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General Interest

Use of Microbiological Indicators for Assessing Hygiene Controls for the Manufacture of Powdered Infant Formula

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ABSTRACT

Microbiological testing for various indicator microorganisms is used extensively as a means of verifying the effectiveness of efforts to ensure the microbiological quality and safety of a wide variety of foods. However, for each use of an indicator organism the underlying scientific assumptions related to the behavior of the target microorganism, the characteristics of the food matrix, the details of the food manufacturing processes, environment, and distribution system, and the methodological basis for the assay must be evaluated to determine the validity, utility, and efficacy of potential microbiological indicator tests. The recent adoption by the Codex Alimentarius Commission of microbiological criteria for powdered infant formulae and related products provides an excellent example of an evidence-based approach for the establishment of consensus microbiological criteria. The present article reviews these criteria and those of various national governments in relation to emerging principles for the evidence-based establishment of effective indicator organisms.

Microbiologists have been employing various indicator tests since the scientific discipline was in its infancy. For example, the use of coliform bacteria as a means of evaluating water for the presence of fecal contamination was originally introduced by Schardinger and Smith in the 1890s. Since that time, the coliform assay has been employed worldwide in conjunction with a variety of food commodities. However, for each use of an indicator organism the underlying scientific assumptions related to the behavior of the target microorganism, the characteristics of the food matrix, the details of the food manufacturing processes, environment, and distribution system, and the methodological basis for the assay must be evaluated to determine the utility and efficacy of these microbiological indicator tests. For example, the purpose and scientific rationale for using coliforms as an indicator test in water are not the same as those for its historic use with pasteurized milk (i.e., indicator of fecal contamination versus indicator of postpasteurization sanitary control) *(8)*.

Traditionally, two types of microbiological indicator assays have been used: index organisms and indicator organisms *(7, 33)*. An index organism is a microorganism, group of microorganisms, or product of microbial metabolism that is predictive of the presence and extent of contamination with a specific pathogen (e.g., the levels of coliforms are directly related to the levels of *Salmonella* in a product). However, index organisms have rarely been effective for this purpose, particularly when the levels of the index organism are low and the pathogen is present sporadically at low levels. Conversely, an indicator organism is a microorganism, group of microorganisms, or product of microbial metabolism that is indicative of a food having been exposed to conditions that pose an increased risk that the food may be contaminated with a pathogen or having been held under conditions that would allow pathogen proliferation. For example, thermotolerant coliforms (e.g., *Escherichia coli* and *Klebsiella* spp.) in uncooked foods often are used as indicators of fecal contamination but are not considered effective index organisms for either *Salmonella enterica* or *E. coli* O157:H7 even though all of these organisms are associated with fecal contamination. The problem again is that contamination by these pathogens is typically at low levels and occurs sporadically. Thermotolerant coliforms are usually not an indicator of fecal contamination in cooked refrigerated foods but instead are indictors of the adequacy of the cold chain and the degree of postprocessing sanitation *(6, 8)*. Various indicator organisms have been used with varying degrees of success.

(i) The total aerobic plate count (TAPC) has been used to indicate general sanitation, effectiveness of intervention steps, microbiological quality, and spoilage in such matrices as cooked ready-to-eat foods, pasteurized milk, and spices. This indicator has also been referred to as mesophilic aerobic bacteria (MAB) or standard plate count.

(ii) Coliforms have been used to indicate fecal contamination and general sanitation in such matrices as raw ground meats, water, and spices.

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(iii) Fecal coliforms (thermotolerant coliforms) have been used to indicate fecal contamination, general sanitation, and temperature abuse in such matrices as raw ground meats, water, and seafood.

(iv) *Enterobacteriaceae* have been used to indicate fecal contamination and effectiveness of sanitation programs, particularly in dry products, in such matrices as powdered infant formula and cooked ready-to-eat foods.

(v) *E. coli* has been used to indicate fecal contamination and effectiveness of sanitation programs in such matrices as seafood, water, and ready-to-eat foods.

(vi) *Staphylococcus aureus* has been used to indicate the extent of human handling in such matrices as foods from food service establishments and fermented meat and dairy products (fermentation failures).

(vii) *Enterococcus* spp. have been used to indicate fecal contamination in such matrices as frozen foods, particularly produce.

(viii) *Clostridium perfringens* has been used to indicate fecal contamination, but such uses are not common.

(ix) *Listeria* spp. have been used to indicate the effectiveness of sanitation programs in such areas as environmental evaluation of food facilities and refrigerated ready-to-eat foods.

(x) *S. enterica* has been used to indicate control of enteric bacteria in raw meat and poultry.

(xi) Male-specific (F+) coliphages have been used to indicate the effectiveness of enteric virus control programs in such matrices as water and shellfish.

