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Effect of Sporulation Medium Content in Carbohydrates, Lipids, and Proteins on Outgrowth of *Bacillus spp* Spores Activated with Heat and Ethanol Solution

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Authors' contributions

This work was carried out in collaboration among all authors. Authors ANT, SLSK and FXE designed the study. Authors ANT and HDN wrote de protocol, managed the analyses of the study and literature searches with author SLSK. Author SLSK performed the statistical analysis with author ANT. Authors ANT, SLSK and FXE wrote the first draft of manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: evaluate the effect of the nutrient content of the medium on the sporulation percentage and the efficiency of ethanolic activation on the outgrowth of *Bacillus* spp.
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Methodology: In order to contribute to the understanding of the effect of the environment of spore origin on the ability to sporulate and outgrow in foods, spores of *Bacillus cereus* and *Bacillus subtilis* were produced in different media differenciated by the carbohydrates, lipids and proteins composition. Mathematical models predicting sporulation rates and germination yields of heat- and ethanol-activated spores were evaluated as a function of the factors selected. The reliability of these models was assessed by comparing the predicted values with those obtained in real food.
Results: It was found that spores originating from high carbohydrates and proteins media had high sporulating and germination rates. It was found that the origin of the spores, mainly the carbohydrate and protein content, resulted in high sporulation rates and that spores from nutrient-rich media germinated faster than those from nutrient-poor media. Germination efficiency varied from strain to strain depending on the method of activation and the composition of the medium in which the spores were produced. The mathematical models obtained were validated in food matrices with correlation of predicted and observed values higher than 0.85.
Conclusion: The ethanolic activation of spore varies depending on the strain and the nutrient content especially the fat content in sporulating medium. The proteins and carbohydrates have significant content for thermal activation.

Keywords: Bacillus spore; activation; ethanol; heat.

1. INTRODUCTION

Contamination of food by microorganisms is a global problem which affects both developed and developing countries. According to the WHO, more than 200 diseases are transmitted through the consumption of food. One in 10 people is affected by foodborne illness [1]. The cost linked to the deterioration of food is in terms of billions of dollars, american industries lose between 3 to 35 billion dollars per year [2]. In the Netherlands, a study estimate loss of about 468 million euros per year [3]. Among these microbial contaminants, sporulating bacteria particularly of the genus *Clostridium* and *Bacillus*, are the most resistant. Indeed, the spores that they produce are considered as one of the most difficult microbial forms to eliminate [4]. Though *Clostridium* is known to be most pathogenic, we still observe that the genus *Bacillus* is the most implicated in food deterioration and food-borne illnesses. They are found in most foods, including rice, bread, fruit juices, dehydrated foods and dairy products [5;6]. If their elimination from food is not easy, their enumeration in food remains a real challenge for most bacteriological analysis laboratories. Food microbiology standards recommend treatment at a sublethal temperature for spore prior to their enumeration. Thermal activation is therefore known as the recommended method for the detection of spores in foods [7]. It has been observed that some vegetative are resistant to the sublethal temperatures and that some conditions of thermal treatment can exted the germination time of spore causing bias in their enumeration [8;9]. However, the problems of thermal-resistance and thermally induced dormancy have led to the search for other alternatives likes chemicals solutions (formaldehyde, alcohol) and high pressure. Among these alternatives, ethanolic activation seems to be more reliable [10;11]. Indeed, it has been demonstrated that the exposure of spores to ethanol combined with agitation improves the kinetic parameters and the germination yields of spores and contrary to thermal activation, there may be no problem of "induced alcohol resistance" [11,12]. [10] observed that the effectiveness of a method varied according to the foods matrix under analysis, thus posing the problem of spore origin on the effectiveness of the activation methods. Many authors have demonstrated that factors such as spore temperature, pH and composition of the spore medium have an impact on spore germination [11,13]. However, very little data showing the effect of the nutrient content of the spore-forming medium on spore germination exists in the literature. The purpose of this work is therefore to evaluate the effect of the nutrient content of the medium on sporulation percentage and the efficiency of ethanolic activation on the outgrowth of *Bacillus* spp.

2. MATERIALS AND METHODS

2.1 Spores Productions

Spore production was carried out in a medium containing, $(NH4)_2SO_4$ 2g, K_2HPO_4,$3H_2O$ (0.08), $MgSO4,7H_2O$ (0.1), $MnSO4,4H_2O$ (0.05) and $CaCl_2$ (0.08). This medium was enriched with sources of carbohydrates (glucose), lipids (margarine) and proteins (yeast extract) based

on a three-factor Central composite design (CCD) with 5 levels each (Table 1). 10^3 cells/mL *Bacillus cereus* and *Bacillus subtilis* were inoculated in different media formulated according to this experimental plan and incubated at 37°C for 7 days. Cells of each condition were harvested by scraping the media surface with a sterile platinum loop. The resulting cells were exposed to ethanol (50% V/V) for 12H at 4°C and several centrifugations (4000tr/min for 15min at 4°C) were performed to separate the spores from the sporangia [14]. The pellet obtained, thus constituting the spores, was suspended in sterile distilled water and stored at 4°C during 1 month for maturation. The spore stock thus produced contained 99.999% sporal cells, as observed under the Ivymen System phase contrast microscope.

2.2 Determination of Sporulation Percentage in the Different Conditions

For each condition, the harvested cells were diluted and seeded, on one hand on nutrient agar to determine the total number of viable cells and on the other hand, these cells were exposed to a temperature of 80°C in a water bath for 10min. Then cooled in an ice bath, maintained at room temperature, and then seeded on nutrient agar to determine the number of spores. The sporulation percentage was thus obtained according to the following formula:

$$\text{Sporulation } \% = \frac{\text{spore count}}{\text{total number of viable cells}} * 100$$

2.3 Activation of Spores

The spores thus produced in different media enriched with carbohydrates, lipids and proteins were being activated using ethanol for the test experiment and using heat as control.

Heat activation:

From the stock of spores produced, serial dilutions were performed in order to have 10^4 spores/mL. The selected dilutions were introduced into a water bath set at 80°C for 10 min. then the tubes were cooled in an ice bath and maintained at room temperature during counting. Decimal dilutions were carried out and 0.1mL of each dilution were seeded by spreading on nutrient agar, and then incubated at 37°C for 24 hours.

Ethanol activation:

The suspensions from each sample of spore and ethanol at a final volume of 20mL were mixed in a sterile 250mL of conical flask with ethanol for a final volume of 20mL and 30%(V/V) ethanol concentration. The resulting mixture containing 10^4 spores/ml were shaken by using an IK H2 260 shaker bath at 70tr/min for 60min. Serial dilution were used to stop the effect of ethanol. Later, decimal dilutions were carried out and sown by spreading on nutrient agar in petri dishes and incubated at 37°C for 24 hours.

2.4 Monitoring of Spore Germination

Germination was monitored by following the individual spore cells of a population on solid medium according to the Protocol described by [11]. Germination time were compared assuming then proportional to the detection time. In fact, it has been observed that if cells of different physiological states are cultured under the same condition, then the difference in latency time is proportional to the difference in their detection time. [15]. Preliminary work has verified that vegetative cells from spores of the same bacterial strain have the same rate during growth as spores during their outgrowth (Fig. 1). Based on this, within a population, while the rate of colony formation represents the rate at which individual spores germinate and outgrowth to visible colonies of about 10^6 cells within a population. These considerations will be guiding interpretation henceforth. Thus, it is here considered that the difference in time taken by two sporal cells to form a visible colony (about10^6 cells), is proportional to their germination time. Thus, the germination kinetics of spores

Table 1. CCD plan for the formulation of culture media

Media	Factors to consider (g/l)	Code	Factor levels				
			2	1	0	-1	-2
Composition	Carbohydrates	G	80	60	40	20	0
	Lipids	L	50	37.5	25	12.5	0
	Proteins	P	10	7.5	5	2.5	0

within a population can be obtained by evaluating the time of appearance of the colonies originating from each individual spore. The germinated spores were therefore counted at their appearance over 24 hours and expressed cumulatively in terms of colonies that appeared over time. The activated spores were sown by spreading (0.1mL) on nutrient agar and incubated for 24H (time obtained after preliminary work after which 99.99% of spores germinated). The number of colonies observed at that time was considered to be the maximum number of spores that was able to germinate and grow. The first colonies that appeared were counted and marked with an indelible marker. Then during incubation, the new colonies were counted every 30 minutes and marked during 24 hours of incubation. Colonies counted in an interval of 30min were considered as the number of colonies with the same detection time which was hence considered as proportional to the germination time. Then kinetics of the colonies that appeared every 30min were plotted as a function of time and adapted to [15] which model to estimate the minimum time of outgrown of a spore in a population (P.lag) and the rate of colony formation. This latency (P.lag) is considered as proportional to the minimum germination time of spore.

