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## ***Bacillus megaterium* Spore Germination and Growth Inhibition by a Treatment Combining Heat with Natural Antimicrobials**

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### **Summary**

Natural antimicrobials are an alternative to the use of chemically synthesized preservatives and other technological treatments. They have the advantage of not being rejected by consumers because of their natural origin. However, prior to their usage at a food factory scale, their precise effects on microorganisms have to be known. Nisin, a bacteriocin, and carvacrol and thymol, phenolic compounds present in the essential oil fraction of *Oreganum* and *Thymus* plants, have been shown to inhibit growth of different bacteria. This research was conducted to show the effect of a thermal treatment, applied previously, on the growth of *B. megaterium* cells in a culture medium with and without nisin, carvacrol and thymol. It has been shown that a thermal treatment able to kill 90 % of the spore population of *B. megaterium* had little effect on the growth of the survivors in nutrient broth at 30 °C. Nisin, in a concentration of 0.05 mM, did not affect greatly the growth of unheated *B. megaterium*. When the same concentration of nisin was applied to the survivors of the heat treatment, lag phase was further increased and growth rate decreased. Thymol, applied at a concentration of 0.6 mM, increased the lag phase duration almost three times, although it did not change the growth rate. Although carvacrol, at the same concentration (0.6 mM), did reduce the growth rate to half its original value, it did not increase the lag phase duration significantly (only twice), resulting in a quite similar growth curve. The combination of thymol and carvacrol at these high doses (0.6 mM) resulted in a further increase of the lag phase and significant decrease of the growth rate. When carvacrol and/or thymol were combined with a previous thermal treatment (able to kill 90 % of the population), the growth of the survivors was inhibited for at least seven days. Therefore a combination of moderate conditions could be effective to control spoilage by spore-forming bacteria.

*Key words:* microorganisms, combined processes, thermal treatment, natural antimicrobials

### **Introduction**

Food manufactures and consumers demand additive-free, fresher and more natural tasting food products (1) while maintaining microbiological safety. This in-

creasing demand could be accomplished by the use of natural antimicrobial systems for preservation of foods.

Nisin is a small protein antibiotic produced by *Lactococcus lactis* subsp. *lactis* (2) and is the most extensively

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studied bacteriocin. The mode of action of nisin is the disruption of membrane function induced by pore formation in the bacterial cytoplasmic membrane (3). This natural substance is bactericidal against many Gram-positive bacteria including *Listeria monocytogenes*, *Staphylococcus aureus* and vegetative cells of *Bacillus cereus* (2,4). Nisin is a Generally Recognized As Safe (GRAS) product and, to date, is the only bacteriocin that has been used as a food preservative (2,5).

Thymol and carvacrol are phenolic compounds, isomers, present in the essential oil fraction of *Origanum* and *Thymus* genera (6) that have been shown to have inhibitory effect on bacteria and fungi, including foodborne pathogens (7–9). Because of their hydrophobicity, they are likely to dissolve in the hydrophobic domain of the cytoplasmic membrane, between the lipid acyl chains, and cause modifications on the membrane permeability and on the activity of enzyme systems (10).

A synergistic effect between nisin and carvacrol or thymol on the growth of *L. monocytogenes*, *Bacillus subtilis* and *B. cereus* has been observed (11–13). Heat can, in some instances, modify this sensitivity to antimicrobials (14).

*Bacillus megaterium* is a Gram-positive spore-forming bacteria. As most of the spore-formers it is usually found in the soil, from which it can easily be transmitted to the foods we consume. It is not a highly heat resistant microbe (compared to other spore-forming heat resistant bacteria), but it shows D values similar to the foodborne pathogen *B. cereus* (15). Hence it could be used as an indicator of the level of inactivation achieved in foods. Although both microbes will be easily destroyed in any food sterilisation process, they will probably survive pasteurisation processes, and so it is necessary to look for combined processes with heat and other lethal agents in order to control the growth of these and other microorganisms when applying such processes.

The measurement of absorbance is a rapid and inexpensive method to monitor bacterial growth. In spite of the controversy arisen by problems related to detection thresholds and correlation with viable counts, numerous techniques and mathematical growth models have been used in recent years for estimation of growth rates and lag times from absorbance data. Firstly, growth rates were determined from the linear part of log-transformed absorbance growth curves (16). Later, absorbance data were fitted directly to sigmoidal growth models, showing accurate results (17). In opinion of Dalgaard and Koutsoumanis (18), absorbance techniques should be limited to conditions where high cell densities are reached, such as those resembling the growth of spoilage bacteria in foods. Even assuming the limitations of absorbance to build growth curves, it may be useful, if not to obtain very precise growth kinetic parameters, at least to compare the growth of different cultures or of the same cultures, but grown in different conditions. Among the models used in predictive microbiology, the model of Baranyi *et al.* (19) is one of the most frequently used by different authors, due to its ability to fit experimental data (20).

