, cf. text.

Table 12.3: Results for feed chain links inoculated with either fat or feed for layers (all feed with high CFU, cf. Table 12.2) (for each type of organic matter: disinfection conditions deteriorating towards the bottom of the table).

Organic matter	TB	TA	DT	S. Enteritidis			S. Senftenberg			Enterococcus faecalis		
				F	B	V	WHO	F	B	V	WHO	V
Fat	30.0 (±0.3)	30.0 (±0.3)	30	4.5/3	3/2	0/0	5/5		1/2	1/1	0/0	3/2
	5.9 (±0.5)	5.9 (±0.5)	30	5/5	5/5	5/5	5/5		3/5	3/5	4/4	4/4
Feed	10.9 (±0.5)	10.9 (±0.5)	30	0/0	5/5	5/5	2/3	0/0	1/0	5/5	5/5	
	5.9 (±0.5) ¹	5.9 (±0.5)	30	0/0	1/0	5/5	4/5	0/0	5/5	5/5	5/5	
	5.9 (±0.5)	5.9 (±0.5)	30	0/0	4/1	5/3	5/5	0/0	1/5	5/5	5/5	
	5.9 (±0.5)	5.9 (±0.5)	15	0/0	1/1	5/5	5/5	0/0	1/1	5/5	5/2	
	5.9 (±0.5)	5.9 (±0.5)	5	0/0	4/4	5/5	5/4	0/1	0/4	4/5	5/4	

¹ The tests in this row were performed with only one feed chain link pair per 250 ml disinfectant (the tests in the other rows were performed with two chain link pairs per 250 ml disinfectant, cf. text).
Legend, cf. Table 12.2.

Table 12.4: Results for wooden dowels inoculated with either fat or feed for layers (all feed with high CFU, cf. Table 12.2) (for each type of organic matter: disinfection conditions deteriorating towards the bottom of the table).

Organic matter	TB	TA	DT	S. Enteritidis			S. Senftenberg			Enterococcus faecalis		
				F	B	V	WHO	F	B	V	WHO	V
Fat	30.0 (±0.3)	30.0 (±0.3)	30	2/1/0	3/2/2	0/2/0	4/3/3	0/0/0	1/0/0	0/0/0	2/0/1	1/0/0
	5.9 (±0.5)	5.9 (±0.5)	30	5/3/3	4/3/3	5/5/5	5/4/5	5/4/2	3/3/3	3/≥2 ¹ /2	4/5/5	3/3/2
Feed	10.9 (±0.5)	10.9 (±0.5)	30	0/0/0	1/1/0	1/1/1	1/1/1	0/0/0	0/0/0	1/0/0	1/2/2	2/3/2
	5.9 (±0.5)	5.9 (±0.5)	30	0/0/0	0/0/0	1/1/1	4/4/3	0/0/0	0/0/0	0/0/0	2/1/1	1/1/2
	5.9 (±0.5)	5.9 (±0.5)	15	0/0/1	0/1/0	1/1/1	2/3/2	0/0/0	0/0/0	0/0/0	1/1/1	1/1/2.5
	5.9 (±0.5)	5.9 (±0.5)	5	2/1/0	0/1/0	2/2.5/2	3/3/3	0/0/0	1/0/0	2/1/2.5	1/1/1	3/3/4
												5/5/5

¹ 10¹ dilution spilled; therefore no dilutions were made in the range 10²-10⁴.
Legend, cf. Table 12.2.

Table 12.5: Results for jute egg belt pieces inoculated with egg yolk (disinfection conditions deteriorating towards the bottom of the table).

CFU ¹	TB	TA	DT	S. Enteritidis			S. Senftenberg			Enterococcus faecalis						
				F	B	V	WHO	F	B	V	WHO	F	B	V	WHO	
Low	10.9 (±0.5)	10.9 (±0.5)	30	0/0/0	1/2/2	2/4/4	4/5/5	0	1	1	1	4				
Low	5.9 (±0.5)	5.9 (±0.5)	30	0	0	4	5	0	1	1	1	3				
Low	5.9 (±0.5)	5.9 (±0.5)	15	0	1	2	5	0	0	0	0	3				
High	10.9 (±0.5)	10.9 (±0.5)	30	0	2	5	5	0	2	4	2	5	2/1	4/3	5/5	5/5
High	5.9 (±0.5)	5.9 (±0.5)	15	0	3	5	5	0	2	2	2	5				
High	5.9 (±0.5)	5.9 (±0.5)	5	3	5	5	5	0	2	4.5	2	5	1/0	5/5	5/5	5/5

¹ Low = ca. 2.9×10^5 - 4.6×10^6 g⁻¹ organic matter; High = ca. 2.9×10^6 - 4.6×10^7 g⁻¹ organic matter (cf. Section 8.3.2.2).

Legend, cf. Table 12.2.

Comparisons of 0 vs. >0 points generally yielded more significant results. For both *S. Enteritidis* and *S. Senftenberg*, F was more effective than B ($p=0.000044$ and 0.00058 , respectively), V ($p<10^{-7}$ and 5×10^{-7} , respectively) and W (both $p<10^{-7}$). For *S. Enteritidis*, B was more effective than V ($p=0.012$) and W ($p=0.000098$). For *S. Senftenberg*, B and V were more effective than W ($p=9\times 10^{-7}$ and 0.00061 , respectively). In pair wise comparisons between the bacteria, *S. Enteritidis* was more resistant than *S. Senftenberg* to V ($p=0.003$), more susceptible than *E. faecalis* to F ($p=0.044$), whereas *S. Senftenberg* was more susceptible than the latter to F ($p=0.0002$), B ($p=0.0056$) and V ($p=0.0026$).

The above tendencies were general for all disinfection series, with one notable exception: when testing feed chain links and fats at 30°C before and after disinfection, V was better than F, B and W, both for SE and EF (Table 12.3). We observed during disinfection that the rapeseed oil bubbled in V, but not in F, B or W, which is probably a result of V's corrosive properties on certain metals, maybe exacerbated by the links being rusty with metal ions enhancing the oxidation process. When disinfecting wooden dowels under the same conditions, no bubbling was observed in any of the disinfectants, and the results for V were more similar to those for F, B and W (Table 12.4).

As several conditions differed between series, poultry house materials and organic matter could not be compared statistically. However, there seemed to be differences between fats and feed (cf. Tables 12.3 and 12.4), as the former were generally more protective, even with higher temperatures and disinfection times applied for these. For each disinfectant and for every combination of poultry house materials and organic matter, there was generally little variation related to the other conditions (numbers of CFU, amount of organic matter on concrete flags, temperatures before and after disinfection and disinfection times), though a somewhat higher survival was seen for the most effective disinfection

tants when conditions deteriorated (Bio Komplet Plus in Table 12.2, formaldehyde for *S. Enteritidis* in Table 12.4, and both these disinfectants for *Salmonella* in Table 12.5).

12.4 Discussion

The lack of scientific literature on disinfection of animal houses, the limited benefit of suspension tests for real-life situations (cf. Section 5.4.4.2), and hundreds of visits to persistently *Salmonella*-infected poultry houses without being able to give much specific advice on disinfection, prompted us to make this study. Surface tests mimicking real-life situations found in the disinfection of badly cleaned poultry houses under low temperatures were important aspects in this study, in spite of the disadvantages of such tests (cf. Section 5.4.5). Nevertheless, “non-standardized” factors, e.g. differences between the roughness in concrete flag surfaces, the degree of rust on feed chain links, or the amount of organic matter sticking to materials, were random for different bacteria and disinfectants, so it is unlikely that the consistent results are due to these. Moreover, dilutions were made, i.e. using an MPN method, to give more detailed results. However, results from dilutions using the organic matter of this study should also be interpreted with caution, as a complete Poisson distribution could probably not be expected. This may explain the few odd results, where growth was not seen in one dilution, but in the dilution being ten times higher. But again, these conditions were the same regardless of disinfectants and bacterial isolates, i.e. it is unlikely that this favoured any of these systematically. Dilutions were also made to avoid the use of neutralizers in the pre-enrichment broth, as there is no common neutralizer for the three disinfectants in this study (cf. Section 5.4.2), and the use of different neutralizers would systematically bias comparisons with regard to disinfectants. Only three samples, all with formaldehyde, did not grow in the 10^0 dilution, but in higher dilutions (data not shown), so we believe dilutions in general compensated for the lack of neutralizers,

probably also supported by the 25 h period after disinfection. Moreover, the disinfectant concentrations in the 10^0 dilutions were calculated to be similar to the MICs of the isolates, but lower than the concentrations at which they still grew in broth (cf. Section 11.3.2). As an example, an amount of 4.0 ml 2% disinfectant solution remaining on a concrete flag after 25 hours would yield $0.04 \text{ ml } 100 \text{ ml}^{-1}$ concentrated disinfectant in the 10^0 dilution, i.e. similar to the lowest MICs which were seen for formaldehyde, but lower than the concentration seen at growth in broth (which was 5.5-12.5 higher than the MIC for formaldehyde, cf. Section 11.3.2). Most published disinfection tests do not incorporate any time span between the disinfection and recovery procedures, probably because a rapid effect is wanted in the food industry and hospital wards, whereas there are normally more than 25 h between poultry house disinfection and the introduction of new chickens. Finally, one obvious way of compensating for the lack of a rigorous standardization is to increase the number of tests, which was done both with different conditions and several replicate tests.

Another point of discussion is the ability of a study like this to mimic worst-case scenarios. Amongst other things, it is difficult to characterize the specific composition of organic matter found in poultry houses. It is well known that organic matter protects bacteria from disinfectants (cf. Section 4.2.2.4), and specific proteins, often albumin, are used in many disinfection tests. Studies on the impact of specific components on the efficacy of disinfection (such as carbohydrates, proteins and fats) have mainly been made with heating of bacteria (cf. Sections 4.1.4 and 4.1.5). However, as many principles are similar between different detrimental conditions with regard to the killing of microorganisms, we presumed that these compounds would also be the most important protectors in chemical disinfection tests. The highest occurrence of proteins, fats and carbohydrates will theoretically be in feed, as this represents the “input” of these compounds to the poultry house. Egg yolk

represents an “output” material with high protein and fat content. Egg white was omitted from the study as it has lower protein content and no fats. These differences explain, at least partly, why bacteria are less heat resistant in egg white than in egg yolk (Doyle and Mazzotta 2000), and this probably also applies to chemical disinfectants. Fats, which we often observed in poultry houses as a layer in badly cleaned feed troughs, probably protect microorganisms effectively from chemical disinfection, as they do during heating. Faeces were not used as organic matter although they are abundant in badly cleaned poultry houses, as the low contents of proteins, fats and carbohydrates probably render them less protective to disinfectants, a tendency also observed in heat disinfection studies (cf. Chapters 8 and 9). Finally, the aspect of biofilms was also omitted from this study, as the occurrence of biofilms in poultry houses has only been reported from water systems where other disinfection procedures than those in the rest of the poultry house are applied (cf. Sections 2.4 and 3.4.4).

It has been alleged, primarily by commercial sources, that glutaraldehyde is an effective disinfectant down to ca. 5 °C whereas formaldehyde needs minimum 16 °C (cf. Table 4.1). It was therefore surprising that formaldehyde did not seem to be less effective than a glutaraldehyde/benzalkonium chloride compound in many test series run at ca. 6 °C. In field studies of persistently *Salmonella*-infected poultry premises, formaldehyde was reported to be more effective than glutaraldehyde and also less susceptible to a bad cleaning standard (Davies and Wray 1995c; Davies *et al.* 1998b; Davies and Breslin 2003a). The low efficacy of the oxidizing compound was less surprising, as these disinfectants are susceptible to organic matter (Russell and Chopra 1996), and the quick reaction times that are often required in in-vitro tests (Gasparini *et al.* 1995) are not necessarily advantageous when disinfecting animal houses where surface disinfectants are left *in situ* rather than be-

ing rinsed away. In one study with boot bath disinfection, a 1% Virkon S solution was effective against bacteria on clean boots, but not when pig manure was present (Amass *et al.* 2001). The favourable results seen with the oxidizing compound when disinfecting feed chain links at 30 °C may be related to its corrosive properties, which apparently were also lethal to the bacteria tested. Fats were generally protective for the bacteria, but the use of Virkon S or a similar compound at high temperatures might be a specific solution in e.g. feed troughs, but more work is needed to confirm this. Moreover, it may be undesirable to promote rapid oxidation of expensive metal equipment.

The only difference in susceptibility between the two *Salmonella* isolates was for Virkon S. *S. Enteritidis* and *S. Senftenberg* represented the lowest and highest minimum inhibitory concentrations (MICs), respectively (cf. Chapter 11). Here, the MICs of the *S. Senftenberg* isolate were two and four times higher for formaldehyde and Virkon S, respectively, than those of the *S. Enteritidis* isolate. It is therefore conspicuous that in this study the only difference in susceptibility was found for Virkon S, but *S. Enteritidis* was more resistant than *S. Senftenberg*. These preliminary results indicate that MIC studies are not reliable indicators of in-use conditions, aspects which have also been discussed in the scientific literature (cf. Section 3.4.7).

Enterococci often occur in higher numbers than *Salmonella*, they have the same habitat, i.e. the intestines, and they are considered to be relatively resistant to various detrimental conditions (also cf. Chapter 9, especially Section 9.4). All these characteristics make them suitable as indicator bacteria for *Salmonella* in field tests, either as naturally occurring bacteria or spiked in organic matter. In our study, *E. faecalis* was generally at least as resistant to both the three disinfectants and the control as the two *Salmonella* isolates, but it

was tested in relatively few series, so more studies and other conditions are needed to validate its use as an indicator bacterium.

In conclusion, the efficacy was (best first): formaldehyde > Bio Komplet Plus > Virkon S > WHO water, with the exception that Virkon S seemed most effective when disinfecting feed chain links with fats at 30 °C. With regard to bacteria, there were no differences between *S. Enteritidis* and *S. Senftenberg*, except for Virkon S that was more lethal to the latter. *E. faecalis* was equally or more difficult to eradicate than the two *Salmonella* isolates, indicating it could be useful as an indicator bacterium.

Chapter 13 – General discussion and conclusions

13.1 Significance of results

The epidemiological broiler house study (cf. Chapter 7) showed that few factors related to poultry house materials, cleaning and disinfection could explain the persistence of the two *Salmonella* types, the main exception being that combined fogging and surface disinfection reduced the likelihood of persistence. Thus, under natural conditions encountered in broiler houses, which are the easiest type of poultry house to clean, the disinfectant type did not seem to influence the results. This is in contrast to the worst-case scenario studies described in Chapter 12 in which the same commonly used disinfectants were applied. Here, formaldehyde was generally the most effective disinfectant, followed by a glutaraldehyde/BC compound and an oxidising compound. The fact that this surface disinfection study illustrated the borderline conditions, and these were not seen under field conditions, suggests that the laboratory test conditions were more worst-case than those encountered in the field, which is an asset for a method that mimics real-life conditions. A visual comparison between the amount of organic matter in the laboratory tests and in the broiler houses also confirmed this assumption, as relatively little organic matter was observed in the latter.

The study on disinfectant resistance (cf. Chapter 11) was the most unrealistic study reported here, but it was primarily a preliminary screening study. The background for the study was the hypothesis that resistance to disinfectants could, at least partly, explain *Salmonella*-persistence, and/or it could explain that certain serotypes tended to persist. Also in this area, too little reference could be made to previous research, especially from the agricultural sector. In general, there was little variation in MICs to five disinfectants

used commonly in the poultry sector, and higher MICs could not be related to persistence or the use of certain types of disinfectants. Thus, the recommendation sometimes given on changing between disinfectants could not be validated. Phenotypic studies on MDR, which has been involved in disinfectant resistance for certain chemical compounds (e.g. triclosan), suggested that this was not involved in resistance to any of the five disinfectants studied. Two isolates, one with low and one with high MICs, were used in the realistic surface disinfection studies (cf. Chapter 12). Interestingly, the isolate with the lowest MICs seemed to be more resistant than the one with the highest MICs, and this illustrates that it may be difficult to extrapolate results from one set of conditions to another, in this case from MIC-studies to more realistic tests.

In the heating studies (cf. Chapters 8,10), moisture seemed to be important in the efficacy of heat. In the laboratory studies, feed, with its high contents of fats, carbohydrates and proteins, was also more protective than faeces. On the other hand, the three serotypes did not differ significantly in their susceptibility. These results indicated that external factors had a higher impact on the outcome than the *Salmonella* serotype, and this was reinforced by the MIC studies in which there were also few variations between the serotypes. The main aim of the heating studies was to find a temperature-humidity-time scheme effective for the elimination of *Salmonella*. Most results showed that a temperature-humidity-time scheme of 60 °C and 100% RH during 24 h was effective, both under controlled conditions in the laboratory and in field studies with naturally occurring *Salmonella*. Moreover, the dosing of formaldehyde in the steam at the beginning to yield 30 ppm (related to the volume of the house) improved the efficacy in the field studies.

Contrary to what was found in the laboratory heating studies, the *Salmonella* type seemed to be more important in the epidemiological field study (cf. Chapter 7) and in the

realistic surface tests (cf. Chapter 12). In both these studies, *S. Enteritidis*, PT8, was more resistant than *S. Typhimurium*, DT66, and *S. Senftenberg*, respectively. These results could partly explain why *S. Enteritidis* has become so widespread and been difficult to eliminate (cf. Section 1.2).

Important information from the field heating studies was gained from the many *Salmonella* samples taken in the layer houses. In spite of all the differences between farms and houses, sites in which *Salmonella* was detected relatively often (i.e. critical control points) were fairly consistent in houses having relatively high numbers of *Salmonella* positive samples. In routine monitoring systems, it is unrealistic to take and process hundreds of samples consistently, so a focus on such critical control points will be advantageous.