2.5 Data Management

The resulting data of germination were transformed and expressed in terms of the growth ratio at a certain time of incubation according to equation (eq1). These data were then transformed according to the Weibull model (eq2). The kinetic data was then adapted to the Weibull model (eq2) where N_{24h} is considered as the initial number of spores (N_0) at t=0min.

Ratio of germinated spores $=Nt/N_{24h}$ (number of spores germinated at time t / number of spores germinated after 24 h) (eq 1)

$$S(t) = Nt/N0 = \exp\lfloor -b * t^n \rfloor \quad \text{(eq 2)}$$

Nt is the number of spores that germinated at a time t, b and n are the parameters to be estimated. In order to calculate the outgrowth distribution times of the population of the sporal cell, the Weibull probability density function was used.

$$PDF = b * n * n * t^{n-1} \exp\lfloor -b * t^n \rfloor \quad \text{(eq 3)}$$

The distribution parameters (mode and mean) were calculated as in [16]. Germination yields were evaluated according to the formula:

$$yield = \frac{\text{number of spores that germinated after 24h}}{\text{number of spores inoculated}} * 100$$

The relationship between germination yields and the composition of the sporulation medium was assessed by a multiple regression and a prediction model obtained.

2.6 Validation of Prediction Model

The validation of sporulation model consisted in evaluating the sporulation percentage of *Bacillus cereus* and *Bacillus subtilis* in food having simillar characteristics of the experimental matrices used. Spores production in food, was done according to the modified protocol of [6]. Vegetative cells of *Bacillus cereus* and *Bacillus subtilis (*10^3 ufc / ml*)* were introduced into culture media made up of foodstuffs (rice; milk; gari; cassava; soya and Ecomba (maize +groundnut) in the proportions 5:50 W/V+ 1.5% of agar . The percentage composition of these food matrix were determined theoriticaly using mean values of nutritional tables. The food matrix thus sown were incubated at 37°C during seven days. After this period, the sporulation percentage were calculated. The Pearson correlation test allowed us to determine the reliability of the prediction models compared to the sporulation percentage obtained in food. Regarding the germination model, the validation consited to évaluate germination yield of spore produce in food milieu after heat and éthanol activation. The Pearson test allowed us to determine the reliability of the germination yield value predicted by the model compared to the germination yield obtained from spore produce in food.

3. RESULTS

3.1 Effect of the Content of Carbohydrates, Lipids and Proteins on the Sporulation Percentage

The sporulation percentage of *Bacillus cereus* and *Bacillus subtilis* produced in media whose content of carbohydrates, lipids and proteins varies are shown in the Table 2. The sporulation percentage varies from 7 to 86.09%, while we observed no direct tendency as function of to the composition of the medium. The lowest sporulation percentage was obtained from the medium containing only lipids (25g/L). Generally, for the two microbial strains, it can be noted that the highest sporulation percentage are obtained in the medium containing very high concentrations of carbohydrates.

Table 2. Sporulation percentage according to the conditions of sporulation

Conditions	Composition of the sporulation medium (g/L)			sporulation percentage (%)	
	Carbohydrates	Lipids	Proteins	*B. cereus*	*B. subtilis*
1	80	25	10	86.09	65.63
2	80	50	5	67.00	78.66
3	80	25	5	82.13	60.27
4	60	12.5	7.5	39.21	48.55
5	60	12.5	2.5	22.00	16.27
6	60	37.5	2.5	44.00	32.35
7	60	37.5	7.5	60.00	38.47
8	40	25	10	70.40	72.76
9	40	50	5	31.43	43.48
10	40	25	5	57.75	26.19
11	40	25	5	50.12	35.48
12	40	25	5	55.08	39.29
13	40	0	5	62.86	64.62
14	40	25	0	19.61	35.20
15	20	12.5	2.5	21.58	20.75
16	20	12.5	7.5	28.09	63.32
17	20	37.5	2.5	25.85	35.05
18	20	37.5	7.5	29.95	57.32
19	0	25	5	18.33	44.17
20	0	0	5	25.33	45.83
21	0	25	0	11.00	7.00

A multiple regression analysis of the sporulation percentage was performed and the mathematical equations reflecting the evolution of the sporulation percentage as a function of the composition of the medium were obtained. Equation 4 and 5 respectively describe the sporulation percentage of *Bacillus cereus* and *Bacillus subtilis* as a function of carbohydrates, lipids and proteins content. The illustration of these models is presented through surface responses in Fig. 2.

$$TS = 0.86 * G + 3.11 * P - 0.01 * G * L \quad \text{(eq 4)}$$

$$TS = 6.73 * P - 0.001891 * P * L * G + 0.0051 * G^2 + 0.01 * L^2 \quad \text{(eq 5)}$$

TS: sporulation percentage; G: carbohydrates, L: lipids, P: proteins

The variability of data expressed by the models (R^2) lies between 73 and 75%. According to the models, it can be noted that in absence of nutriments in the medium there is no sporulation. A combination of increasing concentrations of carbohydrates and proteins leads an increase in the sporulation percentage of *Bacillus cereus* and *Bacillus subtilis* (Fig. 2a and 2b). However, when concentrations of carbohydrate and lipids are combined in the medium, it is observed that *Bacillus cereus* sporulation percentage is proportional to the carbohydrates concentrations in the medium (Fig. 2c). Whereas in *Bacillus subtilis*, the sporulation percentage are proportional to the carbohydrates and lipids concentrations in medium (Fig. 2a). However, the combinations of high concentrations result in a concave evolution of the sporulation percentage as a function of carbohydrates or lipids content of the medium.

3.2 Effect of the Carbohydrates, Lipids and Proteins Content of the Sporulation Medium on Spore Germination Kinetic Parameters

3.2.1 Rate and time of appearance of the first colony

The germination kinetic parameters of spores produced under various conditions were evaluated and the results are presented in Table 3.

It can be seen from this table that the first colony emerging from spores appears more quickly in *B. cereus* than in *B. subtilis*. Meaning that germination is faster for *B. cereus* than for *B. subtilis*. In a general way, germination is faster after heat activation compared to ethanolic activation. From a statistical point of view, it can

be noted that after ethanolic activation the time of appearance of the first colony, proportional to the minimum germination time, varies significantly depending on the bacterial species (*P*=.00) and the proteins content (*P*=.00) of the medium in which the spores were produced. In the case of heat activation, the time of appearance of the first colony varies significantly only as a function of the bacterial species (*P*=.00). From the results presented in Table 3, spore production conditions do not significantly influence the colony emergence rates of ethanol-activated spores. With heat-activated spores, it can be noted that colony appearance rates vary significantly depending on the bacterial species (*P*= .01) and lipids content (*P*=.00) of the medium in which the spores were produced. Fig. 3 a and 3b is an example which show that the spores of *B. cereus* and of *B. subtilis* produced in media richer in lipids form colonies after thermal activation with a high speed compared to those coming from media having a low lipids content

3.2.2 Impact of sporulation media composition on spore germination times

The mode and mean of the distribution of time for colonies to appear from spore are reported as a function of the composition of the medium in which the spores were produced (Table 4). It can be noted that when germination is induced by ethanol, the distribution of time for colonies appear from spore (in other words the distribution of germination times within the spore population) varies significantly from species to species (*P=0.00)* as a function of the lipids content (*P=0.00)* of the medium in which the spores were produced (Figs. 4b and 4d). In the case of mean times of colony formation, there is a significant variation depending on the bacterial species (*P=0.02).* When germination is heat induced, we note that the mode and mean of the germination time of single spore within the population vary significantly depending on the species of activated bacterial spore (*P=0*.00). Fig. 4a and 4c illustrate this and show that the mode of distribution of colony emergence times is shorter in *Bacillus cereus* compared to *Bacillus subtilis*. from a statistical point of view, it can be said that the proteins content (*P=0.00*) of the medium in which the spores are produced, the bacterial species (*P=0.00)* of spore and the activation method (P=0.*00)* are the factors that most influence the mode of germination time distribution. Indeed, the mode of colony distribution is shorter when spores are heat activated. With regard to the mean of the distribution of time for colonies emerge from spore, the bacterial species (*P=0.00*) is the most significant factor.

