EFFECTS OF PROPOLIS EXTRACTS IN CHICKENS DIET AGAINST *SALMONELLA* TYPHIMURIUM DETECTED BY REAL-TIME PCR

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ABSTRACT

The aim of this study was to follow the effect of propolis extractsin chickens feed against colonization of GIT (gastrointestinal tract) with *Salmonella* spp. by Step One real-time PCR. Propolis has been used in folk medicine since ancient times due to its many biological properties, such as antimicrobial, anti-inflammatory, antioxidant, immunomodulatory activities, among others. Propolis extracts was applied to chickens feeds in four groups with different concentration of propolis. We used the PrepSEQ Rapid Spin Sample Preparation Kit for isolation of DNA and MicroSEQ® *Salmonella* spp. Detection Kit for pursuance the real-time PCR(Applied Biosystems). In the investigated samples without incubation we could detect strain of *Salmonella* spp. in twenty of twenty five samples (swabs), as well as internal positive control (IPC), which was positive in all samples. Our results indicate positive effect of propolis against colonization of GIT (gastrointestinal tract) with *Salmonella*spp. in all experimental groups. This Step One real-time PCR assay is extremely useful for any laboratory equipped by real-time PCR. Thus, these results proved real-time PCR to be useful as a rapid diagnostic test for the direct detection of pathogens in food, without the need of enrichment steps.

Keywords: Real-time PCR, *Salmonella* spp., detection kit, chicken, propolis

INTRODUCTION

Propolis (bee glue) exhibits antimicrobial, anti-inflammatory, wound healing and other properties **(Bankova et al., 2000; Burdock, 1998)**. Propolis is a resinous material collected by bees from bud and exudates of the plants, which is transformed in the presence of bee enzymes. Its color varies from green, red to dark brown. Propolis has a characteristic smell and shows adhesive properties because it strongly interacts with oils and proteins of the skin. In general, propolis *in natura* is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen, and other substances **(Burdock, 1998)**. Antibacterial compounds in poplar type (European type) propolis are mainly flavones, flavanones and phenolic acids and esters **(Bankova, 2005)**. Flavonoids are a very diverse group of secondary metabolites, biosynthesized by plants. Bioactivities demonstrated by flavonoids against chronic diseases such as cancer, cardiovascular disease, neurodegenerative disorders **(Havsteen, 2002; Miller et al., 2008; Vanamala et al., 2006; Stanciu et al., 2009; Bastos et al., 2004; Almeida-Muradian et al., 2005; Silva et al., 2006; Mărghitas et al. 2009).** Flavonoids were reported to influence the colonic microflora **(Parkar et al., 2008; Wells et al., 1999)**, suggesting an important role in maintenance of colon health. Antibacterial activity of propolis is bacteriostatic and can be bactericidal in high concentrations **(Mirzoeva et al., 1997; Drago et al., 2000)**. Besides its antibacterial, antifungal and antiviral properties, propolis presents many other beneficial biological activities such as antioxidant, antiinflammatory, antitumor, hepatoprotective, local anesthetic, immunostimulatory, antimutagenic, etc. **(Banskota et al., 2001; Burdock, 1998; Kim et al., 2008; Kujumgiev et al., 1999).**However, the interaction of dietary components such as flavonoids with colonic microflora has not been studied extensively. *Salmonella enterica* serovar Typhimurium is among the leading cause of food-borne diseases. The pathogen colonizes the large intestine and incites gastroenteritis and colitis. *S.* Typhimurium has significant economic burden due to very broad host range and significant morbidity and mortality in human population **(Boyle et al., 2007)**. Some *Salmonella* serovars are intracellular parasites which can survive and replicate within mononuclear or polymorphonuclear phagocytes. The inhibition of phagosome–lysosome fusion is an important factor for *Salmonella* survival within macrophages and for its virulence **(Buchmeier et al., 1991).**

Escherichia coli O157:H7, *Listeria monocytogenes* and *Salmonella* spp. are among the most commonly studied food-borne pathogens and are of major concern because of their association with foods such as poultry, ready-to-eat products, dairy products, fruits and

vegetables **(Kim et al., 2008)**. Real-time PCR methods are often used for quantification of initial target DNA. Unfortunately, amplification efficiencies can be different from sample to sample due to the effects caused by inhibition of amplification, human failures or preparation errors. This implies that quantification, even with external controls, does not always represent a correct calculation of initial amount of target in each sample. To eliminate part of these drawbacks, different approaches of using an internal amplification control (IAC) in each real-time PCR have been described recently.

