Microbiological Quality of Raw Dried Pasta from the German Market, with Special Emphasis on *Cronobacter* Species

Ömer Akineden, Kristina Johanna Murata, Madeleine Gross, and Ewald Usleber

Abstract: The microbiological quality of 132 dried pasta products available on the German market, originating from 11 different countries, was studied. Sample materials included soft or durum wheat products, some of which produced with other ingredients such as eggs, spices, or vegetables. Parameters included hygiene indicators (aerobic plate count, mold count, the presence of Enterobacteriaceae) and pathogenic/toxinogenic bacterial species (*Salmonella* spp., *Staphylococcus aureus*, presumptive *Bacillus cereus*, and *Cronobacter* spp.). The overall results of hygiene parameters indicated a satisfactory quality. Salmonella was not found in any sample. Three samples were positive for *S. aureus* (10^2 to 10^4 colony forming unit (CFU)/g). Presumptive *B. cereus* at levels of 10^3 to 10^4 CFU/g were detected in 3 samples. *Cronobacter* spp. were isolated from 14 (10.6%) products. Of these, 9 isolates were identified as *C. sakazakii*, 2 each as *C. turicensis* and *C. malonaticus*, and 1 as *C. muytjensii*. The isolates were assigned to 9 multilocus sequence typing (MLST) sequence types and to 14 different PFGE profiles. Although pasta products are typically cooked before consumption, some consumers, and children in particular, may also eat raw pasta as nibbles. Raw pasta seems to be a relevant source of exposure to dietary *Cronobacter* spp., although health risks are probably restricted to vulnerable consumers. High numbers of presumptive *B. cereus* as found in some samples may be a risk after improper storage of cooked pasta products because toxinogenic strains are frequently found within this species.

Keywords: *Cronobacter* species, dried raw pasta, multilocus sequence typing, presumptive *Bacillus cereus*, pulsed-field gel electrophoresis

Practical Application: Dried raw pasta products sold on the German market contain considerable levels of microorganisms. Raw pasta seems to be a relevant source of exposure to dietary *Cronobacter* species, although the relevance as a health risk is probably restricted to vulnerable consumers. High numbers of presumptive *B. cereus* as found in some samples may be a health risk after improper storage of cooked pasta products.

Introduction

With an estimated world annual production volume of 13.6 million tons, pasta is a staple food in many parts of the world, with Europe and the Americas being the most important pasta-producing regions. In Germany, the average annual per capita consumption of pasta is 7.9 kg (International Pasta Organization 2013). Dried pasta products are mainly prepared from soft wheat flour or durum wheat flour, but may contain other ingredients such as egg, spices, and vegetables, by a process of sheeting and cutting. Other pasta products are processed from coarse-milled durum wheat semolina by extrusion (Martinez and others 2007; Fu 2008). Because pasta products are no sterile preparations, the quality and safety of the end product directly depends on the microbiological quality of the raw materials. This includes quality of wheat and semolina, and compliance with specific technological process of production (Plavšić and others 2011).

Despite the importance of pasta products in daily nutrition, there is limited recent information available about the general microbiological quality and characteristics of raw, dried pasta. Even fewer data have been reported about the occurrence of emerging bacteria such as *Cronobacter* spp. in pasta. Although the majority of pasta products is cooked for several minutes before consumption, some instant noodle products require only the addition of hot water for preparation. Furthermore, spore-forming bacteria, including toxinogenic *Bacillus (B.) cereus*, may not be eliminated during a short cooking process. Finally, raw dry pasta occasionally is also directly consumed in uncooked form, as a kind of nibbles. According to our observations, children in particular have a tendency to consume small quantities of raw dry pasta, for example, during preparation of lunch or dinner meals by their parents. These aspects show that some concern about the microbiological safety of raw dried pasta is indeed justified.

Some older surveys indicated that microbiological quality of pasta varies within a wide range for parameters such as aerobic plate counts, molds, and Enterobacteriaceae (Rayman and others 1981; Swartzentruber and others 1982; Spicher 1985), but publications describing a direct linkage between pasta and foodborne disease are rare. So far, most reports on pasta-related foodborne outbreaks, as well as survey studies on bacteria in pasta, refer to processed pasta products such as dumplings or lasagne, making it difficult or even impossible to relate bacteria to plain pasta (Woolaway and others 1986). Some evidence has been provided that pasta contaminated with *B. cereus* strains producing emetic toxin may have been the causal factor responsible for some fatal cases of food intoxication,

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although poor household conditions presumably were a cofactor in these cases (Dierick and others 2005; Delbrassinne and others 2011; Naranjo and others 2011).

