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The sterilization of honey with cobalt 60 gamma radiation: a study of honey spiked with spores of *Clostridium botulinum* and *Bacillus subtilis*

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Abstract. Unprocessed honey is a recognized wound-healing remedy. However, to make clinical use of honey acceptable, it should be sterile. To find the lowest dose of irradiation needed for sterilization, six batches of honey (*a*–*f*) were gamma irradiated with 6, 12, 18, 22 and 25 kGy Cobalt-60. After a dose of 25 kGy the antibacterial activity was not altered. Presumably glucose oxidase (EC 1.1.3.4), which produces hydrogen peroxide, is not easily damaged by irradiation. Amylase activity on the other hand was significantly reduced to 19%, 19%, 21%, 22%, 43% in batches *a*), *b*), *c*), *d*) and *f*) respectively, whereas no decrease was observed in batch *e*). All batches spiked with approximately 10^6 spores from *Cl. botulinum* or *B. subtilis* per 50 g honey proved to be sterile after irradiation with a dose of 25 kGy. Honey was also spiked with *Cl. botulinum* at up to 5000 spores per 50 g honey, which is the upper limit of natural contamination. The sterilizing dose in this case was 18 kGy.
Key words. Honey sterilization; irradiation D-values; *Clostridium botulinum*; antibacterial activity.

For 4000 years the wound-healing properties of honey have been recognised: cleansing, absorption of oedema, antimicrobial activity, deodorisation, promotion of granulation, tissue formation, epithelization, and improvement of tissue nutrition [1–4].

In a clinical trial of 104 patients with superficial thermal burns involving less than 40% of the body surface, honey appeared significantly superior to silver-sulfadiazine[5]. Similar results were obtained with pigs in a standard burn wound model[6,7]. Moreover, like many antiseptic agents, silver-sulfadiazine is cytotoxic[8] in contrast to honey, where no toxic or side-effects have been reported. The antibacterial effect of honey has been extensively investigated by Lindner[9] and White et al.[10]. Due to the presence of the enzyme glucose oxidase (EC 1.1.3.4) in the hypopharyngeal glands of the bee, hydrogen peroxide accumulates in diluted honey. In vitro at a concentration of 10^{-6}–10^{-8} mol/l hydrogen peroxide not only inhibits bacterial growth, but also stimulates the proliferation of fibroblasts[11]. In spite of its high osmolarity and its various anti-bacterial factors in addition to hydrogen peroxide[12,13] honey is not sterile, but may contain bacillus and clostridium spores. In 270 samples of unprocessed honey from seven different countries, 8.5% carried up to 40–80 spores of *Clostridium botulinum* per gram honey[14,15].

Although the extremely low a_w value of undiluted honey (<0.45–0.70) excludes bacterial growth or survival of vegetative forms of bacteria[16], in some cases sterile honey is necessary, e.g. when honey is used for the preservation of skin[17]. For other medical purposes sterility is at least highly desirable. The aim of the study was to determine an effective radiation dose for sterilizing honey without impeding its antibacterial activity.

Materials and methods

Honey. Batches *a*), *c*), *d*) and *e*), sold as linden honey, were obtained from various sources. Sample *b*) was identical to *a*) except for the addition of 581 mg ascorbic acid/kg (2.3 mmol/l) as an antioxidant. Sample *f*) was linden-clover honey bought at a local supermarket.

Origin. *a*), *c*) and *d*) came from different well-known beekeepers all located within an area of 12 km^2. Honey *e*) was imported from China. The origin of the linden-clover honey bought in a supermarket was unknown.

Time of honey gathering. Samples of *a*), *c*) and *e*) were gathered in the summer of 1993 and *d*) in the summer of 1992; sample *f*) did not carry a date mark. All samples were kept at 4 °C in the dark in glass or plastic containers until they were spiked with spores in December 1993. According to standard practice, care was taken to avoid contact with metal. Sample *o*), first used in the summer of 1991, came from the same apiary as *a*); it was repeatedly tested and found free of pathogens[18]. A quantity of 20 kg of *o*) was stored and kept as a standard.