Although there is no universal ideal indicator organism, there are characteristics of indicator tests that are considered desirable *(6, 32, 33)*.

(i) Indicator microorganisms are usually nonpathogenic, although in some situations pathogenic microorganisms have been used (e.g., *S. enterica* in meat and poultry).

(ii) Methods for detecting indicators should be rapid, inexpensive, widely available, and easy to interpret.

(iii) Indicator microorganism should occur at a sufficient frequency and/or level when a food safety system is under control such that (a) the baseline levels of the microorganism are clearly identified, (b) out-of-control conditions are reflected in a significant increase in the level of the indicator, and (c) corrective actions can be taken before a designated decision limit is exceeded.

(iv) Whenever possible, the decision criteria should not be set at the lower limit of detection of the methods used.

(v) Decision criteria for the microbiological indicator should be pertinent at multiple points along the food chain.

(v) Growth, inactivation, survival, or resistance of the indicator microorganism in the food should be at least equivalent to or greater than those for the pathogenic microorganisms that are likely to have an increased risk of occurring under conditions leading to elevated levels of the indicator (e.g., growth of an indicator microorganism in temperature abused foods and survival of an indicator in a dry product).

(vii) Selection of indicator tests should be based on a sound understanding of the characteristics and sources of microbiological hazards that the food safety system is being used to control.

PRODUCTION OF POWDERED INFANT FORMULA

Selection of the appropriate indicator tests for verifying the effectiveness of process controls is dependent on an understanding of the characteristics of the food and the steps in its production. The focus of the current review is powdered infant formula (PIF), which is intended for infants 0 to 12 months of age. Much of this discussion is also pertinent to closely related products such as follow-up formula (FUF), which is intended for older infants and young children 6 to 24 months of age, and human milk fortifiers. Such products are formulated to serve as a sole source of nutrition for infants (PIF), a significant part of an older infant's or young child's diet (FUF), or a supplement for infants with special dietary needs (human milk fortifiers). These products are distributed and sold as a shelf-stable food that is rehydrated by the consumer before use. The products' microbiological stability is achieved through dehydration and subsequent packaging, which ensures an effective barrier to uptake of moisture (and oxygen). Although the manufacturer of the product typically employs several steps that reduce the microbial load in the product, PIF is not a commercially sterile product and therefore may have a low level of microorganisms present when produced under controlled conditions. PIF is manufactured differently than is liquid infant formula, which is designed to be a commercially sterile product. Commercial sterility is typically achieved by retorting or aseptically filling a hermetically sealed container with the product. Liquid infant formulas and weaning foods such as infant cereals were not considered in the current evaluation.

Two general approaches are employed in the manufacture of PIF. The most common approach is to prepare the product in a liquid state, subject the formulation to one or more heat treatment steps, spray dry the product, and then dispense the dehydrated product into its final packaging. The other approach is to pretreat the ingredients of the formula to achieve effective pasteurization of the individual components and then dry them separately. The individual dry ingredients are then combined by dry mixing and dispensed into their final packaging. In both these approaches, the product or its ingredients are subjected to a thermal process that is sufficient to effectively eliminate non–spore-forming microorganisms and to reduce the levels of non–heat-resistant spores. Thus, the bioburden of the product would be expected to consist of a low level of sporeformers that survived the thermal treatment and/or non–sporeformers that were reintroduced between dehydration and final packaging. Additional details on the manufacturing of PIF and related products were described by the Food and Agriculture Organization and World Health Organization (FAO-WHO) *(22)* and Cordier *(14)*.

HAZARD IDENTIFICATION

Two primary contamination scenarios can be postulated for microbiological disease associated with PIF: low level

contamination of the product or contamination of the product during rehydration and further handling. The former scenario would be expected to involve spore-forming microorganisms capable of surviving the manufacturing process or microorganisms that are reintroduced before final packaging. The latter scenario would be expected to involve a potentially wider range of microorganisms, including those in the PIF, and microorganisms associated with the water used to rehydrate the PIF, the utensils used to prepare and dispense the rehydrated formula, refrigerator surfaces, and the individuals and environment associated with preparation *(35, 40)*. Similar environmental sources of bacteria have been identified for rehydration and use of powdered enteral feeds *(38)*. With both scenarios, temperature abuse after rehydration will greatly amplify the problem by allowing growth of pathogenic bacteria *(22, 23, 43)*. Laboratory studies have clearly demonstrated that a number of foodborne pathogens are capable of growing when introduced into PIF after rehydration and then held at temperatures that support bacterial growth *(4, 29, 43, 54)*.

