

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

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6 **Barbara Franceschini<sup>1\*</sup>, Maria Paola Previdi<sup>1</sup>, Irene Schianchi<sup>2</sup>**

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9 <sup>1</sup> *Microbiology – Quality, Safety and Pre-Industrialization Area, Stazione Sperimentale delle*  
10 *Conserve Alimentari SSICA-Fondazione di Ricerca, Parma, Italy.*

11 <sup>2</sup> *Strada Terra Amara 12, Langhirano, Parma, Italy.*  
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17 **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 ( $\leq 4.6$ ) 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. subtilis*, *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.

18  
19 *Keywords: [Bacillus, growth limit conditions, pH,  $a_w$ , storage temperature]*

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23 **1. INTRODUCTION**

24  
25 The genus *Bacillus* includes a large group of sporogenic, pathogenic and non-pathogenic microorganisms widespread in  
26 nature (soil, water, dust). Their versatile metabolism and their ability to form spores resistant to heat, drying and

27 chemicals, make these bacteria one of the most important and common group of spoilage organisms affecting food  
28 products [1].

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

30 *B. licheniformis* together with *B. subtilis* belong to mesophiles whereas *B. cereus* is rather a psychrotrophile  
31 microorganism, that proved able to grow in products at cold-storage temperatures and even cause alimentary diseases  
32 [2].

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

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

41 Despite predictive modelling software are available, they can process the growth curves of micro-organisms of greatest  
42 interest on the basis of temperature, pH and  $a_w$  values; however, these elaborations do not consider the effect of the type  
43 of acid or solute used, that play a very important role on microbial development [5,6].

44 Therefore, the aim of this work was to produce data for the most occurring spoilage bacilli (*B. subtilis*, *B. licheniformis*, *B.*  
45 *cereus*) at different pH and water activity range in broth added with different solutes and/or organic acids and to monitor  
46 the potential outgrowth of these spores when stored at different temperatures at non-optimal and optimal conditions.

47 The assessment of the combined effect of the above mentioned physico-chemical parameters, allows to identify the  
48 conditions that could preserve a product by means of a less drastic heat treatment, thus guaranteeing its safety and  
49 commercial stability without remarkably changing its organoleptic characteristics.

## 51 2. MATERIAL AND METHODS

### 53 2.1 Microorganisms

54 Spore mixtures of the following strains of *Bacillus* genus were tested:

55 - 6 strains of *Bacillus cereus*: ATCC® 13061™; ATCC® 9139™; SSICA 768/4 isolated from pesto sauce; SSICA GRA  
56 from cheese; SSICA PM from pesto sauce; *Bacillus weihenstephanensis* SSICA14 from almond milk;

57 - 4 strains of *Bacillus subtilis*: *B. subtilis* subsp. *spizizeni* ATCC® 6633™; *Bacillus atrophaeus* ATCC® 9372™; SSICA  
58 A/904 and SSICA 856/C isolated from pesto sauces;

59 - 3 strains of *Bacillus licheniformis*: ATCC® 14580™; SSICA N isolated from pesto sauce; SSICA A from vegetable cream.

60 Before inoculation, the spores were activated at 80°C for 10 minutes.

### 62 2.2 Preparation of spore suspension

63 Nutrient Agar (Biolife Milano Italia) with the addition of 0.3%  $MnCl_2$  was chosen for strain sporulation. At first, actively  
64 proliferating cells were obtained from several passages in Brain Heart Infusion (BHI – Oxoid Cambridge UK) incubated for  
65 24 hours at 30°C; then spores were obtained by inoculating the proliferating cells in plates incubated for 5-7 days at 30°C.  
66 The spores were then harvested in sterile distilled water by means of a sterile loop, cleaned by centrifugation at 4500 g for  
67 10 min and then suspended in distilled water. Spore suspensions were finally heat shocked at 80°C for 10 minutes and  
68 stored at 4°C until use.

69 Brain Heart Infusion (Oxoid, Cambridge UK) was the medium used for tests. Selected water activities were reached, in the  
70 range  $a_w$  0.93-0.90, by the addition of appropriate amount of sodium chloride (NaCl) (Vwr, Milano Italia), sucrose (Vwr  
71 Milano Italia) or glycerol (Sigma-Aldrich St. Louis, Missouri, USA).

