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Salad dressing spoilage by *Bacillus amyloliquefaciens* with gas formation

Deterioração em molhos para salada com formação de gás causada por Bacillus amyloliquefaciens

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Abstract

B. amyloliquefaciens is a Gram-positive, aerobic, motile rod, often found in soil, which has been described as a plant growth promoter and is used in several industrial processes. This study reports an episode involving the gassy spoilage of salad dressing caused by *B. amyloliquefaciens* in a production facility located in Rio de Janeiro, Brazil. Nine *B. amyloliquefaciens* strains were isolated from spoiled salad dressings, the sugar used as a raw material in the manufacture and from the production plant. A genotypic analysis of the isolates by Rep-PCR generated eight band profiles grouped in five Rep-PCR clusters. When re-inoculated into fresh salad dressing three *B. amyloliquefaciens* isolates belonging to the Rep-PCR clusters A, D and E were able to reproduce the gassy spoilage process, whereas the isolates belonging to the Rep-PCR clusters B and C did not produce any visible spoilage, suggesting that these isolates were not directly involved in the spoilage process. The predominant Rep-PCR cluster, cluster A, included strains isolated from barbecue and passion fruit seed salad dressings and from sugar (raw material), suggesting it is a common source of contamination for such salad dressings.

Keywords: *Microbial spoilage; Molecular typing; Industrial plant contamination.*

Resumo

B. amyloliquefaciens é um bastonete Gram-positivo, aeróbio e móvel, e é encontrado, frequentemente, no solo. Este bastonete foi descrito como um promotor de crescimento de plantas e é usado em diversos processos industriais. Este estudo relata um episódio de deterioração gasosa de molho de salada causada por *B. amyloliquefaciens,* que ocorreu em uma unidade de produção localizada no Rio de Janeiro, Brasil. Foram isoladas nove cepas de *B. amyloliquefaciens* de molhos de salada deteriorados, do açúcar utilizado como matéria-prima na fabricação e da planta de produção. A análise genotípica dos isolados por Rep-PCR gerou oito perfis de bandas, agrupados em cinco *clusters* Rep-PCR. Quando reinoculados em molho de salada fresco, três cepas de *B. amyloliquefaciens*, pertencentes aos *clusters* Rep-PCR A, D e E, foram capazes de reproduzir o processo de deterioração gasosa, enquanto as cepas pertencentes aos *clusters* Rep-PCR B e C não produziram deterioração visível, o que sugere que essas cepas não estavam diretamente envolvidas no processo de deterioração. O grupo predominante, Rep-PCR *cluster* A, incluiu cepas isoladas de molhos de semente de maracujá e barbecue, e do açúcar (matéria-prima), sugerindo uma fonte comum de contaminação para esses molhos.

Palavras-chave: *Deterioração microbiana; Tipagem molecular; Contaminação de planta industrial.*

1 Introduction

The microbiological spoilage of salad dressings and mayonnaises is generally caused by yeasts and bacteria. Yeasts belonging to the genera *Zygosaccharomyces* and *Saccharomyces* have been described as the spoilage agents of starch-based and French salad dressings. In addition, *Bacillus* species, such as *B. subtilis* and *B. vulgatus*, and

Lactobacillus fructivorans have been described as bacteria responsible for salad dressing spoilage (KURTZMAN et al., 1971).

B. amyloliquefaciens is a spore-forming Gram-positive, aerobic, motile rod often found in soil, that belongs to the *B. subtilis* group and is used in several industrial processes (PRIEST et al., 1987). In addition, it has been described as a plant growth promoting Rhizobacterium, known for its ability to produce a biofilm on root surfaces (ZHAO et al., 2015).

The spores of B. *amyloliquefaciens* are well-known contaminants of raw materials used in bread making (ROSENKVIST; HANSEN, 1995; SOROKULOVA et al., 2003), and the spores can potentially even survive the bread baking process (VALERIO et al., 2015). Valerio et al. (2012) found *B. amyloliquefaciens* to be the main species isolated from an outbreak of ropy bread spoilage in southern Italy.

