SYNERGISTIC EFFECT OF PROPOLIS AND ANTIBIOTICS ON THE *SALMONELLA* TYPHI

**Ricardo de Oliveira Orsi[1]*; José Maurício Sforcin[2]; Silvia Regina Cunha Funari[1];
Ary Fernandes Junior[2]; Vassya Bankova[3]**

[1]Departamento de Produção e Exploração Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, SP, Brasil; [2]Departamento de Microbiologia e Imunologia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, SP, Brasil; [3]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

ABSTRACT

The goal of this work was to investigate a possible synergistic effect between ethanolic extracts of propolis from Brazil and Bulgaria and some antibiotics (Amoxicillin, Ampicillin and Cefalexin) against *Salmonella* Typhi. Brazilian and Bulgarian propolis showed an antibacterial action, but the sample from Bulgaria was shown to be more efficient. Both samples showed a similar synergistic effect with these antibiotics. One may conclude that the propolis samples show an important antibacterial action, as well as a synergistic effect with antibiotics against *Salmonella* Typhi.

Key words: propolis, antimicrobial activity, antibiotics, synergistic effect

INTRODUCTION

Salmonella serovars are responsible for human diseases that range from mild gastroenteritis to host-disseminated enteric fever (12).

Bacterial drug resistance is an important world problem (1,23). Poppe *et al*. (14) verified that *Salmonella* serovars, isolated from food or infected animals, were resistant to several antibiotics. Lewin (9) and Stoner *et al*. (19) also reported an increased resistance of *Salmonella* serovars to several antimicrobial drugs.

Propolis is a resinous material produced by bees from plant buds and exudates, showing biological activities such as antibacterial (17). The chemical composition of propolis is very complex and is dependent upon the source plant. The main vegetal source of propolis in Botucatu, São Paulo State, Brazil, is *Baccharis dracunculifolia* DC., followed by *Eucalyptus citriodora* Hook and *Araucaria angustifolia* (Bert.) O. Kuntze (3). The brazilian propolis sample, collected in the Beekeeping Section of the University, was analysed by GC, GC-MS and TLC, revealing that its main components are phenolic compounds (flavonoids, aromatic acids, benzopyranes), di- and triterpenes, essential oils, among others. Seasonal variations in propolis composition are not significant and are predominantly quantitative (2,4,6).

In order to reduce the clinical doses of most antibiotic compounds because of their marked side effects, we investigated a possible synergistic effect of propolis with some antibiotics that act on the bacteria wall, reducing the minimal inhibitory concentration (MIC) of 3 widely-used antibiotics: Amoxicillin, Ampicillin and Cefalexin.

MATERIALS AND METHODS

Propolis samples

Brazilian propolis was collected in the BeeKeeping Section of the School of Veterinary Medicine and Animal Husbandry of Botucatu, UNESP. Bulgarian propolis was given by Dr. Bankova, from the Bulgarian Academy of Sciences, Sofia.

Propolis samples were ground and extracted (30 g of propolis, completing the volume to 100 mL with 70% ethanol). The final concentrations were calculated, obtaining the dry weight of the

*Corresponding Author. Mailing address: Departamento de Produção e Exploração Animal, Faculdade de Medicina Veterinária e Zootecnia, UNESP, Distrito de Rubião Junior, s/n. 18618-000, Botucatu, SP, Brasil. Telefax: (+5514) 3811-7189, E-mail: orsi@fca.unesp.br

solutions (Brazil: 133 mg/mL; Bulgaria: 170 mg/mL). Specific dilutions of these solutions were prepared in appropriate media for each assay.

Salmonella serovars

A standard serovar of *Salmonella* Typhi (00238) was obtained from Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, and maintained in tripticase soya agar (TSA/Oxoid) at room temperature.

Susceptibility test: Propolis

Determination of the minimal inhibitory concentrations (MIC) by the agar dilution method was performed, following the National Committee of Clinical Laboratory Standards Guidelines (11).

Serial concentrations of propolis from Brazil and Bulgaria were achieved (% v/v) in plates containing Mueller Hinton Agar, ranging from 1.0% to 14.0%. Each antimicrobial test also included plates containing the culture medium plus ethanol, in order to obtain a control of the solvent antimicrobial effect.

