ISSN 1062-3590, Biology Bulletin, 2010, Vol. 37, No. 3, pp. 263–267. © Pleiades Publishing, Inc., 2010.
Original Russian Text © A.A. Kupriyanov, A.M. Semenov, A.H.C. Van Bruggen, 2010, published in Izvestiya Akademii Nauk, Seriya Biologicheskaya, 2010, No. 3, pp. 318–323.

MICROBIOLOGY

Transition of Entheropathogenic and Saprotrophic Bacteria in the Niche Cycle: Animals–Excrement–Soil–Plants–Animals

A. A. Kupriyanov[a], A. M. Semenov[a], and A. H. C. Van Bruggen[b]

[a] *Chair of Microbiology, Faculty of Biology, Moscow State University, Leninskie gory 1/12, Moscow, 119992 Russia*
[b] *The Group of Biological Farming Systems, 6709 PG Wageningen University, Marijkeweg str. 22, Wageningen, 78560 the Netherlands*
e-mail: aakupriyanov@mail.ru

Received December 24, 2008

Abstract—The possibility of transition of saprotrophic and enteropathohenic bacterial populations following the chain of naturally related habitats—fodder–animal gastrointestinal tract (GIT)–animals excrement–soil–plants and again animals with a cyclic formation—has been investigated quantitatively. All bacteria used in the experiments have been shown to successfully overcome all the mechanical, physical–chemical, and biological barriers in the food chain and to come out into the environment with a quite high number. It has been demonstrated that the same bacterial population can pass the whole cycle without additional introduction of similar populations from the outside.

DOI: 10.1134/S1062359010030076

INTRODUCTION

The bacteria *Salmonella* spp. and *Escherichia coli* O157:H7 are the most widely spread agents of food poisoning (Brackett, 1999; Rangel et al., 2005). Cattle are traditionally considered to be the main carriers of enteropathogens such as *Salmonella* spp. and *E. coli* O157:H7. More than 10^4 colony forming units per gram (CFU/g) of these bacteria can be found in their excrement, and, thus, cattle excrement can be a source of environmental contamination (Omisakin et al., 2003). The horizontal transition of enteropathogens from an infected animal to a healthy one via excrement spraying, licking of each other, etc., can occur in herds of cattle (McGee et al., 2004).

E. coli O157 can be detected for a long period of time in the manure and soil; *Salmonella enterica* var. Typhimurium can survive for a long time in liquid manure as well (Himathongkham et al., 1999). After applying manure to the soil, *E. coli* O157:H7 and *Salmonella* spp. can survive from two weeks up to several months (Islam et al., 2004). The duration of enteropathogens carriage by animals and the subsequent survival of them in the excrement depend on the animals' genotype, their age (Cray and Moon, 1995), and their diet (Buchko et al., 2000).

Besides cattle, the other wild and domestic animals can be carriers and disseminators of enteropathogens (Rice et al., 2003). The main carriers of enteropathogens are rodents, particularly wild rabbits and rats (Nielsen et al., 2004).

In the overwhelming majority of studies related to the detection of dynamics of these microorganisms in nature, the investigators confined themselves to following their presence and number only in a particular natural substrate or habitat (Natvig et al., 2004). However, as has already been mentioned, there is a problem of microorganism movement from one habitat to another and, moreover, movement through several habitats along the chain and even with cycle formation, when the inoculation of these habitats occurs (Litvin et al., 1997; Semenov et al., 2004; Semenov et al., 2005). This variety of distribution of saprotrophic as well as enterotrophic microorganisms remains poorly studied, though evidence of the existence of such a cycle were found long ago, and the phenomenon itself is undoubtedly more important and interesting than the simple survival of a bacterium in one substrate (Mishustin et al., 1979; Bukharin and Litvin, 1997; Litvin et al., 1997). Thus, the detection of the fact of microorganisms' transition through different, but related habitats in the form of a cycle and the quantitative laws of these transitions is a fundamental as well as applied aspect of the problem.

