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COMPARATIVE STUDIES ON THE NOVEL STERILISATION OF IRISH RETAILED INFANT MILK FORMULA USING ELECTRON BEAM AND PULSED LIGHT TREATMENTS

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Abstract: This constitutes the first study to compare use of electron beam (EB) irradiation and pulsed light (PL) for novel sterilisation of infant mik formula retailed in Ireland. The microbiological quality of 60 powdered infant milk formula (PIMF), representative of two leading brands available in Ireland, were analyzed immediately after reconstitution and were shown to exhibit a total aerobic mesophilic count of $<10^4$ CFU/g (mean 3.3 x10^2 CFU/g) and a *Bacillus cereus* count of $<10^3$ CFU/g powder (mean 2.2x10^2 CFU/g). Only 7 of 60 PIMF samples of Irish PIMF were free of *Bacillus* sp; while the pathogenic bacteria *Cronobacter sakazakii* and *Listeria monocytogenes* were not detected in any samples. Application of EB irradiation at 10 kGy sterilised the aforementioned PIMF. Pulsed light was not suitable for PIMF sterilisation due to turbidity, but did successfully kill *C. sakazakii, L. monocytogenes* and test *Bacillus* species when suspended and treated in saline solution. D_{10} values [dosage required to elicit a one $\log_{10}$ reduction in microbial numbers], for EB varied over the range 1.4 to 2.5 kGy for *Bacillus* species treated. Nutritional studies of EB-treated of PIMF samples at upper 10 kGy revealed no discernible difference in appearance, moisture, protein, ash, vitamin C, total fat and total carbohydrate content compared with untreated controls. The results indicate that EB treatment of Irish retailed infant milk formula at 10 kGy destroyed *Bacillus* endospores in these products without affecting nutritional status or appearance.

Keywords: infant milk formula, electron beam, pulsed light, nutritional status, sterilisation.

I. INTRODUCTION

The global baby and infant food market is projected to grow substantially [1]. In 2015, China imported over 175,000 tonnes of infant formula, this demand is driven by a high level of consumer confidence in product produced outside China. Nielsen Company estimated global

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baby food and formule sales reached 35 billion U.S. dollars in 2015. This market surge is linked with socioeconomic development in these countries commensurate with growing number of working women and policy changes such as the one child policy in China. Ireland currently produces approximately 10% of products for the global infant formula market. However, reconstituted powdered infant formula (PIMF) are considered to be a food class of high risk due to the susceptibility of the infant population to enteric bacterial pathogens such as *Cronobacter sakazakii* and *Clostridium botulinum*, severe response to toxins and increased mortality [2, 3]. Since the first reported *Cronobacter* infection outbreak in 1958, PIMF has been identified as a source of these outbreaks resulting in many recalls of products worldwide. China recently ceased milk powder importants from New Zealand and Australia over concerns with PIMF contamination; Chinese public have grown increasingly distrusful of domestically-produced food since 2008, when ca. 300,000 people were poisoned resulting in 6 infant deaths from consumption of baby formula and milk containing melamine and poisonings from important products [4]. Despite the elevated temperatures employed in the manufacture of PIMF, there have been a number of food related illnesses where PIF has been implicated as the vehicle of infection [5 – 10].

Generally, PIMF are known to be predomintly contaminated with aerobic spore-formers of the genus *Bacillus* [11 – 13], and thus, are not sterile. The application of non-thermal food processing technologies may potentially enable elimination of bacterial endospores in PIMF rendering them sterile. Electron-beam irradiation (EBI) is a novel food decontamination technology that uses low-dose ionizing radiation in the treatment of crops or foods, to eliminate contamination [14]. Pulsed light (PL) has also been applied for water and disinfection of food surfaces owing to its' ability to delivery high-intensity, broad-spectrum biocidal lighr [15]. Irradation has been approved by the United States Food and Drug Administration (USFDA), United Nations Food and Agriculture Organisation (FAO) and World Health Organisation for food decontamination [14]. However, from a perspective of food safety, it must be proven that these food decontamination technologies not only eliminate microorganisms but also exert no adverse effects on the nutrition or residual radiation in the food, before it is applied in food processing industry. The aim of this study was to assess the efficacy of EBI and PL as novel non-thermal methods for sterilising infant milk formula produced in Ireland.

