Contamination of Pasteurized Fruit Juices with *Bacillus licheniformis* in West Azerbaijan Province, Iran

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ABSTRACT

Background and objectives: *Bacillus licheniformis* is a potential cause of spoilage in pasteurized products. The aim of this study was to identify and isolate *B. licheniformis* from commercial pasteurized fruit juices distributed in the West Azarbaijan Province, Iran.

Methods: Sixteen fruit juice samples including four apple juice and 12 orange juice samples were collected from five fruit juice manufacturing companies in Iran. The samples were tested for the presence of *B. licheniformis* by culture in specific media and biochemical testing. Suspected samples were also investigated for the presence of the bacterium by polymerase chain reaction using specific primer for the *gyrB* gene.

Results: Three samples (18.75%) from the 16 tested fruit juice samples were found as positive. In other words, one apple juice sample (25%) and two orange juice samples (16.66%) were contaminated with *B. licheniformis*.

Conclusion: Isolation of this bacterium indicates the unsuitable manufacturing conditions and ineffective bacterial decontamination, which might also be favorable for the growth of other fruit juice spoilage bacteria.

KEYWORDS: *Bacillus licheniformis*, Fruit and Vegetable Juices, Polymerase Chain Reaction.

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INTRODUCTION

Pasteurization of non-alcoholic drinks with acidity of <4 is conducted at temperature of 65 ℃ for 15 minutes (1). In Iran, the pasteurization of fruit juice is commonly performed at 70-75 ℃ for 20 minutes. Thus, only bacteria that are resistant to heat and acidity can survive in the fruit juice after the pasteurization process. Among the *Bacillus* species, spores of *Bacillus licheniformis* can resist high temperatures up to 135 ℃ (2, 3). This soil-borne bacterium is able to grow at 13-60 ℃ and spreads quickly in the culture medium. In industry, it is used for protease production, which is highly important for the production of laundry detergents (4). Important alkaline proteases such as subtilisin BPN and subtilisin carlsberg are also produced from this bacterium (5). Furthermore, *B. licheniformis* is used for controlling some plant diseases, such as mango-related diseases and gray mold of apples (6, 7). This important food-borne pathogen (8) is also a potential source of spoilage in pasteurized foods, particularly in milk (9). The Environmental Conservation Organizations in the United States do not consider this bacterium as a human pathogen (10). However, there are reports on the potential production of toxins by *B. licheniformis* in cases of food poisoning (11). It has been revealed that the bacterium also has the potential to produce considerable amounts of enterotoxin, which could cause vomiting (12). The purpose of this study was to identify and isolate *B. licheniformis* from commercial pasteurized fruit juices distributed in the West Azerbaijan Province, Iran.

MATERIAL AND METHODS

We collected four apple juice samples and 12 orange juice samples from five Iranian fruit juice manufacturing companies that distributed products in the West Azerbaijan Province. After sterilizing the cap of fruit juice containers by alcohol, 0.5 ml of the fruit juice was cultured on potato dexterose agar (containing cycloheximide to inhibit the growth of mold and yeast) and orange serum agar. The culture media were incubated at 45 ℃ for 3-5 days. To provide axenic culture conditions, the colonies appearing on the culture media were streaked on orange serum agar. Later, several biochemical tests were performed to identify *B. licheniformis* (13). *B. licheniformis* PTCC1320 was used as the positive control.

DNA extraction

DNA was extracted from *B. licheniformis* strains cultured in brain heart infusion medium (Merck Co., Germany) using PGEX 2050 kits (Pak gene Yakhteh Company, Iran, Catalog No: PGEX 3050).

Detection of gyrB gene by polymerase chain reaction (PCR)

Specific primers were designed using the primer-BLAST software, and PCR was performed in a solution with final volume of 20 µl, containing 10 µl of sterile deionized water, 2 µl of 10X PCR buffer, 0.6 µl of $MgCl_2$, 0.4 µl of dNTPs, 0.4 µl of each specific primers (Table 1), 1.6 unit of Taq DNA polymerase and 6 µl of extracted DNA. Cycling conditions were as follows: primary denaturation at 94 ℃ for 5 minutes, 35 cycles of denaturation at 94 ℃ for 50 seconds, annealing at 58.5 ℃ for 58 seconds, extension at 72 ℃ for 1 minute and a final extension cycle at 72 ℃ for 7 minutes.

PCR products were electrophoresed on 1.5% agarose gel and then photographed using a Gel Doc system. DNA extracted from *B. licheniformis* PTCC 1320 was used as the positive control. Deionized distilled water was used as the negative control. Samples containing the *gyrB* gene (by presence of a 118 bp fragment) were considered positive for *B. licheniformis*.

