Advances in Medicine and Engineering Interdisciplinary Research, 2024, Volume 2, Issue 2
DOI: 10.32629/ameir.v2i2.2019
ISSN Online: 2972-3825
ISSN Print: 2972-3833

Atypical Spoilage Microorganisms in Argentinean Yogurts: Gas-Producing Molds and Bacteria of the Genus Gluconobacter

María Luján Capra[1], Laura N. Frisón[2], Carolina Chiericatti[2], Ana G. Binetti[1], Jorge A. Reinheimer[1*]

1. Institute of Industrial Lactology (UNL-CONICET), School of Chemical Engineering, Universidad Nacional del Litoral, Santiago del Estero, Santa Fe, Argentina.
2. Chair of Microbiology, Department of Food Engineering and Biotechnology, School of Chemical Engineering, Universidad Nacional del Litoral, Santiago del Estero, Santa Fe, Argentina.
*Corresponding author. Email address: jorreinh@fiq.unl.edu.ar

Abstract: Microbiological spoilage of food leads to products unfit for consumption, and their discarding leads to significant economic losses for the food industry. During storage, fresh foods offer available niches for the survival and growth of undesirable microorganisms. In dairy products, data regarding spoilage and/or pathogenic bacteria is better documented than those for molds and yeasts. Dairy products, due to their refrigerated storage and production from heat-treated milk, are less susceptible to mold contamination compared to fruits and vegetables. The dominant microbiota in fermented dairy products also contributes to acidifying the medium, further reducing the risk of spoilage. However, even cheeses and yogurts may be susceptible to mold contamination. Atypical cases of yogurt samples containing spoilage microorganisms not previously reported (molds producing gas and bacteria of the genus Gluconobacter) in Argentinean fermented milks are presented here. For yogurt, in particular, the "classic" altering organisms were always being yeasts, and in other countries, molds belonging to the genus aspergillus.

Key words: spoilage microorganisms; yogurt; gas-producing molds; mucorales; gluconobacter

Due to their ability to metabolize different compounds present in these foods, a large number of fungal species can survive and even grow in dairy products. In addition, certain species are drought tolerant, acid tolerant, or resistant to mental illness, and can to some extent maintain growth in the presence of chemical preservatives added to these products to extend their effective lifespan [4, 15].

In yogurts, spoilage caused by molds and yeasts can be evidenced by growth (colony or thallus) on the surface of the product, as well as by the presence of unpleasant tastes or odors. These may be caused by the production of metabolites (ethanol, volatile organic compounds) or the action of lipolytic or proteolytic enzymes. There may also be alterations in color or texture and production of gaseous CO_2 [3-5]. Mainly, the presence of yeasts in yogurt is due to post-pasteurization contaminations, although certain species show high thermal resistance [4].

Various molds are responsible for alterations in dairy products. Species belonging to the genera Penicillium, Aspergillus, Mucor, Rhizopus and Thamnidium have been isolated mostly from cheeses with alterations, although they can also contaminate heat-treated products. Similarly to what has been explained in relation to yeast contamination, the appearance of molds is generally due to contamination after the thermal process; in some cases, due to the presence of heat-resistant fungal spores [1].

This study is not a long-term systematic analysis but rather an examination of yogurt samples sent to the Institute of Industrial Lactology (INLAIN) due to defects. Microorganisms responsible for spoilage were isolated from these samples, distinct from the "classic" gaseogenic spoilage agents such as yeasts commonly found in this type of product.

An industry in the region sent to INLAIN samples from 3 productions during the summer of 2016-2017 (Table 1). They were vanilla or strawberry flavored drinkable yogurts and smoothies, with normal pH values and presence of lactic microbiota in all cases. However, in several samples, there were high counts on yeast extract-glucose-chloramphenicol (YGC) agar (incubation: 3 days, 25°C) of different types of contaminants: variety of yeasts (some gaseogenic) (10^2-10^6 CFU/ml), a mold usually isolated as an environmental contaminant (Aspergillus niger, 10 CFU/ml), and, most notably, gaseogenic molds belonging to the order Mucorales (Mucor racemosus and Mucor circinelloides) (10-10^4 CFU/ml). The containers from which a couple of samples from this group came (II.M4 and II.M5) were visibly swollen; in another case (II.M6), the container was collapsed. The production of gas (CO_2) in MRS broth with Dürham hood by certain yeast isolates and fungal isolates corresponding to Mucor was verified. All 3 samples had an unpleasant aroma and taste. Samples II.M4 and II.M5 showed counts of 10^4 CFU/ml of gas-producing molds. Sample II.M6 revealed a count of 10^5 CFU/ml of yeasts.

