ISSN Online: 2456-4192

Listeria Contamination in Chocolate: A Case Report

Roshan Kumar [1]*, Avinash Upadhyay [2], Archana Mehta [1]

[1] Department of Botany, Dr. Hari Singh Gour Vishwavidyalaya, Sagar 470003, MP, India
[2] Hislop School of Biotechnology, Hislop College, Nagpur 440001, Maharashtra, India

Received 10 June 2016; accepted in revised form 04 July 2016

Abstract: A survey was conducted on a branded chocolate bars of company A, which were collected from the local and the nearby places of Sagar, Madhya Pradesh. The samples collected were checked for the presence of microbial growth viz., coliforms, and *Enterobacteriaceae*. 11 % of the sample showed the presence of microbes in wrapped chocolates. Two isolates were pure cultured, which were further characterized morphologically, biochemically and by 16S rRNA gene sequencing. The two isolated were identified as *Listeria denitrificans* and *Kurthia* sp. The outgrowth of these pathogenic microorganisms is a matter of great concern as despite taking care all the precautions these microbes have evolved themselves with the capability which enables them to regrow on milk chocolate and can catastrophically affect human body by causing nervous breakdown. Repeated occurrence also highlight it to be used as an indicator in milk/ food industry.

Key words: Chocolates, *Listeria denitrificans*, *Kurthia.*

Introduction

The modern chocolate manufacturers take all necessary care for the selection of raw materials, both edible and packing. Chocolates are manufactured in a state of the art automated factory untouched by human hands. All the products are stringently checked before leaving the factory. The various processes involved in the manufacturing of chocolates start with the roasting of cocoa beans at 130-140°C. The crushed cocoa nibs are mixed carefully and sweetened using condensed milk. The paste so formed is cooked at 70-80°C for 7 h. The liquid chocolate so made goes through the process of conching at 52-55°C for 6 hours before it goes to mold [1]. Most manufacturers follow the internationally accepted HACCP (Hazard Analysis & Critical Control Point) program, which is the most comprehensive food safety system [2,3]. Although we have cited above the manufacturing process of chocolate followed by most manufacturers, the procedure seems rigorous enough to get rid of contaminating microorganisms, cited below is the data on the growth and survival of *L. monocytogenes* which is quite disturbing. There are seven species of *Listeria*: *L. monocytogenes, L. ivanovii, L. innocua, L. welschimeri, L. seeligera, L. grayi* and *L. murrayi*. Among the above named, the hemolytic species are pathogenic for human beings. These are *L. seeligeri, L. ivanovii,* and *L. monocytogenes* [4,5]. The last named is the main pathogenic species amongst the three. So much so that all colonies of this species must be considered as potentially pathogenic for humans. It is also known that *Listeria monocytogenes* is a common contaminant in milk products and thus it can be one of the contaminants of the milk chocolates [6-8].

In the recent past, there were quite a hue and cry about worms that were found in the milk chocolates manufactured by a particular company. Quite

*Corresponding author (Roshan Kumar)
E-mail: < roshankumar4u@gmail.com >

a bit of newsreel was devoted to this outrage and the print media also gave it a wide coverage. However, the company blamed it on the storage conditions that were not optimum and left much to be desired. It also threw open its state of the art facilities of manufacture to investigate. Quite soon, these charges just lost the novelty value associated with them and the incidence (at least of reporting the findings) of worms in the chocolate also became almost non-existent. The one thing that was missing in this entire episode was the possible microbial contamination.

Materials and methods

Sampling

Twenty seven samples of chocolate bar (dark chocolate) of company A, were collected from the nearby places of Sagar, Madhya Pradesh and were evaluated for the presence of microbial growth on the milk chocolate. Samples were collected randomly from the city and nearby places targeting the product of company A. All samples were kept immediately in the mini-refrigerator at 4^{o}C to avoid changes in temperature.

