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2900 100th Street, Suite 309, Des Moines, IA 50322-3855, USA

Loveskee Ramaswami, Ramona Duchenne-Moutien, Hiteyshini Ramgutty, Manisha Suharye and Hudaa Neetoo*

*Faculty of Agriculture, University of Mauritius, Réduit, Moka, 80837, Mauritius

Prevalence of Emerging Pathogens *Cronobacter* spp. and *Pantoea dispersa* in Low Moisture Foods

ABSTRACT

Until recently, members of the *Cronobacter* genus (formerly known as *Enterobacter sakazakii*) were a relatively unknown cause of human infections. *Cronobacter* spp. has since garnered significant attention as a major microbial contaminant of low moisture foods (LMFs). This study was conducted to survey the worldwide contamination of LMFs with *Cronobacter* spp. from 2002 to 2019 by using HorizonScan (an online surveillance tool) and to assess the prevalence of this pathogen in LMFs in the local food supply chain of Mauritius. LMF samples (n = 302) were collected from local retail outlets and subjected to microbiological analyses and molecular identification. HorizonScan recorded 31 notifications worldwide for LMFs contaminated with *Cronobacter* spp. Presumptive *Cronobacter* spp. were isolated from 35% of local LMF samples but were subsequently confirmed to be *Pantoea dispersa*. This report is the first for *P. dispersa* in LMFs in Mauritius, and this organism is considered an emerging opportunistic human pathogen. Wheat flour was identified as an important vehicle for *P. dispersa* with a contamination rate of 100%. Because LMFs are an integral part of the human diet and are consumed almost daily, these commodities should be monitored for *P. dispersa*.

INTRODUCTION

Traditionally, foodborne pathogens and disease outbreaks have been associated with high moisture foods because water favors microbial growth. As a result, more control interventions have targeted high moisture foods due to the misconception that low moisture foods (LMFs) are generally safe with a low risk of contamination by microbiological hazards *(18)*. LMFs are foods having a water activity (a_w) of < 0.70 *(11)*. Examples of naturally low moisture products are flour, cereals, and nuts. Many factors can influence the exposure of LMFs to microbial hazards in the environment, including soil, air, dust, water (irrigation and rain), wild animals (including birds and pests), people, and equipment *(21)*. Most LMFs are derived from plant material and are therefore in contact with the soil and the environment *(21)*. *Cronobacter*, a gram-negative bacterium of the *Enterobacteriaceae* family (formerly *Enterobacter*

*Author for correspondence: Phone: +1 230.403.7885; Fax: +1 230.465.5743; Email address: s.neetoo@uom.ac.mu

sakazakii), is considered an emerging foodborne pathogen, and plant material is considered its highly probable vehicle of transmission *(14)*. Because this organism is ubiquitous in the environment *(7)*, it may survive for long periods in dry foods, making it a pathogen of concern *(22)*. *Cronobacter* is listed among pathogens under the category "severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration" *(24)*. Because LMFs such as cereals, grains, and spices are widely traded food commodities *(13)*, surveillance of the global LMF supply chain for *Cronobacter* contamination is important. Mauritius is a net food importer *(25)*, importing many essential food requirements, including LMF commodities. However, to our knowledge no studies have been conducted on the occurrence of *Cronobacter* spp. in LMFs in Mauritius. The objectives of this study were (i) to determine the prevalence of *Cronobacter* contamination in the global LMF supply chain through use of an online surveillance database and (ii) to assess the prevalence of *Cronobacter* in LMFs locally available in Mauritius.

MATERIALS AND METHODS

Notifications of *Cronobacter* contamination worldwide

HorizonScan (version 2.1.6, FERA Science, Sand Hutton, York, UK) was the online database of choice for this study of global food contamination issues. This food safety database is a global surveillance and tool for tracking contamination and adulteration of food ingredients and products in international commerce. The database was used to retrieve a history of recalls or other notifications of *Cronobacter* contamination of food products. To perform an unrestricted search and to obtain the maximum number of notifications, "*Cronobacter*" was the search term, the option "all data" was selected, and all other fields were purposely left blank.