Outbreaks of disease associated with PIF have been largely attributed to two microorganisms, *S. enterica* and *Cronobacter* spp. (former *Enterobacter sakazakii*) *(9, 22, 23, 34, 47)*. These two microorganisms were designated by the FAO-WHO *(22)* as category A organisms, i.e., clear evidence of causality. Several other *Enterobacteriaceae* were included as category B organisms, i.e., causality plausible but not yet demonstrated. This group included *Citrobacter freundii (5, 48), Enterobacter hormaechei (10, 52),* and *Serratia marscesens (51),* which have been associated with outbreaks in neonatal intensive care units but for which a link to PIF has not been established. The FAO-WHO *(22)* also established a group of category C organisms, which included *Bacillus cereus*. Low levels of *B. cereus* are commonly found in both PIF and a number of its ingredients (e.g., powdered milk), but this microorganism has not been linked to PIF-associated outbreaks *(19)*.

A number of the *Cronobacter* spp. outbreaks were linked epidemiologically and/or microbiologically to low level contamination of PIF. However, such a link could not be established for other outbreaks and sporadic cases, i.e., it was unclear whether the source of the outbreak was PIF or one of the many other food and environmental sources of *Cronobacter* spp. *(25)*.

SURVEYS OF THE BIOBURDEN OF PIF

A limited number of surveys have been conducted on of the microorganisms that occur in PIF and related products, and most have been conducted in conjunction with surveys to assess the presence of *S. enterica* or *Cronobacter* spp. We could find no specific studies that attempted to examine the impact of process deviations on PIF bioburden; however, in one unpublished study (see below) the researchers evaluated the impact of enhanced sanitation programs *(21, 23)*.

A large early survey of PIF conducted by the U.S. Food and Drug Administration included an examination of 1,574 PIF samples for TAPC, coliforms, and *S. aureus (46)*. Of the 1,329 PIF samples that contained milk, 99.5% had no detectable coliforms (<3 most probable number [MPN]/g), no sample had coliform levels >75 MPN/g, 99.7% had no detectable *S. aureus* (<3 MPN/g), and no sample had >7 MPN/g *S. aureus*. These indicator organisms were present at an even lower frequency in PIF made with a nonmilk formulation. The TAPCs ranged from <100 to 10^6 CFU/g; 98.4% of samples had $\leq 10^3$ CFU/g, 1.3% had $>10^3$ to 10^4 CFU/g, 0.2% had $>10^4$ to 10^5 CFU/g, and 0.1% had $>10^5$ CFU/g.

One of the first surveys conducted to evaluate the correlation between the presence of *Cronobacter* spp. and other *Enterobacteriaceae* in PIF included 141 samples obtained from 35 countries *(37)*. A three-tube MPN assay of 1-, 10-, and 100-g analytical units was employed to allow quantification. *Enterobacteriaceae* were isolated from 52.5% of the samples, and a variety of species were identified, including the relatively frequent isolation of *Enterobacter agglomerans, Enterobacter cloacae, E. sakazakii* (*Cronobacter* spp.), and *Klebsiella pneumoniae*. A lower isolation rate was obtained for *C. freundii, E. coli*, *Klebsiella oxytoca, Citrobacter diversus,* and *Hafnia alvei*. The highest level of *Enterobacteriaceae* contamination was 0.9 CFU/g, with most samples containing <1 CFU/100 g. No correlation between *Cronobacter* spp. and total *Enterobacteriaceae* was discernable. No *S. enterica* was among the *Enterobacteriaceae* detected. These findings are consistent with other surveys and indicate that *S. enterica* is seldom isolated from PIF and FUF *(23)*. The absence of this pathogen likely reflects the extensive control measures and rigorous routine testing employed for this group of products. The same is true of many of the dry ingredients of PIF, such as the milk powders *(23)*. One of the examples of successful *Salmonella* control programs is the dramatic reduction in *S. enterica* contamination of milk powders and their associated products that was achieved during the 1970s and 1980s *(23, 31, 36, 42)*.

Among the 482 food samples examined by Iversen and Forsythe *(31),* 82 PIF samples were evaluated by direct plating for TAPCs, *Enterobacteriaceae,* and *E. sakazakii* (*Cronobacter* spp.). A majority (78%) of samples had TAPCs of $\leq 10^2$ CFU/g, with 14, 6, and 2% having counts of $>10^2$ to 10^3, $>10^3$ to 10^4, and $>10^4$ CFU/g, respectively. With the exception of one sample with 200 CFU/g *Klebsiella ozaenae,* no *Enterobacteriaceae* were detected by direct plating. However, 9 of the 82 PIF samples were positive for *Enterobacteriaceae* after enrichment in *Enterobacteriaceae* enrichment (EE) broth. The isolates identified were *E. cloacae* (one sample), *E. sakazakii* (*Cronobacter* spp.) (two samples), *Pantoea* spp. (two samples), *K. ozaenae* (one sample), *Serratia ficaria* (one sample), *Rahnella aquatilis* (one sample), and *C. freundii* (one sample). A total of 72 samples of milk powder also were examined, and none were positive for *Enterobacteriaceae* upon direct plating. However, a variety of *Enterobacteriaceae* were isolated after enrichment in EE broth. In both products, no *S. enterica* isolates were obtained.