Isolation and identification

The qualitative analysis of the milk chocolate was performed under the laminar flow cabinet in a sterile environment. After unwrapping of chocolate, it is cut into small piece of 1 cm x 1 cm (approx.) using the scalpel and placed on the Nutrient agar medium and maintained at 37°C for 24 h. Pure culture was prepared after repeated sub-cultured in the Nutrient agar medium supplemented with sucrose (5 g/l). Detection of coliforms was checked by growing cultures, and the pure culture was checked by sub-culturing the isolated microorganisms on HiChrome Coliform Agar (Himedia, M1300) and incubated at 37°C for 24 h. Enumeration of *Enterobacteriaceae*, was done by pour plate method by overlaying of Violet Red Bile agar with Glucose (Himedia, M049) with an incubation of 37°C for 24 h [9].

Biochemical and molecular identification

The isolated microorganisms were processed for biochemical characterization and identification. A series of test were performed starting from Gram staining to the most specific tests following the keys stated in the Bergey (1934) [4]. 16S rRNA identification was made by isolation of genomic DNA using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems). PCR and sequencing was done for ~ 550 bp of 16S rRNA gene using illumina sequencer. Bidirectional sequencing was performed with water as a control.

Results and discussion

Chocolate

Out of 27 chocolate bars collected, 11 % of the samples were found to be contaminated in the initial screening and were isolated on Nutrient agar medium. *Enterobacteriaceae*, coliforms or *Escherichia coli* were not detected in the test conducted. The product of company A, when tested, only three chocolate bars seem to be infected. Infected milk bars when plated on the medium showed microbial count in the range of 1.21-1.77 log cfu/g. Each colony when plated on the medium represented three definite colonies. Isolate 1, typically shown arborescent type colony with no pigmentation whereas, the other microbes showed pin-point type colony with yellow pigmentation in Nutrient agar plate.

Biochemical characteristics

Various staining procedures such as Gram-stain, negative stain, capsule stain, endospore stain, and acid fast stain were used to characterize isolated bacteria. Results obtained from such staining techniques are represented in Table 1.

Nutrient agar medium with added carbon sources such as glucose, sucrose, mannitol and fructose, was checked for their effect on the growth of the isolated bacteria. After the incubation of bacteria its effect was checked and is recorded in Table 2.

Media optimization

Optimization of growth medium was performed on Luria Bertani, Trypticase soya agar, Mannitol salt agar and Nutrient agar. After an incubation of 24, 48 and 72 h, growth of the bacteria was observed (Table 3). It is evident from the above results that, sucrose plays an important role in the growth of the bacteria, however, it also helped to regain arborescent pattern of isolate 1, which was

Table 1. Basic morphology and biochemical characteristics of both the isolates

Basic Morphology	**Isolate 1**	**Isolate 2**
Shape	Long rod	Long rod
Morphology	Forms chain usually in 2-3	Forms different irregular shapes
Oxygen requirement	Facultative	Strictly Aerobic
Growth at low temperature	0-4°C	ND
Biochemical Tests	**Isolate 1**	**Isolate 2**
Gram Staining	+	+
Motility	+	+
Negative stain	Long rod	Long rod
Capsule stain	-	-
Endospore	-	-
Acid fast stain	-	-
Catalase test	+	+
Gelatin hydrolysis	-	-
Esculin hydrolysis	+	+
H2S production test	-	-
Blood Agar	-	α-hemolysis
CAMP test (*S. aureus*)	-	ND

Positive (+)
Negative (-)
ND (not determined)

Table 2. Sugar fermentation test

Observation	**Isolate 1**		**Isolate 2**	
	Acid production	**Gas production**	**Acid production**	**Gas production**
Dextrose	+	-	+	-
Fructose	+	-	+	-
Lactose	-	-	+	-
Maltose	-	-	-	-
Mannose	-	-	+	-
Mannitol	-	-	-	-
Sucrose	+	-	+	-
Xyose	+	-	-	-

Positive (+); Negative (-)

lost in sub-culturing on Nutrient agar medium.

