Bulletin of Insectology ***68*** *(2): 233-237, 2015*
ISSN 1721-8861

Preliminary evaluation of glucose oxidase and its products *in vitro* antimicrobial activities on *Paenibacillus larvae* ATCC9545 vegetative form

Simona SAGONA, Barbara TURCHI, Filippo FRATINI, Matteo GIUSTI, Beatrice TORRACCA, Roberta NUVOLONI, Domenico CERRI, Antonio FELICIOLI
Veterinary Sciences Department, University of Pisa, Italy

Abstract

Paenibacillus larvae is the pathogen responsible for American foulbrood disease in honeybees. Today, no useful tool has been identified to contrast its spreading due to the spores diffusion, except for hives burning. The effect of glucose oxidase and its reaction products, hydrogen peroxide and gluconic acid, was evaluated against *P. larvae* ATCC9545. Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by microdilution method. MIC values were employed to evaluate the survival growth rates of *P. larvae* after each treatment in a macromethod test. Results showed an inhibitory effect of glucose oxidase, gluconic acid and hydrogen peroxide at a minimum concentration of 0.28 µg/mL, 3.71 µg/mL and 0.74 µg/mL, respectively. For hydrogen peroxide MIC value corresponded to MBC value, while *P. larvae* survival growth rates at observed MIC was 98.3% and 91.6% for glucose oxidase and gluconic acid, respectively. Glucose oxidase MBC value (74.20 µg/mL) was remarkably higher than its MIC value. Even thought, the present work focused on the *in vitro* inhibitory effect of glucose oxidase and its reaction products only on *P. larvae* ATCC9545 vegetative form, the obtained results were promising and repeatable.

Key words: *Apis mellifera*, *Paenibacillus larvae*, glucose oxidase, American foulbrood disease.

Introduction

As all living organisms, *Apis mellifera* L. is subjected to microorganisms' attack, such as deforming wing virus (Mazzei *et al*., 2015), *Paenibacillus larvae* (Ashiralieva and Genersch, 2006), *Nosema ceranae* (Higes *et al*., 2006); thus beekeepers are constantly looking for new tools to protect honeybees from these pathogens.

P. larvae is the etiological agent of American foulbrood disease (AFB), the most devastating bacterial disease affecting honey bee brood worldwide (Hansen and Brødsgaard, 1999). *P. larvae* is a gram-positive, spore-forming, facultative anaerobic microorganism (Genersch *et al*., 2005), which is transmitted by spore containing honey being fed to newly hatched larvae (Neuendorf *et al*., 2004); moreover adult bees also carry spores (Lindström *et al*., 2008a; 2008b). Spores, which represent the only infectious form, infect only larvae, while adult bees do not become infected upon ingestion of *P. larvae* spores (Genersch, 2010). Spores germinate and proliferate in the midgut lumen (Yue *et al*., 2008). Moreover, it has been shown that larvae can display a different susceptibility, according to their age (Brødsgaard *et al*., 1998) and the infecting *P. larvae* genotype (Genersch *et al*., 2005; Djukic *et al*., 2014).

It is demonstrated that vertical and horizontal transmission of AFB are both possible in daughter swarms (Fries *et al*., 2006) and among apiaries (Lindström *et al*., 2008a). Transmission is possible also by contaminated honey (Lindström *et al*., 2008b). Since the honey bee is a social organism, its health depends not only on the individual immune system, but also on the social immune system (Cremer *et al*., 2007). Spivak and Reuter (2001) showed that colonies selected for their hygienic behaviors were more resistant to AFB, indeed Oliver (2010) states that the honey-bee social immune system takes advantage of hygienic behaviour. The social immune system could also take advantage of several hive products, mainly honey (Gherman *et al*., 2014; Erler *et al*., 2014), but also royal jelly proteins and glucose oxidase, which showed antimicrobial activity (White, 1966; Fujiwara *et al*., 1990; Bilikova *et al*., 2001; Fontana *et al*., 2004, Scarselli *et al*., 2005). Glucose oxidase is secreted by the hypopharyngeal gland (Takenaka *et al*., 1990; Ohashi *et al*., 1999); it has been found in honey (Schepartz and Subers, 1964) and in royal jelly (Furusawa *et al*., 2008). It has been shown that honey glucose oxidase is only active in diluted honey (Carina *et al*., 2014). Glucose oxidase catalyses the oxidation of D-glucose to D-gluconolactone and hydrogen peroxide (Keilin and Hartree, 1948; 1952; Schepartz, 1965). D-gluconolactone in presence of water produces gluconic acid spontaneously (Takahashi and Mitsumoto, 1963). The main function of glucose oxidase is to act as an antibacterial and antifungal agent (Wong *et al*., 2008). Both products of the reaction, gluconic acid and hydrogen peroxide, contribute to the disinfection of the colony, the prevention of the contamination by pathogens and the limitation of diseases diffusion (Alaux *et al*., 2010).

