Original Research Article

Food spoilage by Bacilli: combined effects of pH, a_w and storage temperature on spore germination and growth in cultural broth added with solutes and organic acids.

ABSTRACT

Aims: The control of *Bacillus* spores plays an important role in quality assurance and safety of processed foods. Their growth can be avoided by either sterilization or pasteurization in combination with a lowering of pH (≤4.6) or water activity (a_w<0.90) or with the combined action of both parameters (spore germination inhibition). In some food products, the reduction of pH to values lower than 4.6 or a thermal treatment of sterilization can lead to nutritional and organoleptic changes, that are not always satisfactory for the consumer. The aim of this work was to produce data for a range of spoilage bacilli at a variety of pH and a_w values and to monitor the potential outgrowth of these spores when stored at different temperatures. This study focused on the influence of the type of acid or solute used.

Place and Duration of Study: Quality, Safety and Pre-Industrialization Area, Stazione Sperimentale delle Conserve Alimentari- SSICA, Parma, Italy between March 2018 and November 2019.

Methodology: *B. sublitilis, B. licheniformis, B. cereus* spore's growth was studied in broth under the following conditions: a_w (0.90 to 0.93), temperature (6, 12 and 30°C) and pH (4.9 to 5.5). Selected water activities were reached by the addition of appropriate amount of sodium chloride, sucrose or glycerol; pH was adjusted to various values with lactic acid, acetic acid or citric acid.

Results: A combination of a_w 0.92, with glycerol or sodium chloride, and pH 5.1 inhibits the germination of all strains spores. *B. subtilis* was the species able to grow in the less favorable conditions. Acetic acid proved the strongest antimicrobial action; sodium chloride resulted more effective in inhibiting growth when used in combination with acids tested.

Conclusion: The results achieved can be useful for the food industry, identifying the conditions that could preserve the product by means of a less drastic heat treatment preserving as much as possible the natural organoleptic characteristics.

Keywords: [Bacillus, growth limit conditions, pH, a_w, storage temperature}

1. INTRODUCTION

The genus *Bacillus* includes a large group of sporogenic, pathogenic and non-pathogenic microorganisms widespread in nature (soil, water, dust). Their versatile metabolism and their ability to form spores resistant to heat, drying and chemicals, make these bacteria one of the most important and common group of spoilage organisms affecting food products [1].

Species such as *B. licheniformis, B. subtilis* and *B. cereus* are most commonly isolated from raw products.

B. licheniformis together with *B. subtilis* belong to mesophiles whereas *B. cereus* is rather a psychrotrophile microorganism, that proved able to grow in products at cold-storage temperatures and even cause alimentary diseases [2].

The control of *Bacillus* spores plays an important role in quality assurance and safety of processed foods. Their growth can be avoided by either sterilization, that allows inactivation of spores and vegetative cells, or pasteurization, that allows inactivation of vegetative cells, in combination with a lowering of pH (≤4.6) or water activity (a_w<0.90) or with the combined action of both parameters (spore germination inhibition) [3] [4]. However, in some food products, the reduction of pH to values lower than 4.6 or a thermal treatment of sterilization can lead to nutritional and organoleptic changes, that are not always satisfactory for the consumer.

Social and economic changes over the last few decades have led to new trends in food production. These trends meet consumers demand for minimally processed, healthy products with a limited amount of chemical preservatives.

Despite predictive modelling software are available, they can process the growth curves of micro-organisms of greatest interest on the basis of temperature, pH and a_w values; however, these elaborations do not consider the effect of the type of acid or solute used, that play a very important role on microbial development [5] [6].
Therefore, the aim of this work was to produce data for the most occurring spoilage bacilli (*B. sublitilis, B. licheniformis, B. cereus*) at different pH and water activity range in broth added with different solutes and/or organic acids and to monitor the potential outgrowth of these spores when stored at different temperatures at non-optimal and optimal conditions.
The assessment of the combined effect of the above mentioned physico-chemical parameters, allows to identify the conditions that could preserve the product by means of a less drastic heat treatment, thus guaranteeing its safety and commercial stability without remarkably changing its organoleptic characteristics.

