THE INFLUENCE OF SILVER NANOPARTICLES ON FECAL BACTERIA SUSCEPTIBILITY

E, GORNICKA*[a], J, MIKICIUK[a], A, WRONSKA[a], A, SZTERK[a], J. RACHTAN-JANICKA[a], E, ROSIAK[b], M, MAZUR[c]

[a]*Department of Functional Food and Commodities, Faculty of Human Nutrition and Consumer Sciences, Warsaw University of Life Sciences, Nowoursynowska 159c,02-776 Warsaw, Poland*

[b]*Department of Food Gastronomy and Food, Faculty of Human Nutrition and Consumer Sciences, Warsaw University of Life Sciences, Nowoursynowska 159c,02-776 Warsaw, Poland*

[c]*Department of Chemistry, University of Warsaw, ul. Pasteura 1, 02-093 Warsaw, Poland*

The purpose of this study was to investigatethe potential toxicity of silver nanoparticles tofecalbacteria, on the example of *Lactobacillus*, *Bifidobacterium* and *Peptostreptococcus* isolated from infant's feces on the first and the second day of their life. Bacteria was treated nano-silver solutions and their dilutions with approximate concentration of silver 2 μg ml^{-1}, 0,25 μg ml^{-1} and 0,05 μg ml^{-1} purchased from three different producers. Antibacterial activity of silver nanoparticles was tested using the disc-diffusion method and dilution method. Concentration of silver nanoparticles at level 2 μg ml^{-1} (150nm) had the best and statistically different antibacterial activity to the tested lactic acid bacteria. Nanoparticles of the same concentration (0,05 μg ml^{-1}) and size (80-150) had similar antibacterial properties. In disc-diffusion method opposite to dilution method, the least susceptible bacteria for nanoparticles was *Peptostreptococcus*. The obtained results depended on used method and important limit for disc-diffusion method was observed.

(Received December 22, 2013; Accepted March 8, 2014)

Keywords: Silver nanoparticles, Fecal bacteria, Antimicrobial effect, *Lactobacillus, Bifidobacterium, Peptostreptococcus*

1. Introduction

Silver has been known from its antimicrobial activity from centuries and used for treating burns, wounds and other diseases. After penicillin was introduced the use of silver as antibacterial agent decreased. Today silver nanoparticles (AgNPs) are more and more popular again because of its toxicity to many microorganisms like bacteria, fungi and virii such as *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Aspergillusniger, Candida albicans*, and even Hepatitis B or HIV-1. It is also reported that this toxicity depends on size and shape of silver nanoparticles [1, 2]. There are known possible mechanisms of action of silver in bacteria. Nanoparticles may get attached to the cell membrane and cause it's permeability, or react with proteins and affect proper transport through the membrane. They may also penetrate inside the cell and then generate ROS or release silver ions. All these mechanisms lead to cell damages and death[1-4].Because of these antimicrobial properties they are commonly used not only in medicine but also in many other fields of technology and science [2]. Many consumer products such as cosmetics, chemicals, detergent, machines (eg. washing machines, refrigerators) or even food boxes contain AgNPs. This effect is preferable for pathogenic microflora to extend shelf life of food products but materials for

*Correspondingauthor: gornicka.ewa@gmail.com

food packaging based on nanotechnology bring many more advantages such as environmental or economical. However, using NPs in new food packaging materials may be dangerous because of potential migration of nanoparticles into food and then human's body as an effect of consumption [5, 6].

Consequences of exposure on silver NPs from consumer products are not developed so far and little is known about what happens to nanoparticles in the body but there are known three ways of entrance of NPs to human body: ingestion, inhalation and through skin [6-8]. Due to small size of NPs they are suggested to be toxic to the kerationcytes, fibroblast and mitochondrial activity. Silver has been reported to accumulate in the body, mainly in liver, lung and skin. Described forms of toxicity from silver is e.g. argyria in humans, neurological effects in mice, weight loss and histopatological changes in liver and lungs in rats [2, 9, 10]. According to the theory that nanoparticles are also eliminated from the body via the feces and urine, no matter how it enters, there is a possibility that silver NPs can reach human's intestine and interact with beneficial gastrointestinal bacteria [10, 11].