II. METHODOLOGY

Microbiological quality of reconstituted PIMF samples

The survey was composed of 60 powdered infant milk formula (PIMF), representative of two leading brands available in the Republic of Ireland, were prepared as per methods described previously [13], with slight modifications. Briefly, 25 g of PIMF was reconstituted in 225 ml sterile distilled water at a water temperature 45ºC by shaking 25 times through an excursion of 30 cm. Following a 30 min cooling period triplicate aliquots of 1 ml was removed for total aerobic mesophilic counts (TAMCs) and for other bacteriological enumerations as outlined below The reconstituted PIF were then incubated at either 25ºC and 35ºC for periods up to and including 24 h. Total aerobic mesophilic bacteria in PIF were enumerated and identified at 0, 8, 14 and 24 h sample time intervals by pour and spread plating, decimal diluted samples in buffered peptone water (BPW) and plating on Tryptone Soya Agar supplemented with 0.6% Yeast Extract (TSYEA; Cruinn Diagnostics, Ireland) followed by aerobic incubation of plates at 37ºC for 48 h. This procedure was repeated in duplicate for three separate samples analyzed from the same PIF. *Bacillus* spp present in these PIMF samples were identified as per methods described previously [11]. The identity of each Bacillus isolate was confirmed using the API 50 CHB and API 20 E galleries (bioMérieux Ltd.). PIMF were also examined for the presence of *Cronobacter sakazakii* using conventional isolation method as according to Haughton et al. [13]. Staphyococcal isolates were identified based on Gram reaction, ability to produce catalase and oxidase and coagulase activity, with subsequent use of API Staph (BioMerieux) to confirm identity. Efficacy of bacterial detection was evaluated in PIMF using positive control strains comprising *C. sakazakii* (NCTC 8155), *L. monocytogenes* NCTC 11994, *Salmonella Enteritidis* NCTC 3046, *Escherichia coli* ATCC 29522, *Staphylococcus aureus* ATCC 29523 and *Bacillus cereus* NCTC 11145. PIMF samples were coded based on microbiogical quality for subsequent electron beam irradiation and pulsed light treatments.

Electron-beam irradiation (EBI)

A Mevex high energy electron beam irradiator (combined 10/12 MeV unit, 30 kW) was used to irradiate 25 g samples of IMF at doses of 1.5, 5, 10 and 25 kGy. A range of representative test microorganisms were also artificially-seeded in 25 ml reconstituted IMF (and phosphate buffered saline) samples where starting populations was ca. 10^7 vegetative cells or endospores per millilitre in order to determine the D_{10} values for each test test organism post EB

treatment (average dose to reduce microbial numbers by 1 $\log_{10}$ order expressed in kGy). All samples were irradiated at room temperature in the presence of air at STERIS Advanced Sterilisation Technology plant (Tullamore, Ireland).

Pulsed light (PL) treatments

Powdered and reconstituted IMF samples (25 g/ml) were treated using pulsed light (PL) at UV doses of 4.32, 10.8, 12.98 μJ/cm^2 as described in Garvey et al. [16], with modifications. A pulsed power source (PUV-1, Samtech Ltd., Glasgow) was used to power a low-pressure (60kPa) xenon-filled flashlamp (Heraeus Noblelight XAP type NL4006 series constructed from a clear UV transparent quartz tube) that produced a high-intensity diverging beam of polychromatic pulsed light. This delivery system produced ultra-short duration pulses of an intense broadband emission that is rich in the UV-C germicidal wavelength. PL is produced by storing electricity in a capacitor over relatively long times and releasing it as a short duration pulse using sophisticated pulse compression techniques. The light source has an automatic frequency control function which allows it to operate at 1 pulse per second (pps); this setting was used throughout the study.