Results obtained showed that isolate 1 is a Gram-positive rod, showing no pigmentation in petri plates, with arborescent type colony pattern, facultative, ferment glucose, fructose, sucrose, catalase positive, and esculin positive. Isolate 2 was a Gram-positive rod, yellow pigmenting with small pinpoint colony, strictly aerobic, ferment many sugars such as glucose, catalase positive, and esculin positive. As per the key pro-

Table 3. Effect of various growth media on the growth of isolated bacteria

Culture Media	Growth of bacteria isolate 1			Growth of bacteria isolate 2		
	24 h	48 h	72 h	24 h	48 h	72 h
Luria Bertani Agar	++	++	++	++	++	++
Trypticase soya agar	+	++	++	+	++	++
Mannitol Agar	+	+	+	+	+	+
Nutrient Agar	+++	+++	+++	+++	+++	+++

Heavy growth (+++); Medium growth (++)
Low growth (+)

vided by the information in the Bergey's manual of Determinative Biology, this experiment concluded isolate 1 as *Listeria* sp. whereas isolate 2 is *Kurthia* sp. The same was confirmed when 16S rRNA sequence was blasted. Isolate 1 was found 99 % similar to *Listeria denitrificans* and isolate 2 displayed 97 % similarity to *Kurthia* sp. 8.

It is also known that *Listeria monocytogenes* is a common contaminant in milk products and thus it can be one of the contaminants of the milk chocolates [10,11]. Although the optimum temperature for growth of *L. monocytogenes* is 30-37°C, these pathogens can survive well between 0-45°C. They can survive well anywhere between pH 4.3 to 9.6; there is data available to say that these pathogens can survive 25 days at 4°C with pH 3.6. It grows well in a medium containing 10-12 % of NaCl, and can survive in a medium with 20-30 % NaCl. In a report, it was found that *L. monocytogenes* can survive 132 days at 4°C with 25.5 % of NaCl. The same can be said for the sugar concentration as well. They can develop in vacuum-packed products or when packed under nitrogen. Freezing has no destructive effect on these germs. On the contrary, at low temperature, they can grow and become the major flora in the food product concerned.

Obvious from the above should be the fact that *L. monocytogenes* is a microorganism that can survive well in chocolate and other dairy foods, even when the conditions followed to manufacture them are as rigorous as outlined above. The following is the list of outbreaks of listeriosis due to food contamination in the Western world. The list begins with the first outbreak in 1981, the year that it was first demonstrated that *Listeria* can be transmitted through food.

- 1981 : **41 cases in Canada** (18 deaths) - coleslaw (first demonstration of *Listeria* transmission through foods) [12].
- 1983 : **49 cases in the USA** (14 deaths) - pasteurized milk [13].
- 1985 : **142 cases in the USA** (48 deaths) - fresh pasteurized cheese [14].
- 1983-87 : **122 cases in Switzerland** (34 deaths) Vacherin cheese [15].
- 1987-89: **> 350 cases in GB** (> 90 deaths)- pies [16].
- 1992 : **279 cases in France** (63 deaths) - jellied pork tongue [15].
- 1993 : **38 cases in France** (11 deaths) – rillettes (type of paté) [17].
- 1994 : **45 cases in USA** - milk chocolate [18].
- 1995 : **37 cases in France** - Brie of Meaux (cheese) [19].
- 1997 : **15 cases in France** - Livarot, Pont-l'Evêque, Pavé d'Auge (cheeses) [19]
- 1998 : **98 cases in the USA** (22 deaths) - hot dogs and delicatessen meats [20].

Obvious from the above is the fact that most of these cases are with respect to milk and milk products. There are just a few exceptions. Given above are only a few cases of reported outbreaks of listeriosis. There have been numerous instances where, before any outbreaks could occur, the company concerned recalled the food in which *Listeria* contamination was detected.

