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The inhibiting effect of microwave radiation on *Paenibacillus larvae* spores suspended in water

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Abstract: The aim of this paper was to investigate the effects of microwave radiation on the viability of *Paenibacillus larvae* spores and to study the relationship between the microwave power consumption, the exposure time and the number of spores in the examined suspensions. Sterile distilled water suspensions were made using larval detritus, to contain tens, hundreds and thousands of spores. The suspensions of all the dilutions were gradually exposed to a microwave radiation power of 170, 510 and 850 W. In all the cases, the exposure time was 1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 minutes. After cooling, 0.1 ml of each exposed suspension was inoculated onto three modified MYP (mannitol egg yolk polymyxin) agar plates and incubated aerobically at 37 ± 1 °C for 120 hours. The statistical evaluation of the spore counts decreasing with time was performed with the use of the nonparametric Friedman's variance test using the Unistat Statistical Package v6.5. The results showed that the rate of devitalisation of the spores is dependent on the microwave oven power consumption, but independent of the number of spores. Using a power consumption of 170, 510 and 850 W, the devitalisation of the spores occurred after 15, 3 and 2 min of exposure, respectively.

Keywords: exposure; frequency; microorganisms; resistance; sporulating

Paenibacillus larvae is a Gram-positive spore-forming microorganism causing American foulbrood in honeybees. This disease causes substantial financial losses not only to beekeepers, but also to producers of agricultural crops. It is generally known that spores of the causative agent are resistant to high temperatures and commonly used disinfectants. Consequently, beekeepers and vet-

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erinarians are seeking new methods of sterilisation which could guarantee the complete and rapid destruction of the vegetative forms of the microorganisms and also of the spores. One option is microwave radiation. Microwaves are a form of non-ionising electromagnetic radiation with frequencies ranging between 0.3 and 300 GHz and wavelengths between 1 mm and 1 m (Jones et al. 2013; Jankovic et al. 2014). Microwave ovens operate at radiation frequencies of 900 and 2 450 MHz (Meredith 1998; Jankovic et al. 2014). Microwave radiation has thermal and non-thermal effects upon living organisms. Both effects are dependent on the extent of the radiation absorption, on the dielectric constant and on the electrical conductivity of the target material. Its disintegration effect is time-dependent with the cell damage being directly proportional to the exposure time of the cells to the radiation (Jankovic et al. 2014). The thermal effect of microwave radiation is the consequence of its absorption by cell molecular structures causing them to vibrate and produce heat. The thermal effect is most likely dominant in killing living cells (Jankovic et al. 2014) and manifests itself particularly by the denaturation of proteins and their aggregation in the cytosol (Woo et al. 2000). Non-thermal effects are likely to induce changes in the secondary and tertiary structure of functional protein molecules as a result of rapid changes in the electric field and appear at temperatures lower than the temperatures that cause the destruction of the microbial cells (Jankovic et al. 2014). The non-thermal effects can also alter the structure of chromosomes, and thence the function of the cells. Furthermore, they can affect the tolerance to standard mutagens and the repair of chromosomal lesions, which can lead to mutations (Banik et al. 2003). After short exposures of vegetative microorganisms to the non-thermal effects of microwave radiation, changes in the cell morphology have been reported, especially a dehydrated appearance, the opening of pores in the cell walls and an increased chemical consumption of soluble extracellular oxygen. Extended exposures involve the aggregation of cytoplasmic proteins. Other non-thermal manifestations include the acceleration of enzymatic processes (non-aqueous esterification) and the acceleration of glycopeptide formation (Jankovic et al. 2014). Similar effects of microwave radiation have also been described in bacterial spores which are extremely resistant to various environmental effects that would kill vegetative bacterial cells. These effects include high temperatures, UV radiation, desiccation and exposure to chemicals (Quinn et al. 2011). The bacterial endospore usually consists of multiple layers of coats, including the exosporium, outer spore coats, inner spore coats, the cortex, the germ cell wall and the inner membrane covering the endospore core. It is the layered structure of the endospore wall, its dehydrated state, negligible metabolic activity, high content of small acid-soluble proteins and high content of dipicolinic acid, as well as the high levels of calcium, make it possible for the spores to resist conventional heating for up to 15 min at 121 °C (Quinn et al. 2011). In the case of microwave radiation, for example, *B. licheniformis* spores were damaged after 2 min when exposed to a frequency of 2 450 MHz and a microwave energy of 2 000 W (cortex hydrolysis) and *B. cereus* spores were destroyed after exposure to the microwave power with the same frequency and energy of 600 W after 4 min (Jankovic et al. 2014). Changes observed after exposure were suggestive of spore cortex hydrolysis after breaking its intra-molecular bonds and the insertion of water molecules. This result in the inner membrane swelling and rupture of the spores (Kim et al. 2009). Also, in the case of *P. larvae*, the professional literature highlighted significant differences between vegetative cells and spores in the resistance to heat-induced damage using conventional heating and microwaves. While vegetative *P. larvae* cells were destroyed by simple heating at 60 °C for 15 min, spores were destroyed at 100 °C and higher, depending on the treated material and the number of spores present. The temperature of 100 °C destroyed, e.g., spores in honey and water after 20–160 min, and the temperature of 110 °C had the same effect in honey after 41 minutes. The temperature of 121 °C destroyed spores in honey after 8.6 minutes of exposure, while the same effect in wax was obtained after 30 min of exposure (Hansen and Brodsgaard 1999). Furthermore, in the case of *P. larvae* spores, the use of microwaves has proved to be a highly effective way of their devitalisation. Moline et al. (2015) confirmed that spores in honey samples disappeared completely after 90 s when the microwave power was 800 W. However, after the treatment, undesirable changes in the sensory properties of honey (darkening) were observed, inactivation of the enzymes oc-