The aim of this study was to follow the effect of propolis against colonization of GIT (gastro-intestinal tract) with *Salmonella* spp. by Step One real-time PCR.

MATERIAL AND METHODS

The experiment enrolled 100 pieces of one day chickens hybrid Hubbard JV and was created 5 groups of animals: control (C) and four experimental groups with20 pcs of chickens. Custom feeding insisted 42 days. Chickens were fed to 21th day of age an *ad libitum* with the same starter feed mixture HYD-01 (powdery form) and from 22nd to 42nd day of age fed with the growth feed mixture HYD-02 (powdery form) in the monitored groups. The fed feed mixture HYD-01 and HYD-02 have been produced without antibiotic preparations and coccidiostats.

Chicken feed additives

Propolis dose was administered to both feed mixtures (HYD-01, HYD-02) in various doses:

Control group: compound without the addition of propolis,

1st experimental group: propolis at a dose of 150 mg.kg^{-1},

2nd experimental group: propolis at a dose of 450 mg.kg^{-1},

3rd experimental group: propolis at a dose of 600 mg.kg^{-1},

4th experimental group: propolis at a dose of 800 mg.kg^{-1}.

Appendix samples

Total of 25 samples were analyzed for the presence of *Salmonella spp.* (Table 1). Samples were obtained by taking swabs from the appendix of chickens. After sampling, procedure shown in the Scheme 1 was used.

Tab 1 Samples (swabs) from appendix of chickens according to their experimental groups used in this study

Experimental group	Propolis (mg.kg^{-1})
Control group (5 pcs)	0
1st(5 pcs)	150
2nd(5 pcs)	450
3rd(5 pcs)	600
4th(5 pcs)	800

Sample

(swabs from the appendix of chickens)

↓

Isolation of DNA

(PrepSEQ^{TM} Rapid Spin Sample Preparation Kit)

↓

Pursuance Real Time PCR

(MicroSEQ *Salmonella* spp. Detection Kit)
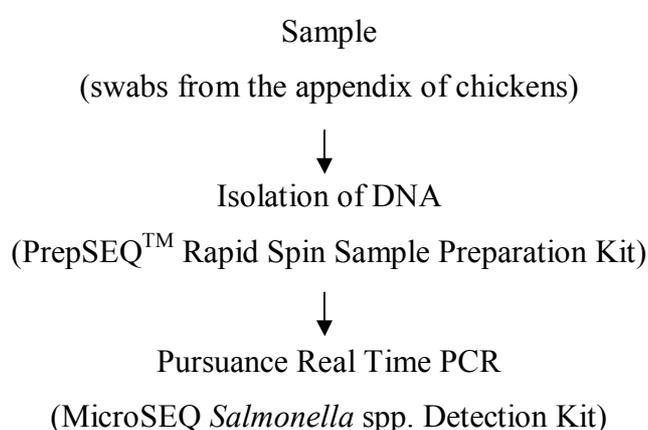
Scheme 1 Real-time PCR procedure for the detection of *Salmonella* spp.

DNA Extraction

As a pre-preparation step for the Step One real-time PCR, DNA extraction was performed using DNA extraction method: PrepSEQ Rapid Spin Sample Preparation Kit (Applied Biosystems, USA). Sample of 750 µL was loaded onto the spin column and microcentrifuged for 3 minutes at maximum speed. Supernatant was discarded and to the pellet was added 50 µL of Lysis Buffer. Samples were incubated for 10 minutes at 95 °C.

MicroSEQ® *Salmonella* spp. Detection Kit

Assay to amplify the polymerase chain reaction (PCR) a unique microorganism specific DNA target sequence and a TaqMan® probe to detect the amplified sequence were

used. 8-tube strips containing assay beads compatible with StepOne™ Systems were used. Samples of 30 µL to the lyophilized beads were loaded. MicroAmp® 48-Well Base and the MicroAmp® Cap Installing Tool to the tubes were used. MicroAmp® Fast 48-Well Tray on the sample block of the StepOne System was performed. Internal positive control – a control in all reaction wells that should always yield amplification.

