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Review

Sampling and testing for pathogens in food: finding the needle in a haystack and the impact of the food microbiome

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Microbiological testing provides food businesses and competent governmental authorities reference points to verify that food safety measures are well implemented. For pathogens, often absence testing is required in one or in several samples of 25 g of food; hence, the test procedure should be able to detect extremely low concentrations of cells. To date, direct detection of these low levels of contaminants is not possible, and adequate detection relies on an enrichment step to increase cell concentrations to detectable levels. The detection chance of pathogens is influenced by intrinsic and extrinsic factors such as an uneven distribution of cells in a food batch, the physiological status of the cells, intraspecies and intrastrain variability, specific food components, and the food microbiome. The advantages and disadvantages of nonselective and selective enrichments are discussed, as well as molecular detection procedures to detect single and multiple pathogens from the food they reside in.

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Introduction

Food safety affects us all, and it is a focus of governmental agencies and the industry. To ensure safety, management systems are put in place by the industry. Hazard Analysis & Critical Control Point is a systematic approach to identify specific hazards and measures for their control to ensure the safety of food [1]. To verify that critical measures are well implemented, microbiological sampling and testing are done by the industry and competent authorities. Sampling and testing are challenging for several reasons. First, the samples tested represent only a fraction of a batch, and absence testing or a satisfactory outcome of a sampling plan does not mean that a pathogen is absent or that the number of contaminated products is negligible. Second, pathogens are often present in low concentrations, and absence is often required in one or in several samples of 10 or 25 g of food, or sometimes in higher quantities for composites (e.g. [2]). Detection of a pathogen in such a sample is challenging, and test outcomes can be negative despite the pathogen being present. Third, multiple pathogens can be present in a food; however, test procedures often target the detection of one pathogen because selective culturing methods are used. Hence, selective procedures may challenge the detection of all pathogens. This review will discuss these challenges in sampling and testing. Furthermore, advances in procedures to reduce the time-to-detection using molecular techniques and to detect multiple pathogens in a sample will be discussed.

Batch sampling

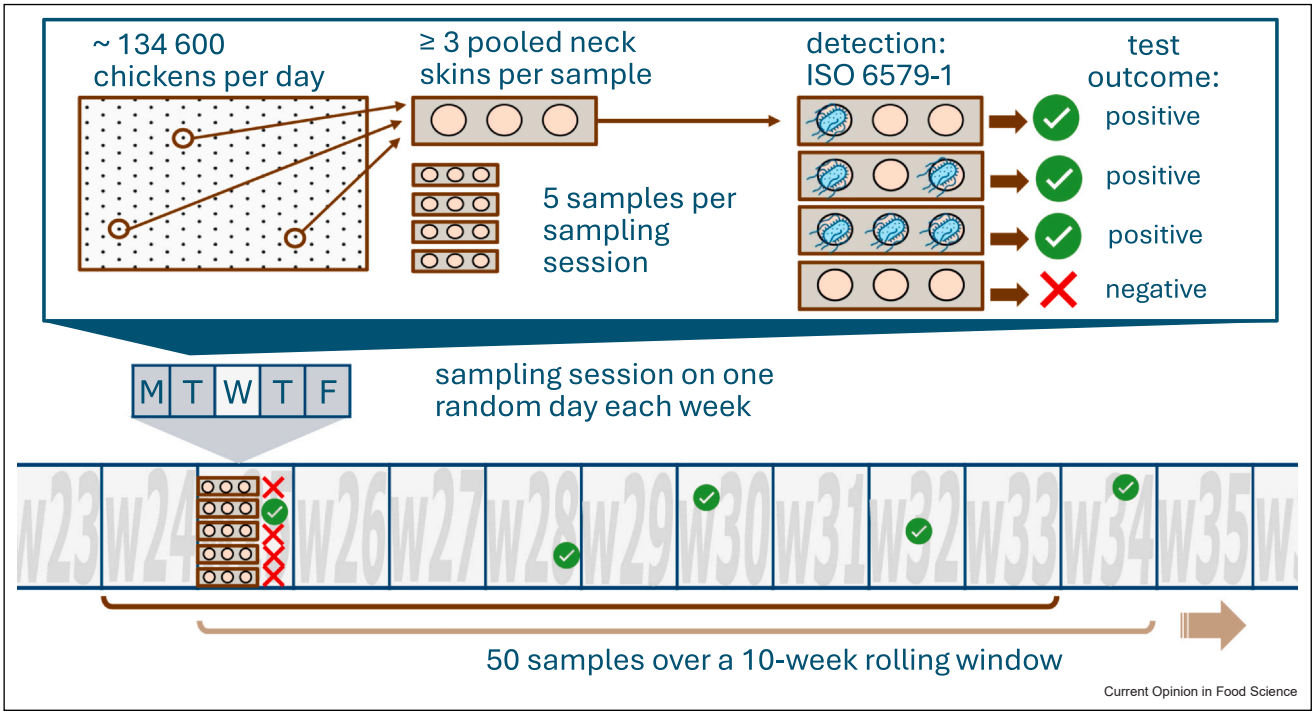
A microbiological criterion can be defined as a risk management metric, which indicates the acceptability of a food at a specified point of the chain [3]. A sampling plan is a component of a criterion and defines the number of samples, the analytical sample size, and the limit(s) of acceptance of a sample. A criterion can be formulated as process hygiene or food safety criterion, and the follow-up actions differ when the result of a sampling plan is unsatisfactory. For a process hygiene criterion, follow-up actions should result in improvement of the hygiene and a review of process controls, whereas for a food safety criterion, the batch will be withdrawn. The criterion for *Salmonella* on poultry carcasses in the EU [4] is an example of a process hygiene criterion where the sampling frequency has been defined; hence, this criterion can be used to evaluate the number of samples in relation to the total number of carcasses. The criterion prescribes that neck skins from a minimum of 15 carcasses shall be sampled at random after chilling