Table 1. Characteristics of yogurt samples with isolation of altering molds and yeasts

Sample	Type of product	Year	Organoleptic and macroscopic analysis	Gas	pH	Counts (CFU/ml)[a]	Identification of microorganisms
I.M1	Drinkable strawberry	2016	Strawberry, normal	nd	4.43	3.2×10^5	Budded yeast, nd
I.M2	Drinkable vanilla	2016	Vanilla, normal	nd	4.40	1.7×10^5	Budded yeast, nd
I.M3	Drinkable strawberry	2016	Strawberry, normal	nd	4.55	4.0×10^6	Ovoid or elongated yeasts, some sprouted, nd
I.M4	Vanilla milkshake	2016	Vanilla, normal	nd	4.50	1.0×10^3	Budded yeast, nd
I.M5	Drinkable strawberry	2016	Strawberry, normal	nd	4.55	4.7×10^6	Budded yeast, nd
I.M7	Drinkable vanilla	2016	Vanilla, weak	nd	4.44	3.5×10^6	Budded yeast, nd
II.M4	Strawberry milkshake	2017	Unpleasant aroma and taste, lumpy texture, lumpy container	+	4.39	1.4×10^4	M. circinelloides
II.M5	Drinkable vanilla	2017	Unpleasant aroma and taste, convex container	+	4.35	1.8×10^4	M. circinelloides
II.M6	Drinkable strawberry	2017	Unpleasant aroma and taste, collapsed container	−	4.35	1.6×10^5	Elongated or circular yeasts, with buds, nd

II.M7	Drinkable strawberry	2017	Strawberry, normal, very liquid	+	4.43	20	A. niger, M. racemosus
II.M8	Drinkable vanilla	2017	Vanilla, normal, liquid	+	4.44	1.1×10^{2b}; 1.0×10^{2c}	M. racemosus, M. circinelloides, ovoid yeasts
II.M10	Drinkable vanilla	2017	Vanilla, weak	−	4.37	8.9×10^{4}	Elongated or circular yeasts, with buds, nd
II.M11	Drinkable vanilla	2017	Vanilla, weak	+	4.52	2.6×10^{2}	Ovoid yeasts, some budded, nd
III.M2	Drinkable vanilla	2017	Vanilla, normal	−	4.42	7.0×10^{4}	Circular yeasts, with buds, nd
III.M3	Drinkable vanilla, with sorbate	2017	Vanilla, normal	+	4.53	10	Elongated yeasts with buds and pseudohyphae
III.M4	Drinkable strawberry, with sorbate	2017	Strawberry, normal	+	4.43	10	M. circinelloides
III.M5	Drinkable vanilla, with sorbate	2017	Strawberry, normal	+	4,42	1.6×10^{2}	A. niger, M. circinelloides

Note: nd: not determined; a: On YGC agar (3 days, 25°C); b: Molds; c: (1)Yeasts. (2) The presence of lactic microbiota characteristic of yogurt ferment was normal in all samples. (3) Sample code: Roman numerals indicate the sample; Arabic numerals indicate the sample number within each sample.

In the sampling corresponding to the last production, 5 samples were analyzed, 3 of which had potassium sorbate added as a preservative (III.M3 to III.M5). Only in one of the samples (III.M1) there was no detection of molds or yeasts, while in 2 others (III.M2 and III.M3) yeasts were detected, at concentrations of 7×10^{4} and 10 CFU/ml without and with addition of the preservative, respectively. In the remaining samples (III.M4 and III.M5), both with added sorbate, the presence of molds was detected in low concentrations (up to 10^{2} CFU/ml). In one of them (III.M5), the 2 genera of molds mentioned (Aspergillus and Mucor) were isolated. Identification of microorganisms was conducted through culturing on specific media and observation using taxonomic keys developed by Pitt and Hocking [10]. Evidently, potassium sorbate did not completely inhibit the molds and yeasts present.

One of the most common contaminants associated with yogurt, but not in our country, is A. niger. Its prevalence is critical for the shelf life of the product, since it is capable of growing abundantly at the yogurt-air interface if conditions are favorable [7]; in general, the origin of the contamination is environmental.