3.3 Impact of Sporulation Composition on Spore Germination Yield

The germination yields of *B. cereus* and *B. subtilis* spores obtained after ethanolic activation and compared to thermal activation are shown in Table 5. It can be noted that germination yields is affected by the method and the species. After thermal activation the best yields are obtained with *B. subtilis* spores, whereas in the case of ethanolic activation the best yields are obtained with *B. cereus* spores. Multiple regression analysis was used to analyse germination yields and polynomial equations derived from it were obtained. Equations 6 and 7 represent the germination yields of heat-activated spores of *Bacillus subtilis* and *Bacillus cereus as a* function of the carbohydrate (G), lipid (L) and protein (P) content of the spore medium. Their surface response representations are shown in Figs. 5a, 5b for *B. subtilis* and 5c, 5d for *B. cereus*.

$$\mathrm{G_{yield}} = 1.22 * \mathrm{L} + 8.68 * \mathrm{P} - 0.08 * \mathrm{P} * \mathrm{G} - 0.133 * \mathrm{L} * \mathrm{P} + 0.006\mathrm{G}^2 \quad \text{(eq 6)}$$

$$\mathrm{G_{yield}} = e^{3.03+0.01*\mathrm{G}+0.05*\mathrm{L}+0.1*\mathrm{P}-0.00435*\mathrm{G}*\mathrm{P}+0.00007*\mathrm{G}*\mathrm{P}*\mathrm{L}-0.0011\mathrm{L}^2} \quad \text{(eq 7)}$$

In addition, equations 8 and 9 represent respectively the germination yields of *B. subtilis* and *B. cereus as a* function of the lipids (L), proteins (P) and carbohydrates (G) content of the spore-forming medium obtained after ethanolic activation. Their representations in the form of surface response are shown in Fig. 6a, b and 6c, d respectively.

$$G_{yield} = 2 * L + 9.24969 * P + 0.0032 * G * L * P - 0.02335 * G * L - 0.36227 * L * P \quad \text{(eq 8)}$$

$$\mathrm{G_{yield}} = 5.47 * \mathrm{P} - 0.11 * \mathrm{G} * \mathrm{P} + 0.007 * \mathrm{G}^2 - 0.06 * \mathrm{L}^2 + 3.3 * \mathrm{L} \quad \text{(eq 9)}$$

Table 3. Rate of spore germination and minimum germination time (P.lag) of single spores produced in different conditions

sporulation medium (g/L)			Rates (colonies/min) **				P.lag (min) **			
			Ethanolic		Heat		Ethanolic		Heat	
carbohydrates	lipids	proteins	*B.C* *	*B.S* *	*B.C* *	*B.S* *	*B.C* *	*B.S* *	*B.C* *	*B.S* *
80	25	10	9.9	4.5	0.1	1	469.6	577.3	247.4	370.1
80	50	5	0.1	3.5	0.6	0.6	235.0	523.7	320.2	422.3
80	25	5	20.6	2.2	0.2	3.2	662.0	558.3	299.9	459.9
60	12.5	7.5	5.3	2.2	3.0	14.3	496.2	538.2	489.1	730.8
60	12.5	2.5	0.2	0.9	0.3	0.4	304.5	550.8	317.4	435.0
60	37.5	2.5	6.5	0.3	0.2	1.2	593.8	487.2	249.7	380.6
60	37.5	7.5	1.1	3.7	1.5	7.3	350.6	514.9	335.0	389.1
40	25	10	0.1	1.9	0.1	4.4	268.4	477.0	282.8	385.5
40	50	5	3.3	0.5	0.1	2.8	524.9	527.6	287.1	655.7
40	25	5	0.1	1.7	0.1	2.8	293.2	511.4	228.4	588.3
40	25	5	0.2	0.8	0.9	0.1	301.3	460.5	379.5	325.3
40	25	5	0.2	1.5	0.1	0.3	327.3	496.5	319.3	422.8
40	0	5	0.1	4.1	0.7	0.2	274.5	536.0	278.9	353.2
40	25	0	4.2	1.2	0.6	0.2	322.4	452.3	286.5	351.3
20	12.5	2.5	2.0	2.2	19.6	4.4	167.2	572.7	353.5	657.9
20	12.5	7.5	0.5	11.0	3.0	9.9	289.5	563.4	289.9	387.8
20	37.5	2.5	2.2	4.1	0.8	4.5	308.1	583.5	309.4	612.9
20	37.5	7.5	2.8	9.4	0.6	3.7	196.4	575.1	311.8	367.8
0	25	5	0.4	20.9	0.9	8.8	143.7	575.0	220.0	333.0
0	0	5	0.8	13.4	0.7	2.6	219.5	488.1	237.5	312.5
0	25	0	0.6	17.3	1.3	7.4	210.6	563.6	279.3	352.4

** BC: Bacillus cereus; BS: Bacillus subtilis; ** the Fitting Baranyi and Roberts (1994) model to the date gave and R^2 > 0.99 in general and permitted the estimation of Rate and Plag in general, parameters estimated were all statistically significant with p<0.05 and standard error of estimation lower than 10% in general*

Table 4. The germination time of distribution parameters (mode, mean) of *B. cereus* and *B. subtilis* spores after thermal and ethanol activation

Sporulation medium (g/L)			Mode (hours) * *				Mean (hours) * *			
			Ethanolic		Heat		Ethanolic		Heat	
Carboh-ydrates	Lipids	Proteins	B.C *	B.S *	B.C *	B.S *	B.C *	B.S *	B.C *	B.S *
80	25	10	6.9	8.0	5.1	6.8	7.3	8.4	6.0	7.6
80	50	5	6.3	7.2	6.7	7.4	8.9	7.6	9.0	9.1
80	25	5	6.8	7.8	5.5	6.8	9.5	8.0	6.8	7.2
60	12.5	7.5	6.9	7.5	7.1	6.6	7.2	7.7	8.9	10.2
60	12.5	2.5	7.0	7.8	6.1	7.9	9.0	8.6	8.4	9.5
60	37.5	2.5	6.8	7.8	4.9	6.6	8.3	10.3	7.3	7.2
60	37.5	7.5	6.4	7.3	5.7	6.8	7.3	7.6	6.2	7.2
40	25	10	5.7	7.4	5.5	6.7	6.9	8.0	7.1	7.3
40	50	5	7.8	8.0	5.6	6.7	10.6	9.1	6.9	8.1
40	25	5	5.5	7.2	5.2	8.5	6.3	7.4	6.3	10.4
40	25	5	6.0	7.2	6.7	6.8	7.4	7.8	7.9	9.5
40	25	5	5.9	7.0	5.9	7.2	7.3	7.4	6.6	8.5
40	0	5	5.4	7.3	6.1	6.7	6.5	7.5	7.5	7.1
40	25	0	5.8	7.1	5.7	6.6	6.7	7.6	6.6	8.1
20	12.5	2.5	4.6	8.2	5.2	7.2	5.7	8.7	5.4	8.6
20	12.5	7.5	5.7	7.7	4.9	6.5	7.4	8.2	5.3	7.0
20	37.5	2.5	5.9	8.1	5.8	7.7	6.8	8.3	7.1	8.4
20	37.5	7.5	4.8	8.0	6.9	6.1	6.4	8.3	9.3	6.4
0	25	5	4.8	8.0	5.2	5.9	7.6	8.2	7.6	6.7
0	0	5	5.3	7.4	5.5	6.1	7.8	7.8	8.1	7.5
0	25	0	5.7	7.8	5.7	6.0	8.0	8.2	6.7	6.4

