ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 3, pp. 373–379. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © Ya.E. Lyapunov, R.Z. Kuzyaev, R.G. Khismatullin, O.A. Bezgodova, 2008, published in Mikrobiologiya, 2008, Vol. 77, No. 3, pp. 421–428.

EXPERIMENTAL ARTICLES

Intestinal Enterobacteria of the Hibernating *Apis mellifera mellifera* L. Bees

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Received March 05, 2007

Abstract—Dynamics of enterobacteria of normal intestinal microflora was studied in *Apis mellifera mellifera* L. bees hibernating under snow in the Western Urals. The cell numbers (*N*) of the predominant species *Klebsiella oxytoca* increased from 10–10^6 CFU/bee in November 2004 to 10^4–10^7 CFU/bee in March 2005; its frequency of occurrence (*P*) increased from 92 to 100%. Increase of *Providencia rettgeri* (11.2004: *N* up to 10^6, P 25%; 03.2005: *N* 10^2–10^6, *P* 80%) was accompanied by the substitution of *Morganella morganii* (11.2004: *N* up to 10^6, *P* 25%) with *Proteus vulgaris* (03.2005: *N* up to 10^5, *P* 8%). By spring, *Hafnia alvei* and *Citrobacter* sp., which are pathogenic to bees, disappeared (11.2004: *N* up to 10^5, *P* 13 and 10%, respectively). Endophytic species *Pantoea agglomerans, Leclecria* sp., and other representatives of the "*Enterobacter agglomerans"* group were present in November and after the first emergence in spring (*N* up to 10^5; November: *P* 15%; April: *P* 23%). In April, the number of enterobacteria decreased to 10^5, and *P. rettgeri* became the predominant species (*P* 54%) instead of *K. oxytoca* (*P* 43%).

Key words: enterobacteria, microflora, intestine, number, frequency of occurrence, endophytes.

DOI: 10.1134/S0026261708030181

For evident reasons, including historical ones, the normal physiology of humans and animals receives much less attention than medicine and veterinary science. In spite of the century-longs history of research in the natural microflora of healthy bees [1], our knowledge is still incomplete and unclear. This statement is true for the members of *Enterobacteriaceae*, although these bacteria play a significant role both in the etiology of bacterial diseases in honey bees [2] and in the microbiological quality of the pollen pellets, the apicultural product second to honey in production quantity [3].

In honey bees, the normal microflora is concentrated mainly in the posterior part of their digestive tract, namely in the middle and posterior intestines with the rectal capsule. Enterobacteria of the genera *Escherichia, Enterobacter, Proteus, Hafnia, Klebsiella*, and *Erwinia* are most commonly isolated from the bee intestine [4–6]. The description of intestinal microflora is usually limited to one of the following characteristics:

(i) occurrence of specific species in individual bees; bacteriological techniques reveal only the dominant culturable species [4–6], while molecular genetic methods result in a broader spectrum of detected species at the expense of a loss of qualitative information [7];

(ii) total microbial numbers for specific physiological groups (mesophilic aerobes and facultative anaerobes, aerobic and anaerobic bacteria, coliforms) in individual bees [6], in combined samples from different sections [8], or from complete intestines of a number of individuals [4].

The most detailed information on bee microflora was published before the 1980s [4, 5]; these results require correction of the taxonomic affiliations of bacterial cultures according to the presently accepted nomenclature.

In the present work, the first attempt was made to perform a qualitative analysis of intestinal microflora in individual bees; bacterial species and numbers were determined in order to elucidate the dynamics of abundance and frequency of occurrence of enterobacteria in bee colonies.