Bacterial strain was grown in Brain Heart Infusion Agar (BHI) (Oxoid) at 37ºC/24h. After incubation, the bacteria was suspended in 5 mL of sterile saline and diluted to yield a final inoculum of approximately 1.0×10^6 CFU/mL.

After the inoculation procedures, using a multiloop replicator, plates were incubated at 37ºC/24h and MIC endpoints were read as the lowest concentration of propolis that resulted in no visible growth or haze on the surface of the culture medium. Populational analyses of data were carried out by calculating the MIC for 90% of the microorganism (17).

Susceptibility test: antibiotic substances

Antibiotic substances acting on the bacterial wall were used: Amoxicillin, Ampicillin and Cefalexin (SIGMA). They were water soluble, dissolved immediately prior to their use and filtered through a 0.22 µm filter into vacuumed sterile pre-sealed vials, to obtain sterile solutions.

The MIC of these antibiotics was determined by serial dilutions in BHI, raging from 0.008 µg/mL to 1024.0 µg/mL.

Aliquots (20 µL) of bacterial suspensions (1.0×10^6 CFU/mL) of *Salmonella* Typhi were added to tubes containing BHI (2.5 mL) plus the antibiotics. Test tubes were incubated for 24 h at 37ºC and MIC endpoints were read as the lowest concentration of antibiotics that resulted in no haze. All assays were carried out in triplicates.

Survival curve and synergism

The survival curve of *Salmonella* Typhi was performed in order to observe the incubation period responsible for propolis antibacterial activity. Thus, 1.0×10^6 CFU/mL were inoculated in BHI plus propolis in the corresponding MIC 90%, obtained previously to each strain.

After 1.5, 3, 6, 9 and 24 h of incubation (37ºC), aliquots of each culture were recovered and plated on Mueller Hinton Agar by the Pour Plate method. Plate counts (CFU/mL) were carried out after 24 h incubation and the survival percentage was calculated (17)

A synergistic effect between propolis and antibiotics was observed, following the behavior of *Salmonella* Typhi incubated with ½ and ¼ of Brazilian and Bulgarian propolis (MIC 90%) plus ½ or ¼ of each antibiotic, respectively.

Statistical analysis

Analysis of variance (ANOVA) was used to examine the treatment effects in the survival curve, according to the incubation period in medium plus Brazilian or Bulgarian propolis or antibiotics. The probability of 0.001 was chosen as the significant level (24).

RESULTS AND DISCUSSION

We could verify that *Salmonella* Typhi is susceptible to propolis from Brazil (MIC = 9.9% v/v) and Bulgaria (MIC = 10.0% v/v) (Table 1). With regards to ethanol 70% effects, used as a solvent for propolis in this assay, its inhibitory action was seen only in the concentration of 12.6% v/v.

Several authors have studied the antimicrobial activity of propolis (7,15). It was reported that propolis shows a marked action against Gram-positive bacteria and a limited activity against Gram-negative ones (17). We could also observe that the MIC for *Salmonella* Typhi was far above the MIC from Gram-positive bacteria (0.5% v/v for *Staphylococcus aureus*) (17).

Brazilian and Bulgarian samples had similar MIC (9.90 and 10.0%, respectively). However, their action on the *Salmonella* survive was significantly different: bacteriostatic activity to Brazilian and bactericidal to Bulgarian propolis (Table 1). Since these samples were produced in distinct geographic regions, differences in their chemical composition are expected, what could be also related to different biological activities.

