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A validated simple and rapid method for the simultaneous detection of both *Cronobacter* spp. and *Salmonella* spp. for infant formula quality control

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Abstract The presence of *Cronobacter* or *Salmonella* in powdered infant formulae represents a serious health risk when fed to newborn infants. The detection of these pathogens by standard food analysis methods takes around 3–6 days to provide an accurate result. In order to reduce the time of analysis to less than 24 h, a real-time PCR-based method for the simultaneous detection of both *Cronobacter* spp. and *Salmonella* spp. in powdered infant formula was designed combining two existing primer-probe sets, and included an internal amplification control to validate the negative results. Inclusivity and exclusivity of the PCR were tested with 70 strains of *Cronobacter*, 13

Marçal Soler and Olaya Ruiz-Rueda contributed equally to this work.

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strains of *Salmonella enterica*, and 75 non-*Cronobacter* non-*Salmonella* strains. The method was validated against the reference standard methods ISO/TS 22964: 2006 for *Cronobacter* and ISO 6579: 2002 for *Salmonella* using powdered infant formula samples, but also with other powdered products which could represent a health risk for infants younger than 6 months, such as follow-up formulae and hydrolyzed cereals. The detection limit of the new method was set at 1 cfu in 10 g of food for *Cronobacter* and 1 cfu in 25 g of food for *Salmonella* after 18 h enrichment in buffered peptone water, fulfilling the requirements of the European Commission.

种有快速检测婴幼儿配方奶粉中克罗诺杆菌(*Cronobacter* spp.)和沙门氏菌(*Salmonella* spp.)

摘要: 婴儿配方粉中含有克罗诺杆菌(*Cronobacter*)和沙门氏菌(*Salmonella*)会对新生儿健康带来严重的危害。这些致病菌的标准检查方法约需要3-6天的时间。为了使分析时间缩短在24 h之内,采用实时聚合酶链反应(PCR)方法并结合两种现有的引物-探针设计用来同时检测婴幼儿配方奶粉中的克罗诺杆菌(*Cronobacter*)和沙门氏菌(*Salmonella*),该方法还包括内部扩增,可以控制阴性结果的准确性。用70株克罗诺杆菌(*Cronobacter*),13株肠道沙门氏菌,75株非克罗诺杆菌和非沙门氏菌来检测聚合酶链反应(PCR)的包容性和排它性。该方法与标准参考方法ISO/TS 22964: 2006(乳和乳制品肠杆菌的检测)和ISO 6579: 2002(食品和动物饲料沙门氏菌的检测)进行对照,证明了其对婴幼儿配方奶粉中致病菌的检测是准确的。但是对其它的粉末产品,如含有谷物水解物的粉末配方产品,有可能对小于6个月的婴幼儿健康存在潜在的危害。在蛋白胨培养液中富集18 h后,该方法对于克罗诺杆菌(*Cronobacter*)检测限是1 cfu/10 g食品,对于沙门氏菌(*Salmonella*)是1 cfu/25 g食品,完全符合欧盟委员会的要求。

Keywords Powdered infant formula · *Cronobacter* spp. · *Salmonella* spp. · Real-time PCR · Validation

关键词 婴幼儿配方奶粉 · 克罗诺杆菌属 · 沙门氏菌属 · 实时聚合酶链反应 · 准确性

1 Introduction

Powdered infant formula (PIF) is a manufactured food able to support the adequate growth of infants under 6 months of age by complementing or substituting breast milk. However, PIF is not sterile, and may easily be colonized by several pathogenic microorganisms. FAO/WHO committee assessed the potential risk of a wide range of microorganisms in PIF and, although they classified some bacteria that might cause causalities in newborns, this relation was only evident for *Cronobacter* spp. and *Salmonella enterica* (FAO/WHOM 2004, 2006).

Cronobacter spp. is a new genus inside the *Enterobacteriaceae* family, which was recently proposed by Iversen et al. (2007a, 2008a). Previously described as *Enterobacter sakazakii* (Farmer et al. 1980), the genus *Cronobacter* includes six species considered as emergent opportunistic human pathogens. They can cause severe infections in infants (especially in immune-depressed, premature, low-birth, or those aged less than 28 days) with poor prognosis and high mortality rates (reviewed by Lehner and Stephan (2004), Drudy et al. (2006) and by Giovannini et al. (2008)). The international reference method ISO/TS 22964: 2006 (ISO International Organization for Standardization 2006) used for the detection of *Cronobacter* spp. is based on

culturing techniques in several media, and it takes from 3 to 6 days to have a result. Moreover, this method has problems to detect *Cronobacter* strains that are not able to grow on modified lauryl sulfate tryptose broth medium supplemented with vancomycin (mLST/vancomycin) (Iversen and Forsythe 2007; Iversen et al. 2008b) and also to detect some strains that do not present yellow pigmentation (Besse et al. 2006).