Most authorities consider the destruction of infected colonies, by burning, as the only effective control measure (Williams, 2000). Antibiotics can only mitigate the disease effects, but do not lead to complete sanification (Reybroeck *et al*., 2012), moreover, they are not allowed in EU apiculture (council regulation EEC n. 2377/90). In order to find alternative measures of control, the antibacterial activity of propolis components,

honey and essential oils were also tested (Albo *et al*., 2003; Roussenova, 2011; Mihai *et al*., 2012; Bilikova, 2013; Erler *et al*., 2014). Fuselli *et al*. (2006) tested essential oil of cinnamon and thyme. As well, they tested thymol compound and the mixture (62.5% of thyme, 12.5% of cinnamon and 25% of thymol) between them. They observed the following results: for cinnamon a minimal inhibitory concentration (MIC) value of 50-100 µg/mL and a minimum bactericidal concentration (MBC) value of 100-125 µg/mL; for thyme a MIC value of 150-250 µg/mL and a MBC value of 200-300 µg/mL; for thymol a MIC and MBC values of 100-150 µg/mL. As concerns the mixture, for both MIC and MBC values a decrease was observed, in particular 67.5 mg/mL and 96.4 mg/mL, respectively. Gende *et al*. (2008) testing *Cinnamon zeylanicum* essential oil also observed an inhibitory activity, with MIC and MBC values of 25-100 µg/mL and 125-250 µg/mL, respectively. Roussenova (2011), by testing eleven essential oils against *P. larvae*, demonstrated a high antimicrobial activity: MIC values recorded for cinnamon, thyme, clove, peppermint, lemongrass, sage and oregano essential oils were ≤ 0.06-0.015% v/v, while marjoram and tea tree oils exhibited a variable activity. Moreover, some reports on the sporicidal effect of natural substances (essential oil and plant extracts) are present in literature (Lawrence and Palombo, 2009; Lau and Rukayadi, 2015) concerning spore forming bacteria other than *P. larvae*.

The aim of this study was to evaluate the *in vitro* effect of glucose oxidase and its products on *P. larvae* growth to establish if they could be used as a fighting measure against AFB.

Materials and methods

Bacterial strain

The type strain ATCC9545 of *P. larvae* was obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium). It was grown in brain heart infusion broth (BHI) (Oxoid, Milano, Italia) at 37 °C for 24 h in aerobic condition or on tryptone soy agar (TSA) (Oxoid, Milano, Italia) at 37 °C for 48-72 h in aerobic condition.

Determination of MIC

Susceptibility of *P. larvae* against glucose oxidase and its products (gluconic acid and hydrogen peroxide) was evaluated by broth microdilution method. Glucose oxidase from *Aspergillus niger* (powder, 10 KU) was purchased from Sigma-Aldrich (Milano, Italia); gluconic acid (50% w/v aqueous solution) from Merck KGaA (Darmstadt, Deutschland) and hydrogen peroxide (40% w/v aqueous solution) from Carlo Erba (Milano, Italy).

MIC values were determined for each potential inhibitor, according to Flesar *et al*. (2010) with modifications. Glucose oxidase was solubilised in a solvent [2 M D (+) glucose (Panreac, Barcelona, Spain) and 100 mM Hepes buffer pH 7]. The obtained solution was thoroughly mixed for 1 hour.