Salmonella Typhi susceptibility to antibiotics is variable, depending on the serovar and the resistance to antibiotic
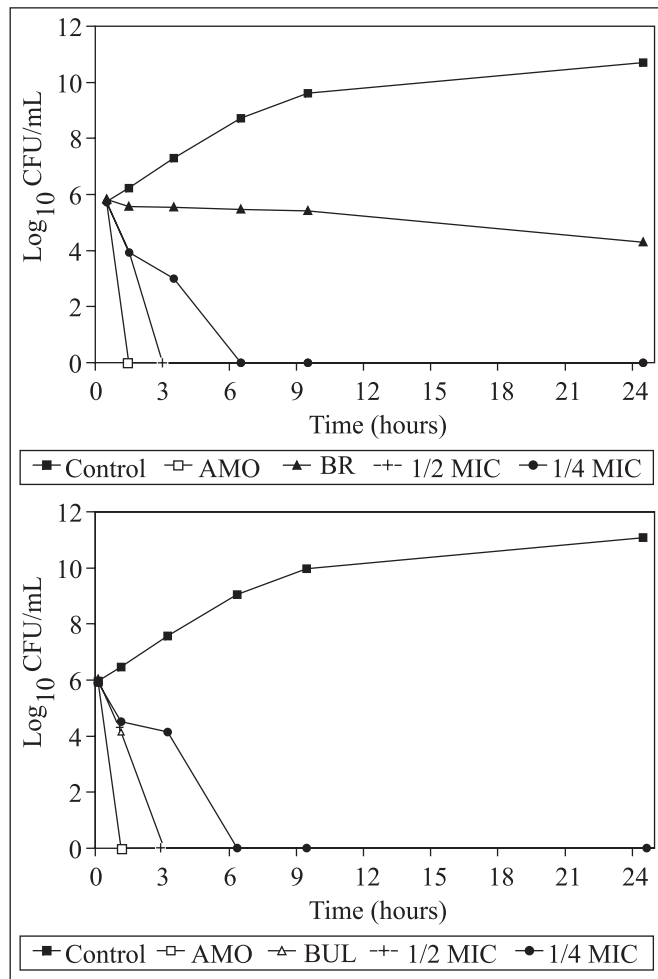
Table 1. Minimal inhibitory concentration (MIC) of Brazilian and Bulgarian propolis and antibiotics (amoxicillin, ampicillin and cefalexin) for *Salmonella* Typhi.

Treatment	MIC 90%	Activity
Brazilian propolis	9.90% v/v (247.5 mg/mL)	bacteriostatic
Bulgarian propolis	10.00% v/v (260 mg/mL)	bactericidal
Amoxicillin	8 µg/mL	bactericidal
Ampicillin	8 µg/mL	bactericidal
Cefalexin	64 µg/mL	bactericidal

substances (5,13). Threlfall (21) related that *Salmonella* Typhi serovars, isolated in India and southwest of Asia, were resistant to several antimicrobial drugs, showing a high level of epidemic in these regions. The data showed that antimicrobial drugs inhibited *Salmonella* Typhi growth, showing a bactericidal effect (Table 1).

In order to verify the synergistic effect, we used the ½ and ¼ of the minimal inhibitory concentration of propolis samples and the antibiotics, obtained previously (Table 1).

The combination of ½ and ¼ of the MIC of Brazilian propolis and Amoxicillin had a remarkable inhibitory effect on *Salmonella* Typhi, after 3 and 6 h of incubation, respectively. A similar result was observed with Bulgarian propolis (Fig. 1).

With regards to Ampicillin, one may observe that ½ and ¼ of Brazilian propolis and antibiotic had an efficient reduction in the CFU of *Salmonella* Typhi (Fig. 2), showing a bactericidal effect after 3 and 6 h, respectively. Bulgarian propolis also reduced the CFU, showing a bactericidal effect for both relations (½ and ¼) after 6 h of incubation (Fig. 2).

The combination of Cefalexin and Brazilian propolis reduced significantly the CFU of *Salmonella* Typhi, having a bactericidal effect after 9 (½) and 24 h (¼). With respect to Bulgarian propolis, there was also a synergistic effect with Cefalexin and a bactericidal effect after 3 h of incubation (½ and ¼) (Fig. 3).

