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FOOD MICROBIOLOGY AND SAFETY

Microbiological and mycotoxicological analyses of processed cereal-based complementary foods for infants and young children from the German market

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Abstract

This study investigated several food safety criteria in 38 different commercial products of processed cereal-based foods (PCF) from the German market. Microbiological assessment, followed by 16S RNA gene sequencing of suspect colonies, included aerobic mesophilic bacteria, moulds, *Enterobacteriaceae*, *Cronobacter* spp., and presumptive *Bacillus cereus*. Mycotoxin analyses were performed by enzyme immunoassays for deoxynivalenol (DON), zearalenone (ZEN), T-2/HT-2 toxins (T-2/HT-2; oat containing products only), ergot alkaloids (EA), and alternariol (AOH). No violative result above existing European Union regulations or international guidelines was obtained. Most samples had very low aerobic mesophilic cell counts ($<2.0 \times 10^1$ CFU/g), the maximum was 9.6×10^2 CFU/g. A few samples contained low numbers of opportunistic pathogens, most notably *Cronobacter sakazakii*, *Acinetobacter* spp., *Pantoea* spp., and enterotoxigenic *Bacillus wiedmannii*. Levels of mycotoxin contamination were very low, well below European Union maximum limits. DON was found in 10 samples, at levels of 9–35 μg/kg. T-2/HT-2 were found in all 15 oat-based products (1–8 μg/kg). All samples were negative for ZEN and EA. A high number ($n = 25$) of samples yielded weakly positive results for the nonregulated AOH (0.4–2 μg/kg), but just three samples exceeded a level of 1 μg/kg. No relationship between cereal composition and analytical findings for microbiological parameters and mycotoxins could be found. As long as PCF meals are freshly prepared and consumed immediately after preparation, the risk from sporadically occurring opportunistic bacteria appears to be minimal.

KEYWORDS
enzyme immunoassay, infant food, mycotoxins, pathogenic bacteria, processed cereal-based foods

1 | INTRODUCTION

Complementary feeding is defined as the period when complementary foods are given together with either breast milk or formula or both. Typically beginning at an age between 4 and 6 months, complementary foods are gradually introduced into the diet of infants, and commercial cereal-based foods play a major role during this period (EFSA, 2019). Processed cereal-based foods (PCF) are defined as containing simple or mixed cereals, which are ready to eat after reconstitution with milk or water (EU, 2013). PCFs are not sterile products, and microbial contamination of cereal grains may occur at various stages of production (Los et al., 2018). With a total revenue in 2020 of, for example, 6.4 billion US$ (United States) and 0.6 billion US$ (Germany), "baby food" (total) constitutes just a minor (≈0.5%) part of the total revenues of food industry in industrialized countries (Pham, 2021). No market data for PCF could be obtained from published literature. However, infant food in general is a highly sensitive market segment, which has to deal with the vulnerability of babies, and with the attitude of parents with regard to food quality and safety. Therefore, the number of companies producing and distributing such products is quite small, just a few different brands dominate the German market, and different brands may in fact have be produced in the same factory.

In contrast to commercial milk-based powdered infant formulae, pathogenic bacteria in PCFs are not specifically regulated within the European Union, except of the more general commitments arising from the "precautionary principle." For example, notorious pathogenic/toxigenic bacteria occurring in cereals are *Cronobacter* spp. and *Bacillus cereus* (Akineden et al., 2015; Kim et al., 2011; Lou et al., 2019), both regulated in infant formulae, but not in PCF, by the European Commission (EC, 2005).

Concerning mycotoxins, a substantial reduction may be achieved during cereal processing for some compounds, but not all mycotoxins can be completely removed (Karlovsky et al., 2016). Maximum levels (ML) have been set for some mycotoxins in PCFs, including aflatoxins (0.1 µg/kg), ochratoxin A (OTA, 0.5 µg/kg), deoxynivalenol (DON, 200 µg/kg), zearalenone (ZEN, 20 µg/kg), fumonisins (200 µg/kg), and, most recently, ergot alkaloids (EA, sum of 12 congeners, 20 µg/kg) (EC, 2006b). With regard to T-2/HT-2 toxins (T-2/HT-2), an "indicative level" of 15 µg/kg, from which onwards further investigations should be performed, was published for PCFs for infants and young children (EC, 2013). No specific regulations have been issued for alternariol (AOH), or other *Alternaria* toxins, in any country of the world. However, risk and exposure assessments published by the European Food Safety Authority (EFSA), underlined the need to further consider AOH as a relevant mycotoxin, and established a preliminary "threshold of toxicological concern" (TTC) of 2.5 ng/kg body weight (b.w.) and day (EFSA, 2011, 2016).

Although the importance of commercial PCF for infant nutrition certainly is high in industrialized countries, the number of published studies dealing with the microbiological and mycotoxicological quality of such products is surprisingly small, and just one study dealt with both (Assunção et al., 2021).