In a small survey of PIF and FUF, Witthuhn et al. *(53)* evaluated 15 samples of PIF and FUF from South Africa for *E. sakazakii* (*Cronobacter* spp.), TAPC, and *Enterobacteriaceae* after enrichment. Almost all total aerobic bacteria

cultures could not be quantified because of the presence of ''spreaders'' (i.e., microorganisms that spread across the surface of culture plates and interfere with the enumeration of individual colonies). *Enterobacteriaceae* were detected postenrichment in 5 of 15 samples, and 1 of the 15 was confirmed to contain *Cronobacter* spp.

A total of 50 PIF samples obtained from markets and pharmacies in Egypt were tested for *S. enterica*, total aerobic bacteria, and *B. cereus (15).* No *S. enterica* was detected. *B. cereus* was detected in seven samples, all at levels between 1×10^2 and 3×10^2 CFU/g. TAPCs ranged from 10^2 to 10^4 CFU/g; approximately half of the samples had levels of 10^2 to $\leq 10^3$ CFU/g and the other half had $>10^3$ to $\leq 10^4$ CFU/g.

Haughton et al. *(29)* evaluated 100 PIF samples from the 10 major brands consumed in Ireland. They did not detect *E. sakazakii* (*Cronobacter* spp.) or *Listeria monocytogenes* in any of the samples. TAPCs were typically in the range of 10^2 to 10^3 CFU/g, and the predominate microorganisms were *Bacillus* spp. Only two PIF samples were positive for *Enterobacteriaceae* by direct plating, which had a lower limit of detection of approximately 10^2 CFU/g. With the exception of one positive sample, it does not appear that the samples were enriched to determine lower levels of contamination.

Giammanco et al. *(26)* examined 75 samples of PIF from five major brands (48 samples) and seven minor brands (27 samples) acquired from drugstores and pharmacies in western Sicily, Italy. *Enterobacteriaceae* were detected in 23 to 62.5% (mean, 48%) of the major brands and 0 to 100% (mean, 50%) of the minor brands. There was no significant difference in the overall frequency of *Enterobacteriaceae* detection between the major and minor brands. The seemingly increased frequency of detection of *Enterobacteriaceae* in the Italian study compared with that in the other studies appears to be a function of the 333-g MPN used in this study, whereas direct plating was used by other investigators. This difference in methodology would result in an approximate 3,000-fold increase in the effective sample size examined. The impact of this difference is evident from the fact that the majority of *Enterobacteriaceae*-positive samples had <10 MPN/g; two samples had 23 MPN/g and 210 MPN/g. Only these two samples would be likely to yield positive results by direct plating, which would have given a detection rate similar to those reported by other researchers. A total of 46 *Enterobacteriaceae* isolates were detected. No *Cronobacter* spp. were confirmed, although several presumptive-positive isolates were subsequently determined to be *C. freundii* and *E. hormaechei*.

As part of an international survey of FUF and other infant foods, Chap et al. *(11)* examined 131 samples of FUF collected from six countries for TAPC and *Cronobacter* spp. They observed TAPCs of $\leq 10^2$, $>10^2$ to 10^3, $>10^3$ to 10^4, $>10^4$ to 10^5, and $>10^5$ CFU/g in 102, 18, 4, 4, and 3 samples, respectively. One of the samples with a TAPC of $>10^5$ CFU/g contained a probiotic culture. Only one sample was confirmed as having *Cronobacter* spp. (*C. sakazakii*).

Members of the *B. cereus* group occur commonly at low levels in PIF *(4, 28, 44).* Becker et al. *(4)* reviewed the reports of *B. cereus* in PIF, FUF, and weaning foods (e.g., infant cereals) from multiple countries. The frequency of detection in PIF and FUF ranged from 0 to 100%, with levels typically being $\leq 10^2$ CFU/g and seldom exceeding 10^3 CFU/g. Pasteurization significantly reduces levels of heat-sensitive spores but is insufficient to inactivate heat-resistant spores. High-temperature pasteurization is likely to induce spore germination, which would be expected to further lead to *B. cereus* reductions during drying. However, Becker et al. *(4)* noted that *B. cereus* levels for prepasteurization formulas and final products were generally similar, and these authors speculated that some of the *B. cereus* spores in PIF and FUF may be the result of postpasteurization recontamination. Similar levels of *B. cereus* were reported by Rowan and Anderson *(44).* The joint Food Standards Authority of Australia and New Zealand *(24)* conducted a formal risk assessment of *B. cereus* in PIF to evaluate potential standards. The agency concluded that low levels of *B. cereus* in PIF and FUF represent a low risk but that higher levels should be avoided. This risk assessment was used to help establish a microbiological criterion (see below) for *B. cereus* in PIF in Australia and New Zealand. In its opinion of the control of *B. cereus* in foods (including PIF and FUF), the European Food Safety Authority [EFSA] *(20)* concluded that

> For the development of new food product, or food product that support growth of *B. cereus*, either by their nature or their conditions of storage (e.g. extended shelf life), processors should ensure that numbers of *B. cereus* between 10^3 and 10^5 per g are not reached at the stage of consumption under anticipated conditions of storage and handling. This should also apply for dehydrated foods reconstituted by hot water before consumption.