The aim of this research was to study the population dynamics of saprotrophic bacteria and analogs of enteropathogenic bacteria, which were introduced to cattle with fodder with the following analysis and registration of them in animals' excrement, soil mixed with excrement, on the plants that were grown in this mixture, and in the excrement of other animals after feeding them these plants.

MATERIAL AND METHODS

Genetically marked bacteria that were able to synthesize green fluorescent protein (GFP) were the sub-

jects of the research: *S. enterica* var. Typhimurium MAE 110 *gfp* (*S.* Typhimurium *gfp*), received from Yu. Remling (microbiological center, Royal Institute, Stockholm, Sweden), *Escherichia coli* O157 : H7 *gfp*, received from A. Van Bruggen laboratory (Wageningen University, the Netherlands), *Pseudomonas fluorescens* 32 *gfp*, received from R. J. Saler (biological faculty, Arkansas University, United States). The investigations were performed with antibiotic resistant and avirulent strains (virulence genes were removed).

S. Typhimurium *gfp* were detected on the medium: yeast extract—5 g/l, bactopeptone—10 g/l, agar—17 g/l, distilled water—1 l, pH 7.2–7.4. The medium was sterilized at a high pressure of 0.5 atm for 30 min with antibiotics (nalidixic acid—50 mg/l). After autoclaving 50 mg/l of antibiotic kanamycin, sterilized by filtration, was added. *E. coli* O157:H7 *gfp* were detected on the medium: yeast extract—5 g/l, bactopeptone—10 g/l, NaCl—10 g/l, agar—17 g/l, distilled water—1 l, pH 7.2–7.4. The medium was sterilized at a high pressure of 0.5 atm for 30 min. After autoclaving 50 mg/l of antibiotic ampicillin, sterilized by filtration, was added. *P. fluorescens* 32 *gfp* was detected on the medium: bactopeptone—2 g/l; K_2HPO_4—1.4 g/l; $MgSO_4 \cdot 7H_2O$ —1.5 g/l; glycerol—15 ml/l; agar—17 g/l; distilled water—1 l; pH 7.0–7.2. The medium was sterilized at a high pressure of 0.5 atm for 30 min. After the autoclaving 50 mg/l of kanamycin and 50 mg/l of rifampicin, sterilized by filtration, were added.

The bacterial biomass for the introduction into the gastrointestinal tract of cattle was cultured in the same, but liquid media. Bacteria were cultured until the achievement of the initial stationary phase of growth (about 24 h for *S* Typhimurium *gfp* and 15 h for *E. coli* O157:H7 and *P. fluorescens* 32 *gfp*). The temperature of the bacteria cultivation was 37°C for *S.* Typhimurium *gfp* and *E. coli* O157 : H7 *gfp* and 25°C for *P. fluorescens* 32 *gfp*.

The experiments were devoted to tracing the survival of the studied bacteria during their passage through the gastrointestinal tract of one-year-old heifer cows of the black-motley breed.

To check the probability of survival of the studied bacteria during their passage through the rodents' gastrointestinal tract, common voles (*Microtus arvalis*) and cavies (*Cavia aperea*) were used.

Cattle excrement was collected on the KRS ZAO Sovkhoz Moskvoretskii farm (Odintsovskii raion, Moskovskaya oblast); the cattle excrement pH was 7.4. The cows' diet included green mass of 2.5 kg/animal/day, hay at 1 kg/animal/day, dry mixed fodder at 3 kg/animal/day, and salt for the green mass at 40 g/animal/day. Freshly collected excrement was used in the experiments. The initial humidity of excrement was 70% on average.