Antibacterial activity. The antibacterial quality of honey was tested by an agar dilution method[18]. In addition to *Staphylococcus aureus* (ATCC29213), as recommended by White et al.[10], *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) and *Clostridium botulinum* (ATCC 19397) were also used as test strains.

Five concentrations of honey (20%, 16%, 12%, 8% and 4% w/w) were tested. The 'inhibine number' indicates the number of the plate with the lowest honey concentration that did not allow visible growth. For example, an inhibine value of 4 means no growth on the fourth plate (8% honey). Control plates without honey were always included. The sum of inhibine values, of the above-mentioned bacterial strains, was used as an indicator for the antibacterial activity of a particular honey batch.

Honey properties. In all samples the pH of diluted honey was measured (1:5, w/w) and the water content was determined with a refractometer[19]. The diastase activity of 1 g of honey, before and after irradiation with 25 kGy, is expressed in units alpha-amylase/kg honey (Dimension^{R}Du Pont, Wilmington, DE, USA). To check roughly the floral origin of the honey the percentage of linden pollen (from *Tilia sp.*) was assessed microscopically.

Preparation of spore suspension. Cultures of *Bacillus subtilis* (ATC 11774) and *Cl. botulinum* (ATCC 19397) were separately prepared by inoculating 1 l brain-heart infusion broth (Oxoid CM 225). The clostridia were incubated under anaerobic conditions and the bacilli under atmospheric conditions for 2 weeks at 37 °C. After incubation the cultures were centrifuged at 18 500 *g* (30 min at 4 °C).

The pellets were washed twice with 100 ml distilled water, centrifuged and then resuspended in 10 ml of water. After heating the suspensions for 20 min at 80 °C they were stored as stock solutions. Honey samples were spiked with *B. subtilis* and *Cl. botulinum* with $10^{4.5}$ spores per g and $10^{5.0}$ per g respectively. A homogeneous mixing of spores with the almost solid honey was obtained by stirring the honey with a speed-controlled electric drill provided with a T-piece as mixing head.

Irradiation. Six portions of 50 and 300 g of each batch were transferred into closed plastic beakers for irradiation. One set was kept as control while the others were irradiated. Irradiation was carried out in a cobalt-60 facility (Proefbedrijf Voor Doorstraling, Wageningen, The Netherlands) at 6, 12, 18, 22 and 25 kGy with a dose rate of 125 Gy per min. Irradiation D values were calculated from the function

$$D = \text{dose (kGy)}/[\log \text{CFU}_x - \log \text{CFU}_y]$$

where x was the CFU (colony forming unit) count before and y the CFU count after irradiation[20].

Sterility after irradiation. To assess sterility, 50 g of honey were diluted with sterile distilled water 1:1 (v/v), centrifuged at 18 500 *g* (30 min, 4 °C) and the supernatant was discarded. After resuspending the residue in 1.0 ml distilled water, CFUs were counted with a spiral plater. The reproducibility of CFU counts with the spiral plater is ± 0.5 log or better. For the enumeration of *Cl. botulinum* plates were incubated anaerobically, whereas atmospheric conditions were used for *B. subtilis*.

Finally, the spores concentrated from 50 g unprocessed honey were transferred into 1000 ml brain-heart infusion broth and incubated aerobically and anaerobically. If no growth occurred within seven days the irradiation batch was considered sterile.

Honey spiked with 5000 spores of *Cl. botulinum* per 50 g was prepared from sample *o*) to find out whether this more naturally occurring spore load could be sterilized by an irradiation dose of 18 kGy, as had been observed in a previous study[18].