On the other hand, *Salmonella* spp. is a well-known genus within the *Enterobacteriaceae* family, responsible of millions of cases of salmonellosis every year that result in thousands of deaths all over the world. Although the risks of *Salmonella* in PIF are less studied than those of *Cronobacter*, *S. enterica* may cause diarrhea, bacteremia, and meningitis in neonates and infants. The international reference method to detect *Salmonella* spp. in food samples (ISO 6579: 2002) (ISO International Organization for Standardization 2002) is based on plate culturing and it takes from 3 to 5 days to give a result. Besides, this method often uses the inability to ferment lactose to identify *Salmonella* spp., which could lead to false negative results due to the fact that most of the strains isolated in PIF are unusual in other foodstuff and sometimes lactose fermenting (Cahill et al. 2008).

Real-time PCR-based molecular methods are relatively less laborious and time-consuming than culture-based methods, providing an outstanding option to complement or replace classical microbiology. To date, there are several real-time PCR-based methods published to detect *Cronobacter* spp. (Derzelle and Dilasser 2006; Derzelle et al. 2007; Krascenicsová et al. 2008; Malorny and Wagner 2005; Mullane et al. 2006; Seo and Brackett 2005; Soler et al. 2011) and *Salmonella* spp. (Calvó et al. 2008; Malorny et al. 2004). Very recently, a new method combining the system developed by Seo and Brackett (2005) for the detection of *Cronobacter*, and the system developed by Malorny et al. (2004) for detecting *Salmonella*, was published (Hyeon et al. 2010). It allows a fast detection of both pathogens, but a proper validation with a diverse set of contaminated PIF samples and several *Cronobacter* species was not performed.

In this work, we present the combination of the method for the detection of *Cronobacter* spp. described by Soler et al. (2011), and the method for detecting *Salmonella* spp. described by Calvó et al. (2008). We designed a real-time PCR-based method to simultaneously detect both pathogens and validated it by comparison with the reference ISO methods for the detection of *Cronobacter* spp. (ISO/TS 22964: 2006; ISO International Organization for Standardization 2006), and *Salmonella* spp. (ISO 6579: 2002; ISO International Organization for Standardization 2002). The method we propose includes an internal amplification control (IAC) to easily discard false negative results. This new real-time PCR-based method allows obtaining reliable results in less than 24 h, which is a great advantage for food industry in terms of costs and stock release compared to standard methods.

2 Materials and methods

2.1 Culture media

Brain heart infusion (BHI; Conda-Pronadisa, Madrid, Spain), brain heart agar (BHA; Conda-Pronadisa), Luria–Bertani broth (LB; Sigma-Aldrich Chemie, Buchs,

Switzerland), buffered peptone water (BPW; Biokar Diagnostics, Allonne, France), Rappaport–Vassiliadis soy broth (RVS; Conda-Pronadisa), Muller–Kauffmann tetrathionate-novobiocine broth (MKTTn; Conda-Pronadisa) supplemented with iodine (Sigma-Aldrich Chemie) and potassium iodide (Sigma-Aldrich Chemie), xylose lysine desoxycholate agar (XLD; Conda-Pronadisa), brilliant green agar (BGA; Conda-Pronadisa), modified lauryl sulfate tryptose broth medium (mLST; Conda-Pronadisa) supplemented with vancomycin (Sigma-Aldrich Chemie), modified *Cronobacter* spp. agar (HiCrome™; Sigma-Aldrich Chemie) and Bolton broth (Oxoid, Basingstoke, UK) were prepared according to manufacturer's indications and according to ISO/TS 22964: 2006 (ISO International Organization for Standardization 2006) and ISO 6579: 2002 (ISO International Organization for Standardization 2002).

2.2 DNA purification

To carry out the inclusivity–exclusivity tests and to determine linearity and efficiency of the PCR reactions, DNA was extracted from isolated colonies resuspended in BHI medium using NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) following the protocol recommended by the supplier. To extract the DNA for the validation and for the detection limit of the assays, 100 µL aliquot of sample enrichment was mixed with 200 µL of DNAREady Lysis Buffer (Microbial, Girona, Spain) and then incubated 30 min at 56 °C, 10 min at 95 °C and finally 5 min at –20 °C.

2.3 Real-time PCR probe and primers design

A previously designed and validated set of specific primers and probes targeting the gene *palF* for *Cronobacter* spp. (Soler et al. 2011) and targeting the gene *bipA* for *Salmonella* spp. (Calvó et al. 2008) were used to compose the *palF*–*bipA*-based assay (Electronic supplementary material (ESM) Fig. 1). Additionally, an artificial 65-bp DNA fragment, formerly used as an IAC in a real-time PCR for the detection of *Salmonella* spp., was included in the method with the corresponding primers and probe (Calvó et al. 2008). To discard putative interactions, all primers and probe combinations were evaluated in silico with the NetPrimer software (Premier Biosoft International, Palo Alto, USA).