In spite of many *Salmonella* samples and the focus on critical control points, there were also houses in which *Salmonella* was detected in low numbers, so the *Salmonella* status *per se* could not be used to systematically monitor the efficacy of disinfection. Therefore, another important aspect of several studies (Chapters 8-10 and 12) was the use of putative indicator bacteria. In the laboratory heating studies, there were high correlations between spiked *Salmonella* and coliforms on one hand and naturally occurring *E. coli* on the other hand. This was used in the field heating studies in which high correlations between coliforms and *Salmonella* were detected on house level. However, if another distribution between sample sites had been applied (e.g. relatively fewer floor samples), such a correlation could have disappeared, as several sites had a poor correlation between coliforms and *Salmonella*. This probably illustrates the complicated dynamics between different bacteria in spite of their occurrence in the same natural habitat. In the field studies, organic indicator samples (feed spiked with *E. coli* or *E. faecalis* and faeces with naturally occurring *E. coli* and enterococci) were also used to monitor the efficacy of the heat treat-

ments, after preliminary heating studies in broth had shown that they were not more heat susceptible than a *Salmonella* isolate. The results of these organic indicator samples generally conformed to the other results, as virtually no bacteria survived above 60 °C and 100% RH during a 24-h period. In the surface disinfection tests, *E. faecalis* was at least as resistant as the two *Salmonella* serotypes, which also suggested its usefulness as an indicator bacterium, e.g. in field studies where it will be hazardous to place samples spiked with *Salmonella*.

Some results can be used directly for recommendations for treatment of persistently *Salmonella*-infected poultry houses. Steam heating, applying 60 °C and 100% RH during a 24-h period, with 30 ppm formaldehyde added to the steam at the beginning, seemed to be effective. As it was difficult to achieve 60 °C near the floor, a supplementary chemical surface disinfection is recommended. The application of formaldehyde gave the best results in the worst-case scenario surface disinfection tests, but the field study in broiler houses also indicated that if little organic matter remains on the floor, other commonly used disinfectants are probably also effective. Moreover, there were indications that other disinfectants may be more effective with other types of organic matter, in this case fats that occur commonly in badly cleaned feed troughs.

13.2 Future perspectives

It is believed there is a need for more studies that mimic worst-case scenarios in the laboratory, followed by full-scale control field intervention studies. These are expensive, but the effect on a range of poultry pathogens, zoonotic organisms, indicator bacteria, viruses, fungi and production parameters could be studied at the same time. Many of the studies reported here are difficult to standardize, and probably the main way of compensating for this is the repetition of tests to evaluate if results are consistent. Other conditions,

such as other types of organic matter and other disinfectants, should also be tested in full factor studies.

For the few types of Gram-negative, vegetative bacteria used in the actual studies, some results suggest that the environmental conditions may be more important than the specific bacterial type or strain, whereas others show bigger differences between the latter. More studies are needed to find well-characterised laboratory strains that can be used as indicator bacteria, i.e. those that are more resistant than *Salmonella*, but represent bacterial types that consistently occur in higher numbers in the same habitats as these. Moreover, these bacteria should be harmless for the animals, so they can also be used in field studies.

The pros and cons of field studies will not be reiterated here. There are, however, characteristic features of the poultry sector that probably minimise biases in such studies. Many commercial poultry houses are very similar, as only a few companies produce and market equipment, and the same applies to the buildings and the materials they are made of. However, the immeasurable factors (i.e. “management”) are often also important for the outcome. In this context, it is advantageous that many farms have two or more identical poultry houses, and the same management procedures are often applied in these, so “immeasurable” biases are minimised. These advantages should be used in future studies, applying various chemical disinfectants and procedures, preferably on broiler farms, where more identical houses are found and which have a higher turn over, so different procedures can be alternated between download periods. Other disinfection procedures than surface disinfection should be investigated, e.g. fogging that is difficult to simulate under laboratory conditions. The use of indicator bacteria, based on sound laboratory studies, should be an inherent part of such studies.

A generally accepted standard monitoring system for disinfection procedures in the field, preferably with the inclusion of relevant indicator bacteria, would be beneficial for farmers and contractors. Such monitoring systems, based on sound scientific studies, should be an inherent part of certification systems run by independent auditing bodies. In food premises, HACCP monitoring systems are widespread. The main obstacle in applying similar systems in the agricultural sector is probably the involvement of only few scientists in this research area. Many resources are needed, and such systems should preferably apply more generally, i.e. not only to *Salmonella*, as tonnes of disinfectants are used for elimination of various microbes without much controlled evidence of their benefit.

It can always be discussed whether more heat disinfection field tests should be performed before a standardised monitoring system can be applied, but most results suggest that the suggested method is effective. Therefore, a certification system with the use of validated temperature and humidity loggers and standardised monitoring and sampling methods should be developed.

More specifically, the following studies will give a more comprehensive picture of the area:

- More worst-case scenario laboratory studies, using various *Salmonella* strains and putative indicator organisms. These should include other conditions, such as different types of organic matter, temperatures and disinfectants (e.g. strong bases).
- The development of standardised, well characterised and reproducible samples containing organic matter and indicator bacteria that can be used in field studies and monitoring systems.
- Biofilm studies that mimic conditions in water lines.

- Chemical disinfection field studies, applying various methods and disinfectant in identical broiler houses, with the use of standardised organic indicator samples to monitor the efficacy.

Results from the above studies should be implemented in standardised monitoring systems. Ideally, independent auditing bodies should certify such systems.

References

- Ababouch, L.H., Grimit, L., Eddafry, R. and Busta, F.F. (1995) Thermal inactivation kinetics of *Bacillus subtilis* spores suspended in buffer and in oils. *Journal of Applied Bacteriology* **78**, 669-676.
- Adams, J.L. and McLean, R.J. (1999) Impact of *rpoS* deletion on *Escherichia coli* biofilms. *Applied and Environmental Microbiology* **65**, 4285-4287.
- Ahmed, N.M., Conner, D.E. and Huffman, D.L. (1995) Heat-resistance of *Escherichia coli* O157:H7 in meat and poultry as affected by product composition. *Journal of Food Science* **60**, 606-610.
- Alder, V.G., Boss, E., Gillespie, W.A. and Swann, A.J. (1971) Residual disinfection of wool blankets treated with formaldehyde. *Journal of Applied Bacteriology* **34**, 757-763.
- Alder, V.G., Brown, A.M. and Gillespie, W.A. (1966) Disinfection of heat-sensitive material by low-temperature steam and formaldehyde. *Journal of Clinical Pathology* **19**, 83-89.
- Aldsworth, T.G., Dodd, C.E. and Stewart, G.S. (1999) Induction of *rpoS* in *Salmonella* Typhimurium by nutrient-poor and depleted media is slower than that achieved by a competitive microflora. *Letters in Applied Microbiology* **28**, 255-257.
- Alekshun, M.N. and Levy, S.B. (1999) The *mar* regulon: multiple resistance to antibiotics and other toxic chemicals. *Trends in Microbiology* **7**, 410-413.
- Alekshun, M.N. and Levy, S.B. (2000) Bacterial drug resistance: response to survival threats. In *Bacterial Stress Responses* ed. Storz, G. and Hengge-Aronis, R. pp. 323-366. Washington D.C.: ASM Press.
- Allison, D.G. and Gilbert, P. (1995) Modification by surface association of antimicrobial susceptibility of bacterial populations. *Journal of Industrial Microbiology* **15**, 311-317.
- Altekruse, S.F., Tollefson, L.K. and Bögel, K. (1993) Control strategies for *Salmonella* Enteritidis in five countries. *Food Control* **4**, 10-16.
- Amass, S.F., Ragland, D. and Spicer, P. (2001) Evaluation of the efficacy of a peroxygen compound, Virkon S, as a boot bath disinfectant. *Journal of Swine Health and Production* **9**, 121-123.
- Angen, Ø., Skov, M.N., Chriél, M., Agger, J.F. and Bisgaard, M. (1996) A retrospective study on *Salmonella* infection in Danish broiler flocks. *Preventive Veterinary Medicine* **26**, 223-237.
- Anonymous (1988) *Vandundersøgelse - Kvalitativ og kvantitativ bestemmelse af Salmonella i vand, slam, sediment og jord (Water Quality - Qualitative and Quantitative Determination of Salmonella in Water, Sludge, Sediment and Soil)*. Dansk Standard (DS), Denmark.

References

- Anonymous (1996) Epi Info, version 6.03. Centers for Disease Control and Prevention, Atlanta, Georgia, USA.
- Anonymous (1997a) Microsoft Access, version 97, 1989-1997. Microsoft Corporation.
- Anonymous (1997b) Microsoft Excel 97 SR-2. 1985-1997. Microsoft Corporation.
- Anonymous (1998) *User's Guide. Neo-Sensitabs[®] Susceptibility Testing*, 10th ed. Rosco, 2630 Taastrup, Denmark.
- Anonymous (1999) SAS STAT, version 8.00. SAS Institute Inc.
- Anonymous (2001) *Salmonella* - Coordinating chemical and thermal control. *Feed International* **December**, 27-34.
- Anonymous (2002a) *Code of Practice for the Prevention and Control of Salmonella in Chickens Reared for Meat on Farm*. Department for Environment, Food & Rural Affairs, UK.
- Anonymous (2002b) *Desinfektion i husdyrbruget*. Landsudvalget for Svin - Danske Slagterier - Primærproduktion - Forsøg og udvikling, Denmark.
- Anonymous (2002c) GraphPad Prism, version 3.03, Copyright 1994-2002. GraphPad Software Incorporated, San Diego, CA 92121, USA.
- Anonymous (2002d) *KRIS 2000 - Redskabsbogen, kvalitetsrengøring i storkøkkener*. Søborg: Danish Veterinary and Food Directorate.
- Anonymous (2002e) *Microbiology of Food and Animal Feeding Stuffs - Horizontal Method for the Detection of Salmonella Spp.* ISO 6579:2002. Brussels, European Committee for Standardization (CEN).
- Anonymous (2002f) S-PLUS 6.1, Release 1. Insightful Corporation, Seattle WA 98109, USA.
- Anonymous (2002g) UNISTAT Statistical Package. [5.5.00]. UNISTAT Ltd., 4 Shirland Mews, London W9 3DY, UK.
- Anonymous (2003) *Annual Report on Zoonoses in Denmark 2002*. Ministry of Food, Agriculture and Fisheries, Denmark.
- Archer, J., Jervis, E.T., Bird, J. and Gaze, J.E. (1998) Heat resistance of *Salmonella* Weltevreden in low-moisture environments. *Journal of Food Protection* **61**, 969-973.
- Asako, H., Nakajima, H., Kobayashi, K., Kobayashi, M. and Aono, R. (1997) Organic solvent tolerance and antibiotic resistance increased by overexpression of *marA* in *Escherichia coli*. *Applied and Environmental Microbiology* **63**, 1428-1433.

References

- Bacon, R.T., Ransom, J.R., Sofos, J.N., Kendall, P.A., Belk, K.E. and Smith, G.C. (2003) Thermal inactivation of susceptible and multiantimicrobial-resistant *Salmonella* strains grown in the absence or presence of glucose. *Applied and Environmental Microbiology* **69**, 4123-4128.
- Bailey, J.S., Cox, N.A. and Berrang, M.E. (1994) Hatchery-acquired salmonellae in broiler chicks. *Poultry Science* **73**, 1153-1157.
- Bains, B.S. and MacKenzie, M.A. (1974) Transmission of *Salmonella* through an integrated poultry organisation. *Poultry Science* **53**, 1114-1118.
- Baird-Parker, A.C., Boothroyd, M. and Jones, E. (1970) The effect of water activity on the heat resistance of heat sensitive and heat resistant strains of salmonellae. *Journal of Applied Bacteriology* **33**, 515-522.
- Bale, M.J., Bennett, P.M., Beringer, J.E. and Hinton, M. (1993) The survival of bacteria exposed to desiccation on surfaces associated with farm buildings. *Journal of Applied Bacteriology* **75**, 519-528.
- Banhazi, T., Murphy, T. and Hartung, J. (2003) Using 'Hygiene-pavers' to evaluate cleaning procedures used on pig farms. In *XI International Congress in Animal Hygiene*, February 23-27, pp. 353-362. International Society for Animal Hygiene, Mexico City, Mexico.
- Barer, M.R. and Harwood, C.R. (1999) Bacterial viability and culturability. *Advances in Microbial Physiology* **41**, 93-137.
- Barker, J. and Bloomfield, S.F. (2000) Survival of *Salmonella* in bathrooms and toilets in domestic homes following salmonellosis. *Journal of Applied Microbiology* **89**, 137-144.
- Barrile, J.C. and Cone, F. (1970) Effect of added moisture on the heat resistance of *Salmonella* Anatum in milk chocolate. *Applied Microbiology* **19**, 177-178.
- Barrow, P.A. (1993) *Salmonella* control - past, present and future. *Avian Pathology* **22**, 651-669.
- Bean, H.S. (1967) Types and characteristics of disinfectants. *Journal of Applied Bacteriology* **30**, 6-16.
- Bean, N.H. and Griffin, P.M. (1990) Foodborne disease outbreaks in the United States, 1973-1987: pathogens, vehicles, and trends. *Journal of Food Protection* **53**, 804-817.
- Bearson, S., Bearson, B. and Foster, J.W. (1997) Acid stress responses in enterobacteria. *FEMS Microbiology Letters* **147**, 173-180.
- Beer, V.K., Methling, W., Mehlhorn, G. and Erwerth, G. (1980) Untersuchungen zur Wirksamkeit der prophylaktischen Reinigung und Desinfektion von Oberflächen in Kälber- und Schweineställen. *Monatshefte für Veterinärmedizin* **35**, 84-90.

References

- Bennett, D.D., Higgins, S.E., Moore, R.W., Beltran, R., Caldwell, D.J., Byrd II, J.A. and Hargis, B.M. (2003) Effects of lime on *Salmonella* Enteritidis survival in vitro. *Journal of Applied Poultry Research* **12**, 65-68.
- Berchieri, A.Jr. and Barrow, P.A. (1996) The antibacterial effects for *Salmonella* Enteritidis phage type 4 of different chemical disinfectants and cleaning agents tested under different conditions. *Avian Pathology* **25**, 663-673.
- Bergan, T. and Lystad, A. (1972) Evaluation of disinfectant inactivators. *Acta Pathologica et Microbiologica Scandinavica. Section B: Microbiology and Immunology* **80**, 507-510.
- Beumer, R.R., Brinkman, E. and Rombouts, F.M. (1991) Enzyme-linked immunoassays for the detection of *Salmonella* spp.: a comparison with other methods. *International Journal of Food Microbiology* **12**, 363-374.
- Bhatia, T.R. and McNabb, G.D. (1980) Dissemination of *Salmonella* in broiler-chicken operations. *Avian Diseases* **24**, 616-624.
- Bisgaard, M. (1992) A voluntary *Salmonella* control programme for the broiler industry, implemented by the Danish Poultry Council. *International Journal of Food Microbiology* **15**, 219-224.
- Blackburn, C.d.W. and McCarthy, J.D. (2000) Modifications to methods for the enumeration and detection of injured *Escherichia coli* O157:H7 in foods. *International Journal of Food Microbiology* **55**, 285-290.
- Blackburn, C.W., Curtis, L.M., Humpheson, L., Billon, C. and McClure, P.J. (1997) Development of thermal inactivation models for *Salmonella* Enteritidis and *Escherichia coli* O157:H7 with temperature, pH and NaCl as controlling factors. *International Journal of Food Microbiology* **38**, 31-44.
- Bloomfield, S.F., Arthur, M., Looney, E., Begun, K. and Patel, H. (1991) Comparative testing of disinfectant and antiseptic products using proposed European suspension testing methods. *Letters in Applied Microbiology* **13**, 233-237.
- Bloomfield, S.F., Arthur, M., van Klingerren, B., Pullen, W., Holah, J.T. and Elton, R. (1994) An evaluation of the repeatability and reproducibility of a surface test for the activity of disinfectants. *Journal of Applied Bacteriology* **76**, 86-94.
- Bloomfield, S.F. and Looney, E. (1992) Evaluation of the repeatability and reproducibility of European suspension test methods for antimicrobial activity of disinfectants and antiseptics. *Journal of Applied Bacteriology* **73**, 87-93.
- Bogosian, G., Morris, P.J. and O'Neil, J.P. (1998) A mixed culture recovery method indicates that enteric bacteria do not enter the viable but nonculturable state. *Applied and Environmental Microbiology* **64**, 1736-1742.
- Bonafonte, M.A., Solano, C., Sesma, B., Alvarez, M., Montuenga, L., Garcia-Ros, D. and Gamazo, C. (2000) The relationship between glycogen synthesis, biofilm formation and virulence in *Salmonella* Enteritidis. *FEMS Microbiology Letters* **191**, 31-36.

