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Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including *Salmonella enteritidis* ☆

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Abstract

Trans-shell infection routes and whole egg contamination of 7 selected bacterial strains; *Staphylococcus warneri*, *Acinetobacter baumannii*, *Alcaligenes* sp., *Serratia marcescens*, *Carnobacterium* sp., *Pseudomonas* sp. and *Salmonella enteritidis*, recovered from egg contents, were studied. The first objective was to correlate bacterial eggshell penetration with various eggshell characteristics and bacterial strains. An agar approach was used to assess the eggshell penetration. The second objective was to assess the contamination of whole eggs with the bacterial strains; whole intact eggs were used in this case. The intact shells of agar-filled and whole eggs were inoculated with 10^3–10^4 cfu of the selected strains. During 3 weeks storage at 20 °C and 60% relative humidity, the bacterial eggshell penetration was regularly monitored. The whole egg contamination was only analyzed after 3 weeks. The eggshell characteristics such as area eggshell, shell thickness and number of pores did not influence the bacterial eggshell penetration. For each individual bacterial strain the mean cuticle deposition was lower for penetrated compared to non-penetrated eggshells. For the individual strain *Carnobacterium* sp. and for the global results of all strains this difference was statistical significantly. The whole egg contamination was not influenced by neither the area of the eggshell nor the porosity of the eggshell. The results of the agar approach indicate that the Gram-negative, motile and non-clustering bacteria penetrated the eggshell most frequently; *Pseudomonas* sp. (60%) and *Alcaligenes* sp. (58%) were primary invaders followed by *S. enteritidis* (43%). All selected strains were able to penetrate; penetration was observed most frequently after ca. 4–5 days. Particularly *S. enteritidis* was a primary invader of whole eggs: the membranes and/or the content of 32% of the whole eggs was contaminated. The remaining bacterial eggshell contamination with the selected strain was determined after 3 weeks storage. Penetrated eggshells and contaminated whole eggs showed a significantly higher bacterial contamination on the eggshell compared to non-penetrated eggshells and non-contaminated whole eggs respectively (global results of all strains). The influence of hen age on bacterial eggshell penetration and egg content contamination was not significant. While the agar approach is suitable to study the influence of the eggshell characteristics on the bacterial eggshell penetration, the intact egg approach gives an estimation of the penetration of the shell followed by the probability of survival and migration in whole eggs.

Keywords: Bacterial eggshell penetration; Egg content contamination; *Salmonella enteritidis*

1. Introduction

The increasing consumer awareness of food safety issues has changed the public perception of a "good egg" from shell cleanliness and physical properties to that of microbial integrity. Microorganisms can contaminate eggs at different stages, from production through processing to preparation and consumption. Transovarian or "vertical" transmission of microorganisms occurs when eggs are infected during their formation in the hen's ovaries. Horizontal transmission occurs when eggs are subsequently exposed to a contaminated environment and microorganisms penetrate the eggshell. Studies conducted by Barrow and Lovell

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(1991) suggest that most of the contamination is due to horizontal transmission, although others do not agree (Humphrey, 1994). The contamination of the contents of whole intact eggs with *Salmonella enteritidis* should be mainly the result of infection of the reproductive tissue (Humphrey, 1994). Different researchers reported on the penetration of bacteria through the eggshell with associated membranes and on whole egg contamination. Some published reports suggest a relationship between eggshell quality and bacterial eggshell penetration and/or whole egg contamination (Sauter and Petersen, 1974; Nascimento and Solomon, 1991). Most research was focused on the penetration of *Pseudomonas* and various salmonellae (Hartung and Stadelman, 1963; Sauter and Petersen, 1974; Nascimento et al., 1992). Bacteria of the genus *Pseudomonas* have been shown to more readily penetrate into whole eggs of poor shell quality (Sauter and Petersen, 1969). Sauter and Petersen (1974) also found that whole eggs with low specific gravity or low shell quality were more likely to be penetrated by *Salmonella*. Berrang et al. (1998) reported on the influence of egg weight, specific gravity, conductance and flock age on the ability of *Salmonella* to penetrate the shell and the membranes. Because shell quality measures did not change greatly in relation to flock age and the *Salmonella typhimurium* penetration patterns did vary, they concluded that it is likely that factors other than just shell quality are involved in bacterial penetration in eggshells. Nascimento et al. (1992) also reported an increasing eggshell penetration from 12.9% (beginning of lay) till 25.0% (end of lay) for *S. enteritidis*. Bruce and Johnson (1978) reported about hatching eggs and found that there was an increasing contamination of eggs as flocks became older.

Until now no attention was given to the connection between bacterial eggshell penetration and whole egg contamination. In this study the influence of hen age and eggshell characteristics on eggshell penetration on the one hand and egg content contamination on the other hand was investigated, using 7 selected bacterial strains isolated from the egg content of consumption eggs.

2. Materials and methods

2.1. Eggs

Eggs from a fixed stable of a commercial conventional housing system, housing ISA-brown laying hens, were collected at the day of lay. Upon storage overnight at 20 °C the eggs were filled with agar and/or inoculated. The laying hens were placed in production at the hen age of 24 weeks and eggs were sampled at the ages of 32, 34, 46, 60, 69 and 74 weeks. Eggs were visually inspected by candling and only intact eggs (no cracks, pin-holes) were included for further analyses.