* BC: *Bacillus cereus*; BS: *Bacillus subtilis; * * Fitting of* $S(t) = \exp(-b*t^{n})$ *to the date gave and* $R^2 > 0.98$ *parameters estimated were all statistically significant with p=.05 and standard error of estimate lower than 10% in general*

Table 5. Germination yields according to the composition of the medium of sporulation

Composition of the sporulation medium (g/L)			Germination yields of the activated spores (%)			
			Heat activation		Alcoholic activation	
Carbohydrates	**Lipids**	**Proteins**	***B subtilis***	***B cereus***	***B subtilis***	***B cereus***
80	25	10	79.6	41.7	68.2	61.2
80	50	5	58.0	43.8	32.3	48.3
80	25	5	66.1	55.4	82.7	71.1
60	12.5	7.5	45.2	38.3	72.7	81.4
60	12.5	2.5	43.9	50.4	38.3	27.0
60	37.5	2.5	78.5	56.6	39.0	47.1
60	37.5	7.5	90.9	57.8	53.4	62.8
40	25	10	90.2	60.3	71.5	73.9
40	50	5	97.1	41.7	45.6	22. 1
40	25	5	42.0	63.3	51.1	65.1
40	25	5	49.0	62.9	56.7	64.5
40	25	5	50.1	61.4	56.1	60.1
40	0	5	79.5	33.3	55.1	22.6
40	25	0	64.1	69.4	35.1	68.6
20	12.5	2.5	72.1	48.4	48.7	77.7
20	12.5	7.5	97.7	56.8	67.4	78.5
20	37.5	2.5	22.9	50.1	62.4	59.8
20	37.5	7.5	91.9	31.1	54.6	72.1
0	25	5	81.4	64.6	58.6	62.1
0	0	5	77.7	47.4	59.5	40.2
0	25	0	85.9	46.0	62.9	58.8

Table 6. Sporulation percentage of *B. cereus* and *B. subtilis* in food according to the prediction model

Foods composition				Sporulation percentage			
Foods	**Carbohydrates**	**Proteins**	**Lipids**	**BS predict**	**BS observed**	**BC predict**	**BC observed**
Ecomba	56.75	9.63	12.63	69.09	64.29	71.57	75.30
Soja	37.00	10.00	21.00	60.59	74.31	55.15	54.17
Gari	80.00	0.50	0.61	49.38	65.66	70.19	72.45
Milk	13.00	8.00	13.00	21.66	23.14	34.37	25.89
Cassava	80.00	2.97	0.20	52.44	66.44	77.55	70.96
Rice	79.00	6.80	0.80	77.24	86.09	88.46	66.67
Correlation percentage between observed and predicted values				0.92		0.94	
determination Coefficient de (R^2)				0.85		0.90	
P. value (P <0.05)				0.009		0.004	

Table 7. Germination yields of spores produced in food after thermal and ethanolic activation

Foods	**Foodcomposition (g/L)**			**Heat activation**				**Ethanolic activation**			
				B. cereus		***B. subtilis***		***B. cereus***		***B. subtilis***	
	Carbohydrates	**Proteins**	**Lipids**	**Food**	**predicted**	**Observed**	**predicted**	**observed**	**predicted**	**observed**	**predicted**
Ecomba	56.75	9.63	12.63	37.26	22.71	60.50	58.41	50.91	47.21	72.66	75.64
Soja	37.00	10.00	21.00	41.27	49.37	61.07	63.10	70.62	66.42	58.25	65.23
Gari	80.00	0.50	0.61	48.13	41.12	37.06	39.77	47.19	43.79	27.65	4.64
Milk	13.00	8.00	13.00	60.46	58.34	65.21	64.16	76.92	66.26	70.72	62.75
Cassava	80.00	2.97	0.20	36.59	22.96	55.00	45.67	43.90	36.90	38.00	27.36
Rice	79.00	6.80	0.80	33.33	9.32	56.30	53.75	34.17	24.39	65.91	62.43
Correlation percentage between observed and predicted data				0.86		0.90		0.98		0.96	
determination Coefficient (R^2)				0.75		0.81		0.96		0.93	
P. value (P≤0.05)				0.02		0.01		0.00		0.00	

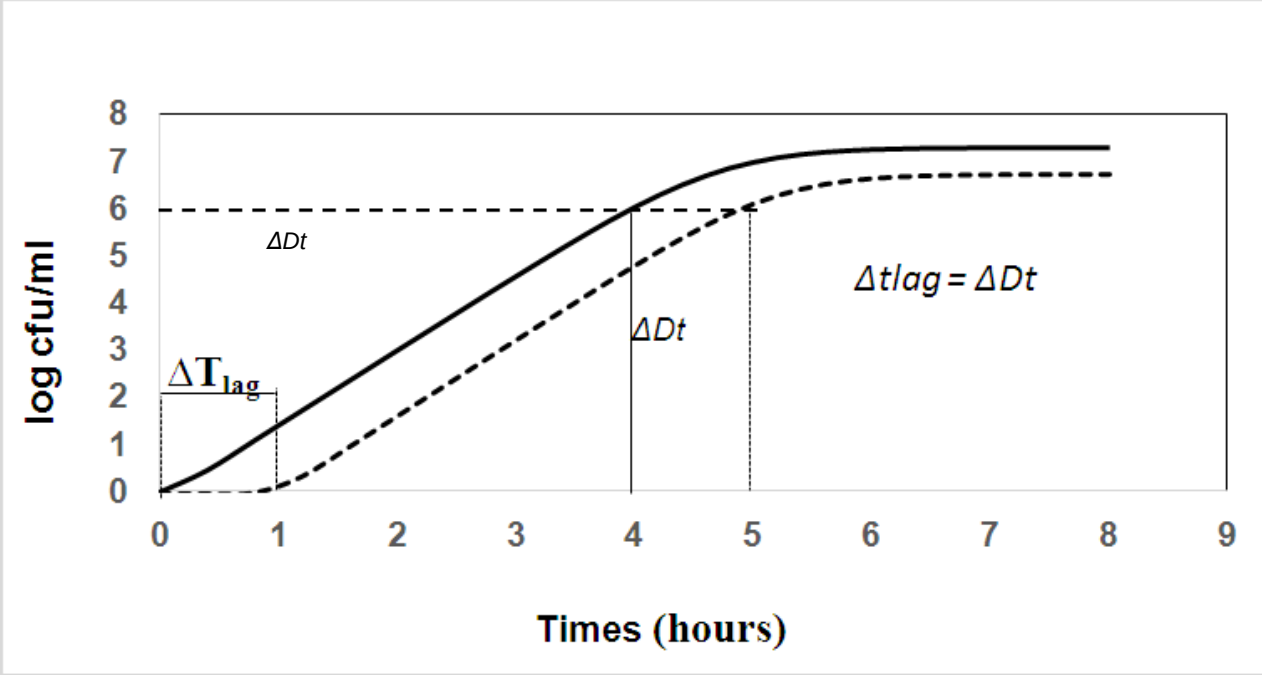

Fig. 1. Germination and outgrowth kinetic of vegetative cell(thick line) and spore (interrupted line) of the same bacteria with the same initial load
Δtlag is the difference between the spore germination +lag time and the vegetative cell lag time; ΔDt is the difference in detection time at 6Log ufc/ml between the two kinetics. (Δtlag = ΔDt)

