BASELINE

SELECTION AND IMPROVING OF FIT-FOR-PURPOSE SAMPLING PROCEDURES FOR SPECIFIC FOODS AND RISKS

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SEVENTH FRAMEWORK PROGRAMME

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Summary

The sampling of eggs and egg products such as table eggs and powdered eggs is not regulated at European level. Current voluntary sampling, performed at National levels, seems not to be adequate for the detection of Salmonella especially in relation to the very low prevalence of the foodborne pathogen in these food matrixes. For table egg sampling, pooling is used extensively coupled with ISO 6579 as analytical method. However, if, from one side, pooling might help laboratories to test a high number of table eggs, on the other side pooling might affect sensitivity of Salmonella detection. Regarding powdered egg sampling, sample units are selected randomly within the lot. However it was described that foodborne pathogens are heterogeneously distributed within powdered food, suggesting the random strategy as not efficient for detection of low prevalence of Salmonella. The aims of this study were 1) to evaluate the effect of pooling on the detection of *Salmonella* by comparing both ISO and real-Time PCR methods and 2) to simulate the best sampling strategy for powdered eggs starting from the assumption of an heterogeneous distribution of Salmonella cells within the egg product.

According to a Monte Carlo simulation on the number of eggs to be tested in order to detect *Salmonella* in positive pooled table eggs with 95% certainty and assuming a Salmonella contamination ranging from 10 to 1000 CFU/pool of table eggs, Real-Time PCR was more sensitive and requires a lower number of eggs to be tested in comparison with the cultural method (160 vs 72 eggs tested in pools of 10 or 9 eggs each) However, with both methods, sampling needs to be very intensive to be sure not to falsely accept a positive lot, even when pooling is used.

According to the Habraken approach for the evaluation of an effective fit-for-purpose sampling plan for powdered eggs, taking more and smaller sample units while keeping the total sample weight constant, improves the performance of sampling plans.

Fit-for-purpose sampling plans for Salmonella detection in table eggs and powdered eggs act as alternative tools to obtain information about the expected prevalence of *Salmonella* in a food lot and how contamination can be distributed within this lot. Nevertheless, it must be pointed out that the intensity required for these egg-by products is not practical so they do not fully serve to discriminate between contaminated and non contaminated lots.

1. Introduction

In this document, BASELINE partners report investigations for fit-for-purpose sampling plans for the presence of *Salmonella* in selected egg and egg products.

From earlier surveys conducted as part of BASELINE it became evident that there is no European legislation for official sampling of whole eggs. Only broad sampling in breeding herds and laying herds during raising and laying period is performed according to Regulation EC 2160/2003. However, there are some voluntary sampling plans done by official authorities (*e.g.* in Germany under administration of the National Institute for Risk Assessment) for *Salmonella* in whole egg. In such sampling plans, the location of sampling is typically on the farm and after/before packing and pooling of samples is used intensively. Most microbiological analysis is performed using standard plating techniques ISO 6579:2002 with correction 1:2004. It is unknown how pooling affects sensitivity and hence the design of the sampling plan in order to give a meaningfull insight into the safety of the eggs.

Baseline partners have developed a sensitive PCR based assay for detection of *Salmonella* on eggs. The real-time assay can be used both in a quantatitive and more user-friendly qualitiative manner (see delivery 2.2). In the current report, this method has been incorporated in studies of fit-for-purpose sampling plans for whole egg using pooling of samples (Chapter 2).

Heat treated egg products, such as pasteurized egg, egg powder, and egg containing ready to eat dishes, on the other hand must comply with EU legislation: Reg. [EC] 853/2004 in connection with 2073/2005, amended in some cases by national law. Also here, culture methods are frequenlty used, however, some industries use PCR methods for culture confirmation.

Powdered egg is a regional (Italian) heat treated and dried egg product. It is produced from dirty and cracked eggs as well as eggs in non-compliance (i.e. coming from a salmonella-positive flock) with *Salmonella* regulation. After pasteurization liquid whole egg is dried and sieved before arriving at the packaging centre. Packages might then be stored for months before commercialisation. The post harvest crucial point where recontamination might occur is at sieving. Sampling for *Salmonella* in this product is very challenging, since contamination is often not evenly distrubuted.

BASELINE WP2 has undertaken an investigation into suitable sampling plans for this product, (Chapter 3).