2.2. Bacterial strains and cultures

Seven bacterial strains, *Staphylococcus warneri* (MB 2792), *Acinetobacter baumannii* (MB 2793), *Alcaligenes* sp. (MB 2794), *Serratia marcescens* (MB 2795), *Carnobacterium* sp. (MB 2796), *Pseudomonas* sp. (MB 2797) and *S. enteritidis* (MB 1409), all own isolates from egg contents, albumen or yolk, were used. The content isolations were obtained from commercial brown eggs from various production units that were analyzed at expiry date after storage at room conditions. The determination of the egg contents contamination was based on successive short flaming of the eggshell with ethanol and aseptical removal of the egg contents and separation of yolk from albumen followed by plating out of both on Nutrient Agar (NA, Oxoid, Hampsire, UK) and incubated at 30 °C for 72 h. Species identification was done by 16S rDNA sequencing (Scheldeman et al., 2004). Strains were selected for resistance to streptomycin. The streptomycin resistant bacteria, stored on Protect Beats at −80 °C, were resuscitated by incubation overnight at 30 °C in Buffered Peptone Water (BPW, Oxoid) with 25 ppm streptomycin (Sigma-Aldrich, S 6501, St. Louis, USA). This culture was plated on NA with 25 ppm streptomycin and again incubated overnight at 30 °C. One colony was grown overnight in 9 ml BPW with 25 ppm streptomycin and 2 ml of the culture was diluted in 200 ml 1/4 Ringer solution (Oxoid) to obtain an immersion solution of 10^5–10^6 colony-forming units (cfu)/ml. Enumeration was done by spiral plating 100 µl on NA with streptomycin (25 ppm).

2.3. Agar method for the assessment of the eggshell penetration

An agar method described by Berrang et al. (1998) was adapted to study and visualize the bacterial eggshell penetration. The egg contents were drained after cutting a hole of ca. 1 cm^2 with a rotary tool (Dremel, S-B Power Tool Company, Chicago, USA) and tweezing. After rinsing the inside of the shell with distilled water in order to remove the albumen adhering to the membranes, the egg was filled with molten (50 °C) NA with 25 ppm streptomycin (Sigma), 50 ppm cycloheximide (Sigma) and 0.1% 2,3,5 triphenyl tetrazolium chloride (TTC, Becton Dickinson and Company, Sparks, USA). After hardening of the agar, the hole was closed with commercial silicone. The addition of streptomycin to the agar assured that only the inoculated streptomycin resistant bacteria were able to grow on the agar thus holding down all other natural flora competitors present on the fresh eggshell and able to penetrate. Cycloheximide was added to prevent yeast and mould growth. Where bacterial penetration occurred organisms grew on the agar and reduced the TTC to formazan which is red in color. Penetration was recorded when red colonies on the agar were visible by candling. Candling was performed daily during the first days of the experiments and three times a week later. Red colonies seen nearby the hole were assumed to result from contamination and not recorded as penetration.

2.4. Inoculation and storage

Agar-filled and whole eggs were inoculated by immersion for 1 min in Phosphate Buffered Saline (PBS, Oxoid) containing 10^5–10^6 cfu/ml of a streptomycin resistant strain of one of the 7 selected species: *S. warneri*, *A. baumannii*, *Alcaligenes* sp., *S. marcescens*, *Carnobacterium* sp., *Pseudomonas* sp. and *S. enteritidis*. This resulted in 10^3–10^4 cfu of the selected bacterium on the eggshell. After drying at ambient conditions the eggs were stored in a climate chamber (Termaks KBP 6395 F, Solheimsvinken, Norway) at 20 °C and 60% relative humidity (RH) for up to

21 days, i.e. the average sell by date in Belgium. This temperature/RH combination resembles the environmental conditions the eggs are exposed to most of the year at the packaging station and the store (data not shown but available).

2.5. Determination of the eggshell contamination

At day 0 and day 21 the eggshell contamination with the selected strains was determined by washing the egg in a plastic bag with diluent and rubbing the eggshell through the bag to detach the bacteria. The diluent was next plated by a spiral-enter on NA with 25 ppm streptomycin. Plates were incubated at 30 °C for 72 h (De Reu et al., 2005a,b).

2.6. Determination of the egg content contamination

To remove the contents of whole eggs aseptically (intact egg approach), a modification of the method described by Himathongkham et al. (1999) was used. Each egg was placed in a petri-dish and sprinkled with 75% ethanol. Rolling the egg in the dish with tweezers the alcohol was burned off at ca. 5 s. After a second successive short flaming the disinfected egg was broken by hand using a sterile blade and sanitized plastic gloves. The whole egg was separated into two fractions: the albumen with yolk and the burned off eggshell with the membranes. Both fractions were enriched (BPW) for 24 h at 30 °C and plated out on NA with 25 ppm streptomycin. Plates were incubated at 30 °C for 72 h.