Real-time PCR

TaqMan® probes labeled with both a fluorophore and a quencher dye were used in real-time PCR assays to detect amplification of specific DNA targets. We used three fluorophore detection chemistries that include FAM^{TM} and VIC® dye-labeled TaqMan® MGB probe-based assays, VIC® and TAMRA^{TM} dye-labeled probe-based assays and ROX^{TM} as passive reference dye. FAM^{TM}, which has an emission of 520 nm, has become the most commonly used fluorophore for singleplex qPCR reactions. TAMRA^{TM} will efficiently quench the fluorescence of FAM^{TM}, until the probe hybridizes to the target and is cleaved by the 5' exonuclease activity of the polymerase. Thermal cycling conditions were as follows: 2 minutes of incubation at 95 °C, followed by 40 cycles of 1 sec. denaturation at 95°C and 20 sec. annealing and elongation at 60 °C. Data were collected during each elongation step. PCR products were detected by monitoring the increase in fluorescence of the reporter dye at each PCR cycle. Applied Biosystems software plots the normalized reporter signal, ΔRn, (reporter signal minus background) against the number of amplification cycles and also determines the threshold cycle (Ct) value i.e. the PCR cycle number at which fluorescence increases above a defined threshold level were used.

RESULTS AND DISCUSSION

The most sensitive detection of *Salmonella* spp. was obtained using PrepSEQ^{TM} Rapid Spin Sample Preparation Kit and MicroSEQ® *Salmonella* spp. Detection Kit compatible with StepOne™ Systems, which was also less time-consuming than the other methods and was relatively easy to use. In the investigated samples without incubation we could detect strain of*Salmonella* spp. in twenty of twenty five samples (swabs), as well as internal positive control (IPC), which was positive in all samples. The results show (Table 2) the positive effect of propolis against *Salmonella* spp. was found in all experimental groups in comparison with the control group.

**Tab 2** The effect of propolis against *Salmonella* spp.

Sample Number	Experimental Group	Ct *Salmonella* spp.	IPC
1	Control	21,44	30,66
2		26,80	30,40
3		25,84	29,90
4		31,44	31,84
5		28,56	30,52
6	1st	33,06	29,66
7		34,42	30,41
8		33,38	31,11
9		negative	31,69
10		25,58	31,24
11	2nd	33,53	30,36
12		26,52	30,02
13		27,91	30,10
14		31,27	29,95
15		33,25	30,56
16	3rd	30,59	30,42
17		24,49	31,00
18		negative	30,38
19		negative	31,86
20		33,28	32,25
21	4th	negative	30,67
22		26,64	30,70
23		22,97	30,11
24		27,88	30,55
25		negative	31,45

The antimicrobial effect of propolis against *Salmonella* spp. was found in all experimental groups whilein 1st experimental group was four of five samples lower than IPC (IPC ≤ 1 CFU) and one sample was negative. In 2nd experimental group (EG) were found three of five samples lower than IPC. Two of five negative samples were found in 3rd and 4th experimental groups while in 3rd EG were two samples lower than IPC and in 4th EG were two of five samples lower than IPC. In control group were found two of five samples lower than IPC. The lowest (Ct) value of positive salmonella samples was reached at 21.44 (control group) and the highest value was 34.42 (1st experimental group) and the lowest IPC value was reached at 29.66 (1st experimental group) and the highest accomplish value was 32.25

(3[rd]experimental group).The antimicrobial effect of propolis against *Salmonella* spp. shows Figure 1-5.
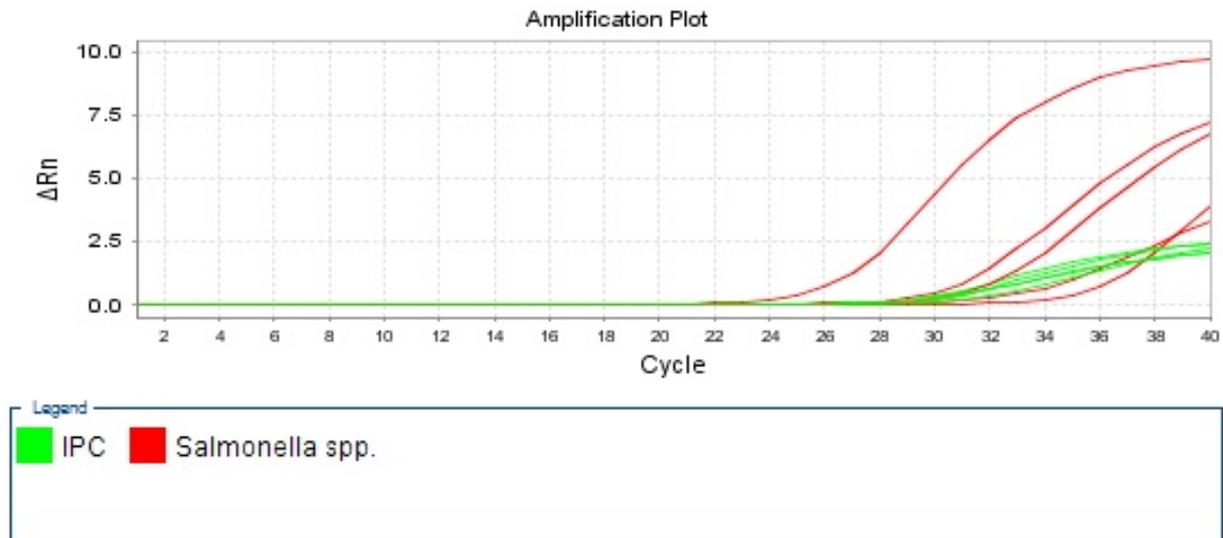