Cronobacter and *Salmonella* probes were both labeled with 6-FAM at the 5'-end, whereas IAC probe was labeled with JOE. Black Hole Quencher 1 was used as a quencher in all probes.

2.4 Real-time PCR conditions

Real-time PCR was performed using an Mx3005P™ thermocycler (Stratagene, Santa Clara, USA) in 20-µL (total volume) reaction mixtures. PCR reaction was optimized testing different concentrations of primers (50, 300 and 500 nmol.L⁻¹ of each primer), and probes (50, 100, 150, 200, and 250 nmol.L⁻¹ of each probe). The optimized reaction was composed of 1× Premix Ex Taq™ (Takara, Otsu, Japan), 300 nmol.L⁻¹ *Cronobacter* forward primer, 900 nmol.L⁻¹ *Cronobacter* reverse primer, 300 nmol.L⁻¹ each *Salmonella* and IAC primers, 250 nmol.L⁻¹ *Cronobacter* probe, 100 nmol.

L^{-1} *Salmonella* and IAC probes, 10^5 copies of IAC DNA and 1 μ L template DNA. PCR conditions were as follows: 95 °C for 10 min and 40 cycles consisting of 95 °C for 15 s and 60 °C for 1 min. Fluorescence values in PCR reactions were analyzed using the Mx3005PTM QPCR Systems software (Stratagene). Positive and non-template control reactions were included in all tests.

2.5 Inclusivity and exclusivity tests

To test both the inclusivity and the exclusivity of the *palF-bipA*-based real-time PCR (Table 1), 10 ng of DNA from 70 *Cronobacter* strains, 13 *S. enterica* strains and 75 non-*Cronobacter* and non-*Salmonella* strains were used. Strains were grown overnight at 37 °C in BHA before DNA extraction. Only *Campylobacter* strains were grown at 42 °C in Bolton media. Non-*Cronobacter* and non-*Salmonella* strains were chosen according to their close phylogenetical relation to *Salmonella* spp., *Cronobacter* spp. or because of their putative capacity to grow in similar environments and conditions.

Strains were isolated at the University of Girona, the University of Barcelona, the Slovakian Food Research Institute or purchased either from the Spanish Type Culture Collection (CECT; Valencia, Spain), the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), the Belgian Coordinated Collections of Microorganisms (BCCM/LMG; Ghent, Belgium), the National Collection of Type Cultures (Colindale, UK) or the Pasteur Institute (IP; Paris, France).

2.6 Determination of the linearity and efficiency of the real-time PCR reaction

DNA for linearity and efficiency evaluation of the real-time PCR reaction was extracted as previously described and quantified using Qubit™ Quantitation Platform (Invitrogen, Carlsbad, USA). Decaplicate tenfold dilutions (ranging from 10^7 to 10^1 genomic copies per reaction) of purified DNA were used to calculate the linearity of the reaction by regression analysis plotting the obtained threshold cycle (Ct) against the logarithm of the number of genomes in the reaction. The efficiency of the real-time PCR assay was calculated using the formula: Efficiency = $[10^{(-1/\text{slope})}] - 1$. The number of equivalent genomes in DNA extractions was calculated based on the genome size of *S. enterica* subsp. *enterica* serovar *Paratyphi B* strain SPB7 (4,858 Kb) and *Cronobacter sakazakii* ATCC BAA-894 (4,368 Kb), considering that the average base pair weight is 652 g.mol⁻¹. Linearity and efficiency were independently assessed for *S. enterica* subsp. *enterica* CECT 4139, *C. sakazakii* DSM 4485, *Cronobacter mytjensii* DSM 21870, *Cronobacter dublinensis* subsp. *dublinensis*, LMG 23823, *Cronobacter malonaticus* LMG 23826, and *Cronobacter turicensis* LMG 23827.