2. MATERIAL AND METHODS

2.1 Microorganisms

Spore mixtures of the following strains of *Bacillus* genus were tested:
- 6 strains of *Bacillus cereus:* ATCC® 13061™; ATCC® 9139™; SSICA 768/4 isolated from pesto sauce; SSICA GRA from cheese; SSICA PM from pesto sauce; Bacillus *weihenstephanensis* SSICA14 from almond milk;
- 4 strains of *Bacillus subtilis: B. subtilis* subsp. *spizizeni* ATCC® 6633™; *Bacillus atropheus* ATCC® 9372™; SSICA A/904 and SSICA 856/C isolated from pesto sauces;
- 3 strains of *Bacillus licheniformis:* ATCC® 14580™; SSICA N isolated from pesto sauce; SSICA A from vegetable cream.
Before inoculation, the spores were activated at 80°C for 10 minutes.

2.2 Preparation of spore suspension

Nutrient Agar (Biolife Milano Italia) with the addition of 0.3% $MnCl_2$ was chosen for strain sporulation. At first, actively proliferating cells were obtained from several passages in Brain Heart Infusion (BHI – Oxoid Cambridge UK) incubated for 24 hours at 30°C; then spores were obtained by inoculating the proliferating cells in plates incubated for 5-7 days at 30°C. The spores were then harvested in sterile distilled water by means of a sterile loop, cleaned by centrifugation at 4500 g for 10 min and then suspended in distilled water. Spore suspensions were finally heat shocked at 80°C for 10 minutes and stored at 4°C until use.
Brain Heart Infusion (Oxoid, Cambridge UK) was the medium used for tests. Selected water activities were reached, in the range a_w 0.93-0.90, by the addition of appropriate amount of sodium chloride (NaCl) (Vwr, Milano Italia), sucrose (Vwr Milano Italia) or glycerol (Sigma-Aldrich St. Louis, Missouri, USA).
Modified media were sterilized for 15 min at 121° C. Each of them was than adjusted to various pH values ranging from 5.5 to 4.9 by adding a 92% Lactic acid solution (Carlo Erba, Milano Italia), a 50% acetic acid solution (J.T. Baker Deventer Holland) or a 50% citric acid monohydrate solution (VWR International Leuven Belgium).
Water activities of the experimental broths were measured by an a_w-meter Aqualab (Steroglass- San Martino in Campo, Perugia Italia).
pH measurement was performed using the Mettler Toledo pH meter (Novate Milanese Italia).
Plate Count Agar (PCA-Oxoid, Cambridge UK) was used for counting *Bacillus;* plates were incubated at 30° C for 24/48 hours.

2.3 Growth test

For each combination and temperature, 20 ml Pyrex® sterile test tubes with screw cap (filled with 10 ml of broth) were separately inoculated with spore mixtures to achieve a final concentration of 10^3 CFU/ml.
Tests were performed in triplicate. Moreover, for each a_w / solutes condition without acids added (pH = 7) three tubes were also inoculated as positive control.
Samples were incubated at 12° and 30° C. In addition, tubes inoculated with B. *cereus* spore mixture were also incubated at 6° C.
Tubes were daily observed for visible growth and analyzed after 14, 28, 60 and 90 days for samples stored at 30° C, whereas the analyzes were performed after 28, 60 and 90 days for samples stored at 12° and 6° C.

3. RESULTS AND DISCUSSION

3.1 Tests at 30°C

Table 1 shows the results of growth tests carried out at 30° C with spore mixture of *B. subtilis, B. licheniformis* and *B. cereus.*
The lowering of the water activity alone, without acidification, was not effective in inhibiting the inoculated spores: all spore mixtures grew (> 10^6 CFU / ml) in 2-28 days, except for *B. cereus* spores that didn't grow at a_w 0.90 in the presence of NaCl.
As can be clearly seen, no spore mixture grew in broths acidified at pH 5.1 with acetic acid.
At a_w 0.93 growth occurred even at the lowest pH values tested (pH 4.9) regardless of the solute used.
In acidified broth only sucrose allowed growth at a_w 0.92-0.91 and 0.90.