This study reports an episode of the gassy spoilage of salad dressing caused by *B. amyloliquefaciens* in a production facility located in Rio de Janeiro, Brazil, and includes the identification and molecular typing of the isolates.

2 Material and methods

2.1 The setting

In July 2011 a salad dressing production facility located in Rio de Janeiro, Brazil reported an episode of gassy spoilage involving several salad dressing flavours. All of them had been manufactured in the same industrial plant. According to information provided by the Production manager, after the production of each salad dressing batch, the production line was cleaned and sanitized with steam (complete data about temperature, pressure and duration of the process not available). Information about the production workflow at the time of the contamination episode was also not provided. During inspection of the production line, biofilm formation was observed on several parts (blender, mixture tank and water connection). In order to determine the microbial agent responsible for the spoilage process, several swollen plastic bottles of different salad dressing flavours were analysed. In an attempt to find the source of contamination, the only raw material common to all salad dressings (sugar) was analysed and different surfaces of the production line screened. These surfaces included the inside of the blender, the mixture tank and the water connection. The compositions of each salad dressing analysed are shown in Table 1.

2.2 Bacterial strains

Nine *B. amyloliquefaciens* strains isolated from spoiled salad dressings, sugar (raw material) and from the production plant were characterized in the framework of this study. In addition, two non-related *B. amyloliquefaciens* strains were included for comparative purposes and as a positive control for the biochemical and physiological tests: LFB-FIOCRUZ 452 (ATCC 23842) and LFB-FIOCRUZ 1703 (isolated from mosquito larvae) (Table 2). All strains were deposited in the culture collection: *Coleção de Culturas do Gênero Bacillus e Gêneros Correlatos* – CCGB, Instituto Oswaldo Cruz – FIOCRUZ, Brazil.

2.3 The isolation and identification of *B. amyloliquefaciens*

Samples of four salad dressings (sesame and ginger, barbecue, papaya seed and passion fruit seed) and samples of the sugar used to manufacture the dressings were analysed. In addition, production line and tank surfaces were swabbed and the material obtained cultured.

One millilitre of each salad dressing sample was pour plated into Plate Count agar (PCA, Oxoid, Basingstoke, Hampshire, England). The plates were incubated at 35 °C under aerobiosis for 24 h and isolated colonies sub-cultured into Trypticase Soy agar (Oxoid, Basingstoke, Hampshire, England).

Table 1. Compositions of the salad dressings.

Salad dressing	Composition
Sesame and ginger	Vegetable oil, water, vinegar, garlic, black pepper, pepperoni pepper, herbs, sugar. Thickener: xanthan gum, guar gum
Barbecue	Concentrated tomato pulp, water, sugar, vinegar, modified starch, shoyu, onion, salt, herbs. Natural pigment: caramel Thickener: guar gum
Papaya seed	Vegetable oil, water, vinegar, sugar, onion, papaya seeds, mustard, salt garlic, herbs. Thickener: xanthan gum
Passion fruit	Vegetable oil, passion fruit pulp, vinegar, sugar, salt, herbs, mustard seeds. Thickener: xanthan gum Acidulant: citric acid Antioxidant: BHT and BHA

Table 2. Rep-PCR clusters and isolation source of the *B. amyloliquefaciens* strains.

Isolate	Source of isolation	Rep-PCR cluster
LFB-FIOCRUZ 1704	Barbecue salad dressing	B
LFB-FIOCRUZ 1705	Barbecue salad dressing	A
LFB-FIOCRUZ 1706	Sugar*	A
LFB-FIOCRUZ 1707	Production tank	C
LFB-FIOCRUZ 1708	Sesame and ginger salad dressing	E
LFB-FIOCRUZ 1709	Sugar*	A
LFB-FIOCRUZ 1710	Papaya seed salad dressing	D
LFB-FIOCRUZ 1711	Passion fruit seed salad dressing	A
LFB-FIOCRUZ 1712	Passion fruit seed salad dressing	A
LFB-FIOCRUZ 452	ATCC	NA
LFB-FIOCRUZ 1703	Mosquito larvae	NA

*Salad dressing raw material; NA not applicable.