Gluconic acid (50% w/v) and hydrogen peroxide (40% w/v) solutions were diluted with sterile water. For each inhibitor, a two-fold serial dilution in BHI broth was performed using microtiter plates. No inhibitory activity against *P. larvae* was detected for these solvents. Tested concentrations ranged from 19000 to 0.035 µg/mL for glucose oxidase, from 118 to 0.000226 µg/mL for gluconic acid and from 95 to 0.000118 µg/mL for hydrogen peroxide. Microtiter plates containing different potential inhibitors were then inoculated with a *P. larvae* culture at a final concentration of 6×10^8 CFU/mL, corresponding to point 2 of the MacFarland turbidity scale. For each microplate, a positive control (containing inoculated BHI broth) and negative control (containing sterile BHI broth) were included. After 24 h of incubation at 37 °C in aerobic conditions and a humid, dark chamber, MIC values for each inhibitor were determined visually as the lowest concentrations at which no growth was observed.

Determination of MBC

After determination of MIC values for each compound, samples contained in the microplate wells corresponding to concentrations equal or higher to the MIC were streaked on TSA plates and incubated at 37 °C for 48 h in aerobic condition to detect cellular proliferation and avoid spores germination. MBC was determined as the lowest concentration which allowed no colonies growth on TSA plates.

Determination of survival growth rate to MIC values

Observed MIC values were employed to evaluate *P. larvae* survival growth rate. A macromethod test in which BHI broth containing inhibitors in a concentration corresponding to the observed MIC values was used. *P. larvae* standard suspension (approximately 6×10^8 CFU/mL) was inoculated in each tube. A control sample consisting of BHI broth inoculated with *P. larvae* standard suspension was also prepared. After incubation at 37 °C for 24 h, each sample was serially diluted in sterile saline solution and pour plated (1 mL) on TSA. After incubation at 37 °C for 48-72 h enumeration of the colonies was performed. Results obtained for each treatment were compared to those of the control sample.

Survival rate of honeybees to gluconic acid

Honey bees have been collected from the experimental apiary of Veterinary Sciences Department of Pisa University. Five concentrations (50, 25, 12.5, 6.25, 3.125% w/v sterile water solution) of gluconic acid and sterile water (control) have been tested. Each concentration has been tested in triplicate on 10 honey bees for replicate. Honey bees were placed in a Petri dish plate with a hole on the cap closed by a netting and fitted with a syringe with honey. An amount of 50 µl of gluconic acid solution was dripped on the thorax of each bee. The survival rate was recorded for 4 days.

Statistical analysis

MIC, MBC values, survival growth rate at the MIC values and survival rate of honeybees to gluconic acid were analyzed by ANOVA and to evaluate possible differences between inhibitors, mean values were tested by Tukey-Kramer HSD post-hoc comparisons.

Results

Table 1 shows MIC and MBC mean values of glucose oxidase and its products determined for *P. larvae* ATCC9545 vegetative form. Moreover, survival growth rates at the MIC values are shown.

Both glucose oxidase, in presence of its substrate, and its products showed an inhibitory effect against *P. larvae* although at different concentrations. Hydrogen peroxide exhibited an inhibitory effect with a MIC value of 0.74 µg/mL, corresponding also to the MBC value. Hydrogen peroxide MBC value resulted the lowest compared to the other MBC values observed ($p < 0.001$). Although both glucose oxidase and gluconic acid showed a high percentage of survival growth rate at the MIC values (98.3 and 91.6%, respectively), a bactericidal effect was observed at reasonably low concentrations, 74.20 µg/mL and 7.42 µg/mL, respectively. In particular, glucose oxidase MIC value resulted significantly lower ($p < 0.001$) than those of other tested inhibitors.

In order to investigate the honeybees' mortality caused by gluconic acid an experiment was carried out. Results (table 2) showed that the survival rate was not significant different in honeybees treated with different gluconic acid concentrations compared to control group.