Surveys on PCF studying microbiological criteria with a broader scope are rare (Kim et al., 2011), but *Cronobacter* spp. (Kim et al., 2011; Ziver et al., 2020) and *B. cereus* (Assunção et al., 2021) have been detected with some frequency. The number of published mycotoxin surveys in PCF is also limited, and even fewer recent data are available (Mallmann et al., 2020). Most studies so far included DON, which was found in up to 50% of PCF samples worldwide (Assunção et al., 2018; Braun et al., 2020; Herrera et al., 2019; Juan et al., 2014; Oueslati et al., 2018; Pereira et al., 2015). The average DON contamination was at 10–100 µg/kg, but in some cases exceeded 200 µg/kg. Depending on the type of cereal, other mycotoxins have been reported with varying frequency over the last 20 years. T-2/HT-2 were predominantly found in oat-containing PCF (Al-Taher et al., 2017; Assunção et al., 2018; Braun et al., 2020; Gotthardt et al., 2019; Juan et al., 2014; Oueslati et al., 2018; Pereira et al., 2015). For AOH and EA, just a small number of published studies is available (Lombaert et al., 2003; Mulder et al., 2015; Reinhard et al., 2008; Scott et al., 2012). Gotthardt et al. (2019) analyzed 25 samples of PCF from the German market for AOH and five other *Alternaria* toxins, the most abundant compound was tenuazonic acid, while low levels (0.76–7.17 µg/kg) of AOH were detected in just six samples.

Considering the scarcity of analytical data, the aim of this study was to elucidate the safety of PCF from the German market with regard to microbiological criteria and mycotoxin contamination, including both known opportunistic pathogens and notorious mycotoxins, plus some less well-studied parameters (Gram-negative bacteria, presumptive *B. cereus*, EA, AOH).

2 | MATERIALS AND METHODS

2.1 | Sample materials and sampling

Between November 2019 and March 2020, 38 dairy-free samples of PCF, recommended age of consumption "after the fourth month" up to "after the sixth month," were purchased from local retail stores, drugstores, and specialized organic retail stores in the area of Giessen, Germany. According to product labels, the samples originated from 12 companies (trade names), most were labeled as

German produce, but 10 samples were from four other countries within the European Union. The samples were collected with the aim that all dairy-free PCF brands from all producers were included in the study. The majority of PCF products is marketed countrywide, so the collection of samples can be attributed as typical for dairy-free PCFs as available from the German market. Nowadays, all brands of PCFs available in Germany are labeled as of "organic produce." Single-grain products ($n = 25$) contained millet, spelt, oats, wheat, barley, rye, rice, or maize. Multigrain products ($n = 13$) contained at least two, up to seven, of these cereals. All products were supplemented with thiamine (vitamin B_1) according to Commission directive 2006/125/EC (EC, 2006a). All products were available in dry form, either as powder or as granulate material, ready for consumption after the addition of 10 volumes of milk, water, or milk diluted with water (1 + 1, by volume). All samples, which contained one to two portions (150–250 g each) of PCF in sealed sachets (paper or aluminum-coated foil), encased in a cardboard package, were stored dry at room temperature until testing. Before opening, each sachet was manually shaken to enhance homogeneity of the material thoroughly homogenized by swiveling the package. The test portion for microbiological analyses was taken out of this sachet under sterile conditions. For microbiological analysis, approximately 50 g were taken from each package under antiseptic conditions, and transferred to a sterile sealable glass bottle. The remaining material was filled into sealable plastic cans and used for mycotoxin analysis.

2.2 | Mycotoxin analysis

2.2.1 | Chemicals, reagents, and buffers

Ergometrine (synonym: ergonovine), ergocristine, DON, and ZEN were purchased from Sigma-Aldrich (Taufkirchen, Germany). AOH was obtained from Cayman Chemicals (Hamburg, Germany). T-2 toxin was from Biopure (Tulln, Austria). Ergotamine D-tartrate (Fluka®) and methanol (Riedel-de Haën®) were from Honeywell (Charlotte, NC, USA). Acetonitrile and ethyl acetate were purchased from Merck (Darmstadt, Germany). All reagents used were at least of analytical grade. For the analysis of EA, a toxin standard mixture of ergometrine, ergocristine, and ergotamine D-tartrate in a ratio of 1:10:14 (w/w/w; Gross et al., 2018) with a total alkaloid concentration of 25 µg/ml was prepared. All mycotoxin stock solutions (except T-2/HT-2) were checked for purity and correct concentration by UV spectroscopy (Shimadzu, Duisburg, Germany), using published spectra and absorption coefficients (Cole et al., 2003). Phosphate-buffered saline (PBS) contained 6.79 g of NaCl, 1.47 g of Na_2HPO_4, and 0.43 g of KH_2PO_4 in 1 L of a. dest. (0.01 mol/L, pH 7.3). For AOH extraction, PBS consisted of 6.79 g of NaCl, 2.94 g of Na_2HPO_4, and 0.86 g of KH_2PO_4 in 1 L of a. dest. (pH 7.2). Sodium bicarbonate buffer contained 1.59 g of Na_2CO_3 and 2.93 g of $NaHCO_3$ in 1 L of a. dest. (0.05 mol/L, pH 9.6). Washing solution contained 8.5 g NaCl and 0.25 ml of Tween in 1 L of a. dest. For the enzyme substrate/chromogen solution, H_2O_2-citrate buffer (8.3 g citric acid, 49 ml KOH (1 mol/L), 72 µl 30% aqueous H_2O_2, 160 ml a. dest; pH 3.9) and TMB solution (50.4 mg 3,3′,5,5′-TMB, 1 ml acetone, 9 ml methanol) were used. The enzyme substrate/chromogen solution was prepared according to Ackermann et al. (2011). Shortly before use, 0.5 ml of TMB was added to 10 ml of H_2O_2-citrate buffer.