2.7. Eggshell characteristics

When the eggshell penetration or egg content contamination experiment was completed different eggshell characteristics were determined: the shell surface area, the shell thickness, the number of pores, the loss of weight at the pores, and the cuticle deposition. The weight of the fresh eggs was measured and the formula $S = 4.67 \times W^{2/3}$ was used to calculate the shell area (Tyler, 1953). S represents the surface area of the egg in cm^2 and W the fresh weight of the egg in g. The shell thickness was determined at three places with a micrometer and the mean value was used for calculations. The number of pores was determined by microscopic counting (ocular ×8, objective ×4) (Olympus BH2-RFCA, Tokyo, Japan) after immersion of the pieces of the eggshell for 25 s in 65% nitric acid solution (Tyler, 1953), rinsing with distilled water and removal of the membranes. 14 places of ca. 11 mm^2 were counted, 7 places at the apex and 7 places at the blunt end. The amount of pores was summed and expressed as the total number of pores of the entire eggshell. Using the intact egg method, the loss of weight was determined for the fresh eggs after exactly 24 h of storage at 20 °C and 60% RH. This weight loss is an indicator for the shell's porosity. The cuticle score was analyzed by dying with an aqueous mixture of 7.2 g Tartrazine and 28 g Green S per liter (Barentz N.V., Zaventem, Belgium) (also referred to as Edicol Pea Green) (Board and Halls, 1973). The cuticle was stained by immersion of the egg for a period of 1 min. The shell

Table 1
Eggshell characteristics and eggshell penetration on day 21

Eggshell penetration (agar approach)							
Strain		Nr[c]	Area eggshell (cm^2)[a]	Shell thickness (mm)[a]	Number of pores[a]	Cuticle score[a]	Shell contamination on day 21 (log cfu/shell)[b]
S. warneri (MB 2792)	T[d]	61	74.3±3.8	0.417±0.036	6300±2300	93±34	2.5±0.9
	Y[e]	9	74.8±5.4	0.407±0.016	5500±1600	120±40	3.5 ± 1.1^{A}
	N[f]	52	74.2±3.5	0.419±0.038	6400±2400	89±32	2.3 ± 0.8^{A}
Carnobacterium sp. (MB 2796)	T	60	74.1±4.6	0.410±0.035	5900±2200	85±40	1.6±1.4
	Y	13	74.8±3.0	0.412±0.035	6000±2800	122 ± 36^{AAA}	2.5±2.1
	N	47	73.9±4.9	0.409±0.036	5800±2100	75 ± 35^{AAA}	1.4±1.1
Alcaligenes sp. (MB 2794)	T	57	75.9±4.2	0.424±0.037	5800±2100	93±34	3.7±2.4
	Y	33	76.8±3.8	0.419±0.037	5900±2000	100±38	5.0 ± 1.9^{CCC}
	N	24	74.7±4.5	0.432±0.036	5800±2400	81±24	1.8 ± 1.7^{CCC}
A. baumannii (MB 2793)	T	62	74.1±5.4	0.418±0.036	5700±2600	84±33	2.0±1.3
	Y	15	74.8±3.3	0.418±0.033	5500±2200	98±41	3.3 ± 1.5^{AA}
	N	47	73.9±5.9	0.418±0.037	5800±2700	79±28	1.7 ± 0.9^{AA}
Pseudomonas sp. (MB 2797)	T	52	75.5±3.8	0.417±0.032	6700±6700	98±36	3.6±2.2
	Y	31	76.1±4.1	0.417±0.035	7600±8400	103±42	4.7 ± 1.7^{DDD}
	N	21	74.7±3.3	0.417±0.028	5400±2800	91±25	2.1 ± 1.8^{DDD}
Salmonella Enteritidis (MB 1409)	T	51	75.0±4.5	0.417±0.029	5800±2400	98±38	2.5±1.8
	Y	22	75.3±4.5	0.426±0.027	5800±2300	107±41	3.4 ± 1.7^{BB}
	N	29	74.9±4.6	0.411±0.029	5700±2500	92±36	1.8 ± 1.6^{BB}
S. marcescens (MB 2795)	T	60	74.7±3.9	0.420±0.033	5800±2400	87±28	1.0±0.7
	Y	8	76.0±3.5	0.425±0.039	6300±2700	96±25	1.9 ± 1.0^{B}
	N	52	74.5±3.9	0.419±0.032	5700±2400	85±28	0.9 ± 0.6^{B}
All bacterial strains	T	403	74.8±4.4	0.418±0.034	6000±3300	91±35	2.3±1.8
	Y	131	75.8±4.0	0.419±0.033	6200±4500	105 ± 39^{BBB}	3.8 ± 1.6^{EEE}
	N	272	74.3±4.5	0.417±0.035	5900±2400	84 ± 31^{BBB}	1.6 ± 1.2^{EEE}

[a]Values are means±SD; [b]values are means±SD after log transformation; [c]number of eggs; [d]total eggshells; [e]penetrated eggshells; [f]non-penetrated eggshells.
$^{A, B, \cdots}$Means with the same letter are significantly different ($P<0.05$); $^{AA, BB, \cdots}$means with 2 same letters are highly significantly different ($P<0.01$); $^{AAA, BBB, \cdots}$means with 3 same letters are extremely significantly different ($P<0.001$).

was then rinsed with distilled water to remove excess dye, followed by drying. The remaining red color, i.e. the color at places were the dye did not bind to, was analyzed with Paint Shop Pro version 8 (Jasc Software, Eden Prairie, MN 55344, USA) using the histogram function. Using this method, the red value score or cuticle score is oppositely correlated with the cuticle deposition.

