Austin Journal of Nutrition and Food Sciences

Open Access

Austin Publishing Group

Special Article – Food Safety

Preliminary Quantitative Risk Assessment of *Cronobacter* Contamination in Powdered Infant Formula and Its Implications for Infant Health in Beijing, China

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Received: May 09, 2019; **Accepted:** July 03, 2019; **Published:** July 10, 2019

Abstract

Objection *Cronobacter*, formerly known as *Enterobacter sakazakii*, is defined as a class A pathogen in powdered infant formula and considered as a 'severe risk for restricted populations, representing threat of death or chronic sequelae of long duration'.

Methods A total of 125 PIF samples were collected from supermarkets, farmers markets, and online shopping, and four domestically manufactured samples were positive for *Cronobacter* with contamination levels ranging from 0.36 MPN/100g to 15 MPN/100g. Based on the contamination data, a probability assessment was carried out.

Results The average risk of *Cronobacter* infection in each meal with water at preparation temperatures of 0°C, 50°C, 60°C, 70°C and 80°C was 3.67×10^{-4}, 3.10×10^{-3}, 1.37×10^{-3}, 1×10^{-8} and 1×10^{-8}. The food microbiological risk classification model matrix shows that the risk score of *Cronobacter* infection in infants through above PIF is 16 (comparatively high), 20 (comparatively high), 20 (comparatively high), 4 (low), and 4 (low).

Conclusion Further study will be focused on families that have infants as target population, and provide a series of risk communication to campaign interventions to reduce exposure to *Cronobacter* in PIF.

Keywords: *Cronobacter*; Risk assessment; Infant; Powdered infant formula

Abbreviations

PIF: Powdered Infant Formula; MPN: Most Probable Number; CFU: Colony Forming Units; ICMSF: International Commission on Microbiological Specification for Foods; WHO: World Health Organization; DNA: Deoxyribonucleic acid; RNA: Ribonucleic Acid; FoodNet: The US Foodborne Diseases Active Surveillance Network; FAO: Food and Agriculture Organization; BPW: Buffered Peptone Water; DFI: Brilliance Enterobacter sakazakii agar; mLST: Modified lauryl sulfate tryptose vancomycin medium; TSA: Tryptone Soya Agar; PCR: Polymerase Chain Reaction; RASFF: European Rapid Alert System for Food and Feed

Introduction

Cronobacter is a Gram-negative, facultative anaerobic rod, and non-spore forming foodborne pathogen of the family *Enterobacteriaceae*. It was identified as a 'yellow-pigmented *Enterobacter cloacae*' in 1961 and renamed as *Enterobacter sakazakii* as a new species of *Enterobacter* based on the results of DNA-DNA hybridization by Farmer et al in 1980 [1,2]. In 2008, it was re-classified as a new genus *Cronobacter*, according to the result of 16S rRNA sequence analysis, DNA hybridization, ribotyping, and fluorescence labeling - amplified fragment length polymorphism fingerprinting. Cronobacter includes seven species: *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, *C. dublinensis*, *C. condiment*, and *C. universalis* [3-5], they had been classified as pathogenic organisms to a restricted population by the International Commission on Microbiological Specification for Foods (ICMSF) in 2002 [6]. According to the World Health Organization (WHO), all seven species of *Cronobacter* could cause life-threatening infections, including necrotizing enterocolitis, bacteremia, neonatal meningitis, and serious neurological sequelae in neonates and immune-compromised infants, and the mortality rate could be as high as 40 -80% [7].

Although *Cronobacter* could cause disease in all ages, a higher incidence was found at a specific age. The US Foodborne Diseases Active Surveillance Network (Food Net) conducted a survey in 2002 and the result showed that the annual invasive incidence of *Cronobacter* in infants (< 12 months) was 1/100,000, and in low birth weight neonates was 8.7/100,000 [8]. Another investigation carried out in 19 neonatal intensive care units reported a similar conclusion; the annual invasive incidence of *Cronobacter* in low birth weight infants (< 1500 g) was 9.4/ 100,000 [6]. Powdered Infant Formula (PIF) is the main source of *Cronobacter* contamination. In 2004, FAO/WHO Expert Consultations categorized *Cronobacter* and *Salmonella* as category a pathogenic bacteria associated with PIF which had 'Clear evidence of causality' with disease due to its harmful effect on infants [6]. Therefore, both China and the European Union stipulate that *Cronobacter* must not be detected in PIF intended for consumption by infants ages between 0 and 6 months.