2. Effect of pooling on sampling for Salmonella on whole eggs.

A review performed under task 2.2 in Month 1-18 (reported in deliverable D2.1) showed that official and producer schemes for sampling of table eggs uses pooling extensively coupled with ISO 6579 method (or less frequently by real-time PCR method) for detection of *Salmonella*. Considering the very low prevalence of *Salmonella* in table eggs (less than 1%) (EFSA and ECDC, 2012), we can assume that when eggs are randomly collected and pooled, no more than one egg per pool is contaminated by *Salmonella*. If we consider a contamination level of 10 to 1000 CFU/egg, then the overall contamination level of the pool might be as low as 10-1000 CFU/pool. Apart from a mere dilution effect, pooling might affect detection in unpredictable way especially in consideration of the analytical method used for detection. In order to detect the contamination in the lot, the sample drawn must contain at least one viable cell and this cell might be detected with the applied detection method. In the example illustrated below, the performance of both methods is considered to be perfect, even though in reality this depends highly on the sensitivity and specificity of the detection method followed.

Objectives

The aim of this study was to evaluate the effect of pooling on the detection of *Salmonella* by comparing both ISO and real-Time PCR. Additionally, to suggest sampling plans we tried to determine the expected number of sample units needed to be tested in order to have a 95% certainty to detect *Salmonella* in a positive sample.

Methodology: Experimental protocol followed and inoculation procedure

Different sizes of pools containing experimentally inoculated table eggs were tested using both the culture based method ISO 6579 and a molecular based Real-Time PCR method. The traditional reference method for the detection of *Salmonella* in surface inoculated shell eggs was performed according to the international reference standard method ISO 6579:2004 (Anonymous, 2004). The Real-Time PCR method used is a 5' nuclease Real-Time PCR assay targeting the ttrRSBCA locus essential for tetrathionate respiration and including an Internal Amplification Control (for details of the Real-Time PCR method see D2.2) (Malorny et al., 2004; Kramer et al., 2010). Egg samples were spiked with *S.* Enteritidis at three levels of contamination (10^0-10^1, 10^2-10^3, 10^3-10^4 CFU/egg) and mixed with 4-9 uninfected eggs. Fifteen replicates per pool size and inoculum level were tested.

Modeling approach

To consider the effect of pooling on sampling size and number of samples needed to test in each lot, different variables were grouped as model inputs:

- N: Number of pooled samples collected from a given lot
- K: Number of eggs in each pooled sample
- n_{eggs}: Total number of eggs collected (N * K)
- X1: Number of positive pools obtained by the ISO method
- X2: Number of positive pools obtained by the qPCR method
- X: Total number of positive pools (X1 + X2)

A Beta distribution was considered to model uncertainty in the probability of finding a positive sample in the pool (P_{pool}). Beta distribution has the following parameters:

Beta (X + 1; N – X + 1)

The probability to find an egg positive within each pool (P_{egg}) depends on the value of K and P_{pool}. The following relationship was established:

$$P_{egg} = 1 - ((1 - P_{pool})^{\frac{1}{K}}) \qquad \text{eq.1}$$

Finally, the required number of eggs to accept the lot at 5% CL ($n_{eggs\ samp}$) was determined basing on the study of Whiting et al. (2006):

$$n_{eggs\ samp} = \frac{\log(P_a)}{\log(1 - P_{egg})} \qquad \text{eq.2}$$

being P_a the probability of accepting the lot (0.05).

To evaluate the effect of each input variable on the model output ($n_{eggs\ samp}$), a MonteCarlo simulation was run in ModelRisk v4.3 (Vose Consulting, Belgium) with 10,000 iterations. Different scenarios were assessed to evaluate the impact of modifying the values of N, X and K.

From the output distributions of $n_{eggs\ samp}$, the 95th percentile was selected in order to estimate the number of samples to achieve a 0.95 probability to detect a positive egg. Alternative pools were randomly simulated.

Results

The results of the Monte Carlo simulation on the number of eggs to be tested in order to detect *Salmonella* in positive pooled table eggs with 95% certainty are reported in Table 1.