One of the largest evaluations of the levels of *Enterobacteriaceae* in PIF and FUF was done in conjunction with the second FAO-WHO risk assessment on PIF safety *(23).* The expert panel reviewed a large volume of industry data on the frequency and levels of *Enterobacteriaceae, Cronobacter* spp., and *S. enterica* in commercially prepared products. The data strongly indicated that implementation of enhanced postpasteurization sanitation programs would reduce the risk associated with the two pathogens, resulting in a demonstrable reduction in the prevalence of *Enterobacteriaceae*. However, the data also indicated no direct correlation between *Enterobacteriaceae* prevalence and that of *Cronobacter* spp. or *S. enterica*. The clear implication is that *Enterobacteriaceae* would make good indicator microorganisms but would not be suitable as index microorganisms. Most of the same data appear to have been used by the EFSA *(21)* to evaluate the use of *Enterobacteriaceae* as an indicator microorganisms for PIF and FUF. Not surprisingly, the FAO-WHO and EFSA reports reached similar conclusions.

A number of surveys have also been conducted to examine the ingredients of PIF, particularly milk powders. Since the 1960s, milk powders have improved dramatically both in terms of indicator microorganisms and pathogens *(4, 23, 42).* Based on the limited number of studies reported, the quality of PIF and milk powders in developing countries

TABLE 1. *Codex Alimentarius microbiological criteria for powdered infant formula, formula for special medical purposes, and human milk fortifiers (13)*[a]

Microorganism	*n*	*c*	*m* (CFU)	*M* (CFU)	Class plan
Enterobacter sakazakii (*Cronobacter* spp.)[b]	30	0	0/10 g	NA	2
Salmonella enterica[c]	60	0	0/25 g	NA	2
Mesophilic aerobic bacteria[d]	5	2	500/g	5,000/g	3
Enterobacteriaceae[e]	10	2	0/10 g	NA	2

[a] *n,* number of samples to be tested; *c,* the number of samples that can be positive and still not exceed the criterion; *m,* the decision limit; *M,* the ''not to exceed'' limit when a three-class plan is used. NA, not applicable.
[b] Mean level detected is 1 CFU in 340 g (when the assumed standard deviation is 0.8 and the probability of detection is 95%) or 1 CFU in 100 g (when the assumed standard deviation is 0.5 and the probability of detection is 99%).
[c] Mean level detected is 1 CFU in 526 g (when the assumed standard deviation is 0.8 and the probability of detection is 95%).
[d] Equivalent to TAPC.
[e] Mean level detected is 1 CFU in 16 g (when the assumed standard deviation is 0.8 and the probability of detection is 95%) or 1 CFU in 10 g (when the assumed standard deviation is 0.5 and the probability of detection is 99%).

is equivalent to that of those products available in developed countries *(1, 15, 27, 45).*

EXISTING MICROBIOLOGICAL CRITERIA FOR PIF

With the emergence of *Cronobacter* spp. as a microorganism of concern, there has been a reconsideration of microbiological criteria for PIF and related products. This new approach is reflected in the recently revised Codex Alimentarius Commission (CAC) criteria within the *Code of Hygienic Practice for Powdered Formulae for Infants and Young Children (13).* The recommendations for PIF and related products include two criteria for pathogen testing and two process control indicators (Table 1). The recommendations for FUF are similar except that no criterion for *Cronobacter* spp. is recommended because of decreased susceptibility in older infants and young children (Table 2). The development of these criteria is an excellent example of the new paradigm in the establishment of microbiological criteria for foods; these criteria are the product of a multinational risk management decision that was informed by three formal international expert consultations that included two formal risk assessments.

Various countries have begun to include *Cronobacter* spp. in their microbiological verification testing programs, but relatively few countries have updated their microbiological criteria for PIF. Some examples of microbiological criteria for PIF and/or FUF are listed here by country (where *m* is the decision limit and *M* is the ''not to exceed'' limit when a three-class system is used).