2.2.2 | Sample extract preparation

In general, sample extract preparation for all mycotoxins was performed as described by Liesener et al. (2010), except for AOH which was extracted by a modification of the procedure described by Ackermann et al. (2011). All procedures had to be adopted to meet the requirements of the highly absorptive PCF matrix. All primary extractions were done with 5 g of sample material mixed with 50 ml of solvent in a beaker and magnetic stirring at full speed (400 rpm) for 30 min. For some toxins, extraction was followed by a centrifugation step (10 min at 3000 × *g* for DON and ZEN; 4 min at 11,000 × *g* for EA). Filtration was done using paper filters.

T-2/HT-2 were extracted with 50 ml of water containing 70% methanol. A 2 ml portion of the filtered extract was mixed with 2 ml of distilled water and extracted twice by liquid–liquid partitioning with each 3 ml portions of ethyl acetate. The two ethyl acetate phases were removed after centrifugation (3000 × *g*, 15 min) and combined, then the solvent was evaporated at 50°C in a rotary evaporator. The residue was dissolved with 0.2 ml of methanol and 1.8 ml of PBS (pH 7.3), using ultra-sonication in a water bath for 2 min. This extract was mixed with 1 ml of n-heptane on a wrist-action shaker. The phases were separated by centrifugation (3000 × *g*, 15 min). The lower aqueous phase was collected and analyzed by enzyme immunoassay (EIA) either directly (sample dilution factor: 10), or after dilution with PBS (pH 7.3) containing 10% methanol.

DON was extracted essentially by the same procedure as for T-2/HT-2, except that extraction solvent was PBS containing 10% methanol, and that no extract defatting step with n-hexane was necessary. After rotary evaporation, the residue was dissolved with 1 ml of PBS (pH 7.3). This extract was analyzed either directly (sample dilution factor: 5), or after dilution with PBS (pH 7.3).

ZEN was extracted with distilled water containing 84% acetonitrile. After centrifugation and filtration, 100 µl of the filtrate was mixed with 1.58 ml PBS (pH 7.3) to obtain a 5% acetonitrile/PBS solution for EIA analysis (sample dilution factor: 168). Further dilutions were made with 5% acetonitrile/PBS.

EAs were extracted with PBS pH 6.0 containing 60% acetonitrile and stirred for 30 min on a magnetic stirrer (400 rpm). After the solid particles had settled, 2 ml of this extract were transferred into a 2 ml Eppendorf vial and centrifuged (11,000 × *g*, 4 min, 20°C). For EIA analysis, 100 µl of this extract was mixed with 0.9 ml PBS, pH 6.0 (sample dilution factor: 100). Further dilutions were made with 5% acetonitrile/PBS pH 6.0.

AOH was extracted with PBS containing 70% methanol, the apparent pH value was adjusted to approximately 7.0 with 3 mol/L NaOH. The extract was filtrated and 2 ml was mixed with 2 ml of distilled water. The mixture was extracted twice by liquid–liquid partitioning with each 3 ml of ethyl acetate followed by centrifugation. The two organic phases were pooled, the solvent evaporated, and the residue dissolved with 1 ml of PBS for 2 min by ultrasonication in a water bath. The resulting extract was analyzed either directly (sample dilution factor: 5), or after dilution with PBS.

Recovery was tested by adding toxin standard solutions to dry sample materials before extraction. Three different toxin levels, and three different sample materials per level, were tested for each mycotoxin. The lowest concentration used for fortification of samples was 3–10 times the calculated LOD of each method. T-2/HT-2 were analyzed only in products, which were labeled as containing oats ($n = 15$). Further, the test samples, which gave the highest toxin results in the enzyme immunoassays (EIAs) for DON, T-2/HT-2, and AOH, were each spiked with the respective toxin at the same level, to double the natural amount of toxin, and then were reanalyzed.

2.2.3 | EIA analyses

All competitive EIAs were performed as microtiter plate (MaxiSorp, Nunc, Roskilde, Denmark) assays as described previously for DON, ZEN, T-2/HT-2, EA, and AOH (Gross et al., 2018). Some important EIA test characteristics are compiled in Table 1. Four replicate wells were tested for each standard concentration and for each dilution of sample extract solution. The resulting EIA absorbance values at 450 nm were measured with a model Sunrise microplate reader (Tecan, Crailsheim, Germany) and evaluated by Magellan EIA calculation software (Tecan). All values were then standardized as percent relative absorbance of the blank (B_0), by dividing the mean absorbance values of standard or sample solutions through the absorbance of B_0, multiplying by 100 ($B/B_0 \times 100$). The standard curve detection limits were set as cut-off values of 70% –80% as described for each EIA in the original studies. Recovery was routinely checked, before the start and during the analyses of each series, by addition of 50–100 µl of toxin standard solution at appropriate concentrations to dry samples before extraction, and allowing for the solvent to evaporate. Each three samples with different matrix composition were tested for each test system. The calculated mean detection limit of each test system for PCF was derived from the mean standard curve detection limit, multiplied by the minimum sample dilution factor, without considering recovery. All toxin concentrations for naturally contaminated samples were reported without correction for the analytical recovery.

2.3 | Microbiological analyses

Microbiological parameters included nonspecific hygiene indicators (aerobic mesophilic plate count (APC), *Enterobacteriaceae*, moulds) and specific pathogenic (*Cronobacter* spp.) or enterotoxin-producing enterotoxigenic bacteria (presumptive *B. cereus*).