during each sampling session. The neck skins from at least three carcasses from the same flock of origin shall be pooled into one sample of 25 g, resulting in five samples per session. In total, 50 samples shall be derived from 10 consecutive sessions. Samples should be taken at least once a week, and the sampling day shall be changed each week to ensure that each day of the week is covered (Figure 1). A sample of 25 g is analyzed according to ISO-6579-1 [5] and results in a qualitative test outcome: absence or presence of *Salmonella* spp. in the sample. In the Netherlands, there are 14 slaughterhouses processing meat from broiler chicken [6]. By taking the total number of chickens slaughtered in 2023 (~490 million) [7], approximately 673 000 animals are processed per week by an average slaughterhouse (i.e. 134 600 per day assuming five operation days per week). Each week, five times three pooled neck skins are taken from different carcasses, so 15 of the 673 000 carcasses, or 0.0022%, are tested per week, exemplifying that a decision is based on only a fraction of the carcasses processed. For a total of 50 samples collected in 10 weeks, the process hygiene criterion is considered unsatisfactory when more than five samples are found to be positive. In other words, five positive samples, of which each originate from three carcasses, is acceptable, resulting in satisfactory outcome when 3.3% (5 of 150; one positive neck skin per sample) to 10% (15 of 150; three positive neck skins per sample) of the tested carcasses is contaminated; ~ 4500–13 500 or fewer carcasses per day per average slaughterhouse would be judged to be an acceptable rate of contamination. This underlines that a satisfactory result does not mean that the number of contaminated products is negligible. The process hygiene criterion, however, helps to verify the level of slaughter hygiene and to reduce the contamination level.