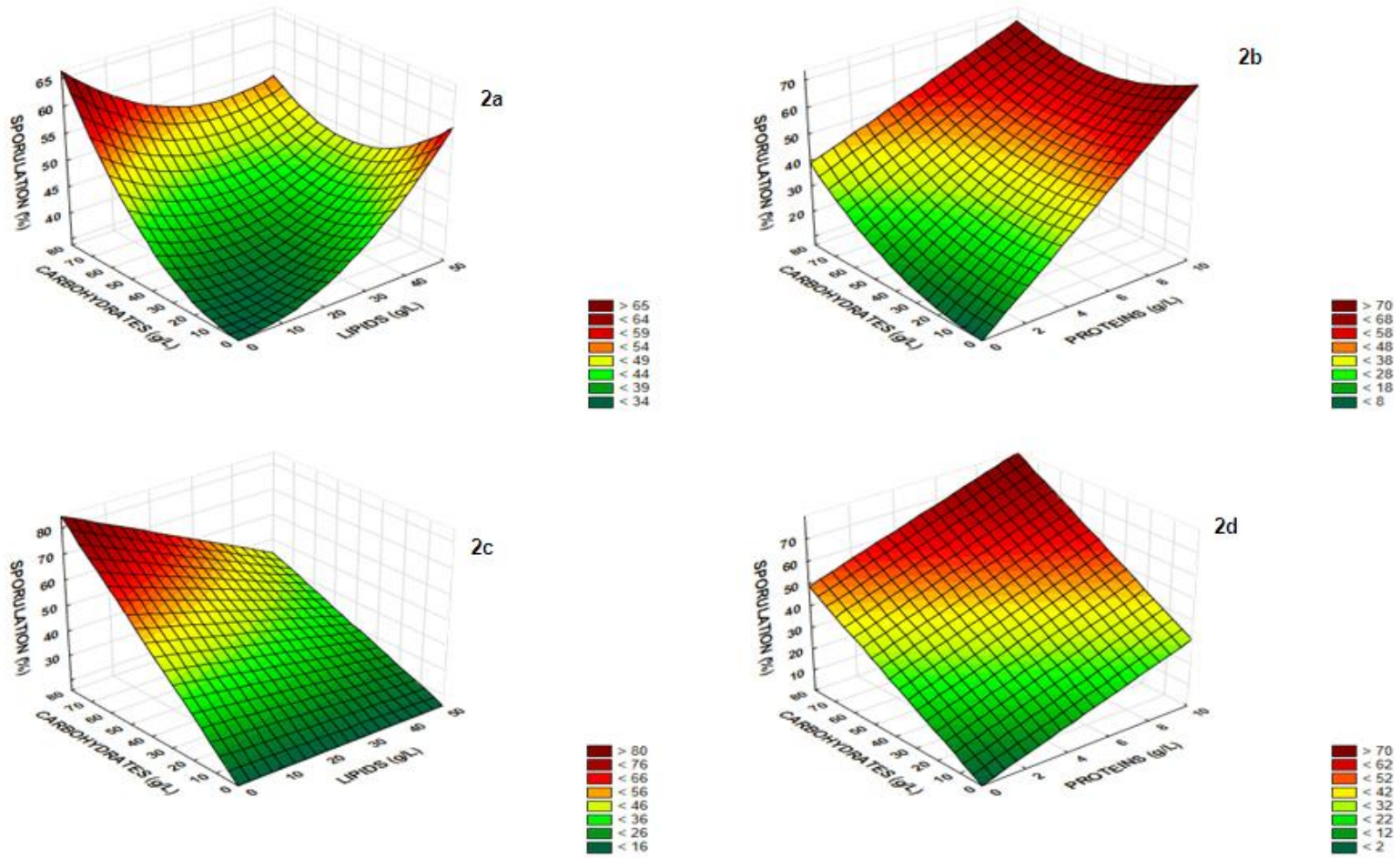

Fig. 2. Sporulation percentage of *B. cereus* (c) and *B. subtilis* (a) as function of carbohydrates and lipids at 5g/L of proteins content of sporulation medium and sporulation percentage of *B. cereus* (d) and *B. subtilis* (b) as function of carbohydrates and proteins at 25g/L of lipids content of sporulation medium

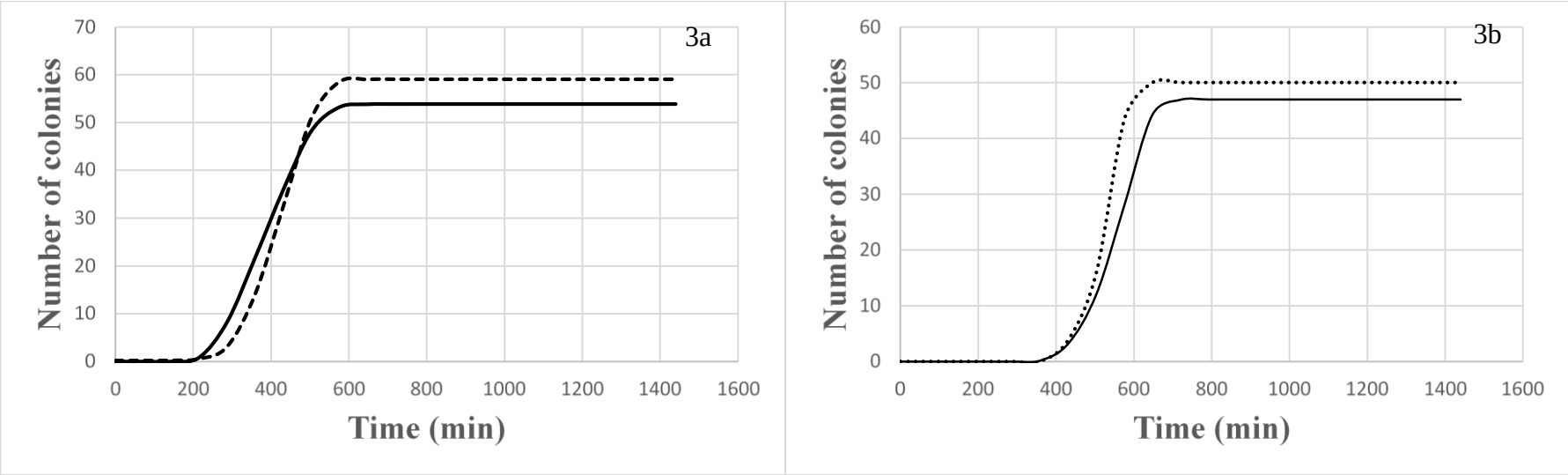

Fig. 3. Kinetic of colony appearance of *B. cereus* spores produced in sporulation medium content low lipid (solid broken line) an hight lipids (solid line) (a) and *B. subtilis* spores after heat activation produced in sporulation medium content low lipid (thin broken line) an hight lipids (thin line) (b)