Discussion and conclusions

Our results not surprisingly showed a marked inhibitory activity of hydrogen peroxide on the vegetative form of *P. larvae* ATCC9545, but the practical application of hydrogen peroxide in the field could pose some problems due to its chemical instability and the possible toxic effect on the bee larvae. Therefore, it would be necessary to test more *P. larvae* strains. Furthermore, it is known that some *P. larvae* strains are weak-delayed catalase positive, depending on age and genotype (Genersch *et al*., 2006), and thus they could be able to dissociate hydrogen peroxide in water and oxygen. The novelty of this investigation is the use of glucose oxidase from *A. niger* and gluconic acid to inhibit *P. larvae* vegetative form *in vitro*. The choice of glucose oxidase from *A. niger* is due to its easy commercial availability and because its antibacterial effectiveness has been demonstrated by Zia *et al*. (2013). The pure enzyme glucose oxidase from *A. mellifera* is not available yet. Although they showed a less marked inhibitory effect, the use of glucose oxidase and gluconic acid could be more practical since they are compatible with the well-being of bees and bee larvae. In fact, glucose oxidase is normally secreted by honeybees on royal jelly and honey (Furusawa *et al*., 2008; Bucekova *et al*., 2014) and gluconic acid is a natural component of honey (Pulcini *et al*., 2004). In addition, glucose oxidase and gluconic acid showed a bactericidal effect at low concentrations. Moreover, since the hive microbial community contributes significantly to the social immunity, it would be important to investigate the effects of glucose oxidase and its products on symbiontic and non symbiontic hive microorganisms (Anderson *et al*., 2011; 2013), in particular those with an inhibitory activity against *P. larvae* (Alippi and Reynaldi, 2006). The main beneficial effect of the employment of glucose oxidase would be a constant production of hydrogen peroxide and gluconic acid, which would then contribute together to the eventual inhibitory activity.

Bucekova *et al*. (2014) showed that glucose oxidase is a regular component of natural honeys and its content varies significantly among honeys. Mato *et al*. (1997) analyzing 20 Spanish honey samples, have shown a gluconic acid content ranging from 3.91 to 11.71 g/kg; later Mato *et al*. (2006), carrying out a research on the determination of honey organic acids content, have highlighted a gluconic acid range from 3.7 up to 14.4 g/kg in 10 Spanish honey samples of different botanical origin. Pulcini *et al*. (2004) analyzed the total gluconic acid content in Italian unifloral honeys and found variable concentrations varying from 2 to 12.3 g/kg, values hundreds of times higher than the MBC value we observed. Moreover, it has been observed that stingless bee honey has a similar gluconic acid content (7.7-11.8 g/kg) (Persano Oddo *et al*., 2008). Erler *et al*. (2014) have already tested honeys with different hydrogen peroxide content against *P. larvae*. Therefore, it would be interesting to

Table 1. MIC, MBC mean values and *P. larvae* survival growth rate at the MIC values observed in 3 replicates of glucose oxidase, gluconic acid and hydrogen peroxide. Different letters into the columns show a statistically significant difference ($p < 0.001$).

Inhibitors	MIC values	MBC values	Survival growth rate at the MIC values (% ± SE)
Glucose oxidase	0.28^{c} ± 0.00 µg/mL	74.20^{a} ± 0.00 µg/mL	98.3^{a} ± 0.3
Gluconic acid	3.71^{a} ± 0.00 µg/mL	7.42^{b} ± 0.00 µg/mL	91.6^{b} ± 0.2
Hydrogen peroxide	0.74^{b} ± 0.00 µg/mL	0.74^{c} ± 0.00 µg/mL	0.0^{c} ± 0.0

Table 2. Survival rate % of honey bees reared *in vitro* with honey and treated with different gluconic acid concentrations. No significant differences at $p < 0.05$.

	Control	3.125% w/v gluconic acid	6.25% w/v gluconic acid	12.5% w/v gluconic acid	25% w/v gluconic acid	50% w/v gluconic acid
Survival rate, mean (SE)	83 (12)	80 (12)	100 (0)	100 (0)	97 (3)	100 (0)

investigate the inhibitory activity against *P. larvae* of honeys containing different gluconic acid and glucose oxidase concentrations, using diluted honeys (Carina *et al*., 2014) so that glucose oxidase would be able to perform its enzymatic activity.