Detection in a test sample: finding a needle in the haystack

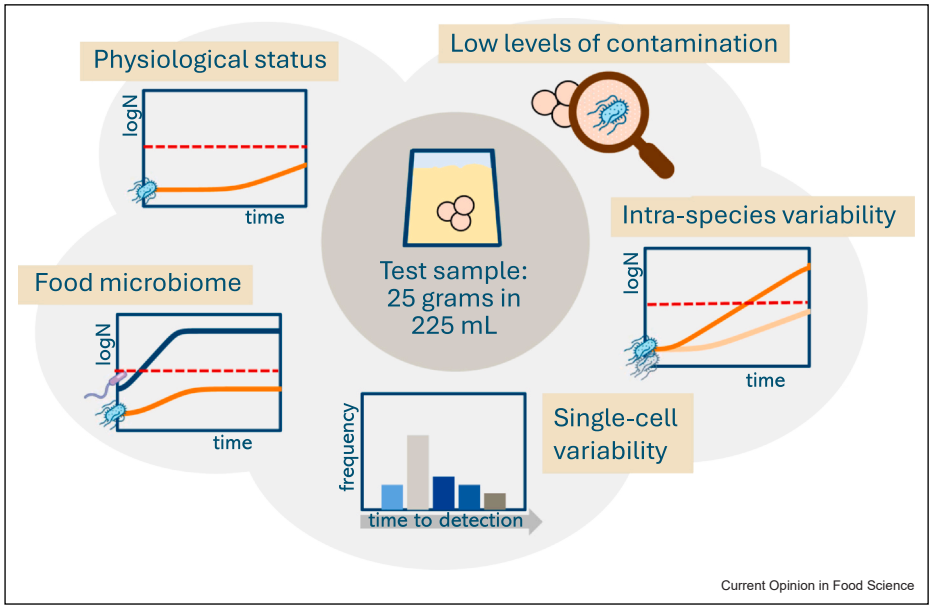
Detection of a pathogen in a sample is challenging for several reasons, among others, low contamination levels and uneven distribution of the pathogen in the food, the presence of other microbiota in the sample, the physiological status of the pathogen, and biological variability within a pathogenic species (i.e. intraspecies variability) and within a strain (i.e. single-cell variability; Figure 2).

Figure 1



Visualization of the sampling plan for the qualitative testing of *Salmonella* spp. in poultry slaughterhouses according to Commission Regulation (EC) No 2073/2005 [4]. Sampling must be carried out at each slaughterhouse once a week, and five samples of at least 25 g (with pooling of three neck skins) must be taken each time at random. The sample is analyzed according to ISO-6579-1 [5], which results in either detection (meaning either one, two, or three of the neck skins in the composite were positive) or no detection (meaning all three neck skins were negative) of *Salmonella* spp. in 25 g. Samples are evaluated over a 10-week rolling window. The sampling plan is considered satisfactory when five or less samples are found to be positive over every 10-week window.

Figure 2



Factors that challenge the detection of a pathogen in a food test sample: low contamination levels of the pathogen, the presence of other food microbiota in the test sample, the physiological status of the pathogen, and biological variability (i.e. intraspecies and single-cell variability).

Low contamination levels

For pathogens, absence testing is often required in one or several samples of 25 g of food. This means that when one or more cells are present in 25 g, the test should result in a positive outcome. The detection of one cell in 25 g is not trivial. Bacteria are extremely small and should be isolated from a relatively enormous volume. Generally, the length and width of a cell are in the range of a few micrometers. A typical cell mass would then be around 5 pg. When finding such a bacterium in 25 g, the methods should detect 1 of 5 trillion parts (Annex A). This is the same as finding a small needle in multiple haystacks. Therefore, analytical methods rely on an enrichment step, where pathogens are enriched in a broth that provides a favorable environment for the pathogen in terms of nutrients and temperature to multiply to detectable levels. Note that when the sample test outcome is negative, this does not mean that the remainder of the batch is free of the pathogen because pathogens are often unevenly distributed in a batch of food.

Physiological status

Foods are often processed or stored at conditions that may induce cell damage or stress. So, the enrichment should support damage repair and/or allow the pathogen to adapt before subsequent outgrowth. The lag phase prepares pathogens for growth and is strain-dependent and affected by the cell's environment and history. Two recent studies conducted on *Campylobacter* and *Listeria monocytogenes* in enrichment broths quantified the lag duration upon preexposure to different conditions for a set of strains that belonged to the same species [8,9]. The lag duration of *Campylobacter* in Bolton broth was extended when cells were previously stored at refrigeration temperatures in comparison to cells precultured in optimal conditions, while the viability was not affected. For some but not all strains, the lag duration of frozen cells was longer than for refrigerated cells, while freezing induced a reduction in viability, indicating that a more severe stress does not always increase the lag duration [8]. For *L. monocytogenes*, no correlation was observed between the lag duration and the heat resistance of strains [9], pointing out that prediction of the lag duration was not possible based on the robustness of cells. In addition, the lag duration is often not a good predictor of the growth rate [10], because they are sometimes highly correlated, and sometimes not at all [10].