Nutritional and cytotoxicity status of irradiated PIMF samples

Powdered IMF samples subjected to EBI were independently analysied for nutritional content changes at ALS Food and Pharmaceutical testing laboratory (Chatteris, Cambridgeshire, England); these tests comprised moisture, energy, protein, ash, vitamin C (as ascorbic acid), total fat and total carbohydrate content. Changes in physical appearance and texture was also examined. Untreated and EBI-treated samples were encoded post treatment and transported to this independent, accredited food testing facility under refrigeration conditions. Cytotoxicity tests of EBI-treated IMF was determined using MTT (3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay following exposure to human HepG2 liver cells as described previously [17]. IMF was reconstituted at 13% w/v (as recommended by manufacturer), 1.0% w/v and 0.1% w/v and exposed to HepG2 cells; release the purple formazan product was measured spectrophotometrically at 490nm after addition of 100ul decolouriser (DMSO).

Statistical analysis

The Fisher's exact test was used to compare the bacteriological quality (where total aerobic counts for 60 PIMF were pooled and compared as a unit under these conditions), differences in treatment technologies (PB, PL) were examined using two-way analysis of variance

(ANOVA, Minitab version 13.1, Minitab Ltd, State College, Pennyylvania, USA). All significant differences were reported at the 95% level of confidence ($p<0.05$).

III. RESULTS

All 60 PIMF examined immediately after reconstitution were of satisfactory bacteriological quality as per new guidelines recommended by Codex Alimentarius Commission code of hygienic practice for powdered formulae for infants and young children [18]. All PIMF had a total aerobic mesophilic counts less than the 1 x 10^4 bacteria per gram (Table 1). While the latter Codex does not recommend an action limit for *B. cereus*, all PIMF were shown to have a count less than 10^3 CFU/g power for this pathogen that is below the safety limit of 10^4 CFU/g recommended by the Association of Dietetic Food Industries of the European Community (IDAEC). The PIMF examined, which were representative of two leading brands currently available in the Republic of Ireland were of similar bacteriological quality ($p<0.05$). The largest concentration of organisms present in any PIMF product was $4.9x10^3$ CFU/g (consisting solely of *B. subtilis*), while the mean total aerobic plate count for all 60 infant foods analyzed was $3.5x10^2$ CFU/g (Table 1). *Bacillus cereus* was present in 38 of 60 PIMF examined (mean *B. cereus* $2.2x10^2$ CFU/g), where the largest number of *B. cereus* recovered from any formulation was 4.7 $x10^2$ CFU/g (Table 1). The microbial flora of PIMF before reconstitution consisted mainly of aerobic spore-formers of the genus *Bacillus*.

Bacillus species exhibited varying D_{10} values (average dose in kGy that caused a reduction in microbial numbers by 1 $\log_{10}$ order) to EB irradiation over the range 0.5 to 2.8 kGy where endospores were shown to be more resistant compared to vegetative cell forms ($p<0.05$) (Table 2). Initial EBI range finding studies was conducted at 25 kGy that was too high a dose for D_{10} determinations. Treatment of *Bacillus* endospores in reconstituted IMF provided protection compared with similar EB irradiated samples in PBS ($p<0.05$). *Cronobacter sakazakii* and *Listeria monocytogenes* exhibited similar EBI sensititiy to that of vegetative cells of *Bacillus* species tested. There was no significant difference between retailed IMF1 and IMF2 products in terms of efficacy of EBI treatments ($p<0.05$). EB irradiation at 10 kGy sterilised retailed powdered IMF of *Bacillus* endospores present.