Consider the following facts

- *Listeria* can be found in a variety of dairy products, vegetables, fish and meat products.
- *Listeria* unlike most other harmful bacteria will grow slowly on foods stored in a refrigerator. This presents the real danger from its contamination.
- *Listeria* can also be spread by contact with an infected product or surface, such as hands or counter tops, during food preparation.
- *Listeria* has been isolated from raw fish, cooked crabs, raw and cooked shrimp, raw lobster, surimi and smoked fish.
- *Listeria* has been found growing in raw milk, supposedly pasteurized fluid milk, cheeses (particularly soft-ripened varieties), ice cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry, raw meats (all types), and raw and smoked fish. Its ability to grow at temperatures as low as 3°C permits multiplication in refrigerated foods.
- Until about 1960, *Listeria* was thought to be associated almost exclusively with infections in animals, and less frequently in humans. However, in the last 30 years, *Listeria* species, including the pathogenic species *L. monocytogenes* and *L. ivanovii* have been isolated from a variety of sources, and they are now recognized to be widely distributed in Nature. In addition to humans, at least 42 species of wild and domestic mammals and 17 avian species, including domestic and game fowl, can harbor Listeria. *Listeria monocytogenes* is reportedly carried in the intestinal tract of 5-10 % of the human population without any apparent symptoms of disease. *Listeria* has also been isolated from crustaceans, fish, oysters, ticks, and flies.
- *L. monocytogenes* can be found as part of the normal flora of many animal species and in humans. In two Danish studies, about 1 % (3/348) of apparently healthy individuals were found to excrete *L. monocytogenes* in faeces, whereas the corresponding figure among patients with listeriosis was 21.6 % (16/74). In household contacts of patients with listeriosis the faecal carrier rate was reported to be 18 %.
- About 10 % of healthy cattle tested in the Netherlands were positive for *L. monocytogenes* in their faeces. Since *Listeria* can survive and grow under many adverse conditions, including low pH, refrigeration temperatures and high salt concentrations, they can easily contaminate food. This is of great concern to the food industry, since a low inoculum can translate into a substantial dose of *Listeria* for the consumer, depending on the shelf life and handling of a particular product.
- As mentioned above, healthy cows can serve as reservoirs for *L. monocytogenes* and secrete the organism in milk. Contamination of milk may also occur through accidental contact with faeces and silage.

The above points give us the entire picture about the danger that *Listeria* poses, especially in the processed, packed foods. In the recent past, a high visibility chocolate manufacturing company was in the midst of a problem when these pathogens were discovered in some of the chocolates. The company concerned blamed it on the faulty storage conditions in which these chocolates existed. While there is no doubt that this indeed was the case, even proper storage cannot negate the existence and growth of *Listeria* in these chocolates. The need is that the company's manufacturing processed and packed food make it completely sure that *Listeria* contamination is taken care of before packaging.

We need to study the growth profile of the pathogenic species of *Listeria* at low temperatures in different food media. Such a study is badly needed, which will be able to advise the manufacturers of these foods cons a case by case basis. We do not know whether the food inspection that is there in India assesses the food for *Listeria* contamination. In view of our results and in view of the previous cases in the Western world, it is necessary that if such a check does not exist currently, it should be put in place. The above points give us the entire picture about the danger that *Listeria* poses, especially in the processed, packed foods. *Enterobacteriaceae* was also found to contaminate the milk chocolate.

Acknowledgment

I am thankful to the Hislop School of Biotechnology, Hislop College, Nagpur and Department of Botany, Dr. Hari Singh Gour University, Sagar for providing me all the necessary facilities.