2.7 Detection limit of the assay

To calculate the detection limit of the assay, *Cronobacter* and *Salmonella* enrichments were carried out separately, per triplicate, using two matrices consisting of PIF and hydrolyzed cereals. *Cronobacter* enrichments were prepared by homogenizing 10 g of food sample in 90 mL BPW, whereas *Salmonella* enrichments were prepared

Table 1 Results of the inclusivity–exclusivity test for the *palF-bipA*-based real-time PCR

Species	Strain number	<i>palF-bipA</i> -based PCR
<i>Cronobacter dublinensis</i>	FRI 546	+
<i>Cronobacter dublinensis</i> subsp. <i>dublinensis</i>	LMG 23823	+
<i>Cronobacter dublinensis</i> subsp. <i>lactaridi</i>	LMG 23825	+
<i>Cronobacter dublinensis</i> subsp. <i>lausannensis</i>	LMG 23824	+
<i>Cronobacter genomospecies 1</i>	NCTC 9529	+
<i>Cronobacter malonaticus</i>	FRI 450	+
<i>Cronobacter malonaticus</i>	FRI 454	+
<i>Cronobacter malonaticus</i>	FRI 536	+
<i>Cronobacter malonaticus</i>	FRI 537	+
<i>Cronobacter malonaticus</i>	FRI 557	+
<i>Cronobacter malonaticus</i>	FRI 563	+
<i>Cronobacter malonaticus</i>	FRI 569	+
<i>Cronobacter malonaticus</i>	FRI 575	+
<i>Cronobacter malonaticus</i>	LMG 23826	+
<i>Cronobacter mytjensii</i>	FRI 447	+
<i>Cronobacter mytjensii</i>	FRI 577	+
<i>Cronobacter mytjensii</i>	DSM 21870	+
<i>Cronobacter sakazakii</i>	CIP 57.33	+
<i>Cronobacter sakazakii</i>	DSM 4485	+
<i>Cronobacter sakazakii</i>	FRI 448	+
<i>Cronobacter sakazakii</i>	FRI 449	+
<i>Cronobacter sakazakii</i>	FRI 451	+
<i>Cronobacter sakazakii</i>	FRI 452	+
<i>Cronobacter sakazakii</i>	FRI 453	+
<i>Cronobacter sakazakii</i>	FRI 455	+
<i>Cronobacter sakazakii</i>	FRI 456	+
<i>Cronobacter sakazakii</i>	FRI 523	+
<i>Cronobacter sakazakii</i>	FRI 524	+
<i>Cronobacter sakazakii</i>	FRI 525	+
<i>Cronobacter sakazakii</i>	FRI 526	+
<i>Cronobacter sakazakii</i>	FRI 527	+
<i>Cronobacter sakazakii</i>	FRI 528	+
<i>Cronobacter sakazakii</i>	FRI 529	+
<i>Cronobacter sakazakii</i>	FRI 530	+
<i>Cronobacter sakazakii</i>	FRI 531	+
<i>Cronobacter sakazakii</i>	FRI 532	+
<i>Cronobacter sakazakii</i>	FRI 533	+
<i>Cronobacter sakazakii</i>	FRI 534	+
<i>Cronobacter sakazakii</i>	FRI 535	+
<i>Cronobacter sakazakii</i>	FRI 538	+
<i>Cronobacter sakazakii</i>	FRI 539	+
<i>Cronobacter sakazakii</i>	FRI 540	+

Table 1 (continued)

Species	Strain number	<i>palF-bipA</i> -based PCR
<i>Cronobacter sakazakii</i>	FRI 541	+
<i>Cronobacter sakazakii</i>	FRI 544	+
<i>Cronobacter sakazakii</i>	FRI 545	+
<i>Cronobacter sakazakii</i>	FRI 547	+
<i>Cronobacter sakazakii</i>	FRI 548	+
<i>Cronobacter sakazakii</i>	FRI 549	+
<i>Cronobacter sakazakii</i>	FRI 550	+
<i>Cronobacter sakazakii</i>	FRI 551	+
<i>Cronobacter sakazakii</i>	FRI 552	+
<i>Cronobacter sakazakii</i>	FRI 553	+
<i>Cronobacter sakazakii</i>	FRI 554	+
<i>Cronobacter sakazakii</i>	FRI 555	+
<i>Cronobacter sakazakii</i>	FRI 558	+
<i>Cronobacter sakazakii</i>	FRI 559	+
<i>Cronobacter sakazakii</i>	LMG 2758	+
<i>Cronobacter sakazakii</i>	LMG 2759	+
<i>Cronobacter sakazakii</i>	LMG 2760	+
<i>Cronobacter sakazakii</i>	LMG 2762	+
<i>Cronobacter sakazakii</i>	LMG 2763	+
<i>Cronobacter sakazakii</i>	LMG 2766	+
<i>Cronobacter sakazakii</i>	LMG 2786	+
<i>Cronobacter sakazakii</i>	LMG 2787	+
<i>Cronobacter sakazakii</i>	LMG 2788	+
<i>Cronobacter sakazakii</i>	LMG 2789	+
<i>Cronobacter sakazakii</i>	LMG 2790	+
<i>Cronobacter turicensis</i>	FRI 566	+
<i>Cronobacter turicensis</i>	FRI 576	+
<i>Cronobacter turicensis</i>	LMG 23827	+
<i>Salmonella enterica</i>	CECT 878	+
<i>Salmonella enterica</i> subsp. <i>arizonae</i>	CECT 4395	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 4000	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 409	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 4139	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 4151	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 4152	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 4153	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 4154	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 4155	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 4296	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 4371	+
<i>Salmonella enterica</i> subsp. <i>enterica</i>	CECT 4482	+
<i>Arcobacter butzleri</i>	DSM 8739	-