Retailed PIMF treated with EB at 10 kGy were not altered in moisture (2.8 g versus 2.8 g treated), ash (2.2 g versus 2.3 g treated), energy (497 kcal versus 497 treated), vitamin C (71.5 mg versus 69.4 mg treated), total fats (23.4 g versus 23.5 treated), total carbohydrates (62.1 versus 61.8 treated) or physical compared with untreated controls. There was no discernible

cytotoxicity difference in untreated and EB irradiated infant formula at 10 kGy when challenged with human HepG2 liver cells post treatments (Fig. 1).

Pulsed light (PL) produced ca. 6 $\log_{10}$ CFU/ml reduction in *Bacillus* endospores when treated in phosphate buffered saline at varying UV doses over range 4.32 to 12.98 μJ/cm^2, but was significantly less effective at destroying these endospores when PL-treated in reconstituted IMF at same UV dose (p<0.05) (Table 3]. Similar to EBI, *Bacillus* in endospore state were more tolerant of UV irradiance compared to vegative cells. The inability of PL to reduce bacterial load in reconstituted IMF is attributed to poor transmissivenss and penetration ability of this system, which is turbid (Fig. 2).

IV. DISCUSSION

The pathogenic bacterium *Cronobacter sakazakii* was not isolated from 60 reconstituted PIMF in this study, findings agree with the work of O'Brien and coworkers [19] where these researchers did not detect *C. sakazakii* in 468 samples representative of 31 different milk and soya-based infant formula products commercially available in European countries. Maximum allowed levels for *B. cereus* in dried infant feeds have also been set in several countries, ranging from an acceptable threshold of 10^3 CFU of *B. cereus* per gramme (e.g., Finland) to 10^4 in Sweden [12]. Veda and co-workers also showed that the most frequently isolated organisms from dried baby formulae in Japan were *B. licheniformis* and *B. subtilis*, while other *Bacillus* recovered included *B. cereus*, *B. pumilus*, *B. megaterium, B. circulans* and *B. coagulans* [cited in 11]. Thus, isolation of aerobic endospores is commonplace for IMF products globally.

Pulsed light is a promising next generation approach for contact surface and water decontamination [15], but is not applicable as sterilisation technology for powdered foods due to lack of ability to penetrate this product. However, irradiation of retailed powdered IMF at dose of 10 kGy can effectively destroy *Bacillus* endospores. There is a significant gap in scientific research on the application of irradiation to powdered foods [14]. Hong et al. [20] reported on the use of EB for inactivation of *C. sakazakii*, *B. cereus* and *Salmonella typhimurium* in powdered weaning food where D_{10} values were 4.83, 1.22 and 0.98 kGy, respectfully. This reported D_{10} value is *C. sakazakii* is approximately 9 times greater to that reported for a different strain of this foodborne pathogen in this study, which highlights the importance of investigating a broad range of species to EBI in powdered infant formula. Sarrias and coworkers [21] revealed that EB can effectively be deployed for rice sterilisation

at 7.5 kGy as it destroys *Bacillus* and *Clostrida* endospores and fungi. Helfinstine et al. [22] previously demonstrated that EB irradiation can effectively destroy *Bacillus atrophaeus* endospores in envelopes under a biodefence study, where D_{10} value of 1.53 kGy was determined for this species that is similar to D_{10} values reported in this study for *Bacillus* species. Other researchers compared the tolerance of multiple bacterial strains to EBI and found variability in tolerances depending on species type such as *Listeria monocytogens* (D10 = 1.09 kGy), *Listeria innocua* (D_{10} – 0.38 kGy), *Salmonella enterica* Poona (D_{10} = 0.38 kGy), *E. coli* O157:H7 (D_{10} = 0.36) and *Salmonella* LT2 (D_{10} = 0.12 kGy) [23]. Lung et al. [14] reported that EBI does not significantly affect nutritional or appearance properties of various treated foods. However, D_{10} value determinations for EB treatments of foods should also consider other varying factors including food composition, water activity, storage temperature and presence of oxygen [24]. While there is gap in current knowledge as to how EB destroys *Bacillus* endospores at molecular level, studies focusing on vegative cell inactivation by Shehata et al. [25] revealed DNA is the principal target govering loss of viability post EB irradiation. Electron beam irradiation has many advantages such as relatively short processing time, in-line process, highly effective, involves few variables, low heat, short release time, low equipment cost and controlled dose [14]. Future studies in this area should consider broadening microbial targets to encompass anaerobic *Clostridia* endospores that may occasionally contaminate powdered infant formula; use of human duodenal Caco2 cells instead of HepG2 cells for cytotoxicity studies; and expand nutritional compostion studies pre and post EBI.