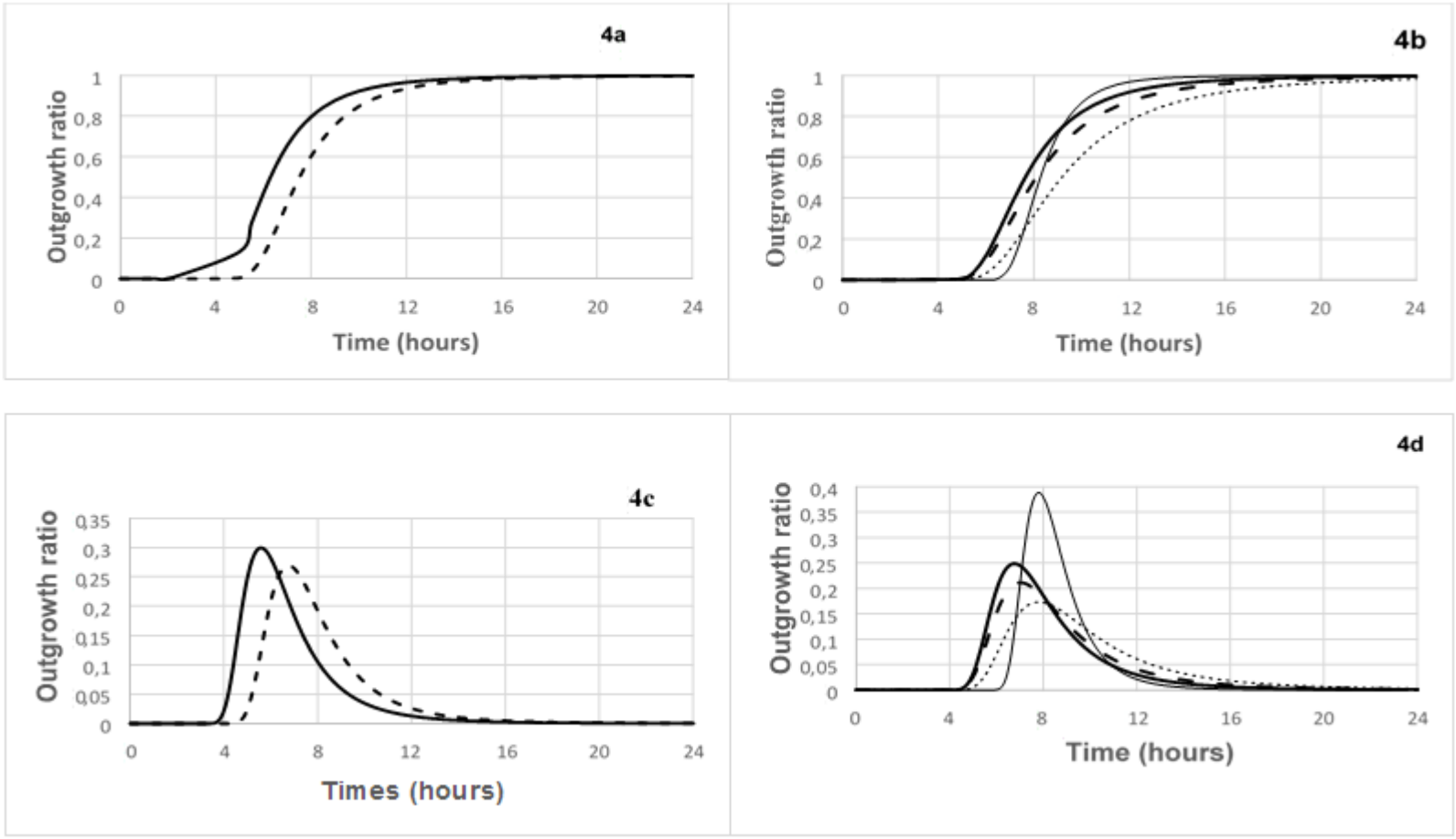

Fig. 4. Outgrowth (a) and mode (c) of the distribution of times for the first colony to emerge from *B. cereus* spore (solid line) and *B. subtilis* spores (solid broken line) produced in the same medium and heat activated. Example of outgrowth (b) and mode (d) of *B. cereus* spores (solid interrupted line) and *B. subtilis* spores (thin interrupted line) produced in the medium contained 60g, 12.5g and 2.5g/L; and for *B. cereus* spores (solid line) and *B. subtilis* (thin line) produced in a medium contained 60g, 37.5g and 2.5g of carbohydrates, lipids and proteins respectively (b and d)

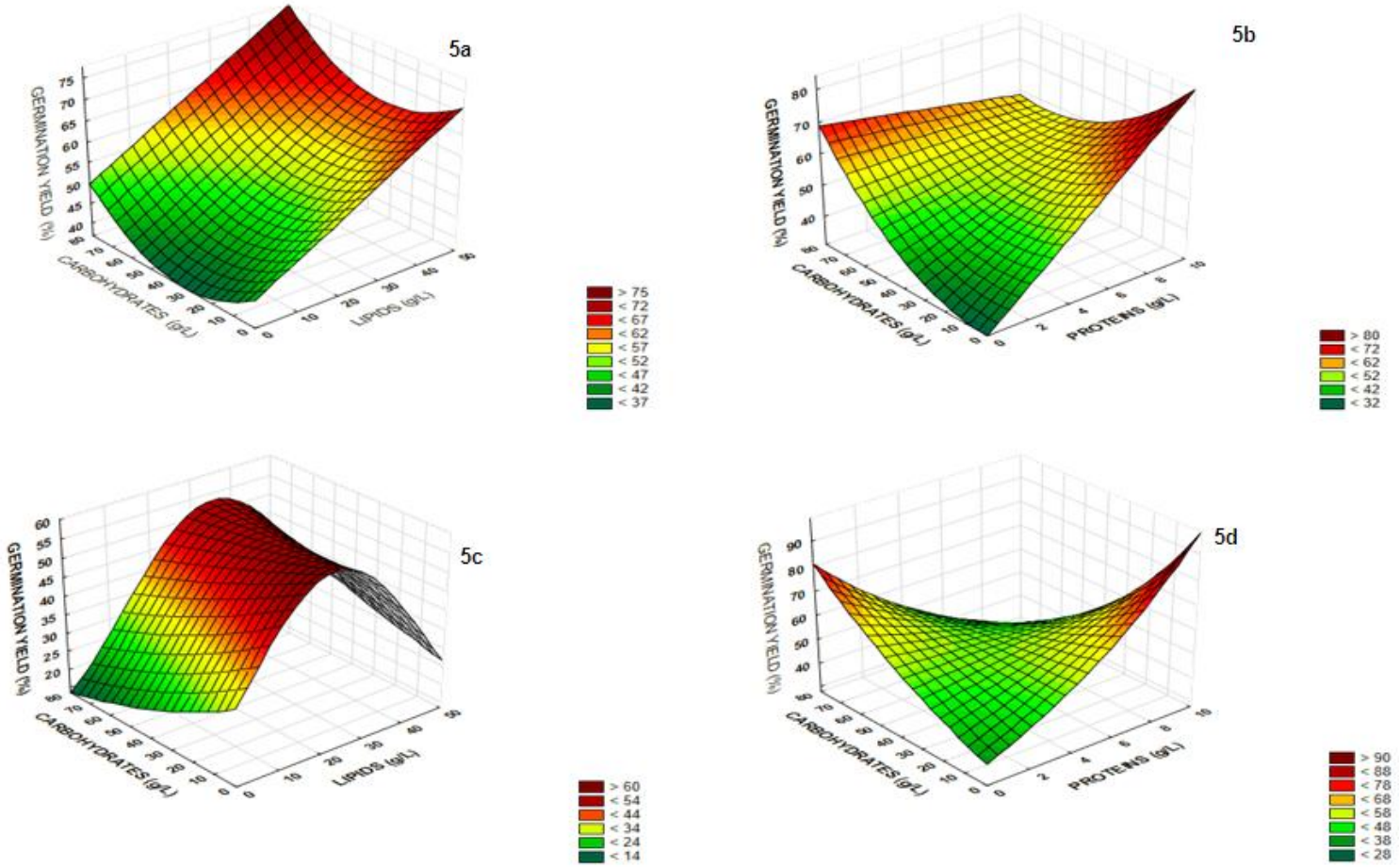

Fig. 5. Germination yields of heat activated spores of *B. subtilis* (a) and *B. cereus* (c) as function of carbohydrates and lipids at 5g/L of proteins content of spores forming medium and germination yield of B.subtilis (b) and *B. cereus* (d) as function of carbohydrates and proteins at 25g/L of lipids content of spores forming medium

