Isolation *of Cronobacter* spp. *(Enterobacter sakazakii)* from artisanal mozzarella

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Abstract

Cronobacter spp. (*Enterobacter sakazakii*) is an opportunistic bacterial pathogen capable of causing disease and even fatalities in newborn infants within the first weeks of life if consumed as part of the diet. Premature and immunocompromised newborn infants are at particular risk. The microorganism has been isolated from a variety of foods including contaminated infant milk formula powder and milk powder substitute. The study aimed to evaluate the level of microbiological contamination in 47 samples of mozzarella cheese made with cow's milk collected from artisan cheese producers in Southern Italy. Samples were collected from commercial sales points and underwent qualitative and quantitative microbiological analyses to test for the bacterial contaminants most commonly found in milk and cheese products. The 47 samples underwent qualitative and quantitative microbiological tests according to ISO UNI EN standards. Analyses focused on *Staphylococcus aures, Salmonella* spp., *Listeria monocytogenes, Pseudomonas* spp., *E. coli, Yersinia* spp., total coliforms and *Cronobacter sakazakii.* The ISO/TS 22964:2006 method was used to investigate possible contamination by *C. sakazakii*. Biochemical identification was carried out using an automated system for identification and susceptibility tests. None of the samples examined resulted positive for *Salmonella* spp. or *Listeria* spp. Only one sample resulted positive for *Staphylococcus aureus. Pseudomonas* spp. was isolated in 10 (21%) of 47 samples. High levels of total coliforms were found in 10 of 47 samples. *Cronobacter* spp. *(Enterobacter sakazakii*) was isolated in one sample.

This is the first study to confirm isolation of *C. sakazakii* in artisan mozzarella cheese made from cow's milk. The presence of *C. sakazakii* could be related to external contamination during the phases of production or to the use of contaminated milk. Since mozzarella is recommended in the diet of children and adults of all ages, this present study helps define it as a potential vehicle for *C. sakazakii* in subjects at particular risk.

Introduction

Mozzarella is a typical Italian cheese product made by stretching and kneading the drained curd mass. It is mainly made from cow's milk, but over recent years mozzarella made with buffalo's milk has becoming increasingly popular. There has been an increase in national demand and important commercial channels have also opened up both in Europe and toward third party countries, in particular the USA and Asia. This has led to significant changes being made in the industrial production system. However, traditional artisan production, even though carried out on a local level, still represents an important proportion of the commercialisation of milk and cheese products.

Like all milk products, mozzarella is subject to microbiological contamination that can lead to organoleptic alteration, or even to episodes of food poisoning. Studies have been carried out to determine the qualitative and quantitative levels of microbiological contamination in mozzarella made with cow's milk produced in traditional artisan systems in some parts of southern Italy. Besides the classical contaminants and pathogens, these studies also looked for *Cronobacter* spp. *(Enterobacter sakazakii*) that is currently considered an emerging opportunistic pathogen of food origin. This genus was originally described as *Enterobacter cloacae*, and after 1980 was named *Enterobacter sakazakii* (Farmer *et al.*, 1980). It has recently been reclassified as a new genus, *Cronobacter* spp. (Iversen *et al.*, 2007, 2008), consisting of seven species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. universalis*, *C. muytjensii*, *C. dublinensis*, and *C. condimenti* (Joseph *et al* muytjensii, dublinensis and genomospecies., 2012). This opportunistic bacterial pathogen is capable of causing disease and even fatalities in newborn infants within the first weeks of life if consumed as part of the diet. Still, not only are premature and immunocompromised newborn infants at particular risk, but also immunocompromised adults, particularly the elderly. Episodes of meningitis, sepsis, and necrotising enterocolitis (NEC) caused by *Cronobacter* spp. are the most frequent pathologies involved (Healy *et al.*, 2010) and they are almost always linked to contaminated milk powder consumption (van Acker *et al.*, 2001).

The microorganism has also been isolated in artificial milk, cereals, fruit and vegetables, meat (Kandhai *et al.*, 2004; Hunter *et al.*, 2008), legumes, herbs and spices, fresh, frozen, cooked and dried food (Chon *Kwang-Young Song* †*et al.*, 2012), drinks and beverages, and water used to prepare food (Friedemann, 2007). *C.sakazakii sakazakii* has also been isolated in cow's (Liu *et al.*, 2006) and donkey's (Conte and Passantino, 2008) milk. In a recent study by Hochel *et al.* (2012), *Cronobacter* spp. was isolated in 53 of 399 samples of commercialised food products and over half of the isolates (53%) were represented by the *sakazakii* species. Isolates from cheeses have been reported in various countries (Chavez-Lopez *et al.*, 2006; Morales *et al.*, 2004; Restaino *et al.*, 2006; Aigbekaen and Oshoma, 2010; Adeyemi, 2012; El-Sharoud *et al.*, 2008). In particular, it has been seen that *C. sakazakii* can survive for up to one month in cheese produced in experimental conditions with contaminated milk powder. This causes considerable concern for the microbiological safety of cheeses prepared with or containing milk powder (El-Sharoud *et al.*, 2008). The pathogen is capable of resisting osmotic pressure, high temperatures and drying (Fang *et al.*, 2012). These characteristics explain why it can survive the processes used in the production of infant milk powder and of other similar products (Nazarowec and Farber, 1997; Breeuwer *et al.*, 2003). Bacterial acid resistance at pH 3 (Edelson-Mammel *et al.*, 2006) has also been demonstrated and this explains why the pathogen can survive during transit in the newborn stomach.