Previous studies show that the *Cronobacter* contamination rate reported by Muytjens in 1988 when analyzing 141 PIF samples

Citation: Gan X, Zhu JH, Fanning S, Butler F, Li FQ. Preliminary Quantitative Risk Assessment of *Cronobacter* Contamination in Powdered Infant Formula and Its Implications for Infant Health in Beijing, China. Austin J Nutri Food Sci. 2019; 7(4): 1121.

collected from 35 countries was 14.2% [9], and Iversen reported a 2.4% positive rate of surveillance in 2004 [10]. The positive rate of Fu yang'Da touwawa' inferior PIF incident in 2004 in China was 12.6% [11]. Lu carried out a surveillance of 88 powdered infant foods in 2008 in Beijing, in his study, one was contaminated by *Cronobacter* in 82 milk based powdered formula, accounted for 1.22%, and 4 of 6 goat based powdered formula were *Cronobacter* positive, accounted for 66.67% [12]. In recent years, with the increase of market supervision and self-management of manufacturers, the contamination rate of *Cronobacter* in PIF was reduced accordingly. However, due to the particularity of the susceptible population and the severity of the damage caused by infection, the objective of this study is to quantitatively monitor the contamination level of *Cronobacter* in retail PIF in Beijing, develop an exposure model of contamination in PIF and its implication on infant health from retail PIF, and to explore the potential intervention measures to reduce the risk of *Cronobacter* to infants during PIF consumption.

Materials and Methods

Hazard characterization

Due to the limited data, the dosage effect assessment method of *Cronobacter* was used to assume that 1 CFU *Cronobacter* was contained in each PIF sample when preparing, and estimated pathogenic rate of PIF. The exponential dose-response model is as follows: $P_{ill} = 1 - \exp -^{rd_c}$ [13] where r is the coefficient of the ingested dosage effect exponential model and d_c is the content of *Cronobacter* in ingested products. The content was calculated from the initial concentration of 1 CFU of *Cronobacter* in each dry product (storage and pre-growth). According to the preparation, storage, and feeding conditions to adjust the initial value of 1 CFU/sample and estimate the ingested dosage. The exponential model is relatively simple, parameters are also easy to read, and there is no data available for model selection. The exponential model is a threshold free model; it is linear at low dose, and only one parameter r in the model, which could explain as the pathogenic rate of single microbial cell.

There is no data that can be used to estimate the coefficient of dosage effect; the risk assessment model contained different infant groups and the coefficient could be different. Thus 6 options are provided as base line values ranging from 1×10^{-5} to 1×10^{-10}. Once selected, the multiplier of the base line value can be calculated, and then by adjusting the r-value, a representation of the relative susceptibility of each infant group can be made. As a system default, since all the susceptibility models can not cover all infant groups, use 1 as a baseline value of r to multiply the multiplier, these options could help to compare numerical values of different assumptions directly, and the relative susceptibility of each infant group by comparing different relative risk. This means the risk is not reflected in absolute values, but the relative value compared by a preset benchmark or operation.

To simulate this model, we need to sample the initial concentration of *Cronobacter* in PIF. This model includes changes of all time periods from PIF preparation to consumption, and to predict the changing of *Cronobacter* contamination as time goes on. The preparation includes preparation time, temperature, and the rate of heating and cooling. At each time step, calculate the temperature of PIF, estimate lag time and growth rate, and calculate any increase or decrease in contamination. In addition, calculate pathogenicity of every million infants and transform it into corresponding risk assessment. Compare the risk assessment between infant groups and preparation phases to confirm which phase could relieve the expectation and realization risk of infant group.