The regular human microflora is a complex ecosystem. At birth intestinal colonization derives from microorganism of the vaginal mucoses of the mother and fecalmicroflora. Diet can influence the microbiota, while breast-feeding promotes an intestine microbiota in which *Bifidobacterium sp*. and *Lactobacillus sp*. predominate, while coliform, enterococci and bacteroides predominate in formula bottle-fed baby [12, 13]. The intestinal microbial flora has numerous functions. Endogenous bacterial microflora inhibit colonization of the intestine by pathogenic microorganism. The intestinal microbial flora influences food digestion, absorption and fermentation, the immune system response, peristalsis, production of vitamins such as B-vitamins, influencing moreover the turnover of intestinal epithelial cells. In addition the metabolism of gut microflora influences hormonal secretion [14-16].

The aim of this study was to investigate the potential toxicity of AgNPs to gastrointestinal bacteria, on the example of *Lactobacillus*, *Bifidobacterium* and *Peptostreptococcus* isolated from infant's feces on the first and the second day of their life.

2. Experiments

2.1 Materials

Three nano-silver solutions were purchased from differentinternational producers: Producer 1:2 μg ml^{-1} (W.1), Producer 2:0,25 μg ml^{-1} (W.2), Producer 3:0,05 μg ml^{-1} (W.3). There were six solutions used in this study: 2 μg ml^{-1} (W.1), dilution of this solution: 0,25 μg ml^{-1}, (W.1.1.) and 0,05 μg ml^{-1} (W.1.2.), 0,25 μg ml^{-1} (W.2) and dilution 0,05 μg ml^{-1} (W.2.1.) and 0,05 μg ml^{-1} (W.3).

2.2 Characterization of NPs

Silver nanoparticles were observed using TEM (Transmission Electron Microscopy) Electron microscope JEM 1400 (JEOL Co., Japan, 2008) equipped with energy- dispersive full range X-ray microanalysis system (EDS INCA Energy TEM, Oxford Instruments, Great Britain), tomographic holder and high resolution digital camera (CCD MORADA, SiS-Olympus, Germany). The studies were performed in the Laboratory of Electron Microscopy, Nencki Institute of Experimental Biology, Warsaw, Poland.

In order to find out the size of NPs, DLS method (Dynamic Light Scattering) was used (Laboratory of Nanostructures for Photonic and Nanomedicine, PAN, Poland).

2.3 Biologic material

Antibacterial properties of nano-silver were tested against bacteria isolated from newbornsfeces. Newbornfecal samples were taken using the sterile swabber in the first or second day after birth. All infants were breastfed exclusively.

Immediately after feces samples transported (5^{o}C) to the laboratory cultures were plated in MRS LAB-AGAR™ (BIOCORP) and Schaedler LAB-AGAR™ medium (BIOCORP)Excipients with material for the isolation of anaerobic bacteria were incubated anaerobically in anareostat

with carbon dioxide for 48 hours at 37°C. Then macroscopic appearance of colonies and Gram stain were conducted.

Lactobacillus sp., Bifidobacterium sp. and *Peptostreptococcus sp*. were isolated and identified from newbornsfeces. Antibacterial activity of silver nanoparticles were tested against these bacteria.

In order to select the optimal dilution of inoculums serial dilutions of bacterial suspensions were made. Bacteria from each dilution were transferred on the plates witch Schaedler LAB-AGAR™. After anaerobically incubation for 24 hours at 37°C optimal dilution of bacteria were selected. In this study bacterial concentration of 10^6cfu ml^{-1} was used.

Antibacterial activity of silver NPs was tested using the disc-diffusion method and dilution method.

2.4 Disc-diffusion method

At the beginning sterility of paper disc was checked. Two paper discs were laid down into petri dish with Blood LAB-AGAR™. After incubation for 24 hours at 37°C microbial growth around the discs was rated.

Bacterial suspensions(100µl) were applied into petri dish with Schaedler LAB-AGAR™ and spread evenly. Then laid down sterile paper discs were moistured with 30µl silver NPs. After anaerobically incubation for 24 hours at 37°C an inhibition zone around the discs was measured.

2.5 Dilution method

All silver NPs (0,5 ml; W.1, W.1.1., W.1.2., W.2, W.2.1, W.3) and bacterial suspensions (0,5ml; *Lactobacillus sp., Bifidobacterium sp.* and*Peptostreptococcus sp.)* weremixed in sterile test tubes.