2.8. Statistical analysis

The penetration and contamination data were analyzed using a generalized linear regression with penetration or contamination (yes/no) as binomial dependent variable and the eggshell characteristics and bacterial survival on the eggshell as continuous independent variables. A logit link function was used to relate the continuous predictors and binomially distributed dependent variable. A simple linear regression was carried out to determine the influence of hen age on eggshell penetration, whole egg contamination and eggshell characteristics. Both analyses were done in Statistica 7 (Statsoft, Tulsa, USA).

There were left and right censored data for bacterial counts simultaneously, as a fraction of the data consisted of values '<10' and '>3000'. However, there were bacterial counts larger than 3000 available as well. Hence, we took a different approach for the left and right censored part. Basically, we assumed that the data that were present are the best guess for the data that have to be reconstructed. We constructed distributions, derived from the available data, from which we sampled in a bootstrap procedure. As there are actual data available above 3000, we constructed an empirical cumulative distribution based on these data. This is equivalent to supposing that the censored data had the same distribution as the available data. This was done separately for each strain inoculated on the agar-filled eggs and on the whole eggs. The values '>3000' were then each replaced by a random sample from the corresponding distribution. Because there are no measured data available for counts '<10', we fitted a distribution to the data (excluding the censored values) of each strain (agar-filled and whole eggs separately) and extrapolated to the '<10 zone'. A normal distribution was fitted to the log-transformed data and then truncated between 0 and 1. The values smaller than 10 were then replaced by random samples from this distribution. Finally, a 10,000 iteration bootstrap was done on the averages of the condensate and control groups where the censored data were sampled from the constructed distributions as outlined above (Manly, 1994).

3. Results

3.1. Effects of egg(shell) characteristics on eggshell penetration and whole egg contamination

Table 1 shows the mean values with standard deviations (SD) for each analyzed eggshell characteristic for all eggshells (T), penetrated eggshells (Y) and non-penetrated eggshells (N) (agar approach). Those data are available for the individually selected bacterial species as well as for all bacterial strains combined.

Table 2
Egg(shell) characteristics and whole egg contamination on day 21

Whole egg contamination (intact egg approach)							
Strain		Nr[c]	Area eggshell (cm^2)[a]	Nr[c]	Loss of weight after 24 h (g)[a]	Nr[c]	Shell contamination on day 21 (log cfu/shell)[b]
S. warneri (MB 2792)	T[d]	55	75.0±3.7	45	0.308±0.151	51	3.0±0.7
	Y[e]	8	76.0±4.1	7	0.324±0.234	7	3.3±0.4
	N[f]	47	74.8±3.7	38	0.304±0.135	44	3.0±0.7
Carnobacterium sp. (MB 2796)	T	53	74.3±4.0	44	0.335±0.166	45	1.1±0.7
	Y	10	73.4±3.3	10	0.263±0.119	8	1.1±0.7
	N	43	74.6±4.1	34	0.356±0.174	37	1.1±0.6
Alcaligenes sp. (MB 2794)	T	55	74.0±3.8	45	0.322±0.119	53	1.3±0.8
	Y	7	75.2±4.8	7	0.311±0.145	7	1.2±0.6
	N	48	73.8±3.7	38	0.324±0.116	46	1.3±0.8
A. baumannii (MB 2793)	T	56	74.8±3.7	46	0.305±0.154	54	1.9±0.7
	Y	8	75.4±3.8	8	0.290±0.175	8	2.0±0.9
	N	48	74.6±3.7	38	0.308±0.152	46	1.9±0.6
Pseudomonas sp. (MB 2797)	T	44	74.2±4.4	43	0.358±0.206	44	1.5±1.0
	Y	5	73.0±3.2	5	0.256±0.080	5	2.6±1.2
	N	39	74.3±4.6	38	0.371±0.214	39	1.4±0.9
Salmonella Enteritidis (MB 1409)	T	45	75.5±4.1	36	0.356±0.220	45	1.3±0.8
	Y	15	75.6±4.7	10	0.402±0.255	15	1.6±0.9
	N	30	75.5±3.8	26	0.338±0.207	30	1.2±0.7
S. marcescens (MB 2795)	T	56	74.8±4.0	46	0.505±1.147	47	1.4±1.0
	Y	5	73.8±3.0	5	0.346±0.137	4	2.4±2.4
	N	51	74.9±4.1	41	0.524±1.215	43	1.3±0.9
All bacterial strains	T	364	74.7±3.9	305	0.356±0.473	339	1.7±1.0
	Y	58	74.8±4.0	52	0.316±0.178	54	1.9±1.1[AA]
	N	306	74.6±3.9	253	0.364±0.513	285	1.7±1.0[AA]

[a]Values are means±SD; [b]values are means±SD after log transformation; [c]number of eggs; [d]total whole eggs; [e]contaminated whole eggs; [f]non-contaminated whole eggs.
[AA, BB, ···]Means with 2 same letters are highly significantly different ($P<0.01$ and >0.001).