Occurrence of Cronobacter in retail PIF samples from Beijing

A total of 125 PIF samples were randomly collected from supermarkets, farm product markets, and E-shops in Beijing from 2014 to 2015. Among these PIF samples, 20 were imported and 105 were domestically produced; 95 were cow milk-based products and 30 were goat milk-based products. All samples were quantitatively assessed for *Cronobacter* based on the method of the China National Food Safety Standard GB4789.40-2010 which was officially issued by the Chinese government and with some modifications in selective enrichment temperature [14]. The detection limit is 0.3 MPN/100 g. Triplicates of 100 g, 10 g, and 1 g test portions were mixed with 900 ml, 90 ml, and 9 ml buffered peptone water (BPW) (Beijing Land Bridge Technology Ltd., Beijing, China), respectively, and incubated at 37 oC for 18±2 h. Inoculated 1 ml cultured broth into 10 ml mLST/ Vancomycin medium (Beijing Land Bridge Technology Ltd., Beijing, China) tubes and incubated at 42°C for 24±2 h. A loopful of cultured medium was streaked on Brilliance *Enterobacter sakazakii* Agar (DFI) (Oxoid, England), and incubated at 42°C for 24±2 h. Five suspected colonies (all suspected colonies will be pick up if the number of colonies less than 5) were selected and streaked onto tryptone soya agar (TSA) (Beijing Land Bridge Technology Ltd., Beijing, China) and incubated at 25°C for 44 h-48 h. Colonies on TSA plates were identified by both VITEK 2 Compact and polymerase chain reaction (PCR) of ITS (ITS-F 5'-GGGTTGTCTGCGAAAGCGAA-3', ITS-R 5'-GTCTTCGTGCTGCGAGTTTG-3') based on the method published by Liu [15]. In total, there were four domestically manufactured PIF samples that were found to be positive for *Cronobacter* and three were contaminated at the level of 0.36 MPN/100 g and one was at the level of 15 MPN/100 g. All these *Cronobacter* positive samples were used for risk assessment.

Infant formula preparation at home

There was no report on the result of systematic surveillance of PIF preparing and feeding habits in China. In order to make the results could be compared internationally, the hypothesis and parameters put forward by WHO/FAO are as follows employed: (a) The contamination level of *Cronobacter* in PIF will not change before preparation with water; (b) To consider the worst situation of the effect of *Cronobacter* in PIF on the health of infants, it is assumed that there are four stages between PIF preparation and consumption, including preparation, cooling, reheating and feeding. The corresponding time for each stage is 0.2, 2, 0.5 and 7 hours, respectively, thereby giving 9.7 hours in total. The temperature for prepared PIF at each stage (the variation in temperature in parenthesis) is 20°C (-0.72), 30°C (-0.72), 30°C (11), and 30°C (0.72), respectively; (c) The maximum contamination level of *Cronobacter* in PIF is 8.3 log CFU/g; (d) The model takes 0.1 hour as interval to analyze the change of both temperature and time on growth of *Cronobacter*.

Exposure assessment

Development of scenario and modeling for Cronobacter

Table 1: Using parameter to assess the increase and decrease of *Cronobacter* in PIF by risk assessment model.

Parameter	Content	Value	Reference
T_{Oopt}	Optimum growth temperature	37°C	[16]
T_{min}	Parameter of growth model	2.5°C	FAO/WHO 'request data', [17]
T_{max}	Parameter of growth model	49°C	FAO/WHO 'request data', [17]
b_G	Parameter of growth model	0.053	FAO/WHO 'request data', [17]
c_L	Parameter of growth model	0.139	FAO/WHO 'request data', [17]
b_L	Parameter of lagging model	4.309	FAO/WHO 'request data', [17]
c_L	Parameter of lagging model	-1.141	FAO/WHO 'request data', [17]
z	z value of *Cronobacter*	5.6°C	[18]
D_{ref}	D value of reference temperature	0.16	[18]
T_{ref}	Reference temperature for determining D value	58°C	N/A
t_i	Length of time	0.02 hour	N/A

assessment: Assuming that preparation, cooling, heating and feeding are all conducted coherently, and to confirm the temperature and time curve of PIF to predict the increase and/or decrease of *Cronobacter* contamination. The process is divided into discontinuous time steps (e.g. 0.1 hour), and we predict the temperature at each time interval to confirm the increase or decrease in *Cronobacter* contamination. If the temperature is lower than the maximum temperature for *Cronobacter* growth, the bacteria will grow. If the temperature is higher than the maximum permitted temperature, the bacterium will die and therefore one can predict that the concentration of *Cronobacter* will decrease. A model describing the change in temperature along with the time in a complex process was given in the following equation.

$$T_i = T_f + (T_{i-1} + T_f)\exp^{(-\hat{a}_{ti})} \quad [13]$$

For each step, T_f is the ambient temperature and T_{i-1} is the temperature of last time interval. The temperature measure at the end of i-1 phase is the starting temperature of next stage. In addition, β is the cooling rate in association with specific stage, e.g. in the time of preparation stage (0.1 hour).