The tame sod-podzol soil was used. It was taken from the Moscow State University Botanical garden near (about 2 m) a recent sea-buckthorn (*Hippophae rhamnoides* L.) plantation from a depth of 0–10 cm. The soil was sifted through a sieve with a cell size of 2 mm, dried, and kept in a plastic bag at a room temperature. After the drying, the humidity was 6%. The soil contained 39.6 mg/g overall carbon, 2.87 mg/g overall nitrogen, 3.75 μg/g ammonia nitrogen, 85.5 μg/g nitrate nitrogen, and 17.7 μg/g phosphorus in the form of PO_4; the soil pH was 6.64. The soil factions (average in volume percent) are clay (11.65), sand (31.8), and silt particles (56.55).

In the experiments oat seeds were used (Avena sativa, Agrofirma Aelita, Moskovskaya oblast, germinating capacity 85%).

To determine the dynamics of the bacterial growth in the mixed fodder suspension, 12 g of the mixed fodder (barley, bran, wheat, rapeseed meal, sunflower cake, salt, and microelements) were mixed with 100 ml of unsterile tap water. Mixed fodder suspensions were inoculated to the corresponding bacteria at a rate of about 10^2 CFU/g of dry substance and incubated using a shaker at 250 rev./min for 30 h at 25°C for *P. fluorescens* 32 *gfp* and 37°C for *S.* Typhimurium *gfp* and *E. coli* O157 : H7 *gfp*. The samples were taken and counted for CFU in terms of the dry mixed fodder gram in dynamics.

Verification of the probability of bacteria survival during passage along the cattle gastrointestinal tract with the fodder was performed by feeding them 1.5 kg of preliminary steamed (500 g of water per 1 kg of the mixed fodder) and cooled mixed fodder, which contained 10^7 CFU/g of the dry fodder of the corresponding bacteria.

The experiment was performed three times with different cows, i.e., all in all 9 cows for each bacterium. The animals were fed in the morning; the mixed fodder was given in the feedboxes for each cow.

In 24 h after the inoculation the samples of cattle excrements were collected and the required bacteria were calculated by inoculation of 100 μl of the prepared suspension in each Petri dish with the selective growth medium and corresponding antibiotics. Dishes with enterobacteria were incubated at 37°C, and those with pseudomonades, at 25°C. The fluorescent colonies were detected and analyzed under a black-light lamp (220 V, 11 wt, PL-S, Philips, Eindhoven, the Netherlands). The amount of CFU was counted per 1 g of the dry substance. The humidity of samples was detected by drying them for several hours at 105°C.

To determine the dynamics of the bacteria survival in cattle excrement, 500 g samples of cattle excrement was put into plastic containers 0.7 l in volume. The containers were covered with a food plastic film and incubated in the dark at a continuous temperature of 18°C.

The moment of mixing of cattle excrement that contained the test bacteria with the soil was determined experimentally, i.e., after achievement of the

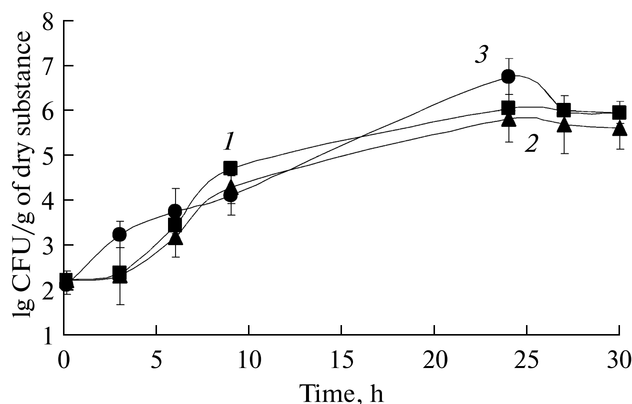

Fig. 1. Growth dynamics of the bacteria studied in the mixed fodder suspension. (*1*)—*S.* Typhimurium MAE 110 *gfp*; (*2*)—*E. coli* O157:H7 *gfp*; (*3*)—*P. fluorescens* 32 *gfp*.