Beta-lactamic antibiotics (Amoxicillin, Ampicillin and Cefalexin) act on a group of proteins called "Penicillin-binding-
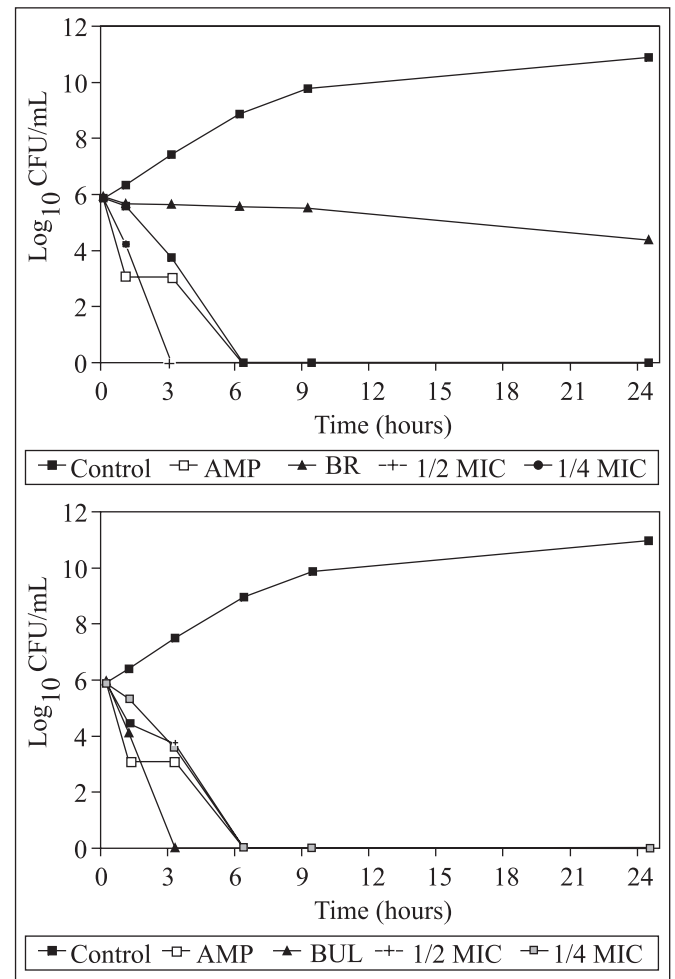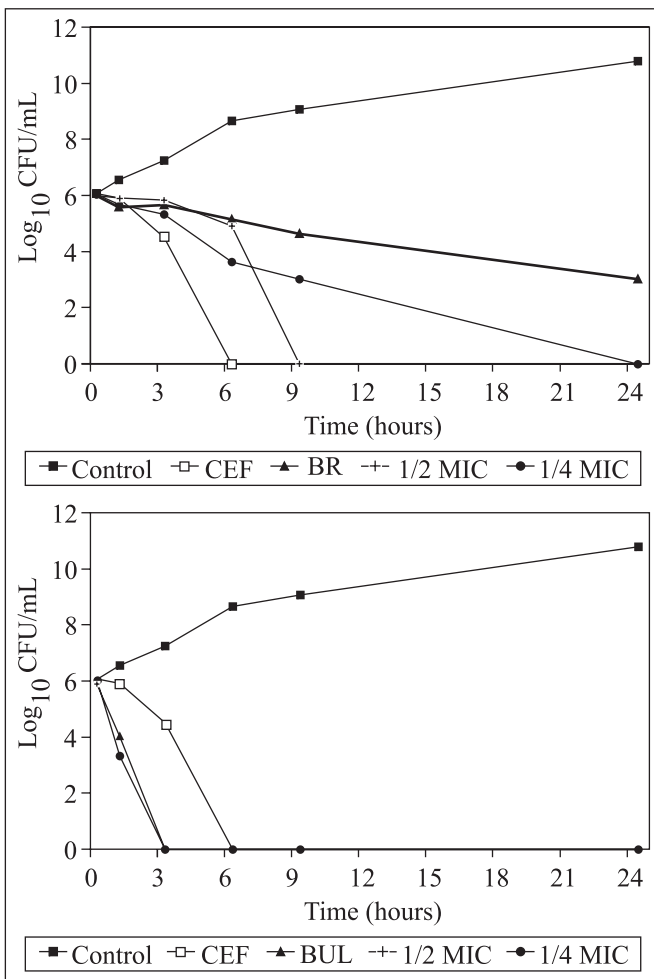

Figure 1. Profile in time (h) of the population analysis of *Salmonella* Typhi according to susceptibility to Brazilian propolis – BR (9.9% v/v), Bulgarian propolis – BUL (10.0%) and Amoxilina - AMO (8.0 µg/mL), with ½ and ¼ of the minimal inhibitory concentration (MIC).

Figure 2. Profile in time (h) of the population analysis of *Salmonella* Typhi according to susceptibility to Brazilian propolis – BR (9.9% v/v), Bulgarian propolis – BUL (10.0%) and Ampicillin - AMP (8.0 µg/mL), with ½ and ¼ of the minimal inhibitory concentration (MIC).