Cronobacter growth prediction: For bacterium increase prediction, the specific growth rate k could lead to the change of *Cronobacter* concentration. Square root model was used in the range of biodynamic temperature, the value of k (ln/hour) could calculate from:

$$\sqrt{k} = b_G - (T - T_{min})\{1 - \exp(C_G(T - T_{max}))\}$$

Where T is the temperature of PIF, T_{min} and T_{max} are the minimum and the maximum temperature, b_G and c_G are the parameter obtained from model fitting. All these data are used to estimate T_{min}, T_{max}, b_G, and c_G. The estimation of model parameters is shown in Table 1.

Calculate the dependence of logarithmic model on temperature by $\log_{10}(\lambda) = C_L \ln(T) + b_L$, is (λ Lag phase in hour, b_L and c_L are parameter obtained from model). The values of b_L and c_L are 4.309 and -1.141, respectively, the temperature of PIF at each time interval is certain, estimate the Lag phase through the percentage of Lag phase: $\%\lambda = \sum_i \frac{t_i}{\ddot{e}} \times 100$, the amplification percentage is up to 100%, Gi is calculated by $\frac{k_i}{\ln(10)} t_i$. .

The prediction of bacterial cell counts decrease happened in any time interval can be obtained from the following equation:

$$R_i = \frac{t_i}{10^{D_{ref}(T_{ref} - T/z_{ES})}} \quad i=0,0.01,0.02...d \quad [13]$$

In this equation, T is temperature of prepared PIF in specific time point, at a specific preparation stage of cooling, T_{ref} and D_{ref} are reference temperature of *Cronobacter* in relation to D and represent the incremental time, t_i represents the incremental time from preparation to the end, and z is the concentration of *Cronobacter*. In consideration of the effects of preparation, holding and feeding, the change of contamination level C in PIF is calculated by the following equation: $C = \sum_i G_i + R_i$.

This model described parameterization of characteristic data of the *Cronobacter* curve. This curve is the most thermally stable in the literature, and the model parameters based on curve characteristics are the worst situation in thermally stable. At this phase, data from all aspects of the model were not enough to explicitly include other curves. The z-value in the curve is 5.6, it is consistent with other studies, for example, z is 5.82 in the study of Nazarowec-White & Faber [19], and 5.7 in two curves in Iversen and Forsythe's study [10]. It is reported that the D value in the curve at 58°C is 0.16 hour (9.6 minutes), other curves at 58°C in the literatures range from 1.3 min to 3.8 min, or 0.4 min to 0.6 min.

Considering the change of *Cronobacter* cell concentration in PIF preparation and feeding steps, suppose the PIF was contaminated before preparation and feeding, and the contamination level is 1 CFU. NEs is defined as the average daily PIF consumption by every 1 million infants, and *Cronobacter* consumed by the infants and its implications on their health are calculated as follows: $N_{Es} = \Theta.C_m.P_{ill}$. In this equation, Θ is the concentration of *Cronobacter* cell in dry PIF products in the preparation stage (should consider the decrease of the bacterium during sampling and storage), P_{ill} is pathogenicity probability of *Cronobacter* in PIF at the preparation stage when the initial contamination level is 1 CFU (growth and activation during preparation and holding), C_m is the daily average intake of PIF by every 1 million infants. The PIF consumption level depends on infant weight. Totally, seven groups of infants were selected as targeted populations for risk assessment according to birth weight or age. For each group in the model, translate daily recommended PIF consumption into weight related ml/Kg form. The model forecasts the number of cases of infants in seven groups in a series of preparation

Table 2: Risk grading matrix of food borne pathogens.

