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The effect of Egyptian honeybee propolis on the growth of *Aspergillus versicolor* and sterigmatocystin biosynthesis in Ras cheese

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Propolis is a natural product collected by honeybee workers. The product was tested for its antifungal effect against *Aspergillus versicolor* ATCC 12996 as well as biosynthesis of sterigmatocystin during ripening of Egyptian Ras cheese. The use of different concentrations of aqueous propolis extract 250, 500 and 1000 part per million (ppm) on the cheese surface was investigated. Mould growth and toxin production were completely inhibited at the highest concentration 1000 ppm, while the lower concentrations exhibited definite fungistatic activity during 90 days of ripening. Control cheese demonstrated that the amount of sterigmatocystin produced was proportional to the growth of *Asp. versicolor* during three months of ripening. It could be concluded that propolis concentration of 1000 ppm could prevent mould growth and sterigmatocystin production in Ras cheese. The economic as well as the public health importance of propolis as a natural preservative in cheese manufacture is discussed.

Keywords: Egyptian honeybee propolis, *Aspergillus versicolor*, Sterigmatocystin, Ras cheese.

Ras cheese is one of the most popular kinds of hard cheese in Egypt. It is an excellent substrate for mould growth. It becomes mouldy during ripening process as well as storage and marketing. Most moulds commonly found on cheese belonged to Genera *Penicillium* and *Aspergillus* (Taniwaki & Dender, 1992; Ominski et al. 1996; Su, 2005). *Asp. versicolor* is the most frequently occurring species found in the tropical environment. It causes economical losses through discolouration, poor appearance, off flavours as well as public health hazards due to production of secondary toxic metabolites known as sterigmatocystin. The action of this toxin may cause liver cancer (Marquadt & Frolich, 1992; Tournas, 1994; Miraglia et al. 1996; Huang et al. 2004; Hemant et al. 2005). Short term high dose exposure gives symptoms of acute liver toxicity leading to liver failure while long term low level exposure leads to liver cirrhosis (Engelhart et al. 2002; Bannasch et al. 2003; Su, 2005). Prevention of mould growth is a challenge for the cheese producers, who commonly use antifungal preservatives such as weak acids or antibiotics. With improvement in living standard, consumers make higher demands for the safety food. The use of natural preservatives to control fungal growth and mycotoxin production has been the subject of concern in recent years (Fuglsang et al. 1995; Brul & Coote, 1997; Varanda et al. 1999). Propolis, a natural resinous bee product collected by honeybee workers, has gained popularity as an alternative medicine or food for health amelioration and disease prevention in various parts of the world, including the United States of America, the European Union and Japan (Greenaway et al. 1990; Cafarchia et al. 1999; Wohrl, 2003; Tan et al. 2006). It possesses many biological activities, such as antitumor, antioxidant, anti-inflammatory, anticancer, and has the ability to reduce blood pressure, prevent allergies, sore throats, respiratory and skin disorders (Ghaly et al. 1998; Koo et al. 2000; Teraki & Shiohara, 2001; Lombardi, 2003; Mani et al. 2005; Scazzocchio et al. 2006).

Because of the current market needs for good quality cheese, this work was planned to investigate the effect of different concentrations of propolis, on the growth of *Asp. versicolor* ATCC 12996 artificially grown on Ras cheese and sterigmatocystin production during 90 d ripening period.

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Materials and Methods

Collection of propolis

Propolis samples were collected from the hybrid honeybee colonies at Fayoum Governorate, Egypt. Collected samples were weighed and stored separately in the refrigerator until used as described by Pepeljnjak et al. (1982) and Muszynska et al. (1993).

Preparation of propolis

Propolis sample (100 g) was treated with hexane for 3 h in Soxhlet apparatus. A second extraction in Soxhlet followed, with methanol, for 3 h. Waxes from the propolis extract were eliminated by three consecutive steps of cooling in freezer, filtration, concentration of the filtrate and dissolution of the residue in 10 ml methanol. The methanol extract was concentrated under reduced pressure and transferred to glass vials with a small volume of methanol. The solvent was evaporated and the residue was dissolved in distilled water at concentrations of 1000 ppm. (resin mass and propolis). This was diluted to the concentration of 250 and 500 ppm in distilled water according to. Muszynska et al. (1993).

Ras cheese manufacture

Blocks of Ras cheese, 20 cm diameter and 15 cm height without plastic coat, were made from fresh whole buffaloes' milk obtained from Dairy Processing Pilot Plant, Faculty of Agriculture, Fayoum University. The cheese samples were divided into four blocks: Block I: covered with 250 ppm propolis. Block II: covered with 500 ppm propolis. Block III: covered with 1000 ppm propolis. Block IV: untreated with propolis (control) and did not receive any other treatment Three replicates were prepared from each treatment. Surfaces of cheese blocks were varnished and treated at once with propolis solutions. All cheese surfaces were covered completely with propolis using brushes. The cheese blocks were stored at 10 °C with 80% relative humidity for 90 d according to Hofi et al. (1970). All samples were analysed for *Asp. versicolor* and sterigmatocystin production when fresh (at zero time), 7, 14, 21, 28, 35, 42, 50, 60, 70, 80 and 90 d ripening according to Berngard (1976).