Results

Pollen analysis revealed that only batch *e*) was real linden honey. The local honeys, sold as linden honey,

Table 1. Some physico-chemical properties of six different unirradiated honey batches and their antibacterial activity according to the inhibine score (i).

	a	*b*	*c*	*d*	*e*	*f*
pH	4.49	3.94	4.04	4.05	4.45	3.96
% H_2O (w/w)	16.9	16.9	18.7	16.5	18.9	17.2
μS/cm*	320	-	895	700	520	310
% lime pollen**	<1	-	1	<1	22	11
amylase (U/kg)	1738	1693	3727	3506	860	777
E. coli	3	3	4	3	1	0
P. aeruginosa	3	3	4	4	2	1
Staph. aureus	4	4	5	5	2	0
Enteroc. faecalis	3	3	4	3	0	0
Cl. botulinum	4	4	4	5	1	2
Total inhibine value (Max. score: 25)	17	17	21	20	6	3

The batches *a*), *c*) and *d*) were sold under the name of linden honey. All three came from different local apiaries within the area of Maastricht. The Chinese linden honey *e*) and the supermarket lime-clover honey *f*) were from unknown sources. Note: to raise the antioxidant concentration of honey, *b*) was prepared by adding 581 mg ascorbic acid/kg to batch *a*).

*Electrical conductivity.

**Usually genuine linden honey contains at least 20% pollen of the lime tree.

turned out to be a multifloral summer honey gathered from linden, privet, sweet chestnut, white clover and forget-me-not. In table 1 a number of physico-chemical properties of six different honey batches are summarized.

The summer honey of apiary *c*) had the highest anitbacterial activity, whereas *e*) and *f*) were scoring low. Adding the antioxidant vitamin C up to 581 mg per kg *b*) did not alter the inhibine (i) score. The *i*) score of unprocessed multifloral summer honey samples from apiary *a*), studied in 1990, 1991, 1992, 1993 and 1994 were identical (data not shown). Similar results were obtained with honey from beekeeper *c*).

Irradiation effects. After irradiation gas evolution occurred, for bubbles were seen in all samples. This has also been observed by others[20]. The antibacterial activity remained unchanged after an irradiation dose of 25 kGy (data not shown). However, this dose inactivated the amylase activity significantly. In batches *a*), *b*), *c*), *d*) and *f*) the amylase activity was reduced to 19%, 19%, 21%, 22% and 43% respectively, whereas no decrease was observed in batch *e*). The sterilizing effect of the cobalt gamma-radiation on the number of added spores is given in the figure.

The electrical conductivity data, as shown in table 1, could not be correlated with the gamma-irradiation dose and its spore killing effect. In this study they appeared irrelevant. Samples of 50 g spiked with a high spore dose of either *Cl. botulinum* or *B. subtilis* appeared sterile after 25 kGy applied with a dose rate of 125 Gy per min. With 18 kGy, the CFU reduction of the antioxidant-enriched batch *b*) differed from *a*). In addition to a high antioxidant level, *b*) had a lower pH than *a*) (table 2).

A honey batch with an almost natural spore load was made up by spiking batch *o*) with 5000 spores *Cl. botulinum* per 50 g. Batch *o*) became sterile after irradiation with 18 kGy, and this observation agreed with an earlier report [18].

Table 2. Effect of irradiation on spores of *Clostridium botulinum* in six different batches of undiluted, unprocessed honey.

Honey	log CFU/50 g irradiation dose		D value (18 kGy)
	none	18 kGy	
a	6.68 (6.20)	3.13 (2.50)	5.07 (4.86)
b	6.68 (6.20)	1.60 (1.60)	3.54 (3.91)
c	6.68 (6.20)	1.48 (1.84)	3.46 (4.31)
d	6.68 (6.20)	2.00 (2.04)	3.84 (4.33)
e	6.68 (6.20)	1.00 (1.69)	3.17 (3.99)
f	6.68 (6.20)	1.00 (2.57)	3.17 (4.96)

The log CFU/50 g and the D values for *Bacillus subtilis* are given in brackets.