Probability level (score)	Hazard level (score)				
	Extremely low (1)	**low (2)**	**Medium** (3)	**High** (4)	**Serious** (5)
≥1×10^{-3} (5)	Low (5)	Medium (10)	comparatively high (15)	high (20)	high (25)
1×10^{-4}~<1×10^{-3} (4)	Low (4)	Medium (8)	comparatively high (12)	comparatively high (16)	high (20)
1×10^{-5}~<1×10^{-4} (3)	Extremely low (3)	Low (6)	Medium (9)	Higher (12)	comparatively high (15)
1×10^{-6}~<1×10^{-5} (2)	Extremely low (2)	Low (4)	Low (6)	Medium (8)	Medium (10)
<1×10^{-6} (1)	Extremely low (1)	Extremely low (2)	Extremely low (3)	Low (4)	Low (5)

Table 3: Prediction of the level of *Cronobacter* consumed every meal and health risk for infants at different water temperatures for PIF preparation obtained by modeling.

Water temperature (°C) for PIF preparation	Estimated mean of Cronobacter consumed per meal (median,MPN)	Estimated average risk of Cronobacter infection per meal (median)	Estimated average cases of Cronobacter infection per million meals(median)
40	0.073(0.000)	3.67×10^{-4} (1×10^{-8})	366.7(0.01)
50	0.101(0.000)	3.10×10^{-3} (1×10^{-8})	3102.0(0.01)
60	0.091(0.000)	1.37×10^{-3} (1×10^{-8})	1371.5(0.01)
70	0.000(0.000)	1×10^{-8} (1×10^{-8})	0.01 (0.01)
80	0.000(0.000)	1×10^{-8} (1×10^{-8})	0.01 (0.01)

situation of PIF. The weight of infant and daily intake of PIF used in present study is given in Table 2. The @risk software was used for risk assessment, iteration 10,000 times, simulate at different preparation temperatures, the quantitative contamination level of *Cronobacter* in retail PIF in Beijing according to this study, the recommended intake of every meal of different PIF and the exposure assessment model proposed by WHO/FAO.

Results

Cronobacter contamination in PIF

This study analyzed a total of 125 PIF samples collected from supermarkets, farm product markets, and E-shop between 2014 and 2015 in China. Approximately 3.2% (4/125) of all samples were contaminated by *Cronobacter*. All *Cronobacter* positive samples were isolated from domestic milk-based products, which accounted for 3.8% (4/105) of all domestic PIF and 4.2% (4/95) of all milk-based PIF. In four *Cronobacter* positive samples, three were contaminated at the level of 0.36 MPN/100 g, and one was at the level of 15 MPN/100 g.

Cronobacter exposure via PIF and its implications on infant health

According to the food microbiological risk classification model matrix reported by Zhu [20], the hazard of *Cronobacter* through contaminated PIF prepared at different water temperatures was classified as five levels on the basis of its severity and scored as 5, 4, 3, 2, and 1 representing very serious harm to general population, very serious harm to particular population, very harmful, moderately hazardous and other. The risk of target population infected by *Cronobacter* through PIF would be negligible with if the probability of *Cronobacter* infection via each meal were less than 1×10^{-6}. Change in an order of magnitude is defined as significant risk in onset of the disease. The probability of disease at the level less than1×10^{-6}, between 1×10^{-6} and less than 1×10^{-5} (includes 1×10^{-6} but excludes 1×10^{-5}, the following are the same), between 1×10^{-5} and less than 1×10^{-4}, between 1×10^{-4}, and less than1×10^{-3} and ≥1×10^{-3}, respectively, were correspondingly scored as 1, 2, 3, 4, and 5. The score of the risk= score of matrix column variable (hazard level) × score of row variable (initiation potential), and 1~3, 4~5, 6~10, 12~16, and 20~25 are defined as very low, low, medium, high, and high risk, respectively (Table 2). Based on the approach mentioned above, risk assessment results expressed by the mean and median of infants' populations infected by *Cronobacter* through the PIF monitored by our study is shown in Table 3. The results illustrated that when these PIF are prepared with water at temperatures of 40°C, 50°C, 60°C, 70°C, and 80°C, the average intake of *Cronobacter* exposed by infants through PIF are 0.073, 0.101, 0.091, 0, and 0 MPN per meal, and the risk of *Cronobacter* infection are 3.67×10^{-4}, 3.10×10^{-3}, 1.37×10^{-3}, 1×10^{-8}, and 1×10^{-8}, respectively. The risk assessment scores of infants infected by PIF contaminated with *Cronobacter* obtained in our study at different temperatures of preparing water are 16 (comparatively high), 20 (comparatively high), 20 (comparatively high), 4 (low), and 4 (low), respectively. According to the definition of the risk matrix, the probability scores of the disease are 4, 5, 5, 1, and 1. ICMSF considered that the infection caused by *Cronobacter* endangers life or causes serious chronic sequelae, or the duration of disease is much longer and belongs to very serious pathogen to the specific population if the score of hazard is 4. It is estimated that 366.7, 3,102, 1371.5, 0.01, and 0.01 cases of *Cronobacter* infection could occur in every million meals.