Sampling and a_w determination of commercially available LMFs

Samples of LMFs (n = 302) for 10 broad food categories (cereals, dried adult milk, dried noodles, flour, infant foods, nuts, pulses, spices, seasonings, and tea) were purchased from supermarkets in Mauritius. The samples were kept in their original packaging at room temperature away from sunlight to prevent degradation or change in the microbiota of the food. The a_w of the samples was determined with a calibrated a_w meter (Novasina, Lachen, Switzerland) with triplicate measurements and two independent replicates.

Microbiological analysis of LMFs for *Cronobacter* spp.

Cronobacter spp. isolates were recovered according to the protocol of Berthold-Pluta et al. *(6)*, and the sensitivity of the method was improved by increasing the analytical sample size from 10 to 25 g. The 25-g samples of each product were weighed aseptically, mixed with 225 mL of buffered peptone water, and incubated at 37°C for 18 to 24 h. The primary mixture was then enriched in *E. sakazakii* enrichment broth *(26)* for 24 h, plated on selective and chromogenic HiCrome *E. sakazakii* agar (HiMedia, Mumbai, India), and incubated at 37°C for 24 h. The selectivity of the chromogenic medium is based on the activity of α-glucosidase, which is produced by all strains of *Cronobacter (16)*. Deep blue colonies were streaked onto tryptic soy agar (TSA; HiMedia) and incubated at 26°C for up to 72 h. Yellow colonies on TSA were suspected to be *Cronobacter* spp. and were picked up for Gram staining and microscopic examination. Pale yellow colonies that appeared after 72 h were incubated for another 24 h until colonies appeared deep yellow. According to Garbowska et al. *(16)*, the yellow pigmentation is characteristic of the majority of *Cronobacter* strains. Growth of yellow colonies on TSA is currently used as a criterion for identification of presumptive *E. sakazakii* isolates *(29)*, but the intensity of pigmentation differs among strains *(12)*.

Molecular identification of presumptive *Cronobacter* isolates

Presumptive *Cronobacter* isolates were identified based on the formation of uniform deep blue colonies on HiCrome *E. sakazakii* agar and intense yellow colonies on TSA. Five isolates from wheat flour, black pepper, masala powder, black tea powder, and dried milk were randomly selected for further identification by a PCR assay. Genomic DNA was extracted according to the phenol-chloroform protocol of Ausubel et al. *(5)* followed by DNA quantification by UV spectrophotometry (Spectronic 1201, Milton Roy, Lenexa, KS). For PCR identification of the isolates at the genus level, a pair of primers were designed targeting a 438-bp fragment of the gyrase B gene (*gyrB*) of *Cronobacter*: gyrB-F: 5′-ATGGATAAAGAGGGCTACAG-3′ and gyrB-R: 5′-GCCTGATTCTTACGGTTAC-3′ *(1, 2)*. The PCR thermocycling program was predenaturation at 95°C for 5 min; amplification in 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s; and final elongation at 72°C for 10 min *(8)*. The amplification products were analyzed by electrophoresis on a 1.5% agarose gel with a DNA size ladder. The gel was stained with ethidium bromide, visualized with a UV transilluminator, and photographed. PCR products for the *gyrB* gene were subsequently sent to Inqaba Biotec (Pretoria, South Africa) for purification and sequencing with the Sanger dideoxy sequencing technology. The forward and reverse sequences obtained from Inqaba Biotec were processed with the BioEdit sequence alignment editor (version 7.0.5.3, Informer Technologies, https://www.informer.com/) to yield a consensus sequence. The consensus sequences were verified and manually corrected where needed. A BLAST search on the NCBI website (https://blast.ncbi.nim.nih.gov/Blast.cgi) was done to compare the partial *gyrB* sequence with sequences in the NCBI GenBank database.