In the present study, the inhibitory effect of nisin, thymol and carvacrol, alone and combined, on the ger-

mination and growth of *B. megaterium* spores, unheated or previously heated, was investigated in a growth medium to evaluate their potential use as food preservatives. Absorbance growth curves were used through this research in order to compare the inhibitory effect of these antimicrobials.

## Material and Methods

### *Bacterial strain and spore suspension preparations*

The type strain of *B. megaterium* (CECT 4313) was provided by the Spanish Type Culture Collection (CECT).

Spores were prepared on Petri dishes containing plate count agar (PCA, Scharlau Microbiology, Barcelona, Spain) supplemented with 3 mg/L of manganese sulphate (Scharlau Microbiology). Agar surface was inoculated with 0.4 mL of a 24-hour culture grown in nutrient broth (Scharlau Microbiology) at 30 °C. After incubation at 30 °C for 4 days, a sporulation rate of more than 80 % was achieved, as determined by phase contrast microscopy (Leica, Wetzlar, Germany).

Spores were collected by flooding the agar plate with pH=7 citrate phosphate McIlvaine buffer (21), rubbing the surface with a glass spatula. After harvesting, spores were washed twice by centrifugation at 2500 × g for 15 min and resuspended in pH=7 McIlvaine buffer. The concentration of spores in the final suspension was assessed by microscopic count with a Thoma counting chamber and was adjusted to 10<sup>9</sup> spores/mL with pH=7 McIlvaine buffer. The spore suspension was stored frozen at –18 °C until used. Spore viability was observed to be unchanged after storage under our conditions during the time in which the experiments were performed. Vegetative cells were eliminated during this procedure, as assessed by plate counts prior to and after applying a heat shock to the spore suspension.

### *Chemicals*

Carvacrol and thymol (Sigma Aldrich Chemie, Steinheim, Germany) stock solutions (0.5 M) were made in 95 % ethanol and stored at 4 °C. Nisin (Sigma Aldrich Chemie) stock solution (0.3 mM) was made in 50 % ethanol, filter sterilized and kept at –20 °C.

### *Heat treatment*

Thermal treatments aimed to inactivate 90 % of the initial spore population and consisted of exposing the spore suspension at 90 °C for 25 min in a glass tube immersed in an oil bath.

### *Growth conditions*

Bacterial spores were germinated and grown at 30 °C in 4.5 mL of nutrient broth (Scharlau Microbiology). Initial concentration of spores in the growth medium was adjusted to 10<sup>6</sup> CFU/mL, which corresponds to the concentration when the absorbance of the culture starts to increase. Even when a previous thermal treatment was applied, this initial number was calculated to be 10<sup>6</sup> viable cells/mL.

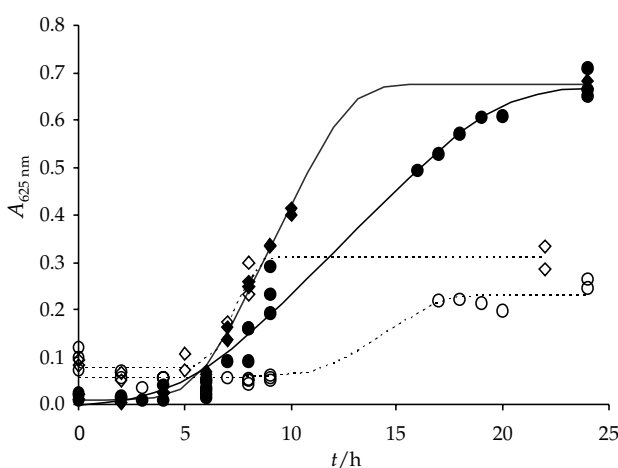
The effect of nisin, carvacrol and thymol on the growth of vegetative cells, at 30 °C in nutrient broth, was

studied. Cell cultures were exposed to nisin (0.05 mM) and different concentrations of carvacrol (0.2 and 0.6 mM) or thymol (0.2 and 0.6 mM), alone or combined, during at least 24 h at 30 °C. To explore the effect of a previous thermal treatment on the growth of the survivors, the spore suspension was exposed to 90 °C for 25 min prior to its incubation in nutrient broth, with or without antimicrobials. Samples were taken at different time intervals during growth, and their absorbance at 625 nm ( $A_{625\text{nm}}$ ) was immediately measured using a spectrophotometer (ZUZI 4110RS, Auxilab, Beriaín, Spain). Three replicate experiments per condition were performed.

