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Influence of feed additives used in prevention of coccidiosis on the quality of enteric microflora of fast-growing and slow-growing chickens[1]

Wpływ wybranych dodatków paszowych stosowanych w profilaktyce kokcydiozy na jakość mikroflory jelit kurcząt szybko i wolno rosnących

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Keywords: slow- and fast-growing chickens, coccidiostats, enteric microflora
Słowa kluczowe: kurczęta wolno i szybko rosnące, kokcydiostatyki, mikroflora jelit

Abstract
The experiment was conducted on 480 Hubbard Flex chickens (fast-growing) reared to 42 days of age and 480 Hubbard JA 957 chickens (slow-growing) reared to 63 day of age. Day-old chicks were randomly assigned to the three following groups according to the type of coccidiostat: C (control - no coccidiostat in the diet and birds not vaccinated against coccidiosis), A (plant coccidiostat adiCox® AP), and M (monensin coccidiostat). At the end of rearing period the results of the controlled production were presented, the chickens were slaughtered and samples of their intestines were collected for microflora composition analyses.
The obtained results show that rearing time influenced the composition of enteric microflora (small intestine and blind gut). Moreover, a higher total count of bacteria was stated in intestinal digesta of the slow-growing chickens that were kept for three weeks longer than the Hubbard Flex chickens.
The study also proved a positive influence of the diet on the quantitative composition of enteric microflora. The lowest count of mesophilic bacteria and those from the *Enterobacteriaceae* family was observed in the chickens receiving adiCox® AP compared to the chickens of the control group and those receiving monensin.

Streszczenie
Doświadczenie przeprowadzono na 480 kurczętach szybko rosnących Hubbard Flex, które utrzymywano do 42 dnia życia i 480 kurczętach wolno rosnących Hubbard JA 957, odchowywanych do 63 dnia życia. Jednodniowe pisklęta przydzielono losowo do 3 grup żywieniowych w 4 powtórzeniach: K (kontrolna – nie zawierała kokcydiostatyku w diecie i kurczęta nie były szczepione przeciwko kokcydiozie), A (kokcydiostatyk roślinny – adiCox® AP), M (kokcydiostatyk w paszy – monenzyna). Po zakończeniu odchowu, skontrolowano wyniki produkcyjne oraz od kurcząt z każdej grupy pobrano wycinki jelit do dalszych badań mikrobiologicznych. Wykazano wpływ czasu odchowu kurcząt na skład mikroflory treści jelita cienkiego i ślepego; ponadto stwierdzono większą ogólną liczebność bakterii w treści jelit kurcząt wolno rosnących, które utrzymywane były dłużej o 3 tygodnie, w porównaniu z kurczętami Hubbard Flex.
Zaobserwowano pozytywny wpływ diety na stan ilościowy mikroflory jelita cienkiego. Najmniejszą liczebność bakterii mezofilnych oraz z rodziny *Enterobacteriaceae* wykazano w grupie otrzymującej w diecie preparat roślinny, w porównaniu z kurczętami z grupy kontrolnej i otrzymującej monenzynę.

1. INTRODUCTION

Coccidiosis of poultry is a parasitic disease induced by protozoa of the genus *Eimeria*. Birds become infected through the ingestion of oocysts with contaminated feed and water. The optimal conditions for oocysts growth include temperature of 25°C and humidity of 31–62%. They may be transmitted mechanically by wild fowl, insects, rodents, contaminated shoes, clothes, appliances or dust [Gussem 2007; McDougald, 2003].
Oocysts are resistant to environmental changes and to disinfectants (they are capable of surviving for many months in litter). In contrast, they are negatively influenced by temperatures above 56°C and below 0°C, but still these temperatures do not cause their complete destruction [McDougald, 2003].
Coccidia are capable of rapid multiplication inside cells that coat intestines. Damage of tissues as a result of their proliferation causes a reduced feed intake and poorer absorption of nutrients, lesser body weight gains, dehydration, blood loss and increased susceptibility to infections [Hafez, 2008; Elmusharaf and Beynen, 2007].
The qualitative and quantitative composition of enteric microflora depends on many factors, e.g. age of birds, maintenance conditions, environmental stress, composition and processing method of feed mixtures, and feed additives applied (probiotics, prebiotics, synbiotics, coccidiostats, enzymes).
The prevention of hazards posed by the impaired functioning of the gastrointestinal tract includes the application of probiotics, prebiotics, synbiotics, organic acids and phytobiotics.
The aim of this research was to determine the effect of a ionophore coccidiostat and a natural plant preparation on the quality of enteric microflora of Hubbard Flex and Hubbard JA 957 broiler chickens.