References

- Borg, K. (1985) Smittspridning genom vilda djur - 35 års studier redovisas. *Svensk Veterinär Tidning* **37**, 111-128.
- Borneff, J. and Werner, H.P. (1977) Entwicklung einer neuen Prüfmethode für Flächendesinfektionsverfahren. *Zentralblatt für Bakteriologie und Hygiene, I. Abteil., Originale B* **165**, 97-101.
- Bremer, E. and Krämer, R. (2000) Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria. In *Bacterial Stress Responses* ed. Storz, G. and Hengge-Aronis, R. pp. 79-97. Washington DC: ASM Press.
- Brown, D.D., Duff, R.H., Wilson, J.E. and Ross, J.G. (1973) A survey of the incidence of infections with salmonellae in broilers and broiler breeders in Scotland. *British Veterinary Journal* **129**, 493-500.
- Brown, M.R., Allison, D.G. and Gilbert, P. (1988) Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *Journal of Antimicrobial Chemotherapy* **22**, 777-780.
- Brown, M.R., Collier, P.J. and Gilbert, P. (1990) Influence of growth rate on susceptibility to antimicrobial agents: modification of the cell envelope and batch and continuous culture studies. *Antimicrobial Agents and Chemotherapy* **34**, 1623-1628.
- Brown, M.R. and Williams, P. (1985) Influence of substrate limitation and growth phase on sensitivity to antimicrobial agents. *Journal of Antimicrobial Chemotherapy* **15 Suppl A**, 7-14.
- Brown, M.R.W. and Barker, J. (1999) Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. *Trends in Microbiology* **7**, 46-50.
- Brown, M.R.W., Costerton, J.W. and Gilbert, P. (1991) Leading article: extrapolating to bacterial life outside the test tube. *Journal of Antimicrobial Chemotherapy* **27**, 565-567.
- Buchanan, R.L. and Edelson, S.G. (1999) Effect of pH-dependant, stationary phase acid resistance on the thermal tolerance of *Escherichia coli* O157:H7. *Food Microbiology* **16**, 447-458.
- Bunning, V.K., Crawford, R.G., Tierney, J.T. and Peeler, J.T. (1990) Thermotolerance of *Listeria monocytogenes* and *Salmonella* Typhimurium after sublethal heat shock. *Applied and Environmental Microbiology* **56**, 3216-3219.
- Caro, A., Got, P., Lesne, J., Binard, S. and Baleux, B. (1999) Viability and virulence of experimentally stressed nonculturable *Salmonella* Typhimurium. *Applied and Environmental Microbiology* **65**, 3229-3232.
- Casadei, M.A., Ingram, R., Skinner, R.J. and Gaze, J.E. (2000) Heat resistance of *Paenibacillus polymyxa* in relation to pH and acidulants. *Journal of Applied Microbiology* **89**, 801-806.

References

- Casella, M.L. and Schmidt-Lorenz, W. (1989) Disinfection with gaseous formaldehyde. First part: bactericidal and sporicidal effectiveness of formaldehyde with and without formation of a condensing layer. *Zentralblatt für Hygiene und Umweltmedizin* **188**, 144-165.
- Cason, J.A., Cox, N.A. and Bailey, J.S. (1994) Transmission of *Salmonella* Typhimurium during hatching of broiler chicks. *Avian Diseases* **38**, 583-588.
- Cerf, O. (1977) Tailing of survival curves of bacterial spores. *Journal of Applied Bacteriology* **42**, 1-19.
- Christensen, J.P., Brown, D.J., Madsen, M., Olsen, J.E. and Bisgaard, M. (1997) Hatchery-borne *Salmonella enterica* serovar Tennessee infection in broilers. *Avian Pathology* **26**, 155-168.
- Christman, M.F., Morgan, R.W., Jacobson, F.S. and Ames, B.N. (1985) Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella* Typhimurium. *Cell* **41**, 753-762.
- Cizek, A., Literak, I., Hejlícek, K., Treml, F. and Smola, J. (1994) *Salmonella* contamination of the environment and its incidence in wild birds. *Journal of Veterinary Medicine* **41**, 320-327.
- Cogan, T.A. and Humphrey, T.J. (2003) The rise and fall of *Salmonella* Enteritidis in the UK. *Journal of Applied Microbiology* **94 Suppl 1**, 114-119.
- Committee on Formaldehyde Disinfection of the Public Health Laboratory Service (1958) Disinfection of fabrics with gaseous formaldehyde. *Journal of Hygiene Cambridge* **56**, 488-515.
- Compeau, G., Al Achi, B.J., Platsouka, E. and Levy, S.B. (1988) Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. *Applied and Environmental Microbiology* **54**, 2432-2438.
- Corry, J.E.L. (1973) The water relations and heat resistance of micro-organisms. *Progress in Industrial Microbiology* **12**, 73-108.
- Corry, J.E.L. (1974) The effect of sugars and polyols on the heat resistance of salmonellae. *Journal of Applied Bacteriology* **37**, 31-43.
- Corry, J.E.L. and Allen, V.M. (2000) Biofilm formation and machine cleaning. *International Food Safety News* **9**, 2-6.
- Corry, J.E.L. and Roberts, T.A. (1970) A note on the development of resistance to heat and gamma radiation in *Salmonella*. *Journal of Applied Bacteriology* **33**, 733-737.
- Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987) Bacterial biofilms in nature and disease. *Annual Review of Microbiology* **41**, 435-464.

References

- Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Laapin-Scott, H.M. (1995) Microbial biofilms. *Annual Review of Microbiology* **49**, 711-745.
- Cotterill, O.J. and Glauert, J. (1969) Thermal resistance of salmonellae in egg yolk products containing sugar or salt. *Poultry Science* **48**, 1156-1166.
- Coulson, J.C., Butterfield, J. and Thomas, C. (1983) The herring gull *Larus argentatus* as a likely transmitting agent of *Salmonella* Montevideo to sheep and cattle. *Journal of Hygiene Cambridge* **91**, 437-443.
- Cox, N.A., Bailey, J.S., Mauldin, J.M. and Blankenship, L.C. (1990) Presence and impact of *Salmonella* contamination in commercial broiler hatcheries. *Poultry Science* **69**, 1606-1609.
- Cox, N.A., Berrang, M.E. and Cason, J.A. (2000) *Salmonella* penetration of egg shells and proliferation in broiler hatching eggs - a review. *Poultry Science* **79**, 1571-1574.
- Cremieux, A. and Fleurette, J. (1991) Methods of testing disinfectants. In *Disinfection, Sterilization and Preservation* ed. Block, S.S. pp. 1009-1027. Philadelphia & London: Lea & Febiger.
- Croshaw, B. (1981) Disinfectant testing - with particular reference to the Rideal-Walker and Kelsey-Sykes tests. In *Disinfectants: Their Use and Evaluation of Effectiveness* ed. Collins, C.H., Allwood, M.C., Bloomfield, S.F. and Fox, A. pp. 1-15. London: Academic Press Inc. Ltd.
- Cross, G.L. and Lach, V.H. (1990) The effects of controlled exposure to formaldehyde vapour on spores of *Bacillus globigii* NCTC 10073. *Journal of Applied Bacteriology* **68**, 461-469.
- D'Aoust, J.Y. (1989) *Salmonella*. In *Foodborne Bacterial Pathogens* ed. Doyle, M.P. pp. 327-445. New York and Basel: Marcel Dekker Inc.
- D'Aoust, J.Y. (1994) *Salmonella* and the international food trade. *International Journal of Food Microbiology* **24**, 11-31.
- Danese, P.N., Pratt, L.A., Dove, S.L. and Kolter, R. (2000) The outer membrane protein, antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. *Molecular Microbiology* **37**, 424-432.
- Davies, D.G., Parsek, M.R., Pearson, J.P., Igilewski, B.H., Costerton, J.W. and Greenberg, E.P. (1998a) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**, 295-298.
- Davies, R.H. (2001) Quantitative estimation of *Salmonella* at VLA. In *Report on the Sixth Workshop Organised by CRL-Salmonella*, 11 and 12 June, pp. 29-30. Community Reference Laboratory for *Salmonella*, Bilthoven, the Netherlands.

References

- Davies, R.H. and Breslin, M. (2003a) Observations on *Salmonella* contamination of commercial laying farms before and after cleaning and disinfection. *Veterinary Record* **152**, 283-287.
- Davies, R.H. and Breslin, M. (2003b) Persistence of *Salmonella* Enteritidis phage type 4 in the environment and arthropod vectors on an empty free-range chicken farm. *Environmental Microbiology* **5**, 79-84.
- Davies, R.H., Breslin, M., Bedford, S. and Wray, C. (1998b) Observations on *Salmonella* contamination of turkey farms. In *1st International Symposium on Turkey Diseases*, 19-21 February, pp. 274-290. German Branch of the World Veterinary Poultry Association and Institute of Poultry Diseases, Berlin.
- Davies, R.H., Breslin, M., Corry, J.E.L., Hudson, W. and Allen, V.M. (2001) Observations on the distribution and control of *Salmonella* species in two integrated broiler companies. *Veterinary Record* **149**, 227-232.
- Davies, R.H., Nicholas, R.A.J., McLaren, I.M., Corkish, J.D., Lanning, D.G. and Wray, C. (1997) Bacteriological and serological investigation of persistent *Salmonella* Enteritidis infection in an integrated poultry organisation. *Veterinary Microbiology* **58**, 277-293.
- Davies, R.H. and Wray, C. (1994) An approach to reduction of *Salmonella* infection in broiler chicken flocks through intensive sampling and identification of cross-contamination hazards in commercial hatcheries. *International Journal of Food Microbiology* **24**, 147-160.
- Davies, R.H. and Wray, C. (1995a) Contribution of the lesser mealworm beetle (*Alphitobius diaperinus*) to carriage of *Salmonella* Enteritidis in poultry. *Veterinary Record* **137**, 407-408.
- Davies, R.H. and Wray, C. (1995b) Mice as carriers of *Salmonella* Enteritidis on persistently infected poultry units. *Veterinary Record* **137**, 337-341.
- Davies, R.H. and Wray, C. (1995c) Observations on disinfection regimens used on *Salmonella* Enteritidis infected poultry units. *Poultry Science* **74**, 638-647.
- Davies, R.H. and Wray, C. (1996a) Determination of an effective sampling regime to detect *Salmonella* Enteritidis in the environment of poultry units. *Veterinary Microbiology* **50**, 117-127.
- Davies, R.H. and Wray, C. (1996b) Persistence of *Salmonella* Enteritidis in poultry units and poultry food. *British Poultry Science* **37**, 589-596.
- Davies, R.H. and Wray, C. (1996c) Studies of contamination of three broiler breeder houses with *Salmonella* Enteritidis before and after cleansing and disinfection. *Avian Diseases* **40**, 626-633.
- Davies, R.H. and Wray, C. (1997) Distribution of *Salmonella* contamination in ten animal feedmills. *Veterinary Microbiology* **51**, 159-169.

References

- Davison, S., Benson, C.E., Munro, D.S., Rankin, S.C., Ziegler, A.E. and Eckroade, R.J. (2003) The role of disinfectant resistance of *Salmonella enterica* serotype Enteritidis in recurring infections in Pennsylvania egg quality assurance program monitored flocks. *Avian Diseases* **47**, 143-148.
- De las, C.E., Pomeroy, B.S. and Harein, P.K. (1968) Infection and quantitative recovery of *Salmonella* Typhimurium and *Escherichia coli* from within the lesser mealworm, *Alphitobius diaperinus* (panzer). *Poultry Science* **47**, 1871-1875.
- Dega, C.A., Goepfert, J.M. and Amundson, C.H. (1972) Heat resistance of salmonellae in concentrated milk. *Applied Microbiology* **23**, 415-420.
- Denyer, S.P. and Maillard, J.Y. (2002) Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. *Journal of Applied Microbiology* **92 Suppl**, 35S-45S.
- Dhir, V.K. and Dodd, C.E. (1995) Susceptibility of suspended and surface-attached *Salmonella* Enteritidis to biocides and elevated temperatures. *Applied and Environmental Microbiology* **61**, 1731-1738.
- Doesburg, J.J., Lamprecht, E.C. and Elliott, M. (1970) Death rates of salmonellae in fish-meals with different water activities. I. During storage. II. During heat treatment. *Journal of the Science of Food and Agriculture* **21**, 632-640.
- Donlan, R.M. and Costerton, J.W. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews* **15**, 167-193.
- Dougherty, T.J. (1976) A study of *Salmonella* contamination in broiler flocks. *Poultry Science* **55**, 1811-1815.
- Doyle, M.E. and Mazzotta, A.S. (2000) Review of studies on the thermal resistance of Salmonellae. *Journal of Food Protection* **63**, 779-795.
- Doyle, M.P. and Cliver, D.O. (1990) *Salmonella*. In *Foodborne Diseases* ed. Cliver, D.O. pp. 186-204. San Diego: Academic Press Inc.
- Duffy, G., Ellison, A., Anderson, W., Cole, M.B. and Stewart, G.S. (1995) Use of bioluminescence to model the thermal inactivation of *Salmonella* Typhimurium in the presence of a competitive microflora. *Applied and Environmental Microbiology* **61**, 3463-3465.
- Duguid, J.P. and North, R.A. (1991) Eggs and *Salmonella* food-poisoning: an evaluation. *Journal of Medical Microbiology* **34**, 65-72.
- Edel, W. (1994) *Salmonella* Enteritidis eradication programme in poultry breeder flocks in the Netherlands. *International Journal of Food Microbiology* **21**, 171-178.
- Edwards, C. (2000) Problems posed by natural environments for monitoring microorganisms. *Molecular Biotechnology* **15**, 211-223.

References

- Eginton, P.J., Holah, J., Allison, D.G., Handley, P.S. and Gilbert, P. (1998) Changes in the strength of attachment of micro-organisms to surfaces following treatment with disinfectants and cleansing agents. *Letters in Applied Microbiology* **27**, 101-105.
- Eisenstadt, E., Carlton, B.C. and Brown, B.J. (1994) Gene mutation. In *Methods for General and Molecular Bacteriology* ed. Gerhardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R. pp. 297-316. Washington D.C.: American Society for Microbiology.
- Euden, P.R. (1990) *Salmonella* isolates from wild animals in Cornwall. *British Veterinary Journal* **146**, 228-232.
- Favero, M.S., McDade, J.J., Robertson, J.A., Hoffmann, R.K. and Edwards, R.V. (1968) Microbiological sampling of surfaces. *Journal of Applied Bacteriology* **31**, 336-343.
- Feld, N.C., Ekeröth, L., Gradel, K.O., Kabell, S. and Madsen, M. (2000) Evaluation of a serological *Salmonella* mix-ELISA for poultry used in a national surveillance programme. *Epidemiology and Infection* **125**, 263-268.
- Foschino, R., Nervegna, I., Motta, A. and Galli, A. (1998) Bactericidal activity of chlorine dioxide against *Escherichia coli* in water and on hard surfaces. *Journal of Food Protection* **61**, 668-672.
- Fossum, K., Grønstøl, H., Kapperud, G., Schaller, G. and Skjerve, E. (1996) Salmonellainfeksjoner hos dyr og mennesker. *Norsk Veterinærtidsskrift* **108**, 639-645.
- Foster, J.W. (2000) Microbial responses to acid stress. In *Bacterial Stress Responses* ed. Storz, G. and Hengge-Aronis, R. pp. 99-116. Washington DC: ASM Press.
- Foster, J.W. and Spector, M.P. (1995) How *Salmonella* survive against the odds. *Annual Review of Microbiology* **49**, 145-174.
- Fournier, G., Drouin, P., Valancony, H. and Toux, J.Y. (2001) *Etude de la Decontamination des Batiments de Poules Pondeuses en Cages - Rapport Final*. AFSSA, Unité de Recherche en Epidémiologie et Qualité en Aviculture, Ploufragan, France.
- Frank, J.F. and Koffi, R.A. (1990) Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *Journal of Food Protection* **53**, 550-554.
- Fricker, C.R. (1987) The isolation of salmonellas and campylobacters. *Journal of Applied Bacteriology* **63**, 99-116.
- Fris, C. and Van den Bos, J. (1995) A retrospective case-control study of risk factors associated with *Salmonella enterica* subsp. *enterica* serovar Enteritidis infections on Dutch broiler breeder farms. *Avian Pathology* **24**, 255-272.
- Fry, R.M. and Greaves, R.I.N. (1949) The survival of bacteria during and after drying. *Journal of Hygiene Cambridge* **51**, 220-246.

References

- Gasparini, R., Pozzi, T., Magnelli, R., Fatighenti, D., Giotti, E., Polisenio, G., Pratelli, M., Severini, R., Bonanni, P. and De Feo, L. (1995) Evaluation of in vitro efficacy of the disinfectant Virkon. *European Journal of Epidemiology* **11**, 193-197.
- Gawande, P.V. and Bhagwat, A.A. (2002a) Inoculation onto solid surfaces protects *Salmonella* spp. during acid challenge: a model study using polyethersulfone membranes. *Applied and Environmental Microbiology* **68**, 86-92.
- Gawande, P.V. and Bhagwat, A.A. (2002b) Protective effects of cold temperature and surface-contact on acid tolerance of *Salmonella* spp. *Journal of Applied Microbiology* **93**, 689-696.
- George, S.M., Richardson, L.C.C., Pol, I.E. and Peck, M.W. (1998) Effect of oxygen concentration and redox potential on recovery of sublethally heat-damaged cells of *Escherichia coli* O157:H7, *Salmonella* Enteritidis and *Listeria monocytogenes*. *Journal of Applied Microbiology* **84**, 903-909.
- Gibson, G.L. (1977) Processing urinary endoscopes in a low-temperature steam and formaldehyde autoclave. *Journal of Clinical Pathology* **30**, 269-274.
- Gibson, H., Taylor, J.H., Hall, K.E. and Holah, J.T. (1999) Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. *Journal of Applied Microbiology* **87**, 41-48.
- Gilbert, P., Allison, D.G. and McBain, A.J. (2002) Biofilms in vitro and in vivo: do singular mechanisms imply cross-resistance? *Symposium Series (Society for Applied Microbiology)* 98S-110S.
- Gilbert, P., Collier, P.J. and Brown, M.R. (1990) Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. *Antimicrobial Agents and Chemotherapy* **34**, 1865-1868.
- Gilbert, P., Das, J. and Foley, I. (1997) Biofilm susceptibility to antimicrobials. *Advances in Dental Research* **11**, 160-167.
- Gilbert, P., Evans, D.J. and Brown, M.R. (1993) Formation and dispersal of bacterial biofilms in vivo and in situ. *Journal of Applied Bacteriology* **74 Suppl**, 67S-78S.
- Goepfert, J.M. and Biggie, R.A. (1968) Heat resistance of *Salmonella* Typhimurium and *Salmonella* Senftenberg 775W in milk chocolate. *Applied Microbiology* **16**, 1939-1940.
- Goepfert, J.M., Iskander, I.K. and Amundson, C.H. (1970) Relation of the heat resistance of salmonellae to the water activity of the environment. *Applied Microbiology* **19**, 429-433.
- Goossens, H., Wauters, G., de Boeck, M., Janssens, M. and Butzler, J. (1984) Semisolid selective-motility enrichment medium for isolation of salmonellae from fecal specimens. *Journal of Clinical Microbiology* **19**, 940-941.
- Gorman, S.P., Scott, E.M. and Russell, A.D. (1980) A review - antimicrobial activity, uses and mechanism of action of glutaraldehyde. *Journal of Applied Bacteriology* **48**, 161-190.