References

1. **Beckett, S.T. (2009).** Conching. In Industrial Chocolate Manufacture and Use, (pp. 192-223): Wiley-Blackwell.
2. **Bryan, F.L. and Organization, W.H. (1992).** Hazard analysis critical control point evaluations: A guide to identifying hazards and assessing risks associated with food preparation and storage.
3. **Ropkins, K. and Beck, A.J. (2000).** Evaluation of worldwide approaches to the use of HACCP to control food safety. Trends in Food Science & Technology, 11(1): 10-21.
4. **Bergey, D.H. (1934).** Bergey's Manual of Determinative Bacteriology. The American Journal of the Medical Sciences, 188(2): 282.
5. **Holt, J.G., Krieg, N.R., Sneath, P.H., Staley, J.T. and Williams, S.T. (1994).** Bergey's Manual of determinate bacteriology.
6. **Farber, J. and Peterkin, P. (1991).** *Listeria monocytogenes*, a food-borne pathogen. Microbiological reviews, 55(3): 476-511.
7. **Kenney, S.J. and Beuchat, L.R. (2004).** Survival, growth, and thermal resistance of Listeria monocytogenes in products containing peanut and chocolate. Journal of Food Protection, 67(10): 2205-2211.
8. **Rosenow, E.M. and Marth, E.H. (1987).** Growth of *Listeria monocytogenes* in skim, whole and chocolate milk, and in whipping cream during incubation at 4, 8, 13, 21 and 35°C. Journal of Food Protection, 50(6): 452-463.
9. **Jeffrey, L.K., Joshua, B.G. and Bradley, A.S. (2013).** Enterobacteriaceae, Coliforms, and *Escherichia coli* as Quality and Safety Indicators. In Compendium of Methods for the Microbiological Examination of Foods): American Public Health Association.
10. **Nayak, D., Savalia, C., Kshirsagar, D. and Kumar, R. (2016).** Isolation, Identification and Molecular characterization of *Listeria* species from milk and milk products in Navsari city of South Gujarat. Journal of Veterinary Public Health, 13(1): 19-23.
11. **Yehia, H.M., Ibraheim, S.M. and Hassanein, W.A. (2016).** Prevalence of *Listeria* species in some foods and their rapid identification. Tropical Journal of Pharmaceutical Research, 15(5): 1047-1052.
12. **El Emam, M.M. (2013).** Developing an integrated phage-PCR assay for rapid detection of *Listeria monocytogenes* in foods. University of Nottingham.
13. **Cartwright, E.J., Jackson, K.A., Johnson, S.D., Graves, L.M., Silk, B.J. and Mahon, B.E. (2013).** Listeriosis outbreaks and associated food vehicles, United States, 1998-2008. Emerg. Infect. Dis., 19(1): 1-9.
14. **Ryser, E.T. (2012).** Safety of dairy products. In: Microbial Food Safety, (pp. 127-145): Springer.
15. **Bell, C. and and Kyriakides, A. (2012).** *Listeria:* a practical approach to the organism and its control in foods: Springer Science & Business Media.
16. **Nawaz, F. (2013).** Exploring the Actors and Factors Involved in Public Policy Implementation in Bangladesh: A Case Study of Reproductive Health Policy. Available at SSRN 2228582.
17. **Girard, D., Leclercq, A., Laurent, E., Lecuit, M., de Valk, H. and Goulet, V. (2014).** Pregnancy-related listeriosis in France, 1984 to 2011, with a focus on 606 cases from 1999 to 2011. Euro Surveill, 19: 19.
18. **Chevalley, J. (1994).** Chocolate flow properties. In Industrial chocolate manufacture and use, (pp. 139-155): Springer.
19. **De Buyser, M.L., Dufour, B., Maire, M. and Lafarge, V. (2001).** Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. International Journal of Food Microbiology, 67(1): 1-17.
20. **Slutsker, L., Ries, A.A., Maloney, K., Wells, J.G., Greene, K.D. and Griffin, P.M. (1998).** A nationwide case-control study of *Escherichia coli* O157: H7 infection in the United States. Journal of Infectious Diseases, 177(4): 962-966.