[1] This study was carried out as part of research project no. NN311 405239 MNiSW in the years 2010–2012.

2. MATERIAL AND METHODS

The experiment was conducted at the experimental station of the Warsaw University of Life Sciences (RZD Wilanów – Obory) in the springtime of 2010. The experimental material were fast-growing Hubbard Flex chickens (480 pc.), reared until 42 days of life, and 480 slow-growing Hubbard JA 857 chickens, reared until 63 days of life. Starting from the first week, weekly measurements were conducted of husbandry conditions in the facility the birds were reared. In-house temperature, air relative humidity and concentration of gases (CO_2, NH_3 and H_2S) were measured. A three-stage feeding program was applied throughout the rearing period.

One-day chicks were randomly allocated to the 3 feeding groups: C, A and M, 160 birds each, in 4 replications × 40 birds. A differentiating factor of diets was the content of a complementary loose feed mixture containing a plant coccidiostat (adiCox® AP – A) with the following composition: hardened palm oil, paprika fruit, seeds of white mustard; sensory additives (herbs 98%, thymol 0.5%), or a ionophore coccidiostat (monoensin – M). In the control group (C), no coccidiostat was added to diet and the birds were not vaccinated against coccidiosis. In both groups, stock density in a pen reached 15 birds per m^2. At the end of rearing period the results of controlled production were presented. On day 42 and 63 of rearing, 12 cocks and 12 hens with body weights similar to the mean body weight in a group, were selected from each feeding group (C, A, M), which gave a total of 144 birds. The chickens were slaughtered in a poultry abattoir, and their small intestines and ceca were collected for microbiological analyses.

The coproscopic method of feces analysis, i.e. floatation method, was applied in the study. This method was proposed by Fullebron in the 1920s for diagnosis of the invasion of gastrointestinal tract parasites including protozoa and most of nematodes. It is based on the phenomenon of floatation of light oocysts, cysts or eggs of parasites on the surface of a high-density fluid [Gundłach, 1995].

A sample of feces (ca. 0.5 g) was placed in a test tube with a small volume of saturated NaCl solution. The sample was mixed with a glass rod until achieving homogenous suspension, and filled up with the NaCl solution in order to obtain convex meniscus. Next, the test tube was covered with a cover glass and left for 15-20 min. Afterwards, the glass was taken off and placed on a microscopic slide [Gundłach, 2004, Ziomko, 1999]. Then, the preparation was observed under a microscope, beginning from the minimal 4× magnification, followed by 10× to the final 40× magnification.

In some cases, centrifugation was applied (MPW-310 centrifuge with cooling, 3000 rpm, 5 min) in order to accelerate the floatation of eggs, oocysts and cysts onto the surface.

Samples of feces were collected in the morning hours in special plastic containers. Until analyses, the samples were kept in a refrigerator at a temperature of +4°C (for 24 h).

Qualitative analyses were carried out in order to determine the total bacteria count in intestines of broiler chickens.

In turn, qualitative analyses were conducted with the use of Bergey's Manual of Systematic Bacteriology [Brenner et al. 2005 ; De Vos et al. 2009] in order to identify bacteria isolated from intestinal digesta of birds to the *Enterobacteriaceae* and *Lactobacillaceae* families.

The quantitative analyses of bacteria in intestinal digesta of the chickens were carried out in sterile conditions, on a 0.1 g weighed sample of small intestine digesta and a 0.1 g weighed sample of cecum digesta. The samples were suspended in 100 ml of sterile distilled water, which enabled reaching 10^{-3} dilution. Subsequent dilutions were prepared using sterile tips, by transferring 10 ml of the suspension from the 10^{-3} dilution into a bottle with 90 ml of sterile distilled water, thus obtaining 10^{-4} dilution. Further serial dilutions were prepared following the same procedure. Then, 10 ml of culture medium (nutrient agar, Endo and Sabouraud) were poured onto sterile Petri dishes and 0.1 ml of the suspension of the earlier prepared dilution was instilled with sterile tips on each dish with the medium. The suspension was spread with a sterile bacteria spreader until the first resistance. Each of the samples was inoculated in three replications and incubated for 24–72 h, under aerobic conditions, at a temperature of 30°C. Afterwards, the grown bacterial colonies were counted. In order to achieve microbial count per 1 ml, results were multiplied by the converse of the dilution of a suspension that was instilled on Petri dish, and the result was multiplied by 10, according to the following formula:

Y = X × converse of dilution × 10, where:
Y – total bacteria count; X – number of colonies on Petri dish.