References

- Gould, G.W. (1989) Heat-induced injury and inactivation. In *Mechanisms of Action of Food Preservation Procedures* ed. Gould, G.W. pp. 11-42. London & New York: Elsevier Applied Science.
- Gradel, K.O. (1998) Et foderanlæg skilles ad. *Dansk Erhvervsfjerkræ* **10**, 322.
- Gradel, K.O., Andersen, J.S. and Madsen, M. (2002) Comparisons of sampling procedures and time of sampling for the detection of *Salmonella* in Danish infected chicken flocks raised in floor systems. *Acta Veterinaria Scandinavica* **43**, 21-30.
- Griffith, C.J., Cooper, R.A., Gilmore, J., Davies, C. and Lewis, M. (2000) An evaluation of hospital cleaning regimes and standards. *Journal of Hospital Infection* **45**, 19-28.
- Griffiths, P.A., Babb, J.R., Bradley, C.R. and Fraise, A.P. (1997) Glutaraldehyde-resistant *Mycobacterium chelonae* from endoscope washer disinfectors. *Journal of Applied Microbiology* **82**, 519-526.
- Guerin-Mechin, L., Dubois-Brissonnet, F., Heyd, B. and Leveau, J.Y. (1999) Specific variations of fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by quaternary ammonium compounds and relation with resistance to bactericidal activity. *Journal of Applied Microbiology* **87**, 735-742.
- Hammond, S.A., Morgan, J.R. and Russell, A.D. (1987) Comparative susceptibility of hospital isolates of Gram-negative bacteria to antiseptics and disinfectants. *Journal of Hospital Infection* **9**, 255-264.
- Hansen, N.H. and Riemann, H. (1963) Factors affecting the heat resistance of nonsporing organisms. *Journal of Applied Bacteriology* **26**, 314-333.
- Harry, E.G. and Hemsley, I.A. (1964) Factors influencing the survival of coliforms in the dust of deep litter houses. *Veterinary Record* **76**, 863-867.
- Hengge-Aronis, R. (2000) The general stress response in *Escherichia coli*. In *Bacterial Stress Responses* ed. Storz, G. and Hengge-Aronis, R. pp. 161-178. Washington DC: ASM Press.
- Henken, A.M., Frankena, K., Goelema, J.O., Graat, E.A. and Noordhuizen, J.P. (1992) Multivariate epidemiological approach to salmonellosis in broiler breeder flocks. *Poultry Science* **71**, 838-843.
- Henzler, D.J. and Opitz, H.M. (1992) The role of mice in the epizootiology of *Salmonella* Enteritidis infection on chicken layer farms. *Avian Diseases* **36**, 625-631.
- Herendeen, S.L., VanBogelen, R.A. and Neidhardt, F.C. (1979) Levels of major proteins of *Escherichia coli* during growth at different temperatures. *Journal of Bacteriology* **139**, 185-194.
- Higgins, R., Malo, R., René, R.R. and Gauthier, R. (1982) Studies on the dissemination of *Salmonella* in nine broiler-chicken flocks. *Avian Diseases* **26**, 26-33.

References

- Himathongkham, S., Nuanualsuwan, S. and Riemann, H. (1999) Survival of *Salmonella* Enteritidis and *Salmonella* Typhimurium in chicken manure at different levels of water activity. *FEMS Microbiology Letters* **172**, 159-163.
- Himathongkham, S., Pereira, M.G. and Riemann, H. (1996) Heat destruction of *Salmonella* in poultry feed: effect of time, temperature and moisture. *Avian Diseases* **40**, 72-77.
- Himathongkham, S., Riemann, H., Bahari, S., Nuanualsuwan, S., Kass, P. and Cliver, D.O. (2000) Survival of *Salmonella* Typhimurium and *Escherichia coli* O157:H7 in poultry manure and manure slurry at sublethal temperatures. *Avian Diseases* **44**, 853-860.
- Hinton, M. (1988) *Salmonella* infection in chicks following the consumption of artificially contaminated feed. *Epidemiology and Infection* **100**, 247-256.
- Hinton, M. and Linton, A.H. (1988) Control of *Salmonella* infections in broiler chickens by the acid treatment of their feed. *Veterinary Record* **123**, 416-421.
- Hogue, A., White, P., Guard-Petter, J., Schlosser, W., Gast, R., Ebel, E., Farrar, J., Gomez, T., Madden, J., Madison, M., McNamara, A.M., Morales, R., Parham, D., Sparling, P., Sutherlin, W. and Swerdlow, D. (1997) Epidemiology and control of egg-associated *Salmonella* Enteritidis in the United States of America. *Revue scientifique et technique/Office international des épizooties* **16**, 542-553.
- Holah, J.T., Taylor, J.H., Dawson, D.J. and Hall, K.E. (2002) Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and *Escherichia coli*. *Journal of Applied Microbiology* **92 Suppl**, 111S-120S.
- Holah, J.T. and Thorpe, R.H. (1990) Cleanability in relation to bacterial retention on unused and abraded domestic sink materials. *Journal of Applied Bacteriology* **69**, 599-608.
- Hood, S.K. and Zottola, E.A. (1997a) Adherence to stainless steel by foodborne microorganisms during growth in model food systems. *International Journal of Food Microbiology* **37**, 145-153.
- Hood, S.K. and Zottola, E.A. (1997b) Growth media and surface conditioning influence the adherence of *Pseudomonas fragi*, *Salmonella* Typhimurium and *Listeria monocytogenes* cells to stainless steel. *Journal of Food Protection* **60**, 1034-1037.
- Hopper, S.A. and Mawer, S. (1988) *Salmonella* Enteritidis in a commercial layer flock. *Veterinary Record* **123**, 351.
- Hoxey, E.V. (1984) *Bacterial Spores as Biological Indicators for Sterilization by Low Temperatures Steam and Formaldehyde*. Thesis, University of Bath, UK.
- Hoxey, E.V. and Thomas, N. (1999) Gaseous sterilization. In *Principles and Practice of Disinfection, Preservation and Sterilization* ed. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. pp. 703-732. London: Blackwell Science Ltd.

References

- Hugo, W.B. and Russell, A.D. (1999) Types of antimicrobial agents. In *Principles and Practice of Disinfection, Preservation and Sterilization* ed. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. pp. 5-94. London: Blackwell Science Ltd.
- Humbert, F. (2001) Numeration/quantification of *Salmonella*. In *Report on the Sixth Workshop Organised by CRL-Salmonella*, 11 and 12 June, pp. 25-27. Community Reference Laboratory for *Salmonella*, Bilthoven, the Netherlands.
- Humpheson, L., Adams, M.R., Anderson, W.A. and Cole, M.B. (1998) Biphasic thermal inactivation kinetics in *Salmonella* Enteritidis PT4. *Applied and Environmental Microbiology* **64**, 459-464.
- Humphrey, T.J. (1990) Heat resistance in *Salmonella* Enteritidis phage type 4: the influence of storage temperatures before heating. *Journal of Applied Bacteriology* **69**, 493-497.
- Humphrey, T.J., Chapman, P.A., Rowe, B. and Gilbert, R.J. (1990) A comparative study of the heat resistance of salmonellas in homogenized whole eggs, egg yolk and albumen. *Epidemiology and Infection* **104**, 237-241.
- Humphrey, T.J., Slater, E., McAlpine, K., Rowbury, R. and Gilbert, R.J. (1995) *Salmonella* Enteritidis Phage type 4 isolates more tolerant of heat, acid and hydrogen peroxide also survive longer on surfaces. *Applied and Environmental Microbiology* **61**, 3161-3164.
- Humphrey, T.J., Wilde, S.J. and Rowbury, R.J. (1997) Heat tolerance of *Salmonella* Typhimurium DT104 isolates attached to muscle tissue. *Letters in Applied Microbiology* **25**, 265-268.
- Hunter, A.G., Linklater, K.A. and Scott, J.A. (1976) Rodent vectors of *Salmonella*. *Veterinary Record* **99**, 145-146.
- Ingraham, J.L. and Marr, A.G. (1996) Effect of temperature, pressure, pH and osmotic stress on growth. In *Escherichia coli and Salmonella - Cellular and Molecular Biology* ed. Neidhardt, F.C., Curtiss, R. and Lin, E.C. pp. 1570-1578. Washington DC: ASM Press.
- Jackson, T.C., Hardin, M.D. and Acuff, G.R. (1996) Heat resistance of *Escherichia coli* O157:H7 in a nutritient medium and in ground beef patties as influenced by storage and holding temperatures. *Journal of Food Protection* **59**, 230-237.
- James, G.A., Beaudette, L. and Costerton, J.W. (1995) Interspecies bacterial interactions in biofilms. *Journal of Industrial Microbiology* **15**, 257-262.
- Janning, B., in 't Veld, P.H., Notermans, S. and Kramer, J. (1994) Resistance of bacterial strains to dry conditions: use of anhydrous silica gel in a desiccation model system. *Journal of Applied Bacteriology* **77**, 319-324.
- Jepsen, Å. (1960) *Salmonella*-levnedsmiddelinfektioner. In *Diagnostisk Bakteriologi og Levnedsmiddelbakteriologi* ed. Jepsen, Å. pp. 365-380. Copenhagen, Denmark: A/S Carl Fr. Mortensen.

References

- Johnston, M.D., Simons, E.A. and Lambert, R.J.W. (2000) One explanation for the variability of the bacterial suspension test. *Journal of Applied Microbiology* **88**, 237-242.
- Jones, F.T., Axtell, R.C., Rives, D.V., Scheideler, S.E., Tarver Jr., F.R., Walker, R.L. and Wineland, M.J. (1991) A survey of *Salmonella* contamination in modern broiler production. *Journal of Food Protection* **54**, 502-507.
- Jones, K. and Bradshaw, S.B. (1997) Synergism in biofilm formation between *Salmonella* Enteritidis and a nitrogen-fixing strain of *Klebsiella pneumoniae*. *Journal of Applied Microbiology* **82**, 663-668.
- Jones, P.W., Rennison, L.M., Lewin, V.H. and Redhead, D.L. (1980) The occurrence and significance to animal health of salmonellas in sewage and sewage sludges. *Journal of Hygiene London* **84**, 47-62.
- Joseph, B., Otta, S.K., Karunasagar, I. and Karunasagar, I. (2001) Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *International Journal of Food Microbiology* **64**, 367-372.
- Juneja, V.K., Snyder, O.P.J. and Marmer, B.S. (1997) Thermal destruction of *Escherichia coli* O157:H7 in beef and chicken: determination of D- and z-values. *International Journal of Food Microbiology* **35**, 231-237.
- Juven, B.J., Cox, N.A., Bailey, J.S., Thomson, J.E., Charles, O.W. and Shutze, J.V. (1984) Survival of *Salmonella* in dry food and feed. *Journal of Food Protection* **47**, 445-448.
- Kang, D.H. and Fung, D.Y. (2000) Application of thin agar layer method for recovery of injured *Salmonella* Typhimurium. *International Journal of Food Microbiology* **54**, 127-132.
- Katsui, N., Tsuchido, T., Takano, M. and Shibasaki, I. (1981) Effect of preincubation temperature on the heat resistance of *Escherichia coli* having different fatty acid compositions. *Journal of General Microbiology* **122**, 357-361.
- Katsui, N., Tsuchido, T., Takano, M. and Shibasaki, I. (1982) Viability of heat-stressed cells of micro-organisms as influenced by pre-incubation and post-incubation temperatures. *Journal of Applied Bacteriology* **53**, 103-108.
- Kaur, J., Ledward, D.A., Park, R.W.A. and Robson, R.L. (1998) Factors affecting the heat resistance of *Escherichia coli* O157:H7. *Journal of Applied Microbiology* **26**, 325-330.
- Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R. and Barer, M.R. (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie Van Leeuwenhoek* **73**, 169-187.
- Kinde, H., Read, D.H., Ardans, A., Breitmeyer, R.E., Willoughby, D., Little, H.E., Kerr, D., Gireesh, R. and Nagaraja, K.V. (1996a) Sewage effluent: likely source of *Salmonella* Enteritidis, phage type 4 infection in a commercial chicken layer flock in Southern California. *Avian Diseases* **40**, 672-676.

References

- Kinde, H., Read, D.H., Chin, R.P., Bickford, A.A., Walker, R.L., Ardans, A., Breitmeyer, R.E., Willoughby, D., Little, H.E., Kerr, D. and Gardner, I.A. (1996b) *Salmonella* Enteritidis, phage type 4 infection in a commercial layer flock in Southern California: bacteriologic and epidemiologic findings. *Avian Diseases* **40**, 665-671.
- Kirby, R.M. and Davies, R. (1990) Survival of dehydrated cells of *Salmonella* Typhimurium LT2 at high temperatures. *Journal of Applied Bacteriology* **68**, 241-246.
- Klijn, N., Herrewegh, A.A. and de Jong, P. (2001) Heat inactivation data for *Mycobacterium avium* subsp. *paratuberculosis*: implications for interpretation. *Journal of Applied Microbiology* **91**, 697-704.
- Klimek, J.W. and Bailey, J.H. (1956) Factors influencing the rate of killing of *Escherichia coli* exposed to benzalkonium chloride. *Applied Microbiology* **4**, 53-59.
- Kolter, R., Siegele, D.A. and Tormo, A. (1993) The stationary phase of the bacterial life cycle. *Annual Review of Microbiology* **47**, 855-874.
- Korber, D.R., Choi, A., Wolfaardt, G.M., Ingham, S.C. and Caldwell, D.E. (1997) Substratum topography influences susceptibility of *Salmonella* Enteritidis biofilms to trisodium phosphate. *Applied and Environmental Microbiology* **63**, 3352-3358.
- Krabisch, P. and Dorn, P. (1980) Zur epidemiologischen Bedeutung von Lebendvektoren bei der Verbreitung von Salmonellen in der Geflügelmast. *Berliner und Münchener Tierärztliche Wochenschrift* **93**, 232-235.
- Kumar, C.G. and Anand, S.K. (1998) Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology* **42**, 9-27.
- Kummerle, N., Feucht, H.H. and Kaulfers, P.M. (1996) Plasmid-mediated formaldehyde resistance in *Escherichia coli*: characterization of resistance gene. *Antimicrobial Agents and Chemotherapy* **40**, 2276-2279.
- Kunonga, N.I., Sobieski, R.J. and Crupper, S.S. (2000) Prevalence of the multiple antibiotic resistance operon (*marRAB*) in the genus *Salmonella*. *FEMS Microbiology Letters* **187**, 155-160.
- Käsbohrer, A. and Blaha, T. (1997) Field experiences with monitoring and reducing *Salmonella* in a poultry meat production chain. In *Salmonella and Salmonellosis*, May 20-22, pp. 625-630. Zoopôle, Ploufragan, France.
- Köhler, B. (1993) Beispiele für die Anreicherung von Salmonellen in der Umwelt. *Deutsche Tierärztliche Wochenschrift* **100**, 264-274.
- Lahellec, C. and Colin, P. (1985) Relationship between serotypes of salmonellae from hatcheries and rearing farms and those from processed poultry carcasses. *British Poultry Science* **26**, 179-186.

References

- Lahellec, C., Colin, P., Bennejean, G., Paquin, J., Guillerm, A. and Debois, J.C. (1986) Influence of resident *Salmonella* on contamination of broiler flocks. *Poultry Science* **65**, 2034-2039.
- Lambert, R.J.W. and Johnston, M.D. (2000) Disinfection kinetics: a new hypothesis and model for the tailing of log-survivor/time curves. *Journal of Applied Microbiology* **88**, 907-913.
- Lange, R. and Hengge-Aronis, R. (1991) Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Molecular Microbiology* **5**, 49-59.
- Lange, R. and Hengge-Aronis, R. (1994) The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes and development* **8**, 1600-1612.
- Langsrud, S. (1998) *Resistance to Disinfectants used in the Food Processing Industry, with Emphasis on Resistance to Benzalkonium Chloride*. Thesis, Department of Biotechnological Sciences, Agricultural University of Norway.
- Langsrud, S. and Sundheim, G. (1999) Factors contributing to the survival of poultry associated *Pseudomonas* spp. exposed to a quaternary ammonium compound. *Journal of Applied Microbiology* **82**, 705-712.
- Le Bacq, F., Louwagie, B. and Verhaegen, J. (1993) *Salmonella* Typhimurium and *Salmonella* Enteritidis: changing epidemiology from 1973 until 1992. *European Journal of Epidemiology* **10**, 367-371.
- LeChevallier, M.W., Cawthon, C.D. and Lee, R.G. (1988a) Factors promoting survival of bacteria in chlorinated water supplies. *Applied and Environmental Microbiology* **54**, 649-654.
- LeChevallier, M.W., Cawthon, C.D. and Lee, R.G. (1988b) Inactivation of biofilm bacteria. *Applied and Environmental Microbiology* **54**, 2492-2499.
- Lee, A.C. and Goepfert, J.M. (1975) Influence of selected solutes on thermally induced death and injury of *Salmonella* Typhimurium. *Journal of Milk and Food Technology* **38**, 195-200.
- Lee, C.A. and Falkow, S. (1990) The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4304-4308.
- Lemaux, P.G., Herendeen, S.L., Bloch, P.L. and Neidhardt, F.C. (1978) Transient rates of synthesis of individual polypeptides in *E. coli* following temperature shifts. *Cell* **13**, 427-434.
- Lemcke, R.M. and White, H.R. (1959) The heat resistance of *Escherichia coli* cells from cultures of different ages. *Journal of Applied Bacteriology* **22**, 193-201.