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curred and the amount of hydroxymethylfurfural (HMF) increased. However, microwave radiation could still be successfully used in beekeeping for the sterilisation of different types of materials and equipment due to the relatively low purchase price of a microwave oven, the low operating costs and the absence of residues.

This paper deals with the effects of high-energy and high-frequency microwave radiation on *P. larvae* spores, investigates the relationships between the microwave power, exposure time and the number of spores in water suspensions and considers the possibilities of use of this radiation in practice.

MATERIAL AND METHODS

Spores of a field strain of *P. larvae* obtained from several liquefied larvae which had died during a severe clinical form of the American foulbrood were used in this study. The infected cells were unsealed and their contents were pipetted into microtubes. The material was stored in a refrigerator at 4 °C for 6 months.

The presence of spores was verified by microscopy using Schaefer-Fulton staining (Bzdil 2010). Subsequently, the material was diluted with sterile distilled water by ten-fold dilution from 1×10^{-1} to 1×10^{-9}. Each dilution was heat treated at 90 ± 2 °C for 10 min to remove the vegetative forms of the microorganisms. After the cooling, suspension volumes of 0.1 ml from each of them were inoculated on 5 plates with a modified mannitol egg yolk polymyxin (MYP) agar and aerobically incubated at 37 ± 1 °C for 120 h in a BT 120 thermostat (EKOM s.r.o., Polna, Czech Republic). The modified MYP agar was composed of meat peptone (10.0 g), NaCl (10.0 g), D-mannitol (10.0 g), beef extract (1.0 g), phenol red (0.025 g), agar (12.0 g), sterile water (900.0 ml), after autoclaving sterile D-glucose (1.0 g), sterile egg yolk emulsion (100.0 ml) and 2 vials of polymyxin B, each 50 000 UI (5.0 + 5.0 ml), were added to reach a total pH = 7.1 ± 0.2. After incubation, the colonies were counted and the number of colony-forming units (CFU) per millilitre of suspension was calculated. The *Paenibacillus larvae* colonies had a typical orange colour and musty odour. The grown strains were typed using a catalase test with a negative result and subsequently by the phenotype molecular method MALDI-TOF MS (Bruker Daltoniks GmbH, Bremen, Germany) as *Paenibacillus larvae* (high identification score: 2.210–2.250). The number of spores in 1 ml of the starting material was approximately 150 million. Dilutions of 1×10^{-5}, 1×10^{-6} and 5×10^{-7} were then used for our experiment. Thus, suspensions containing three different spore concentrations (tens, hundreds and thousands of spores) in precisely defined liquid volumes were formed. The exact initial concentrations were 1.965×10^{3} CFU/ml, 1.37×10^{2} CFU/ml and 7.2×10^{1} CFU/ml of water suspension. A total of 50 g of each final suspension was transferred to a sterile polypropylene tissue-sampling vial (Gama Group a.s., Dalecin, Czech Republic) and the vial with the contents was weighed on a scale with a precision of 0.1 g (HELAGO HF-1200G; A&D Instruments Limited, Oxford, UK). Subsequently, the suspensions were exposed to microwave radiation in an LG model