Results correspond to the number of CFU (colony forming units) per 1 g of fresh weight of intestinal digesta. The results were then expressed on dry matter basis and as values of decimal logarithms. In order to isolate pure cultures and to identify bacteria isolated from enteric digesta, several reductive cultures were prepared as follows: for *E. coli* bacteria from the *Enterobacteriaceae* family on nutrient agar Columbia Agar (with 5% addition of defibrinated ram blood), McConkey, Endo and Sabouraud medium for bacteria of the genus *Lactobacillus* from the *Lactobacillaceae* family. Isolates of strains were cultured onto slants with an appropriate agar medium and stored at a temperature of 4°C. Selected isolates were subjected to morphological examinations in terms of colonies appearance, including colour, size, shape, surface, and consistency. Next, microscopic slides were prepared by staining the isolated bacterial cultures with the Gram method. Microscopic observations were conducted with a Nikon E 600 light microscope coupled with a digital camera. The qualitative evaluation of bacterial cultures was carried out with the use of selected biochemical tests for the *Enterobacteriaceae* family (GN-ID A+B PANEL for *E. coli*) and for the *Lactobacillaceae* family, based on Bergey's Manual of Systematic Bacteriology [Brenner et al. 2005; De Vos et al. 2009].

Statistical computations were carried out with SPSS 19.0 statistical package (2010). Normality of traits distribution was verified with the Kołmogorow–Smirnow test. Distributions of all traits differed from the normal distribution. After logarithmic transformation (decimal logarithm), distributions of traits (apart from results of the total count of mesophilic bacteria grown on nutrient agar) did not differ from normal distribution. Differences between groups and genetic lines were determined with the use of two-way analysis of variance with interaction – GLM procedure.

3. RESULTS AND DISCUSSION

The highest body weight on day 42 and 63 was recorded in birds who were administered the feed supplement adiCox® AP (2548 g and 3582 g, respectively) (Table 1). Bitter substances, terpenes and phenolic acids stimulate the production of digestive juices rich in endogenous enzymes, which could contribute to the weight gain recorded in group A. The intake of feed in both experimental groups was comparable with the control group. However, there was a tendency towards a higher feed intake in group A in birds with the highest body weight at the end of rearing. The statistical analysis demonstrated significant differences ($P \leq 0.05$) in mortality between the fast-growing and slow-growing chickens. The lowest mortality rate was recorded in chicken broilers that were administered the feed mixture adiCox® AP, while the highest one was recorded in the birds of the control group (Table 1).

Table 1. Productivity parameters in the fast-growing (6 weeks) and slow-growing chickens (9 weeks).

Group	Hubbard Flex			Hubbard JA 957		
	BW (g)	Mortality (%)	Feed conversion ratio kg/kg BW	BW (g)	Mortality (%)	Feed conversion ratio kg/kg BW
C	2477^{A}	5.72^{a}	1.55	3543	4.33^{a}	2.01
A	2548^{B}	1.82^{b}	1.60	3582	2.60^{b}	2.22
M	2541^{B}	3.38^{b}	1.50	3526	3.16^{b}	2.00
SE	145.6	1.1	0.7	145.6	1.1	0.7

$^{A, B}$ $P \leq 0.01$; $^{a, b}$ $P \leq 0.05$

The results indicate the feasibility of supplementing feed mixtures with natural plant extracts acting as plant coccidiostats without compromising the productivity of birds. In the chickens from group A (plant preparation), there was even an increase in the efficacy of rearing indicated by a reduced mortality rate, which points to prospective economic benefits of this product administration.