Table 1 (continued)

Species	Strain number	<i>palF-bipA</i> -based PCR
<i>Bacillus cereus</i>	UDG 65	–
<i>Bacillus megaterium</i>	UDG 66	–
<i>Bacillus sp.</i>	CECT 40	–
<i>Bacillus subtilis</i> subsp. <i>spizizenii</i>	CECT 482	–
<i>Campylobacter jejuni</i>	UB 12	–
<i>Campylobacter sputorum</i> subsp. <i>sputorum</i>	DSM 10535	–
<i>Citrobacter freundii</i>	CECT 401	–
<i>Enterobacter aerogenes</i>	UDG 69	–
<i>Enterobacter aerogenes</i>	CECT 684	–
<i>Enterobacter amnigenus</i>	CECT 4078	–
<i>Enterobacter asburiae</i>	DSM 17506	–
<i>Enterobacter cancerogenus</i>	DSM 17580	–
<i>Enterobacter cloacae</i>	CECT 194	–
<i>Enterobacter cowanii</i>	DSM 18146	+
<i>Enterobacter gergoviae</i>	CECT 857	–
<i>Enterobacter kobei</i>	DSM 13645	–
<i>Enterobacter ludwigii</i>	DSM 16688	–
<i>Enterobacter pyrinus</i>	DSM 12410	–
<i>Enterobacter radicincitans</i>	DSM 16656	–
<i>Enterobacter turicensis</i>	DSM 18397	–
<i>Enterococcus avium</i>	CECT 968	–
<i>Enterococcus durans</i>	CECT 411	–
<i>Enterococcus faecalis</i>	CECT 481	–
<i>Enterococcus faecalis</i>	UB FS1	–
<i>Enterococcus faecium</i>	CECT 410	–
<i>Enterococcus faecium</i>	UB FM4	–
<i>Enterococcus gallinarum</i>	CECT 970	–
<i>Enterococcus gallinarum</i>	UB GL17	–
<i>Enterococcus mundtii</i>	CECT 972	–
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	CECT 225	–
<i>Escherichia coli</i>	CECT 100	–
<i>Escherichia coli</i>	CECT 101	–
<i>Escherichia coli</i>	CECT 105	–
<i>Escherichia coli</i>	CECT 405	–
<i>Escherichia coli</i>	CECT 4084	–
<i>Escherichia coli</i>	CECT 4201	–
<i>Escherichia coli</i>	CECT 831	–
<i>Escherichia coli</i>	UDG 50	–
<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i>	CECT 143	–
<i>Kocuria rhizophila</i>	CECT 241	–
<i>Kocuria rhizophila</i>	UDG 71	–
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	CECT 984	–

Table 1 (continued)

Species	Strain number	<i>palF-bipA</i> -based PCR
<i>Listeria innocua</i>	CECT 910	–
<i>Listeria monocytogenes</i>	CECT 4031	–
<i>Micrococcus luteus</i>	CECT 245	–
<i>Mycobacterium phlei</i>	CECT 3009	–
<i>Pantoea agglomerans</i>	CECT 5392	–
<i>Proteus mirabilis</i>	CECT 170	–
<i>Pseudomonas aeruginosa</i>	CECT 532	–
<i>Pseudomonas fluorescens</i>	CECT 378	–
<i>Pseudomonas mendocina</i>	UDG 17	–
<i>Pseudomonas putida</i>	CECT 324	–
<i>Serratia liquefaciens</i>	DSM 30125	–
<i>Serratia marcescens</i>	UDG 14	–
<i>Shigella sonnei</i>	UDG 64	–
<i>Shigella</i> spp.	UDG 63	–
<i>Staphylococcus aureus</i>	UDG 20	–
<i>Staphylococcus aureus</i>	UDG 57	–
<i>Staphylococcus epidermidis</i>	CECT 231	–
<i>Streptococcus agalactiae</i>	CECT 183	–
<i>Streptococcus anginosus</i>	CECT 948	–
<i>Streptococcus equi</i> subsp. <i>equi</i>	CECT 989	–
<i>Streptococcus equinus</i>	CECT 213	–
<i>Streptococcus intermedius</i>	CECT 803	–
<i>Streptococcus mutans</i>	CECT 479	–
<i>Streptococcus oralis</i>	CECT 907	–
<i>Streptococcus pneumoniae</i>	CECT 993	–
<i>Streptococcus pyogenes</i>	CECT 985	–
<i>Streptococcus salivarius</i>	CECT 805	–
<i>Streptococcus sanguinis</i>	CECT 480	–
<i>Streptococcus sobrinus</i>	CECT 4034	–
<i>Streptococcus suis</i>	CECT 958	–
<i>Streptococcus thermophilus</i>	CECT 986	–
<i>Streptococcus uberis</i>	CECT 994	–