72 Modified media were sterilized for 15 min at 121° C. Each of them was than adjusted to various pH values ranging from  
73 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  
74 Holland) or a 50% citric acid monohydrate solution (VWR International Leuven Belgium).

75 Water activities of the experimental broths were measured by an  $a_w$ -meter Aqualab (Steroglass- San Martino in Campo,  
76 Perugia Italia).

77 pH measurement was performed using the Mettler Toledo pH meter (Novate Milanese Italia).

78 Plate Count Agar (PCA-Oxoid, Cambridge UK) was used for counting *Bacillus*; plates were incubated at 30° C for 24/48  
79 hours.

### 81 2.3 Growth test

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

#### 92 3.1 Tests at 30°C

95 Table 1 shows the results of growth tests carried out at 30° C with spore mixture of *B. subtilis*, *B. licheniformis* and *B.*  
96 *cereus*.

97 The lowering of the water activity alone, without acidification, was not effective in inhibiting the inoculated spores: all spore  
98 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  
99 NaCl.

100 As can be clearly seen, no spore mixture grew in broths acidified at pH 5.1 with acetic acid.

101 At  $a_w$  0.93 growth occurred even at the lowest pH values tested (pH 4.9) regardless of the solute used.

102 In acidified broth only sucrose allowed growth at  $a_w$  0.92-0.91 and 0.90.  
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**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.

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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)	-	-	-	109
	0.92	Bs-Bl-Bc (7-14)	-	-	-	-	-	-	-	-	-	110
	0.91	Bs-Bl-Bc (7-14)	-	-	-	-	-	-	-	-	-	111
	0.90	Bs-Bl-Bc (14-28)	-	-	-	-	-	-	-	-	-	112
	0.90	Bs-Bl-Bc (14-28)	-	-	-	-	-	-	-	-	-	113
NaCl	0.93	Bs-Bl-Bc (2-3)	Bs (14)	Bs (14)	Bs (14)	Bs-Bc (14-20)	Bs-Bc (14-23)	Bc (38)	-	-	-	114
	0.92	Bs-Bl-Bc (3-8)	-	-	-	-	-	-	-	-	-	115
	0.91	Bs-Bl-Bc (10)	-	-	-	-	-	-	-	-	-	116
	0.90	Bs-Bl-Bc (10)	-	-	-	-	-	-	-	-	-	117
	0.90	Bs-Bl-Bc (10)	-	-	-	-	-	-	-	-	-	118
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)	-	-	-	119
	0.92	Bs-Bl-Bc (5-14)	Bs-Bl (14-28)	Bs (14)	-	Bs-Bl (28-60)	Bs (60)	-	-	-	-	120
	0.91	Bs-Bl-Bc (14)	Bs-Bl (14)	Bs (14)	-	Bs-Bl (14)	Bs (14)	-	-	-	-	121
	0.90	Bs-Bl-Bc (14-28)	Bs (14)	-	-	-	-	-	-	-	-	122
	0.90	Bs-Bl-Bc (14-28)	Bs (14)	-	-	-	-	-	-	-	-	123

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

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### 3.2 Tests at 12°C

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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).

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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.

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Growth at pH 5.3 was observed only with Sucrose.

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**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.

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Solutes	$a_w$	pH						144
		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	Bs	-	-	-	-	148
		(27-28)*	(60)	-	-	-	-	149
	0.92	Bs	-	-	-	-	-	150
		(60)	-	-	-	-	-	151
		-	-	-	-	-	-	152
0.91	-	-	-	-	-	-	153	
0.90	-	-	-	-	-	-	154	
NaCl	0.93	Bs-Bc	-	-	-	-	-	155
		(42-60)	-	-	-	-	-	156
	0.92	-	-	-	-	-	-	157
		-	-	-	-	-	-	158
		-	-	-	-	-	-	159
0.91	-	-	-	-	-	-	160	
Sucrose	0.93	Bs-Bc	Bs-Bc	Bc	Bs-Bc	Bs-Bc	-	161
		(28-60)	(60)	(90)	(60)	(60)	-	162
	0.92	-	-	-	-	-	-	163
		-	-	-	-	-	-	164
		-	-	-	-	-	-	165
0.91	-	-	-	-	-	-	166	
0.90	-	-	-	-	-	-	166	

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

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178 **3.3 Tests at 6°C**  
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180 At 6°C *B. cereus* spore mixture did not grow in 90 days of incubation even at  $a_w$  0.93 and pH 7.