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Table 1: Variation in aerobic plate counts for named bacterial species in 60 Powdered IMF (30 IMF1 & 30 IMF2) enumerated immediately after reconstitution at a water temperature of 45ºC

Bacterial spp.	**Number of PIF where named bacterial spp had total aerobic count (log_{10} CFU/g) in the below ranges**								**Total Aerobic Count (log_{10} CFU/g) Mean[a]**	
	< 2		**2**		**2 - 3**		**3 - 4**			
	IMF1	**IMF2**	**IMF1**	**IMF2**	**IMF1**	**IMF2**	**IMF1**	**IMF2**	**IMF1**	**IMF2**
B. licheniformis	8	5	11	7	11	6	3	1	2.1±1.1	2.2±1.4
B. subtilis	6	7	6	7	5	5	0	1	2.2±0.6	2.4±0.5
B. pumilus	1	1	3	3	2	2	0	1	2.0±1.0	2.4±0.5
B. cereus	7	7	7	8	6	5	1	1	2.4±0.4	2.5±0.3
B. mycoides	2	1	2	1	1	2	0	0	2.0±0.7	1.9±0.5
B. megaterium	4	2	3	3	2	2	0	0	2.4±0.7	2.3±0.4
B. sphaericus	1	3	3	4	1	1	0	0	1.9±0.3	2.0±0.6
B. circulans	2	0	2	0	1	0	0	0	2.0±0.4	2.0±0.4
Staphylococcus spp.	3	2	2	1	3	3	0	0	1.8±0.5	1.8±0.3
Listeria spp.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cronobacter spp.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Mean ± SD refer to variation in total aerobic counts (log_{10} CFU/g) among PIMF in each brand prepared at water temperature of 45ºC (n=30). No significant difference in microbial numbers was observed between brands of PIMF. ND, not detected.

Table 2: D_{10} value determinations for EB irradiated (kGy) test bacteria in vegetative and endospore state suspended in reconstituted infant milk formula (RIMF) or phosphate buffered saline (PBS)

Test Bacterium	Morphological State	D_{10} value (kGy)*		
		PBS	IMF1	IMF2
C. sakazakii NCTC 8115	Vegetative cell	0.488±0.04	0.492±0.07	0.531±0.06
L. monocytogenes NCTC 11994	Vegetative cell	0.554±0.04	0.705±0.04	0.562±0.05
B. cereus NCTC 11145	Vegetative cell	0.771±0.03	0.632±0.04	0.618±0.05
B. cereus NCTC 11145	Endospore	1.588±0.04	1.674±0.11	1.666±0.14
B. cereus NR3	Vegetative cell	0.513±0.04	0.816±0.08	0.926±0.12
B. cereus NR3	Endospore	1.658±0.05	1.236±0.24	1.257±0.17
B. cereus EMF2	Vegetative cell	0.504±0.06	0.554±0.08	0.611±0.07
B. cereus EMF2	Endospore	1.018±0.08	1.294±0.06	1.898±0.31
B. cereus NCTC 11143	Vegetative cell	0.501±0.03	0.536±0.07	0.528±0.06
B. cereus NCTC 11143	Endospore	1.506±0.10	2.155±0.28	2.08±0.24
B. cereus NB40	Vegetative cell	0.678±0.11	0.690±0.09	0.674±0.13
B. cereus NB40	Endospore	1.526±0.04	1.722±0.19	2.51±0.05
B. cereus NB51	Vegetative cell	0.561±0.05	0.554±0.08	0.601±0.12
B. cereus NB51	Endospore	1.466±0.06	1.309±0.16	2.281±0.15
B. coagulans NB11	Vegetative cell	0.466±0.05	0.528±0.08	0.518±0.05
B. coagulans NB11	Endospore	1.521±0.04	1.239±0.14	1.428±0.06
B. licheniformis NCTC 10341	Vegetative cell	0.540±0.11	0.590±0.06	0.612±0.08
B. licheniformis NCTC 10341	Endospore	1.408±0.10	1.622±0.24	1.630±0.13
B. subtilis NCTC 3610	Vegetative cell	0.582±0.06	0.602±0.04	0.598±0.06
B. subtilis NCTC 3610	Endospore	1.455±0.03	1.612±0.11	1.550±0.8