References

- Leriche, V. and Carpentier, B. (1995) Viable but nonculturable *Salmonella* Typhimurium in single- and binary species biofilms in response to chlorine treatment. *Journal of Food Protection* **58**, 1186-1191.
- Levy, S.B. (2002) Active efflux, a common mechanism for biocide and antibiotic resistance. *Journal of Applied Microbiology* **92 Suppl**, 65S-71S.
- Lewis, K. (2001) Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy* **45**, 999-1007.
- Leyer, G.J. and Johnson, E.A. (1993) Acid adaptation induces cross-protection against environmental stresses in *Salmonella* Typhimurium. *Applied and Environmental Microbiology* **59**, 1842-1847.
- Lidwell, O.M. and Lowbury, E.J. (1950) The survival of bacteria in dust. II. The effect of atmospheric humidity on the survival of bacteria in dust. *Journal of Hygiene Cambridge* **48**, 21-27.
- Linton, A.H., Hugo, W.B. and Russell, A.D. (1987) Practical aspects of disinfection and infection control. In *Disinfection in Veterinary and Farm Animal Practice* ed. Linton, A.H., Hugo, W.B. and Russell, A.D. pp. 144-167. London: Blackwell Scientific Publications.
- Lisle, J.T., Broadaway, S.C., Prescott, A.M., Pyle, B.H., Fricker, C. and McFeters, G.A. (1998) Effects of starvation on physiological activity and chlorine disinfection resistance in *Escherichia coli* O157:H7. *Applied and Environmental Microbiology* **64**, 4658-4662.
- Mackey, B.M. (1999) Injured bacteria. In *The Microbiological Safety and Quality of Food* ed. Lund, B.M., Baird-Parker, T.C. and Gould, G.W. pp. 315-341. Gaithersburg: Aspen Publishers, Inc.
- Mackey, B.M. and Derrick, C.M. (1986) Elevation of the heat resistance of *Salmonella* Typhimurium by sublethal heat shock. *Journal of Applied Bacteriology* **61**, 389-393.
- Mackey, B.M. and Derrick, C.M. (1987a) Changes in the heat resistance of *Salmonella* Typhimurium during heating at rising temperatures. *Letters in Applied Microbiology* **4**, 13-16.
- Mackey, B.M. and Derrick, C.M. (1987b) The effect of prior heat shock on the thermoresistance of *Salmonella* Thompson in foods. *Letters in Applied Microbiology* **5**, 115-118.
- MacKinnon, I.H. (1974) The use of inactivators in the evaluation of disinfectants. *Journal of Hygiene London* **73**, 189-195.
- Mafart, P. (2000) Taking injuries of surviving bacteria into account for optimising heat treatments. *International Journal of Food Microbiology* **55**, 175-179.
- Maillard, J.Y. (2002) Bacterial target sites for biocide action. *Journal of Applied Microbiology* **92 Suppl**, 16S-27S.

References

- Mañas, P., Pagan, R., Sala, F.J. and Condon, S. (2001) Low molecular weight milk whey components protect *Salmonella* Senftenberg 775W against heat by a mechanism involving divalent cations. *Journal of Applied Microbiology* **91**, 871-877.
- Maris, P. (1995) Modes of action of disinfectants. *Revue scientifique et technique/Office international des épizooties* **14**, 47-55.
- Marthedal, H.E. (1960) *Salmonella Gallinarum, Salmonella Pullorum og Salmonella Typhimurium - Kulturelle, serologiske og epizootiologiske undersøgelser samt studier over O-antigenstrukturen*. Thesis, The Royal Veterinary and Agricultural University, Copenhagen, Denmark.
- Martin, S.W., Meek, A.H. and Willeberg, P. (1987) *Veterinary Epidemiology - Principles and Methods*. Ames, USA: Iowa State University Press.
- Mattick, K.L., Jørgensen, F., Legan, J.D., Lappin-Scott, H.M. and Humphrey, T.J. (2000) Habituation of *Salmonella* spp. at reduced water activity and its effect on heat tolerance. *Applied and Environmental Microbiology* **66**, 4921-4925.
- Mattila-Sandholm, T. and Wirtanen, G. (1992) Biofilm formation in the food industry: a review. *Food Reviews International* **8**, 573-603.
- McCann, M.P., Kidwell, J.P. and Matin, A. (1991) The putative sigma factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. *Journal of Bacteriology* **173**, 4188-4194.
- McCoy, J.H. (1975) Trends in *Salmonella* food poisoning in England and Wales 1941-72. *Journal of Hygiene London* **74**, 271-282.
- McDade, J.J. and Hall, L.B. (1964) Survival of Gram-negative bacteria in the environment. I. Effect of relative humidity on surface-exposed organisms. *American Journal of Hygiene* **80**, 192-204.
- McDonnell, G. and Russell, A.D. (1999) Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews* **12**, 147-179.
- McEldowney, S. and Fletcher, M. (1988) The effect of temperature and relative humidity on the survival of bacteria attached to dry solid surfaces. *Letters in Applied Microbiology* **7**, 83-86.
- Meroz, M. and Samberg, Y. (1995) Disinfecting poultry production premises. *Revue scientifique et technique/Office international des épizooties* **14**, 273-291.
- Milner, K.C. and Shaffer, M.F. (1952) Bacteriological studies of experimental *Salmonella* infections in chicks. *Journal of Infectious Diseases* **90**, 8186-8189.
- Mitchener, H.D., Thompson, P.A. and Lewis, J.C. (1959) Search for substances which reduce the heat resistance of bacterial spores. *Applied Microbiology* **7**, 166-173.

References

- Mitscherlich, E. and Marth, E.H. (1984) *Microbial Survival in the Environment - Bacteria and Rickettsiae in Human and Animal Health*. Berlin: Springer-Verlag.
- Miura, S., Sayo, G. and Miyamae, T. (1964) Occurrence and survival of *Salmonella* organisms in hatcher chick fluff from commercial hatcheries. *Avian Diseases* **8**, 546-554.
- Moats, W.A., Dabbah, R. and Edwards, V.M. (1971) Interpretation of nonlogarithmic-survivor curves of heated bacteria. *Journal of Food Science* **36**, 523-526.
- Moken, M.C., McMurry, L.M. and Levy, S.B. (1997) Selection of multiple-antibiotic-resistant (*mar*) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the *mar* and *acrAB* loci. *Antimicrobial Agents and Chemotherapy* **41**, 2770-2772.
- Molin, G. (1977) Dry-heat resistance of *Bacillus subtilis* spores in contact with serum albumin, carbohydrates or lipids. *Journal of Applied Bacteriology* **42**, 111-116.
- Monaghan, P., Shedden, C.B., Ensor, K., Fricker, C.R. and Girdwood, R.W.A. (1985) *Salmonella* carriage by herring gulls in the Clyde area of Scotland in relation to their feeding ecology. *Journal of Applied Ecology* **22**, 669-680.
- Moore, G. and Griffith, C. (2002) A comparison of surface sampling methods for detecting coliforms on food contact surfaces. *Food Microbiology* **19**, 65-73.
- Morgan-Jones, S.C. (1981) Cleansing and disinfection of farm buildings. In *Disinfectants: Their Use and Evaluation of Effectiveness* ed. Collins, C.H., Allwood, M.C., Bloomfield, S.F. and Fox, A. pp. 199-212. London: Academic Press.
- Morris, G.K., McMurray, B.L., Galton, M.M. and Wells, J.G. (1969) A study of the dissemination of salmonellosis in a commercial broiler chicken operation. *American Journal of Veterinary Research* **30**, 1413-1421.
- Mossel, D.A.A. and Corry, J.E.L. (1977) Detection and enumeration of sublethally injured pathogenic and index bacteria in foods and water processed for safety. *Alimenta* 19-34.
- Munro, P.M., Flatau, G.N., Clement, R.L. and Gauthier, M.J. (1995) Influence of the RpoS (KatF) sigma factor on maintenance of viability and culturability of *Escherichia coli* and *Salmonella* Typhimurium in seawater. *Applied and Environmental Microbiology* **61**, 1853-1858.
- Murano, E.A. and Pierson, M.D. (1992) Effect of heat shock and growth atmosphere on the heat resistance of *Escherichia coli* O157:H7. *Journal of Food Protection* **55**, 171-175.
- Murase, T., Senjyu, K., Maeda, T., Tanaka, M., Sakae, H., Matsumoto, Y., Kaneda, Y., Ito, T. and Otsuki, K. (2001) Monitoring of chicken houses and an attached egg-processing facility in a laying farm for *Salmonella* contamination between 1994 and 1998. *Journal of Food Protection* **64**, 1912-1916.
- Murray, C.J. (1991) Salmonellae in the environment. *Revue scientifique et technique/Office international des épizooties* **10**, 765-785.

References

- Møretrø, T., Midtgaard, E.S., Nesse, L.L. and Langsrud, S. (2003) Susceptibility of *Salmonella* isolated from fish feed factories to disinfectants and air-drying at surfaces. *Veterinary Microbiology* **94**, 207-217.
- Nakamura, M. (1962) The survival of *Shigella sonnei* on cotton, glass, wood, paper, and metal at various temperatures. *Journal of Hygiene Cambridge* **60**, 35-39.
- Neidhardt, F.C. and VanBogelen, R.A. (1987) Heat shock response. In *Escherichia coli and Salmonella Typhimurium - Cellular and Molecular Biology* ed. Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M. and Umberger, H.E. pp. 1334-1345. Washington DC: American Society for Microbiology.
- Ng, H., Bayne, H.G. and Garibaldi, J.A. (1969) Heat resistance of *Salmonella*: the uniqueness of *Salmonella* Senftenberg 775W. *Applied Microbiology* **17**, 78-82.
- Nikaido, H. and Vaara, M. (1987) Outer membrane. In *Escherichia coli and Salmonella Typhimurium - Cellular and Molecular Biology* ed. Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M. and Umberger, H.E. pp. 7-22. Washington DC: ASM Press.
- Nordgren, G. (1939) Investigations on the sterilization efficacy of gaseous formaldehyde. *Acta Pathologica et Microbiologica Scandinavica, supplementum XL* 1-165.
- Oliver, J.D. (1993) Formation of viable but nonculturable cells. In *Starvation in Bacteria* ed. Kjelleberg, S. pp. 239-272. New York and London: Plenum Press.
- Opitz, H.M. (1992) Progress being made in *Salmonella* Enteritidis reduction on the farm. *Poultry Digest* **March**, 16-22.
- Petersen, K.E. and James, W.O. (1998) Agents, vehicles, and causal inference in bacterial foodborne disease outbreaks: 82 reports (1986-1995). *Journal of the American Veterinary Medical Association* **212**, 1874-1881.
- Petersen, L. (1997) A comparison of EF-18 agar and modified brilliant green agar with lutensit for isolation of *Salmonella* from poultry samples. *Acta Veterinaria Scandinavica* **38**, 79-85.
- Pfeifer, J. and Kessler, H.G. (1994) Effect of relative humidity of hot air on the heat resistance of *Bacillus cereus* spores. *Journal of Applied Bacteriology* **77**, 121-128.
- Placha, I., Venglovsky, J., Sasakova, N. and Svoboda, I.F. (2001) The effect of summer and winter seasons on the survival of *Salmonella* Typhimurium and indicator microorganisms during the storage of solid fraction of pig slurry. *Journal of Applied Microbiology* **91**, 1036-1043.
- Plym-Forsell, L. and Ekesbo, I. (1996) Survival of salmonellas in urine and dry faeces from cattle - an experimental study. *Acta Veterinaria Scandinavica* **37**, 127-131.
- Popoff, M.Y., Bockemuhl, J. and Gheesling, L.L. (2003) Supplement 2001 (no. 45) to the Kauffmann-White scheme. *Research in Microbiology* **154**, 173-174.

References

- Popoff, M.Y. and Le Minor, L. (1997) *Antigenic Formulas of the Salmonella Serovars*. Insitut Pasteur, WHO Collaborating Centre for Reference and Research on *Salmonella*, Paris, France.
- Poppe, C. (1999) Epidemiology of *Salmonella enterica* serovar Enteritidis. In *Salmonella Enterica Serovar Enteritidis in Humans and Animals - Epidemiology, Pathogenesis, and Control* ed. Saeed, A.M., Gast, R.K., Potter, M.E. and Wall, P.G. pp. 3-18. Ames: Iowa State University Press.
- Prince, J. and Ayliffe, A.J. (1972) In-use testing of disinfectants in hospitals. *Journal of Clinical Pathology* **25**, 586-589.
- Profe, D. and Trenner, P. (1994) Experimental investigations on nebulizing disinfectants in large poultry houses. In *8th International Congress on Animal Hygiene*, September 12-16. International Society on Animal Hygiene, St. Paul, Minnesota, USA.
- Rabsch, W., Hargis, B.M., Tsois, R.M., Kingsley, R.A., Hinz, K.H., Tschape, H. and Baumler, A.J. (2000) Competitive exclusion of *Salmonella* Enteritidis by *Salmonella* Gallinarum in poultry. *Emerging Infectious Diseases* **6**, 443-448.
- Rambach, A. (1990) New plate medium for facilitated differentiation of *Salmonella* spp. from *Proteus* spp. and other enteric bacteria. *Applied and Environmental Microbiology* **56**, 301-303.
- Randall, L.P., Cooles, S.W., Sayers, A.R. and Woodward, M.J. (2001) Association between cyclohexane resistance in *Salmonella* of different serovars and increased resistance to multiple antibiotics, disinfectants and dyes. *Journal of Medical Microbiology* **50**, 919-924.
- Randall, L.P. and Woodward, M.J. (2001a) Multiple antibiotic resistance (*mar*) locus in *Salmonella enterica* serovar Typhimurium DT104. *Applied and Environmental Microbiology* **67**, 1190-1197.
- Randall, L.P. and Woodward, M.J. (2001b) Role of the *mar* locus in virulence of *Salmonella enterica* serovar Typhimurium DT104 in chickens. *Journal of Medical Microbiology* **50**, 770-779.
- Randall, L.P. and Woodward, M.J. (2002) The multiple antibiotic resistance (*mar*) locus and its significance. *Research in Veterinary Science* **72**, 87-93.
- Read, R.B., Bradshaw, J.G., Dickerson, R.W. and Peeler, J.T. (1968) Thermal resistance of salmonellae isolated from dry milk. *Applied Microbiology* **16**, 998-1001.
- Refsum, T. (2003) *Salmonella Infections in Wild-living Birds and Hedgehogs in Norway*. Thesis, The Norwegian School of Veterinary Science and National Veterinary Institute, Norway.

References

- Reilly, W.J., Forbes, G.I., Paterson, G.M. and Sharp, J.C. (1981) Human and animal salmonellosis in Scotland associated with environmental contamination, 1973-79. *Veterinary Record* **108**, 553-555.
- Reybrouck, G. (1999) Evaluation of the antibacterial and antifungal activity of disinfectants. In *Principles and Practice of Disinfection, Preservation and Sterilization* ed. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. pp. 124-144. London: Blackwell Science, Inc.
- Riemann, H. (1968) Effect of water activity on the heat resistance of *Salmonella* in "dry" materials. *Applied Microbiology* **16**, 1621-1622.
- Rigby, C.E., Pettit, J.R., Baker, M.F., Bentley, A.H., Salomons, M.O. and Lior, H. (1980) Sources of salmonellae in an uninfected commercially-processed broiler flock. *Canadian Journal of Comparative Medicine* **44**, 267-274.
- Rigby, C.E., Pettit, J.R., Bentley, A.H., Spencer, J.L., Salomons, M.O. and Lior, H. (1982) The relationships of salmonellae from infected broiler flocks, transport crates or processing plants to contamination of eviscerated carcasses. *Canadian Journal of Comparative Medicine* **46**, 272-278.
- Robertshaw, R.G. (1983) Low temperature steam and formaldehyde sterilization. *Journal of Hospital Infection* **4**, 305-314.
- Robertson, M.H. (1972) Survival of *S. Typhimurium* in floor dust - a possible reservoir of infection in institutions. *Public Health* **87**, 39-45.
- Rodrigue, D.C., Tauxe, R.V. and Rowe, B. (1990) International increase in *Salmonella* Enteritidis: a new pandemic? *Epidemiology and Infection* **105**, 21-27.
- Rolfe, D.L., Riemann, H.P., Farver, T.B. and Himathongkham, S. (2000) Drag swab efficiency factors when sampling chicken manure. *Avian Diseases* **44**, 668-675.
- Ronner, A.B. and Wong A.C.L. (1993) Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella* Typhimurium on stainless steel and Buna-n rubber. *Journal of Food Protection* **56**, 750-758.
- Rose, N., Beaudreau, F., Drouin, P., Toux, J.Y., Rose, V. and Colin, P. (1999) Risk factors for *Salmonella enterica* subsp. *enterica* contamination in French broiler-chicken flocks at the end of the rearing period. *Preventive Veterinary Medicine* **39**, 265-277.
- Rose, N., Beaudreau, F., Drouin, P., Toux, J.Y., Rose, V. and Colin, P. (2000) Risk factors for *Salmonella* persistence after cleansing and disinfection in French broiler-chicken houses. *Preventive Veterinary Medicine* **44**, 9-20.
- Roszak, D.B. and Colwell, R.R. (1987) Survival strategies of bacteria in the natural environment. *Microbiological Reviews* **51**, 365-379.
- Roszak, D.B., Grimes, D.J. and Colwell, R.R. (1984) Viable but nonrecoverable stage of *Salmonella* Enteritidis in aquatic systems. *Canadian Journal of Microbiology* **30**, 334-338.