MATERIALS AND METHODS

Dark European honey bees *Apis mellifera mellifera* L. were colleted from a clinically healthy bee colony hibernating freely (under snow) at the apiary of the Predural'e wild life preserve of Perm' State University

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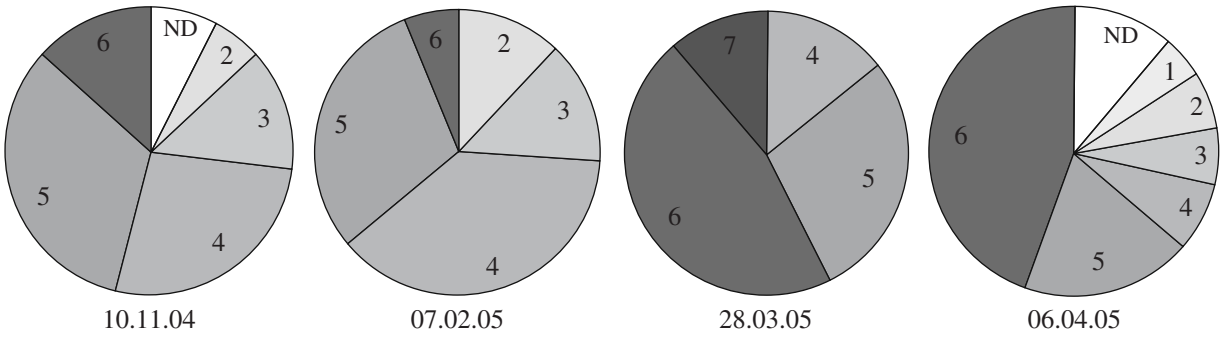

Fig. 1. Dynamics of the total number of enterobacteria in the normal microflora of bee intestines. 1–7 are log (CFU/bee) values; ND, not detected.

(Kishert raion, Perm' krai). The bees were put away with chloroform; abdominal segments were cleaned with 70% ethanol and the middle and posterior intestinal sections were removed with sterile tweezers and suspended in 1.0 ml of 0.8% NaCl solution. Tenfold dilutions (up to 10^{-6}) were prepared from the suspension; 0.2 ml of each dilution was spread inoculated on Endo and Chromocult agarized media (Merck, Germany). Isolated colonies of each type were counted and transferred to agar slants for subsequent identification. Gram-negative oxidase-positive rods capable of fermenting glucose were identified as members of the family *Enterobacteriaceae*.

Primary identification of enterobacteria was carried out according to Bergey's Manual of Systematic Bacteriology [10] using conventional methods (CM) [9] and micromethods (MM) in plates for enzyme immunodetection. The following characteristics were determined: motility; gas production via glucose fermentation (CM); indole and acetoin production; methyl red reaction (CM); fermentation of carbohydrates (D-adonitol, L-arabinose, dulcitol, *m*-inositol, D-xylose, lactose, maltose, D-mannitol, D-mannose, melibiose, alpha-methyl-D-glucoside, L-rhamnose, D-raffinose, salicin, sucrose, D-sorbitol, sorbose, trehalose, and cellobiose) (MM); utilization of acetate, malonate, and citrate (CM); urea and gelatin hydrolysis (CM); esculin hydrolysis (MM); lysine decarboxylase, arginine dehydrolase, ornithine decarboxylase (MM under paraffin oil); and phenylalanine deaminase and lipase (CM).

For micromethods, media without agar were prepared according to the standard formulations. In 96-well sterile plates for enzyme immunodetection, 11 rows were filled with 150 μl of the media. Seven wells of the last row were filled with 180 μl of bacterial suspensions; from these, 15 μl of inoculum was transferred to sterile media. One row was used as an uninoculated control. All the transfers were carried out with an eight-channel dispenser. The work was carried out in a class II laminar flow hood.

The redox conditions of microbial growth change with decreased volume of the medium. The conditions in a 150-μl well of multiwell plate are identical to those in the upper 3 mm of the 25-mm high liquid medium in a test tube; the results for the lower layers are therefore excluded from analysis. Thus micromethods can not be exclusively used for identification; however, the cultures can be revealed with the same ratio of consumption rates for proteins and carbohydrates under aerobic and anaerobic conditions. For this purpose, carbohydrate utilization was determined in two variants, i.e., with and without an overlay of paraffin oil. In the case of ambiguous results, the test were repeated in the conventional variant (in test tubes).

The results were read twice (after 18–20 and 42–44 h of cultivation at 36°C) by scanning the plates and storing their digital images.