(i) The United States proposed establishment of PIF microbiological criteria that included TAPC ($<10^4$ CFU/g); coliforms, fecal coliforms, and *E. coli* (absence based on a 0.1, 0.01, and 0.001 three-tube MPN, <3 MPN/g); *Salmonella* (absence in 60 25-g samples); *L. monocytogenes* (absence in five 25-g samples); *S. aureus* (<3.01 MPN/g); and *B. cereus* (100 CFU/g). However, the proposed good manufacturing practices regulation that included these criteria has not been finalized *(49). Cronobacter* spp. (absence based on a 333-g MPN assay) began to be routinely included in 2002.

TABLE 2. *Codex Alimentarius microbiological criteria for follow-up formulae and formulae for special medical purposes for young children (13)*[a]

Microorganism	*n*	*c*	*m* (CFU)	*M* (CFU)	Class plan
Salmonella enterica[b]	60	0	0/25 g	NA[c]	2
Mesophilic aerobic bacteria[c]	5	2	500/g	5,000/g	3
Enterobacteriaceae[d]	10	2	0/10 g	NA	2

[a] *n,* number of samples to be tested; *c,* the number of samples that can be positive and still not exceed the criterion; *m,* the decision limit; *M,* the ''not to exceed'' limit when a three-class plan is used. NA, not applicable.
[b] Mean level detected is 1 CFU in 526 g (when the assumed standard deviation is 0.8 and the probability of detection is 95%).
[c] Equivalent to TAPC.
[d] Mean level detected is 1 CFU in 16 g (when the assumed standard deviation is 0.8 and the probability of detection is 95%) or 1 CFU in 10 g (when the assumed standard deviation is 0.5 and the probability of detection is 99%).

(ii) The European Union initial microbiological criteria for PIF and FUF were established under Commission Regulation 2073/2005 *(16)* and included *S. enterica, Cronobacter* spp., and *Enterobacteriaceae* for PIF and *S. enterica* and *Enterobacteriaceae* for FUF. With this two-stage criterion, the product was initially tested for *Enterobacteriaceae*; if they were detected, additional analyses for *S. enterica* and *Cronobacter* spp. were conducted. However, the EFSA *(21)* subsequently questioned the scientific basis of the two-stage approach because of the lack of correlations between the pathogens and the indicator microorganisms and the differential in sample sizes examined for *Enterobacteriaceae* versus the pathogens. The PIF and FUF microbiological criteria were subsequently modified by Commission Regulation 1441/2007 so that each step is performed independently *(17, 18).*

(iii) The microbiological criteria for PIF in Canada currently include TAPC ($m = 10^2$ CFU/g, $M = 10^4$ CFU/g), *E. coli* ($m = 1.8$ CFU/g, $M = 10^1$ CFU/g), *Salmonella* (absence in 20 25-g samples), *S. aureus* ($m = 10^1$ CFU/g, $M = 10^2$ CFU/g), *B. cereus* ($m = 10^2$ CFU/g, $M = 10^4$ CFU/g), and *C. perfringens* ($m = 10^2$ CFU/g, $M = 10^3$ CFU/g); however, these criteria are currently under review to determine whether they should be replaced with the CAC criteria *(39).*

(iv) The initial microbiological criteria in Australia and New Zealand included *C. perfringens* ($m < 1$ CFU/g, $M = 10$ CFU/g) and *L. monocytogenes* (absence in five 25-g samples) *(2).* However, those criteria were replaced by

TAPC ($m = 10^3$ CFU/g, $M = 10^4$ CFU/g), *Salmonella* (absence in 10 25-g samples), coliforms ($m < 3$ MPN/g, $M = 10$ MPN/g), *S. aureus* ($m = 0$ CFU/g, $M = 10$ CFU/g), and *B. cereus* ($M = 10^2$ CFU/g) *(3)*.

(v) China's new microbiological criteria for PIF include TAPC ($m = 10^3$ CFU/g, $M = 10^4$ CFU/g), coliforms ($m = 10^1$ CFU/g, $M = 10^2$ CFU/g), *Cronobacter* spp. (absence in three 100-g samples), and *S. enterica* (absence in five 25-g samples) *(41)*. These standards are similar to those of the CAC except that they substitute coliforms for *Enterobacteriaceae* and the stringency of the *Salmonella* sampling plan is reduced substantially ($n = 5$ versus 60).

These few examples highlight the substantial differences among countries regarding microbiological testing currently recommended for PIF.

SCIENTIFIC SUPPORT FOR DIFFERENT INDICATOR TESTS FOR PIF

The CAC provides guidelines for establishing and applying criteria for microbiological testing programs *(12)*. Some of the general guidance on whether to establish criteria includes the following questions: (i) is there a definite need, (ii) is the criterion practical, (iii) is there epidemiological evidence that the food under consideration represents a public health risk, and (iv) is the criterion meaningful for consumer protection?