References

- Rozen, Y. and Belkin, S. (2001) Survival of enteric bacteria in seawater. *FEMS Microbiology Reviews* **25**, 513-529.
- Ruckaberle, E., Sting, R. and Hafez, H.M. (1999) Studies on the efficacy of disinfection of turkey houses and a slaughter house to combat foodborne pathogens. In *2nd International Symposium on Turkey Diseases*, 24-27 March, pp. 190-198. German Branch of the World Veterinary Poultry Association, Berlin, Germany.
- Russell, A.D. (1981) Neutralization procedures in the evaluation of bactericidal activity. In *Disinfectants: their Use and Evaluation of Effectiveness* ed. Collins, C.H., Allwood, M.C., Bloomfield, S.F. and Fox, A. pp. 45-59. London: Academic Press.
- Russell, A.D. (1997) Plasmids and bacterial resistance to biocides. *Journal of Applied Microbiology* **82**, 155-165.
- Russell, A.D. (1998) Mechanisms of bacterial resistance to antibiotics and biocides. *Progress in Medicinal Chemistry* **35**, 133-197.
- Russell, A.D. (1999a) Bacterial resistance to disinfectants: present knowledge and future problems. *Journal of Hospital Infection* **43 (Supplement)**, S57-S68.
- Russell, A.D. (1999b) Factors influencing the efficacy of antimicrobial agents. In *Principles and Practice of Disinfection, Preservation and Sterilization* ed. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. pp. 95-123. London: Blackwell Science Ltd.
- Russell, A.D. (1999c) Heat sterilization - sterilization and disinfection by heat methods. In *Principles and Practice of Disinfection, Preservation and Sterilization* ed. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. pp. 629-639. London: Blackwell Science Ltd.
- Russell, A.D. (2000) Do biocides select for antibiotic resistance? *Journal of Pharmacy and Pharmacology* **52**, 227-233.
- Russell, A.D. and Chopra, I. (1996) Antiseptics, disinfectants and preservatives: their properties, mechanisms of action and uptake into bacteria. In *Understanding Antibacterial Action and Resistance* ed. Russell, A.D. and Chopra, I. pp. 96-149. London: Ellis Horwood.
- Russell, A.D. and Gould, G.W. (1988) Resistance of Enterobacteriaceae to preservatives and disinfectants. *Journal of Applied Bacteriology - Symposium supplement*, 167S-195S.
- Russell, A.D. and McDonnell, G. (2000) Concentration: a major factor in studying biocidal action. *Journal of Hospital Infection* **44**, 1-3.
- Ryu, J.H. and Beuchat, L.R. (1999) Changes in heat tolerance of *Escherichia coli* O157:H7 after exposure to acidic environments. *Food Microbiology* **16**, 317-324.
- Sackett, D.L. (1992) A primer on the precision and accuracy of the clinical examination. *Journal of the American Medical Association*. **267**, 2638-2644.

References

- Salmond, G.P., Bycroft, B.W., Stewart, G.S. and Williams, P. (1995) The bacterial 'enigma': cracking the code of cell-cell communication. *Molecular Microbiology* **16**, 615-624.
- Savov, D., Dimitrov, N., Slavkov, I., Tsonev, T. and Duparinov, I. (1976) [Experience in controlling the *Salmonella* carrier state on a pedigree poultry breeding farm] (in Bulgarian, with English abstract). *Veterinarno-meditsinski nauki* **13**, 28-35.
- Senhaji, A.F. (1977) The protective effect of fat on the heat resistance of bacteria (II). *Journal of Food Technology* **12**, 217-230.
- Shivaprasad, H.L., Timoney, J.F., Morales, S., Lucio, B. and Baker, R.C. (1990) Pathogenesis of *Salmonella* Enteritidis infection in laying chickens. I. Studies on egg transmission, clinical signs, fecal shedding, and serological responses. *Avian Diseases* **34**, 548-557.
- Sidhu, M.S. (2001) *Disinfectant- and Antibiotic Resistant Bacteria from Food and Clinical Environments*. Thesis, Department of Chemistry and Biotechnology, Agricultural University of Norway.
- Sidhu, M.S., Sørum, H. and Holck, A. (2002) Resistance to quaternary ammonium compounds in food related bacteria. *Microbial Drug Resistance* **8**, 393-399.
- Siegele, D.A. and Kolter, R. (1992) Life after log. *Journal of Bacteriology* **174**, 345-348.
- Skaarup, H. (2002) *Kvalitetskontrolprogram for Mølbjerg Rugeri. Rengøring og desinfektion af avlshuse* (internal report).
- Skov, M.N., Carstensen, B., Tornøe, N. and Madsen, M. (1999) Evaluation of sampling methods for the detection of *Salmonella* in broiler flocks. *Journal of Applied Microbiology* **86**, 695-700.
- Smith, P.J., Jones, F. and Watson, D.C. (1978) *Salmonella* pollution of surface waters. *Journal of Hygiene London* **81**, 353-360.
- Snoeyenbos, G.H., Carlson, V.L., McKie, B.A. and Smyser, C.F. (1967) An epidemiological study of salmonellosis of chickens. *Avian Diseases* **11**, 653-667.
- Somers, E.B., Schoeni, J.L. and Wong, A.C.L. (1994) Effect of trisodium phosphate on biofilm and planktonic cells of *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* Typhimurium. *International Journal of Food Microbiology* **22**, 269-276.
- Sondossi, M., Roosmoore, H.W. and Wireman, J.W. (1985) Observations of resistance and cross-resistance to formaldehyde and a formaldehyde condensate biocide in *Pseudomonas aeruginosa*. *International Biodeterioration* **21**, 105-106.
- Spiner, D.R. and Hoffmann, R.K. (1971) Effect of relative humidity on formaldehyde decontamination. *Applied Microbiology* **11**, 1138-1140.

References

- St Louis, M.E., Morse, D.L., Potter, M.E., DeMelfi, T.M., Guzewich, J.J., Tauxe, R.V. and Blake, P.A. (1988) The emergence of grade A eggs as a major source of *Salmonella* Enteritidis infections. New implications for the control of salmonellosis. *Journal of the American Medical Association*. **259**, 2103-2107.
- Stewart, P.S. and Costerton, J.W. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135-138.
- Stickler, D.J. and King, J.B. (1999) Bacterial sensitivity and resistance - intrinsic resistance. In *Principles and Practice of Disinfection, Preservation and Sterilization* ed. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. pp. 284-296. London: Blackwell Science Ltd.
- Stickler, D.J. and Thomas, B. (1980) Antiseptic and antibiotic resistance in Gram-negative bacteria causing urinary tract infection. *Journal of Clinical Pathology* **33**, 288-296.
- Storz, G. and Hengge-Aronis, R. (2000) *Bacterial Stress Responses*. Washington DC: ASM Press.
- Storz, G. and Zheng, M. (2000) Oxidative stress. In *Bacterial Stress Responses* ed. Storz, G. and Hengge-Aronis, R. pp. 47-59. Washington DC: ASM Press.
- Sundahl, A.M. (1975) Cleanability of building materials. *Farm Building Progress* **40**, 19-21.
- Sutton, S.V., Proud, D.W., Rachui, S. and Brannan, D.K. (2002) Validation of microbial recovery from disinfectants. *PDA Journal of Pharmaceutical Science and Technology* **56**, 255-266.
- Swift, S., Bainton, N.J. and Winson, M.K. (1994) Gram-negative bacterial communication by N-acyl homoserine lactones: a universal language? *Trends in Microbiology* **2**, 193-198.
- Søeberg, H.P. and Pedersen, N. (1998) *Rengøring og desinfektion af fjerkræstalde*. Landskontoret for Fjerkrærådgivning, Denmark.
- Takeo, Y., Oie, S., Kamiya, A., Konishi, H. and Nakazawa, T. (1994) Efficacy of disinfectants against biofilm cells of *Pseudomonas aeruginosa*. *Microbios* **79**, 19-26.
- Tanner, R.S. (1989) Comparative testing and evaluation of hard-surface disinfectants. *Journal of Industrial Microbiology* **4**, 145-154.
- Taylor, J.H. and Holah, J.T. (1996) A comparative evaluation with respect to the bacterial cleanability of a range of wall and floor surface materials used in the food industry. *Journal of Applied Bacteriology* **81**, 262-266.
- Thompson, W.S., Busta, F.F., Thompson, D.R. and Allen, C.E. (1979) Inactivation of salmonellae in autoclaved ground beef exposed to constantly rising temperatures. *Journal of Food Protection* **42**, 410-415.

References

- Toma, B., Vaillancourt, J., Dufour, B., Eloit, M., Moutou, F., Marsh, W., Bénét, J., Sanaa, M. and Michel, P. (1999) *Dictionary of Veterinary Epidemiology*. Iowa, USA: Iowa State University Press/Ames.
- Tucker, J.F. (1967) Survival of salmonellae in built-up litter for housing of rearing and laying fowls. *British Veterinary Journal* **123**, 92-103.
- Turnbull, P.C.B. and Snoeyenbos, G.H. (1973) The roles of ammonia, water activity, and pH in the salmonellacidal effect of long-used poultry litter. *Avian Diseases* **17**, 72-86.
- Turner, K., Porter, J., Pickup, R. and Edwards, C. (2000) Changes in viability and macromolecular content of long-term batch cultures of *Salmonella* Typhimurium measured by flow cytometry. *Journal of Applied Microbiology* **89**, 90-99.
- Tuschewitzki, G.J., Exner, M. and Thofern, E. (1983) Induktion einer mikrobiellen Wandbesiedlung in Kunststoffschläuchen durch Trinkwasser. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene [B]* **178**, 380-388.
- Vaara, M. (1992) Agents that increase the permeability of the outer membrane. *Microbiological Reviews* **56**, 395-411.
- Valancony, H., Fournier, G., Drouin, P., Danguy, R., Toux, J.Y. and Balaine, L. (2001) Le nettoyage et la désinfection des bâtiments de poules pondeuses en cages vis à vis de *Salmonella* Enteritidis: 1^{re} partie/bilan des prélèvements bactériologiques. *Sciences et Techniques Avicoles* **36**, 5-14.
- van de Giessen, A.W., Ament, A.J.H.A. and Notermans, S.H.W. (1994) Intervention strategies for *Salmonella* Enteritidis in poultry flocks: a basic approach. *International Journal of Food Microbiology* **21**, 145-154.
- van Klingeren, B. (1995) Disinfectant testing on surfaces. *Journal of Hospital Infection* **30 Suppl**, 397-408.
- van Klingeren, B., Leussink, A.B. and van Wijngaarden, L.J. (1977) A collaborative study on the repeatability and the reproducibility of the Dutch Standard-suspension-test for the evaluation of disinfectants. *Zentralblatt für Bakteriologie und Hygiene, I. Abteile, Originale B* **164**, 521-548.
- van Klingeren, B. and Pullen, W. (1993) Glutaraldehyde resistant mycobacteria from endoscope washers. *Journal of Hospital Infection* **25**, 147-149.
- Veldman, A., Vahl, H.A., Borggreve, G.J. and Fuller, D.C. (1995) A survey of the incidence of *Salmonella* species and *Enterobacteriaceae* in poultry feeds and feed components. *Veterinary Record* **136**, 169-172.
- Verrips, C.T., Kwast, R.H. and de Vries, W. (1980) Growth conditions and heat resistance of *Citrobacter freundii*. *Antonie Van Leeuwenhoek* **46**, 551-563.

References

- Vigh, L., Maresca, B. and Harwood, J.L. (1998) Does the membrane's physical state control the expression of heat shock and other genes? *Trends in Biochemical Sciences* **23**, 369-374.
- von Löhren, U. (1994) Maßnahmen zur Desinfektion und Entwesung von Geflügelställen nach dem Auftreten von *S. Enteritidis*. *Deutsche Tierärztliche Wochenschrift* **101**, 290-292.
- Walters, A.H. (1967) Hard surface disinfection and its evaluation. *Journal of Applied Bacteriology* **30**, 56-65.
- Waltman, W.D., Horne, A.M. and Pirkle, C. (1993) Influence of enrichment incubation time on the isolation of *Salmonella*. *Avian Diseases* **37**, 884-887.
- Wang, G., Zhao, T. and Doyle, M.P. (1996) Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. *Applied and Environmental Microbiology* **62**, 2567-2570.
- Ward, L.R., de Sa, J.D. and Rowe, B. (1987) A phage-typing scheme for *Salmonella* Enteritidis. *Epidemiology and Infection* **99**, 291-294.
- Wegener, H.C., Hald, T., Wong, D.L., Madsen, M., Korsgaard, H., Bager, F., Gerner-Smidt, P. and Mølbak, K. (2003) *Salmonella* control programs in Denmark. *Emerging Infectious Diseases* **9**, 774-780.
- Welsh, D.T. and Herbert, R.A. (1999) Osmotically induced intracellular trehalose, but not glycine betaine accumulation promotes desiccation tolerance in *Escherichia coli*. *FEMS Microbiology Letters* **174**, 57-63.
- Werner, H.P. (1975) Investigations on the efficacy of surface disinfection and surface cleaning procedures. 2. Laboratory testing of the efficacy under conditions simulating those of real-life. *Zentralblatt für Bakteriologie und Hygiene, I. Abteile, Originale B*, **B 160**, 568-578.
- White, D.G., Goldman, J.D., Demple, B. and Levy, S.B. (1997) Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA*, *soxS*, or *robA* in *Escherichia coli*. *Journal of Bacteriology* **179**, 6122-6126.
- Wilde, S., Jørgensen, F., Campbell, A., Rowbury, R. and Humphrey, T. (2000) Growth of *Salmonella enterica* serovar Enteritidis PT4 in media containing glucose results in enhanced RpoS-independent heat and acid tolerance but does not affect the ability to survive air-drying on surfaces. *Food Microbiology* **17**, 679-686.
- Wilkoff, L.J., Westbrook, L. and Dixon, G.J. (1969) Persistence of *Salmonella* Typhimurium on fabrics. *Applied Microbiology* **18**, 256-261.
- Wille, B. (1976) Möglichkeiten einer Resistenzentwicklung von Mikroorganismen gegen Desinfektionsmittel. *Zentralblatt für Bakteriologie [Originale B]* **162**, 217-220.
- Williams, J.E. and Benson, S.T. (1978) Survival of *Salmonella* Typhimurium in poultry feed and litter at three temperatures. *Avian Diseases* **22**, 742-747.

References

Willinghan, E.M., Sander, J.E., Thayer, S.G. and Wilson, J.L. (1996) Investigation of bacterial resistance to hatchery disinfectants. *Avian Diseases* **40**, 510-515.

Winans, S.C. and Zhu, J. (2000) Roles of cell-cell communication in confronting the limitations and opportunities of high population densities. In *Bacterial Stress Responses* ed. Storz, G. and Hengge-Aronis, R. pp. 261-272. Washington DC: ASM Press.

Winfield, M.D. and Groisman, E.A. (2003) Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Applied and Environmental Microbiology* **69**, 3687-3694.

World Health Organization (1988) *Salmonellosis Control: the Role of Animal and Product Hygiene*. WHO Expert Committee on salmonellosis control, Technical Report Series 774.

Wray, C. (1995) Salmonellosis: a hundred years old and still going strong. *British Veterinary Journal* **151**, 339-341.

Wray, C. and Davies, R.H. (1997) Reflections on the epidemiology of *Salmonella*: a challenge for disease control. In *Salmonella and Salmonellosis*, May 20-22, pp. 309-314. Ploufragan, France.

Wright, A.M., Hoxey, E.V., Soper, C.J. and Davies, D.J. (1996) Biological indicators for low temperature steam and formaldehyde sterilization: investigation of the effect of change in temperature and formaldehyde concentration on spores of *Bacillus stearothermophilus* NCIMB 8224. *Journal of Applied Bacteriology* **80**, 259-265.

Yamamori, T. and Yura, T. (1980) Temperature-induced synthesis of specific proteins in *Escherichia coli*: evidence for transcriptional control. *Journal of Bacteriology* **142**, 843-851.

Yura, T., Kanemori, M. and Morita, M.T. (2000) The heat shock response: regulation and function. In *Bacterial Stress Responses* ed. Storz, G. and Hengge-Aronis, R. pp. 3-18. Washington DC: ASM Press.

Zottola, E.A. and Sasahara, K.C. (1994) Microbial biofilms in the food processing industry - should they be a concern? *International Journal of Food Microbiology* **23**, 125-148.

Appendix A – Quantitative heat experiments

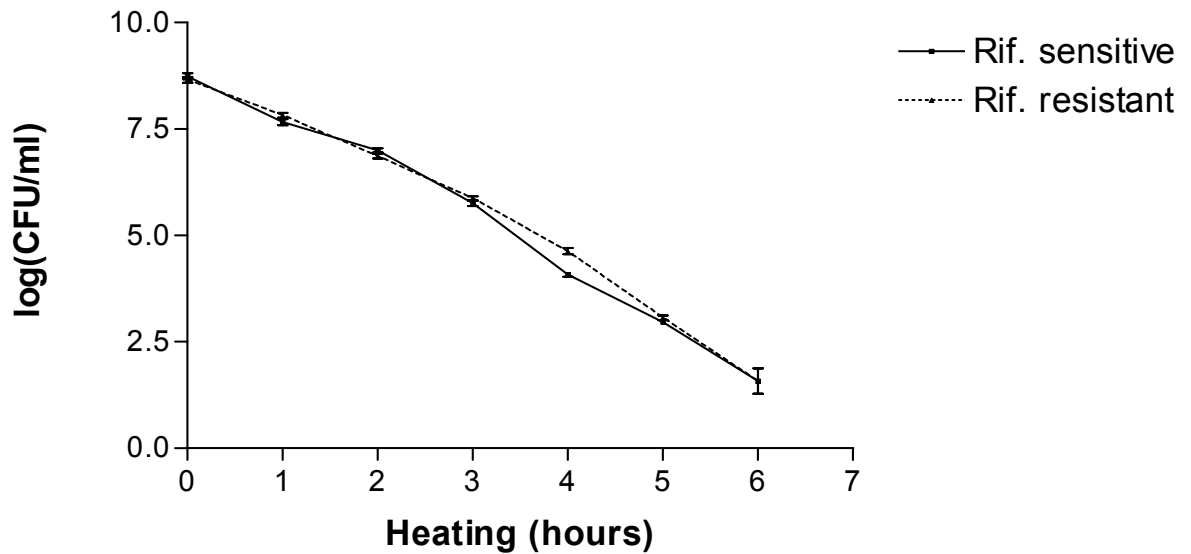


Figure A1: Heating of *Salmonella Enteritidis*, phage type 8, from floor sample taken in an empty poultry house (isolate 9981190-21).