Fig 1 Real-time PCR detection of *Salmonella* spp. without incubation and internal positive control (IPC) in control group
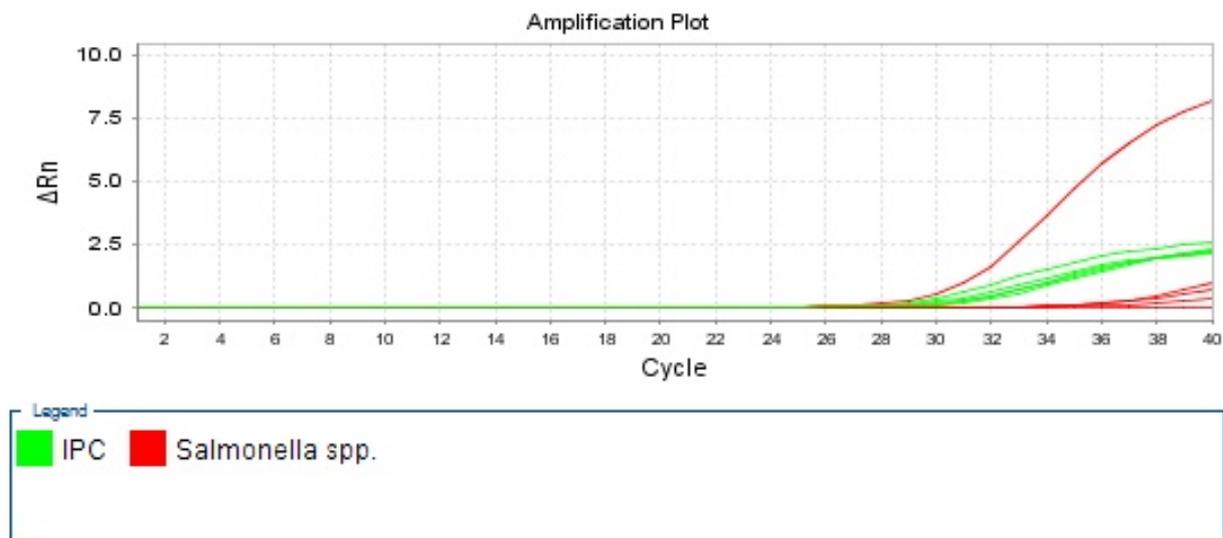