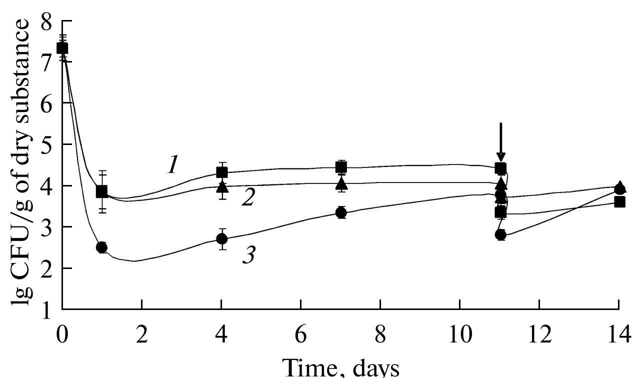

Fig. 2. Dynamics after the feeding of cattle of the bacteria studied in cattle excrement and the excrement–soil mixture. Arrow shows the time of mixing of cattle excrement with soil. (*1*) *S.* Typhimurium MAE 110 *gfp*; (*2*) *E. coli* O157:H7 *gfp*; (*3*) *P. fluorescens* 32 *gfp*.

quasi-stationary state (exit to the plateau) of the population density of the plants in the studied substrate.

Half of gram of oat seeds was sown in the containers with cattle excrement and the soil mixture containing the corresponding test bacterium. The containers were covered with a food plastic film to maintain humidity and kept under a daylight lamp. Seven days after the sprouts appeared, plants were counted in each pot, 1 g of plants were cut off (overground part of plants, 5–6 cm), and the studied bacteria were counted in the oat phylosphere by inoculation on the selective medium.

To determine the bacteria on the oat roots, the roots were carefully pulled out of the soil, soil was shaken off, and soil was washed off with water from 1 g of the rhizosphere. Then the bacteria were counted in the oat rhizosphere by inoculation in the selective medium.

To feed voles and cavies with oat plants, three voles and one cavy were put into sterile plastic containers. The animals were not fed 1 day prior to the experiment. One gram of oats, which was cut off in the same container from which the number of bacteria was detected in the phylosphere and the rhizosphere, was added into each container. In 4–5 h and after 24 h, collection of the voles and cavy excrement was performed. The amount of CFU/g excitements was detected by the inoculation method, which was repeated three times.

RESULTS

The most evident type of infection of animals with microorganisms is receipt of microorganisms with fodder and, particularly, with a mixed fodder. Mixed fodders are given to animals in a different form, as dry, moist, or sometimes quite watery suspension. We studied the growth dynamics of two enteropathogen analogs and one saprotroph bacterium in the mixed fodder suspension (Fig. 1). The initial inoculation dose for all bacteria during introduction into the mixed fodder suspension was very low (10^2 of cells per gram of the dry mixed fodder).

S. Typhimurium and *E. coli* had a short lag period, while *P. fluorescens* 32 *gfp* did not have one. The traditional growth curve with the exit to the steady state in a day was shown for almost all bacteria with some variations. By this time the number of cells of corresponding bacteria was 1.2×10^6 for *S.* Typhimurium *gfp*, 5.8×10^6 for *P. fluorescens* 32 *gfp*, and 6.9×10^5 for *E. coli* O157 : H7 *gfp* per gram of the dry mixed fodder.

Thus, the mixed fodder is a suitable substrate for bacteria growth. In everyday practice the conditions similar to the previously described experiment appear quite often and, therefore, active increase of the number of bacteria in the fodder will occur, and due to feeding animals with such fodder, the infection of these animals will happen.

To determine the number of the labeled bacteria in the cattle excitement and in the excrement and soil mixture, the collection of the cattle excrement was performed once every 24 h after feeding. In freshly collected samples 7.0×10^3, 3.2×10^2, and 7.1×10^3 CFU/g of dry substance for *S.* Typhimurium *gfp*, *P. fluorescens* 32 *gfp*, and *E. coli* O157 : H7 *gfp*, respectively, were found. The collected excrement was incubated for 11 days in pots (Fig. 2). During the incubation of excrement some increase of the number of labeled bacteria occurred, and in 11 days it became 2.5×10^4, 5.9×10^3, and 1.1×10^4 CFU/g of dry substance for *S.* Typhimurium *gfp*, *P. fluorescens* 32 *gfp*, and *E. coli* O157 : H7 *gfp*, respectively.