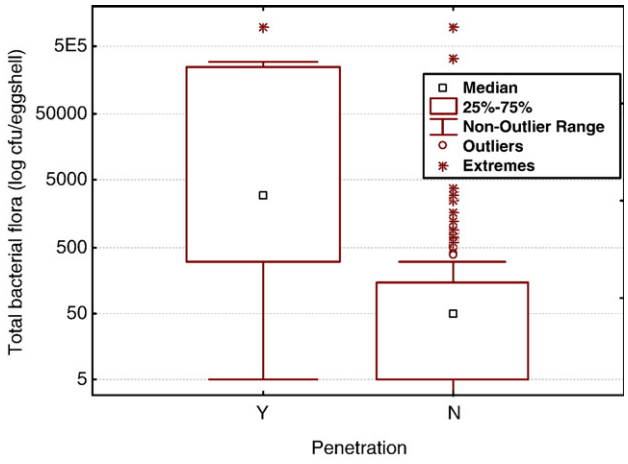

Fig. 1. Total count of inoculated species on the eggshell of penetrated (*Y*) and non-penetrated eggshells (*N*) considering all strains.

Table 2 shows the data for the whole egg contamination: all whole eggs (T), contaminated whole eggs (Y) and non-contaminated whole eggs (N) (intact egg approach).

Evaluation of the data (Table 1) showed no significant difference between the area eggshell, shell thickness and number of pores and the presence or absence of bacterial eggshell penetration. For each individual strain and for the global results of all strains the mean eggshell area of the penetrated eggshells was higher, but not significant, compared to the non-penetrated eggshells. The mean cuticle score was higher for penetrated compared to non-penetrated eggshells (individual strain and all strains). For the individual strain *Carnobacterium* sp. and for the global result of all strains this difference was significant ($P<0.001$). Using our method, the cuticle score is oppositely correlated with cuticle deposition; the higher cuticle score corresponds with a lower cuticle deposition. Table 2 shows that the whole egg contamination was not influenced by either the area of the eggshell or by the porosity of the eggshell (loss of weight after 24 h).

3.2. Effects of storage time on eggshell penetration

Independent of the selected strain, the eggshell penetration was observed most frequently at ca. day 4–5 (data not shown). At day 6 and day 14, respectively, up till 80% and more than 95% of the total eggshell penetration was observed. The histograms (not shown) of the penetration days for each individual strain are comparable; most penetration spots appeared before day 6.

3.3. Effect of bacterial survival on the eggshell on eggshell penetration and whole egg contamination

The individual data per selected strain (MB) and the global data (all bacterial strains) obtained with the agar approach, showed a higher count of the inoculated strain on the eggshell at day 21 (shell contamination on day 21) for penetrated eggshells (Y) compared to non-penetrated eggshells (N) (Table 1). This higher count was even significant for the global data ($P<0.001$) and for six of the seven selected strains (MB), respectively for *S. warneri*, *Alcaligenes* sp., *A. baumannii*, *Pseudomonas* sp., *S. enteritidis* and *S. marcescens* (respectively $P<0.001$, <0.001, 0.0018, <0.001, 0.0016 and 0.0038). Fig. 1 shows the box plot of the bacterial count on the eggshell for penetrated compared to non-penetrated eggshells, considering all selected strains. The count of bacteria on the shell of whole eggs was on average 0.6 log cfu/shell lower compared to agar-filled shells, respectively 1.7 versus 2.3 log cfu/shell (Table 2). For five of the seven selected strains the contaminated whole eggs had a higher count of the inoculated strains on the eggshell at day 21; this was significant for none of the strains. The overall data of all strains showed that the count on the eggshell of the contaminated whole eggs was significantly higher ($P=0.0029$): 1.89 log cfu/shell versus 1.66 log cfu/shell for non-contaminated whole eggs.