In parallel control samples were prepared by mixing 0.5 ml of bacterial suspensions with 0.5 ml of sterile PBS.

Material was incubated anaerobically for 24 hours at 37°C. Mixtures were applied into petri dish with Schaedler LAB-AGAR™. After anaerobic incubation for 24 hours at 37°C macroscopic growth of colonies of microorganisms was evaluated.

2.6 Statistical analyses

The statistical package was Statgraphics version 15.2.11.0. All data were tested for multifactor ANOVA, Multiple Range Test.

3. Results

3.1 Characterisation of NPs

From the TEM images presented in **Fig.. 1, 3, 5** and DLS method **(Fig. 2, 4, 6)**it was found that average size of W.1 NPs was 150 nm, W.2 was 80 nm in 80% and 13 nm in 20% and W.3 –100 nm.According to TEM images all these nanoparticles tended to gather bigger clusters. Because of the fact that all these solutions were stable gravimetrically this occurrence probably happened during sample preparation when drop was drying.

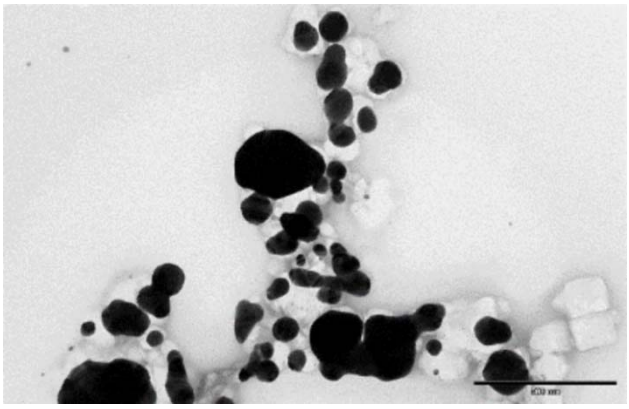

Fig. 1. TEM image of W.1 NPs

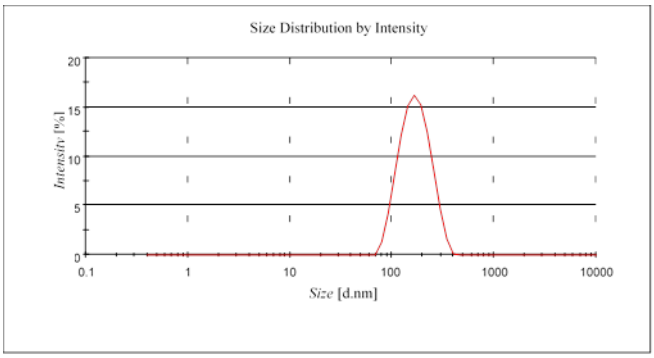

Fig. 2. Size distribution by intensity of W.1 NPs (DLS method)

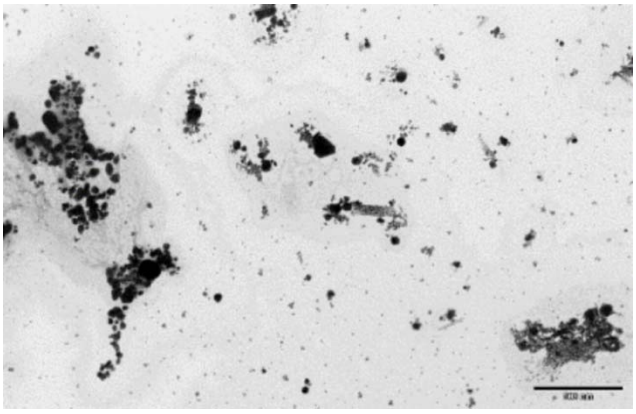

Fig. 3. TEM image of W.2 NPs

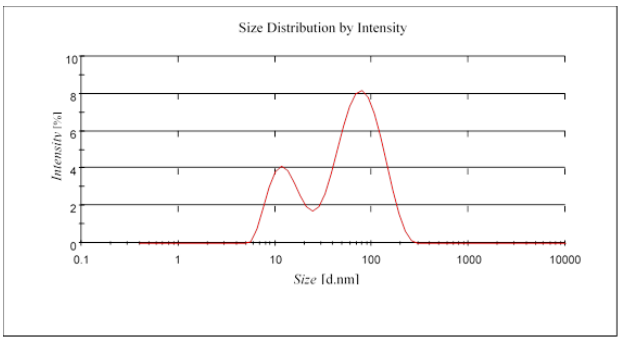

Fig. 4. Size distribution by intensity of W.2 NPs (DLS method)