The genus *Cronobacter* (formerly: *Enterobacter sakazakii*) has been associated worldwide with rare but life-threatening diseases in newborns and premature infants, characterized by meningitis, septicemia, and necrotizing enterocolitis (Biering and others 1989; van Acker and others 2001; Block and others 2002; Himelright and others 2002). Because powdered infant formula (PIF) have been suspected as important vehicle for the disease, most surveys performed so far have dealt with the presence of *Cronobacter* spp. in PIF and other infant foods (Chap and others 2009). However, Patrick and others (2014) in a recent study report that the overall rates of isolation of *Cronobacter* spp. from ill persons in the United States were higher than anticipated, and that the very young and very old were disproportionately affected. Due to their ubiquitous nature, *Cronobacter* spp. have been frequently isolated not only from PIF but also from environmental samples, water, plant material, and various food products (Friedemann, 2007; Baumgartner and others 2009; Chon and others 2012; Cetinkaya and others 2013). A survey performed in Prague (Czech Republic) included a few pasta samples ($n = 8$): *Cronobacter* was found in oat-based flakes but not in wheat-based infant food or pasta (Hochel and others 2012). More recently, Lou and others (2014) reported a *Cronobacter* contamination rate of 100% for wheat flour and dry noodles (5 samples) from China. In another studies from this country, *Cronobacter* spp. were also detected with higher frequency in cereal and cereal products using a traditional culture method (Lee and others 2012; Li and others 2014). In this context, the capacity of *Cronobacter* to adapt to, survive and persist under desiccated environmental conditions as shown for PIF probably also applies to dry pasta. To the best of our knowledge, a larger survey on the occurrence of *Cronobacter* spp. specifically in dry raw pasta has never been performed. However, such data seems to be necessary, to evaluate the possible risk for children and other vulnerable groups of consumers.

Therefore, this study aimed at evaluating the overall microbiological quality of dry raw pasta as available at the German market, including domestic and international produce. A special focus was on the characterization of *Cronobacter* spp. isolated from these samples.

Materials and Methods

Sample material

Between August and November 2010, a total of 132 packages of dried pasta samples were purchased from retail stores in Germany. Sampling followed the relative presence of individual brands and products on the market, but included niche products from minor producers as well. The term pasta is used in this study because of the lack of a clear and internationally accepted means to differentiate between pasta, noodles, and other regional names such as "spätzle." Table 1 gives an overview of characteristics and composition of the pasta samples under study. Because of the dominance of products made from durum wheat on the German market, two thirds of samples were durum wheat pasta, although pasta made from soft wheat accounted for about 20%, and pasta from other cereals about 10% of all samples. According to product labeling, the majority of samples (71%) was German and Italian produce, but some products originated from other European countries. A few samples ($n = 16$) were of Asian or American produce. All products were raw, dried pasta products, and none was of the precooked, ready-to-eat type. Most of the samples ($n = 129$) were pasta products of different composition but without fillings, 3 samples were pasta made with soy mince or cheese fillings. About half of the samples were plain cereal products made from durum or soft wheat with only water and salt added, although the other half contained additional ingredients such as egg, spices, and vegetables.

Microbiological analyses

For analysis of aerobic mesophilic bacteria, molds, presumptive *B. cereus*, and coagulase-positive staphylococci, a 10 g test portion of each sample was aseptically taken and diluted with 90 mL of Ringer's solution quarter strength (Oxoid, Wernigerode, Germany). After soaking for 15 min, the mixture was homogenized using a Stomacher Lab-Blender 400 (Seward Medical, London, U.K.). A $\log_{10}$ dilution series was made with Ringer's solution quarter strength, and duplicate 100 μL portions of each dilution spread-plated onto appropriate media. For coagulase-positive staphylococci and presumptive *B. cereus*, 1 mL of the 1:10 dilution was additionally streaked on each 3 petri dishes. Aerobic mesophilic bacteria were determined by using standard procedure, as given by German food standard (DIN 10192–5; microbiological analysis of milk—colony count—part 5: spatula method), on plate count agar (Oxoid) after incubation for 72 h at 30 °C. Mold count was determined on Malt Extract Agar (Oxoid), plates were incubated aerobically at 25 °C for 3 to 5 d. Coagulase-positive staphylococci were determined following method ISO 6888-1:1999/Amd. 1: 2003 by surface plating on Baird Parker agar (Oxoid), supplemented with egg yolk tellurite emulsion, and incubating plates at 37 °C for 30 to 48 h. Presumptive *B. cereus* were determined according to ISO 7932:2004, using surface plating on mannitol egg yolk polymixin agar (Oxoid) and incubation at 30 °C for 24 h. Presence of *Salmonella* spp. in 25 g sample was checked following the method described in ISO 6785:2001.

The isolation of *Cronobacter* spp. from 10 g test portions was conducted using a modified version of the method proposed by the Intl. Organization for Standards Technical Specification on the detection of *E. sakazakii* (ISO/TS 22964). This method was also used to qualitatively detect the presence of Enterobacteriaceae in 10 g test portions. In brief, 10 g of sample was soaked in 90 mL buffered peptone water (Oxoid) in a Stomacher bag, homogenized and incubated for 24 h at 37 °C. Then, 1 mL of the preenriched sample was added to 9 mL of Enterobacteriaceae enrichment broth (EE broth; Oxoid) and incubated for another 24 h at 37 °C. Using a loop, the enrichment broth was streaked in parallel onto Brilliance Enterobacter sakazakii agar (Druggan-Forsythe-Iversen [DF] formulation Oxoid) and on Violet Red Bile Glucose agar (VRBGA; Oxoid), and incubated for 24 h at 37 °C. From each plate, a typical colony was checked by oxidase test, further preliminary identification of *Cronobacter* spp. and other Enterobacteriaceae was performed using the API32E biochemical identification system (bio-Mérieux, Marcy l'Etoile, France). Isolates identified as *Cronobacter* spp. were further characterized based on biochemical tests according to Farmer (1980) and Iversen and others (2007, 2006). The reference strains *Cronobacter (C.) sakazakii* (DSM 4485), *C. malonaticus* (DSM 18702), *C. turicensis* (DSM 18703), *C. universalis* (NCTC 9529), *C. muytjensii* (DSM 21870), *C. dublinensis* ssp. *dublinensis* (DSM 18705), *C. dublinensis* ssp. *lactaridi* (DSM 18707), *C. dublinensis* ssp. *lausannensis* (DSM 18706), and *C. condimenti* (LMG 26250) were used as controls.