By international agreement, the establishment of microbiological criteria should be limited to either pathogens that represent a significant risk for the food product in question or indicators that measure the control of conditions that are pertinent to the pathogens identified as being of concern for the food product. Because microbiological testing represents a cost that will ultimately be passed along to the consumer, the use of redundant or ineffective testing should be avoided. The stringency of a microbiological criterion is then established by specifying the number of samples to be tested (n), the number of samples that can be positive and still not exceed the criterion (c), the size of the analytical unit (s), the decision limit (m), and when a three-class system is being used, the ''not to exceed'' limit (M) *(30)*. The frequency of the testing also influences the stringency of the testing.

An in-depth evaluation of potential microbiological criteria for specific pathogens and potential indicator microorganisms was conducted as part of both the first and second FAO-WHO expert consultations on the microbiological safety of PIF *(22, 23)*. The recommendations for pathogen testing criteria for *S. enterica* and *Cronobacter* spp. were based on clear epidemiological evidence that these microorganisms cause disease that can be directly related to their presence in the product as provided to the consumer. No criteria were recommended for *B. cereus, S. aureus, C. perfringens,* or *L. monocytogenes*, in part because they were designated as category C microorganisms *(22, 23)*. Likewise, no microbiological criteria were recommended for category B microorganisms, which are (with one exception) all *Enterobacteriaceae (22, 23)*.

***Enterobacteriaceae*.** The FAO-WHO experts *(22, 23)* evaluated a number of potential process hygiene indicator microorganisms. The experts in the second consultation concluded that *Enterobacteriaceae* are ''the ideal tool to assess the effectiveness of preventative measures and to detect the occurrence of recontamination'' *(23),* the primary risk factor associated with the microbiological safety of PIF and FUF. That conclusion was based on the fact that the primary pathogens of concern are members of the *Enterobacteriaceae* family and there was clear evidence that *Enterobacteriaceae* levels were responsive both to conditions that would increase the risk of pathogens of concern (e.g., the introduction of water into certain areas of the processing facility) and to enhanced sanitation programs that reduce the risk. Other factors that contributed to the conclusion were the simplicity and timeliness of the methods and the low levels of *Enterobacteriaceae* that occur in PIF and FUF when these products are produced under controlled conditions. The ability to establish baseline levels means that *Enterobacteriaceae* can be used for process control verification programs, which greatly enhance the effectiveness of sampling programs by allowing corrective actions to be taken before rejection criteria are exceeded. The EFSA *(19, 21)* reached similar conclusions regarding the use of *Enterobacteriaceae* as the key microbiological indicator for PIF and FUF. Manufacturers have concurred in the use of *Enterobacteriaceae* as a highly effective tool for verifying the efficacy of hygiene programs *(19, 23)*. In summary, testing for *Enterobacteriaceae* fulfills all of the requirements for an effective indicator assay as defined here.

However, the utility of *Enterobacteriaceae* is as indicator organisms only. The lack of a correlation between *Enterobacteriaceae* levels and those of *Cronobacter* spp. and *S. enterica* prevents *Enterobacteriaceae* from being used as index organisms *(21, 23)*. This lack of correlation is not surprising considering that such associations become highly uncertain at the lower limits of detection, and any association becomes even more uncertain when the stringency of the sampling plan (e.g., total amount sampled) for *Enterobacteriaceae* is substantially less than that for the pathogens. Both of these factors are major reasons why index microorganisms have found limited utility in food safety.

MAB and TAPCs. The TAPC is one of the most commonly used and simplest indicators of microbial contamination. It is typically used to assess quality attributes associated with raw ingredients or finished products for foods that can support bacterial and fungal growth. It usefulness is based on the ability of a broad range of microorganisms to grow on a standard, nonselective medium at a set temperature (typically 35 to 37°C). The assay provides a nonspecific estimate of the total bioburden in a product but, in the absence of supplemental tests, not much else. Some supplemental information can be acquired by examination of colony morphology, but the identification of microorganisms without laboratory confirmation is very uncertain. For PIF and FUF, microorganisms isolated in a TAPC assay would largely represent spore-forming bacteria

that survived thermal treatments and/or non–spore-forming bacteria that recontaminated the product after the heat treatments. TAPC assays often favor the isolation of *Enterobacteriaceae,* in part because of the media and incubation temperatures and times employed.

The CAC microbiological criteria for PIF and FUF *(13)* include an MAB criterion, as do many of the criteria that have been established by national governments. The CAC criteria for pathogens and *Enterobacteriaceae* had strong scientific underpinnings based on expert consultations and risk assessments provided by the FAO-WHO *(22, 23),* which were further augmented by the scientific advice provided by the EFSA *(19, 21)* and the U.S. Food and Drug Administration Food Advisory Committee (FAC) *(50).* The supporting scientific rationale for the CAC MAB criterion for PIF and FUF *(13)* is less clear cut because the subject was not covered to any degree in the FAO-WHO, EFSA, or FAC consultations. The rationale stated in the code of practice *(13)* was

> The proposed criteria for mesophilic aerobic bacteria are reflective of Good Manufacturing Practices and do not include microorganisms that may be intentionally added such as probiotics. Mesophilic aerobic bacteria counts provide useful indications on the hygienic status of wet processing steps. Increases beyond the recommended limits are indicative of the build-up of bacteria in equipment such as evaporators or contamination due to leaks in plate-heat exchangers (refer to Annex III).