In order to isolate spore-forming bacteria from the sugar used in the dressings, 50 g of sugar was diluted with 450 ml of sterile distilled water and heated to 80 °C for 30 min. Serial dilutions were prepared by adding 10 ml, 1ml and 0.1 ml of the solution to 100 ml of PCA for pour plating. All plates were incubated at 35 °C under aerobiosis for 24 h.

Sterile cotton swabs were used to sample the surfaces of the industrial plant to detect microbial contamination. The swabs were transported in distilled sterile water under refrigeration and immediately cultured. Bacterial isolation was carried out by pour plating 1 ml of the water into PCA and the plates incubated at 35 °C under aerobiosis for 24 h.

The *B. amyloliquefaciens* isolates were identified according to their cell morphology, and the following biochemical tests applied: glucose, arabinose, xylose and mannitol utilization, tyrosine decomposition, nitrate reduction, starch and casein hydrolysis, production of acetyl methyl carbinol, haemolytic activity, catalase production, citrate utilization, growth in an anaerobic medium and growth in 5%, 7% and 10% NaCl (VASCONCELOS; RABINOVITCH, 1994; RHODEHAMEL; HARMON, 2001; CLAUS; BERKELEY, 1986). One representative isolate (LFB-FIOCRUZ 1705) was selected for identification by 16S rRNA gene sequencing, according to previously described methodology (LANE, 1991).

2.4 Water activity (a_w) and pH measurements

The a_w values of the salad dressings were determined using an AquaLab 3TE water activity meter (Decagon Devices Inc., Pullman, Washington) according to the manufacturer's instructions, and the pH was determined in a pH meter. Each test was repeated three times for each sample and the mean values calculated.

2.5 Bacillus *Rep-PCR*

Repetitive element sequence polymorphism-PCR (Rep-PCR fingerprinting) was used for chromosomal comparisons of the *B. amyloliquefaciens* strains. The primers and the conditions for amplification were those previously described (REYES-RAMIREZ; IBARRA, 2005). Briefly, amplifications were made with the GeneAmp System 9700 (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µL containing: 300 ng of each primer, 200 µM of dNTP, 5 mM of $MgCl_2$, 2.5 U of Taq Polymerase (Invitrogen, São Paulo, Brazil) and 1 µL of template DNA. To visualize the band patterns, amplicons were separated by electrophoresis into 1.2% agarose gels in Tris–borate EDTA buffer (Tris–borate 89 mM; EDTA 2 mM pH 8.0) at 2 V/cm for four hours. The amplicons were visualized under ultraviolet light, after treatment of the gels with a 0.5 µg/mL Ethidium Bromide solution for 15 min. As a reference, 100 bp DNA molecular size marker (Invitrogen, São Paulo, Brazil) was used. The banding patterns were analysed by visual inspection and by a computer-assisted analysis with GelCompar II (version 1.5) software (Applied Maths, Kortrijk, Belgium) using 0.40% optimization and 1% position tolerance. Similarity of the banding patterns was assessed using the Dice index and the unweighted pair group method with the arithmetic average (UPGMA) and 80% of similarity was used for the cluster analysis (ABRIOUEL et al., 2007; MANZANO et al., 2009).