Despite that the lag phase has been known for decennia, little is known of the molecular events which characterize it [11]. It was demonstrated for *Salmonella* that the lag phase involved transient accumulation of metals, including iron, which may play a role in oxidative stress adaptation [12]. Upregulation of genes involved in metal transport was also observed during the lag phase for *L. monocytogenes* in half Fraser broth [13], and those authors speculated that metals may play a role in the regulation of metabolic enzymes where they function as co-factors.

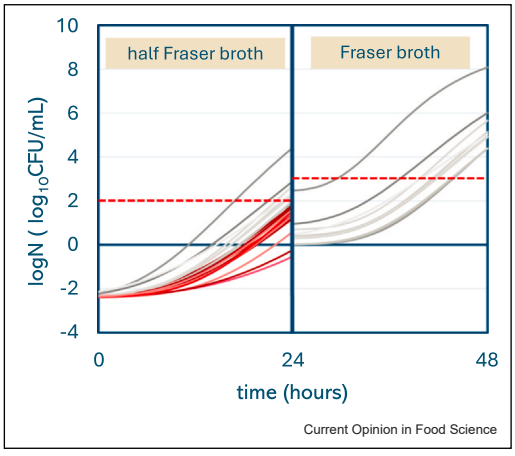
Food microbiome

As the pathogen often only represents a fraction of the food microbiota, the selection of a suitable medium to favor the growth of the pathogen is not trivial. Enrichment media have been developed historically based upon expertise and decided upon by international consensus and are specified in the analytical method. The enrichment medium should preferably favor the growth of the pathogen relative to other microorganisms present, but competitors may grow faster than the pathogen and mask its presence when the enrichment broth is subsequently streaked on plates for identification. For example, *Cronobacter* was overgrown by background microbiota when powdered infant formula was enriched in nonselective Buffered Peptone Water [14]. The authors attributed this to the Jameson effect, where in mixed cultures, growth suppression occurs by a dominant species when it reaches its maximum concentration. On the other hand, enrichment in a nonselective broth may favor damage repair of cells and shorten the lag phase. For some foods (e.g. powdered infant formula), pooling of samples is a common procedure to reduce cost and for greater convenience [14]. For example, in these cases, 10 samples of 10 g are taken and pooled to 100 g and mixed with 900 ml enrichment broth, instead of mixing each 10 g sample with 90 ml. Pooling of samples can reduce the initial concentration of the pathogen in the larger volume and may also affect the background microbiota and reduce the detection chance [14].

Several studies observed inhibitory phenomena in pathogen-specific selective broth. For example, *Campylobacter* was outcompeted in Bolton broth because certain strains of *Escherichia coli* were not susceptible to the antimicrobials present in it, which allowed them to overgrow *Campylobacter* [15,16]. The growth of *E. coli* could be inhibited by adding potassium clavulanate to Bolton broth [15,17]; however, this may increase the lag duration [15]. Also for *L. monocytogenes*, the maximum concentration reached in selective medium was influenced by competitive strains [18,19]. Extension of the lag phase due to the selective nature of the medium may, however, also introduce an additional complicating factor because pathogens may recover too slowly. It has been demonstrated for *L. monocytogenes* that damaged cells may not reach the detection limit during the incubation period [9], even in modified medium that supports a shorter lag phase [13], and this may result in false-negative detection outcomes (Figure 3). Also, other changes could be considered to increase the detection chance, like changing the temperature or, ultimately, a longer incubation period.

The food microbiome composition can change dynamically during enrichment and affects the chance of pathogen detection [21–23]. Analyzing the food microbiome during enrichment can reveal the most dominant species that may suppress the pathogen [21,23]. For example, Ottesen et al. demonstrated that the abundance of *L. monocytogenes* during enrichments was dynamic and decreased during the first 12 hours of enrichment of naturally contaminated ice cream, after which it increased to be the dominant species after 48 hours [23]. Two moderate thermophilic species, *Anoxybacillus* and *Geobacillus*, appeared to have an advantage over *L. monocytogenes* during early incubation at 30°C, indicating that these bacteria were able to adapt more readily. Insight into the enrichment ecology can give direction for a rational design of medium components in concert with judicious use of additives and possible alterations in protocols [21–23].