181 So the two psychrotrophic strains, *Bacillus cereus* SSICA GRA and *B. weihenstephanensis* SSICA14, were separately  
182 inoculated to verify their growth ability in long storage times (10 months): they did not grow, they grew only in non-  
183 modified BHI broth ( $a_w = 0.98$ , pH = 7) in 2 months.  
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186 Tests carried out clearly show that combined action of pH,  $a_w$ , temperature, type of acid/solute, strongly influence  
187 germination and growth of microbial spores;  $a_w$  values tested were chosen to achieve safety against *Clostridium botulinum*  
188 spores [8].

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

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

194 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)  
195 inhibited the germination of all strains spores; *B. cereus* did not growth also when sucrose was used as solute.

196 About *B. cereus*, growth limit conditions for Combbase predictor System are  $a_w$  0.94, pH 4.9, even at 5°C [5]. The mixture  
197 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.

198 Quintavalla evaluated the effect of two acids (citric and o-phosphoric acid) and two solutes (NaCl and glycerol), used  
199 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  
200 agreement with the present study; but, no growth was observed with glycerol at  $a_w$  lower than 0.92 and pH 7 [11].

201 More recently, Franceschini tested *Bacillus* growth in BHI modified with glycerol and acidified with HCl: in this paper *B.*  
202 *subtilis* grew up to  $a_w$  0.92 and pH 5.1: hydrochloric acid showed a lower inhibiting effect than organic acids [12].

203 The variability of results obtained in different studies clearly indicates how substrates, environmental conditions and  
204 strains tested, could play a pivotal role in assessing microbial growth.

205 At 30° and 12°C, acetic acid showed an inhibiting effect regardless of the  $a_w$  values tested; It could therefore provide a  
206 contribution to the shelf life of food products, but its use is limited, since it proved to changes the organoleptic  
207 characteristics of foods. The inhibitory effect of acids followed the order: acetic acid > lactic acid > citric acid.

208 The impact on the growth due to the solute was greater for NaCl, according to the order: NaCl > Glicerol > Sucrose.

209 Also William H. Sperber observed that the type of solute used to control  $a_w$  had some influence on growth patterns [10]; in  
210 his data, sodium chloride, potassium chloride, glucose or sucrose showed similar patterns whereas glycerol allowed  
211 growth at lower  $a_w$  values.

212 *B. subtilis* was the species able to grow in the less favorable conditions at 30° and 12°C, followed by *B. licheniformis* and  
213 *B. cereus*; *B. subtilis* could therefore be the reference species to keep control in products which have similar  $a_w$  and pH  
214 values.

215 The behavior of *B. subtilis* and *B. cereus* at 12°C proved to be similar.

216 Table 3 summarize the combinations of  $a_w$ , pH and temperatures that inhibited spore's germination according to acid and  
217 solute used.  
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**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	0.93	5.1	Acetic acid
	NaCl			
	Sucrose			
30°C	Glicerol	0.92	5.1	Lactic acid
	NaCl			Citric acid
				Acetic acid
12°C	Glicerol	0.92	5.5	Lactic acid
				Citric acid
				Acetic acid
12°C	NaCl	0.92	7	-
	Sucrose			

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#### 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.

About organic acids tested, acetic acid proved the strongest antimicrobial action. Among solutes used, NaCl resulted more effective in inhibiting growth in combination with acids tested; on the contrary, sucrose, allowed growth also in the extreme conditions of  $a_w$  and pH tested

The results obtained in cultural broth could be useful for food preservation. Clearly it is necessary to check the results in the product; if they are confirmed, it will be possible to safety storage products like sauces and vegetable creams, condiments, mousses, velvets, preserving and improving as much as possible the natural organoleptic characteristics.

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#### COMPETING INTERESTS

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All authors declared that no competing interests exist.

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#### AUTHORS' CONTRIBUTIONS

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'Barbara Franceschini and Maria Paola Previdi' designed the study, wrote the protocol, and wrote the first draft of the manuscript. 'Irene Schianchi' managed the analyses of the study and the literature searches.

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All authors read and approved the final manuscript.

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