2.6 SDS-PAGE whole-cell protein pattern analysis

The SDS-PAGE whole-cell protein pattern analysis was carried out as previously described (KIM et al., 2010). Strains were incubated aerobically overnight at 30 °C in 5 ml of LB broth (Difco, Spaks, MD, USA) and centrifuged at 12,000 × g for 3 min at 4°C. Each pellet was washed twice with deionized water and suspended in 50µl of 50 mM Tris-HCl buffer (pH 8.0). Fifty milligrams of glass beads (diameter, 425 µm to 600 µm; Sigma, St. Louis, MO, U.S.A.) were added to each tube, and the bacteria vortexed for 5 min. The pellet was then re-suspended in 50µl of sample buffer [2× SDS sample buffer: 25 ml of 4× Tris-HCl/SDS (pH 6.8), 20 ml of glycerol, 4 g of SDS, 2 ml of 2-mercaptoethanol, 1 mg of bromophenol blue, and water to complete the volume to 100 ml] and heated for 5 min at 95 °C. The samples were centrifuged and the supernatant collected for analysis in a 12% SDS-polyacrylamide vertical slab gel. After electrophoresis, the gel was stained for 2 h with 0.05% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, VA, U.S.A.) and then de-stained with a 10% acetic acid and 30% methanol solution for 2 h. The de-stained gels were scanned for further analysis.

2.7 *B. amyloliquefaciens* Inoculation of *B. amyloliquefaciens* into fresh salad dressings

In order to evaluate if the *B. amyloliquefaciens* isolates were able to reproduce the gassy spoilage when inoculated into fresh salad dressing, five isolates (LFB-FIOCRUZ 1704, LFB-FIOCRUZ 1707, LFB-FIOCRUZ 1708, LFB-FIOCRUZ 1710 and LFB-FIOCRUZ 1711) were tested. These isolates were recovered from different salad dressings and from the production tank, and were representative of the Rep-PCR profiles found. The experiment was carried out as follows: the isolates were grown in nutrient broth (Difco, Sparks, MD, USA) at 33 °C until producing turbidity equivalent to 0.5 on the MacFalrland scale. Twenty microliters of bacterial suspension were inoculated into 40mL of each salad dressing (final concentration of 10^5 CFU/mL) in triplicate. All bottles were incubated at 33 °C and observed daily for up to two weeks.

3 Results and discussion

In this study *B. amyloliquefaciens* was shown to be the causative agent of gassy spoilage in four different types of salad dressing produced in the same industrial plant.

Table 3 shows the mean values for the pH and water activity of the salad dressings. All the samples presented acidic environments with pH values ranging from 3.22 to 4.50 and a_w values ranging from 0.938 to 0.986. All the contaminated products presented high a_w values and acidic environments, both propitious for supporting the growth of *B. amyloliquefaciens*.

Nine *B. amyloliquefaciens* isolates were recovered, six from four different salad dressings, two from sugar and one from the production tank (Table 2). This species was the only microorganism isolated from the samples. All isolates were identified as *B. amyloliquefaciens* according to their cell morphology and their biochemical and physiological characteristics. The isolates presented rough colonies and a characteristic odour. Wet mount and Gram stain microscopy showed Gram-positive motile sporulating rods, non-deforming sporangia with elliptic, para-central spores. Short chains were observed.

The SDS-PAGE whole-cell protein pattern analysis was carried out as an auxiliary methodology for identification. All the strains, including LFB-FIOCRUZ 452 (ATCC 23842), presented characteristic bands of 118, 53, 50, 48 and 46 KDa (KIM et al., 2010), confirming the species identification (Figure 1).

Table 3. Water activity and pH values found for the different types of salad dressing.

Salad dressing	pH	Water activity	Temperature (°C)
Passion fruit seed	3.84	0.986	24.5
Barbecue	4.50	0.953	24.4
Papaya seed	3.22	0.938	24.6
Sesame and ginger	3.48	0.948	24.5

The results of the 16S rDNA sequencing of LFB-FIOCRUZ 1705 showed 98% of similarity with the *B. amyloliquefaciens* sequences from the GenBank database, confirming the biochemical and SDS-PAGE based identification.