After the detected stabilization of the *gfp*-bacteria, the cattle excrement was mixed with soil in a 1 : 16 ratio according to the dry substance. The number of bacteria after mixing was 2.2×10^3, 6.4×10^2, and 5.2×10^3 CFU/g of dry substance for *S.* Typhimurium *gfp*, *P. fluorescens* 32 *gfp*, and *E. coli* O157 : H7 *gfp*, respectively (Fig. 3).

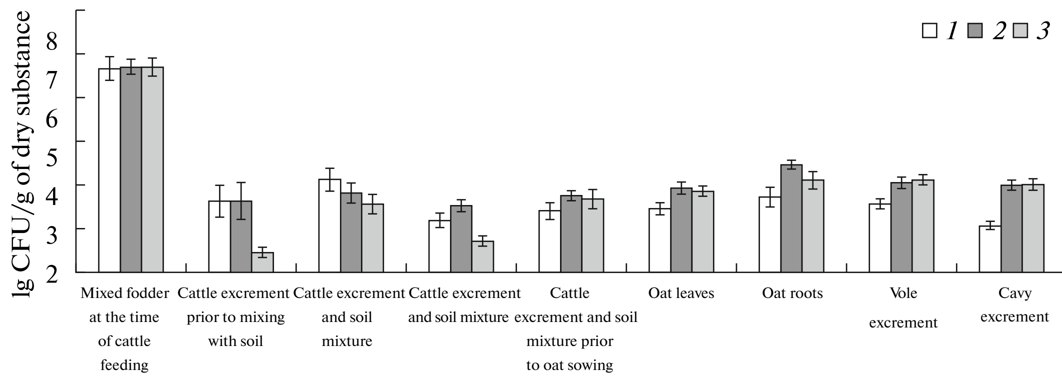

Fig. 3. Survival of the bacteria studied in a series of natural substrates after the feeding of cattle. (*1*) *S.* Typhimurium MAE 110 *gfp*; (*2*) *E. coli* O157:H7 *gfp*; (*3*) *P. fluorescens* 32 *gfp*.

To determine the number of *gfp*-bacteria on the plants, oats were sown in the mixture of the cattle excrement with soil three days after the mixing. Before the oat sowing, the number of bacteria in the excrement and soil mixture was 3.8×10^3, 8.0×10^3, and 9.5×10^3 CFU/g of dry substance for *S.* Typhimurium *gfp*, *P. fluorescens* 32 *gfp*, and *E. coli* O157 : H7 *gfp*, respectively. Seven days after the emergence of seedlings, the number of bacteria in the philosphere and rhizosphere of oat germs was calculated.

On the surface of oat plants 4.4×10^3, 1.3×10^4, and 1.5×10^4 CFU/g of dry leaves were found for *S.* Typhimurium *gfp*, *P. fluorescens* 32 *gfp*, and *E. coli* O157 : H7 *gfp*, respectively, and in the rhizosphere, 8.9×10^3, 2.4×10^4, and 6.2×10^4 CFU/g of dry roots were detected for *S.* Typhimurium *gfp*, *P. fluorescens* 32 *gfp*, and *E. coli* O157 : H7 *gfp*, respectively (Fig. 3).