Statistical analysis

An analysis of variance (ANOVA) with significance defined at $P \leq 0.05$ was used to determine whether there were significant differences in the a_w and in the prevalence of pathogen contamination among the study samples. The Tukey-Kramer honestly significant difference post-hoc test was conducted to determine which pairwise differences in the a_w of samples were significant at $\alpha = 0.05$. We used Prism software (version 9.0.0, GraphPad, San Diego, CA) for the analysis. Associations between food product type and a_w (explanatory or predictor variables) or pathogen contamination (explained or dependent variable) were explored with a binary logistic regression analysis. Logistic regression is most commonly used to model such a binary response (i.e., presence or absence). For this analysis, we dichotomized the dependent variable, where absence was coded as 0 and presence was coded as 1. Because the explanatory variable of food product type was categorical, it was converted to a numeric variable by assigning a code to each product type. For the 22 categories (i.e., 22 product types), we used 21 dummy variables to contrast the different categories and used powdered infant formula as the reference category. Differences were considered significant when the model chi-square *P*-value was ≤ 0.05, and all reported *P*-values were two tailed. The resulting unstandardized coefficient B and the standardized regression coefficient EXP(B) were reported to quantify the type of association between the predictor and the dependent variable. Regression analyses were performed with SPSS for Windows (release 19, SPSS, IBM, Armonk, NY).

RESULTS

Notifications of *Cronobacter* contamination worldwide

The HorizonScan review resulted in 31 incidences of recalls or border rejections worldwide related to food contaminated with *Cronobacter* spp. from 2002 to 2019. The only LMFs incriminated were infant formulae, cereals, milk, and baby food. The countries of origin for these products were identified as Belgium, Denmark, Germany, Israel, The Netherlands, Poland, Spain, Switzerland, Turkey, and Uganda.

Laboratory analyses of local LMFs

The a_w of LMF samples was 0.226 to 0.615; instant cereal had the lowest a_w (0.226) and Kalia spice mix had the highest a_w (0.615) *(Table 1)*. Presumptive *Cronobacter* isolates were recovered from cereals, dried milk, pulses, seasonings, spices, tea powder, and wheat flour based on the formation of characteristic deep blue and intense yellow colonies on HiCrome *E. sakazakii* agar and TSA, respectively. However, the organism was not recovered from dried noodles, nuts, and powdered infant formula. PCR amplification of genomic DNA extracted from the five presumptive *Cronobacter* isolates resulted in amplicons of the expected size of 438 bp (data not shown). PCR products that yielded very discrete and pronounced bands corresponding to 438 bp were selected for purification and sequencing. BLAST sequence analysis identifies sequences that are identical or similar *(40)*. Upon sequence comparison, 100% similarity was noted between the isolate sequences and the *gyrB* gene of *Pantoea dispersa*, another member of the *Enterobacteriaceae* family, whereas similarity was only 89% with *C. sakazakii*. The *gyrB* gene present in *Pantoea* and *Cronobacter* probably descended from a common ancestor. To our knowledge, this report is the first of *P. dispersa* in LMFs in Mauritius. The prevalence of this novel organism in the broad food categories was in the following decreasing order: flour (100%) > tea (70%) > cereals (55%) > seasonings (40%) > spices (29%) > dried milk (30%) > pulses (20%) *(Table 1)*. However, the samples of noodles, nuts, and infant foods were free of *P. dispersa*. The prevalence of this microorganism in the various food categories and specific products is summarized in *Table 1*.

The one-way ANOVA revealed a significant difference ($P \leq 0.05$) in the prevalence of *P. dispersa* contamination in the samples in this study. Results of the logistic regression analysis indicated that only the predictor "food product type" was statistically reliable ($P \leq 0.05$) for predicting the presence of *P. dispersa*. The logistic regression model was significant ($P \leq 0.05$), with a Nagelkerke pseudo R^2 value of 0.756, indicating that the model explained ca. 75% of the data. The estimates of regression coefficients B, Wald statistic, *P*-values, and EXP(B) for the predictor are presented in *Table 2* for each product type. Because powdered infant formula was chosen as the baseline or reference category, all categories were contrasted with this baseline. The EXP(B) values, also known as the odds ratios, for wheat flour and wheat biscuits were as high as 323,094,969. Therefore, we infer that with respect to powdered infant formula the likelihood of *P. dispersa* contamination of wheat flour and wheat biscuits was multiplied by a factor of 323,094,969. However, all other food products had EXP(B) values (odds ratios) of < 1; therefore, the likelihood of contamination of these products was less than that of powdered infant formula. In other words, certain food product types were more likely than others to become contaminated with *P. dispersa*.