All sample materials were prepared for analysis according to ISO 6887-4:2017. For all tests, a 10 g test portion was mixed with 190 ml of 0.1% sterile peptone water (Oxoid, Wernigerode, Germany) in a sterile plastic bag and homogenized in a paddle blender (Stomacher). Two further decimal dilutions (2×10^{-2}; 2×10^{-3}) were prepared with sterile peptone water (Oxoid). Each 100 µl of the original homogenate and the two dilutions were spread onto two plates and 1 ml of the original homogenate onto four plates of the appropriate media. All sample homogenates were analyzed in duplicate. Taking into account the minimum dilution factor, the detection limit was 2.0×10^1 colony forming units (CFU)/g. All incubation of plates were done under aerobic conditions.

The number of aerobic mesophilic bacteria was determined on plate count agar (PC agar, Oxoid) plates according to ISO 4833-2:2014, incubation was at 30°C for 24–48 h.

The enumeration of *Enterobacteriaceae* was done on Violet Red Bile Glucose (VRBG) agar plates according to ISO 21528-2:2017, incubation was 37°C for 24–48 h.

Mould counts were determined on Sabouraud agar plates with chloramphenicol 0.5 g/L (Oxoid), with an incubation at 25°C for 3–5 days.

Presumptive *B. cereus* were isolated by surface plating on polymyxin egg yolk mannitol bromthymol blue agar, PEMBA (Oxoid) according to ISO 7932:2004, and incubation at 30°C for 24 h. Colonies showing typical

TABLE 1 Overview of enzyme immunoassays (EIAs) used in this study and test performance in relationship to benchmark values

EIA system			Test sensitivity achieved		
Name	Known relevant cross-reactions	Reference	Mean LOD, standard curve cut-off value (ng/ml)	LOD in PCF (µg/kg)	Benchmark value, µg/kg
DON	DON and its 8-ketotrichothecene analogues	(Usleber et al., 1991; Curtui et al., 2003)	2 ± 0.5	10	200[a]
ZEN	ZEN and their analogues	(Usleber et al., 1992; Seidler, 2007)	0.04 ± 0.02	7	20[a]
T-2/HT-2	T-2, HT-2	(Esgin et al., 1989)	0.05 ± 0.01	0.5	15[b]
EA	All EAs and isomers	(Gross et al., 2018)	0.3 ± 0.05	30	20[a]
AOH	None	(Ackermann et al., 2011)	0.4 ± 0.05	0.3	1[c]

[a]EC (2006b).
[b]EC (2013).
[c]Estimated from threshold of toxicological concern (TTC) value (2.5 ng/kg body weight (b.w.) and day; EFSA, 2011), assuming a b.w. of 10 kg and a daily consumption of 25 g dry product.
Abbreviations: DON, deoxynivalenol; ZEN, zearalenone, EA, ergot alkaloids; AOH, alternariol.

morphology, blue color, and a precipitation zone were transferred to Columbia agar supplemented with 5% of sheep blood (30°C, 24 h). Isolates identified as presumptive *B. cereus* on PEMBA were tested for toxin production (hemolysin BL [Hbl]; nonhemolytic enterotoxin [Nhe]) with the Duopath® Cereus Enterotoxins immunoassay (Merck).

Cronobacter spp. were qualitatively detected by method ISO 22964:2017. A 10 g portion of the sample was reconstituted with 190 ml buffered peptone water (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) according to ISO 6887-4:2017, and pre-enriched at 37°C for 18 h. Then, 0.1 ml was added to 10 ml of *Cronobacter* Selective Broth (CSB; Oxoid Thermo Fisher) and cultured at 41.5°C for 24 h. A 10 µl portion was streaked onto Chromogenic *Cronobacter* Isolation agar (CCI Agar; Oxoid Thermo Fisher) with a loop, and incubated at 41.5°C for 24 h. Colonies which were identified as tentative *Cronobacter* were further characterized by a commercial biochemical identification system (API32E; bio-Mérieux, Marcy l'Etoile, France), and then identified on species level by analyzing the *fusA* gene sequences as described previously by Akineden et al. (2017).

Further characterization of randomly chosen isolates was done if a noticeable colony growth was observed on PC agar, VRBG agar, PEMBA, or CCI Agar, excluding typical aerobic spore forming bacteria on PC agar, which frequently grew on PC agar in low densities. One or more typical, morphologically distinct colony was selected and purified by streaking onto Columbia blood agar. The genomic DNA was extracted from single colonies with a commercial test system (DNeasy blood and tissue kit, Qiagen). Species identification of isolates was done by amplification and sequencing of the 16S rRNA gene (Kuhnert et al., 1996). For species identification, sequences were compared using the "Basic Local Alignment Search Tool" (BLAST) at the National Centre for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3 | RESULTS AND DISCUSSION

3.1 | Microbiological analyses

The vast majority of samples were negative for *Enterobacteriaceae*, moulds, and for presumptive *B. cereus* (Table 2). Each sample yielded visible colony growth on VRBG (sample #6) and PEMBA (#20), respectively. Four samples had mould counts at the detection limit of (2.0–4.0 × 10^1 CFU/g), and only one sample (#17) was moderately positive for moulds at 2.0 × 10^2 CFU/g. On CCI agar, two samples (#6, #17) resulted in colony growth, indicative for *Cronobacter* spp.