Moreover, the evaluation of *P. larvae* wild strains resistance against glucose oxidase and gluconic acid would be needed in order to understand the effective antimicrobial potential.

Although we studied the inhibitory effect of glucose oxidase and its reaction products only on *P. larvae* ATCC9545 vegetative form, and not on the spore, nonetheless we obtained promising and repeatable results *in vitro*.

Moreover, Dingman (2011) showed that hydrogen peroxide/peroxyacetic acid biocide is able to inactivate *P. larvae* endospores and thus could be employed as a sanitizing agent of beekeeping equipment. It would be then interesting to investigate the effects of glucose oxidase and its products on *P. larvae* endospores.

This work represents a preliminary investigation; however, the employment of glucose oxidase/gluconic acid could be a new alternative tool against *P. larvae*, compatible with honeybee well-being and beekeepers economy.

Acknowledgements

We would like to thank the anonymous reviewers for their valuable comments and suggestions to improve the quality of the paper. Authors' contribution: SS, BTu, contributed equally to this study; AF, DC, SS, conceived and designed the experiments; SS, BTu, FF, BTo, performed the experiments; MG, RN, AF, analyzed the data; AF, RN, DC, contributed reagents/material/analysis tools; SS, BTu, FF, BTo, AF, wrote the paper.

References

ALAUX C., DUCLOZ F., CRAUSER D., LE CONTE Y., 2010.- Diet effects on honeybee immunocompetence.- *Biology Letters*, 6 (4): 562-565.

ALBO G. N., HENNING C., RINQUELET J., REYNALDI F. J., DE GIUSTI M. R., ALIPPI A. M., 2003.- Evaluation of some essential oils for the control and prevention of American foulbrood disease in honey bees.- *Apidologie*, 34 (5): 417-427.

ALIPPI A. M., REYNALDI F. J., 2006.- Inhibition of the growth of *Paenibacillus larvae*, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore-forming bacteria isolated from apiarian sources.- *Journal of invertebrate pathology*, 91 (3): 141-146.

ANDERSON K. E., SHEEHAN T. H., ECKHOLM B. J., MOTT B. M., DEGRANDI-HOFFMAN G., 2011.- An emerging paradigm of colony health: microbial balance of the honey bee and hive (*Apis mellifera*).- *Insectes Sociaux*, 58: 431-444.

ANDERSON K. E., SHEEHAN T. H., MOTT B. M., MAES P., SNYDER L., SCHWAN M. R., WALTON A., JONES B. M., CORBY-HARRIS V., 2013.- Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (*Apis mellifera*).- *PloS ONE*, 8 (12): e83125.

ASHIRALIEVA A., GENERSCH E., 2006.- Reclassification, genotypes and virulence of *Paenibacillus larvae*, the etiological agent of American foulbrood in honeybees - a review.- *Apidologie*, 37 (4): 411-420.

BILIKOVA K., 2013.- New anti-*Paenibacillus larvae* substances purified from propolis.- *Apidologie*, 44 (3): 278-285.

BILIKOVA K., GUSUI W., SIMUTH J., 2001.- Isolation of a peptide fraction from honeybee royal jelly as a potential anti-foulbrood factor.- *Apidologie*, 32: 275-283.

BRØDSGAARD C. J., RITTER W., HANSEN H., 1998.- Response of in vitro reared honey bee larvae to various doses of *Paenibacillus larvae* larvae spores.- *Apidologie*, 29 (6): 569-578.

BUCEKOVA M., VALACHOVA I., KOHUTOVA L., PROCHAZKA E., KLAUDINY J., MAJTAN J., 2014.- Honeybee glucose oxidase-its expression in honeybee workers and comparative analyses of its content and H_2O_2- mediated antibacterial activity in natural honeys.- *Naturwissenschaften*, 101 (8): 661-670.