Weekly analyses of feces, collected every day from birds of all groups, did not demonstrate the presence of *Eimeria* family parasites. The diets were found to influence the composition of microflora of small intestine and cecum digesta at the end of rearing of both Hubbard Flex (day 42) and Hubbard JA 957 (day 63) broiler chickens. Respective results are presented in Tables 2 and 3.

A higher bacteria count was demonstrated in intestinal digesta of the slow-growing chickens that were reared for 3 weeks longer compared to the Hubbard Flex birds (Tab. 2 and 3). This difference was statistically significant ($P \leq 0.01$ or $P \leq 0.05$) in all feeding groups, apart from the number of *Enterobacteriaceae* family bacteria in the control group.

Table 2. The composition of small intestine microflora of Hubbard Flex and Hubbard JA 957 chickens.

Bacteria	Group	Hubbard Flex		Hubbard JA 957		P between genotypes
		Mean	SE	Mean	SE	
Mesophiles grown on nutrient agar	C	1.2×10^{6}	1.90×10^{6}	$3.3 \times 10^{9\,A}$	5.8×10^{9}	0.000
$P_1 = 0.353$	A	0.8×10^{6}	1.28×10^{6}	$0.3 \times 10^{9\,C}$	7.5×10^{9}	0.000
$P_2 = 0.000$	M	1.4×10^{6}	3.76×10^{6}	$0.4 \times 10^{9\,B}$	1.0×10^{9}	0.000
Enterobacteriaceae	C	$2.1 \times 10^{6\,A}$	6.14×10^{6}	$2.2 \times 10^{9\,a}$	4.2×10^{6}	0.863
$P_1 = 0.008$	A	$0.3 \times 10^{6\,C}$	4.67×10^{6}	$1.1 \times 10^{9\,c}$	3.7×10^{8}	0.000
$P_2 = 0.017$	M	$0.7 \times 10^{6\,B}$	2.17×10^{6}	$1.8 \times 10^{9\,b}$	6.8×10^{8}	0.040
Lactobacillaceae	C	0.9×10^{6}	3.09×10^{6}	$0.2 \times 10^{9\,c}$	9.8×10^{9}	0.000
$P_1 = 0.173$	A	1.1×10^{6}	3.95×10^{6}	$9.1 \times 10^{9\,a}$	9.8×10^{9}	0.000
$P_2 = 0.022$	M	1.3×10^{6}	1.78×10^{6}	$2.2 \times 10^{9\,b}$	3.5×10^{9}	0.000

Values denoted with different letters A, B, C – differ significantly in columns at $P \leq 0.01$;
Values denoted with different letters a, b, c – differ significantly in columns at $P \leq 0.05$;
P_1 – within a group of nutrition, Hubbard Flex
P_2 – within a group of nutrition, Hubbard JA 957

Table 3. The composition of cecal microflora of Hubbard Flex and Hubbard JA 957 chickens.

Bacteria	Group	Hubbard Flex		Hubbard JA 957		P between genotypes
		Mean	SE	Mean	SE	
Mesophiles grown on nutrient agar	C	$6.3 \times 10^{8\,A}$	1.42×10^{8}	$0.3 \times 10^{9\,C}$	8.5×10^{9}	0.000
$P_1 = 0.000$	A	$0.7 \times 10^{8\,C}$	1.1×10^{8}	$0.4 \times 10^{9\,B}$	6.2×10^{9}	0.000
$P_2 = 0.000$	M	$1.8 \times 10^{8\,B}$	4.8×10^{8}	$1.8 \times 10^{9\,A}$	2.3×10^{9}	0.000
Enterobacteriaceae	C	$6.2 \times 10^{8\,A}$	1.5×10^{8}	$1.6 \times 10^{9\,b}$	3.6×10^{6}	0.019
$P_1 = 0.002$	A	$3.6 \times 10^{8\,B}$	1.4×10^{8}	$0.2 \times 10^{9\,c}$	4.5×10^{8}	0.003
$P_2 = 0.041$	M	$1.1 \times 10^{8\,C}$	1.8×10^{8}	$2.0 \times 10^{9\,a}$	5.0×10^{8}	0.002
Lactobacillaceae	C	$9.5 \times 10^{8\,b}$	1.1×10^{8}	1.1×10^{9}	3.4×10^{9}	0.000
$P_1 = 0.029$	A	$10 \times 10^{8\,a}$	1.5×10^{8}	9.3×10^{9}	2.4×10^{9}	0.001
$P_2 = 0.826$	M	$5.5 \times 10^{8\,c}$	7.4×10^{8}	6.3×10^{9}	1.0×10^{9}	0.000