With regard to aerobic mesophilic bacteria, the majority of samples (24 of 38) were negative, six were weakly positives (10^1–10^2 CFU/g). However, eight samples had colony counts of ≥10^2 CFU/g, highest result was obtained for sample #6 (9.6 × 10^2 CFU/g). As far as we know, no specific regulation for aerobic colony counts in PCF exist, neither in the European Union nor elsewhere. However, this parameter has been addressed as a "useful testing" criterion by the International Commission on Microbiological Specifications for Foods (ICMSF), and limits/g in a range of m = 1 × 10^3−5 × 10^3 to M = 1 × 10^4−5 × 10^4 have been suggested (ICMSF, 2011). The ML obtained in our study were 1–2 orders of magnitude lower than that,

TABLE 2 Quantitative results for aerobic mesophilic bacteria, Enterobacteriaceae, moulds, and presumptive *Bacillus cereus* in processed cereal-based foods (PCF) ($n = 38$), number of sample per interval

CFU/g interval	Aerobic mesophilic bacteria	*Enterobacteriaceae*	Moulds	Presumptive *Bacillus cereus*
Negative, $< 2.0 \times 10^1$	24	37	33	37
10^1–$< 10^2$	6	0	4	1[b]
10^2–$< 10^3$	8	1[a]	1	0

[a]Identified as *Acinetobacter baumannii* (non-Enterobacteriaceae).
[b]Identified as *Bacillus wiedmannii*.

TABLE 3 Identification of isolates from plate count agar (PC agar) of processed cereal-based foods (PCF) based on partial sequencing of the 16S rRNA gene

No. of sample and isolate	No. of isolate analyzed	Phylogenetic affiliation	Designation of isolate	Sequence length (bp)	Identity % (query coverage)	Closest species (NCBI accession number)
3	3	*Moraxella osloensis*	BBK 10/20	1304	99.23 (99)	*Moraxella osloensis* DSM 6998^T (NR_104936.1)
		Moraxella osloensis	BBK 14/20	1304	99.69 (100)	*Moraxella osloensis* DSM 6998^T (NR_104936.1)
		Moraxella osloensis	BBK19/20-pca2	1310	99.54 (99)	*Moraxella osloensis* DSM 6998^T (NR_104936.1)
2	2	*Acinetobacter baumannii*	BBK 6/20	1308	99.85 (99)	*Acinetobacter baumannii* DSM 30007^T (NR_117677)
		Acinetobacter nosocomialis	BBK12/20-cci	1312	99.62 (99)	*Acinetobacter nosocomialis* DSM 102856^T (NR_117931.1)
1	1	*Pantoea brenneri*	BBK17/20	1181	99.41 (100)	*Pantoea brenneri* DSM 24232^T (NR_116748.1)
1	1	*Microbacterium zeae*	BBK13/20	1294	99.07 (99)	*Microbacterium zeae* DSM 100750^T (NR_149816.1)
1	1	*Chryseobacterium hominis*	BBK18/20-pca2	1238	98.54 (100)	*Chryseobacterium hominis* DSM 30866^T (NR_042517.2)
1	1	*Janibacter melonis*	BBK19/20-pca1	1293	99.61 (100)	*Janibacter melonis* DSM 16063^T (NR_025805.1)
1	1	*Micrococcus aloeverae*	BBK18/20-pca1	1295	99.77 (99)	*Micrococcus aloeverae* DSM 27472^T (NR_134088.1)
4	1	*Bacillus subtilis*	BBK12/20-pca	1324	99.85 (99)	*Bacillus subtilis* DSM 10^T (NR_027552.1)

supporting the conclusion that for this parameter, a good overall quality of all products could be assumed. Nevertheless, each one to two characteristic isolates representing the dominant type of colony morphology per PC agar plate, from a total of 14 positive samples, were further identified, using biochemical methods and 16S rDNA sequencing, to obtain some preliminary information about the spectrum of bacterial species in PCF (Table 3). The dominant isolates from four samples were identified as *Bacillus subtilis*. For colony growth on the remaining 10 PC agar plates, non-spore forming bacteria dominated. The identified species presented a very diverse spectrum of bacteria and included Gram-negative (*Acinetobacter nosocomialis*, *Chryseobacterium hominis*, *Moraxella osloensis*, *Pantoea brenneri*) and Gram-positive (*Janibacter melonis*, *Microbacterium zeae*, *Micrococcus luteus* [syn. *M. aloeverae*]) species (Table 3). All these species have been isolated from various environmental habitats including plants, but also from infant formulae and from clinical specimen, some as commensal bacteria, but some have been involved in nosocomial human infectious diseases (Rajilić-Stojanović & de Vos, 2014).

Species identification after 16S RNA gene analysis of one suspect colony growth on VRBG (sample #6) revealed that this was not caused by a member of the *Enterobacteriaceae* family but by *Acinetobacter baumannii*.

Interestingly, the same species was identified from plate count agar of sample #6, indicating that *A. baumannii* was a major bacterial species in this particular sample material. Furthermore, species identification of suspect *Cronobacter* spp. from CCI of sample #6 showed that it was *C. sakazakii*, thereby confirming co-contamination with both opportunistic pathogens, which both have a history of causing foodborne infectious disease in newborns and infants <6 months of age (Amorim & Nascimento, 2017; Taylor et al., 2021).