Figure 3



Strains with a longer lag phase/and or lower growth rate may fail to reach the detection threshold for transfer to the secondary enrichment step. After the first enrichment in half Fraser broth, 100 µl is transferred into 10 ml Fraser broth; after the second enrichment in Fraser broth, 10 µl is spread onto selective agar plate. Dashed line in red is the detection limit that allows transfer of at least one cell to the following up step with 99.995% probability, assuming a Poisson distribution for low cell numbers (i.e. 1-Poisson(x=0, mean=10)) [20]. The growth curves in red originate from those strains that fail to reach the detection limit in half Fraser broth within 24 hours, assuming one cell in 250 ml at the start of the enrichment and therefore are not transferred to the second enrichment in Fraser broth.

Biological variability

Food can be contaminated with different subtypes of the same pathogen [24,25], resulting in intrasample strain diversity [26]. Increasing evidence suggests that selective test methods may introduce a differential recovery of pathogenic subtypes [27] or variants [28]. Indeed, surveillance and epidemiological data analyses demonstrate that while food is assumed to be an important vehicle for infection, there is a discrepancy in subtype distribution among clinical and food isolates. This may point to a selection bias toward specific subtypes

[27,29] and/or be attributed to differences in strains' virulence, robustness, and fitness. This bias of selective procedures hampers root cause analysis of outbreaks and complicates accurate risk assessments.

The behavior at the single cell can also be variable, and this can affect the detection efficacy of enrichment-based detection methods. Single-cell heterogeneity may be higher in selective broth compared to nonselective broth, as demonstrated for *L. monocytogenes* when grown in half Fraser broth and nonselective Brain Heart Infusion broth [30]. Single-cell heterogeneity is also strain dependent [30] and may increase when cells are stressed [30,31] and reduces the chance to detect stressed cells at low levels, also in cases where enrichments are used as initial step of the procedure.

Molecular-based detection

For most important pathogens, international standardized procedures by the International Standardization Organization (ISO) or the Association of Official Analytical Collaboration are available. These are accepted as the golden standard to detect a specific pathogen (i.e. isolation of the pathogen followed by confirmation). The confirmation most often relies on culturing-based and biochemical analyses that are applied after an initial and/or secondary enrichment. The golden standard procedures are lengthy workflows and can take more than 5 days. Advances in molecular identification and confirmation methods have significantly shortened procedures. The most widely accepted alternative confirmation methods rely on nucleic acid amplification, with polymerase chain reaction (PCR) and real-time PCR (RT-PCR) as techniques that are most often applied. Some of these molecular-based detection procedures have been validated in interlaboratory validation studies (e.g. [32–34]) supporting the uptake in testing programs. The sensitivity is theoretically one genome/PCR reaction, but because reaction volumes are low and food is often solid, detection limits are around 10^2–10^3 per gram food, underlining the need of an enrichment. To increase the sensitivity, immunomagnetic separation and filtration methods have been developed to concentrate the pathogen before molecular-based detection. Some studies reported remarkable sensitive detection using filtration, DNA concentration and RT-PCR with detection limits as low as ~few to 25 cells per 25 g of foods [35,36], but differences in sensitivity were demonstrated between strains and foods. The disadvantage of DNA detection is that DNA can originate from nonviable cells. For that reason, (RT-) PCR has been combined with propidium monoazide staining. This dye penetrates the compromised membranes of dead cells, allowing cross-linking to DNA to inhibit amplification [37]. Alternatively, nucleic acid amplification can be preceded with reverse transcription to target RNA, and reverse transcription PCR has been mainly developed for the detection of RNA viruses [38]. More recently, a novel method has been developed to ease the detection of amplified nucleic acid products using clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas. A CRISPR-cas12a system detects amplified DNA fragments and was applied to detect *L. monocytogenes* in diluted pork in a simultaneous amplification and detection procedure [39] and *E. coli* O157:H7 in milk [40]. Testing procedures that start with solid foods, and where only part of the diluted suspension is used for analysis, necessitate careful bookkeeping of cells and sample volume to determine the detection limit in the original solid sample; hence, a detailed description of the methods applied is imperative. The Cas13a system is similar to Cas12a but targets RNA instead of DNA, and the RNA recognition function allows for detection of viral and bacterial RNA. Nevertheless, these molecular-based methods are pathogen-specific unless more than one primer set is used (i.e. multiplex PCR) and multi-pathogen detection has not been demonstrated yet for CRISPR-cas-based detection.