Table 1–Overview of composition and origin of raw dried pasta samples.

Pasta made from	Additional ingredients	Number of samples	Number of manufacturers	Origin of products[b]
Durum wheat semolina	None	30	28	TR (2), IT (11), DE (9), RUS (1), NL (1), not declared (6)
	Millet flour	1	1	DE
	egg	47	28	DE (42), IT (2), not declared (3)
	Egg and bear's garlic	1	1	DE
	Egg and pesto spice	1	1	DE
	Egg, tomato, pepper, cayenne pepper and garlic	1	1	DE
	Red pepper	2	1	DE
	Spinach	2	1	IT (1), not declared (1)
	Chili and sepia	1	1	IT
	Boletus	1	1	IT
	Lemon extract and curcuma	1	1	IT
	Sepia	1	1	IT
	Basil, red mangold, curcuma	1	1	IT
	Milk, cheese, palm oil, marine salt, oats, soya lecithin, pepper	1	1	not declared
Soft wheat flour	None	9	9	DE (3), CN (2), KZ (1), TW (1), not declared (2)
	Egg	9	8	DE (2), TR (3), CN (1), not declared (2)
	Curcuma and spice	1	1	PL
	Turkey egg, curcuma	1	1	PL
	Egg, soya, onion and pepper	1	1	TR
	Egg, soya	1	1	TR
	Sugar, glutamate, scallop and maize flour	1	1	HK
	Unripe spelt grain flour	1	1	DE
	Egg, beetroot, curcuma, tomato, spinach	1	1	DE
Kamut semolina	–	2	2	Not declared
rice flour	–	3	3	DE (1), IT (1), BO (1)
Whole spelt flour/semolina	–	6	6	DE (4), not declared (2)
	Egg	4	3	DE

[a]–, without ingredient.
[b]In parenthesis given the number of samples analyzed.
BO, Bolivia; CN, VR China; DE, Germany; HK, Hong Kong; IT, Italy; KZ, Kazakhstan; NL, The Netherlands; PL, Poland; RUS, Russia; TR, Turkey; TW, Taiwan.

Table 2–Microbial counts of aerobic mesophilic bacteria, molds, presumptive *Bacillus cereus*, and *Staphylococcus aureus* in 132 dried pasta samples.

CFU/g interval	Aerobic mesophilic bacteria (%)	Molds (%)	Presumptive *Bacillus cereus* (%)	*S. aureus* (%)
$<10^2$	35.4	74.2	95.4	97.7
10^2–$<10^3$	25.7	22.0	2.3	0.8
10^3–$<10^4$	20.5	3,8	2.3	1.5
10^4–$<10^5$	9.9	0	0	0
$>10^5$	7.6[a]	0	0	0

[a]Maximum value 2.3×10^6 CFU/g.

Identification of *Cronobacter* isolates

For species identification of *Cronobacter* isolates, a partial β subunit of RNA polymerase encoding gene (*rpoB*) of all isolates was performed. Genomic DNA was prepared with the DNeasy Blood Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The oligonucleotide primers and the conditions for the amplification of *rpo*B gene were used as described previously by Mollet and others (1997). The PCR reaction (total volume 30 μL) was performed in a 0.2 mL reaction vial and consisted of 3 μL GeneAmp 10× PCR Gold Buffer (150 mmol/L Tris-HCl, 500 mmol/L KCl; pH 8.0; Applied Biosystem, Darmstadt, Germany), 1.8 μL $MgCl_2$ (25 mmol/L; Applied Biosystem), 1.0 μL of each primer (10 pmol/μL), 0.6 μL dNTP-mix (10 mmol/L; MBI Fermentas, St Leon-Rot, Germany), 0.2 μL AmpliTaq Gold® polymerase (5 U/μL, Applied Biosystem), 19.9 μL sterile Aqua dest., and 2.5 μL DNA template. PCR products were determined by electrophoresis of 10 μL of the reaction product in a 2% agarose gel (Biozym, Hessisch-Oldendorf, Germany) at 120 Volt in 1× Tris–acetate-electrophoresis buffer (TAE; [0.04 mol/L Tris, 0.001 mol/L EDTA; pH 7.8]). The molecular marker GeneRuler 50 bp DNA ladder (MBI Fermentas) was used. The purification of amplicons from PCR reactions was performed using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The purified PCR products were sequenced by SEQLAB Sequence Laboratories (Göttingen, Germany) under standard sequencing conditions according to the manufacturer's protocol.

The *rpoB* sequences of 14 *Cronobacter* isolates from pasta were aligned to those of 63 assigned *rpoB* alleles of *Cronobacter* spp. strains from the open access online *Cronobacter* MLST database (www.pubMLST.org/cronobacter) using clustal W tool in MEGA 6. A phylogenetic tree based on these *rpoB* gene sequences was constructed using the Neighbor-Joining method with the following multiple alignment parameters: gap open penalty: 15, gap extention penalty: 6.66, Transition Weight: none, DNA weight matrix: UIB.