Growth curves were obtained by plotting the  $A_{625\text{nm}}$  against the exposure time. Growth curves were fitted using the function of Baranyi *et al.* (19) to estimate the main growth parameters, *i.e.* specific growth rate and lag phase. Since the initial concentration of spores ( $10^6$  CFU/mL) corresponded approximately to that when the absorbance of the culture started to increase (data not shown), no corrections had to be made in order to calculate real lag phases from the absorbance measurements. Only growth curves with at least 10 data points were used for modelling, as suggested by the authors.

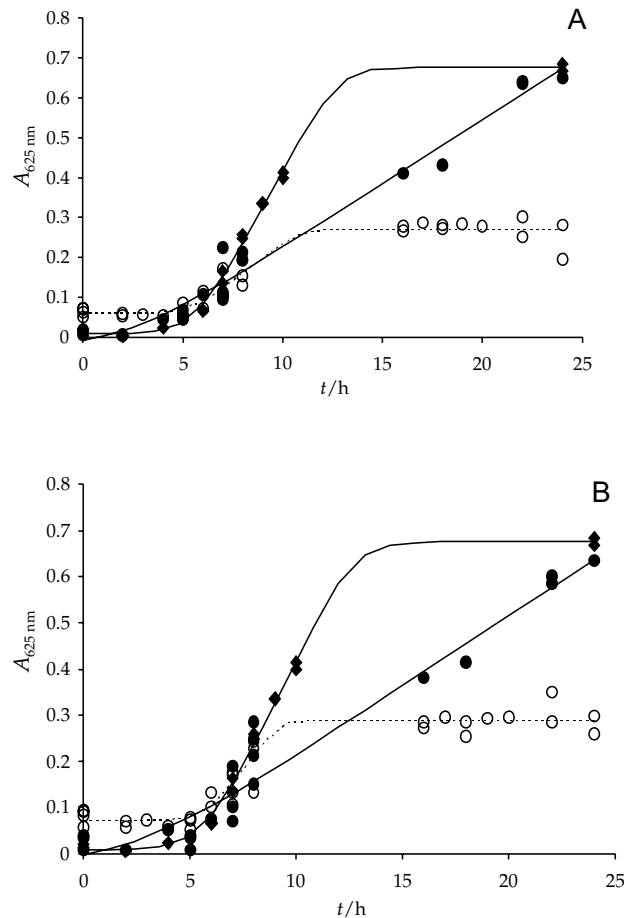
## Results

Growth of *B. megaterium* was characterised, in optimal conditions, by a lag phase of ( $5.6 \pm 0.2$ ) h and a growth rate of ( $0.093 \pm 0.005$ ) AU/h. Table 1 summarizes the results obtained in this investigation. For each condition tested, the growth rate and lag phase duration ( $\pm$  standard deviation) are shown, as well as the coefficients of correlation ( $r_0$ ) obtained when fitting the experimental values to the growth curves derived from the model of Baranyi (19). Figs. 1, 2 and 3 show the most relevant results.



**Fig. 1.** Effect of a previous thermal treatment and of nisin on the growth of *Bacillus megaterium* ATCC 14581 in nutrient broth at 30 °C

(◆) unheated spores with no antimicrobials in the growth medium (blank), (●) unheated spores with 0.05 mM nisin in the growth medium, (◇) spores preheated at 90 °C during 25 min, and (○) spores preheated at 90 °C during 25 min with 0.05 mM nisin in the growth medium (solid lines: unheated spores, dotted lines: spores preheated at 90 °C during 25 min)



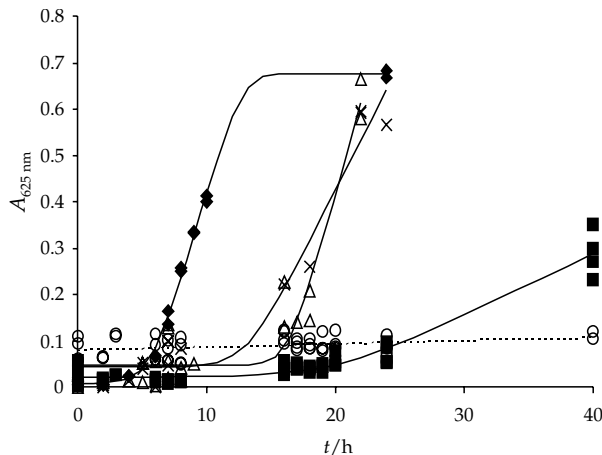
**Fig. 2.** Effect of a previous thermal treatment and of 0.2 mM carvacrol (A) and 0.2 mM thymol (B) on the growth of *Bacillus megaterium* ATCC 14581 in nutrient broth at 30 °C