According to this simulation, Real-time PCR was more sensitive and requires a lower number of eggs to be tested. In real conditions we do not know the exact *Salmonella* contamination-level on the egg, but assuming this is in a range from 10 to 1000 CFU/pool, then 155 are the minimum number of eggs to be tested by ISO method in order to detect *Salmonella* with

95% certainty in pools of 10 eggs contaminated by a *Salmonella* contamination level in the range of 10-1000 CFU/pool. Since this result is related to pools of 10 eggs each, 155 eggs might be round up to 160 eggs in order to test 16 pooled samples of 10 eggs per pool. By Real-Time PCR, approximately half of the eggs need to be tested. The minimum number of eggs is 67 corresponding to pools of 9 eggs each. This value might be round up to 72 eggs in order to test 8 samples of pools of 9 eggs per sample. Anyway, the results with both methods showed that sampling needs to be very intensive to be sure not to falsely accept a positive lot, even when pooling is used.

Table 1. MonteCarlo simulation results on the number of eggs to be tested for 95% certainty to detect Salmonella (neggs-samp) when the contamination level is 10, 100 or 1000 CFU/pool. Combinations highlighted in grey correspond to the optimal combinations of pools flowing ISO and RTPCR detection methods.

Contamination level	K	n_{eggs}	X1	X2	Real-Time PCR $n_{eggs\ samp}$	ISO $n_{eggs\ samp}$
10 cfu/pool	10	150	6	10	51	116
	9	135	2	10	46	484
	8	120	2	11	34	442
	7	105	0	0	6872	6962
	6	90	1	2	333	748
	5	75	0	3	162	4644
100 cfu/pool	10	150	5	9	60	**155**
	9	135	7	8	**67**	83
	8	120	7	6	93	74
	7	105	5	5	106	108
	6	90	7	7	55	55
	5	75	7	9	31	47
1000 cfu/pool	10	150	12	6	117	35
	9	135	11	9	56	38
	8	120	13	6	93	23
	7	105	13	7	65	20
	6	90	12	9	37	21
	5	75	12	7	46	18

The output distributions for ISO and qPCR methods are illustrated in the Figure below. Regarding the qPCR method, the distribution is skewed to the left indicating a lesser number of eggs to be taken from the lot in order to detect a positive *Salmonella* cell in the pool.

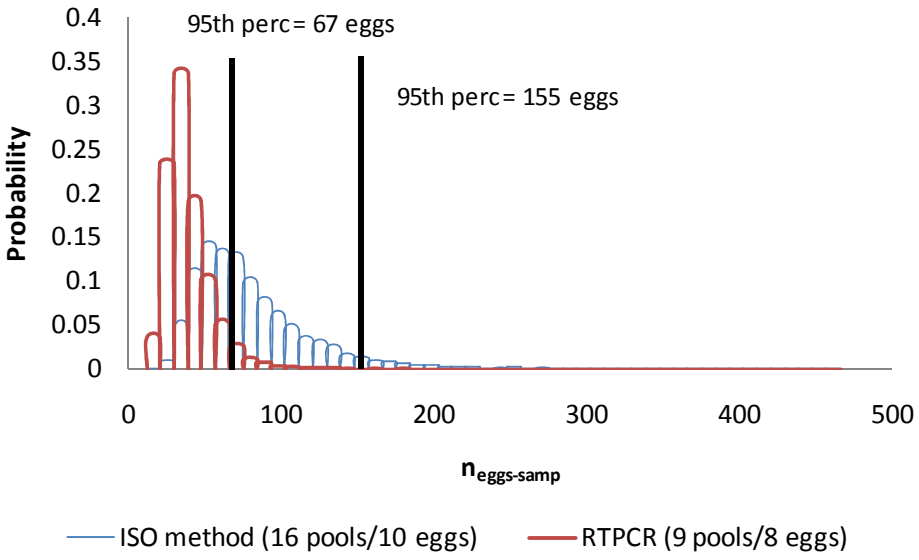

Figure 1. Output distributions of the required number of eggs to accept the lot at 5% CL (neggs-samp) considering the ISO method (16 pools/10 eggs per pool) and qPCR method (9 pools/8 eggs per pool)

3. Sampling plan for powdered eggs

Powdered egg is a regional product, mainly produced and used for preparation of ice cream, egg-based pasta and bakery products at industrial level. A major problem in relation to sampling of this product type is an uneven, distribution of contamination. In the current project, the influence of this on fit for purpose sampling schemes was simulated.