Values denoted with different letters A, B, C – differ significantly in columns at $P \leq 0.01$;
Values denoted with different letters a, b, c – differ significantly in columns at $P \leq 0.05$;
P_1 – within a group of nutrition, Hubbard Flex
P_2 – within a group of nutrition, Hubbard JA 957

The genotype × diet interaction (P = 0.026) was observed for bacteria of the family *Lactobacillaceae* present in small intestine digesta. The diet, applied to birds, was found to influence the total count of small intestine microflora. The lowest number of mesophilic bacteria and those from the *Enterobacteriaceae* family was demonstrated in the group receiving the plant coccidiostat in diet, compared to the birds from the control group and those receiving monoensin. The group that was administered the plant coccidiostat was characterized by a decrease in the count of *Enterobacteriaceae* family bacteria and, simultaneously, by a beneficial increase in the count of *Lactobacillaceae* family bacteria. In the case of Hubbard JA 957 chickens, statistically significant differences were demonstrated across the feeding groups in the total bacteria count, whereas in the case of Hubbard Flex chickens difference was noted only in the count of *Enterobacteriaceae* family bacteria. Investigations by Michalczuk et al. [2008] showed a positive, stimulating effect of a plant preparation (cinnamon, oregano and red paprika) on the status of enteric microflora and on the increase in the count of lactic acid bacteria able to reduce colonization of pathogenic bacteria. Active substances of cinnamon and oregano possess antioxidative properties, which assures the protection of intestinal villi. Owing to this protection, the size of villi does not increase, which results in the greater surface available for nutrients absorption. Also the study by Giannenas et al. [2003] confirmed the positive effects of applying a plant extract from oregano in a feed mixture at a dose of 300 mg/kg on reduced damage of intestines and excretion of oocyte in chickens infected with *E. tenella*.

Statistically significant differences were noted in the count of mesophilic bacteria grown on the nutrient agar (P ≤ 0.01) and in the count of bacteria of the *Enterobacteriaceae* family (P ≤ 0.05) in cecal digesta of Hubbard JA 957 chickens, depending on the diet applied.

Investigations by Idoui et al. [2009] demonstrated the antagonistic activity of different strains of *Lactobacillus* sp., that contributed to growth inhibition of bacteria of the *Enterobacteriaceae* family. In turn, Messaoudi et al. [2011] showed that bacteria of the *Lactobacillus* sp., present in digesta of small intestine and cecum of chickens as a result of their being administered with feed mixtures such as probiotics, were antagonistic against pathogenic bacteria, e.g. those belonging to *Salmonella* and *Campylobacter* genera.

In cocciodiosis prophylaxis, the greatest interest amongst feed additives is observed for herbal extracts. Investigators have already proved the coccidiostatic effects of sweet sagewort (*Artemisia annua*), administered to birds in the form of dried leaves [Allen et al. 1997], an extract from grape stones inhibiting the growth of *E. tenella* [Wang et al. 2008], and popular coneflower (*Echinacea*), affecting a reduction in pathologic lesions in intestines of chickens [Allen, 2003].

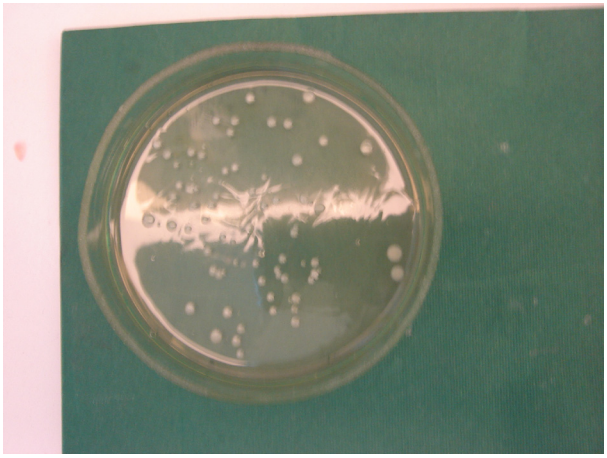

Photo 1. The colonies of lactic acid *Lactobacillus* sp. bacteria from *Lactobacillaceae* family, grown in Sabouraud's medium after 2 hours of incubation.