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Materials and Methods

This study aimed to evaluate the risk of bacterial pathogenic contamination of mozzarella made with cow's milk. The study examined 47 samples from small traditional cheese producers in a well-defined area of southern Italy. These are characterised by small-scale, artisan

cheese production methods and a limited marketing area.

The production process uses cow milk either in crude form or heated to approximately 85°C for 10-15 seconds. Natural fermented milk whey is added to promote acidification and natural veal rennet is used for coagulation. The curd is then broken up into pieces, kneaded and stretched so as to make samples of mozzarella cheese of different weight and shape.

The product is then placed in brine, and packaged and sold in its original liquid or whey (water, salt and serum diluted acid). If stored in its original whey and kept in a refrigerator at a constant temperature, the product maintains its natural characteristics for 3-4 days.

The study was carried out over a 6-month period between November 2012 and April 2013. One sample from each producer was collected together with its original whey directly from the retail sales point. Samples underwent qualitative and quantitative microbiological tests according to ISO regulations for the following parameters: *Staphylococcus aures* (UNI EN ISO 6888-1:2004), *Salmonella* spp. (UNI EN ISO 6579:2008), *Listeria monocytogenes* (UNI EN ISO 11290-1:2005), *Pseudomonas* spp. (ISO/TS 1059:2009), *E. coli* (ISO 16649-2:2001), *Yersinia* spp. (UNI EN ISO 10273:2005), total coliforms (ISO 4832:2006) and *Cronobacter sakazakii*. Samples were examined for *C. sakazakii* using the ISO/TS 22964:2006 *Milk and milk products-Detection of Enterobacter sakazakii* isolation method involving the following phases: i) pre-enrichment in buffered peptone water and incubated at 37±1°C for 16-20 h; ii) enrichment in liquid medium with 0.1 mL inoculum in modified lauryl sulfate tryptose (mLST) broth/vancomycin medium and incubated at 44°C for 24 h; iii) addition in solid medium of selective *Cronobacter sakazakii* (ESIA) chromogene at 44°C for 24 h; iv), typical colonies were isolated and purifed on Trypticase Soy Agar (TSA) medium. To conclude, isolated strains are identified through single biochemical tests or through an automated system. In the present study the automated system has been used Vitek® (BioMérieux, Marcy l'Etoile, France).

Results

The study was carried out in the context of the typical problems and limitations of artisan food production systems. However, in spite of this, results from microbiological tests showed a generally acceptable level of health and hygiene for this type of cheese production. Table 1 shows the general results of the tests carried out on the 47 samples of mozzarella cheese, one sample from each of the artisan cheese producers taking part in the study.

Cronobacter sakazakii was isolated in only one mozzarella sample (2.1%), in association with other contaminants such as *Citrobacter freundii* and *Aeromonas hydrophila.* None of the samples tested positive for *Salmonella* spp. or *Listeria monocytogenes*. Only one sample tested positive for *Staphylococcus aureus* (>10^5 ufc/g) in the absence of enterotoxins. However, samples frequently tested positive for other pathogens: *Pseudomonas* spp (21%, represented by the species *P. aeruginosa, P. fluorescens* and *P. putida*), *Citrobacter* spp (21%, *C. braakii* and *C. freundii*), *Enterobacter* spp (17%, *E. cloacae, E. aerogenes* and *E. amnigenus*) and *Enterococcus* spp (36%, *E. faecium, E. faecalis, E. casseliflavus* and *E. gallinarum*). Samples also tested positive for *Klebsiella* spp, *Serratia* spp and *Aeromonas* spp.

Total coliform values of over 10^5 ufc/g were found in 29 (61.7%) of 47 samples analyzed while 13 of 47 samples (27%) had values of *E. coli* over 10^3 ufc/g. One sample of 47 was contaminated by *C. sakazakii* with 6.2 mg/100 g of furosin (-N-2-furoylmethyl-l-lysine), a molecule commonly used as an indicator to evaluate the effects of heat treatments applied to milk or the addition of milk powder or UHT to crude or pasteurised milk. The European Union maximum limit for mozzarella has been set at 10 mg in 100 g of protein substance (Regulation 2527/98/CE).