CARINA L., SOLEDAD V., MARINA B., 2014.- Antibacterial activity of honey: A review of honey around the world.- *Journal of Microbiology and Antimicrobials*, 6 (3): 51-56.

CREMER S., ARMITAGE S. A. O., SCHMID-HEMPEL P., 2007.- Social immunity.- *Current Biology*, 17: R693-R702.

DINGMAN D. W., 2011.- Inactivation of *Paenibacillus larvae* endospores by a hydrogen peroxide/ peroxyacetic acid biocide.- *Journal of Apicultural Research*, 50 (1): 173-175.

DJUKIC M., BRZUSZKIEWICZ E., FÜNFHAUS A., VOSS J., GOLLNOW K., POPPINGA L., LIESEGANG H., GARCIA-GONZALEZ E., GENERSCH E., DANIEL, R., 2014.- How to kill the honey bee larva: genomic potential and virulence mechanisms of *Paenibacillus larvae*.- *PLoS ONE*, 9 (3): e90914.

ERLER S., DENNER A., BOBIŞ O., FORSGREN E., MORITZ R. F. A., 2014.- Diversity of honey stores and their impact on pathogenic bacteria of the honeybee, *Apis mellifera*.- *Ecology and evolution*, 20 (4): 3960-3967.

FLESAR J., HAVLIK J., KLOUCEK P., RADA V., TITERA D., BEDNAR M., STROPNICKY M., KOKOSKA L., 2010.- In vitro growth-inhibitory effect of plant-derived extracts and compounds against *Paenibacillus larvae* and their acute oral toxicity to adult honey bees.- *Veterinary Microbiology*, 145 (1-2): 129-133.

FONTANA R., MENDES M. A., MONSON DE SOUZA B., KONNO K., MARCONDES CÉSAR L. M., MALASPINA O., PALMA M. S., 2004.- Jelleines: a family of antimicrobial peptides from the royal jelly of honeybees (*Apis mellifera*).- *Peptides*, 25: 919-928.

FRIES I., LINDSTRÖM A., KORPELA S., 2006.-Vertical transmission of American foulbrood (*Paenibacillus larvae*) in honey bees (*Apis mellifera*).- *Veterinary Microbiology*, 114 (3-4): 269-274.

FUJIWARA S., IMAI J., FUJIWARA M., YAESHIMA T., KAWASHIMA T., KOBAYASHI K., 1990.- A potent antibacterial protein in royal jelly. purification and determination of the primary structure of royalisin.- *The journal of biological chemistry*, 265: 11333-11337.

FURUSAWA T., RAKWAL R., NAM H. W., SHIBATO J., AGRAWAL G. K., KIM Y. S., OGAWA Y., YOSHIDA Y., KOUZUMA Y., MASUO Y., YONEKURA M., 2008.- Comprehensive royal jelly (RJ) proteomics using one- and two-dimensional proteomics platforms reveals novel RJ proteins and potential phospho/glycoproteins.- *Journal of Proteome Research*, 7 (8): 3194-3229.

FUSELLI S. R., GARCÍA DE LA ROSA S. B., GENDE L. B., EGUARAS M. J., FRITZ R., 2006.- Inhibition of *Paenibacillus larvae* employing a mixture of essential oils and thymol.- *Revista Argentina de Microbiologia*, 38 (2): 89-92.

GENDE L. B., FLORIS I., FRITZ R., EGUARAS M. J., 2008.- Antimicrobial activity of cinnamon (*Cinnamomum zeylanicum*) essential oil and its main components against *Paenibacillus larvae* from Argentine.- *Bulletin of Insectology*, 61 (1): 1-4.

GENERSCH E., 2010.- American foulbrood in honeybees and its causative agent, *Paenibacillus larvae*.- *Journal of Invertebrate Pathology*, 103: S10-S19.

GENERSCH E., ASHIRALIEVA A., FRIES I., 2005.- Strain- and genotype-specific differences in virulence of *Paenibacillus larvae* subsp. *larvae*, a bacterial pathogen causing American foulbrood disease in honeybees.- *Applied and Environmental Microbiology*, 71 (11): 7551-7555.