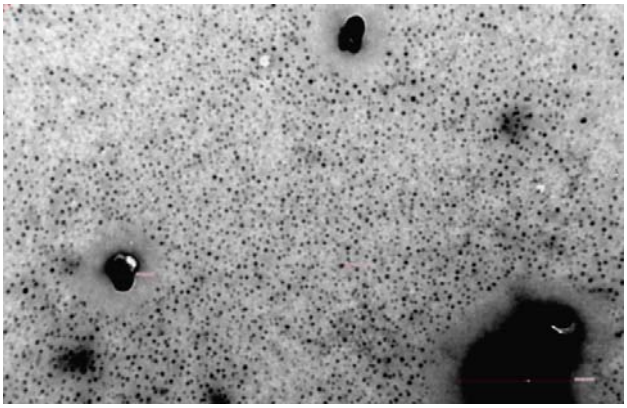

Fig. 5. TEM image of W.3 NPs

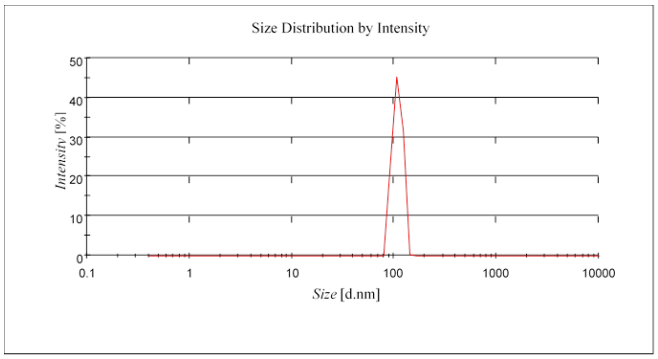

Fig. 6. Size distribution by intensity of W.3 NPs (DLS method)

3.2 Disc-diffusion method

Fig. 7. shows inhibition zone diameter of bacteria isolated from newbornfeces. The obtained results showed that growth inhibition of bacteria depends on type and dilution of silver NPs and also bacteria species ($p<0,05$).

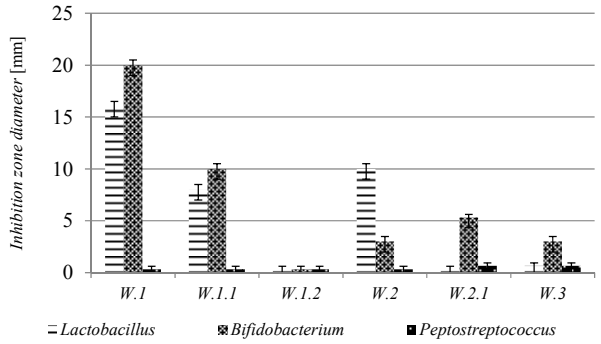

Fig. 7. Inhibition zone diameter [mm] of bacteria observed with different NPs. Error bars represent standard error of the mean n = 36, A-B groups of statistically different values (α = 0,05) for bacteria species as factor, a-c groups of statistically different

Statistical analysis indicated that the less susceptible for NPs bacterium was *Peptostreptococcus.* Its growth was inhibited by none of tested silver NPs and inhibition zone diameter was significantly different from *Lactobacillus* and *Bifidobacterium* inhibition growth. There were no significant different between *Lactobacillus* and *Bifidobacterium* inhibition growth.

The influence of NPs on bacteria was also tested. Results showed that W.1.2, W.3 and W.2.1 silver NPs(0,05 μg ml^{-1}) had the weakest inhibition effect and there were no significant difference between their effectiveness. The influence of W.2, W.2.1 and W.3 NPs on bacterial growth is quite interesting. The W.2 sample (0,25 μg ml^{-1}) is much more concentrated than W.2.1

(0,05 μg ml^{-1}) and W.3 (0,05 μg ml^{-1}) but there were no significant differences observed. W.1 (2 μg ml^{-1}), W.1.1 (0,25 μg ml^{-1}) and W.2 (0,25 μg ml^{-1}) samples revealed the best inhibition effect on tested bacteria and there were also no significant differences observed between them.