In-silico model to estimate the probability of detection of *Salmonella* positive samples in powdered eggs

In this product, there is relatively little knowledge about how microorganisms are distributed so sampling strategies should be based on maximizing the probability of detection of a positive sample. In many cases, a default assumption is that the product is handled in the same conditions thus producing lots with homogeneous contamination (theoretically). In several approaches, the binomial distribution can be applied to calculate the probability of accepting a product lot, which is based on (i) the number of sample units (n) drawn from the lot is predetermined, (ii) the sample units are independent of each other, (iii) the sample units are either contaminated or not contaminated (alternatively, the contamination of sample unit falls above or below some prespecified limit), and (iv) the probability of a sample unit being contaminated is the same for each sample unit.

The difference occurs when lots are heterogeneous, i.e., when a part of the lot is free from contamination and other parts are contaminated; hence, the fourth binomial assumption does not hold. Instead, when sampling from the contaminated part of the lot, the probability of detecting contamination is calculated according to a Poisson distribution with a given average rate (per unit weight, area, or volume).

For powdered eggs, we considered that microbial contamination is not homogeneous, but localized in a small part of the lot.

Habraken et al. (1986) derived the following formula for the probability of accepting a lot under zero tolerance.

$$P_a = \left[1 - p + p\exp\left(-\frac{cw}{Np}\right)\right]^n \qquad \text{eq.3}$$

where n is the number of samples, w is the weight (grams) of each sample, N is the weight (kg) of the lot, p is the proportion of the lot that is contaminated, and c is the number of organisms in the contaminated part of the lot (CFU/g). The concentration of organisms in the contaminated part of the lot is represented by λ= c/(Np), so the formula can be rewritten as:

$$P_a = \left[1 - p + p\exp(-\lambda w)\right]^n \qquad \text{eq.4}$$

Results

Different scenarios were simulated considering as variables the lot size, the number of samples taken from the lot, the sample size, the microbial concentration in the contaminated part of the lot and the proportion of lot contaminated.

In the first sampling scheme, A (N=10000; n=20; w=50; c=5; p=0.01) the probability of accepting (P_a) the lot by taking 20 samples is 0.83, which is not restrictive enough to discriminate between contaminated and non contaminated lot. In Fig. I-A it can be seen that even when taking 100 samples, P_a is higher than 0.4 by what the sampling scheme cannot used as a verification tool for lot contamination. In Fig. II-A the influence of increasing sample size does not affect significantly the performance of the sampling scheme.