Molds belonging to the order Mucorales have previously been found in yogurt from other countries [3, 13]. They are able to alternate between 2 growth phases (dimorphism). Their predominant growth form is filamentous, but under specific conditions (e.g., low oxygen tension), they can grow as yeasts. It is in this yeast-like growth phase that CO_2 production occurs, leading to the effervescence detected in yogurts and to the bulging of the containers [13]. Figure 1 shows the macroscopic appearance of the colonies and includes microscopic observations of the gasogenic molds isolated from the contaminated samples. The latter showed growth characteristics typical of Mucor species, i.e. aerial non-septate hyphae decorated with sporangia containing thousands of sporangiospores (asexual spores).

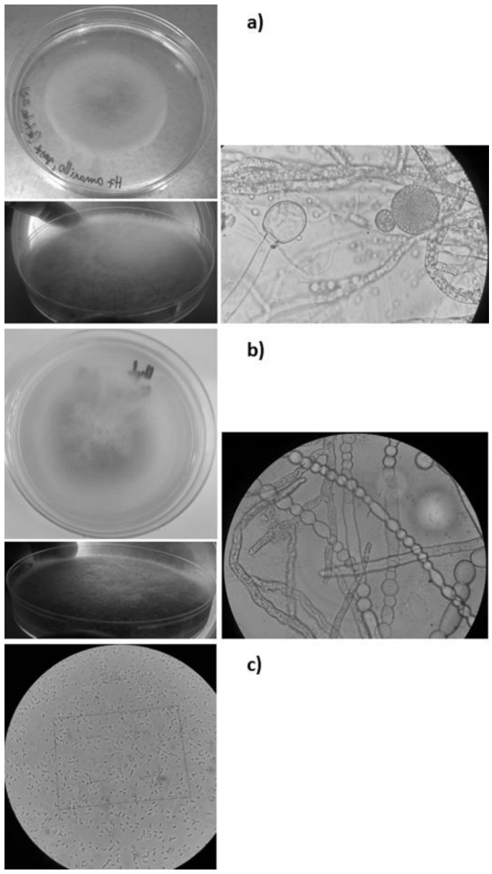

Figure 1. Appearance of contaminating microorganisms in altered yogurt samples. Macroscopic observation of the colony (left) and microscopic (100x) phase contrast (right) of the molds Mucor racemosus (a) and Mucor circinelloides (b) on YGC agar at 2 and 5 days of incubation at 28°C; microscopic (100x) phase contrast observation of Gluconobacter sp. (c).

The genus Mucor is recognized as a causal agent of food spoilage, but it is not usually considered a food pathogen, as its ingestion is not associated with gastrointestinal illness, especially among healthy individuals [13]. M. circinelloides is a filamentous mold by nature; although dimorphic, it is mostly present in soil, plants, and decaying fruits (apples, pears, and strawberries).

In 2013, an outbreak occurred in the United States related to the consumption of a commercial product contaminated with M. circinelloides. Puffy pots and presence of bubbles were observed in a Greek-type yogurt due to CO_2 produced by the contaminating mold [13]. As reported by the Food and Drug Administration, more than 200 consumers complained of digestive distress, including abdominal cramps, vomiting, nausea, and diarrhea, and the batch was recalled. The authors who reported this finding indicated that it is difficult for M. circinelloides to survive pasteurization, so its presence is due to contamination after heat treatment. Once in the container and due to the metabolites generated by the fermentation of lactic acid bacteria in yogurt, yeast growth with CO_2 production can occur. M. circinelloides is recognized as an opportunistic pathogen and is one of the etiologic causative agents of mucormycosis, a potentially fatal infection in humans and immunocompromised animals [9, 13].

Molds and yeasts can grow in the humid environments of dairy plants and establish themselves in different areas, surfaces and equipment, if these are not adequately sanitized. They can also enter along with the ingredients and raw materials used, and even by personnel, and pass into packaging. Air is a very important entry route and vehicle, especially for the dispersion of fungal spores. Particularly in yogurt, fruit preparations can be a source of entry for molds and yeasts [1, 5, 14, 15].

Temperature control is critical to the quality and shelf life of dairy products, although low temperatures and even frozen storage do not eliminate microorganisms. Differences in growth capacity during refrigerated storage of yogurt were found when comparing 12 fungal species; isolates corresponding to Penicillium commune (7.6°C) and A. niger (9.6°C) showed growth at lower temperatures [6]. Species of the genus Mucor grow adequately at refrigeration temperatures and under very low oxygen tension conditions [15].