3.3 Dilution method

Fig. 8. shows the average growth of tested bacteria in dilution method. It was proved that bacteria species (p=0,0001) and type of NPs (p<0,05) have significant influence on their growth.

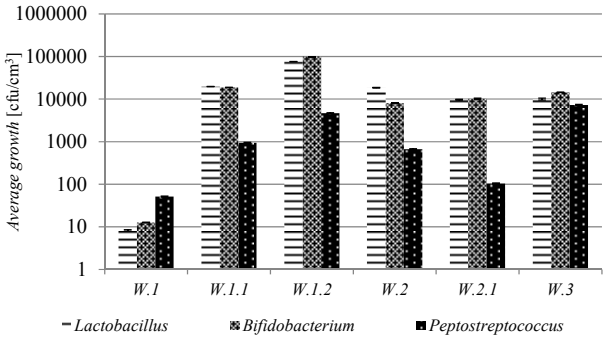

Fig. 8. The average growth of chosen bacteria inhibited by tested NPs. Error bars represent standard error of the mean n = 36, A-B groups of statistically different values (α = 0,05) for bacteria species as factor, a-d groups of statistically different values

In this method the most susceptible bacteria for tested NPs was *Peptostreptococcus* and its growth was significantly different from other tested bacteria. This is an opposite result comparing to this one obtained in disc-diffusion method when none of tested NPs was able to inhibit *Peptostreptococcus*.

The lowest and significantly different inhibiting effect NPs towards bacteria was proved for sample W.1.2. The best inhibiting effect showed W.1 sample which was the most concentrated (2μg ml^{-1}).

There were also revealed no significant differences between samples: W.2, W.2.1 and W.3 despite the fact that W.2 sample is more concentrated (0,25 μg ml^{-1}) than other (0,05 μg ml^{-1}). Results connected with NPs effectiveness obtained in this method are similar to these obtained in disc-diffusion method.

4. Discussion

In our study concentration of silver at level 2μg ml (150 nm) had the best and statistically different antibacterial properties to the tested lactic acid bacteria. According to Hadrup et.al (2012), Ag-NPs and Ag-acetate in dose up to 9mg Ag/kg bw/day in size 14nm, did not disturb the microbiological balance of the gastrointestinal environment at the phyla level [9].

Dziendzikowska et al., (2012) who examined biodistribution and excretion of silver NPs in rats treated with 5mg ml^{-1} 20 and 200 nm Ag-NPs found that silver is excreted in feces [7].This indicates NPs may have negative influence on beneficial gastrointestinal bacteria.

In this study NPs in size between 80 nm (W.2.1), 100 nm (W.3) and 150 nm (W.1.2) in the same concentration (0,05 μg ml^{-1}) had similar antibacterial properties. These results are consistent with findings of Carlson et al., (2008) who reported that toxicity of NPs (15, 30 and 55nm) was size-dependent [17].

According to disc-diffusion method the less susceptible bacteria from all of analysed microorganisms was *Peptostreptococcus*. Noneof analysed Ag-NPs inhibited the growth of this bacteria. In contrast, in dilution method there was observed significant inhibition growth of *Peptostreptococcus*. The reason of this effect can be connected with the size of *Peptostreptococcus*cell. According to Croze et.al (2011), pore size of agar is about 1μmand ismore than *Peptostreptococcus*cell diameter (depending on species) 0,5-1,2 μm [18-20] which could penetrated deeply into agar.Move of NPs took place on the agar surface only and as a result there

was no inhibition zone observed in diffusion method**.**This kind of penetration into agar pores could not happened to *Bifidobacterium* and *Lactobacillus* because of their rod-shape and bigger size. *Bifidobacterium* is 3-8μm in length [21] and *Lactobacillus* 3-6μm [22] and even 10– 30 μm when species were isolated from human stomach [23]. It has never been discussed before and probably there is a serious limit of the disc-diffusion method. Results obtained through this method may be proper only to bacteria in size bigger than pore diameter, namely bigger than 2 μm. It confirms necessity of using more than one method to verify the effect.

For *Lactobacillus* and *Bifidobacterium*there were similar results obtained in dilution and disc-diffusion methods. Silver nanoparticles inhibited growth of these bacteriato the same extent.