homogenizing 25 g of food sample in 225 mL BPW. Samples were checked for the absence of naturally occurring *Cronobacter* spp. and *Salmonella* spp. contamination and then enrichments were inoculated with 1 mL of a liquid suspension of *C. sakazakii* DSM 4485, *C. muytjensii* DSM 21870, *C. dublinensis* subsp. *dublinensis* LMG 23823, *C. malonaticus* LMG 23826, *C. turicensis* LMG 23827 or *S. enterica* subsp. *enterica* CECT 4155. Each of the six strains was tested separately and at three different levels of contamination, which were analyzed per triplicate. Inoculum concentrations in the enrichments were of 1, 5, and 10 cfu/10 g of food sample in

the case of *Cronobacter*, and 1, 5, and 10 cfu/25 g of food sample in the case of *Salmonella*. All enrichments were incubated at 37 °C for 18 h and then analyzed by the *palF-bipA*-based real-time PCR. Inocula were quantified by plate counting in BHA.

2.8 Food matrices and validation

Validation of the *palF-bipA*-based real-time PCR method was carried out with a total of 70 food enrichments consisting of 33 PIF samples for neonates (including lactose-free PIF used in intolerance to lactose cases, hypoallergenic PIF used in cases with allergy to milk proteins plus intolerance to lactose, PIF made of soy proteins used in severe cow milk allergies plus intolerance to lactose, and PIF suitable for neonates with colic and other digestive disorders like constipation, regurgitation or gastroesophageal reflux disease), 13 follow-up formulae (FUF) samples for infants older than 6 months, and 24 hydrolyzed cereals samples for infants older than 4–6 months. Food samples were from different brands available in the European market.

A total of 70 food enrichments were carried out homogenizing 25 g of food sample in 225 mL BPW, and incubating at 37 °C for 18 h. After this shared step, samples were analyzed in parallel by ISO/TS 22964: 2006, ISO 6579: 2002 (ISO International Organization for Standardization 2002, 2006) and *palF-bipA*-based real-time PCR (ESM Fig. 2). Naturally contaminated samples were actively sought out. However, to increase the number of positive samples, matrices initially free of *Cronobacter* spp. and *Salmonella* spp. were inoculated with 1 mL of a liquid suspension containing both 100 cfu of *C. sakazakii* DSM 4485 and 100 cfu of *S. enterica* subsp. *enterica* CECT 878 and then analyzed again. Inoculums were quantified by plate count in BHA. Presumptive *Cronobacter* colonies isolated from naturally contaminated food samples were confirmed by 16S rRNA sequencing with the primers for *Enterobacteriaceae* and PCR conditions described by Lane (1991).

The number of negative results correctly assigned (specificity), the percentage of positive results rightfully assigned (sensitivity) and the percentage of samples correctly assigned (efficiency) of the *palF-bipA*-based method were measured against the reference methods as specified in the ISO 16140: 2003 (ISO International Organization for Standardization 2003) for the validation of alternative methods.

3 Results

3.1 Inclusivity and exclusivity tests

All tested *Cronobacter* and *Salmonella* strains were correctly detected by the *palF-bipA*-based real-time PCR method. The rest of strains remained undetected with the exception of *Enterobacter cowanii* (Table 1), which was detected but, to our knowledge, it has been never isolated in PIF, FUF, or hydrolyzed cereals for infants.

3.2 Linearity and efficiency of the PCR reaction

In order to assess the linearity, the efficiency and the amplification limit of the *palF-bipA*-based method, genomic DNA from *C. sakazakii* DSM 4485, *C. muytjensii* DSM 21870, *C. dublinensis* subsp. *dublinensis*, LMG 23823, *C. malonaticus* LMG 23826, *C. turicensis* LMG 23827 and *S. enterica* subsp. *enterica* CECT 4139 was used for the real-time PCR reactions (Table 2). In all cases, the amplification limit was set at 10^2 genomes per reaction and the efficiency of the reaction was above 90% with a correlation value higher than 0.99.