Table 3–Summary of identification and differentiation of *Cronobacter* spp. isolates from 14 dried raw pasta samples.

Isolate	Source	Ingredients	Origin	Manufacturer	API32E ID (%)	Biotype[c]	MLST type	PFGE type	Species[d,e]
N47	Egg noodles	Durum wheat semolina, egg	DE	A	*E. sakazakii (99.9)*	1	17	XII	*C. sakazakii*
N51	Wok egg noodle	Durum wheat semolina, egg	DE	B	*E. sakazakii (99.9)*	1	13	II	*C. sakazakii*
N90	Egg noodle	Whole spelt semolina, egg	DE	C	*E. sakazakii (99.9)*	1	40	V	*C. sakazakii*
N96	Swabian spaetzle	Durum wheat semolina, egg	DE	C	*E. sakazakii (99.9)*	1	12	III	*C. sakazakii*
N153	Tagliatelle	Durum wheat semolina, egg	DE	D	*E. sakazakii (99.9)*	1	40	IV	*C. sakazakii*
N132	Egg noodle	Durum wheat semolina, egg	DE	E	*E. sakazakii (99.9)*	1	292	XIII	*C. sakazakii*
N105	Egg noodle	Durum wheat semolina, egg	DE	E	*E. sakazakii (99.9)*	2	1	VIII	*C. sakazakii*
N73	Vermicelli	Durum wheat semolina	IT	F	*E. sakazakii (99.9)*	1	96	VI	*C. sakazakii*
N94	Noodle with vegetable	Durum wheat semolina, basil, red mangold, curcuma	IT	G	*E. sakazakii (99.9)*	2	1	VII	*C. sakazakii*
N30	Japanese somen noodle	Wheat flour	CN	I	*E. sakazakii (99.9)*	9	291	XI	*C. malonaticus*
N6	Kazakh vermicelli	Wheat flour	KZ	H	*E. sakazakii (99.9)*	5	291	IX	*C. malonaticus*
N40	Egg noodle with basil	Durum wheat semolina, egg, basil	DE	J	*E. sakazakii (99.9)*	15	n.d.	XIV	*C. muytiensii*
N35	Red pepper noodle	Durum wheat semolina, red pepper	DE	K	*E. sakazakii (99.9)*	16	251	X	*C. turicensis*
N160	Red pepper noodle	Durum wheat semolina, red pepper	DE	K	*E. sakazakii (99.9)*	16	293	I	*C. turicensis*

[a]KZ, Kazakhstan; CN, China; IT, Italy; DE, Germany.
[b]n.d., not determined.
[c]According to Farmer (1980) and Iversen and others (2006).
[d]According to the biochemical tests: indole production, malonate utilization, acid production from methy-alpha-D-glucose, and dulcitol (Iversen and others 2007).
[e]According to the *fusA* gene sequencing.

Multilocus sequence typing

The multilocus sequence typing (MLST) method was applied as published by Baldwin and others (2009). Amplification and nested sequencing primers for the MLST loci and PCR conditions were used according to protocol available at MLST *Cronobacter* web database (http://pubmlst.org/cronobacter/). The oligonucleotide primers used in this study were synthesized by Eurofins MWG/Operon (Ebersberg, Germany). The amplified products were then purified using the QIAquick PCR Purification Kit (Qiagen) following the manufacturers protocol. The sequencing was performed using the purified PCR products as described above. Finally, allele number was assigned by tools available at MLST *Cronobacter* web (http://pubmlst.org/cronobacter/).

Macrorestriction analysis

Macrorestriction analysis of chromosomal DNA was carried out by preparation of the whole bacterial DNA of the *Cronobacter* isolates in agarose gel plugs and subsequent digestion of the bacterial DNA with the *Xba*I restriction enzyme and with the pulse time suggested by Caubilla-Barron and others (2007). The fragment pattern was finally studied by the Chef-Dr II pulsed-field electrophoresis system (Bio-Rad, Munich, Germany). Interpretation of the restriction patterns was performed using Bionumerics software 5.1 (Applied Maths, Belgium).

Results and Discussion

This study was designed to get a comprehensive overview of the microbiological quality of dry raw pasta products, and covered a broad variety of both domestic and international produce as available at the German market.

Regarding the total bacteria count, 63.7% of the samples had an aerobic mesophilic plate count above 10^2 colony forming units (CFU)/g. Most samples (46.3%) had a count ranging from 10^2 to 10^4 CFU/g, but 17.5% of the samples exceeded 10^5 CFU/g (Table 2). These results were comparable to those reported in earlier studies by Rayman and others (1981), Swartzentruber and others (1982), Spicher (1985), and Massa and others (1986) for pasta products. The only specific regulation we could found for this parameter in raw, dried pasta is a tolerance value of 10^5 CFU/g, issued by Swiss Authorities (Eidgenössisches Departement des Inneren 2005). A corresponding value (10^6 CFU/g) was recommended by the Intl. Commission on Microbiological Specifications for Foods (ICMSF 2011) as an *In*-process criterion for unfilled pasta and noodles. We assume that higher levels of aerobic mesophilic microorganisms, as found in 7.6% of pasta samples in this study, at least reflects a suboptimal quality of raw materials or suboptimal processing.