Table 1- Sequence of the specific primers used for the detection of the *gyrB* gene (14)

Gene	Sequence	PCR product size (bp)
gyrB	**5′-AAAGCTGATTTGAAAGTCATTGGAGAT-3′** **5′-GAGTGGCGAGCGTATCATAGTC-3′**	**118**

RESULTS

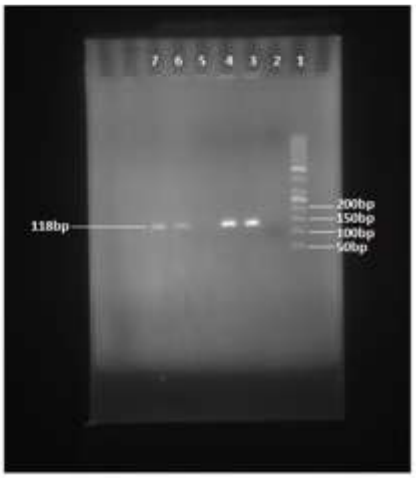
Presence of *B. licheniformis* was confirmed in four samples, including an apple juice sample and three orange juice samples. Table 2 shows the results of the biochemical tests used for the detection of *B. licheniformis* in fruit juice samples.

After 24 hours of incubation at 43 ℃, the bacterium significantly spread on the orange serum agar and almost the whole surface of medium was occupied by the bacterial colonies. Although none of the tested samples were collected from expired products, all positive samples were related to juice containers with a swollen appearance before being opened. Among the four positive samples in the culture method, three samples (18.75%) were identified as *B. licheniformis* in the PCR test (Figure 1). In other words, the results of PCR showed that one apple juice sample (25%) and two orange juice samples (16.66%) were contaminated with *B. licheniformis*.

Table 2- Results of the biochemical tests used for the detection of *B. licheniformis*

Biochemical tests	Nitrate reduction	H_2S	Indole	Catalase	Lactose	Voges-Proskauer	Methyl red	Urease	Growth in anaerobic Jar	Growth at 60 ℃	Growth at 65 ℃	Growth at 13 ℃	Acid production from glucose	Gas production from glucose
Results	+	-	-	+	-	+	+	+	+	+	-	+	+	-

Figure 1-Results of amplification of the *gyrB* gene for identification of samples positive for *B. licheniformis*. Production of the 118 bp fragment confirms presence of the *gyrB* gene. Lane 1: DNA marker (50bp); lane 2: negative control (deionized distilled water); lane 3: positive control (*B. licheniformis* PTCC 1320); lanes 4,6 and 7: samples positive for *B. licheniformis*; lane 5: negative samples



DISCUSSION

The sources of contamination of fruit juices with microorganisms include raw fruits, soil, birds, insects and contaminated instruments. The total count of microorganisms in a healthy fruit varies from 1000 to 1000000 per gram, and may be higher in damaged and moldy fruits. Proper washing of fruits reduces the level of primary contamination by up to 90% (15). This is the first study in Iran that has identified and isolated *B. licheniformis* in fruit juices based on a molecular method. We found that 25% of apple juice samples and 16.66% of orange juice samples were contaminated with *B. licheniformis*. Overall, four samples (18.75%) in our study were contaminated with the bacterium. Similar to our study, in a study by Motamedi and Tajbakhsh (2014), 12.5% of commercial pasteurized orange juices tested were contaminated with *B. licheniformis* (16). Spores of *B. licheniformis* have also been isolated from carrot juice (17) and milk (18) samples. Sadeghi et al. (2015) found *B. licheniformis* contamination in 4% of traditional ice creams and 4.76% of hand-made carrot and cantaloupe juices in Gorgan, Northeast of Iran (19). In this study, the *gyrB* gene was used for the molecular detection of

B. licheniformis. The gene has been used for primer design and specific probing of the bacterium, with a favorable accuracy (20). In the culture method, four samples (25%) under investigation were positive, while in the PCR method, three samples were positive for *B. licheniformis*. This demonstrates the high accuracy of the molecular technique compared to the culture methods. The contamination of pasteurized fruit juice with *B. licheniformis* indicates that other bacteria resistant to the pasteurization process can also survive and spoil the product if given the suitable conditions.

CONCLUSION

Bacterial contamination of fruit juice could be due to various factors including the production environment, preservation time, transportation conditions, unsterile equipment and use of damaged containers. Therefore, ensuring the sanitary conditions in all these stages will dramatically reduce the possibility of microbial contamination of these products.

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CONFLICT OF INTEREST

There is no conflict of interest.

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