(◆) unheated spores with no antimicrobials in the growth medium (blank), (●) unheated spores with 0.2 mM carvacrol (A) / thymol (B) in the growth medium, and (○) spores preheated at 90 °C during 25 min with 0.2 mM carvacrol (A) / thymol (B) in the growth medium (solid lines: unheated spores, dotted lines: spores preheated at 90 °C during 25 min)

Fig. 1 shows the growth curves (absorbance versus time) of *B. megaterium* in nutrient broth at 30 °C in optimal conditions (blank), after a thermal treatment at 90 °C during 25 min, with 0.05 mM nisin, and combining both the nisin and the thermal treatment.

As it can be seen, the application of a thermal treatment prior to the incubation in the growing medium changed only slightly the lag phase and the growth rate of the microorganism, although the final absorbance reached by the culture was significantly lower. This lower final culture concentration was observed in all growth curves performed in this investigation when a previous heat treatment was applied.

The addition of nisin did not increase the lag phase duration and only reduced the growth rate to one half (Table 1). However, when nisin was applied to the survivors of a thermal treatment, lag phase increased by about twofold and growth rate decreased to one third of the value attained in optimal conditions (Table 1).



**Fig. 3.** Effect of a previous thermal treatment and of 0.6 mM carvacrol and/or thymol on the growth of *Bacillus megaterium* ATCC 14581 in nutrient broth at 30 °C (◆) unheated spores with no antimicrobials in the growth medium (blank), (×) unheated spores with 0.6 mM carvacrol in the growth medium, (Δ) unheated spores with 0.6 mM thymol in the growth medium, (■) unheated spores with 0.6 mM carvacrol and 0.6 mM thymol in the growth medium, and (O) spores preheated at 90 °C during 25 min with 0.6 mM carvacrol and 0.6 mM thymol in the growth medium (solid lines: unheated spores, dotted line: spores preheated at 90 °C during 25 min)

Fig. 2 shows the effect of the addition of 0.2 mM carvacrol (A) and 0.2 mM thymol (B) with or without a previous thermal treatment. Both carvacrol and thymol, at a concentration of 0.2 mM, did not increase the lag phase duration, but reduced the growth rate in a similar way, to  $(0.032 \pm 0.002)$  and  $(0.030 \pm 0.003)$  AU/h respectively, one third of the value of the blank (Table 1). When a heat treatment was applied before the exposure to the antimicrobials at this concentration, although the growth started without further lag, again the absorbance reached by the cultures was lower (Fig. 2).

Fig. 3 shows the effect of the addition of 0.6 mM carvacrol and 0.6 mM thymol, separately and combined, and after a previous thermal treatment. Both carvacrol and thymol, at this higher concentration increased the lag phase duration to  $(13.0 \pm 1.2)$  and  $(16.3 \pm 0.5)$  h respectively, about two to three times the blank. Thymol alone had no influence on the growth rate, but carvacrol reduced it to almost one half the value of the blank (Table 1), resulting in quite similar growth curves. When both antimicrobials were combined, lag phase was further increased to  $(20.1 \pm 1.3)$  h and growth rate decreased considerably to  $(0.013 \pm 0.001)$  AU/h. When a heat treatment was applied before exposure to both antimicrobials, interestingly, growth was inhibited for at least seven days.