The detection rates for molds were 25.8% and the largest population distribution for molds was found in the range of 10^2 to 10^3 CFU/g (Table 2). In these samples, the mycoflora was dominated by species of *Aspergillus* (27 samples) followed by *Penicillium* (15 samples) and *Alternaria* (1 sample). *Aspergillus* and *Penicillium* cooccurred in 9 samples. None of the samples exceeded the guidance level for molds in raw, dried pasta of 10^3 CFU/g as given in the recommendations the German Society for Hygiene and Microbiology (DGHM 2011). We conclude that in aspects of

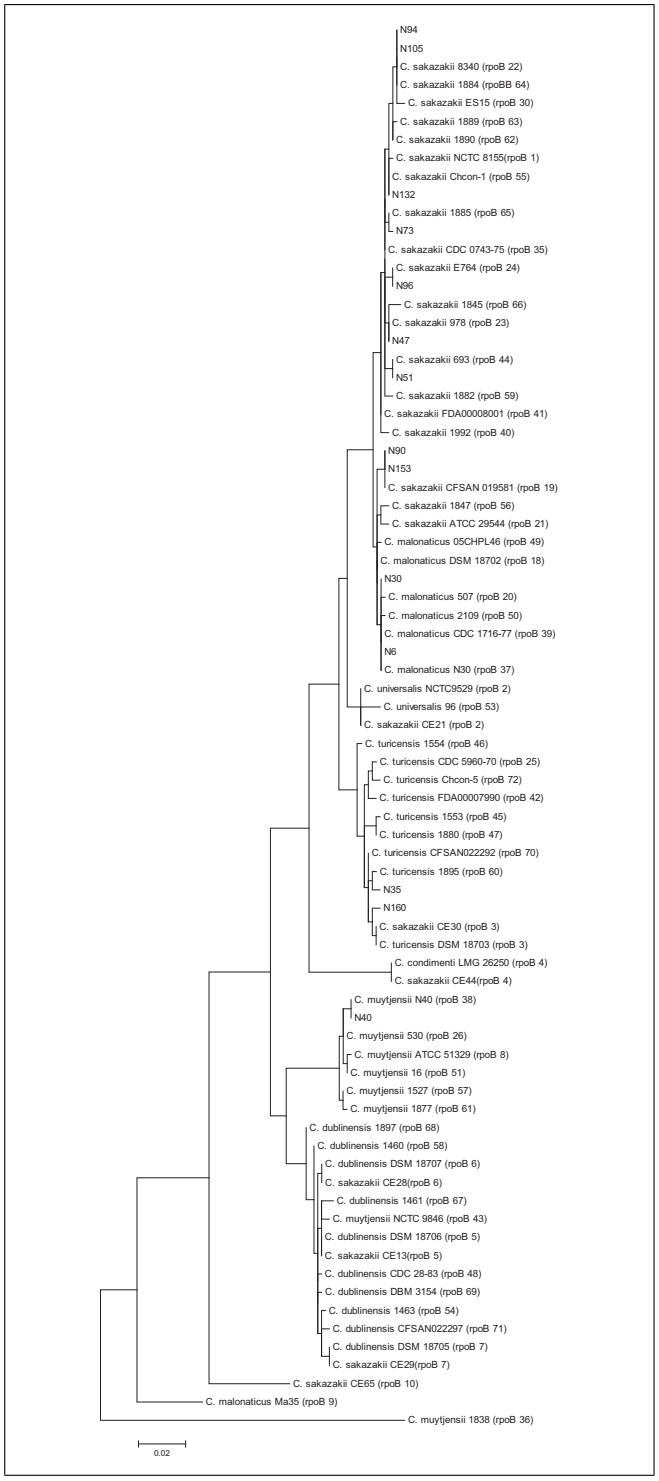


Figure 1–Neighbor-joining tree of the *rpoB* alleles (637 bp) of 14 *Cronobacter* isolates and the Cronobacter MLST data set ($n = 63$). The numbers in the parenthesis indicate the *rpoB* allelic profiles.

molds, the overall quality of samples under study was satisfactory. This study did not investigate the presence of mycotoxin in the samples; because it seems to be very unlikely that fungi, including mycotoxinogenic species, could grow in the final dried pasta products under proper storage conditions. However, it cannot be excluded that, for example, *Fusarium* mycotoxins such as deoxynivalenol are present at significant levels, because pasta products are a major source of cereal-borne mycotoxins in the food chain (Weidenbörner and others 2000; Curtui and others 2006). These toxins, however, originate from a field contamination of cereal ingredients and cannot be attributed to the pasta-making process as such. Presumptive *B. cereus* was detected in 4.6% of samples above the detection limit of 10^2 CFU/g (Table 2). This spore-forming organism is a common soil saprophyte and is easily spread to many types of foods, especially of plant origin (EFSA 2005). Dried pasta products may contain *B. cereus* spores that can survive in dry food and possess sufficient heat resistance to withstand the heat treatment (Parry and Gilbert 1980; EFSA 2005). However, the organism has been implicated in a number of emetic food poisoning outbreaks which were mainly associated with starchy foods such as rice and pasta (Ehling-Schulz and others 2004; EFSA 2005). In our study, the ability to produce emetic toxin by the *B. cereus* isolates was not determined. However, one recent study from Germany showed that about 5% of pasta product samples contained emetic *B. cereus* (Messelhäusser and others 2014). Higher numbers exceeding the guidance value of $>10^3$ CFU/g recommended by the DGHM (2011) for presumptive *B. cereus* in raw, dried pasta were obtained in 3 samples, but the corresponding DGHM (2011) action level (10^4 CFU/g) was not exceeded. Although the risk from such levels of *B. cereus* in unprocessed pasta seems to be low, Granum and Lund (2006) stated that such levels cannot be considered completely safe for consumption. In a worst-case scenario, improper storage of such pasta after cooking for a prolonged time may provide favorable conditions for germination of spores. Considering that toxinogenic strains are frequently found within this species (Dierick and others 2005; Delbrassinne and others 2011; Naranjo and others 2011), critical levels may be reached in such a case.