MS-2334B microwave oven (LG Electronics CZ, Prague, Czech Republic). This type of microwave oven operates at a wavelength of 12.17 cm and a frequency of 2 450–2 665 MHz. The vials were placed on a white porcelain plate covered with one layer of filter paper and separately exposed to 170, 510 and 850 W. In all the cases, the exposure time was 1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 minutes. After exposure, the vials were removed from the microwave oven. After the spontaneous cooling of each suspension to room temperature, each vial with the contents was weighed again and the volume of the evaporated liquid was refilled with distilled water to the original weight so that after the culture, the number of spores was comparable to the control. Each test was performed in five replicates. After thorough mixing, each exposed sample was cultured on 3 MYP agar plates, 90 mm in diameter (Trios s.r.o., Prague, Czech Republic), in an amount of 0.1 ml per plate. The inocula were spread over the agar surface with a curved sterile plastic rod (VWR International s.r.o, Roznov pod Radhostem, Czech Republic). After drying, the labelled plates were incubated aerobically at 37 ± 1 °C for 120 h in a BT 120 thermostat (EKOM s. r. o., Polna, Czech Republic). The colonies were counted and the results recorded in tables. The average numbers of colonies were compared. The statistical evaluation of the results was carried out using the Unistat Statistical Package v6.5 (Unistat Ltd., London, UK).

Due to the fact that, in our experiments, the method of repeated observations of the analysed samples at 8 time-intervals (exposure times) was

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used and the sample numbers (n) in the individual groups were low, the data evaluation was subjected to the non-parametric Friedman's analysis of variance (ANOVA). Subsequently, Friedman's ANOVA multiple comparisons with the control group was performed by Dunnett's test using rank sums (Zar 1999) in order to assess the statistical significance of the differences between the compared groups. The value of $P \leq 0.05$ was the level of statistical significance when evaluating the tests.

RESULTS

The decrease in the numbers of viable spores of *P. larvae* after exposure to the microwave radiation in the samples suspended in water with the initial concentrations of 7.2×10^1 CFU/ml of water suspension, 1.37×10^2 CFU/ml and 1.965×10^3 CFU/ml of water suspension are shown in Tables 1–3.

At the microwave power consumption of 170 W, the spores decreased in numbers in all the suspensions with different concentrations after 1 min when compared to the control non-exposed suspension. This decrease continued until 10 min of exposure and, after 15 min, no viable spores were detected, again, in any of the suspensions. At the microwave power consumption of 510 W, a reduction in the viable spore numbers was observed in all the dilutions after 1 min of exposure. After 3 min, no viable spores were detected and no growth on the agar plates was observed. At the microwave power consumption of 850 W, the numbers of viable spores also declined after 1 min exposure, and no spores capable of germination were found after 2 minutes.