To determine bacteria survival during their passing through the gastrointestinal tract of rodents, they were fed the infected oat plants. One part of oat plants was given to voles, and the other, to cavies. In vole excrement 5.8×10^3, 2.5×10^4, and 2.1×10^4 CFU/g of dry excrement were found for *S.* Typhimurium *gfp*, *P. fluorescens* 32 *gfp*, and *E. coli* O157:H7 *gfp*, respectively. In cavy excrement 1.6×10^3, 1.9×10^4, and 1.8×10^4 CFU/g of dry excrement were observed for *S.* Typhimurium *gfp*, *P. fluorescens* 32 *gfp*, and *E. coli* O157 : H7 *gfp*, respectively (Fig. 3).

DISCUSSION

The results of this investigation concern different aspects of microorganisms' ecology and epidemiology, as well as a problem of the food industry. It was shown that the mixed fodder can be a favorable substrate for bacteria reproduction, including pathogenic, and lead to infection of farm animals. With the infection of the mixed fodder suspension with a very small amount of bacteria, their number can reach a considerable density from 10^5 to 10^6 CFU/g of dry mixed fodder in a day, which is more than enough to infect cattle (Fig. 1). The appearance of the mixed fodder and other fodder in the drinking bowls during feeding is a quite common event. There are published data about *E. coli* O157 survival for at least 245 days in the sediment of animal drinking bowls (McGee et al., 2004).

Appearing with fodder in the animals' gastrointestinal tract, bacteria pass through the physical–chemical and biological barriers and appear in the environment. In the present experiments, we incubated cattle excrement for only 11 days after its collection with the studied bacteria. During this time there was a stable number of *E. coli* and salmonella or even a marked increase in the number of pseudomonades (Fig. 2). These results, concerning the stabilization of the population number at some stage after their introduction, mainly coincide with the results of our previous experiments, where we introduced high numbers of these bacteria directly into cattle excrement and followed the dynamics of their survival (Kupriyanov, et al., 2009). In those experiments after some decrease in the number of bacteria, stabilization in the number of bateria also occurred, which is in agreement with common laws for saprotrophic bacteria determined by other researchers (Kozhevin, 1989).

The animals' excrement, which contain bacteria, including pathogenic, appear in the soil, water, and more often on the plants, which are fodder for other graminivorous animals and thereby they finish the cycle. So, it is quite possible to talk about the existence of rotation or microorganism cycle, and not only about the biogeochemical cycles of elements. The other scientists were very close to this conclusion before, but they did not state it directly due to the absence of valuable experiments, such as those performed by us (Litvin et al., 1997).

We showed that just one and the same bacterial population can pass through the whole cycle without the introduction of the same population additionally from outside, though the possibility of passing additional inoculation of one natural substrate or another can realistically be suggested. At the same time in some niches, particularly on the plants and in the rodents' gastrointestinal tract, though not so signifi-

cant, a quite evident increase of the number of *E. coli* O157 : H7 *gfp* and pseudomonades, unlike salmonella, occurs. This interesting fact undoubtedly requires further investigations, particularly taking into account the following two important aspects. Firstly, during the survival of enteropathogens in the gastrointestinal tract of rodents, taking into consideration the high mobility of them, there is a danger of contamination of large areas with these bacteria. Secondly, there is a very high mortality due to infection with the enterohemorrhagic strain of *E. coli* O157:H7.

ACKNOWLEDGMENTS

This study was supported by NWO—Russia collaborative grants (Dossier no. 047.014.001 and 047.0147.011) (A.H.C. Van Bruggen and A.M. Semenov).

REFERENCES

Brackett, R.E., Incidence, Contributing Factors, and Control of Bacterial Pathogens in Produce, *Postharvest Biol. Technol.*, 1999, vol. 15, pp. 305–311.

Buchko, S.J., Holley, R.A., Olson, W.O., et al., The Effect of Fasting and Diet on Fecal Shedding of *Escherichia coli* O157:H7 by Cattle, *Can. J. Anim. Sci.*, 2000, vol. 80, pp. 741–744.

Bukharin, O.V. and Litvin, V.Yu., *Patogennye bakterii v prirodnykh ekosistemakh* (Pathogenic Bacteria in Natural Ecosystems), Yekaterinburg: Izd. UrO RAN, 1997.