For *Klebsiella* species identification, additional tests were carried out: the ability to grow at 5, 10, and 44.5°C; utilization of hydroxybenzoate and gentisate; D-arabinose fermentation; and gas production by lactose fermentation at 44°C [10]. For *Citrobacter* species identification, the ability to ferment maltitol and palatinose was determined [11].

RESULTS AND DISCUSSION

The bees were collected in late autumn, after the winter club formation (November 10, 2004); in winter and early spring, prior to the first cleaning flight (February 7 and March 28, 2005); and shortly after the flight (April 6, 2005). From 178 bees, 598 enterobacterial strains were isolated. In the course of wintering (prior to the first cleaning flight), the ratio of bees containing enterobacteria increased from 92.3 to 100%; the number of enterobacteria per bee increased from 0–10^6 to 10^4–10^7 CFU (Fig. 1). The average intestinal mass was ca. 50 mg. Thus, the intestinal content contained up to 10^8 enterobacterial cells per gram. These values are much higher than those reported by Tysset and Durand [4] (the average number of mesophilic aerobic and facultatively anaerobic microorganisms determined for 10 bees was 2.5×10^5–75×10^5 CFU/g intestinal content) and Gilliam et al. [6] (6×10^5 CFU of gram-negative rods per gram intestinal content; this value is calculated from the CFU/bee values provided by the

Table 1. Identification and practical importance of enterobacteria from healthy bee intestines

Results of identification	Number of strains (number of bees)	Occurrence in pollen load [27]	Agents of bee septicemia	Sanitary indicators (coliform bacteria) [30]
Klebsiella oxytoca	297 (139)	++	–	+
Providencia rettgeri	177 (100)	–	+ [19]	–
Morganella morganii	39 (32)	–	–	–
Proteus vulgaris	27 (19)	–	+ [19]	–
Hafnia alvei	15 (11)	+	+ [2]	+
Citrobacter sp.	12 (10)	+	+ [2]	+
Pantoea agglomerans	11 (8)	+++	–	–
Enterobacter agglomerans	4 (4)	+++	–	+
Leclercia sp.	3 (3)	+++	–	+
Serratia sp.	2 (2)	+	*S. marcescens* [29]	*S. marcescens, S. liquefaciens*
Escherichia coli	1 (1)	+	+ [2]	+

Note: +, often, ++, moderate, +++, seldom.

authors). According to our preliminary estimates, enterobacteria constituted almost 100% of aerobic microflora in the bees under study. Thus, for the average values at each sampling, the content of enterobacteria increasing from 10^6 to 10^7 CFU/g is in good correlation with the values presented in [8] (3×10^6 CFU/g for aerobic microorganisms in combined intestinal samples of hibernating bees.

Tropholaxis (mutual feeding), common winter feedstuff, and close contacts between bees in the winter club seem to promote the uniformity of intestinal microflora. However, our results confirm the differences in the microflora of individual bees. In the beginning of hibernation, the numbers of enterobacteria in bees may vary by six orders of magnitude. The microflora became more uniform at the time of the first cleaning flight, with the difference in enterobacterial content decreasing to four orders of magnitude (10^4–10^7 CFU/bee).

In the study of dynamics of the species composition of enterobacteria in hibernating bees, species identification of 571 and genus identification of 17 bacterial cultures was performed; 10 isolates were not identified (Table 1). The absence of pronounced differences between some biotypes of *Citrobacter freundii* and *C. braakii* [11] prevented reliable identification of *Citrobacter* species. Their net characteristics are close to those of biotypes A and B of these species. A similar situation has been described in the study of the agent of bee citrobacteriosis [13]; its species affiliation was also not determined.

Table 2 presents the qualitative and occurrence characteristics of the enterobacterial intestinal microflora of the bee family under study: content of individual bacterial species in the intestine of a single bee and the ratio of bees containing the specified amount of these species.