DISCUSSION

This study focused on the contamination of LMFs by *Cronobacter*, an emerging human pathogenic microorganism that persists in dry food processing environments *(19, 27)*. HorizonScan, an online surveillance database, was used to find international food recalls and border rejections linked to *Cronobacter* spp. in LMFs. The analysis revealed only 31 notifications since 2002, and all incriminated LMFs were infant foods, cereals, and milk products. This low prevalence is congruent with data retrieved by the EU-RASFF alert system for 2010 to 2014 *(10)*; only one recall or border rejection was noted incriminating cereals and grains *(13)*.

TABLE 1. Prevalence of *P. dispersa* in local LMFs by food product types. Mean water activity values with the same letters are not significantly different ($P \leq 0.05$)

Food product	Water activity	No. of positive samples	No. of samples tested	% positive samples
Cereals		22	40	55
Wheat biscuit	0.557 ± 0.0014 EF	10	10	100
Cornflakes	0.549 ± 0.0031 DE	2	10	20
Instant cereal	0.226 ± 0.0000 A	3	10	30
Oatmeal	0.512 ± 0.0141 DE	7	10	70
Flour				
Wheat flour	0.581 ± 0.0219 EF	40	40	100
Spices		12	42	29
Masala powder	0.435 ± 0.0131 C	4	6	67
Chili powder	0.345 ± 0.0233 B	0	6	0
Tandoori mix	0.433 ± 0.0007 C	2	6	33
Haleem mix	0.325 ± 0.0276 B	1	6	17
Kalia mix	0.615 ± 0.0283 F	5	6	83
Turmeric powder	0.609 ± 0.0016 EF	0	6	0
Coriander powder	0.549 ± 0.0042 DE	0	6	0
Seasonings		8	20	40
Black pepper	0.604 ± 0.0156 EF	8	10	80
Noodle seasonings	0.409 ± 0.0163 C	0	10	0
Pulses		4	20	20
Red lentils	0.455 ± 0.0240 C	3	10	30
Split yellow chickpeas	0.494 ± 0.0043 CD	1	10	10
Milk				
Dried adult milk	0.355 ± 0.0049 B	12	40	30
Noodles				
Dried noodles	0.474 ± 0.0068 CD	0	10	0
Tea				
Black tea powder	0.607 ± 0.0240 F	7	10	70
Infant foods				
Powdered infant formula	0.373 ± 0.0077 B	0	40	0
Nuts				
Almonds	0.573 ± 0.0057 EF	0	20	0
Peanuts	0.607 ± 0.0035 F	0	20	0
TOTAL		105	302	35

In this study, the a_w of LMF samples from local retail outlets was 0.226 to 0.615. As noted by Farakos and Frank *(11)*, these dry products could also be considered low water activity foods because the readings were consistently < 0.700. Low a_w renders food unfavorable for growth as most gram-negative bacteria, which require an a_w of ≥ 0.95 to be metabolically active *(23)*.

Contrary to our expectations, presumptive *Cronobacter* isolates from LMF products were subsequently identified as *P. dispersa* after sequencing part of the gene *gyrB*, which is highly conserved in the *Enterobacteriaceae* and acts as a phylogenetic marker *(28, 37, 39)*. Analysis and comparison of sequences revealed 89% similarity with the *gyrB* of *C.*

TABLE 2. Prediction of *P. dispersa* in low moisture foods with binary logistic regression

Food products	B	Wald	Significance	EXP(B)
Cereals				
Wheat biscuit	19.593	0.000	0.999	323,094,968.600
Cornflakes	–2.996	4.917	0.027	0.050
Instant cereal	–2.457	3.601	0.058	0.086
Oatmeal	–0.762	0.347	0.556	0.467
Flour				
Wheat flour	19.593	0.000	0.998	323,094,968.600
Spices				
Masala powder	–0.916	0.431	0.512	0.400
Chili powder	–22.812	0.000	0.999	0.000
Tandoori mix	–2.303	2.719	0.099	0.100
Haleem mix	–3.219	4.317	0.038	0.040
Kalia mix	–22.812	0.000	0.997	0.000
Turmeric powder	–22.812	0.000	0.999	0.000
Coriander powder	–22.812	0.000	0.999	0.000
Seasonings				
Black pepper	–0.223	0.027	0.869	0.800
Noodle seasonings	–22.812	0.000	0.999	0.000
Pulses				
Red lentils	–2.457	3.601	0.058	0.086
Split yellow chickpeas	–3.807	6.270	0.012	0.022
Milk				
Dried adult milk	–2.457	4.576	0.032	0.086
Noodles				
Dried noodles	–22.812	0.000	0.999	0.000
Tea				
Black tea powder	–0.762	0.347	0.556	0.467
Nuts				
Almonds	–22.812	0.000	0.998	0.000
Peanuts	–22.812	0.000	0.998	0.000