Discussion

Cronobacter show ability for heating, drying and osmotic tolerance, and to form biofilm, which could lead a long-term survival in environment. The *Cronobacter* positive PIF could contaminate utensils for infants such as feeding bottle and spoon, and incorrect operation may also cause cross-contamination in kitchen. Once *Cronobacter* persist on PIF preparation facilities, it will seriously affect the health of infants. Persistent strain has a significant impact on PIF manufacturers, it could store on the surface of processing facilities and survive in raw material, and cause a long-term contamination in factory and end products. For the sake of reducing the risk from this bacterium, the WHO issued guidelines on the safe preparation,

storage, and handling PIF, including preparation of PIF in care settings [21] and preparation at home [22]. The WHO recommended preparing PIF at 70°C, and in our study, the average intake of *Cronobacter* exposed by infants through *Cronobacter* positive PIF we detected is 0 MPN per meal at 70°C, the risk of *Cronobacter* infection per meal is 1×10^{-8} and 0.01 case of *Cronobacter* infection could occur in every million meals. Therefore, it is suggested that the preparation of PIF should be carried out by follow the WHO recommendation.

PIF is not a sterilized product, and *Cronobacter* infection has been linked to consumption of contaminated PIF according to epidemiological research. As a foodborne pathogen, *Cronobacter* could cause life-threatening and invasive diseases, including meningitis, necrotizing enterocolitis and septicemia in neonates and infants. From 2003 to 2009, 544 cases of *Cronobacter* infection were identified in Mexico [23], and between 2002 and 2008 European Rapid Alert System for Food and Feed (RASFF) had reported 11 lots of PIF were contaminated by *Cronobacter* and lead to infection [1]. Studies showed that a trace contamination such as 3 CFU/100g in PIF could cause infant *Cronobacter* infection, and in Pagotoo's research after a 10 h incubating in reconstituted PIF at room temperature, 1 CFU/ml of initial *Cronobacter* contamination could grow up to 10^7 [24]. Therefore, many countries take measures to control the contamination of *Cronobacter* in PIF. The Chinese government pay more attention to reduce the infection risk of infants via PIF and the regulation of *Cronobacter* for PIF intended for consumption by infants at the age of less than 6 months is not allowed to be detected.

There are many uncertainties in the risk assessment carried out in the present study due to a variety of assumptions, models and parameters, and only cover the stage from retail to feeding. The exposure assessment model set up in this study includes microbial growth model, microbial lag phase model and microbial inactivation model. For the data used in these models, some were based on models in Combase database, and some were based on models in references. It is necessary to further improve the model parameters by joint laboratory data. This quantitative risk assessment of the impact of *Cronobacter* contamination in PIF to infant health in China only involves two stages, from retail to consumption. Although the contamination of *Cronobacter* in PIF from retail to feeding stage is most closely related to the population health, however, few public health interventions could be provided to reduce the onset risk, and it is not possible to made effective intervention to the processing link which mainly cause PIF contamination.

Conclusion

In conclusion, *Cronobacter* was identified in 4 out of 125 PIF samples in China between 2014 and 2015 with a contamination level from 0.36 MPN/100 g to 15 MPN/100 g. The quantitative risk assessment showed the threat of consuming these contaminated PIF to infants. Further research on nationwide survey on contamination of *Cronobacter* in PIF at the retail level as well as along the PIF production chain, and making a systematic risk assessment of infant exposure to *Cronobacter* via PIF is needed. In order to put these interventions into practice, it is necessary to provide a series risk communication targeting infants' family and to publicize interventions to reduce the exposure to *Cronobcter* via PIF.

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