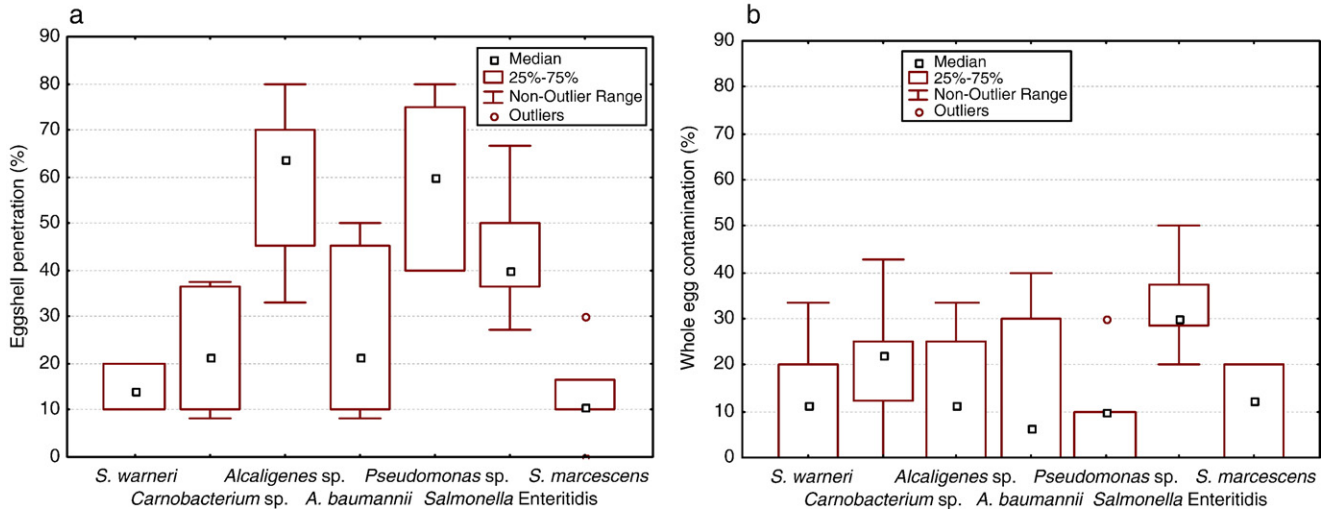

Fig. 2. a: Percentage of eggshell penetration for each individual bacterial strain. b: Percentage whole egg contamination for each individual bacterial strain.

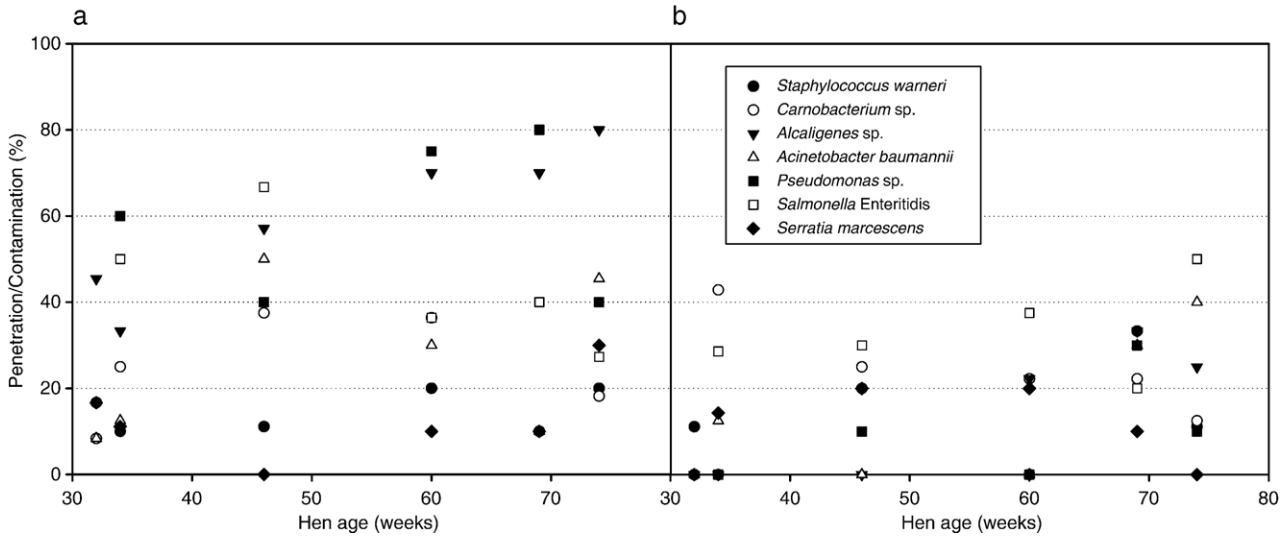

Fig. 3. a: Bacterial eggshell penetration of each selected strain during laying period. b: Bacterial whole egg contamination of each selected strain during laying period.

3.4. Effect of bacterial strain on eggshell penetration and whole egg contamination

Fig. 2a shows the percentage of eggshell penetration (agar approach) for all strains used, after 21 days of incubation. *Pseudomonas* sp. and *Alcaligenes* sp. followed by *S. enteritidis* penetrated most frequently the eggshell. They accounted for 60, 58 and 43% of the agar-filled eggs penetration, respectively. Fig. 2b shows the percentages of whole egg contamination (intact egg approach). The contents of whole eggs were most frequently contaminated by *S. enteritidis* (33%) followed by *Carnobacterium* sp. (17.5%). All strains were able to penetrate in agar-filled eggs (eggshell penetration) as well as contaminate whole eggs (egg content contamination). Of the 403 agar-filled eggs, 131 (33%) were penetrated by the selected strains compared to a content contamination of 16% (60 on 385) whole eggs. The fraction albumen and yolk from whole eggs was contaminated for 11% (42 on 385) while 15% (56 on 385) of the eggshells (outside decontaminated) with membranes attached was positive.

3.5. Effect of hen age on eggshell penetration, whole egg contamination and eggshell characteristics

Bacterial eggshell penetration and egg content contamination for all 7 selected strains were studied on eggs laid at 34, 46, 60, 69 and 74 weeks of hen age using the agar approach and the intact egg approach (Fig. 3a and b). The results showed that the bacterial eggshell penetration remained almost constant during the entire laying period. At week 34, 46, 60, 69 and 74 average penetration percentages for all selected strains together were respectively 30, 39, 41, 33 and 37%. The whole egg contamination increased slightly with hen age from respectively 13%, 13% and 15% in week 34, 46 and 60 till 26% and 20% in week 69 and 74 (not significant; $P=0.167$). The egg(shell) characteristics, shell thickness and shell area, were significantly influenced by hen age, albeit very weak; shell thickness decreased while shell area increased.