Antibiotic susceptibility was tested using a Kirby-Bauer agar disc diffusion method (Bauer *et al*., 1966). The strain isolated was sensitive to streptomycin, tetracycline, kanamycin, ofloxacyn and cephalothin, and was resistant to amoxicillin, vancomycin, rifampicin, ampicillin and lincomycin.

Discussion

Isolation of *C. sakazakii* in fresh mozzarella cheese produced in an artisan system sheds more light on food contamination by the bacterium from an epidemiological perspective. Indeed, its isolation shows to what extent consumers are exposed to the risk of diseases supported by the microorganism. Mozzarella is light and easy to digest, and because of this, is often recommended in the diet of subjects of all ages. It is particularly recommended for children, adults and bedriddens affected by a whole range of pathologies. Thus, tese subjects could be even more exposed to *C. sakazakii* infection if they eat contaminated mozzarella.

The mozzarella sample that was found to be contaminated with *C. sakazakii* had been produced with raw milk, collected and analyzed in the summer of 2012. Although this is a product that is eaten throughout the year, the biggest consumer demand is concentrated above all in the summer months because of the seasonal preference for fresh, light and easily digested foods. In these cases, mozzarella producers must, therefore, respond to the notable increase in demand precisely at that time of year when milk production is more seriously compromised by a series of negative factors, such as climate and seasonal food preferences (Mariani *et al.*, 1998; Summer *et al.*, 1998).

In the present study, isolation of *C. sakazakii* in a sample of mozzarella has also made it necessary to evaluate the origin of contamination.

The cheese factory involved was an authorised producer who had been approved according to EU regulations in force.

The producer declared that only fresh locally produced cow's milk is used. However, there have been repeated reports of the fraudulent addition of milk powder for zootechnical use reconstituted in the production of cheeses. This is an illegal practice carried out for economic reasons. But it can be proven through laboratory tests that can show the qualitative and quantitative modifications of some indicators, such as furosin, in commercialised milk – cheese products (Resmini and Pellegrino, 2003). The mozzarella sample that tested positive for *C. sakazakii* showed furosin levels within normal values (6.2 mg/100 g). This, therefore, excluded the possibility of contamination through the use of milk powder. Other possible origins of contamination by *C. sakazakii* were hypothesised to lie in the production process and the milk used.

Mozzarella production requires a large quantity of water, both during the actual production phase and in maintaining the natural conditions of the product for sale, by immersion in a mixture of water and whey.

Therefore, the use of water contaminated with *C. sakazakii* could be a probable hypothesis (Friedemann, 2007), also given the ability of some strains to stick to work surfaces made of silicon, latex, polycarbonates and steel (Iversen *et al.*, 2004).

The temperatures used during the production process of mozzarella from *not heat treated* milk are not usually able to affect any C. sakazakii contamination that may be present in the raw material, from where could also have origin the contamination of the final product.

In general, the qualitative and quantitative microbiological tests conducted on the 47 mozzarella samples showed microbial contamination as an expression of health and hygiene conditions (total coliforms, *E. coli*) in approximately 70% of the samples already at the beginning of the sales phase. Therefore, this seems to confirm that the origin of the contamination by *C. sakazakii* lies in the contamination of the milk destined for transformation or to external contamination during the production phases.

Table 1. Results of the microbiological tests performed on 47 samples of mozzarella produced and marketed by artisan dairies in Southern Italy.

Microbiological parameters	Number of positive samples (%)
Salmonella spp.	0
Cronobacter sakazakii	1 (2.1)
Listeria monocytogenes	0
Yersinia enterocolitica	0
E. coli	10 (21)
Staphylococcus aureus	1 (2.1)
Pseudomonas spp.	10 (21)
Total coliforms	29 (61.7)

Conclusions

All health and food agencies around the world agree that *C. sakazakii* represents a serious threat to subjects at risk (FDA, 2002; FAO/WHO, 2008). The same European Union regulation concerning food safety, and in particular Regulations (CE) 2073/2005 as amended by Regulation (CE) 365/2010, expressly require tests be carried out for *Enterobacter sakazakii* on infant milk powder. This has so far found contamination by this bacterium in 13 countries from among those where targeted investigations have been carried out. In order to evaluate the risk *C. sakazakii* presents for food health and safety, it is important to identify the food products that can act as a vehicle for this microorganism. Results of our research confirm that mozzarella cheese produced in artisan systems can potentially be subject to contamination by *C. sakazakii.*

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