Metagenomics and quasi-metagenomics

It is recognized that food can be contaminated with multiple pathogens [41]. Hence, testing foods for specific pathogens with selective testing procedures can keep other pathogens that require other favorable conditions under the detection radar. The advances in high-throughput sequencing and metagenomic analyses open avenues to gain insights into the food microbiome, including (multiple) pathogenic species (e.g. [42]). Metagenomics is defined as the untargeted sequencing of the whole genomic content of a sample (e.g. [43]). Classification of the microorganisms can be performed by analyzing short or longer DNA sequences, obtained through shotgun metagenomic sequencing (e.g. Illumina platform) or long-read metagenomic sequencing (e.g. Oxford Nanopore platform), respectively. Application of metagenomics for food safety is limited for several reasons. First, for low-abundant species, such as pathogens, metagenomic analyses result in just a few or no sequencing reads of pathogens. Host depletion protocols can reduce food DNA, leading to a greater proportion of reads from microorganisms but is no guarantee that reads of low-abundant pathogens are detected. Second, for some pathogens like pathogenic *E. coli* and *Bacillus cereus*, pathogenic strains can be differentiated from nonpathogenic only by detection of virulence factors or toxin genes, underlining the need for deep sequencing. Altogether, the application of culturing-free direct metagenomic analyses is not realistic when low-abundance testing is required as is the case for regulatory food safety testing. On the other hand, researchers can employ metagenomics to obtain insights into the microbial dynamics during enrichments [44]. This latter

approach is referred to as quasi-metagenomics, where metagenomics is applied on enriched cultures. Quasi-metagenomics has been applied is some recent studies for source tracking of a pathogen from food [45–48] or to analyze swab samples [49]. This hybrid approach where enrichments are directly sequenced can provide high-resolution source tracking sequence data and is significantly faster (more than twice as fast) than culturing and sequencing of individual isolates [48]. When quasi-metagenomics is combined with a nonselective medium, multipathogen detection is possible. Townsend et al. used Tryptic Soy Broth to co-enrich *S. enterica* and *E. coli* O157:H7, followed by immunomagnetic separation to concentrate the pathogens before sequencing, resulting in a detection limit of a few cells per 25 g [50]. In the ideal scenario, detection of pathogens is unbiased and allows detection of multiple pathogens from the same sample, including pathogens such as *Campylobacter* that require specific microaerobic enrichment conditions. Obviously, further research efforts are needed to elucidate how to optimize the enrichment, as a first critical step for multipathogen detection from food.

Conclusions

Food sampling is relevant for verifying that food safety management systems work as intended and to collect baseline data. Due to the very limited quantity of sample material that is being tested, sampling will not stand on its own to prove appropriate control. Moreover, the target levels of many pathogens are so low that instantaneous methods are not possible. With advanced molecular methods detection times can be reduced but only for the detection part that has to be preceded with an enrichment step. Furthermore, molecular methods can have the advantage of being much more specific and being able to detect and identify multiple targets simultaneously. These tools can also be supportive to investigate the correlations between the target pathogen(s) and the food microbiome to further optimize methods.

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Author Contributions

Heidy M. W. den Besten: Conceptualization, Methodology, Writing – original draft. **Johanna Mentani:** Conceptualization, Methodology, Writing – original draft. **Marcel H. Zwietering:** Conceptualization, Methodology, Writing – review & editing.

Data Availability

Data will be made available on request.

Declaration of Competing Interest

The authors have no competing interests to declare.

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Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cofs.2025.101332.

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- •• of outstanding interest

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