Only 3 samples yielded low colony counts for *S. aureus*, at levels ranging from 10^2 to 10^4 CFU/g (Table 2), and complied with DGHM and ICMSF action level of 10^4 CFU/g. It is nevertheless noteworthy that further PCR testing for enterotoxins (Akineden and others 2008) revealed that these *S. aureus* isolates were enterotoxigenic and possessed the gene for Staphylococcus enterotoxin H (data not shown). Contamination of pasta with *S. aureus* may occur from various ingredients and *S. aureus* may grow in pasta dough processed at warm temperatures for extended periods of time before it is dried, and sufficient growth of *S. aureus* may occur to produce enterotoxin (Troller and Stinson 1978; Valik and Görner 1993). *S. aureus* has been occasionally recovered in pasta products like macaroni, spaghetti, egg-noodles, etc. (Lee and others 1975; Eschment and Steuer 1977; Rayman and others 1981; Swartzentruber and others 1982; Woolaway and others 1986).

A high percentage (38.6%) of samples was qualitative positive for Enterobacteriaceae, which are indicators of hygiene and post process contaminatio. Further biochemical identification showed that *Citrobacter freundii*, *Enterobacter cloacae*, *Pantoea agglomerans*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Serratia* spp. were the predominant species. Although numerous samples contained Enterobacteriaceae, *Salmonella* spp. were not found in any of the samples.

However another pathogenic Genus within the family Enterobacteriaceae, *Cronobacter* spp. could be recovered from 14 samples (10.6%). Ten samples were domestic produce from Germany, 2 from Italy, and 1 each from Kazakhstan and China (Table 3). Each one isolate per sample was recovered for further investigations. Using a commercially available identification kit (API32E strips), the isolates were all identified at genus level as *Cronobacter* (*Enterobacter sakazakii*) with 99.9% ID (Table 3). Additional biochemical tests facilitated the classification of all presumptive *Cronobacter* isolates

into 6 different biogroups according to the scheme used by Farmer (1980) and Iversen and others (2006; Table 3). However, biochemical panels are not sufficient to correctly identify *Cronobacter* isolates (Iversen and Forsythe, 2004), therefore further identification using the *rpoB* sequence and MLST was employed. *C. sakazakii* was found to be the dominant species which is consistent with previous studies on occurrence of *Cronobacter* isolates from plant based dried foods (Iversen and Forsythe, 2004; Turcovský and others 2011; Chon and others 2012; Cetinkaya and others 2013). However, 2 isolates were *C. turicensis* and 1 was *C. muytijensii*, species which are somewhat less common. Furthermore, the species identification of 14 isolates was determined by the phylogenetic trees constructed by the neighbor-joining method based on the *rpoB* gene sequences (Figure 1), and in all cases the isolates clustering identical with the *Cronobacter* species delineating cluster. Among the 63 different *rpoB* alleles in the MLST database, some allelic