There are not many research connected with lactic acid bacteria and their susceptibility to NPs. Emamifar et al., (2011) described effect of nanocomposite packaging with Ag and ZnO on inactivation of *Lactobacillus plantarum,* one of major contaminants of orange juice [24]. Authors found significant decrease of *Lactobacillus plantarum* in juice packed in containing silver NPs material [24].Some researchers focus their attention on tendency ofdifferent *Lactobacillus* species to accumulate and reduce Ag^{+}. Silver was associated with the biomass of many species of lactic acid bacteria [25].

The antibacterial propertiesof NPs were examined by Sarkar et al., (2007) against seven devastating bacterial strains: *E coli (ATCC 10536)*, *S. aureus* (three different strains: ML422, ATCC 29737, ML 276), *S. typhymurium(NCTC 74)*, *V. 353holera* and S. *flexneri(80903)*. Authors observed growth inhibition of*V. cholera* and *S. aureus* (ML 422) in 2 μg ml^{-1} concentration of Ag nanoparticles in agar solution [26]. Curiously the growth of this two strains were observed when penicillin wastaken as the reference drug. Concentration of nanoparticles at level 4 μg ml^{-1} inhibited growth of seven bacterial strains. Also, the antibacterial activity of nanoparticles have been shown by Arshi et al. (2011). In this study AuNPs synthesized using the microwave irradiation method show significant antibacterial behaviour to *E. coli*.The observed inhibition zone was 22 mm [27].

He et al. (2012) who examined antibacterial activity of silver stabilized on tannin grafted collagen fiber demonstrated high antibacterial activity Ag-NPs to *E. coli* and *S. aureus*. Minimal inhibitory concentration of Ag-NPs stabilized on tannin grafted collagen fiber against *E. coli*, *S. aureus* , *P. glaucum* and *S. cerevisiae* was 2 μg ml^{-1}, 4 μg ml^{-1}, 6 μg ml^{-1} and 12 μg ml^{-1} Ag, respectively [28]. In another study Raffi et al. (2008) reported that 16 nm AgNPs which was generated using gas condensation in concentration at 60 μg ml^{-1} had excellent inhibited growth colony forming units *E. coli*[29]. Antibacterial experiment of influence AgNPs to bacterial strain *E. coli* was conducted by Chen et. al (2010) which demonstrated nanoparticles of Ag synthesized as a nanocomposite shows significant antibacterial activity with dosage as 10μg ml^{-1} Ag_2TiO_2 [30].

5. Conclusion

To summarise, silver nanoparticles inhibited bacterial growth of *Peptostreptococcus*, *Lactobacillus* and *Bifidobacterium*isolated frominfant's feces.In executed study silver nanoparticles with concentration at level 2 μg ml^{-1} (150nm) had the best and statistically different antibacterial activity to the tested lactic acid bacteria. Antibacterial characterization has been demonstrated against *Peptostreptococcus* in dilution method opposite to disc-diffusion method. The reason of that effect was smaller diameter of *Peptostreptococcus* than size of pores in agar and consequently the bacteria penetrated deeply into agar so that, inhibition effect was not possible. In addition size of pores in agar was bigger than the size of rod-shaped *Bifidobacterium* and *Lactobacillus* and antibacterial activity of silver nanoparticles have been demonstrated in both disc-diffusion and dilution method. It confirms necessity of using more than one method to verify the effect.

Acknowledgements

This work has been supported byt he Laboratory of Electron Microscopy, Nencki Institute of Experimental Biology, Warsaw, Poland.

We would like to gratefully thank for guidance, useful discussions,advice and the extreme patience shown by Professor Wanda Kawecka during this research.