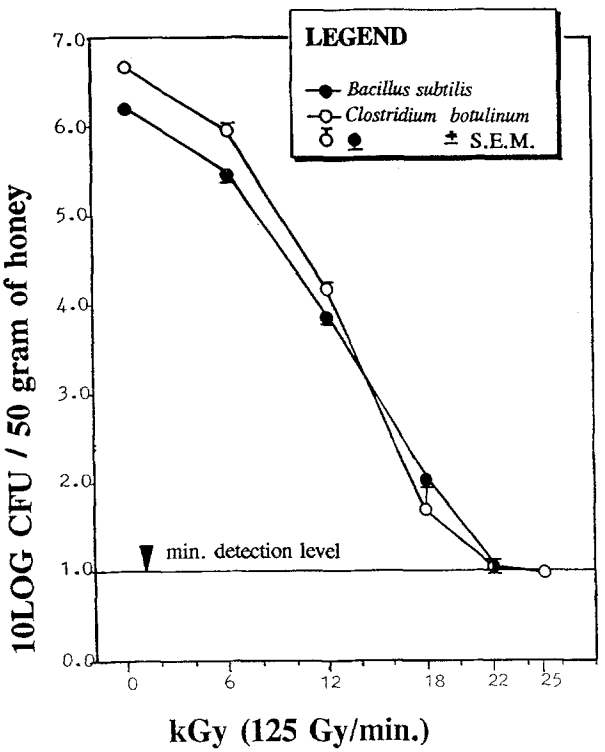

Figure. Reduction of the initial CFU count by killing of the spores of *Bacillus subtilis* and *Clostridium botulinum* is almost linear between 6 and 18 kGy. A dose of 22 kGy brings the CFUs below the minimum detection level. Each point represents the mean of 6 different samples of honey.

Discussion

The antibacterial activity of honey was high in four and low in two of the batches tested. The very low inhibine score of the Chinese linden honey *e*) and that of the linden-clover honey *f*) might be due to overheating. A low amylase activity, such as was found in *e*) and *f*), is often associated with overheating during processing or storage.

In an earlier study we reported that lime-honey, which is synonymous with linden honey, had a high antibacterial activity[18]. Because gamma irradiation effects have been reported to vary with floral sources of honey[21,22], only one type of honey was selected from our experiments. We decided to use linden honey, with in addition linden-clover honey as a close variant of linden honey. Unfortunately the linden honey *a*), *c*) and *d*), which we bought from local beekeepers, was not linden honey at all. According to table 1, only batch *e*) can be called linden honey. Batches *a*), *c*) and *d*) are, generally speaking, multifloral summer honey and are quite different from each other.

In e.g. batches *c*) and *d*) 60% of the pollen was from various plants not shared, whereas only 40% came from plants such as maple, sweet chestnut and linden. Hence *c*) and *d*) are not identical.

The pH of all batches was within a normal range of 3.94–4.04 and similar values were reported by Lindner[9].

The decrease of the pH in *b*) by a value of ca 0.5 is caused by the added ascorbic acid but did not affect the antibacterial activity. The resistance of bacterial spores in honey to gamma radiation may be influenced by the presence of free radical scavengers such as ascorbic acid, fumarate and glutamate[21].

Honey may also show a slight change in colour and a strong reduction in amylase activity when exposed to doses of 0 to 25 kGy[22].

In all batches used, the water content of honey was between 16.5 and 18.9% and this may explain why we observed an almost similar spore killing per irradiation dose and per batch. With 25 kGy all honey batches, highly contaminated with spores, were sterilized. Though after irradiation the amylase activity was reduced to a mere 20–40%, the antibacterial activity remained unaltered. Only the Chinese lime honey had an unchanged amylase activity. With 25 kGy an almost unchanged amylase activity was also observed in an Australian study[22]. Why amylase in European honey becomes inactivated after irradiation and not in Chinese or Australian honey remains unclear.

Honey usually contains 22–24 mg vitamin C per kg (0.19 mmol/l); only thyme honey has an exceptionally high level of 581 mg/kg (2.3 mmol/l)[23]. In our study the spore-killing effect of gamma irradiation was increased in the presence of 2.2 mmol vitamin C/l. As can be seen in table 2, at 18 kGy the vitamin C effect on *Clostridium* is shown by a drop from log 3.13 to 1.6 and for *B. subtilis* a drop from 2.50 to 1.60 per gram. Because the reproducibility of the method is within a log value of ± 0.5, the observed decrease was considered as significant.

During irradiation spores are killed directly by DNA damage and indirectly by oxidative breakdown of polyunsaturated fatty acids (PUFAs), oligosaccharides and proteins[24]. The breakdown of PUFAs is damaging to the lipid membranes of the procaryotic and eucaryotic cell.