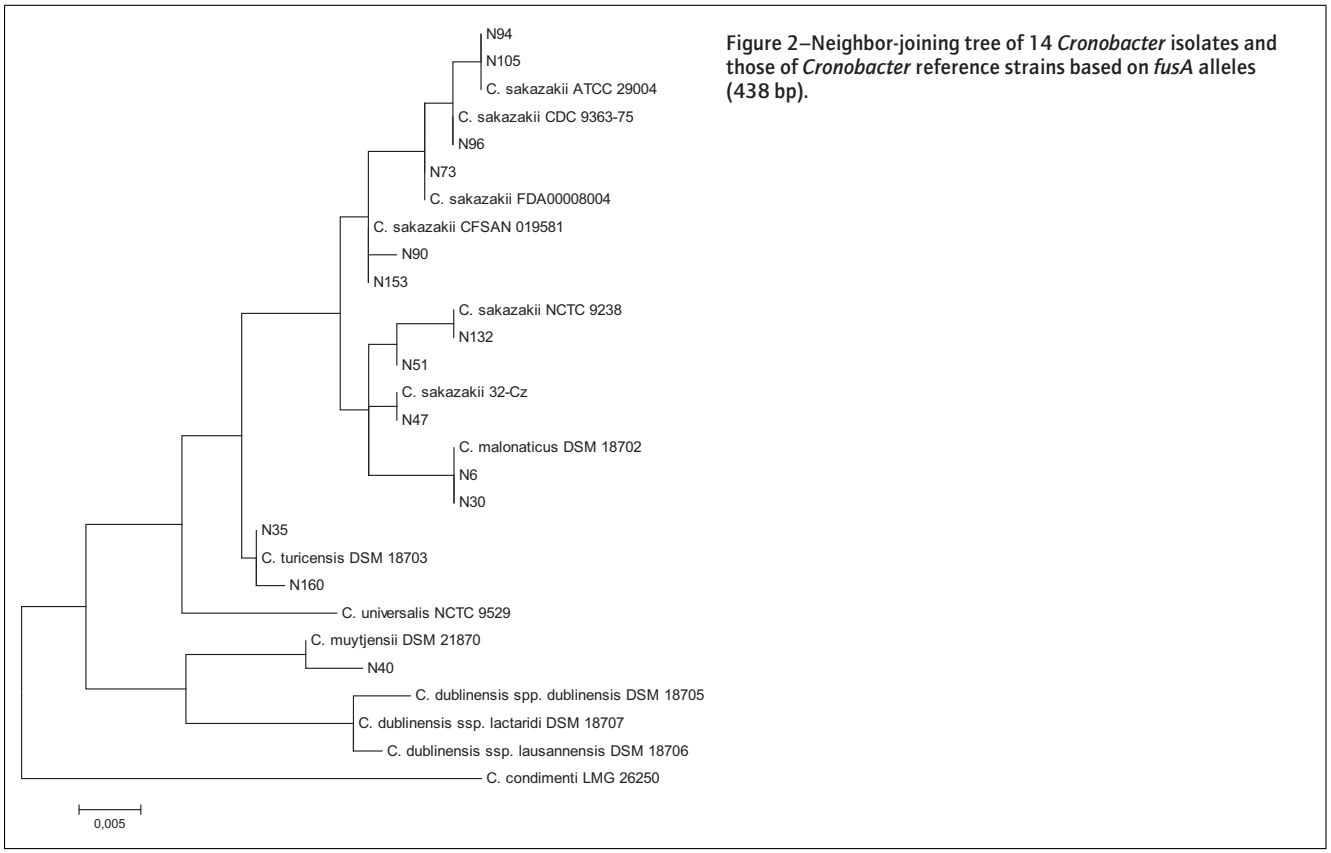
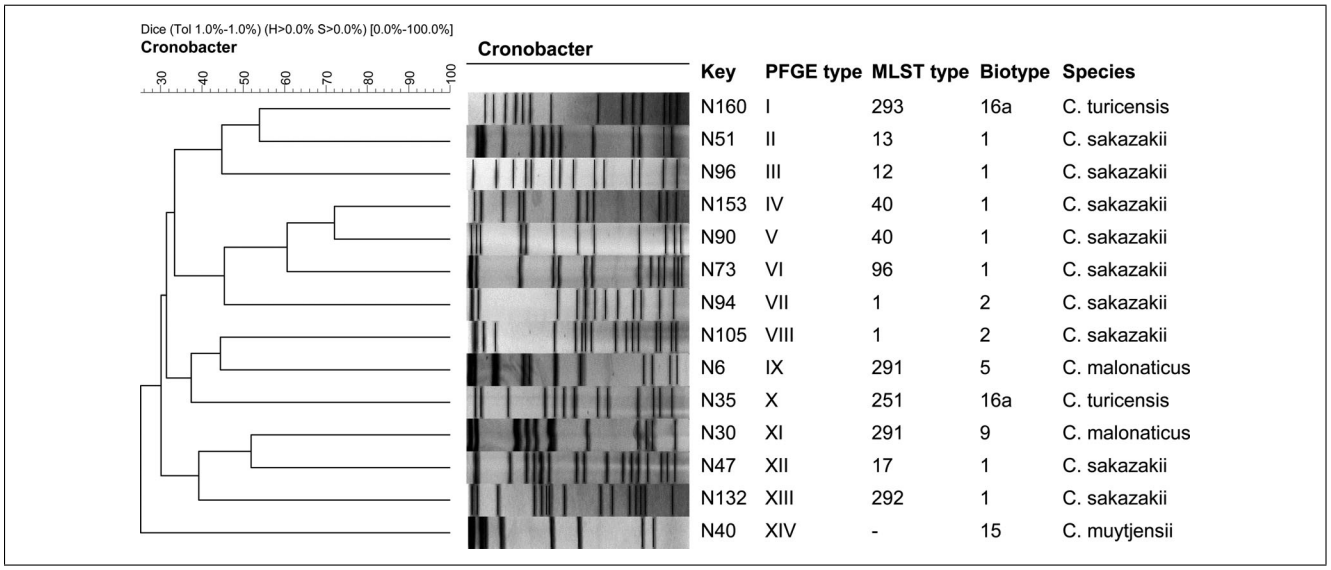


Figure 2–Neighbor-joining tree of 14 *Cronobacter* isolates and those of *Cronobacter* reference strains based on *fusA* alleles (438 bp).