The specifics related to the use of MAB were not covered in Annex III of the document. However, based on the above statement, the rationale would likely include either sporeformers that survived thermal processing and then contaminated the postpasteurization equipment or postpasteurization recontamination of the type that the *Enterobacteriaceae* criterion is intended to indicate. For national governments that have a TAPC hygiene criterion in addition to criteria for *B. cereus* and *Enterobacteriaceae,* the TAPC indicator test may be redundant to the other two. Either *B. cereus* counts or TAPCs could be eliminated without a substantial loss of critical information.

Other indicators. The use of alternative indicators must be considered in light of (i) the characteristics of a good microbiological indicator assay, (ii) the principles for establishing microbiological criteria, and (iii) the microbiological survey data described above. Currently, scientific support for the use of any microorganism as an index microorganism for directly predicting the levels of *S. enterica* and/or *Cronobacter* spp. is not available nor is it likely to become available in the foreseeable future. Issues related to the potential use of other indicators are listed below.

(i) *B. cereus*. Although *B. cereus* traditionally was used as a ''pathogen criterion,'' this use was not supported by the risk assessments. It could be used as an indicator of the adequacy of the heat treatments or the outgrowth of endospores that have germinated postpasteurization; however, the CAC criterion for MAB also would highlight such deviations.

(ii) Coliforms, fecal coliforms, and *E. coli*. These microorganisms are traditionally used as indicators of fecal contamination and, to a lesser extent, of general sanitation in foods that have received a substantial thermal treatment. Because fecal contamination is not a hazard of concern in PIF and FUF, the use of these bacteria would be limited to indicators of recontamination. However, the coliform and fecal coliform tests are less effective than *Enterobacteriaceae* counts. *E. coli* contamination is highly sporadic and at very low levels, making it ineffective as a process hygiene indicator. Replacement of coliform testing with *Enterobacteriaceae* testing as a process control indicator was recommended by both FAO-WHO consultations *(22, 23)* because the *Enterobacteriaceae* family includes the pathogens of concern and are found more commonly and consistently than are coliforms,

(iii) *S. aureus*. The traditional use of the *S. aureus* criterion is as a hygiene indicator for the degree of control in human handling of foods. Surveys have indicated that *S. aureus* occurs only sporadically and at very low levels, making it ineffective as a process control indicator. Modern manufacturing facilities and practices make human contact with PIF and FUF highly unlikely. The greatest risk of *S. aureus* infection would likely be a combination of recontamination in the home after the product has been opened and gross temperature abuse.

(iv) *Enterococcus* spp. and *C. perfringens*. Both organisms have been traditionally used as indicators of fecal contamination. Neither is considered a microorganism of public health concern in PIF or FUF *(23).* No data in the scientific literature have supported the use of these microorganisms as microbiological quality indicators for PIF and FUF. Although *C. perfringens* could be used as an indicator of spore-forming bacteria, the need for anaerobic techniques makes this organism a much less desirable indicator than *B. cereus* or MAB.

(v) *Listeria* spp. and male-specific (F+) coliphages. These assays are used as indicators for conditions that favor the presence of *L. monocytogenes* and enteric viruses, respectively. However, because PIF and FUF have not been identified as vehicles for the transmission of these pathogens, the use of these indicators would not meet the ''best practices'' standard for the establishment of microbiological criteria.

In conclusion, the newly recommended CAC microbiological criteria for PIF and FUF represent a significant advancement in how microbiological criteria are established and implemented. The selection of these criteria involved an evidence- and risk-based approach to the establishment of two pathogen-specific assays (for *S. enterica* and *Cronobacter* spp.) and one microbiological indicator assay (for *Enterobacteriaceae*). An additional microbiological indicator, MAB, also was included. Although useful, the MAB criterion did not have the degree of scientific support provided for the other three indicators. In light of these efforts and the principles for the establishment of effective microbiological testing programs, it is recommended that (i) industry and governments should adopt the CAC criteria for

PIF and FUF; (ii) industry and governments should reevaluate alternate microbiological criteria and eliminate them if they are redundant, ineffective, or not supported by scientific reasoning and data; and (iii) the CAC, in conjunction with the FAO-WHO, should provide a stronger scientific rationale for the MAB criteria for PIF and FUF to provide the same high level of scientific justification as exist for the other criteria in this international code of practice.

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