From a statistical viewpoint, the numbers of *P. larvae* spores or their viability in all the concentrations in the water suspensions after exposure to 170 W were highly significantly reduced ($P < 0.01$) after 5 min; at 510 W, the viability was significantly decreased ($P < 0.05$) after 2 min and highly significantly decreased ($P < 0.01$) after 3 min of exposure; at 850 W, a highly significant decrease in the viability ($P < 0.01$) was observed after 2 min of exposure. Zero viability of spores was found in all the concentrations after exposure to the same microwave power consumption for the same amount of time. This occurred after 15, 3 and 2 min of irradiation at 170, 510 and 850 W, respectively.

Table 1. The decrease in the *Paenibacillus larvae* spores in time after exposure to microwave radiation with a power consumption of 170, 510 and 850 Watts (tens of spores per millilitre of water suspension)

Power consumption/time	Control	1 min	2 min	3 min	4 min	5 min	10 min	15 min
	72	70	63	30	8	4	1	0
	72	68	65	30	12	2	0	0
170 W	72	72	62	27	11	1	0	0
	72	70	62	33	16	1	0	0
	72	66	59	22	11	3	2	0
Median	72	70	62	30	11	2	0	0
Friedman's test	*P*-value	0.995 2	0.708 0	0.272 1	0.064 4	0.009 7**	0.000 4**	0.000 2**
	72	62	19	0	0	0	0	0
	72	55	12	0	0	0	0	0
510 W	72	67	16	0	0	0	0	0
	72	52	21	0	0	0	0	0
	72	66	9	0	0	0	0	0
Median	72	62	16	0	0	0	0	0
Friedman's test	*P*-value	0.469 4	0.038 5*	0.000 7**	–	–	–	–
	72	70	0	0	0	0	0	0
	72	70	0	0	0	0	0	0
850 W	72	68	0	0	0	0	0	0
	72	65	0	0	0	0	0	0
	72	66	0	0	0	0	0	0
Median	72	68	0	0	0	0	0	0
Friedman's test	*P*-value	0.198 3	0.003 1**	–	–	–	–	–

*Statistically significant decrease in the spore viability, **statistically highly significant decrease in the spore viability

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Table 2. The decrease in the *Paenibacillus larvae* spores in time after exposure to microwave radiation with a power consumption of 170, 510 and 850 Watts (hundreds of spores per millilitre of water suspension)

Power consumption/time	Control	1 min	2 min	3 min	4 min	5 min	10 min	15 min
	137	135	122	116	95	52	2	0
	137	136	120	115	85	41	0	0
170 W	137	129	118	107	85	54	2	0
	137	133	120	113	88	49	2	0
	137	134	123	112	93	50	3	0
Median	137	134	120	113	88	50	2	0
Friedman's test	*P*-value	0.982 7	0.660 0	0.240 5	0.054 4	0.007 8**	0.000 6**	0.000 1**
	137	130	57	0	0	0	0	0
	137	121	30	0	0	0	0	0
510 W	137	132	10	0	0	0	0	0
	137	127	7	0	0	0	0	0
	137	129	22	0	0	0	0	0
Median	137	129	22	0	0	0	0	0
Friedman's test	*P*-value	0.469 4	0.038 5*	0.000 7**	–	–	–	–
	137	134	0	0	0	0	0	0
	137	124	0	0	0	0	0	0
850 W	137	129	0	0	0	0	0	0
	137	129	0	0	0	0	0	0
	137	127	0	0	0	0	0	0
Median	137	129	0	0	0	0	0	0
Friedman's test	*P*-value	0.198 3	0.003 1**	–	–	–	–	–

*Statistically significant decrease in the spore viability, **statistically highly significant decrease in the spore viability

Table 3. The decrease in the *Paenibacillus larvae* spores in time after exposure to microwave radiation with a power consumption of 170, 510 and 850 Watts (thousands of spores per millilitre of water suspension)