Spoilage of yogurts by contamination with M. circinelloides represents an unusual spoilage event. Most Mucorales are saprophytes found in decaying organic matter. Commonly, M. circinelloides causes spoilage of fresh fruits and vegetables. The processes of contamination and spoilage of food products by this mold have been very poorly studied in comparison with those caused by many of the members of Ascomycota (Penicillium, Aspergillus, Saccharomyces), which are commonly associated with food spoilage.

As already mentioned, yeasts have been the "classic" alterants in Argentine yogurts. However, at INLAIN in mid-2018, 3 yogurt samples were received (whipped with fruit mattress, whipped with pulp and drinkable) with evident alteration of organoleptic characters (intense odor with unpleasant background, bitter taste and rust orange color, without evidence of gas production), suspected of containing alterants of microbial origin. The pH values were very acidic for this type of sample: 3.68 to 3.98; normal values are in the range of 4.3-4.5. From routine microbial counts, it was possible to detect the presence of bacteria capable of growing on YGC agar, a medium suitable for detecting molds and yeasts and inhibiting bacterial growth (due to the presence of 0.1 g/l chloramphenicol). These acid ($pH < 4$) and chloramphenicol-resistant bacteria were found to be motile short bacilli, grouped in chains and diplos or isolated, catalase (+) and gram-negative, and were present at high levels in the yogurt samples (10^6-5×10^7 CFU/g) (Fig. 1). After isolation and DNA extraction, by PCR amplification of a fragment (1,500 bp) of the 16S rRNA [2] gene and subsequent sequencing (Macrogen service, Seoul, Korea), the alterant was identified as belonging to the genus Gluconobacter. With the exception of the microbiota characteristic of the yogurt ferment, only Gluconobacter cells were detected in high concentration in the various culture media seeded. Other cell forms were not observed, i.e., what was observed under the microscope coincided with what was detected by culture applying classic routine microbiological methodologies for this type of samples.

Gluconobacter is a genus of the group of acetic acid bacteria (BAA). They are acidophilic (resistant to high acidity), gram-negative, strict aerobic bacilli, closely related to human life. They belong to the family Acetobacteriaceae, which currently includes 12 genera, with Gluconobacter and Acetobacter as the most industrially relevant. Naturally, they are found in environments with high availability of sugars, such as fruits, bee honey and flowers, or in foods such as beverages (cider, beer, wine or vinegar). They are characterized by rapid and incomplete oxidation of a wide range of sugars, alcohols and acids (such as D-glucose, glycerol, D-sorbitol, ethanol, D-gluconic acid), from which they accumulate large amounts of oxidation products. Examples of this metabolism are the production of acetic acid from ethanol or gluconic acid from D-glucose. Most oxidative reactions are catalyzed by membrane-bound dehydrogenases. This implies that transport of substrates into the cell is not necessary and that the accumulation of oxidation products in the medium is very rapid and almost quantitative. Because of these metabolic characteristics, BAA are widely used in the food industry for applications such as vinegar production and cocoa bean fermentation. Acetobacter aceti is the most relevant species, capable of completely oxidizing acetic acid to carbon dioxide and water. The genus Gluconobacter is involved in the production of L-sorbose from D-sorbitol, D-gluconic acid from D-glucose and dihydroxyacetone from glycerol. However, its industrial relevance is due to its participation in the vinegar industry, since it produces large concentrations of acetic acid from ethanol [11, 12].

In certain regions of the Caucasus, a yogurt with low acidity and high viscosity, containing strains of Lactococcus lactis sp. cremoris, Leuconostoc sp. and Gluconobacter sp. or Acetobacter orientalis, is consumed [8]. This yogurt is fermented by hand at low temperature (25-30°C) and, due to its easy way of preparation, was introduced in recent years in Japan for home preparation and consumption; it is known as "Caspian yogurt" and has gained rapid acceptance by the population.