Aim: comparison of heat resistances between rifampicin sensitive and resistant isolates.

Media: physiologic saline (0.85% NaCl) for tenfold dilutions; Rambach agar for plating.

Conditions: 55 °C (air temperature).

Conclusion: no difference in heat resistances between rifampicin sensitive and resistant isolates.

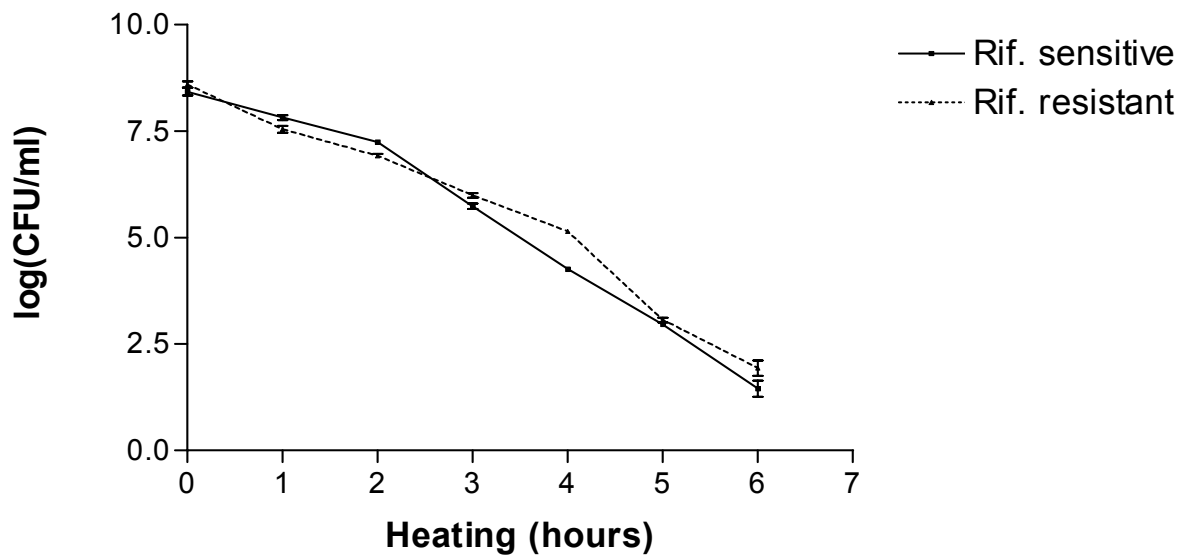


Figure A2: Heating of *Salmonella Enteritidis*, phage type 8, from hen (isolate 7260281-2).

Aim, media, conditions, conclusion: as for Figure A1.

Appendix A

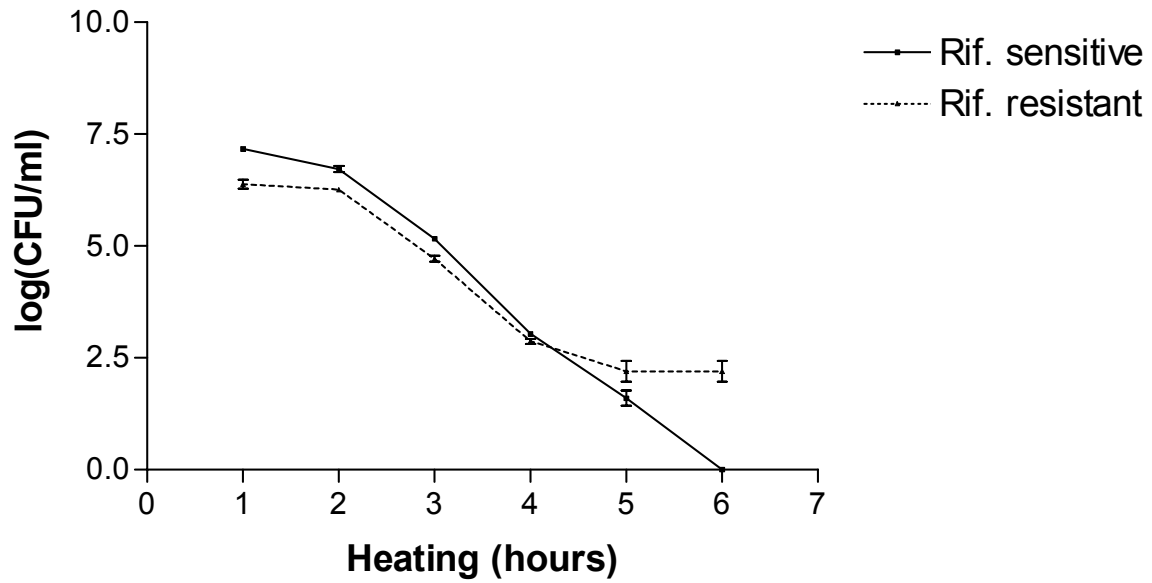


Figure A3: Heating of *Salmonella Enteritidis*, phage type 8, from faecal sample (isolate 7278230-1).

Aim, media, conditions, conclusion: as for Figure A1.

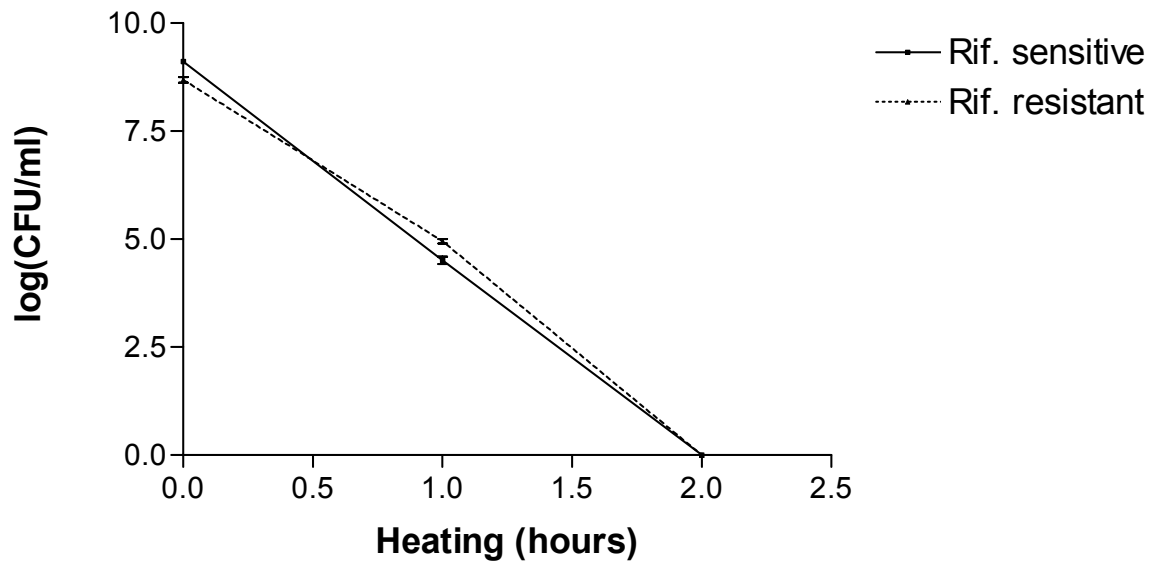


Figure A4: Heating of *Salmonella Infantis*, from faecal sample (isolate 9879483-1).

Aim, media, conditions, conclusion: as for Figure A1.

Appendix A

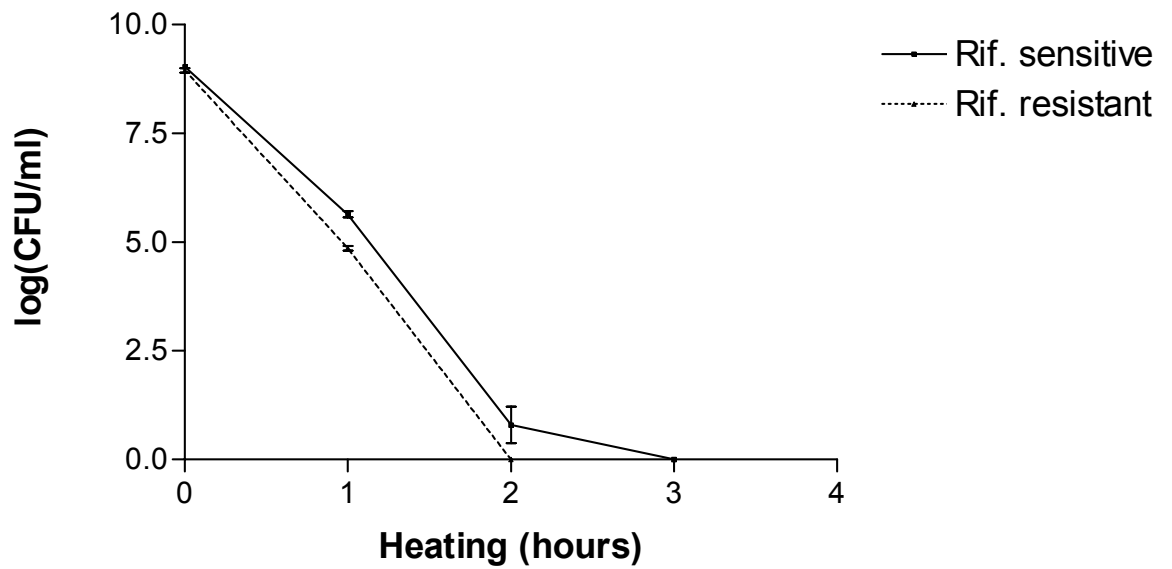


Figure A5: Heating of *Salmonella Infantis*, from environmental sample taken in an empty poultry house (isolate 9888954-7).

Aim, media, conditions, conclusion: as for Figure A1.

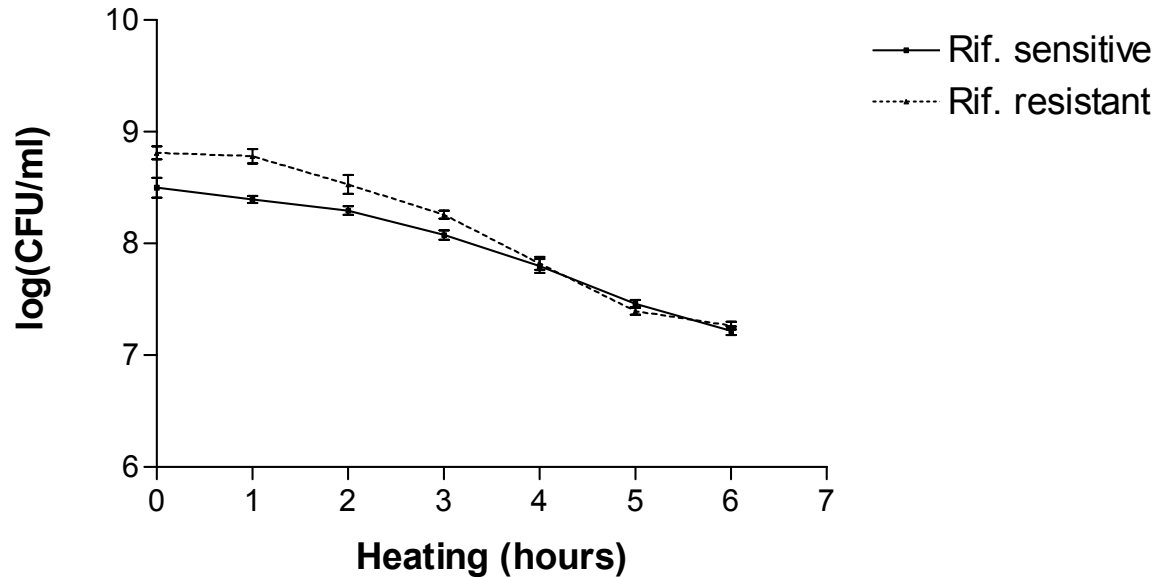


Figure A6: Heating of *Salmonella Typhimurium*, definitive type 110, from environmental sample taken in an empty poultry house (isolate 9976240-4).

Conditions: 50 °C (air temperature).

Aim, media, conclusion: as for Figure A1.

Appendix A

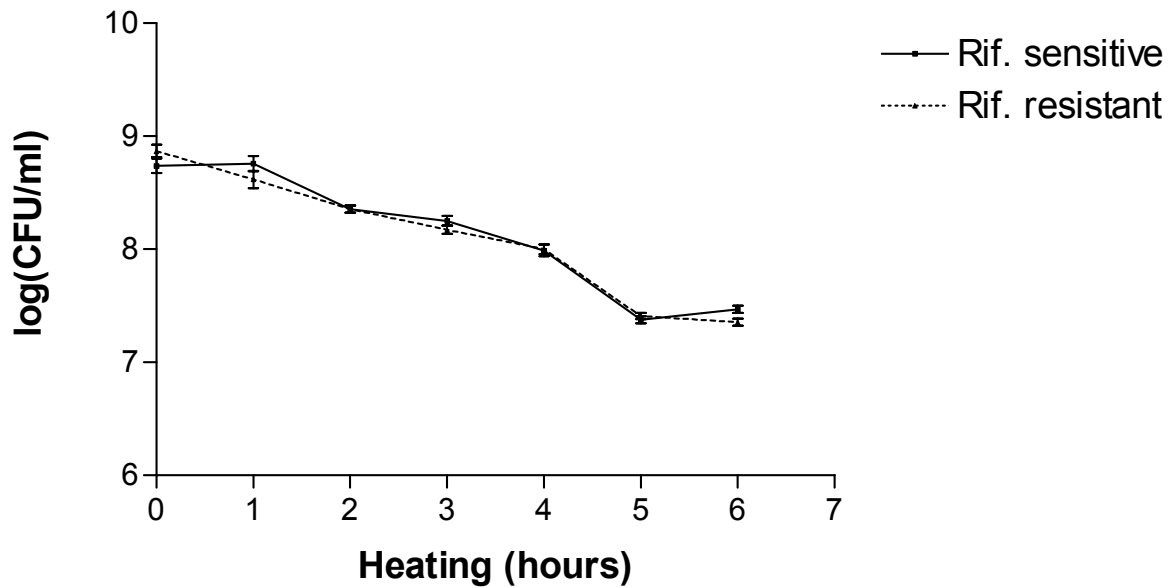


Figure A7: Heating of *Salmonella Typhimurium*, definitive type 110, faecal sample (isolate 9974037-1).

Conditions: 50 °C (air temperature).

Aim, media, conclusion: as for Figure A1.

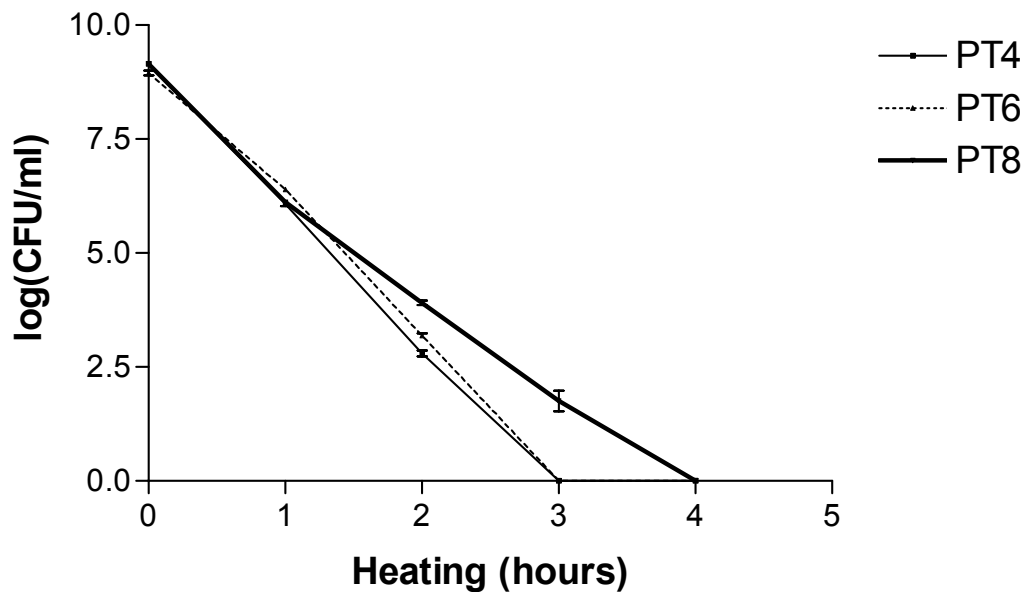


Figure A8: Heating of three *Salmonella Enteritidis* phage types (PT4 (isolate 7278447-1), PT6 (isolate 9979724-1), PT8 (isolate 7278230-1)), all from faecal samples.

Aim: comparison of heat resistances between three *S. Enteritidis* phage types found commonly in the Danish poultry sector to see if one phage type could substitute the other two.

Media, conditions: as for Figure A1.

Conclusion: PT8 was at least as heat resistant as PT4 and PT6.