*The D_{10} value indicates the average dose (kGy) it takes to reduce the bacterial load by 1-Log

Table 3: Log_{10} reduction in microbial numbers (CFU/ml) of test bacteria treated in phosphate buffered saline (PBS) at different UV doses ($\mu J/cm^2$) produced by pulsed light

Test Bacterium	**Morphological State (Vegetative cell or Endospore)**	**Log_{10} reduction (CFU/ml) of test bacteria at varying UV doses ($\mu J/cm^2$)**		
		4.32 $\mu J/cm^2$	**10.8 $\mu J/cm^2$**	**12.96 $\mu J/cm^2$**
C. sakazakii NCTC 8115*	Vegetative cell	5.6±0.3	7.2±0.6	7.4±0.3
L. monocytogenes NCTC 11994*	Vegetative cell	5.3±0.6	6.9±0.5	7.3±0.3
B. cereus NCTC 11145	Vegetative cell	4.2±0.2	6.0±0.5	6.5±0.4
B. cereus NCTC 11145	Endospore	2.5±0.5	5.6±0.4	5.9±0.3
B. cereus NR3	Vegetative cell	5.4 ±0.4	6.4 ±0.3	6.9±0.2
B. cereus NR3	Endospore	3.4 ±0.1	5.3±0.5	5.6±0.5
B. cereus EMF2	Vegetative cell	5.1±0.3	6.2±0.4	6.7±0.4
B. cereus EMF2	Endospore	2.3±0.5	5.0±0.4	5.3±0.3
B. cereus NCTC 11143	Vegetative cell	5.0±0.5	5.8±0.3	6.7±0.2
B. cereus NCTC 11143	Endospore	3.2±0.4	5.1±0.6	5.5±0.2
B. cereus NB40	Vegetative cell	4.9±0.1	5.5±0.5	6.4±0.4
B. cereus NB40	Endospore	2.2±0.3	5.1±0.3	5.5±0.2
B. cereus NB51	Vegetative cell	5.3±0.2	6.2±0.2	7.0±0.1
B. cereus NB51	Endospore	3.2±0.5	5.4±0.5	5.8±0.3
B. coagulans NB11	Vegetative cell	4.3±0.4	6.0±0.4	6.4±0.4
B. coagulans NB11	Endospore	2.4±0.4	5.4±0.5	5.7±0.3
B. licheniformis NCTC 10341	Vegetative cell	4.6±0.5	6.6±0.4	6.9±0.2
B. licheniformis NCTC 10341	Endospore	3.9±0.1	5.7±0.3	6.1±0.5
B. subtilis NCTC 3610	Vegetative cell	4.3±0.5	6.1±0.6	6.7±0.2
B. subtilis NCTC 3610	Endospore	3.3±0.3	5.3±0.5	5.6±0.3
*Non-endospore forming pathogenic bacteria				