GENERSCH E., FORSGREN E., PENTIKÄINEN J., ASHIRALIEVA A., RAUCH S., KILWINSKI J., FRIES I., 2006.- Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation.- *International Journal of Systematic and Evolutionary Microbiology*, 56: 501-511.

GHERMAN B. I., DENNER A., BOBIŞ O., DEZMIREAN D. S., MĂRGHITAŞ L. A., SCHLÜNS H., MORITZ R. F. A., ERLER S., 2014.- Pathogen-associated self-medication behavior in the honeybee *Apis mellifera*.- *Behavioral Ecology and Sociobiology*, 68 (11): 1777-1784.

HANSEN H., BRØDSGAARD C. J., 1999.- American foulbrood: a review of its biology, diagnosis and control.- *Bee World*, 80 (1): 5-23.

HIGES M., MARTÍN R., MEANA A., 2006.- *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe.- *Journal of Invertebrate Pathology*, 92: 93-95.

KEILIN D., HARTREE E. F., 1948.- Properties of glucose oxidase (Notatin).- *Biochemical journal*, 42 (2): 221-229.

KEILIN D., HARTREE E. F., 1952.- Specificity of glucose oxidase (Notatin).- *Biochemical Journal*, 50 (3): 331-341.

LAU K. Y., RUKAYADI Y., 2015.- Screening of tropical medicinal plants for sporicidal activity.- *International Food Research Journal*, 22 (1): 421-425.

LAWRENCE H. A., PALOMBO E. A., 2009.- Activity of essential oils against *Bacillus subtilis* spores.- *Journal of Microbiology and Biotechnology*, 19 (12): 1590-1595.

LINDSTRÖM A., KORPELA S., FRIES I., 2008a.- Horizontal transmission of *Paenibacillus larvae* spores between honey bee (*Apis mellifera*) colonies through robbing.- *Apidologie*, 39 (5): 515-522.

LINDSTRÖM A., KORPELA S., FRIES I., 2008b.- Distribution of *Paenibacillus larvae* spores in adult bees and honey and larval mortality, following the addition of American foulbrood diseased brood or spore-contaminated honey in honey bee (*Apis mellifera*) colonies.- *Journal of Invertebrate Pathology*, 99 (1): 82-86.

MATO I., HUIDOBRO J. F., SÁNCHEZ M. P., MUNIATEGUI S., FERNÁNDEZ-MUIÑO M. A., SANCHO M. T., 1997.- Enzymatic determination of total D-gluconic acid in honey.- *Journal of Agricultural and Food Chemistry*, 45 (9): 3550-3553.

MATO I., HUIDOBRO J. F., SIMAL-LOZANO J., SANCHO M. T., 2006.- Rapid determination of nonaromatic organic acids in honey by capillary zone electrophoresis with direct ultraviolet detection.- *Journal of Agricultural and Food Chemistry*, 54 (5): 1541-1550.

MAZZEI M., CARROZZA M. L., LUISI E., FORZAN M., GIUSTI M., SAGONA S., TOLARI F., FELICIOLI A., 2014.- Infectivity of DWV associated to flower pollen: experimental evidence of a horizontal transmission route.- *PLoS ONE*, 9 (11): e113448.

MIHAI C. M., MĂRGHITAŞ L. A., DEZMIREAN D. S., CHIRILĂ F., MORITZ R. F. A., SCHLUNS H., 2012.- Interactions among flavonoids of propolis affect antibacterial activity against the honeybee pathogen *Paenibacillus larvae*.- *Journal of Invertebrate Pathology*, 110 (1): 68-72.

NEUENDORF S., HEDTKE K., TANGEN G., GENERSCH E., 2004.- Biochemical characterization of different genotypes of *Paenibacillus larvae* subsp. *larvae*, a honey bee bacterial pathogen.- *Microbiology*, 150 (7): 2381-2390.

OHASHI K., NATORI S., KUBO T., 1999.- Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (*Apis mellifera* L.).- *European Journal of Biochemistry*, 265 (1): 127-133.

OLIVER R., 2010.- Sick bees - part 3. The bee immune system.- *American Bee Journal*, 150 (10): 967-971.