Cray, W.C. and Moon, H.W., Experimental Infection of Calves and Adult Cattle with *Escherichia coli* O157:H7, *Appl. Environ. Microbiol.*, 1995, vol. 61, pp. 1586–1590.

Himathongkham, S., Bahari, S., Riemann, H., and Cliver, D., Survival of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in Cow Manure and Cow Manure Slurry, *FEMS Microbiol. Letts.*, 1999, vol. 178, pp. 251–257.

Islam, M., Doyle, M.P., Phatak, S.C., et al., Persistence of Enterohemorrhagic *Escherichia coli* O157:H7 in Soil and on Leaf Lettuce and Parsley Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water, *J. Food Prot.*, 2004, vol. 67, pp. 1365–1370.

Kozhevin, P.A., *Mikrobnye populyatsii v prirode* (Microbial Populations in Nature), Moscow: Mosk. Gos. Univ., 1989.

Kupriyanov, A.A., Semenov, A.M., Kunenkova, N.N., et al., Multistep Introduction of Bacteria to Natural Substrates at Different Initial Inoculation Doses, *Pochvovedenie*, 2009, no. 9, pp. 1–7.

Litvin, V.Yu., Gintsburg, A.L., Pushkareva, V.I., et al., *Epidemiologicheskie aspekty ekologii bakterii* (Epidemiological Aspects of Bacterial Ecology), Moscow: Farmarus-Print, 1997.

McGee, P., Scott, L., Sheridan, J.J., et al., Horizontal Transmission of *Escherichia coli* O157:H7 during Cattle Housing, *J. Food Prot.*, 2004, vol. 67, pp. 2651–2656.

Mishustin, E.N., Pertsovskaya, M.I., and Gorbov V.A., *Sanitarnaya mikrobiologiya pochvy* (Sanitary Microbiology of Soil), Moscow: Nauka, 1979.

Natvig, E.E., Ingham, S.C., Ingham, B.H., et al., *Salmonella enterica* Serovar *typhimurium* and *Escherichia coli* Contamination of Root and Leaf Vegetables Grown in Soils with Incorporated Bovine Manure, *Appl. Environ. Microbiol.*, 2004, vol. 68, pp. 2737–2744.

Nielsen, E.M., Skov, M.N., Madsen, J.J., et al., Verocytotoxin-Producing *Escherichia coli* in Wild Birds and Rodents in Close Proximity to Farms, *Appl. Environ. Microbiol.*, 2004, vol. 70, pp. 6944–6947.

Omisakin, F., MacRae, M., Ogden, I.D., and Strachan, N.J.C., Concentration and Prevalence of *Escherichia coli* O157 in Cattle Faeces at Slaughter, *Appl. Environ. Microbiol.*, 2003, vol. 69, pp. 2444–2447.

Rangel, J.M., Sparling, P.H., Crowe, C., et al., Epidemiology of *Escherichia coli* O157 : H7 Outbreaks, United States, 1985–2002, *Emerg. Infect. Dis.*, 2005, vol. 11, pp. 603–609.

Rice, D.H., Hancock, D.D., and Besser, T.E., Faecal Culture of Wild Animals for *Escherichia coli* O157:H7, *Vet. Rec.*, 2003, vol. 152, pp. 82–83.

Semenov, A.M., Trophic Grouping and Dynamics of Development of Microbial Communities in Soil and Rhizosphere, *Extended Abstract of Doctoral (Biol.) Dissertation*, Moscow: Mosk. Gos. Univ., 2005.

Semenov, A.M., Van Bruggen, A.H.C., Kunenkova, N.N., et al., Experiments for the Risk Analysis of the Spread of Enteropathogens in the Environment within the Microbial Cycle, *Proc. Sec. Int. Conf. "Biotechnology—To Environm. Preserv.,"* Part II, Moscow: Sport, Culture, 2004, P. 163–168.