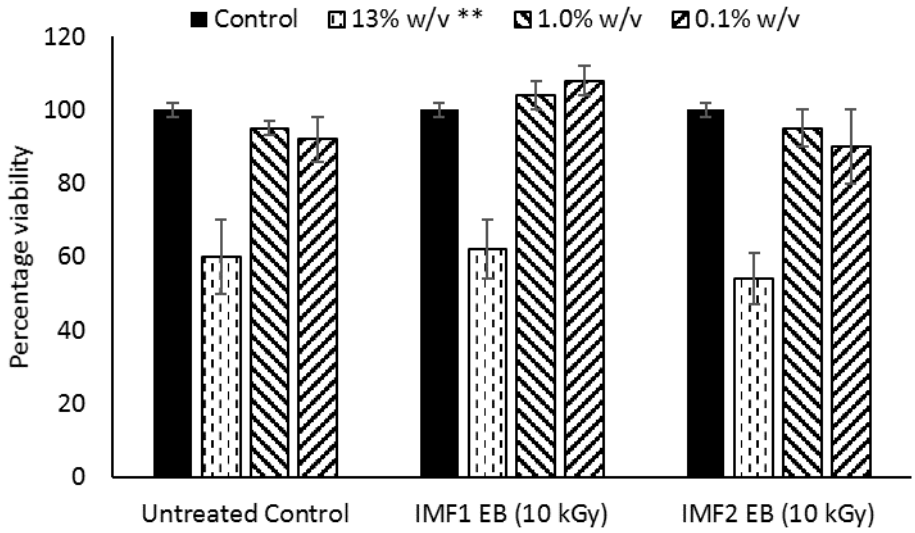

Figure 1 Percentage viability of human HepG2 liver cells exposed to varying dilutions of IMF1 and IMF2 pre and post EB irradiation at 10 kGy. **intimates recommended preparation mix for reconstituting powdered IMF products

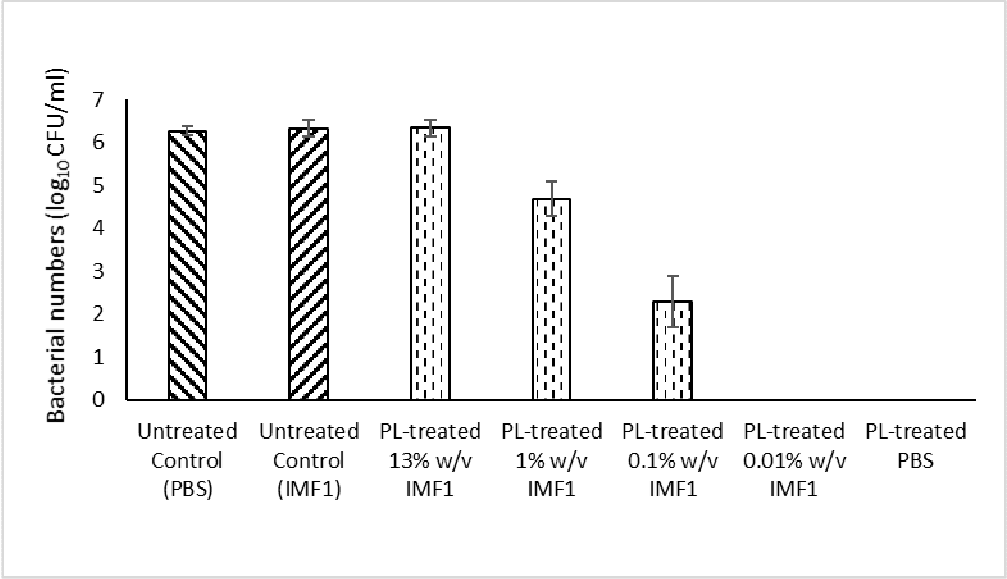

Figure 2 Log_{10} order reduction, expressed in CFU/ml of *Bacillus cereus* (NB3), for serially diluted infan t formula and PBS treated with a pulsed light UV dose of 12.98 μJ/cm^2.