Inoculation of Asp. versicolor *on Ras cheese*

Lyophilized strain of *Asp. versicolor* ATCC 12 996 was kindly provided by Dr. Hans Diekmann, Institute of Microbiology, Hannover, Germany. The culture was maintained on potato dextrose agar for 7 d at 25 °C until they were sporulated. The spores were harvested in 10 ml sterile tween 80 (100 g/l) to give a final spore concentration of approximately 1×10^3 spores/ml. The spore suspension was filtered through filter paper to remove mycelial debris. One ml spore suspension (1×10^3 spores/ml) was inoculated and spread onto the upper surface of Ras cheese blocks as described by Filtenberg et al. (1983).

Mycological analysis

All Cheese samples were prepared according to the method recommended by APHA (1990). The samples were examined for *Asp. versicolor* count by growing on Dichloran Rose Bengal chlorotetracyclin agar (Oxoid). Inoculated plates were incubated at 25 °C for 7 d. The identification was based on characteristic morphology according to Pitt & Hocking (1997). The results were expressed in terms of cfu/g cheese.

Extraction of sterigmatocystin

All cheese blocks were extracted in triplicate samples according to AOAC (1990) and Schneider et al. (1991). The clean up was applied according to Scott (1995).

Determination of sterigmatocystin

Sterigmatocystin was determined in Ras cheese in triplicate samples using ELISA according to the method applied by Scott, (1995) and Riedel de Haen (1997). The detection limit was 50 ng/kg. The recovery rate was 90%.

Statistical analysis

Statistical analysis of the data was performed using statistical equation as described by Steel & Torrie (1980). Results were expressed as the mean and standard errors, differences were considered to be significant when $P<0{\cdot}05$. Correlation coefficient among studied traits and variants components were calculated according to SAS (2000).

Results and Discussion

Comparisons were made between the growth of *Asp. versicolor* in the control cheese samples and cheeses treated with the different concentrations of propolis, Data presented in Tables 1a & 1b and Fig. 1 revealed that *Asp. versicolor* was able to grow and produce sterigmatocystin in the inoculated control cheese samples. *Asp. versicolor* count in the control batches increased to a mean value of 5×10^6 during 90 d ripening. Moreover, sterigmatocystin increased approximately three fold during ripening. The concentration of sterigmatocystin in control cheese samples, regardless of *Asp. versicolor* count, would properly be considered hazardous to human health (FAO/WHO, 2000; Healther, 2004). The growth of mould and sterigmatocystin production in control cheese is in agreement with El-Dieb et al. (1997) and Aly (1999) who concluded that cheese is a good medium for *Asp. versicolor*

Table 1a. Effect of different concentrations of propolis on the mean growth of *Aspergillus versicolor* inoculated on Ras cheese CFU/g

Values are means ± SD for $n=3$

Ripening Period/day	Control (Untreated) CFU/gram	Propolis concentration 250 ppm	500 ppm	1000 ppm
Zero time	$1\times10^{3}\pm0{\cdot}01^{a}$	$1\times10^{3}\pm0{\cdot}02^{a}$	$1\times10^{3}\pm0{\cdot}01^{a}$	$1\times10^{3}\pm0{\cdot}05^{a}$
7	$1{\cdot}9\times10^{3}\pm0{\cdot}20^{a}$	$1{\cdot}7\times10^{3}\pm0{\cdot}61^{a}$	$1{\cdot}2\times10^{3}\pm0{\cdot}20^{a}$	$40\pm0{\cdot}01^{b}$
14	$3{\cdot}8\times10^{3}\pm0{\cdot}04^{a}$	$2{\cdot}8\times10^{3}\pm0{\cdot}02^{a}$	$1{\cdot}1\times10^{3}\pm0{\cdot}03^{b}$	0
21	$6{\cdot}2\times10^{3}\pm0{\cdot}10^{a}$	$5\times10^{3}\pm0{\cdot}30^{a}$	$9\times10^{2}\pm0{\cdot}10^{b}$	0
28	$9{\cdot}7\times10^{3}\pm0{\cdot}03^{a}$	$7{\cdot}8\times10^{3}\pm0{\cdot}01^{a}$	$6{\cdot}6\times10^{2}\pm0{\cdot}20^{b}$	0
35	$3\times10^{4}\pm0{\cdot}02^{a}$	$9\times10^{3}\pm0{\cdot}10^{a}$	$7\times10^{2}\pm0{\cdot}15^{b}$	0
42	$5{\cdot}4\times10^{4}\pm0{\cdot}05^{a}$	$28\times10^{3}\pm0{\cdot}03^{a}$	$5{\cdot}9\times10^{2}\pm0{\cdot}01^{b}$	0
50	$9\times10^{4}\pm0{\cdot}02^{a}$	$30\times10^{3}\pm0{\cdot}05^{a}$	$2{\cdot}0\times10^{2}\pm0{\cdot}01^{b}$	0
60	$20\times10^{4}\pm0{\cdot}06^{a}$	$59\times10^{3}\pm0{\cdot}01^{a}$	$90\pm0{\cdot}01^{b}$	0
70	$76\times10^{4}\pm0{\cdot}01^{a}$	$9\times10^{4}\pm0{\cdot}07^{a}$	$30\pm0{\cdot}01^{b}$	0
80	$38\times10^{5}\pm0{\cdot}02^{a}$	$11\times10^{4}\pm0{\cdot}01^{a}$	$20\pm0{\cdot}05^{b}$	0
90	$5\times10^{6}\pm0{\cdot}03^{a}$	$3\times10^{5}\pm0{\cdot}02^{a}$	$10\pm0{\cdot}01^{b}$	0