Cronobacter spp. was isolated from the CCI plate of a second sample (#17), and this isolate also turned out to be *C. sakazakii*. Another isolate from this sample, collected from PC agar, was identified as *Pantoea brenneri*. Most *Pantoea* spp. are ubiquitous on plants and in water, but also have been frequently isolated from infant formulae (Estuningsih et al., 2006), and have been associated with very rare but severe cases of septicaemia in newborn or immunocompromised infants (Bergman et al., 2007).

Cronobacter spp., specifically *C. sakazakii*, probably have been the most notorious foodborne pathogens in commercial, milk-based powdered infant formulae in the last decades (Muytjens et al., 1988; Taylor et al., 2021). *Cronobacter* spp. have also been isolated from various cereal-based foods (Akineden et al., 2017; Friedemann, 2007; Lou et al., 2019; Silva et al., 2019), but little information about the frequency in PCF is available. In our study, colony forming units of *Cronobacter* spp. were obtained only by using 10 g test portions, and including a pre-enrichment step. Low levels of contamination, usually lower than 1 CFU/g (Al-Holy et al., 2011; Muytjens et al., 1988), seem to by typical for *Cronobacter* in dried foods. In the European Union, PCF for infants and young children are regulated under the Commission Directive 2006/125/EC (EC, 2006a), but this does not include microbiological criteria. *Cronobacter* spp., presumptive *B. cereus*, and *Enterobacteriaceae* in milk-based commercial formulae for infants <6 months of age are covered by European Union regulation No. 2073/2005 (EC, 2005), but this regulation does not apply to PCF.

Four samples yielded a weakly positive result for moulds, all near the detection limit (2.0×10^1 CFU/g) of the method. By microscopy, all were tentatively identified as *Penicillium* spp. No further attempt was made to identify and characterize these colonies at the species level.

Only one sample (#20) yielded colony growth on PEMBA (2.0×10^1 CFU/g), indicative for presumptive *B. cereus*. Further 16S rDNA sequence analysis identified this isolate as *Bacillus wiedmannii*, which is a member of the *B. cereus* group (Miller et al., 2016). This isolate also showed hemolytic activity on blood agar and was positive for Nhe and Hbl in the Duopath® Cereus Enterotoxins test (Merck). Toxigenic strains of the *B. cereus* group species have previously been reported in infant foods, including cereal-based products (Kim et al., 2011; Sadek et al., 2018). Severe outbreaks of intoxication associated with enterotoxigenic *B. cereus* spp. in food consumed by children have been reported (Delbrassinne et al., 2015; Dierick et al., 2005), but these cases were also characterized by improper food handling. At such a low level of contamination, which would have been well below even the European Union requirements for infant formulae (EC, 2005), this does not appear to be a food safety issue.

Summarizing the results of the microbiological analyses, all samples had low or very low colony counts, and five out of 38 PCF products were found to be positive, at low level, for one or two bacterial species that have been associated with rare cases of newborn or preterm infant disease. Nine other samples contained moulds or commensal bacteria at low numbers. We agree with the opinion expressed by the ICMSF (2011) that findings in such a range cannot be regarded as a direct threat to the health of infants ≥4 months of age, as long as the product is prepared and handled according to the recommendations. Further, no clear association between a specific PCF ingredient or mixture of ingredients and the occurrence of specific bacteria could be detected, also because the majority of products contained mixed cereals. These products yielded most remarkable findings, including both *C. sakazakii* isolates, *A. baumannii*, *P. brenneri*, and *B. wiedmannii* (Table 4).

3.2 | Mycotoxin analyses

3.2.1 | Method validation

All PCF products under study were dried powdery or granular products of similar appearance, the ingredients presented a wide variety of different cereals as ingredients or ingredient mixtures. The majority of products in this study contained cereal mixtures, similar as observed from the display of products in eight local retail shops. Product labels listed up to seven different cereals, providing multiple potential sources for mycotoxin contamination. Out of the *Fusarium* mycotoxins under study, DON and ZEN were analyzed in all samples, T-2/HT-2 were analyzed in oat-containing products only, because oats appear to be by far the most relevant source in middle European cereals (EFSA, 2017). For each mycotoxin EIA, the mean limit of detection (LOD) as indicated by Liesener et al. (2010) was checked and verified, using spiked PCF sample material. The recovery experiments showed that the mean recovery rates for DON, T-2/HT-2, and AOH in spiked samples were in the range of 69%–120% (Table 5). For EA and ZEN, which were also analyzed in all samples, higher recoveries ranging from 115% to 160% were

TABLE 4 Compilation of samples which yielded highest or else remarkable results, with regard to either microbiological contamination or maximum mycotoxin levels

	Sample #						
Parameter	**6**	**12**	**17**	**20**	**24**	**30**	**34**
Composition	Oats, wheat, barley, spelt, rye	Wheat	Oats, einkorn wheat, barley, rye	Oats, wheat, rye, barley, spelt	Rice, maize, sorghum	Wheat, oats, rye, barley, sorghum, rice, maize	Wheat
Recommended age, months	≥6	≥4	≥6	≥6	≥4	≥6	≥4
Microbiology							
Aerobic mesophilic bacteria, CFU/g	9.6×10^2	3.6×10^2	1.2×10^2	$<2.0 \times 10^1$	$<2.0 \times 10^1$	$<2.0 \times 10^1$	$<2.0 \times 10^1$
Specific isolates	*C. sakazakii, A. baumannii*	*A. nosocomialis, B. subtilis*	*C. sakazakii, P. brenneri*	*B. wiedmannii*	–	–	–
Mycotoxin EIA results, μg/kg							
DON	<9	<9	15	<9	<9	11	35
T-2/HT-2	4	n.a.	5	5	n.a.	8	n.a.
AOH	1	<0.3	0.5	0.7	2	2	<0.3

Abbreviations: AOH, alternariol; DON, deoxynivalenol; EIA, enzyme immunoassay; n.a., not analyzed.