Fig 2 Real-time PCR detection of *Salmonella* spp. without incubation and IPC in 1[st]experimental group
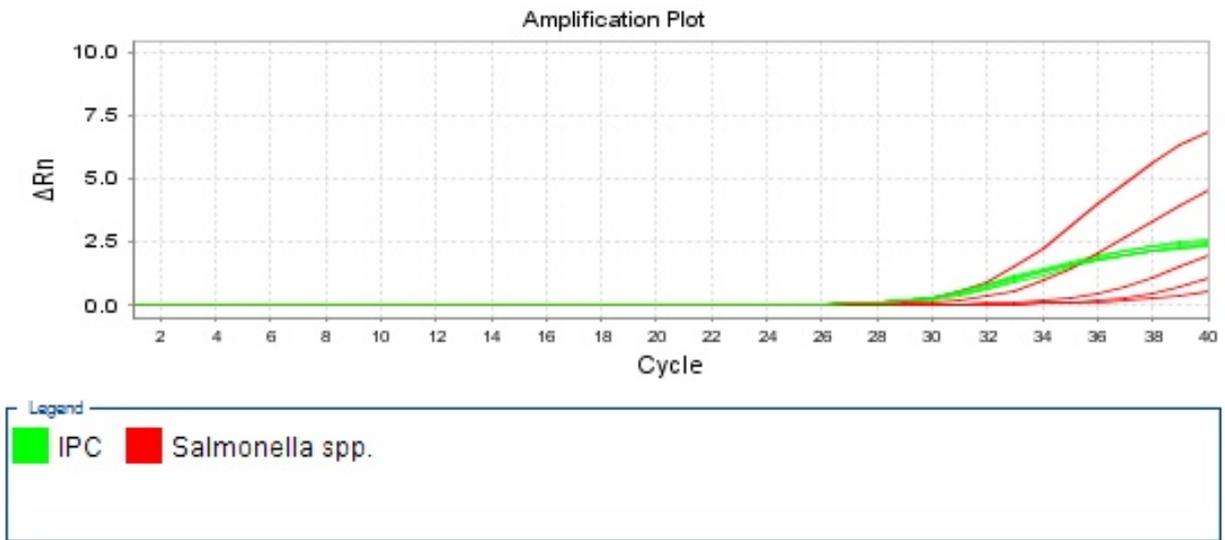
Fig 3 Real-time PCR detection of *Salmonella* spp. without incubation and IPC in 2[nd]experimental group
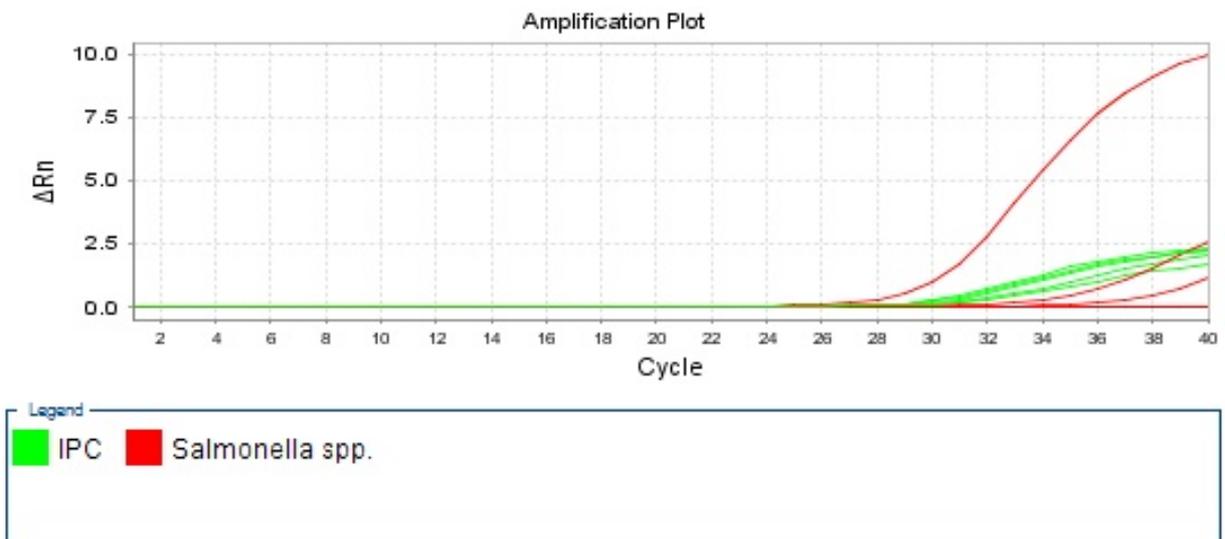
Fig 4 Real-time PCR detection of *Salmonella* spp. without incubation and IPC in 3[rd]experimental group
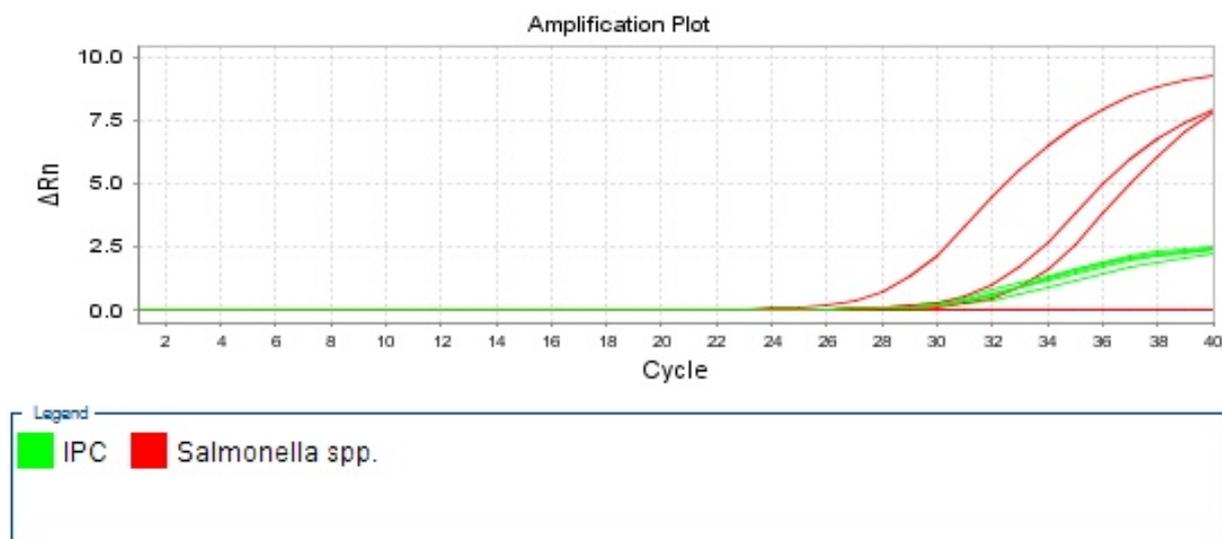
Fig 5 Real-time PCR detection of *Salmonella* spp. without incubation and IPC in 4thexperimental group