PERSANO-ODDO L., HEARD T. A., RODRÍGUEZ-MALAVER A., PÉREZ R. A., FERNÁNDEZ-MUIÑO M., SANCHO M. T., SESTA G., LUSCO L., VIT P., 2008.- Composition and antioxidant activity of *Trigona carbonaria* honey from Australia.- *Journal of Medicinal Food*, 11 (4): 789-794.

PULCINI P., PIAZZA M. G., ALLEGRINI F., 2004.- Contenuto di acido gluconico totale (AGT) nei mieli uniflorali italiani.- *Industrie Alimentari*, 43: 263-268.

REYBROECK W., DAESELEIRE E., DE BRABANDER H. F., HERMAN L., 2012.- Antimicrobials in beekeeping.- *Veterinary Microbiology*, 158 (1-2): 1-11.

ROUSSENOVA N., 2011.- Antibacterial activity of essential oils against the etiological agent of American foulbrood disease (*Paenibacillus larvae*).- *Bulgarian Journal of Veterinary Medicine*, 14 (1): 17-24.

SCARSELLI R., DONADIO E., GIUFFRIDA M. G., FORTUNATO D., CONTI A., BALESTRERI E., FELICIOLI R., PINZAUTI M., SABATINI A. G., FELICIOLI A., 2005.- Towards royal jelly proteome.- *Proteomics*, 5: 769-776.

SCHEPARTZ A. I., 1965.- The glucose oxidase of honey. III. Kinetics and stoichiometry of the reaction.- *Biochimica et Biophysica Acta*, 99 (1): 161-164.

SCHEPARTZ A. I., SUBERS M. H., 1964.- The glucose oxidase of honey. I. Purification and some general properties of the enzyme.- *Biochimica et Biophysica Acta*, 85 (2): 228-237.

SPIVAK M., REUTER G. S., 2001.- Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior.- *Apidologie*, 32 (6): 555-565.

TAKAHASHI T., MITSUMOTO M., 1963.- Transformation and hydrolysis of D-glucono-γ and δ-lactone.- *Nature*, 199: 765-767.

TAKENAKA T., ITO H., YATSUNAMI K., ECHIGO T., 1990.- Changes of glucose oxidase activity and amount of gluconico acid formation in the hypopharingeal glands during the lifespan of the honey bee workers (*Apis mellifera* L.).- *Agricultural and Biological Chemistry*, 54 (8): 2133-2134.

WHITE J. W., 1966.- Inhibine and glucose oxidase in honey - a review.- *American Bee Journal*, 106 (6): 214-216.

WILLIAMS D. L., 2000.- A veterinary approach to the European honey bee (*Apis mellifera*).- *Veterinary Journal*, 160 (1): 61-73.

WONG C. M., WONG K. H., CHEN X. D., 2008.- Glucose oxidase: natural occurrence, function, properties and industrial applications.- *Applied Microbiology and Biotechnology*, 78 (6): 927-938.

YUE D., NORDHOFF M., WIELER L. H., GENERSCH E., 2008.- Fluorescence in situ-hybridization (FISH) analysis of the interactions between honeybee larvae and *Paenibacillus larvae*, the causative agent of American foulbrood of honeybees (*Apis mellifera*).- *Environmental Microbiology*, 10 (6): 1612-1620.

ZIA M. A., RIAZ A., RASUL S., ABBAS R. Z., 2013.- Evaluation of antimicrobial activity of glucose oxidase from *Aspergillus niger* EBL-A and *Penicillium notatum*.- *Brazilian Archives of Biology and Technology*, 56 (6): 956-961.

Authors' addresses: Antonio FELICIOLI (corresponding author, antonio.felicioli@unipi.it), Simona SAGONA, Barbara TURCHI, Filippo FRATINI, Matteo GIUSTI, Beatrice TORRACCA, Roberta NUVOLONI, Domenico CERRI, Department of Veterinary Science, University of Pisa, viale delle Piagge 2, 56124 Pisa, Italy.

Received January 29, 2015. Accepted September 21, 2015.