[a,b]Values in the same column with common superscript letters were not significantly different $P<0{\cdot}05$

Table 1b. The effect of propolis on mean sterigmatocystin production during cheese ripening

Values are means ± SD for $n=3$

Period/day	Control	Propolis concentration 250 ppm	500 ppm	1000 ppm
	Toxin concentration µg/kg			
Zero time	ND	ND	ND	ND
7	$0{\cdot}32\pm0{\cdot}20^{a}$	$0{\cdot}30\pm0{\cdot}10^{b}$	$0{\cdot}18\pm0{\cdot}01^{b}$	ND
14	$0{\cdot}39\pm0{\cdot}03^{a}$	$0{\cdot}28\pm0{\cdot}01^{b}$	$0{\cdot}07\pm0{\cdot}01^{b}$	ND
21	$0{\cdot}44\pm0{\cdot}05^{a}$	$0{\cdot}21\pm0{\cdot}03^{b}$	$0{\cdot}06\pm0{\cdot}01^{b}$	ND
28	$0{\cdot}60\pm0{\cdot}10^{a}$	$0{\cdot}20\pm0{\cdot}01^{b}$	$0{\cdot}05\pm0{\cdot}01^{b}$	ND
35	$0{\cdot}65\pm0{\cdot}02^{a}$	$0{\cdot}19\pm0{\cdot}05^{b}$	ND	ND
42	$0{\cdot}69\pm0{\cdot}01^{a}$	$0{\cdot}17\pm0{\cdot}03^{b}$	ND	ND
50	$0{\cdot}71\pm0{\cdot}10^{a}$	$0{\cdot}14\pm0{\cdot}01^{b}$	ND	ND
60	$0{\cdot}77\pm0{\cdot}05^{a}$	$0{\cdot}12\pm0{\cdot}20^{b}$	ND	ND
70	$0{\cdot}85\pm0{\cdot}01^{a}$	$0{\cdot}11\pm0{\cdot}10^{b}$	ND	ND
80	$0{\cdot}92\pm0{\cdot}03^{a}$	$0{\cdot}09\pm0{\cdot}05^{b}$	ND	ND
90	$1{\cdot}50\pm0{\cdot}01^{a}$	$0{\cdot}03\pm0{\cdot}01^{b}$	ND	ND

[a,b]Values in the same column with common superscript letters were not significantly different $P<0{\cdot}05$
ND, Not detected

growth and sterigmatocystin production. Treatment of cheese blocks treated with 250 ppm propolis had a significant effect ($P<0{\cdot}05$) in decreasing the production of sterigmatocystin but no significant difference in the growth of mould was observed. Increasing the concentration to 500 and 1000 ppm propolis had a significant inhibitory effect ($P<0{\cdot}05$) on the growth of mould, which was reduced to zero by treatment at the higher level (Table 1). Furthermore, at 500 ppm, sterigmatocystin was only detected during the intial 28 d ripening, while 1000 ppm concentration showed powerful anti-mycotoxigenic activity on mould and sterigmatocystin biosynthesis (Tables 1a & 1b).