Propolis shows antimicrobial activities, and its effects may occur through a direct action on microorganisms killing. Our results indicate positive effect of propolis against colonization of GIT (gastro-intestinal tract) with *Salmonella* spp in all experimental groups. All tested experimental groups were able to inhibit and eliminate genus *Salmonella* spp. Species-dependent differences in antibacterial activity of the bee glue have been found within aerobic/facultative bacteria, and *Enterococcus* and *Salmonella* species have been found to be the most resistant species within the Gram-positive and Gram-negative bacteria, respectively **(Stepanovic et al., 2003).** In aerobic/facultative bacteria, the bee glue is usually more active against Gram-positive species and yeasts than against Gram-negative species**(Drago et al., 2000)** The multiplex real-time PCR developed in this study was the first to detect all *Salmonella* spp. possibly related with meats and to differentiate simultaneously *S.* Typhimurium from *S.* Enteritidis in meats. Previously, real-time PCR assays had been applied for *Salmonella* spp. and other food-borne pathogens **(Malorny et al., 2004; Rossmanith et al., 2006; Sails et al., 2003).** The multiplex real-time PCR developed in this study was the first to detect all *Salmonella* spp. possibly related with meats and to differentiate simultaneously *S.* Typhimurium from *S.* Enteritidis in meats. Previously, real-time PCR assays had been applied for *Salmonella* spp. and other food-borne pathogens. **(Lee et al., 2009; Rossmanith et al., 2006).**According to **Grange et al. (1990)** propolis is more

activeagainstGrampositivebacteriathanGramnegative.Studies have reported that Gram-positive bacteria were moresusceptible to ethan ol-extracted propolis than Gram-negative bacteria**(Fernandes et al., 2001; Grange, 1990)**. **Grange (1990)** showed that propolis has a preferentialinhibitory effect on both Gram-positive cocci and rods.**Park and Kim (1996) and Marcucci (1995)**have reported antibacterial activity ofpropolis against Gram-positive bacteria including *B. cereus, Bacillus subtilis, L. monocytogenes* and *S. typhimurium*. The constituents, the doseapplication and the probable presence of nonvolatile componentsof extract all seem to contribute to the variation in the antimicrobialeffect of propolis on microorganisms **(Bankova et al., 2005; Bonvehi et al., 1994; Castaldo et al., 2004).**The antimicrobial effects of propolis may be influenced bymany factors such as the propolis origin, extract preparation,chemical composition which exhibit considerable geographic differencesas well. Flavonoids and esters of phenolic acids have beenreported to be associated with the antimicrobial activity of European propolis **(Grangeand Davey, 1990).**

CONCLUSION

Our results indicated that the Step One real-time PCR assay developed in this study could sensitively detect *Salmonella* spp. in ready to eat food. This will not only prevent many people from becoming infected with *Salmonella*, it will also benefit food manufacturing companies by extending their product's shelf-life by several days and saving them the cost of warehousing their food products while awaiting pathogen testing results. This will not only prevent many people from becoming infected with *Salmonella*, it will also benefit food manufacturing companies by extending their product's shelf-life by several days and saving them the cost of warehousing their food products while awaiting pathogen testing results.

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