3.3 Detection limit in matrices

Two different matrices, PIF and hydrolyzed cereals, were used to calculate the detection limit of the *palF-bipA*-based assay. Food was inoculated per triplicate at different levels with *C. sakazakii* DSM 4485, *C. muytjensii* DSM 21870, *C. dublinensis* subsp. *dublinensis* LMG 23823, *C. malonaticus* LMG 23826, *C. turicensis* LMG 23827 and *S. enterica* subsp. *enterica* CECT 4155. For all food enrichments, detection limit was set at 1 cfu/10 g of food sample for the *Cronobacter* strains assayed, whereas detection limit for *S. enterica* subsp. *enterica* was found to be 1 cfu/25 g of food sample.

3.4 Validation

The *palF-bipA*-based real-time PCR, the ISO/TS 22964: 2006 (ISO International Organization for Standardization 2006) and the ISO 6579: 2002 (ISO International Organization for Standardization 2002) were tested in parallel using 70 diverse food enrichments from PIF, FUF and hydrolyzed cereals. Of the 70 samples of PIF, FUF and cereals analyzed, only one sample of cereals presented a natural contamination of

Table 2 Linearity and efficiency results for the *palF-bipA*-based real-time PCR

	<i>palF-bipA</i> -based real-time PCR			
	Amplification limit	Reaction efficiency (%)	PCR amplification equation	Correlation value
<i>C. sakazakii</i> DSM4485	10^2	103.1	Ct=37.83–3.25 log NG	0.99
<i>C. muytjensii</i> DSM21870	10^2	92.7	Ct=37.99–3.51 log NG	0.99
<i>C. dublinensis</i> subsp. <i>dublinensis</i> LMG23823	10^2	90.3	Ct=39.76–3.58 log NG	0.99
<i>C. malonaticus</i> LMG23826	10^2	92.3	Ct=39.73–3.52 log NG	0.99
<i>C. turicensis</i> LMG23827	10^2	96.8	Ct=38.50–3.40 log NG	0.99
<i>S. enterica</i> subsp. <i>enterica</i> CECT4139	10^2	96.8	Ct=37.91–3.40 log NG	0.99

Ct cycle threshold; NG number of genomes

Cronobacter according to ISO/TS 22964: 2006 (ISO International Organization for Standardization 2006), whereas no samples contaminated with *Salmonella* were found when analyzed by the ISO 6579: 2002 (ISO International Organization for Standardization 2002) method. To increase the number of positive samples, 35 matrices were artificially inoculated with both pathogens.

Sensitivity, specificity, and efficiency of the *palF-bipA*-based method were calculated by comparing the obtained results against the reference methods (Table 3). The comparison of the *palF-bipA*-based method after BPW enrichment with the ISO/TS 22964: 2006 (ISO International Organization for Standardization 2006) showed a sensitivity of 100%, a specificity of 97.1% and an efficiency of 98.6%. The comparison of the *palF-bipA*-based method with the ISO 6579: 2002 (ISO International Organization for Standardization 2002) showed a sensitivity of 100%, a specificity of 94.4% and an efficiency of 97.1%. Fisher's exact test was conducted to compare the PCR-based method against the reference methods, thus confirming the equivalence of the data obtained with all methods ($P < 0.001$ for each comparison).

4 Discussion

In this work, a real-time PCR-based method targeting the genes *palF* and *bipA* was used for the simultaneous detection of *Cronobacter* spp. and *Salmonella* spp. in PIF samples. This method had a detection limit after BPW enrichment of 1 cfu of *Cronobacter* in 10 g of food sample, and 1 cfu of *Salmonella* in 25 g of food sample, which satisfies the requirements of the European Commission (EC-2073: 2005, EC-1441: 2007; EC Commission of the European Communities 2005, 2007) for the analysis of these pathogens in food. To date, both the *palF*-based PCR and the *bipA*-based PCR are the published methods with highest inclusivity (Calvo et al. 2008; Soler et al. 2011). Therefore, the combination of both methods may ensure the detection of rare *Cronobacter* and *Salmonella* strains found in PIF. In this study, some FUF and hydrolyzed cereals samples were included in the analysis because they are non sterile products that could represent a health risk, especially when used in infants younger than 6 months. In this sense, the FAO/WHO committee (FAO/WHO Food and Agriculture Organization/World Health Organization 2008) reported some *Cronobacter* infections in children older than 4–6 months, when children start to get fed with foods other than PIF.