References

[1] C. Marambio-Jones, E.M.V. Hoek, J. Nanopart. Res., **12**, 1531 (2010).
[2] M. Rai, A. Yadav, A. Gade, Biotechnology Advances, **27**, 76 (2009).
[3] J. Gromadzka-Ostrowska, K. Dziendzikowska, A. Lankoff, M. Dobrzyńska, Ch. Instanes, G. Brunborg, A. Gajowik, J. Radzikowska, M. Wojewódzka, M. Kruszewski, ToxicologyLetters, **214**, 251 (2012).
[4] A. Pourjavadi, R. Soleyman, Materials Research Bulletin, **46**, 1860 (2011).
[5] Q. Chaudhry, M. Scotter, J. Blackburn, B. Ross, A. Boxall, L. Castle, R. Aitken, R. Watkins, Food Additives and Contaminants, **25**(3), 241 (2008).
[6] C. Silvestre, D. Duraccio, S. Cimmino, Progress in Polymer Science, **36**, 1766 (2011).
[7] K. Dziendzikowska, J. Gromadzka-Ostrowska, A. Lankoff, M. Oczkowski, A. Krawczyńska, J. Chwastowska, M. Sadowska-Bratek, E. Chajduk, M. Wojewódzka, M. Dusinska, M. Kruszewski, Journal of Applied Toxicology, **32**, 920 (2012).
[8] Y.S. Kim, M.Y. Song, J.D. Park, K.S. Song, H.R. Ryu, Y.H. Chung, H.K. Chang, J.H. Lee, K.H. Oh, B.J. Kelman, I.H. Hwang, I.J. Yu, Particle and FibreToxicology, **7**, 20 (2010).
[9] N. Hadrup, K. Loeschner, A. Bergstrom, A. Wilcks, X. Gao, U. Vogel, H.L. Frandsen, E.H. Larsen, H.R. Lam, A. Mortensen, Arch. Toxicol., **86**, 543 (2012).
[10] K.H.O. Pelkonen, H. Heinonen-Tanski, O.O.P. Hanninen, Toxicology, **186**, 151 (2003).
[11] E. Casals, S. Vazquez-Campos, N.G. Bastus, V. Puntes, Trends in Analytical Chemistry, **27**(8), 672 (2008).
[12] H.K. Park, S.S. Shim, S.Y. Kim, J.H. Park, S.E. Park, H.J. Kim, B.C. Kang and C.M. Kim, The Journal of Microbiology, **43**(4), 345 (2005).
[13] L. Morelli, J Nutr., **138** (9), 1791S (2008).
[14] A.J. Macpherson, and N. L. Harris, Nat. Rev. Immunol., **4**(6), 478 (2004).
[15] S.C. Resta, The Journal of Physiology, **587**, 4169 (2009).
[16] E.F. Verdu, NeurogastroenterolMotil, **21**, 477 (2009).
[17] C. Carlson, S.M. Hussain, A.M. Schrand, L.K. Braydich-Stolle, K.L. Hess, R. Jones, J.J. Schlager, J. Phys. Chem., 112(43), 13608-13619 (2008).
[18] O.A. Croze, G.P. Ferguson, M.E. Cates, W.C.K. Poon, Biophys. J., **101**(3), 525 (2011).
[19] J.G. Holt, D.H. Bergey, Gram-positive Cocci in: Bergey's Manual of Determinative Bacteriology, Lippincott Williams and Wilkins (Eds.); 9th Revised Edition, Philadelphia(1994).
[20] D.A. Murdoch, Clinical Microbiology Reviews, **11**(1), 81 (1998).
[21] L. Zhu, W. Li, X. Dong, International Journal of Systematic and Evolutionary Microbiology, **53**(5), 1619 (2003).
[22] K.L. Simpson, B. Pettersson, F.G. Priest, Microbiology, **147**(4), 1007 (2001).
[23] S. Roos, L. Engstrand, H. Jonsson, International Journal of Systematic and Evolutionary Microbiology, **55**(1), 77 (2005).
[24] A. Emamifar, M. Kadivar, M. Shahedi, S. Soleimanian-Zad, Food Control., **22**, 408 (2011).
[25] L. Sintubin, W.D. Windt, J. Dick, J. Mast, D. van der Ha, W. Verstraete, N. Boon Applied Microbial and Cell Physiology, **84**, 741 (2009).
[26] S. Sarkar, A.D. Jana, S.K. Samanta, G. Mostafa, Polyhedron, **26**, 4419 (2007).
[27] N. Arshi , F. Ahmed , S. Kumar , M.S Anwar, J. Lu, B.H. Koo, C.G. Lee, Current Applied Physics, **11**, 360 (2011).
[28] L. He, S. Gao, H. Wu, X. Liao, Q. He, B. Shi, Materials Science and Engineering C, **32**, 1050 (2012).
[29] M. Raffi, F. Hussain, T. Bhatti, J. Akhter, A. Hameed, M. Hasan, Journal Mater Sci. Technol., **24**, 192 (2008).
[30] F.S. Chen, J.P. Li, K. Qian, W.P. Xu, Y. Lu, W.X. Huang, S.H. Yu,Nano Res., **3**, 244 (2010).