Data correlation matrix (Table 2) showed a strong positive correlation (+0·968) between *Asp. versicolor* count and sterigmatocystin production in control samples, while strong negative correlation (−0·971) was obtained between mould count and toxin production with 250 ppm propolis which reflect the inhibitory effect of propolis on sterigmatocystin biosynthesis. Moderate positive correlation (+0·650) was found between mould count and toxin production with 500 ppm propolis. Moreover the coefficient determination indicated that *Asp. versicolor* was responsible for up to 94% of variants affect sterigmatocystin production in control cheese blocks as well as cheese treated with 250 ppm propolis while 42% in samples treated with 500 and 1000 ppm. This may be due to the lack of sterigmatocystin detected after 28 d and very little data is actually available to calculate the coefficient. This is in agreement with the findings of others (Bankova et al. 1997; Kujumgiev et al. 1999). The

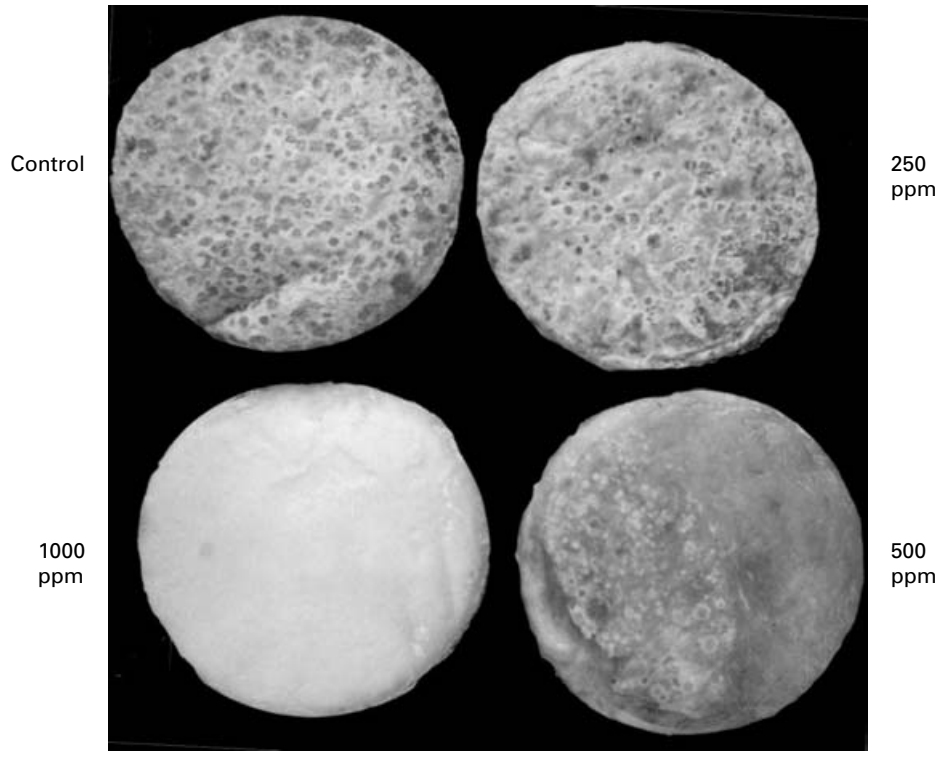

Fig. 1. Macroscopic appearance after the ripening period of control and treated cheese blocks, varnished with 250, 500 or 1000 ppm propolis.

Table 2. Correlation between *Asp. versicolor* count (log/g) and toxin production in treated and untreated (control) Ras cheese blocks

Asp. versicolor count	Correlation matrix	Coefficient of determination (%)
Control	0·968	94
250 ppm propolis	−0·971	94
500 ppm propolis	0·650	42
1000 ppm propolis	NA	NA

NA, Not available

obtained results declared that propolis applied to cheese surface exerted a significant inhibitory effect on growth and toxin production. Propolis has antibacterial, antifungal and antiviral activities (Marcucci et al. 2001; Bankova, 2005). The most important active constituents in Egyptian propolis are phenolic acid esters (72·7%), phenolic acid (1·1%), aliphatic acids (2·4%), dihydrochalcones (6·5%), chalcones (1·7%), flavonoids (1·9%), flavones (4·6%) and tetrahydrofuran derivatives (Abdelhady, 1994; Hegazi & Abdelhady, 1997; Bankova et al. 1997; Banskota et al. 1998: Nagy et al. 2004). However, several investigators have examined the relationship between flavonoids and antimicrobial activity of propolis (Matsuno et al. 1997; Nieva et al. 2005; Piccinelli et al. 2005). Flavonoids, phenolic acids and flavones such as galangin and quercetin are thought to account for much biological activity. The action of quercetin has been attributed to inhibition the protein synthesis, DNA and cytoplasmic membrane function of the mould and subsequent toxin production (Burdock, 1998; Bratter et al. 1999; Hirota et al. 2000; Midorikawa et al. 2001; Cushnie & Lamb, 2005; Tan et al. 2006).

In conclusion, it is advisable to use propolis as a natural preservative in the field of Ras cheese making as a coat at 1000 ppm concentration to safe-guard the consumer from the fungi and its toxic metabolites.

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