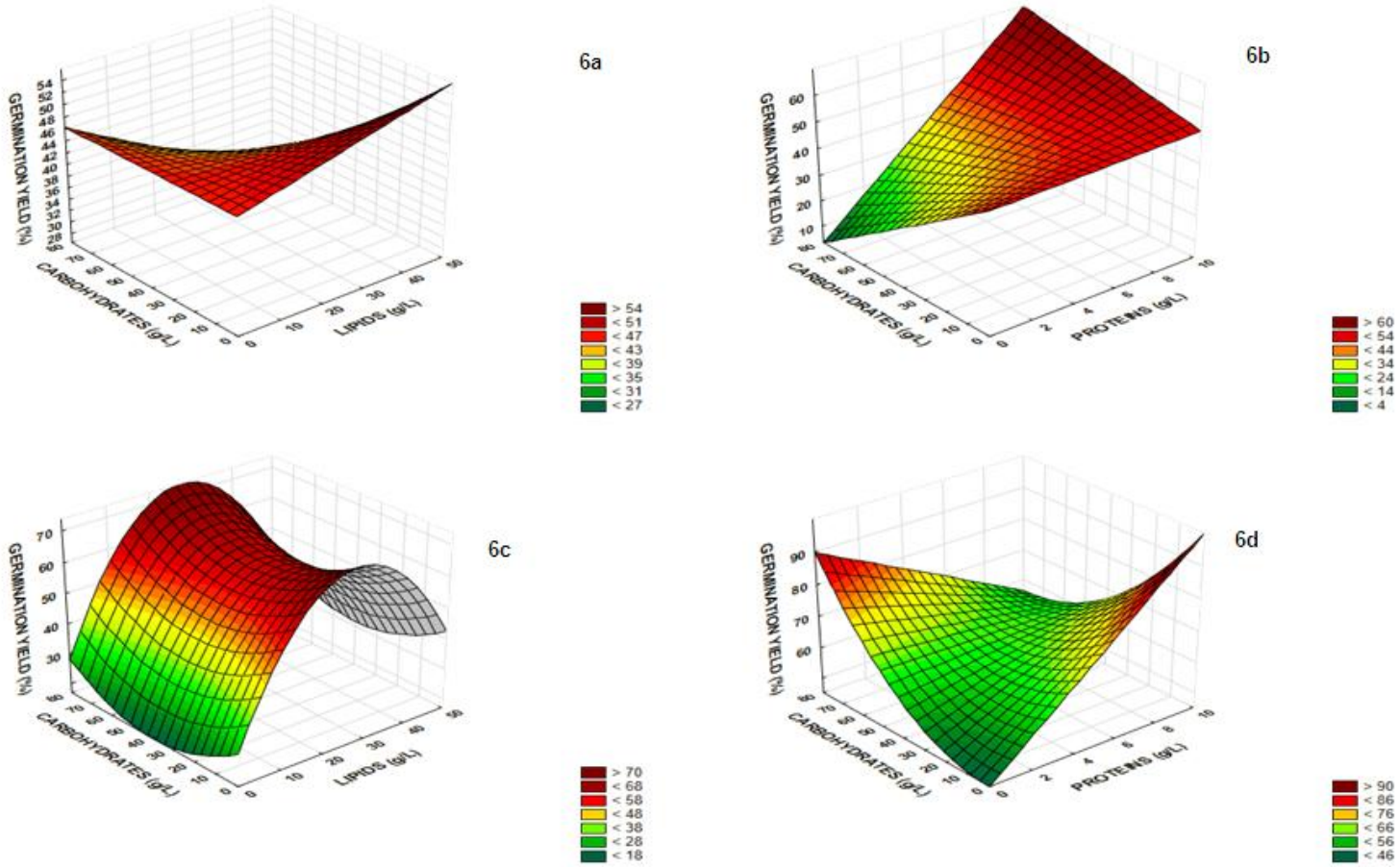

Fig. 6. Germination yields of ethanolic activated spores of *B. subtilis* (a) and *B. cereus* (c) as function of carbohydrates and lipids at 5g/L of proteins content of spores forming medium and germination yield of *B. subtilis* (b) and *B. cereus* (d) as function of carbohydrates and proteins at 25g/L of lipids content of spores forming medium

The variability of the experimental data expressed by these models ranges from 60 to 80% and it is also noted that the highest spore germination yield values are obtained after ethanolic activation. According to the models obtained, at a fixed protein content (5g/L) of the medium in which the spores were produced, it can be seen that after thermal activation the germination yields of *B. subtilis* spores are proportional to the lipids concentrations of the sporulation medium and show concavity as a function of the carbohydrates content (Fig. 5a). For *B. cereus spores* produced in media with a fixed proteins concentration (5g/L), an optimal lipids value (30g/L) is noted for the best yields as a function of the carbohydrate content of the spore-forming medium (Fig. 5c). For a fixed lipids concentration, germination yields are proportional to the carbohydrates and proteins concentrations of the sporulation medium (Fig. 5b and 5d). However, for *B. cereus* spores a combination of the highest carbohydrate and protein values of the medium induces a decrease in germination yields after heat activation (Fig. 5d).

With ethanol-activated spores, it is noted that for a fixed lipid concentration (25g/L) the germination yields for the two strain are proportional to the carbohydrate and protein concentrations of the sporulation medium (Fig. 6b an 6d). For a fixed protein concentration (5g/L), the germination yields of *B. subtilis are* proportional to the lipid concentration of the sporulation medium. However, a combination of the highest lipid and carbohydrate of the sporulation medium induces a decrease in germination yield (Fig. 6a). With *B. cereus* spores, after ethanolic activation at a fixed protein content (5g/L) of the medium in which the spores were produced, it can be seen that an optimal lipid value (30g/L) is noted for the best yields as a function of the carbohydrate content of the sporulation medium (Fig. 6b).

3.4 Validation of Sporulation and Germination Prediction Model

3.4.1 Sporulation prediction models

In order to evaluate the reliability of models for predicting sporulation percentage of *B. cereus* and *B. subtilis*, spores were produced in different food products and sporulation percentage were compared to those predicted by the models. The result obtained are presented in Table 6. The Pearson correlation test carried out shows that the sporulation percentage obtained from food are correlated at 92.1% with the sporulation percentage predicted by the *B. subtilis* model (eq5), there is also a correlation of 94.1% of the sporulation of *B. cereus* obtained from food with those predicted by the model (eq4).

3.4.2 Germination yield predicted by models

In order to determine the effectiveness of models for predicting spore germination yields, the germination yields of spores produced in different foods were evaluated after thermal and ethanolic activation. A correlation coefficient between the germination yields of the spores produced in these foods and those of the prediction models were determined. The results obtained are presented in Table 7.

After heat activation one notes that the germination yield of *B cereus* spores, obtained starting from food are correlated at 86% with the value predicted by the germination model (eq7). In the case of *B subtilis,* one observes a correlation of 90% between the values of the germination yield obtained from food and those predicted by the model (eq6). In the case of the spores activated with ethanol one observes a correlation of 98% between the germination yield of *B cereus* (eq9) and a correlation of 96% between germination yields of *B. subtilis*(eq8). These correlations take into account with-less the 75% of the variability of the experimental data.

4. DISCUSSION

This work was aimed at assessing the effect of sporulation media composition on the outgrowth of single spore germination within a spore population. In this regard, it was earlier demonstrated that the difference in outgrowth time of two single spores is proportional to their difference in germination time. Depending on the nutrient of the germination medium or the origin of spores, germination times can vary considerably [17,18]. These differences explain why colonies from outgrowth of spores in a population appear at different times during incubation. Many authors have shown that factors such as temperature, pH, composition of the medium and incubation time have an influence on the speed and sporulation percentage [13,19,20]. In this work, according to the sporulation percentage prediction models obtained, it is observed that in the absence of nutrients in the environment there is no sporulation. Sporulation is generally initiated by

nutrient limitation [21,22], however, the phenomenon of bacterial sporulation is an energy-intensive process, which generally takes place at the end of the exponential growth phase [13,23]. The vegetative cells involved in this process must have sufficient energy, which is not the case in the absence of nutrients. The highest sporulation percentage are obtained when spores are produced in environments with a high nutrient content, especially carbohydrates (glucose), which are considered the preferred carbon source of microorganisms [24]. Under these conditions, media containing high carbohydrate concentrations are those in which microbial growth will be more important [24,25]. When cell density becomes important, some peptides are secreted by bacteria. These peptides activate through receptors located on the surface of the bacteria the differentiation factor SpoOA which is at the origin of sporulation [21,26]. In the medium, the greater the cell density, the more abundant these peptides produced are and consequently the sporulation percentage is also high.