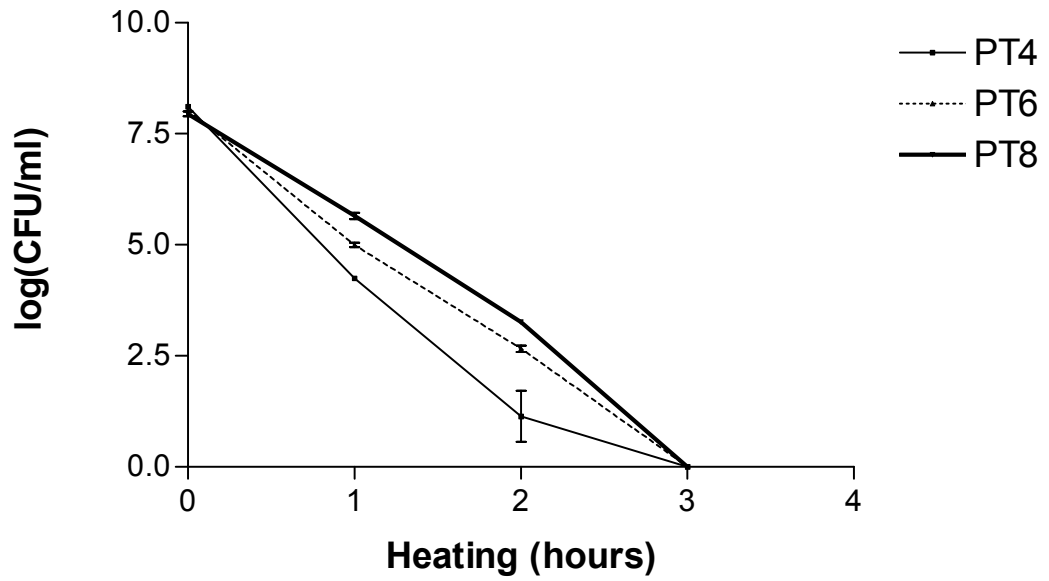


Figure A9: Heating of three *Salmonella Enteritidis* phage types (PT4 (isolate 9969695-1), PT6 (isolate 7260731-16), PT8 (isolate 9981190-21)), all from environmental samples. Media, conditions: as for Figure A1. Aim, conclusion: as for Figure A8.

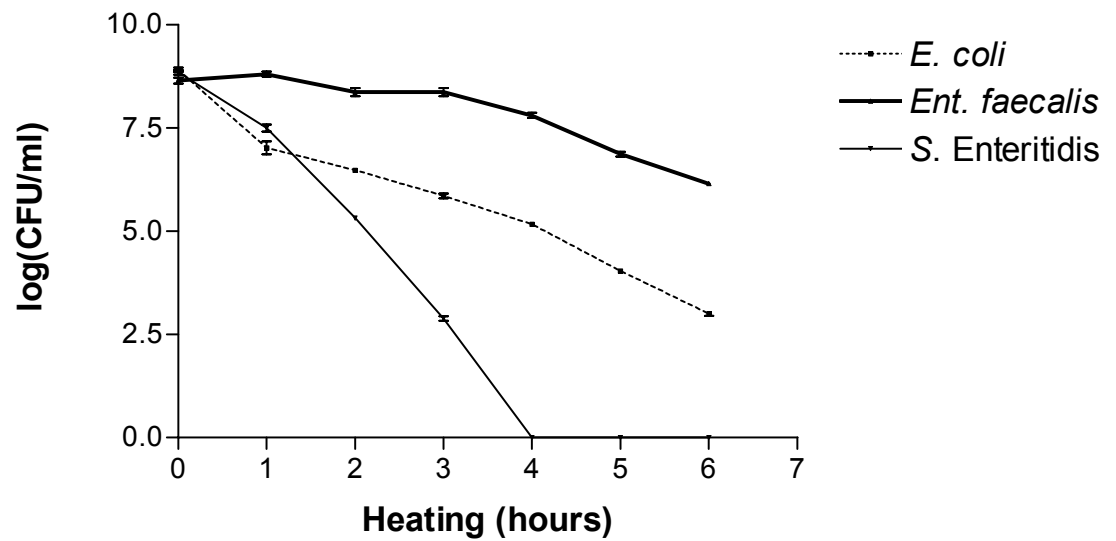


Figure A10: Heating of *Escherichia coli* (isolate 7330455), *Enterococcus faecalis* (isolate 7330481) and *Salmonella Enteritidis*, phage type 8 (isolate 7278230-1) in air incubator. Aim: to see if *E. coli* and *Ent. faecalis* could be used as indicator bacteria for *Salmonella* in heating experiments. Media: physiologic saline (0.85% NaCl) for tenfold dilutions; BA for plating. Conditions: as for Figure A1. Conclusion: *E. coli* and *Ent. faecalis* were more heat resistant than *S. Enteritidis*, PT8.

Appendix A

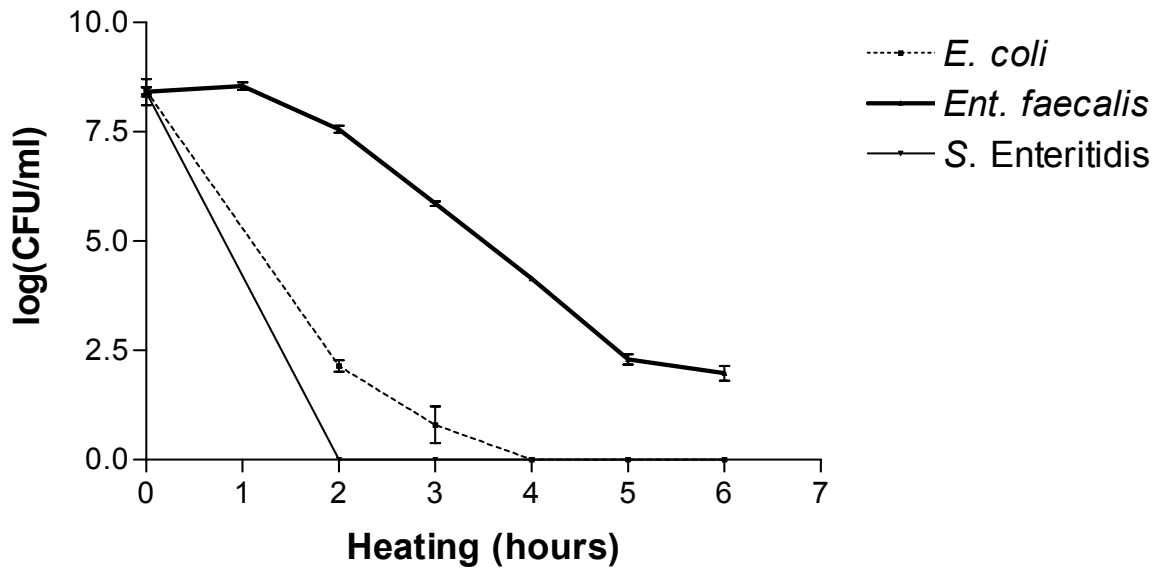


Figure A11: Heating of *Escherichia coli* (isolate 7330455), *Enterococcus faecalis* (isolate 7330481) and *Salmonella Enteritidis*, phage type 8 (isolate 7278230-1) in a water bath.

Conditions: 55 °C, water bath.

Aim, media, conclusion: as for Figure A10.

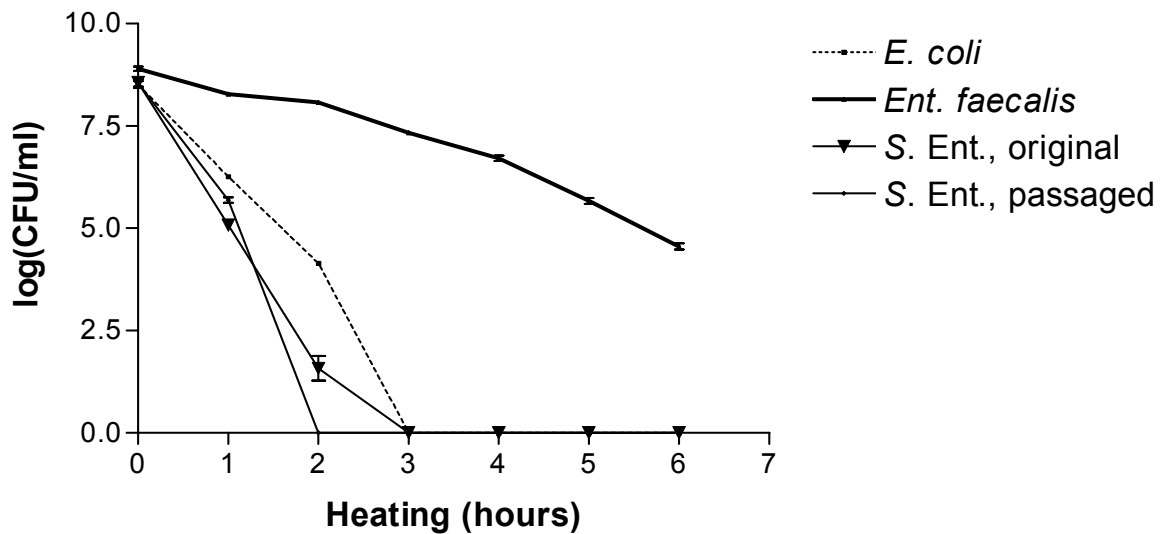


Figure A12: Heating of *Escherichia coli* (isolate 7330455), *Enterococcus faecalis* (isolate 7330481) and *Salmonella Enteritidis*, phage type 8 (original and passaged isolate 7278230-1) in a water bath.

Aims: as for Figure A10. In addition, heat resistances between an original and a passaged *Salmonella* isolate were compared.

Media: BPW for tenfold dilutions; BA for plating.

Conditions: as for Figure A11.

Conclusion: all isolates, except *Ent. faecalis*, had similar heat resistances.

APPENDIX B – AUTHOR’S PUBLICATIONS

Peer-reviewed publications, published:

Feld, N.C., Ekeröth, L., **Gradel, K.O.**, Kabell, S. and Madsen, M. (2000) Evaluation of a serological *Salmonella* mix-ELISA for poultry used in a national surveillance programme. *Epidemiology and Infection* **125**, 263-268.

Gradel, K.O., Feld, N.C. and Andersen, J.S. (2001) Serologic reactions against *Salmonella* in samples from broiler parent stock with and without preceding colibacillosis: a case-control study. *Avian Diseases* **45**, 486-491.

Wedderkopp, A., **Gradel, K.O.**, Jørgensen, J.C. and Madsen, M. (2001) Pre-harvest surveillance of *Campylobacter* and *Salmonella* in Danish broiler flocks: a 2-year study. *International Journal of Food Microbiology* **68**, 53-59.

Gradel, K.O., Andersen, J.S. and Madsen, M. (2002) Comparisons of sampling procedures and time of sampling for the detection of *Salmonella* in Danish infected chicken flocks raised in floor systems. *Acta Veterinaria Scandinavica* **43**, 21-30.

Gradel, K.O. and Rattenborg, E. (2003) A questionnaire-based, retrospective field study of persistence of *Salmonella* Enteritidis and *Salmonella* Typhimurium in Danish broiler houses. *Preventive Veterinary Medicine* **56**, 267-284. **Modified version in Chapter 7.**

Gradel, K.O., Jørgensen, J.C., Andersen, J.S. and Corry, J.E.L. (2003) Laboratory heating studies with *Salmonella* spp. and *Escherichia coli* in organic matter, with a view to decontamination of poultry houses. *Journal of Applied Microbiology* **94**, 919-928. **Modified version in Chapter 8.**

Bang, D.D., Scheutz, F., **Gradel, K.O.**, Nielsen, E.M., Pedersen, K., Engberg, J., Gerner-Smidt, P., Handberg, K. and Madsen, M. (2003) Detection of seven virulence and toxin genes of *Campylobacter jejuni* and *Campylobacter coli* isolates from different sources and Cytolethal Distending Toxin production suggest potential diversity of pathogenic properties among isolates. *Genome Letter* **2**, 62-72.

Gradel, K.O., Jørgensen, J.C., Andersen, J.S. and Corry, J.E.L. (2004) Monitoring the efficacy of steam and formaldehyde treatment of naturally *Salmonella*-infected layer houses. *Journal of Applied Microbiology*, **96**, 613-622. **Modified version in Chapter 9.**

Peer-reviewed publications, submitted:

Gradel, K.O., Randall, L., Sayers, A.R. and Davies, R.H. (2003) Possible associations between *Salmonella* persistence in poultry houses and resistance to commonly used disinfectants and a putative role of *mar*. **Modified version in Chapter 11.**

Gradel, K.O., Sayers, A.R. and Davies, R.H. (2003) Surface disinfection tests with *Salmonella* and a putative indicator bacterium, mimicking worst-case scenarios in poultry houses. **Modified version in Chapter 12.**

Non peer-reviewed publications, all in Danish:

Gradel, K.O. (1998) Et foderanlæg skilles ad [A feed system is dismantled]. *Dansk Erhvervsfjerkræ* **10**, 322.

Gradel, K.O. (2000) Varme og Salmonella [Heat and *Salmonella*]. *Dansk Erhvervsfjerkræ* **4**, 110-112.

Gradel, K.O. (2000) Bakteriefund efter varmebehandling af en burstald [Bacterial findings after heat treatment of a battery hen house]. *Dansk Erhvervsfjerkræ* **6**, 183-184. **Modified version in Chapter 10.**

Olsen, C.B., **Gradel, K.O.** and Olsen, T.B. (2000) Undersøgelse af drabseffekten på salmonella ved opvarmning af en burhønestald [Investigation of the elimination effect on *Salmonella* by heating a battery hen house]. *Dansk Erhvervsfjerkræ* **11**, 16-23.

Gradel, K.O., Jessen, B. and Nielsen, B.L. (2000) Søges: Deltagere til varmedesinfektionsprojekt [Wanted: participants for the heat disinfection project]. *Dansk Erhvervsfjerkræ* **12**, 360-361.

Gradel, K.O. (2001) Salmonellafund før og efter damp- og formalinbehandling af en burstald [*Salmonella* findings before and after steam and formalin treatment of a battery cage house]. *Dansk Erhvervsfjerkræ* **4**, 114-116. **Modified version in Chapter 10.**

Gradel, K.O. (2002) Varmedesinfektionsprojektet: Resultater fra laboratorieforsøg [The heat disinfection project: results from laboratory trials]. *Dansk Erhvervsfjerkræ* **7**, 208-211.

Gradel, K.O., Nielsen, B.L., Jessen, B. and Knudsen, V. (2002) Varmedesinfektionsprojektet: Resultater fra feltforsøg [The heat disinfection project: results from field trials]. *Dansk Erhvervsfjerkræ* **8**, 238-244.

Gradel, K.O. (2003) Desinfektionsmiddeltolerance og stationære salmonellainfektioner i fjerkræstalde: Er der en sammenhæng? [Disinfectant tolerance and stationary *Salmonella* infections in poultry houses: does an association exist?] *Dansk Erhvervsfjerkræ* **6**, 181-183.

Gradel, K.O. (2003) Varmedesinfektion er effektiv i stalde [Heat disinfection is effective in animal houses]. *Landbrugsavisen* **7. november**, 36.

Gradel, K.O. (2003) Fokus på rengøring og desinfektion af stalde [Focus on cleaning and disinfection of animal houses]. *Effektivt Landbrug*, **20. oktober**, 13.

Gradel, K.O. (2003) Desinfektionstests på overflader, som simulerer de værst tænkelige forhold i tomme fjerkræstalde [Disinfection tests on surfaces that simulate worst-case scenarios in empty poultry houses]. *Dansk Erhvervsfjerkræ*, **1**, 16-18.

Congresses and symposia, selected:

Gradel, K.O., Andersen, J.S., Jorgensen, J.C. and Madsen, M. (2000) Comparison of dung samples and sock samples for surveillance of *Salmonella* in poultry under the Danish *Salmonella* Control Programme. In *Report on the Fifth Workshop Organised by CRL-Salmonella*, 18 and 19 September, Community Reference Laboratory for *Salmonella*, Bilthoven, the Netherlands, pp. 19-20.

Gradel, K.O. (2002) Heat treatment of persistently *Salmonella* infected poultry houses. In *Salmonella and Salmonellosis*, May 29-31, ISPAIA, Saint-Brieuc, France, p. 595 (+ poster).

Gradel, K.O. and Randall, L.P. (2003) Minimum inhibitory concentrations of and adaptation to five disinfectants commonly used towards *Salmonella* in the poultry industry. In *XI International Congress in Animal Hygiene*, 23-27 February, International Society for Animal Hygiene, Mexico City, Mexico, pp. 339-344 (+ 10 min talk).

Gradel, K.O. (2003) Heat as a disinfection method for poultry houses persistently infected with *Salmonella* – an outline of methods and results. In *Disinfection in Animal Production – Cleaning and Disinfection Projects under the Danish Salmonella Control Programme for Poultry, with a view to Virus Disinfection*, 18 November, Danish Veterinary Institute, Scandinavian Congress Center, Århus, Denmark (paper + 30 min talk).

Gradel, K.O. (2003) Chemical disinfectants commonly used in the poultry sector and their impact on *Salmonella* – an outline of methods and results. In *Disinfection in Animal Production – Cleaning and Disinfection Projects under the Danish Salmonella Control Programme for Poultry, with a view to Virus Disinfection*, 18 November, Danish Veterinary Institute, Scandinavian Congress Center, Århus, Denmark (paper + 30 min talk).

Gradel, K.O. (2003) Disinfection of housing systems, with special reference to *Salmonella*. In *Nordic Poultry Consultant and Veterinary Conference 2003*, 20-22 November, Svensk Fågel, Frösundavik, Stockholm, Sweden (paper + 45 min talk).