**Table 1.** Growth parameters (lag phase and growth rate  $\pm$  standard deviations) and correlation coefficients of the growth curves ( $r_0$ ) of *Bacillus megaterium* ATCC 14581 in nutrient broth at 30 °C, unheated or previously heat-treated at 90 °C for 25 min, and exposed to different concentrations of nisin, carvacrol and thymol added separately or simultaneously

Growth conditions <sup>a</sup>	Growth rate $\pm$ s.d. (AU / h)	Lag phase $\pm$ s.d. h	$r_0$
Blank	0.093 $\pm$ 0.005	5.6 $\pm$ 0.2	0.998
HT	0.117 $\pm$ 0.035	6.4 $\pm$ 0.4	0.962
N	0.045 $\pm$ 0.004	4.9 $\pm$ 0.8	0.991
HT + N	0.031 $\pm$ 0.078	11.5 $\pm$ 1.4	0.950
0.2 mM C	0.032 $\pm$ 0.002	2.7 $\pm$ 1.4	0.987
0.6 mM C	0.054 $\pm$ 0.008	13.0 $\pm$ 1.2	0.976
HT + 0.2 mM C	0.040 $\pm$ 0.020	5.5 $\pm$ 1.2	0.968
HT + 0.6 mM C	–	> 7 days	–
0.2 mM T	0.030 $\pm$ 0.003	2.9 $\pm$ 2.0	0.972
0.6 mM T	0.099 $\pm$ 0.012	16.3 $\pm$ 0.5	0.952
HT + 0.2 mM T	0.064 $\pm$ 0.021	5.8 $\pm$ 0.7	0.959
HT + 0.6 mM T	–	> 7 days	–
N + 0.2 mM C	0.033 $\pm$ 0.006	4.5 $\pm$ 2.9	0.972
HT + N + 0.2 mM C	0.022 $\pm$ 0.002	22.1 $\pm$ 1.8	0.970
N + 0.2 mM T	0.033 $\pm$ 0.005	5.8 $\pm$ 2.6	0.970
HT + N + 0.2 mM T	0.025 $\pm$ 0.003	21.9 $\pm$ 1.8	0.955
0.2 mM C + 0.2 mM T	0.030 $\pm$ 0.005	3.2 $\pm$ 2.9	0.973
HT + 0.2 C + 0.2 mM T	0.017 $\pm$ 0.003	4.8 $\pm$ 3.2	0.984
N + 0.2 mM C + 0.2 mM T	0.032 $\pm$ 0.006	5.8 $\pm$ 2.7	0.970
HT + N + 0.2 mM C + 0.2 mM T	0.029 $\pm$ 0.007	24.0 $\pm$ 3.7	0.938
0.6 mM C + 0.6 mM T	0.013 $\pm$ 0.001	20.1 $\pm$ 1.3	0.969
HT + 0.6 mM C + 0.6 mM T	–	> 7 days	–

<sup>a</sup>HT – previously heat-treated at 90 °C for 25 min, N – 0.05 mM nisin, C – carvacrol and T – thymol

## Discussion and Conclusion

When *B. megaterium* was exposed to only one of the antimicrobials studied (nisin, carvacrol, thymol) at low doses (less than 0.2 mM), growth parameters were scarcely changed: lag phase duration was kept constant (it was even reduced, attending to the values given by Baranyi model (19)) and growth rate diminished only to some extent (Table 1). Under any of these conditions, the culture reached the maximum absorbance in 24 h, only 10 h later than the blank (Figs. 1 and 2). Also, the previous thermal treatment applied to the dormant spores did not modify the growth rate and increased only slightly the lag phase of the survivors (Table 1). Although the heating killed the majority of the spores (90 % of the initial population; data not shown), at the same time, it could have helped to activate the germination and growth of the survivors, even if they had suffered some kind of damage. It is, however, more difficult to explain why the final absorbance reached by the culture was significantly lower, compared to that of unheated cells (Fig. 1). Perhaps, preheated bacteria, as an expression of the damage, could change their metabolism and inhibit growth at a previous stage than when unheated.

When carvacrol or thymol were added at higher doses (0.6 mM) to the growth medium, lag phase duration increased considerably (more than twice the value obtained under optimal conditions), although growth rate was kept almost constant in these cases, being higher than the growth rate under low doses of the same antimicrobials (Table 1). These higher doses of carvacrol and thymol could somehow inhibit the germination of most of the spores present in the suspension. For those spores that are able to develop (*i.e.* the fraction of the population that is naturally resistant to the antimicrobials), it could take a longer time to adapt to growth under such hostile conditions, but once started, growth would be almost as fast as without these substances.