TABLE 5 Recovery of mycotoxins from artificially contaminated processed cereal-based foods (PCF) samples. Each level was spiked in triplicate

		Toxin found	
Test system for	**Spiked level (μg/kg)**	**Mean recovery (%)**	**RSD (%)**
DON	30	69	4
	50	78	4
	150	70	9
ZEN	20	155	9
	50	133	10
	100	127	5
T-2/HT-2	10	99	10
	20	120	19
	50	96	17
EA	100	160	29
	200	130	18
	500	115	9
AOH	3	72	18
	5	85	3
	10	75	10

Abbreviations: AOH, alternariol; DON, deoxynivalenol; EA, ergot alkaloids; RSD, relative standard deviation; ZEN, zearalenone.

determined. The high recoveries obtained for ZEN may be explained by some remaining matrix interference in the three selected materials, because the blank materials used for spiking were clearly below the LOD but yielded absorbance values of 93%–98% $B/B_0 \times 100$. Likewise, the high recovery rates for EA may also be explained by some remaining matrix effects. Since both tests yielded toxin-negative results for all samples, no attempt was made to further improve sample extract preparation. Further studies will aim at optimizing the overall analytical strategy, including sample extraction and confirmation of positive results by, for example, an LC-MS/MS reference method.

Relative standard deviations (RSDs) of ≤10% were found for DON and ZEN, whereas RSDs <20% could be achieved for all other mycotoxins except for the 100 μg/kg spiking level of EA (29%). Further, three samples containing each of the highest toxin level (Table 4) of DON (#34), T-2/HT-2 (#30), and AOH (#24) were spiked with the respective toxin and reanalyzed. After subtracting the measured toxin content of the nonspiked sample, recoveries of 77%–106% were obtained, which demonstrates a toxin-dependent additive effect and further indicates the suitability of the EIA methods.

While the sample extraction methods, and the overall method performance were considered to be sufficient, the relative cross-reactivities of the EIAs, specifically for T-2/HT-2 and EA may present some underestimate of the true toxin content, while the DON and AOH results could be an overestimate due to reactivity with toxin analogues. The LOD of the EIA method for EAs (30 μg/kg) did not fully meet the recently published European Union ML for this group of toxins in PCF (20 μg/kg), but still are at a very similar level.

3.2.2 | Mycotoxin frequency and levels

All samples, even rye-containing products, yielded clearly EA-negative results, which indicates that this group of mycotoxins does not play a relevant role in PCF from the German market (Table 6). EAs in cereals, even if present in the harvested lots, can easily be removed during the cleaning process, allowing selection of low-contamination batches for production of PCF. It could also be assumed that PCF producers were already aware of "soon to come" European Union regulations for EAs in 2019/2020. This could explain why the contamination situation as found in our study was better than that reported in a few previous studies. For example, breakfast cereals, biscuits, and cookies from the Dutch market 2010–2014 had mean total EAs levels of 10 μg/kg, with some products exceeding 100 μg/kg (Mulder et al., 2015). Apparently, dedicated PCF products were not included in this study. Similar results were reported in a 1997–1999 survey for Canadian products (Lombaert et al., 2003).

Like EAs, ZEN was also not detected in any sample. Although ZEN is a common contaminant in most cereals at the time of harvest, it is largely eliminated during the grain cleaning processes. Our results on the absence of ZEN at <7 μg/kg are supported by others who also found no ZEN, or just traces near 1 μg/kg, in PCF from other European countries (Braun et al., 2020; Juan et al., 2014), while slightly higher concentrations were reported for such products from the United States (Al-Taher et al., 2017).

DON was found with relatively high frequency (26%) but at low levels (maximum concentration: 35 μg/kg) in PCF products, regardless of cereal composition (Table 6). While most samples were negative (<9 μg/kg), the majority of positive samples contained levels of just DON, probably the most frequent *Fusarium* toxin in cereals in Germany, and, in contrast to ZEN, removal during cereal processing is not fully efficient (Karlovsky et al., 2016), which may explain our findings. Similar findings have been reported by others (Herrera et al., 2019; Juan et al., 2014; Pereira et al., 2015).

T-2/HT-2 were found in all 15 oat-containing products, but again at very low concentrations between 1 and 8 μg/kg (Table 6). This was not unexpected, as previous surveys have shown that oat is the major, if not only, relevant cereal in Europe in aspects of T-2/HT-2 contamination (Curtui et al., 2009; Gottschalk et al., 2009; Kirinčič et al., 2015). None of the samples exceeded the guideline value of 15 μg/kg. Our results are consistent with the findings of Al-Taher et al. (2017), who reported low levels of T-2/HT-2 (<10 μg/kg) in oat-based and mixed-grain infant cereals from the U.S. market.