sakazakii and 100% similarity with the *gyrB* of *P. dispersa*, indicating that these two genera once shared a common ancestor. Other authors had also similarly isolated putative *Cronobacter* or *C. sakazakii* from food samples, which were subsequently confirmed as *Pantoea* spp. *(9, 16)*.

Findings of this study clearly point to the wide distribution and prevalence of *Pantoea dispersa* in dry foods commercially available in Mauritius; 105 (35%) of 302 samples tested positive. The prevalence of *P. dispersa* in these sampled commodities was ranked in the following decreasing order: wheat flour = wheat biscuits (100% of sample contaminated) > black tea powder (70%) > cereals (55%) > seasonings (40%) > dried adult milk (30%) > spice mixes (29%) > pulses (20%). Statistical analysis revealed that *P. dispersa* contamination was significantly associated with food product type and that wheat flour and wheat biscuits were far more likely to become contaminated with *P. dispersa* than were other products. The ubiquitous nature of *Pantoea* in the food supply thus makes it an emerging pathogen of potential concern. In other studies, *P. dispersa* has been isolated

from plants *(17)*, fruits and vegetables *(38)*, infant formula *(30)*, and flour *(15)*. However, in contrast to the findings of Oonaka et al. *(33)* and Mardaneh and Dallal *(30)*, we did not detect *P. dispersa* in infant formula, suggesting that the factories producing this product likely conformed to all hygienic and food safety standards, including adequate pasteurization of raw materials, ensuring absence of microbiological contaminants in heat-labile ingredients, and adequate cleaning and sanitization of processing equipment.

Pantoea dispersa has been described as an opportunistic or secondary pathogen; therefore, infections are likely to be very mild or self-limiting in most individuals *(35)*. Nevertheless, recent studies have revealed the presence of this pathogen in health care settings *(3, 4, 20, 32, 34, 36)*, where it has been associated with various pathologies including lung infection, bacteremia, neonatal infections, and septicemia. Hence, caution should be exercised during the handling of LMFs in neonatal care, health care settings, and homes for the elderly. Because this pathogen was frequently isolated from wheat flour and cereals, public health authorities should institute hygiene education programs at the consumer level. These programs should be used to raise awareness of the health risks associated with *P. dispersa* in LMFs, especially ready-to-eat cereals that are often consumed without a heat-killing step. Currently, no microbiological standards have been developed for *Pantoea* spp. in any of the food products mentioned in the Mauritius Food Regulations *(31)*. Because *P. dispersa* is an emerging foodborne pathogen, a review of the Mauritius food standards is needed.

CONCLUSIONS

HorizonScan was a suitable database for monitoring international food contamination events linked to *Cronobacter* spp. in a variety of LMFs. In all, a relatively small number of notifications *(31)* were recorded from 2002 to 2019, suggesting that current processes for control or elimination of *Cronobacter* spp. are effective. However, contrary to our expectations presumptive *Cronobacter* isolates from local products in Mauritius were identified as *P. dispersa* after molecular analysis. This study is the first to report the presence of *P. dispersa* in the dry food supply chain of Mauritius. Overall, 35% of food samples were positive for *P. dispersa*, with 100% prevalence in wheat flour. *P. dispersa* is an emerging pathogen worthy of concern, and findings of this study underscore the need for routine microbiological testing of LMF products for *Pantoea* spp. The Mauritius food regulations should be revised to include microbiological reference criteria for *P. dispersa* for specific foods such as flour, dried milk, and cereals.

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