A combination of carbohydrates and proteins in the medium results in high sporulation percentage. The source of carbon and nitrogen are important factors in the production of bacterial spores. Glucose and yeast extract used in this work as a source of carbohydrate and protein, respectively, are considered and recommended as the best culture media inputs for spore production [25,27,28]. Indeed, the work of [29], on spore production as a function of nutrients showed that a carbon/nitrogen source ratio of 0.5 resulted in an optimum biomass in the medium and consequently very high sporulation percentage. The sporulation percentage vary considerably as function of species; *B. cereus* producing more spores than *B. subtilis*. The generation time of *B. cereus (*15-17min) is less important than that of *B. subtilis (*27-32min) [30]. Therefore, under the same culture conditions the cell density will be higher in *B. cereus* which will lead in principle to a higher sporulation percentage as this phase will be initiated earlier [13]. [31,32], in the framework of the study of the *B. subtilis* genome observed that in terms of sporulation genes *B. subtilis* has 5 gerA operons among which only three are expressed during sporulation. In the case of *B. cereus*, seven gerA operons were observed and all are expressed during sporulation [33].

Spore germination is a heterogeneous phenomenon that depends on many factors. Spore production conditions contribute largely to this observed heterogeneity [34,35]. This heterogeneity is an additional challenge in the search and enumeration method for spores in food safety. In this work, we observe that when spores are heat activated, there is a variation in the time of appearance of the first colony emerging from spore depending on the proteins content of the medium in which the spores were produced and the bacterial species. In other word, spore germination time depends on the history of it's production. The proteins content of the sporulation medium has an influence on the quality and number of germination receptors [36]. Indeed, the higher the proteins concentration during sporulation, the higher the number of germination receptors [34]. Spore activation increases the sensitivity of the germination receptors, which reduces germination times [37]. According to the work of [37] on bacterial spore germination receptors, the sensitivity of these receptors to germinants varies from one species to another depending on thermal activation.

In the case of colony appearence rate, which is proportional to the spore germination rate, it is observed that, the rate of colony formation after ethanolic activation does not appear to be influenced by the composition of the spore-forming medium. With heat-activated spores, lipids content of the spore-forming medium and the bacterial species affect spore germination rate within a population. The difference in sensitivity of the two strains to heat treatment could explain the variability in the observed rates. [38], showed that *B. cereus* had a better sensitivity to heat treatment, this sensitivity would thus facilitate the accessibility of germinants to germination receptors, which also translates into the fact that the highest speed is obtained with *B. cereus*. Lipids content has an influence on the structure of bacterial spores, especially at the level of the spore envelopes which have a fundamental role in the resistance of bacterial spores [39,40]. During bacterial sporulation, many lipids membranes such as phosphadyl ethanolamine, Cardiolipin and glycolipids such as diglucosyl diacylglycerol are produced [41]. [42], showed that these membrane lipids modulate germination receptor activity and spore germination rate. Although the mechanisms are not clearly established, these authors nevertheless observed that the spore germination rate is proportional to the quantity and state of the membrane lipids. According to the work of [43], on spore composition, it is observed that the spore structure is specific to

the environment in which the spores are formed. In lipid-rich environments, an increase in the quantity of membrane lipids is observed [44], This explains the impact of lipids on the speed of colony formation after thermal activation.

The observation of spore germination dynamics allowed to evaluate the time when most of the colonies emerge from spore (mode) and the average time of distribution of colony appearance times. From these dynamics, it can be observed that the mode The mode of the distribution of time for colonies to appear is weaker in *B. cereus* than in *B. subtilis,* which shows that *B. cereus* spores germinate faster than those of *B. subtilis*. This is one of the reasons why *B. cereus* is considered to be a major spoiling bacteria compared to *B. subtilis*. With ethanol-activated spores there is a significant variation in mode depending on the lipids content of the medium in which the spores were produced and the bacterial species. In the case of heat-activated spores, variability is observed only as a function of the bacterial species. Ethanol is a polar organic solvent by its nature is very lipophilic, which allows it to easily solubilize membrane lipids. The heterogeneous distribution of membrane lipids that vary from one strain to another as suggested by [39], may explain the difference in the mode of distribution observed. [43], mentioned the influence of lipid composition of the sporulation medium on spore germination speed, notably by modulating the activity of germination receptors.

Mathematical models are increasingly used to predict the behaviour of bacterial spores [45]. According to the germination yield models obtained in this work, it was observed that thermal activation induces very high germination yields in *B. subtilis*. In the case of *B. cereus* spores, it is observed that ethanolic activation induces the highest germination yields compared to thermal activation. Depending on their structure, the susceptibility of spores to activation methods may vary. Based on the work of [46], and [38], on the susceptibility of spores to heat treatment, it has been shown that spores of *B. subtilis* are less sensitive to heat treatment compared to those of *B. cereus,* but the opposite is observed for chemicals such as hydrogen peroxide or formaldehyde. It should also be noted that during the activation of bacterial spores, three possibilities are generally observed, the spores can be damaged, inactivated (dead spores) or viable following activation [9]. Depending on the susceptibility of the spores to treatment, there could therefore be several fractions within the same spore population. This degree of heterogeneity varies from one species to another depending on the method of activation [8,37]. Due to their higher thermal sensitivity, *B. cereus* spores may have a higher fraction of heat-sensitive spore that is either eliminated or damaged by heat, which may explain why germination yields of *B. cereus spores are* lower after thermal activation.

The origin of the spores is a very important factor to consider when looking for spores in food. [47], observed a substantial change in the peptidoglycan of the cortex of spores produced in a medium containing high concentrations of carbohydrates and proteins compared to poor media. This structure gives the spore these properties of resistance and impermeability [48]. In these protein- and carbohydrate-rich media, spores produced there have a very high number of germination receptors compared to spores produced in poor media and the germination yields obtained are also the highest [34,49]. Similar results were obtained in this work. Indeed, according to the spore germination yield models obtained, it is observed that germination yields are proportional to the carbohydrates and proteins concentrations of the medium in which they were produced. According to these models, it is observed for example that after thermal activation, the highest germination yields are obtained when *B. subtilis* spores come from nutrient-rich media (carbohydrates, proteins and lipids). [34], showed that spores produced in a nutrient-rich medium had germination receptors 3.5 times greater than those of spores produced in a poor medium. The richness of the germinosome (set of germination receptors) of the spores under these conditions thus allows the spores to quickly repair any damage caused by activation and to germinate in large quantities. In the case of lipids contained in sporulation media, the concavity observed in the evolution of spore germination yields as a function of the lipids content of the sporulation medium in this work suggests that at a certain threshold (30g/L), lipids could significantly influence spore susceptibility to activation methods as well as germination yields.

The importance of predictive microbiology has already been demonstrated in several areas of food microbiology. Mathematical models for predicting the behavior of microorganisms are generally developed from laboratory environments. However, the composition of food

does not always match that of laboratory culture media, it is therefore necessary to validate these models by determining the reliability of the model, in particular by evaluating the degree of correlation between the values predicted by the model and those observed in food. In this work, the model for predicting sporulation percentage of *B. cereus* showed a correlation of 0.92 with food obtained from markets and analyzed for their spore content. In the case of *B. subtilis* there is a correlation of 0.94. The sporulation models obtained in this work therefore have good reliability as demonstrated by validation. In the case of germination models in food, it is observed that the germination yields of spores produced in food have a correlation of more than 95% with the forecast models obtained after ethanolic activation and this percentage of correlation takes into account almost 90% of the variability in the data. In the case of thermal activation, these yields are correlated to 86% in *B. cereus* and 90% in *B. subtilis*. These results thus show that the yield forecasts obtained after ethanolic activation are closer to reality than those obtained after thermal activation.

5. CONCLUSION

Foods rich in carbohydrates and proteins are favorable environments for the formation of spores. The spores from these foods have a high germination capacity and therefore more likely to germinate quickly and cause food spoilage. The choice of the activation method varies depending on the strain and the nutrient content including the fat content for ethanolic activation and the proteins and carbohydrates content for thermal activation. The factors taken into account in this work have a significant influence on the sporulation rates and the germination capacity of the spores, as shown by the correlation coefficients between the values predicted by the model and those obtained in food.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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