Key	PFGE type	MLST type	Biotype	Species
N160	I	293	16a	C. turicensis
N51	II	13	1	C. sakazakii
N96	III	12	1	C. sakazakii
N153	IV	40	1	C. sakazakii
N90	V	40	1	C. sakazakii
N73	VI	96	1	C. sakazakii
N94	VII	1	2	C. sakazakii
N105	VIII	1	2	C. sakazakii
N6	IX	291	5	C. malonaticus
N35	X	251	16a	C. turicensis
N30	XI	291	9	C. malonaticus
N47	XII	17	1	C. sakazakii
N132	XIII	292	1	C. sakazakii
N40	XIV	-	15	C. muytjensii

Figure 3–A dendrogram obtained from cluster analysis (dice similarity coefficient setting both tolerance and optimization at 1%; unweighted pair-group average method) of PFGE patterns (*Xba*I) of 14 *Cronobacter* spp. isolates from 14 different samples of raw dried pasta.

profiles (rpoB 2, rpoB 3, rpoB 4, rpoB 5, rpoB 6, and rpoB 7) were shared between more than 1 species. This was observed for strain pairs from the following species: *C. sakazakii*/*C. muytjensii*, *C. sakazakii*/*C. turicensis*, and *C. sakazakii*/*C. condimenti* (Figure 1). As a consequence of the high degree of homology between these *Cronobacter* species, the *rpoB* sequence analysis was not sufficient to clearly distinguish between these species. Therefore, the isolates were further analyzed using MLST analysis, which correlates with whole genome phylogeny and provides a further means to differentiate between *Cronobacter* species. The MLST scheme for the entire *Cronobacter* genus is based on 7 housekeeping genes (*atpD, fusA, glnS, gltB, gyrB, infB, ppsA*), and *fusA* allele has been found to follow the 7 loci MLST phylogeny (Joseph and others 2012). The *fusA* alleles of 14 isolates were further compared by the phylogenetic trees constructed by the neighbor-joining method with those of the *Cronobacter* reference strains in the MLST database, and was found to be suitable for the unambiguous differentiation between species. Indeed, none of the profiles shown in Figure 2 was shared between 2 or more species. Nine isolates were grouped into the same cluster along with the *C. sakazakii* strains, each 2 isolates with the *C. malonaticus* type strain and the *C. turicensis* type strain, and 1 isolate with *C. muytjensii* type strain. The species identities of the isolates inferred by *fusA* alleles were in accordance with those obtained by biotyping and, except of isolates N90, N153, and N160 also by the partial *rpoB* sequences. The 14 isolates were heterogeneous by MLST and could be assigned into 9 different MLST sequence types (ST). Only partial overlap was observed between ST and biotypes, as reported by Baldwin and others (2009) and Joseph and others (2013), because several ST covered multiple biotypes and vice versa. Two *C. sakazakii* isolates (N94 and N105) which were isolated from different pasta samples manufactured in Italy and Germany, respectively, were assigned to the prevalent sequence type ST1. Furthermore, 1 isolate (N96) belonged to the sequence type ST12 which has been associated with necrotizing enterocolitis (Forsythe and others 2014). Other strains isolated in our study belonged to rare sequence types (ST13, ST17, ST40) which are represented by strains in the MLST database covering food, environmental, and clinical isolates, or by newly established sequence types (ST251, ST291, ST292, ST293). One *C. muytiensii* isolate (N40) could not be assigned to any ST type, because the *gltB* gene could not be amplified by primers given in the Cronobacter MLST database. The 2 isolates of biogroup 16, identified *C. turicensis,* were from 2 different packages of red pepper pasta (N35 and N160), and showed 2 distinct, newly established sequence types (ST251 and ST293). The PFGE results revealed a high degree of diversity between isolates, and a total of 14 different PFGE profiles for each isolate were obtained, with patterns of 9 to 15 DNA fragments ranging in size from 48.5 to 630.5 kb (Figure 3). In this study, 2 molecular typing methods, MLST and PFGE, were applied to further characterize the *Cronobacter* isolates showed the high diversity of the *Cronobacter* spp. isolates indicates the largely diverse environmental and geographic sources of contamination.

The ability of *Cronobacter* spp. to survive in dried pasta products may be due to their desiccation and heat tolerance traits (Breeuwer and others 2003; Dancer and others 2009). Our results are in agreement with previous reports (Iversen and Forsythe 2004; Friedemann 2007; Schmid and others 2009; Turcovský and others 2011; Cetinkaya and others 2013; Lou and others 2014) on the occurrence of *Cronobacter* species in plant-based foods such as grains, bread, sorghum, rice, seed, herbs, and spices. Presently there are no regulations for *Cronobacter* spp. in foods except of PIF, and there is also no published evidence for foodborne disease causation by *Cronobacter* spp. from other types of food. However, the recent studies of Patrick and others (2014) and Alsonosi and others (2015) suggest that there may be other clinically relevant sources for *Cronobacter* infections in elderly adults, and plant-based foods currently cannot be excluded. No clear evidence for a correlation between microbiological results and pasta type (soft wheat, durum wheat), other ingredients, or country of origin could be found, although *Cronobacter* spp. were more frequently isolated from durum wheat pasta than from soft wheat pasta. However, the number of samples was too small to draw definite conclusions.

Conclusions

Results for most microbiological parameters studied in raw dried pasta products from the German market indicated a satisfactory overall quality. Two findings seem to require further consideration, though. The levels of *B. cereus* found in a few samples are not far below of critical levels, in particular when considering toxinogenic strains. The frequent occurrence of *Cronobacter* spp. in raw dried pasta may justify a further risk assessment, including vulnerable consumers other than infants.

Authors' Contributions

Ömer Akineden contributed substantially for the study design, all of the laboratory study stage, data analysis and interpretation of the results, and manuscript writing and designing. Kristina J. Murata collected samples and performed microbiological tests except molecular study, data analysis, and interpretation of the results data with Ömer Akineden. Madeleine Gross supported language correction and evaluation of the molecular study and writing levels of this section and tables. Ewald Usleber designed the study, interpreted the results and revised the manuscript.

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