4. Discussion

The area of the shell of penetrated eggshells or contaminated whole eggs was not significantly higher compared to non-penetrated shells or non-contaminated whole eggs. Smeltzer et al. (1979), using the agar method, reported also a shell penetration that was independent of the shell surface area.

In agreement with our results, Williams et al. (1968) reported that shell thickness did not significantly affect the penetration with *S. typhimurium*. Smeltzer et al. (1979) concluded the same, using agar-filled eggs and the bacterial eggshell flora of nest box and floor eggs. Orel (1959) and Sauter and Petersen (1969, 1974), using whole eggs, reported the opposite. Eggs with shells of high quality, i.e. high specific gravity (sp. gr.), were more resistant to penetration by *Pseudomonas fluorescens* (Orel, 1959; Sauter and Petersen, 1969) and *S. enteritidis* (Sauter and Petersen, 1974). The sp. gr. measurements gave an indication of the shells' thickness.

The primordial route for bacteria to penetrate intact eggs is the pores with diameters in the range of 6–65 μm (Tyler, 1953, 1956), far above the bacterial dimensions. We did not find a correlation between the number of pores and the bacterial eggshell penetration and between the loss of weight at the pores and the whole egg contamination. Fromm and Monroe (1960) and Board and Halls (1973) correlated porosity with bacterial penetration; Reinke and Baker (1966) refuted this view. The studies of Hartung and Stadelman (1963) and Nascimento et al. (1992) also supported that bacterial eggshell penetration is not pore dependent. The fact that some pores do not extend through the thickness of the shell but end abruptly (Silyn-Roberts, 1983) and cuticular capping and plugs often present on/into pores preventing microbial penetration (Board and Halls, 1973), may contribute to these conflicting opinions.

The cuticle on the eggshell serves as a water proofing agent and as a barrier of primary importance for particle, bacterial and fungal invasion (Board and Halls, 1973). In our study a significant lower cuticle deposition was found on penetrated eggshells compared to non-penetrated eggshells. Alls et al. (1964) found that cuticle removal increased microbial contamination from 20 to 60%.

Drysdale (1985) found also a significantly higher bacterial contamination in eggs which had a poor cuticle (40%) compared to eggs with a medium or good quality cuticle (26%). The defense of the cuticular layer has on the other hand been questioned by Nascimento et al. (1992).

A correlation was found between bacterial eggshell contamination with the inoculated strain on day 21 and shell penetration and whole egg contamination with the strain(s). This corresponds with ample evidence in the literature that highly contaminated eggs suffer more from bacterial spoilage on the eggshell. Smeltzer et al. (1979) found that floor eggs had a higher incidence of bacterial penetration (15.3%) compared to nest eggs (10.5%). Making comparison between eggs laid in roll away cages (2.6×10^4 cfu/eggshell) and laid in nests (3.4×10^5 cfu/eggshell), Harry (1963) found higher contamination of whole eggs suffering from more bacterial eggshell contamination. As different researchers (Board and Halls, 1973; Board et al., 1979) showed that bacteria such as *Pseudomonas* spp., *Alcaligenes brookeri* and *Streptomyces* can only digest the cuticle when humidity approaches 100%, the minor cuticle deposition we found for all strains at day 21 could not be caused by the higher bacterial loading on penetrated eggshells. The count of inoculated bacteria on day 21 on the shell of whole eggs was on average 0.6 log cfu/shell lower compared to agar-filled eggs. This may suggest that nutrients available from the agar favor the survival and growth on the agar-filled eggshells and/or that the antimicrobial components of the egg content of whole eggs may reduce the survival and growth.

Using the agar approach *Pseudomonas* sp., *Alcaligenes* sp. and *S. enteritidis* penetrate most frequently (Fig. 2a), respectively for 60, 58 and 43% of the inoculated eggs. The higher shell contamination (on day 21) with *Pseudomonas* sp. and *Alcaligenes* sp. (Table 1) can explain the higher fraction of penetrated eggshells. Notwithstanding the comparable eggshell contamination with *S. enteritidis* and *S. warneri* (both 2.5 log CFU/eggshell) on day 21, penetration prevalence with *Salmonella* was higher (43% versus 18%). It is likely that the motile, non-clustering properties of *Salmonella* favor the eggshell penetration; *Pseudomonas* sp. and *Alcaligenes* sp. also have these properties. Berrang et al. (1998), using an agar approach, found 67% penetration with *S. typhimurium* for eggs sampled at hen ages ranging from week 29 till 56. Eggs were dipped into a 10^4 cfu/ml suspension.