Figure 3. Profile in time (h) of the population analysis of *Salmonella* Typhi according to susceptibility to Brazilian propolis – BR (9.9% v/v), Bulgarian propolis – BUL (10.0%) and Cefalexina - CEF (64.0 µg/mL), with ½ and ¼ of the minimal inhibitory concentration (MIC).

proteins" (PBP), found in the periplasmatic space of Gram-negative bacteria. These PBP are involved in the peptideoglican synthesis - an important structure of bacteria wall (18).

Although little is known about the mechanisms of propolis antibacterial action, Takaisi-Kikuni and Schilder (20) verified that ethanolic extract of propolis acted on *S. agalactie* growth, inhibiting the protein synthesis. Mirzoeva *et al.* (10) reported that propolis and some of its cinnamic and flavonoid components were found to uncouple the energy transducing cytoplasmic membrane and to inhibit bacterial motility. Koo *et al.* (7) suggested that propolis could act on the enzymatic activity of *S. mutans* and *S. sangui*.

Krol *et al.* (8) related that propolis had a marked synergistic effect on the antibacterial activity of streptomycin and cloxacillin

towards *Staphylococcus aureus*. Scheller *et al.* (16) observed a synergistic effect between an ethanolic extract of propolis and antibiotics used against mycobacteria.

In our work, propolis seemed to aid beta-lactamic antibiotics in PBP inhibition, what could explain the synergistic effects. Besides, propolis could also diminish the resistance of the bacteria wall to antibiotics, as suggested by Mirzoeva *et al.* (10), promoting beta-lactamics action on PBP.

CONCLUSION

We conclude that Brazilian and Bulgarian propolis had an antibacterial action on *Salmonella* Typhi, although the Bulgarian propolis shows to be more efficient that the Brazilian sample. Both samples show a similar synergistic effect with some antibiotics acting on the cell wall. This result is important to reduce the antibiotic clinical doses and their marked side effects.

RESUMO

Efeito sinérgico da própolis e antibióticos sobre *Salmonella* Typhi

O objetivo do presente trabalho foi investigar um possível efeito sinérgico entre extrato alcoólico de própolis do Brasil e Bulgária com alguns antibióticos (Amoxilina, Ampicilina e Cefalexina) utilizados contra *Salmonella* Typhi. Própolis do Brasil e Bulgária mostraram uma atividade antibacteriana, embora a amostra da Bulgária tenha sido mais eficiente. Ambas as amostras apresentaram um efeito sinérgico com os antibióticos estudados. Pode-se concluir que as amostras de própolis possuem atividade antibacteriana, bem como apresentam efeito sinérgico com antibióticos utilizados contra *Salmonella* Typhi.

Palavras-chave: própolis, atividade antibacteriana, antibióticos, efeito sinérgico

REFERENCES

1. Asna, S.M.Z.H.; Haq, J.A. Decrease of antibiotic resistance in *Salmonella* Typhi isolated from patients attending hospitals of Dhaka City over a 3 years period. *Int. J. Antim. Agents*, 16, 249-51, 2000.
2. Bankova, V.; Boudourova-Krasteva, G.; Popov, S.; Sforcin, J.M.; Funari, S.R.C. Seasonal variations of the chemical composition of Brazilian propolis. *Apidologie*, 29, 361-7, 1998a.
3. Bankova, V.; Boudourova-Krasteva, G.; Sforcin, J.M.; Frete, X.; Kujumgiev, A.; Maimoni-Rodella, R.; Popov, S. Phytochemical evidence for the plant origin of Brazilian propolis from São Paulo State. *Z. Naturforsch.*, 54c, 401-5, 1999.
4. Bankova, V.; Boudourova-Krasteva, G.; Sforcin, J.M.; Frete, X.; Kujumgiev, A.; Rodella-Maimoni, R.; Popov, S. Phytochemical evidence for the plant origin of brazilian propolis from São Paulo State. *Z. Naturforsch.*, 54c, 401-5, 1998b.
5. Boonmar, S.; Bangtrakulnonth, A.; Pornruangwong, S.; Samosornsuk, S.B.; Kaneko, K; Ogawa, M. Significant increase in the antibiotic

resistance of *Salmonella* isolates from human beings and chicken meat in Thailand. *Vet. Microbiol.*, 62, 73-80, 1998.