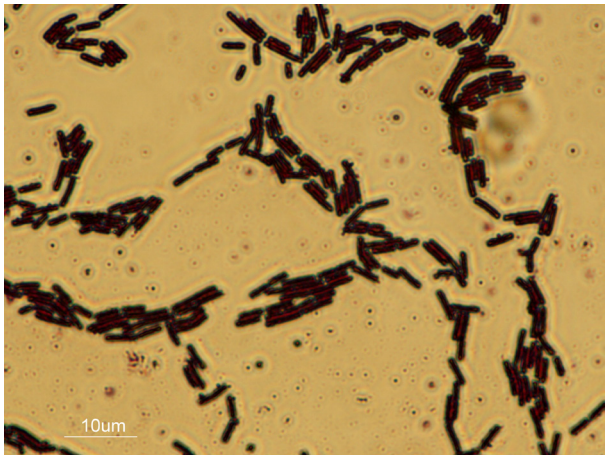

Photo 2. *Lactobacillus* sp. bacteria grown on Sabouraud's medium after 72 hours of incubation; Gram-stained preparation, 1100× magnification.

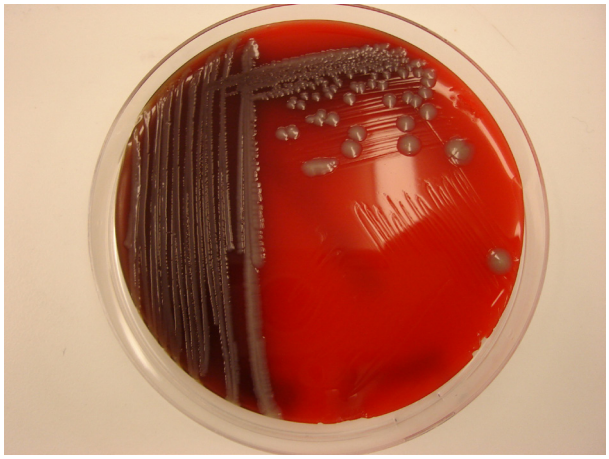

Photo 3. The colonies of *Escherichia coli* from *Enterobacteriaceae* family, grown after 72 hours on Columbia Agar medium.

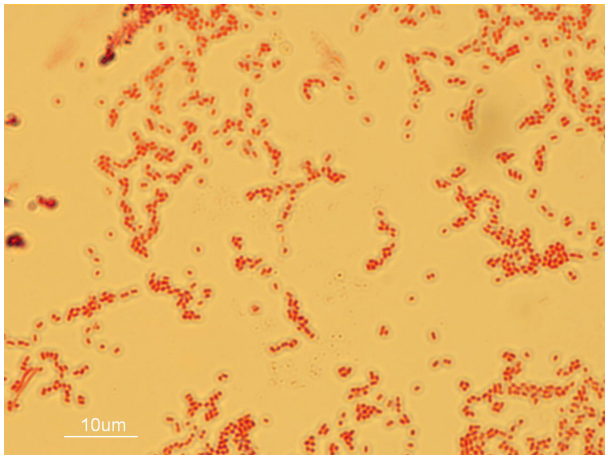

Photo 4. *Escherichia coli* bacteria grown on Endo medium after 48 hours of incubation; Gram-stained preparation, 1100× magnification.

4. CONCLUSIONS

1. No parasites of the genus Eimeria were detected in feces of the chickens.
2. Rearing time affected the composition of microflora in small intestine and cecum digesta of Hubbard Flex and Hubbard JA 957 chickens. A higher total count of bacteria was stated in intestinal digesta of the slow-growing chickens that were kept for three weeks longer than the Hubbard Flex chickens.
3. The diets influenced the total count of microflora in the small intestine. The lowest number of mesophilic bacteria and those from the *Enterobacteriaceae* family was observed in the chickens receiving the plant preparation, compared to the chickens from the control group and those receiving monensin.
4. The group that was administered the plant preparation was characterized by a decrease in the count of the *Enterobacteriaceae* family bacteria and by a beneficial increase in the count of the *Lactobacillaceae* family bacteria.

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