The vitamin C effect on the killing of spores, noted at 18 kGy level, may partly be explained by the Fenton reaction, which causes the oxidative breakdown of PUFAs by $OH^{\cdot}$ radicals[25]. The reaction ($Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH-$) is fueled by hydrogen peroxide, formed during the radiolysis of water, and the reduction of Fe^{3+} into Fe^{2+} by vitamin C.

Because trace amounts of iron and vitamin C are always found in honey, a potentiation of a lipid peroxidation process might be expected as described by Bast[25]. A similar vitamin C effect may enhance the antibacterial activity of unirradiated honey, both in vitro and in vivo.

Conclusion

Honey can be sterilized without loss of antibacterial properties. Even at 25 kGy the antibacterial activity remained unaffected. However, irradiation may also change the chemical composition of honey, and further research is needed to prove that sterilized honey is equally effective in wound treatment as unprocessed honey.

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1 Efem, S. E. E., Br. J. Surg. *75* (1988) 679.
2 Dany-Mazeau, M., and Pautard, G., Krankenpflege/Soins infirmiers *6* (1991) 54.
3 Bergman, A., Yanai, J., Weiss, J., Bell, D., and David, M. P., Am. J. Surg. *145* (1983) 374.
4 Efem, S. E. E., Surgery *113* (1993) 200.
5 Subrahmanyam, M., Br. J. Surg. *78* (1991) 497.
6 Hoekstra, M. J., Hupkens, P., Dutrieux, R. P., Bosch, M. M. C., and Kreis, R. W., Br. J. plast. Surg. *46* (1993) 585.
7 Postmes, T., Bosch, M. M. C., Hoekstra, M. J., and Dutrieux, R. P., (1995) submitted.
8 Teepe, R. G. C., Koebrugge, E. J., Löwik, C. W. G. M., Petit, P. L. C., Bosboom, R. W., Twiss, I. M., Boxma, H., Vermeer, B. J., and Ponec, M., J. Trauma *35* (1993) 8.
9 Lindner, K. E., Zentbl. Bakt. Parasitkde. Allg. landw. tech. Microbiol. *115* (1959) 720.
10 White, J. W., Subers, M. H., and Shepartz, A. I., Biochem. biophys. Acta *73* (1963) 57.
11 Schmidt, R. J., Chung, L. Y., Andrews, A. M., and Turner, T. D., in: Second European Conference in Advances in Wound Management. Proc. Int. Conf. Center Harrogate Oct. 20–23 1992.
12 Obaseiki-Ebor, E. E., and Afonya, T. C. A., J. Pharm. Pharmac. *36* (1984) 283.
13 Molan, P. C., and Russell, K. M., J. apicult. Res. *27* (1988) 62.
14 Nakano, H., Okabe, T., Hashimoto, H., and Sakaguchi, G., Jap. J. Med. Sci. Biol. *43* (1990) 183.
15 Midura, T. F., Snowden, S., Wood, R. M., and Arnon, S. S., J. clin. Microbiol. *9* (1979) 282.
16 Chirife, J., Scarmato, G., and Herszage, L., Lancet *i* (1982) 560.
17 Subrahmanyam, M., Lancet *341* (1993) 63.
18 Postmes, T., Bogaard van den A., and Hazen, M., Lancet *341* (1993) 756.
19 Codex Alimentarius Commission CAC, vol. 3, edn 1, suppl. 2. FAO/WHO, Rome 1989.
20 Shimanuki, H., Herbert, E. W., and Knox, D. A., Am. Bee J. *124* (1984) 865.
21 Huhtanen, C. N., J. Food Prot. *54* (1991) 894.
22 Wootton, M., Hornitzky, M., and Beneke, L., J. apicult. Res. *24* (1985) 188.
23 Kerkvliet, J., Bijen *1* (1992) 163.
24 Grecz, N., Rowley, D. B., and Matsuyama, A., in: Preservation of Food by Ionizing Radiation, vol. 2, p. 171. Eds. E. S. Josephson and M. S. Peterson. CRP Press, 1983.
25 Bast, A., Haenen, G.R.M.M., and Doelman, C. J. A., Am. J. Med. *91* (1991) 3C.