Table 3 Results of the *palF-bipA*-based real-time PCR after BPW enrichment compared to the results obtained using the classical microbiological methods described in ISO/TS 22964: 2006 for *Cronobacter* spp. and ISO 6579: 2002 for *Salmonella* spp

		ISO/TS 22964: 2006			ISO 6579: 2002		
		Presence	Absence		Presence	Absence	
<i>palF-bipA</i> -based real-time PCR	Presence	35	1	36	34	2	36
	Absence	0	34	34	0	34	34
		35	35	70	34	36	70

The validation of the *palF-bipA*-based approach showed similar performance when compared to the reference method ISO/TS 22964: 2006 (ISO International Organization for Standardization 2006) for detecting *Cronobacter* spp. with just one sample in disagreement. This sample presented a weak positive signal in the PCR and a negative signal when using the ISO/TS 22964: 2006 method (ISO International Organization for Standardization 2006). This sample was also positive when analyzed using the *palF*-based PCR after BPW enrichment described by Soler et al. (2011), but was negative when analyzed using the *palF*-based PCR after BPW and mLST/vancomycin enrichment (data not shown). Enrichment in mLST/vancomycin is currently used in ISO/TS 22964: 2006 (ISO International Organization for Standardization 2006), but some *Cronobacter* strains cannot grow in this media (Iversen and Forsythe 2007; Iversen et al. 2007b, 2008b; Lehner et al. 2006). All together, these data suggest that the additional PCR-positive sample was indeed a true *Cronobacter* positive sample that was probably missed with the ISO/TS 22964: 2006 method (ISO International Organization for Standardization 2006) due to lower *Cronobacter* cell density in the BPW enriched sample and the lack of growth during the mLST/vancomycin enrichment step. These findings also suggest that real-time PCR methods, which can be coupled to a less selective enrichment procedure to detect particular bacteria in a food suspension, might be slightly more sensitive, being less affected by the growth of competitors and background microflora than are culture methods.

The validation of the *palF-bipA*-based method against the reference standard for detecting *Salmonella* spp. showed only two samples in disagreement, being positive using the PCR-based method, and negative using ISO 6579: 2002 (ISO International Organization for Standardization 2002). These two additional PCR-positive samples were not true false positive results but an indication that both samples were contaminated with *Cronobacter* instead of *Salmonella*. One of them was a naturally contaminated sample detected with the ISO/TS 22964: 2006 method (ISO International Organization for Standardization 2006). The other was the PCR-positive but ISO/TS 22964: 2006 negative sample discussed above. This discrepancy observed, that leads to lower specificity and efficiency values calculated for the real-time PCR-based approach compared to the reference ISO 6579: 2002 (ISO International Organization for Standardization 2002) method, would be avoided by using differently labeled probes for both targets.

Inclusivity and exclusivity tests of the *palF-bipA*-based PCR were satisfactory, although it also detected *Enterobacter cowanii*. Little is known about *E. cowanii*, except that most strains are isolated from clinical specimens (Inoue et al. 2000), and that some strains can be found in the internal parts of *Eucalyptus* tissue (Brady et al. 2009). Thus, *E. cowanii* should be regarded as a putative false positive result when using the *palF-bipA*-based real-time PCR aiming to simultaneously detect *Cronobacter* spp. and *Salmonella* spp. However, as far as we know, this microorganism has never been isolated in PIF, FUF or hydrolyzed cereals for infants, pointing that this cross detection should not represent a serious limitation of the method.

Very recently, Hyeon et al. (2010) presented a method that allows distinguishing between *Cronobacter* and *Salmonella*. Although the method was not completely validated according to ISO 16140: 2003 (ISO International Organization for Standardization 2003), and that distinction is only possible at genus level, this trait

could be very useful for some specific purposes. However, not all thermocyclers are able to read data using three or more different wavelength channels. Besides, the predominance of one pathogen in the food matrix could mask the presence of the other. The *palF-bipA*-based method hereby presented only needs two channels (one for *Cronobacter* and *Salmonella* and the other for the IAC), so it is not possible to distinguish the pathogens at genus level. On the contrary, using only two probes, the method could be easily used for most of the thermocyclers in the market. Moreover, for fast stock liberation in food industry, distinction of both pathogens is not necessary, which makes the *palF-bipA*-based method easily applicable in any laboratory, with simple procedures and reducing operational costs.

In conclusion, *palF-bipA*-based method hereby presented proved to satisfactorily check for the presence–absence of *Salmonella* spp. and *Cronobacter* spp. specimens in a large collection of samples of PIF, FUF and hydrolyzed cereals for infants. The detection limit of this method fulfills all the legal requirements from the European Commission (EC-2073: 2005, EC-1441: 2007; EC Commission of the European Communities 2005, 2007), and considerably reduces the time of analysis from 3 to 6 days to less than 24 h, allowing food industry a faster release of the stocks for commercialization. Moreover, the performance of this PCR-based method is not limited to the detection of *Cronobacter* strains that grow in mLST/vancomycin or the *Salmonella* strains that are not able to ferment lactose. Finally, this method has been satisfactorily validated compared to the current reference methods, thus demonstrating equivalence with ISO/TS 22964: 2006 (ISO International Organization for Standardization 2006) for detecting *Cronobacter* and ISO 6579: 2002 (ISO International Organization for Standardization 2002) for detecting *Salmonella*.

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