Using the intact egg approach *S. enteritidis* followed by *Carnobacterium* sp. seemed to penetrate, survive and eventually grow most frequently (Fig. 2b), respectively 33% and 17.5% of the inoculated eggs. Sauter and Petersen (1974) found a contamination average of 47.5% for various salmonellae using whole eggs of poor shell quality (sp. gr. 1.070) and 21.4% and 10.0% for whole eggs of intermediate (sp. gr. 1.080) and excellent shell quality (sp. gr. 1.090), respectively. Eggs were dipped for 3 min. into solutions containing ca. 1.0×10^4 *Salmonella* cfu/ml. Sauter and Petersen (1969) challenged eggs (challenge suspension 1.1×10^6 cfu/ml) with different specific gravity with *P. fluorescens* and found an incidence of fluorescent spoilage for eggs of high, medium and low levels of shell quality (sp. gr. of 1.085, 1.077 and 1.070 respectively) of 6.3, 19.4 and 29.1% after 8 weeks of storage. In addition, microbiological examination of the eggs that did not show fluorescence by eight weeks indicated that 45% of the eggs also contained viable microorganisms. In our study 10.5% of the whole eggs was contaminated with *Pseudomonas* sp. Despite the antimicrobial defenses of the membranes and the albumen all selected bacterial strains were able to penetrate the membranes and remain viable up till 21 days in the albumen. The high prevalence of *S. enteritidis* and even of the Gram-positive *Carnobacterium* sp. indicates that the antimicrobial aspects have only limited inhibitory effect after their penetration of the shell. Recent research shows a higher resistance of *S. enteritidis* to egg albumen compared to other salmonellae. Lu et al. (2003) reported the identification of *yafD* as a gene essential for resistance of *S. enteritidis* to egg albumen. Mayes and Takeballi (1983) reported especially Gram-negative bacteria such as *Alcaligenes*, *Pseudomonas* and *Aeromonas* as the most common natural contaminants of whole eggs. In our study *Alcaligenes* sp. contaminated 14% of the whole eggs. Notwithstanding *S. warneri* counts on the eggshell on day 21 were higher compared to all other selected strains (Table 2); this did not result in higher whole egg contamination prevalence. As our data showed that horizontal transmission of e.g. *S. enteritidis* and *Pseudomonas* sp. in whole eggs is possible; respectively the absence of the pathogen and the reduction of the spoilage organisms on the eggshell is important.

Independent of the selected strain, the eggshell penetration was observed most frequently at day 4 and 5 after inoculation of the eggs. Taking into account the necessary time for growth of the bacteria on the agar to initiate the appearance of the red spots (formazan) we can that conclude most eggshell penetration occurred within 0–2 days after inoculation. Williams et al. (1968) demonstrated that penetration of the cuticle and the shell by salmonellae occurred almost immediately in some eggs. Other researchers have demonstrated bacterial penetration in 25 to 60% of inner membranes and in 5 to 15% of albumen in eggs on the first day of inoculation (Muira et al., 1964; Humphrey et al., 1989, 1991). Using whole eggs, on day 21 we found 15% of the (outside disinfected) eggshells with membranes being contaminated compared to 11% of the egg contents (albumen and yolk).

Nascimento et al. (1992) reported, using an agar approach, an increasing eggshell penetration from 12.9% (beginning of lay) till 25.0% (end of lay) for *S. enteritidis* (challenge suspension 3×10^3 cfu/ml). In our study (agar approach), eggshell penetration with *S. enteritidis* even decreased from 50% and 66.7% respectively in week 34 and 46 till 40% and 27%, respectively, in week 69 and 74. Berrang et al. (1998), using *S. typhimurium*, found an upward correlation between the number of penetrated eggs and flock age approaching significance. Our obtained results of all strains (agar approach) showed an almost constant bacterial eggshell penetration during the entire laying period.

The study of Bruce and Johnson (1978) reported about hatching eggs and found an increasing contamination of eggs as flocks became older. Data from Jones et al. (2002), when using whole eggs and *S. enteritidis* and *P. fluorescens* (challenge suspension 10^6 cfu/ml), suggest also that bacterial contamination of air cells, shell membranes and egg contents is more easily achieved in eggs from older hens than from younger hens. In our study whole egg contamination slightly increased, respectively, from 13, 13 and 15% in week 34, 46 and 60 till 26 and 20% in week 69 and 74. Fajardo et al. (1995) reported 43% of whole eggs

positive for *S. enteritidis* after incubation of the inoculated eggs, from 72-week-old hens, for 48 h at 32 °C.

Wells (1968) found that old hens lay bigger eggs which have a lower specific gravity and thinner shells. In our study shell thickness also decreased while shell area increased. Those two changing eggshell characteristics during flock age did not influence the eggshell penetration. Berrang et al. (1998), using an agar approach, did not observe a decline in eggshell quality through flock life, but *S. typhimurium* penetration patterns varied. They concluded it was likely that other factors other than specific gravity and conductance are involved in the bacterial penetration of the eggshell.

The agar approach seemed to be most suited to study the influence of the egg(shell) characteristics but it gives no estimation of the contamination of whole eggs. The intact egg approach gave an estimation of the penetration of the shell followed by the probability of survival and migration in whole eggs. Comparison of different studies gave sometimes conflicting conclusions. As most studies are old, differences in animal feeding, rearing, genetic deposition, methodological possibilities, methodology, groups of hens, flock ages, measured shell characteristics, incubation times and conditions, viability of inoculated bacteria, etc. and differences in eggshell membranes and the albumen (having a pivotal role in exclusion of bacteria from the inside of an egg) can explain this.

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