Power consumption/time	Control	1 min	2 min	3 min	4 min	5 min	10 min	15 min
	1 965	1 864	1 758	1 590	1 258	886	392	0
	1 965	1 920	1 706	1 656	1 214	722	52	0
170 W	1 965	1 930	1 716	1 644	1 292	782	88	0
	1 965	1 948	1 758	1 596	1 284	634	38	0
	1 965	1 900	1 739	1 590	1 252	772	19	0
Median	1 965	1 920	1 739	1 596	1 252	772	52	0
Friedman's test	*P*-value	0.982 7	0.660 0	0.240 5	0.054 4	0.007 8**	0.000 7**	0.000 1**
	1 965	1 832	154	0	0	0	0	0
	1 965	1 825	143	0	0	0	0	0
510 W	1 965	1 782	106	0	0	0	0	0
	1 965	1 817	197	0	0	0	0	0
	1 965	1 822	133	0	0	0	0	0
Median	1 965	1 822	143	0	0	0	0	0
Friedman's test	*P*-value	0.469 4	0.038 5*	0.000 7**	–	–	–	–
	1 965	1 800	0	0	0	0	0	0
	1 965	1 784	0	0	0	0	0	0
850 W	1 965	1 782	0	0	0	0	0	0
	1 965	1 766	0	0	0	0	0	0
	1 965	1 798	0	0	0	0	0	0
Median	1 965	1 784	0	0	0	0	0	0
Friedman's test	*P*-value	0.198 3	0.003 1**	–	–	–	–	–

*Statistically significant decrease in the spore viability, **statistically highly significant decrease in the spore viability

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DISCUSSION

Several studies have, so far, dealt with the effective inactivation of spores by microwave radiation. However, these studies have focused rather more on the spores of *Bacillus* sp. and *Clostridium* sp. (Celandroni et al. 2004; Kim et al. 2009) and only a few were focused on *P. larvae* spores (Moline et al. 2015).

The results of the tests carried out in our study showed that the microwave radiation is highly effective in terms of the devitalisation of the *P. larvae* spores. The radiation obviously penetrates the walls of the spores easily and destroys them (Woo et al. 2000; Jankovic et al. 2014), which then allows the core to be heated. This leads to the devitalisation of the spores. Moreover, non-thermal effects can certainly come into play in this process (Banik et al. 2003; Jankovic et al. 2014). Therefore, large differences in the decrease in viable spore numbers can be observed between the use of conventional boiling and microwaves. While ordinary boiling (100 °C) destroyed the spores in honey and water after 20 to 160 min (Hansen and Brodsgaard 1999), the microwaves killed the spores suspended in water after 2–15 min depending on the input power of the microwave generator. It was also shown that the spore concentration of 7.2×10^1 CFU/ml, 1.37×10^2 CFU/ml and 1.965×10^3 CFU/ml of water suspension in the samples did not affect the rate of devitalisation of the spores, but the input power of the microwave oven was a critical factor in this process. This result is in accordance with data published earlier, which have shown that microwaves can be an effective method for the destruction of various microbial spores and that they are more effective than conventional conductive heating (Kim et al. 2009; Ojha et al. 2016).

The above-mentioned experiences confirm that it would be possible to use microwave radiation for the sterilisation of non-metallic beekeeping devices and equipment, industrial wax, the packaging of the beehive products, and also in other fields of human activity, such as in agriculture and industry. However, it is impossible to sterilise the bee products themselves using microwave radiation because it biologically deactivates the active substances and causes changes to the sensory properties and other changes at the molecular level in such products (Woo et al. 2000; Jankovic et al. 2014; Moline et al. 2015).

We assume that the use of microwave radiation in beekeeping could be a new alternative to sterilisation and could be faster, cheaper, more efficient and more environmentally friendly than using chemicals. Given that microwave radiation easily penetrates into biological materials, the thorough mechanical cleaning of the treated materials and surfaces could become unnecessary.

However, further tests and studies are needed to verify the use of microwaves in beekeeping practice in the cause of stability and quality of the individual materials and equipment. The pending publication of our first experiments showed that some dry materials, namely paper and wood, ignite at higher microwave oven power consumptions. Further research on the effects of microwaves on the spores in dry materials is therefore needed.

Conflict of interest

The authors declare no conflict of interest.

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