At low doses, the combinations of two or all three antimicrobials did not affect the lag and reduced only to some extent the growth rate of *B. megaterium* (Table 1), which would mean neither synergistic nor even additive effect. However, when carvacrol and thymol were applied simultaneously at higher doses (0.6 mM; Fig. 3), lag phase was further increased and growth rate decreased significantly, denoting a stronger inhibition (synergism). The synergism between antimicrobials like nisin, carvacrol, thymol or cymene has already been shown on the viability of *L. monocytogenes* (11,22) and *B. cereus* (11, 13,14). However, Lambert *et al.* (23) found only an additive effect of carvacrol and thymol against *Pseudomonas aeruginosa* and *S. aureus*. To complicate more the overall picture, Delaquis *et al.* (24) found that fractions of cilantro and eucalyptus essential oils, when mixed in various combinations, resulted in additive, synergistic or antagonistic effects, depending on the individual microorganism being tested and on the proportion of the mixture. Other authors (25) have found synergy between oregano essential oils and EDTA on *Escherichia coli* O157:H7, while EDTA alone had no antibacterial action.

When *B. megaterium* spores were exposed to a heat treatment (90 °C during 25 min) and then grown in the presence of antimicrobials, growth rate was reduced in

most of the cases, lag phase increased considerably and the final concentration reached by the cultures was always lower (Table 1). When the concentration of carvacrol and/or thymol was increased to 0.6 mM, growth of the survivors of the heat treatment was even inhibited for at least seven days (Table 1). Probably the survivors of the heat treatment, although able to grow in optimal conditions (with no antimicrobials added; Fig. 1 and Table 1), were somehow injured and these damages hampered their repair mechanisms in the presence of antimicrobials. One of the most suitable targets for heat destruction of bacterial spores are the spore membranes (26). The germinating spore undergoes very important transport phenomena through its membrane, as it has to excrete dipicolinic acid and other typical sporulation compounds and uptake the nutrients that will enable its development and outgrowth (27). If such germinating spore, which may have already become damaged by heat on its membranes, is incubated in the presence of antimicrobials, known to cause modifications on the membrane permeability (3,10), it is not surprising to find a synergy. Moreover, other possible targets for bacterial heat damage are core enzymes (28), and essential oils can also influence the activity of enzyme systems (10). Although the mechanisms of action may be completely different, similar synergisms combining heat and nisin have been obtained for vegetative cells of *B. cereus* (14) and for *Lactobacillus plantarum* (3), and even for spores of *Alicyclobacillus acidoterrestris* (29).

Although it has generally been found that a greater concentration of antimicrobials is needed to achieve the same effects in foods (6) and it would be necessary to perform trials prior to drawing any conclusions for the food industry, the application of these antimicrobials could help to inhibit growth of the survivors of the heat treatments applied to preserve foods.

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## Sprečavanje klijanja spora i rasta *Bacillus megaterium* kombinirajući toplinsku obradu i prirodne antimikrobne spojeve

### Sažetak

Prirodna antimikrobna sredstva su alternativa kemijski sintetiziranim konzervansima i drugim tehnološkim postupcima. Njihova je prednost u tome što su zbog svoga prirodnog podrijetla prihvatljivi potrošačima. Prije njihove primjene u prehrambenoj industriji, potrebno je znati kako djeluju na mikroorganizme. Nisin, bakteriocin, te karvakrol i timol, fenolni spojevi prisutni u frakcijama eteričnog ulja biljaka *Oreganum* i *Thymus*, inhibiraju rast raznih bakterija. Opisano je istraživanje provedeno da bi se utvrdio učinak prethodno provedene toplinske obrade na rast stanica *B. megaterium* u podlozi s nisinom, karvakrolom i timolom te bez njih. Pokazalo se da toplinska obrada može usmrtniti 90 % populacije spora *B. megaterium*, a gotovo nikako ne utječe na rast preživjelih u hranjivoj podlozi pri 30 °C.

Nisin koncentracije od 0,05 mM nije bitno utjecao na rast toplinski neobrađenog *B. megaterium*. Kada se ista koncentracija nisina primijeni na preživjele spore, produži se lag faza, a smanji brzina rasta. Timol koncentracije od 0,6 mM produžio je trajanje lag faze skoro 3 puta, a nije promijenio brzinu rasta. Iako je karvakrol iste koncentracije (0,6 mM) smanjio brzinu rasta na polovinu originalne vrijednosti, dužinu lag faze produžio je samo za 2 puta, što je rezultiralo skoro jednakom (0,6 mM) krivuljom rasta. Kombinacijom tih velikih koncentracija timola i karvakrola znatno je produljena lag faza i smanjena brzina rasta. Kada se karvakrol i/ili timol kombiniraju s prethodnom toplinskom obradom (pri čemu je uništeno 90 % populacije), rast preživjelih bio je inhibiran barem 7 dana. Stoga kombinacija jednostavnih uvjeta može biti djelotvorna u kontroli onečišćenja sa sporulirajućim bakterijama.