When inoculated into fresh salad dressings, the isolates LFB-FIOCRUZ 1708, LFB-FIOCRUZ 1710 and LFB-FIOCRUZ 1711 were able to reproduce the gassy spoilage in all the salad dressing samples. On the other hand, the isolates LFB-FIOCRUZ 1704 and LFB-FIOCRUZ 1707 did not produce any visible alteration in the salad dressings, suggesting that these isolates were not directly involved in the spoilage process.

The Rep-PCR typing of the *B. amyloliquefaciens* isolates generated eight band profiles, of which seven were represented by only one strain. Computer assisted analysis revealed that five patterns presented more than 80% similarity. Five Rep-PCR clusters were observed amongst the *B. amyloliquefaciens* strains. The predominant cluster (cluster A) included LFB-FIOCRUZ 1705, LFB-FIOCRUZ 1711 and LFB-FIOCRUZ 1712 (isolated from the barbecue and passion fruit seed salad dressings) and LFB-FIOCRUZ 1706 and LFB-FIOCRUZ 1709 (isolated from the raw material sugar) (Figure 2).

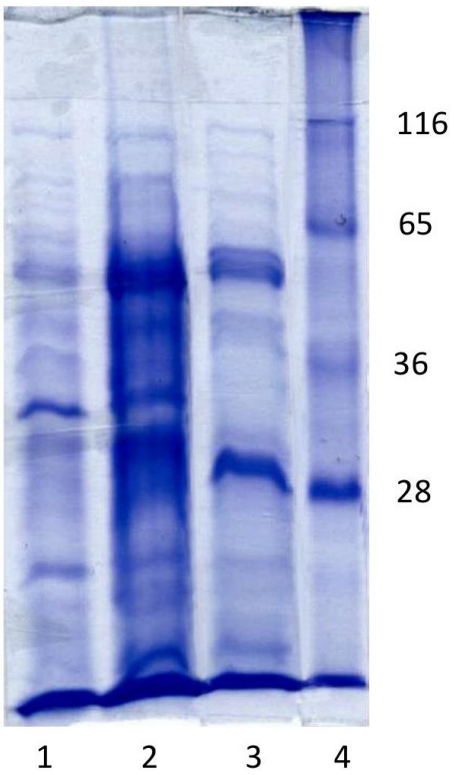

Figure 1. The SDS-PAGE whole-cell protein pattern of the *B. amyloliquefaciens* strains. LANE 1, LFB-FIOCRUZ 452; LANE 2, LFB-FIOCRUZ 1705; LANE 3, LFB-FIOCRUZ 1709; LANE 4, PROTEIN MOLECULAR MASS MARKER.