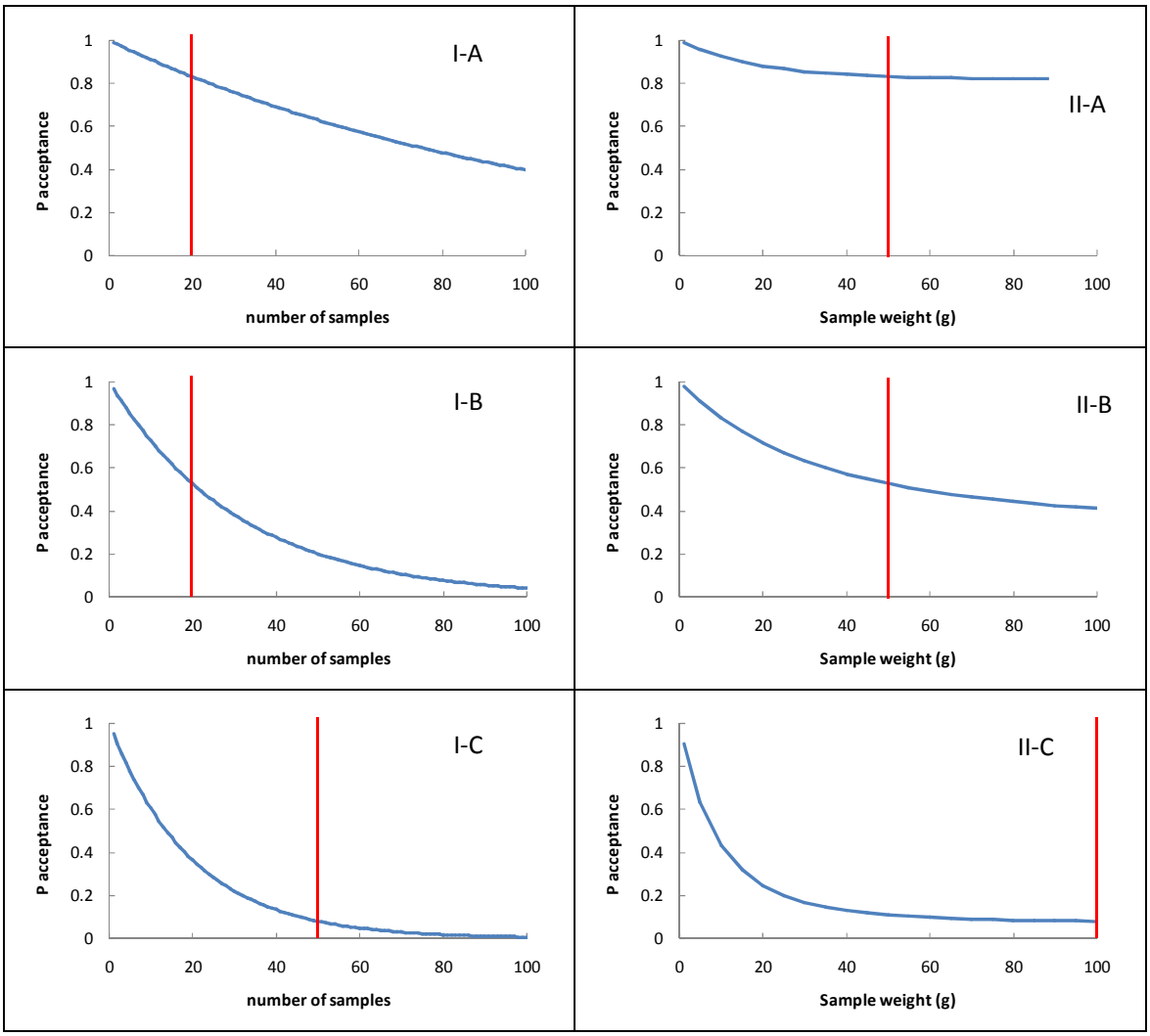

Figure 2. Application of sampling schemes according to the Habraken approach and influence of the lot size (N, Kg), number of samples taken (n), sample weight (w, g), microbial concentration in the contaminated part (c, CFU/g) and proportion of contaminated lot (p, %) on the number of samples (0-100) (Fig. I) and sample weight (0-100g) (Fig. II).

In the second sampling schemes (B), parameters were as shown below.

A- N=10000; n=20; w=50; c=5; p=0.01

B- N=10000; n=20; w=50; c=10; p=0.05

C- N=5000; n=50; w=100; c= c=10; p=0.05

The sampling scheme B represents a higher concentration in the contaminated part (10 CFU/g) and higher proportion of lot contaminated (0.05). By taking 20 samples P_a is equal to 0.53. To obtain a P_a of 0.05, 94 samples must be collected from the lot. Likewise, there is more influence of the sample size, as represented in Fig. II-B. However, increasing the sample size does not improve the values of P_a since they are higher than 0.4.

Finally, the intensity of the sampling scheme is represented in scenario C, where N=5000 kg; n=50 and w=100. By taking 20 samples P_a is equal to 0.08. To obtain a P_a of 0.05, 60 samples must be collected from the lot.

4. Conclusions

In the light of these results it is evident that sampling of whole egg does not constitute a suitable tool for control of *Salmonella,* even when pooling is performed. The sample size than be dramatically reduced from 16 pools of 10 eggs to 9 pools of 8 eggs if a more sensitive PCR method is employed instead of the ISO culture method.

The results were produced using artificially contaminated eggs. It is currently unknown how the distribution of *Salmonella* is on positive eggs, and in lack of this, we opted for two relatively low levels of contamination. However, for sampling plans to be really evaluated it is important that information on normal shell contamination is obtained.

From our studies of powdered eggs, it can be concluded that taking more and smaller sample units while keeping the total sample weight constant, improves the performance of sampling plans. However, in most cases contamination occurs at very low levels, thus being undetectable by conventional analytical techniques unless an impractical number of samples is collected. This premise makes that sampling plans act as alternative tools to obtain information about the expected prevalence of *Salmonella* in a food lot and how contamination can be distributed within this lot. Nevertheless, it must be pointed out that the intensity required for these egg-by products is not practical so they do not fully serve to discriminate between contaminated and non contaminated lots.

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