Table 1 Germination and growth ability of *B. subtilis* (Bs)*, B. licheniformis* (Bl) and *B. cereus* (Bc) spore mixtures in cultural broth under different combinations of a_w and pH values at 30°C.

Solutes	a_w	pH									
		pH 7	Lactic acid			Citric acid			Acetic acid		
			5.1	5	4.9	5.1	5	4.9	5.1	5	4.9
Glycerol	0.93	Bs-Bl-Bc (2-4)*	Bs-Bl-Bc (6-14)	Bs-Bl-Bc (6-14)	Bs-Bl-Bc (14)	Bs-Bl-Bc (7-14)	Bs-Bl-Bc (10-14)	Bs-Bl (12-14)	-	-	-
	0.92	Bs-Bl-Bc (7-14)	-	-	-	-	-	-	-	-	-
	0.91	Bs-Bl-Bc (7-14)	-	-	-	-	-	-	-	-	-
	0.90	Bs-Bl-Bc (14-28)	-	-	-	-	-	-	-	-	-
NaCl	0.93	Bs-Bl-Bc (2-3)	Bs (14)	Bs (14)	Bs (14)	Bs-Bc (14-20)	Bs-Bc (14-23)	Bc (38)	-	-	-
	0.92	Bs-Bl-Bc (3-8)	-	-	-	-	-	-	-	-	-
	0.91	Bs-Bl-Bc (10)	-	-	-	-	-	-	-	-	-
	0.90	Bs-Bl (10)	-	-	-	-	-	-	-	-	-
Sucrose	0.93	Bs-Bl-Bc (2-3)	Bs-Bl (4-7)	Bs-Bl (4-7)	Bs-Bl (11-14)	Bs-Bl-Bc (4-14)	Bs-Bl-Bc (4-14)	Bs-Bl-Bc (7-14)	-	-	-
	0.92	Bs-Bl-Bc (5-14)	Bs-Bl (14-28)	Bs (14)	-	Bs-Bl (28-60)	Bs (60)	-	-	-	-
	0.91	Bs-Bl-Bc (14)	Bs-Bl (14)	Bs (14)	-	Bs-Bl (14)	Bs (14)	-	-	-	-
	0.90	Bs-Bl-Bc (14-28)	Bs (14)	-	-	-	-	-	-	-	-

*: numbers show minimum and maximum growth time (days)

3.2 Tests at 12°C

The trials were previously carried out at the same pH values tested at 30° C (pH 5.1-4.9); as growth was never observed (data not reported), tests were carried out at higher pH values (pH = 5.5 and 5.3).
Table 2 shows the results. As reported in literature [5] [7] *B. licheniformis* did not grow at 12° C in any of the tested conditions; *Bacillus subtilis* was less sensitive to low temperature; only this microorganism grew at a_w 0.92 with glycerol (pH 7) after 2 months of storage.
Growth at pH 5.3 was observed only with Sucrose.

Table 2 Germination and growth ability of *B. subtilis* (Bs)*, B. licheniformis* (Bl) and *B. cereus* (Bc) spore mixtures in cultural broth under different combination of a_w and pH values at 12°C.

Solutes	a_w	pH						
		pH 7	Lactic acid		Citric acid		Acetic acid	
			5.5	5.3	5.5	5.3	5.5	5.3
Glycerol	0.93	Bs-Bc (27-28)*	Bs (60)	-	-	-	-	-
	0.92	Bs (60)	-	-	-	-	-	-
	0.91	-	-	-	-	-	-	-
	0.90	-	-	-	-	-	-	-
NaCl	0.93	Bs-Bc (42-60)	-	-	-	-	-	-
	0.92	-	-	-	-	-	-	-
	0.91	-	-	-	-	-	-	-
	0.90	-	-	-	-	-	-	-
Sucrose	0.93	Bs-Bc (28-60)	Bs-Bc (60)	Bc (90)	Bs-Bc (60)	Bs-Bc (60)	-	-
	0.92	-	-	-	-	-	-	-
	0.91	-	-	-	-	-	-	-
	0.90	-	-	-	-	-	-	-

*: numbers show minimum and maximum growth time (days)

3.3 Tests at 6°C

At 6°C *B. cereus* spore mixture did not grow in 90 days of incubation even at a_w 0.93 and pH 7.