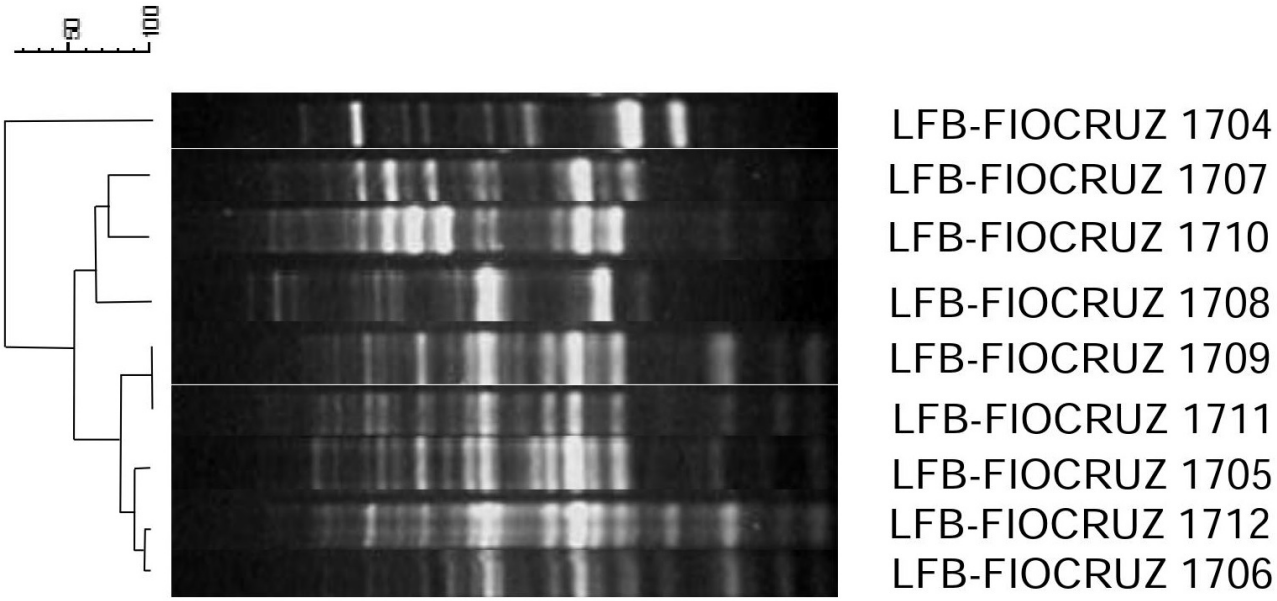

Figure 2. Computer assisted analysis of the Rep-PCR profiles of the *B. amyloliquefaciens* isolated from salad dressing, raw materials and the production tank.

A specific Rep-PCR cluster was found to be responsible for the spoilage of two types of salad dressing: barbecue and passion fruit seed. The same strain was isolated from the sugar used to make both salad dressings, suggesting that the sugar was the source of contamination. In addition, two other unrelated strains, belonging to different Rep-PCR clusters, were found in the papaya seed and sesame and ginger salad dressings. This suggests that the source of contamination was not the same for the three salad dressings. In addition, the three different strains isolated may have different nutritional requirements and, therefore, were able to grow in different types of salad dressings with different compositions.

Information about the pre-treatment of raw materials or post-treatment of salad dressings by pasteurization or Ultra High Temperature was not provided by the production facility. However, it is to be expected that either the raw materials or the final product would go through some form of heat treatment, since most of them do not include conservatives in the composition. It has been demonstrated that the presence of the *spoVA* operon correlates with the heat resistance of the *B.amyloliquefaciens* and *B.licheniformis* spores. Strains harbouring the *spoVA* Operon produced spores that required significantly longer heating times to achieve one decimal reduction than those without this transposon (BERENDSEN et al., 2016). It is possible that the strains causing the deterioration were able to resist any heat treatment applied, such as pasteurization and UHT, but further experiments to detect the presence of the *spoVA* operon would be necessary to confirm this.

B. amyloliquefaciens is known as a biofilm producer. These biofilms play an important role in the colonization of plant roots and the intestine of farm animals such as pigs and chickens. In addition, it has been reported that *B. amyloliquefaciens* may spoil bread, degrading the starch and generating extracellular polysaccharides (VALERIO et al., 2012, 2015). A *B. amyloliquefaciens* strain belonging to a different Rep-PCR cluster, unrelated to those that included the strains isolated from the salad dressings was found in a biofilm layer in the production tank. A possible explanation for this was that the strain colonizing the tank was not the one causing the contamination, and that this strain may not be able to grow in the substrates that compose the salad dressings since it was not able to grow when inoculated into fresh salad dressing. In addition, it is possible that the strains coming from the sugar were in greater numbers and better adapted to the substrates, and hence overcame the biofilm strains.

It is noteworthy that other types of salad dressing were being produced in the same industrial plant which did not present any type of microbial spoilage. This may be because these other products presented preservatives in their compositions, such as sodium benzoate and potassium sorbate, which were not present in the dressings that presented contamination.

To the authors' knowledge, this is the first report in the literature of the gassy spoilage of salad dressing by *B. amyloliquefaciens*.

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