BAA are often present on the surface of damaged fruits, making them rancid; this is often the source of wine contamination and the cause of spoilage. In addition, these bacteria can trigger other adverse reactions that contribute to losses in the food industry. They can also influence food quality as ingredients causing spoilage, such as rancidity in beer and other alcoholic beverages. Yogurt is an ancestrally popular food and its consumption has increased in recent years mainly due to its recognized health benefits. However, yogurt contaminated with BAA can cause large losses to the industry. In this dairy matrix, BAA can produce acetic acid, mainly by consuming the nutrients in yogurt, and can completely oxidize acetic acid to carbon dioxide and water. Consequently, BAA can cause significant alterations by generating rancidity, discoloration, swelling of the container (due to CO_2 accumulation) and unpleasant flavors [12]. These alterations were evident in the samples analyzed, but the presence of gas was not evident, possibly because the acetic acid formed was not completely oxidized.

Although the presence of BAA does not represent a common contamination in yogurts, due to the characteristics of the environment generated in the food (acidity, presence of oxygen and low temperatures), its timely and effective detection in yogurt during production avoids serious economic losses.

A possible source of entry of BAA into the yogurt production process is the ingredients used, such as fruits, which represent a normal habitat for these microorganisms. Thus, an adequate control of the fruit pulps used could avoid serious economic losses. Accordingly, the isolation of Gluconobacter sp. as a "new" alterant of Argentine yogurts could be correlated to the presence of fruits in the samples analyzed.

The alterations in yogurt caused by gaseous molds and bacteria of the genus Gluconobacter are novel events in the Argentine reality and are linked to the microbial diversity that threatens the processes and products (fermented milks). In yogurts, lactose-fermenting yeasts, which produce gas and changes in aroma, flavor and appearance, have always been considered as traditional microbial alterants. However, in this work we have shown that gasogenic defects can also be attributed to molds that probably come from the environment, and that certain color and flavor changes can originate from the activity of gluconobacteria, whose source could be the fruit added to the yogurt.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

References

[1] Dijksterhuis J. Fungal spores: Highly variable and stress resistant vehicles for 275 distribution and spoilage. *Food Microbiol*. 2019;81:2-11.

[2] Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res*. 1989;17:7843-53.

[3] Foschino R, Garzaroli C, Ottogalli G. Microbial contaminants cause swelling and inward collapse of yoghurt packs. *Le Lait.* 1993;73:395-400.

[4] Garnier L, Valence F, Mounier J. Review diversity and control of spoilage fungi in dairy products: An update. *Microorganisms*. 2017;5:1-33.

[5] Garnier L, Valence F, Pawtowski A, Auhustsinava-Galerne L, Frotté N, Baroncelli R, Deniel F, Cotona E, Mounier J. Diversity of spoilage fungi associated with various French dairy products. *Int J Food Microbiol*. 2017;241:191-7.

[6] Gougouli M, Kalantzi K, Beletsiotis E, Koutsoumanis KP. Development and application of predictive models for fungal growth as tools to improve quality control in yoghurt production. *Food Microbiol*. 2011;28:1453-62.

[7] Gougouli M, Koutsoumanis KP. Risk assessment of fungal spoilage: A case study of Aspergillus niger on yogurt. *Food Microbiol*. 2017;65:264-73.

[8] Kiryu T, Kiso T, Nakano H, Ooe K, Kimura T, Murakami H. Involvement of Acetobacter orientalis in the production of lactobionic acid in Caucasian yogurt («Caspian Sea yogurt») in Japan. *J Dairy Sci*. 2009;92:25-34.

[9] Lazar SP, Lukaszewicz JM, Persad KA, Reinhardt JF. Rhinocerebral Mucor circinelloides infection in immunocompromised patient following yogurt ingestion. *Del Med J*. 2014;86:245-8.

[10] Pitt J, Hocking A. Fungi and food spoilage. 3th Ed. New York, EE. UU.: Springer; 2009. p. 512.

[11] Saichana N, Matsushita K, Adachi O, Frébort I, Frebortova J. Acetic acid bacteria: A group of bacteria with versatile biotechnological applications. *Biotechnol Adv*. 2015;33:1260-71.

[12] Sengun I, Karabiyikli S. Importance of acetic acid bacteria in food industry. *Food Control*. 2011;22:647-56.

[13] Snyder AB, Churey JJ, Worobo RW. Characterization and control of Mucor circinelloides spoilage in yogurt. *Int J Food Microbiol*. 2016;228:14-21.

[14] Snyder AB, Worobo RW. Mini-review fungal spoilage in food processing. *J Food Prot*. 2018;81:1035-40.

[15] Sorhaug T. Spoilage molds in dairy products. *Encyclopedia of Dairy Sciences*. 2011;4:780-4.