6. Boudourova-Krasteva, G.; Bankova, V.; Sforcin, J.M.; Nikolova, N.; Popov, S. Phenolics from Brazilian propolis. *Z. Naturforsch.*, 52c, 676-9, 1997.

7. Koo, H.; Rosalen, P.L.; Cury, J.A.; Park, Y.K.; Bowen, W.H. Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity. *Antimicrob. Agents Chemoth.*, 46, 1302-9, 2002.

8. Krol, W.; Scheller, S.; Shani, J.; Pietsz, G.; Zcuba, Z. Synergistic effect of ethanolic extract of propolis and antibiotics on the growth of *Staphylococcus aureus*. *Drug Res.*, 43, 607-9, 1993.

9. Lewin, C.E. Treatment of multiresistant *Salmonella* infection. *The Lancet*, 337, 47, 1991.

10. Mirzoeva, O.K.; Grishanin, P.C.; Calder, P.C. Antimicrobial action of propolis and some its components: the effects on growth, membrane potential and motility of bacteria. *Microbiol. Res.*, 152, 239-46, 1997.

11. National Committee for Clinical Laboratory Standards. (NCCLS). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M7- A. NCCLS., Villanova, Pa. 1997.

12. Ohl, M.E.; Miller, S.I. *Salmonella*: a model for bacterial pathogenisis. *Ann. Rev. Med.*, 52, 259-274, 2001.

13. Pang, T. Genetic dynamics of *Salmonella* Typhi – divestity in clonality. *Trends Microbiol.*, 6, 339-42, 1998.

14. Poppe, C.; Aryoud, M.; Ollis, G.; Chirino-Trejo, M.; Smart, N.; Quessy, S.; Michel, P. Trends in antimicrobial resistance of *Salmonella* isolated from animals, foods of animal origin, and the environment of animal production in Canada, 1994-1997. *Microbial Drug Resistance-Mechanisms Epidemiol. Dis.*, 7, 197-212, 2001.

15. Santos, F.A.; Bastos, E.M.A.; Uzeda, M.; Carvalho, M.A.R.; Farias, L.M.; Moreira, E.S.A.; Braga, F.C. Antibacterial activity of Brazilian propolis and fractions against oral anaerobic bacteria. *J. Ethnopharmacol.*, 80, 1-7, 2002.

16. Scheller, S.; Dworniczak, S.; Klimmek, K.W.; Rajca, M.; Tomczyk, A.; Shani, J. Synergism between ethanolic extract of propolis (EEP) and anti-tuberculosis drugs on growth of Mycobacteria. *Z. Naturforsch.*, 54, 549-53, 1999.

17. Sforcin, J.M.; Fernandes-Jr., A.; Lopes, C.A.M.; Bankova, V.; Funari, S.R.C. Seasonal effect on Brazilian propolis antibacterial activity. *J. Ethonopharmacol.*, 73, 243-9, 2000.

18. Silva, J. Mechanisms of antibiotic resistance. *Curr. Ther. Res.*, 57, 30-5, 2000.

19. Stoner, M.C.; Forsythe, R.; Mills, A.S.; Ivatury, R.R.; Broderick, T.J. Intestinal perforation secondary to *Salmonella* Typhi: case reported and review of the literature. *Amer. Surg.*, 66, 219-22, 2000.

20. Takaisi-Kikuni, N.B.; Schilder, H. Electron microscopic and microcalorimetric investigations of the possible mechanism of the antibacterial action of a defined propolis provenance. *Planta Med.*, 60, 222-227, 1994.

21. Threlfall, E.J. Antimicrobial drug resistance in *Salmonella*: problems and perspectives in food and water-borne infections. *FEMS Microbiol. Rev.*, 26, 141-8, 2002.

22. Wolf, D.C.; Giannella, R.A. Antibiotic therapy for bacterial enterocolotis: a comprehensive review. *Am. J. Gastroenterol.*, 88, 1667-83, 1993.

23. World Health Organization (WHO). Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe. Sixth report 1990-1992, 32-38. 1995.

24. Zar, J.H. Biostatistical analysis. New Jersey: Pretince Hall, 1996, 718p.