So the two psychrotrophic strains, *Bacillus cereus* SSICA GRA and *B. weihenstephanensis* SSICA14, were separately inoculated to verify their growth ability in long storage times (10 months): theydid not growthey grew only in non-modified BHI broth (a_w = 0.98, pH = 7) in 2 months.

Tests carried out clearly show that combined action of pH, a_w, temperature, type of acid/solute, strongly influence germination and growth of microbial spores; a_w values tested were chosen to achieve safety against *Clostridium botulinum* spores [8].

Maximum incubation time (90 days) was considered since previous tests showed that, if environmental conditions allowed spore germination, growth occurred at 30° C within 45/60 days (data not published). Moreover, it must be taken into account that most refrigerated foods (REPFED food) have a maximum shelf life of 90 days [9].

The temperature of 12°C was chosen as a temperature of strong thermal abuse for products intended to be stored refrigerated (4/5° C). The results show that this temperature heavily affects the growth of the microorganisms studied.

At 30°C, a combination of a_w 0.92, with glycerol or NaCl, and pH 5.1 (acidified with the three different organic acids) inhibits the germination of all strains spores; *B. cereus* did not growth also when sucrose was used as solute.

About *B. cereus*, growth limit conditions for Combase predictor System are a_w 0.94, pH 4.9, even at 5°C [5]. The mixture of six strains tested in this study grew also at a_w 0.93 (sucrose or glycerol added) at pH 4.9 only at 30°C.

B. subtilis was the species able to grow in the less favorable conditions.

About organic acids tested, acetic acid proved the strongest antimicrobial action: it did not allow growth in any of the tested conditions. It could therefore provide a contribution to the shelf life of food products, but its use is limited, since it proved to changes the organoleptic characteristics of foods.

Among solutes used, NaCl resulted more effective in inhibiting growth when used in combination with acids tested (especially against *B. licheniformis*); on the contrary, sucrose, allowed growth also in the extreme conditions of a_w and pH tested.
Also William H. Sperber observed that the type of solute used to control a_w had some influence on growth patterns [10]; in his data, sodium chloride, potassium chloride, glucose or sucrose showed similar patterns whereas glycerol allowed growth at lower a_w values.
Quintavalla evaluated the effect of two acids (citric and o-phosphoric acid) and two solutes (NaCl and glycerol), used separately or in combination, on eight *Bacillus* strains at 30°C; all strains were inhibited at pH 5.0 and a_w 0.92 in agreement with the present study; but, no growth was observed with glycerol at a_w lower than 0.92 and pH 7 [11].
More recently, Franceschini tested *Bacillus* growth in BHI modified with glycerol and acidified with HCl: in this paper *B. subtilis* grew up to a_w 0.92 and pH 5.1: hydrochloric acid showed a lower inhibiting effect than organic acids [12].
The variability of results obtained in different studies clearly indicates how substrates, environmental conditions and strains tested, could play a pivotal role in assessing microbial growth.
It is important to underline that the results obtained in cultural broth, need confirmation in food products.
Table 3 summarize the combinations of a_w, pH and temperatures that could allow the preservation of a food product with a pasteurization heat treatment.

Table 3 Combinations of chemical-physical parameters evaluated that proved effective in inhibiting spore germination and growth in 90 days.

Temperature	Solutes	a_w	pH	Organic acid
30°C	Glicerol NaCl Sucrose	0.93	5.1	Acetic acid
30°C	Glicerol NaCl	0.92	5.1	Lactic acid Citric acid Acetic acid
12°C	Glicerol	0.92	5.5	Lactic acid Citric acid Acetic acid
12°C	NaCl Sucrose	0.92	7	-

4. CONCLUSION

The results obtained by monitoring behavior of *Bacillus* spores, at different pH and a_w values, using different solutes and organic acids, at non-optimal and optimal temperature conditions allow to detect the combinations that inhibit spore germination in three months of storage.
The confirmation of the results in food products will permit to safety storage products like sauces and vegetable creams, condiments, mousses, velvets, preserving and improving as much as possible the natural organoleptic characteristics.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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