It was not surprising that a considerable part of the samples (66%) from each of the six product categories was positive for AOH (0.4–2 μg/kg) (Table 6), because AOH is the most frequent occurring *Alternaria* mycotoxin in food (EFSA, 2011). Similar levels of AOH (<10 μg/kg) in single grain as well as in multi-grain baby foods had also been reported by Scott et al. (2012) and Gotthardt et al. (2019). However, the AOH levels in positive samples consistently were very low. In the absence of a full toxicological risk assessment, it is difficult to evaluate the relevance of these findings. Applying (i) the TTC recommended for AOH by EFSA (2.5 ng/kg b.w. and day; EFSA, 2011), (ii) assuming that 25 g PCF (dry product) is a reasonable daily serving size, and (iii) using a b.w. range (6–12 months) of 5–10 kg, the critical AOH concentration in PCF would be at 0.5–1 μg/kg. This means that several samples would have approached or even exceeded this critical concentration, similar as estimated by EFSA in 2011 (EFSA, 2011). It has to be emphasized that the TTC approach for AOH is affected by several uncertainties, including the lack of in vivo toxicological data.

Co-occurrence of DON and AOH was observed in five samples (13%). Out of 15 oat-containing samples, which were all positive for T-2/HT-2, one additionally contained DON (oat-based product) and eight contained AOH (four oat-based products + four mixed-grain products). All three toxins were found in three oat-containing samples (mixed-grain products). This is not surprising, as the majority ($n = 7$) of these 12 co-contaminated oat-containing samples were composed of mixed cereals, providing different sources for contamination of fungal toxins, and similar

TABLE 6 Mycotoxin contamination in processed cereal-based foods (PCF) for infants and young children ($n = 38$) from the German market based on six product categories

	DON	ZEN	T-2/HT-2	EA	AOH
Spelt-based cereals ($n = 5$)					
Positive (%)	40	–	n.a.	–	60
Mean[a] ± SD	12 ± 3	<7	n.a.	<30	0.7 ± 0.2
Range[a]	11–14	<7	n.a.	<30	0.5–0.9
Wheat-based cereals ($n = 5$)					
Positive (%)	20	–	n.a.	–	40
Mean[a] ± SD	35	<7	n.a.	<30	0.7 ± 0.2
Range[a]	–	<7	n.a.	<30	0.6–0.8
Oat-based cereals ($n = 6$)					
Positive (%)	17	–	100	–	67
Mean[a] ± SD	17	<7	4 ± 1	<30	0.6 ± 0.1
Range[a]	–	<7	3–6	<30	0.4–0.7
Millet-based cereals ($n = 3$)					
Positive (%)	–	–	n.a.	–	100
Mean[a] ± SD	<9	<7	n.a.	<30	0.6 ± 0.1
Range[a]	<9	<7	n.a.	<30	0.5–0.7
Rice-based cereals ($n = 6$)					
Positive (%)	17	–	n.a.	–	33
Mean[a] ± SD	11	<7	n.a.	<30	1 ± 0.2
Range[a]	–	<7	n.a.	<30	1.1–1.5
Mixed-grain cereals ($n = 13$)					
Positive (%)	38	–	100[b]	–	85
Mean[a] ± SD	13 ± 2	<7	4 ± 2	<30	0.9 ± 0.6
Range[a]	11–15	<7	1–8	<30	0.4–2

Note: Concentration values as mean ± standard deviation (SD) expressed in μg/kg.

Abbreviations: AOH, alternariol; DON, deoxynivalenol; EA, ergot alkaloids; n.a., not analyzed; ZEN, zearalenone.

[a]Positive samples only.

[b]$n = 9$.

findings have been reported for PCF products previously (Juan et al., 2014; Zhang et al., 2018).

Concluding the results of the mycotoxin analyses, contamination frequency of PCF with DON, T-2/HT-2, and AOH was within a span, which was expected from previous studies, but levels of contamination were all in the low range, well below the ML. The complete absence of detectable levels of ZEN and EAs may be explainable by careful cereal cleaning and selection and procedures.

4 | CONCLUSION

This is the first analysis of the PCFs from the German market, and the second one worldwide (Assunção et al., 2021), in which two major food safety categories have been studied, namely microbiological quality and contamination with mycotoxins. Of course pathogenic, opportunistic, or spoilage bacteria have no direct relationship with fungal toxins. However, it may be argued that an overall deficit in maintaining effective measures to ensure food safety and quality in PCF production facilities may negatively impact both areas. Our study, although limited in sample number, included products of all major companies offering PCF products on the German market. All products fully complied with present European Union regulations of microbiological criteria and mycotoxin contamination. Very few samples (Table 4) yielded results which, in one or more parameters, surfaced the generally unremarkable data set of analytical findings. The risk from low levels of *Cronobacter* spp. and enterotoxigenic *Bacillus* spp., which are a major concern in infant formulae for preterm infants and neonates, can easily be avoided if PCF are prepared freshly for each meal and consumed immediately thereafter.

AUTHOR CONTRIBUTIONS

Christina Rehagel: Conceptualization; formal analysis; investigation; validation; visualization; writing – original draft; writing – review & editing. **Ömer Akineden**: Conceptualization; investigation; validation; visualization; writing – original draft; writing – review & editing. **Ewald Usleber**: Funding acquisition; project administration; resources; supervision; writing – review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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