During all the wintering period prior to the first flight, *Klebsiella* were the most frequent bacteria (82–100% of the bees studied). Although the characteristics of most of our isolates correlated well with *K. oxytoca* species description, the scarcity and vagueness of the biochemical differences between *K. oxytoca, K. planticola*, and *K. pneumonia* subsp. *pneumonia* [10] made additional tests necessary [11]. They were even more important in the case of *K. oxytoca* variants with the methyl red reactions different from the species standard. All the isolates showed no growth at 5 and 44°C, grew at 10°C, produced acid from sorbose and D-arabinose, and utilized 3-hydroxybenzoate; most of the isolates utilized gentisate. These characteristics enabled their classification as *K. oxytoca.* Although this bacterial species has been isolated by Vassart et al. [14] from a diseased bee family, the source has not been characterized. The authors did not claim that *K. oxytoca* was the causative agent. Gilliam et al. [6, 15] reported the presence of *K. pneumonia* in healthy bees in families, as well as the presence of *K. oxytoca* in intestines of the bees kept in holding cages but not in families. Other researches have also reported *Klebsiella* of an undefined species affiliation in healthy bees [4, 16].

For *Providencia rettgery* which has been previously detected in the intestines of healthy bees [17] and in the hemolymph in septicemia cases [18], the frequency of occurrence increased during hibernation from 25 to 83% of the bees under study. After the first spring flight, apart from a significant decrease in the number of enterobacteria (Fig. 1), the dominant (most frequently detected) species also changed; *K. oxytoca* and

Table 2. Dynamics of CFU numbers and occurrence of enterobacterial species in intestines of hibernating bees

CFU per bee	% ratio of bees containing enterobacteria of the species							
	K. oxytoca	*Providencia rettgeri*	*M. morganii*	*Proteus vulgaris*	*H. alvei*	*Citrobacter* sp.	"*Enterobacter agglomerans*"*	Other species
				10.11.2004				
Less than 10	7.7	75.0	75.0	98.1	88.5	90.4	86.5	92.3
10	0.0	1.9	0.0	0.0	0.0	0.0	0.0	1.9
10^2	7.7	3.8	5.8	0.0	1.9	0.0	0.0	0.0
10^3	13.5	5.8	5.8	0.0	1.9	1.9	5.8	0.0
10^4	30.8	3.8	9.6	1.9	5.8	3.8	3.8	3.8
10^5	32.7	3.8	1.9	0.0	1.9	3.8	3.8	1.9
10^6	7.7	5.8	1.9	0.0	0.0	0.0	0.0	0.0
				07.02.2005				
Less than 10	18.0	20.0	78.0	90.0	92.0	94.0	100.0	96.0
10	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
10^2	8.0	12.0	4.0	4.0	2.0	0.0	0.0	0.0
10^3	24.0	20.0	8.0	0.0	2.0	0.0	0.0	0.0
10^4	30.0	20.0	8.0	4.0	4.0	6.0	0.0	4.0
10^5	18.0	22.0	2.0	2.0	0.0	0.0	0.0	0.0
10^6	2.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0
				28.03.2005				
Less than 10	0.0	20.0	82.9	91.4	94.3	97.1	100.0	100.0
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10^2	0.0	2.9	0.0	0.0	0.0	0.0	0.0	0.0
10^3	0.0	2.9	2.9	2.9	2.9	0.0	0.0	0.0
10^4	17.1	20.0	8.6	0.0	0.0	0.0	0.0	0.0
10^5	37.1	31.4	2.9	5.7	2.9	2.9	0.0	0.0
10^6	34.3	25.7	2.9	0.0	0.0	0.0	0.0	0.0
10^7	11.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
				06.04.2005				
Less than 10	57.1	45.7	97.1	68.6	97.1	100.0	77.1	97.1
10	5.7	2.9	2.9	2.9	0.0	0.0	2.9	0.0
10^2	5.7	11.4	0.0	8.6	2.9	0.0	2.9	0.0
10^3	5.7	8.6	0.0	2.9	0.0	0.0	5.7	0.0
10^4	5.7	11.4	0.0	5.7	0.0	0.0	8.6	2.9
10^5	20.0	20.0	0.0	11.4	0.0	0.0	2.9	0.0

* The "*Enterobacter agglomerans*" group comprises epi- and endophytic species *P. agglomerans, Leclercia* sp., and other members of the "*Erwinia herbicola–Enterobacter agglomerans*" group.

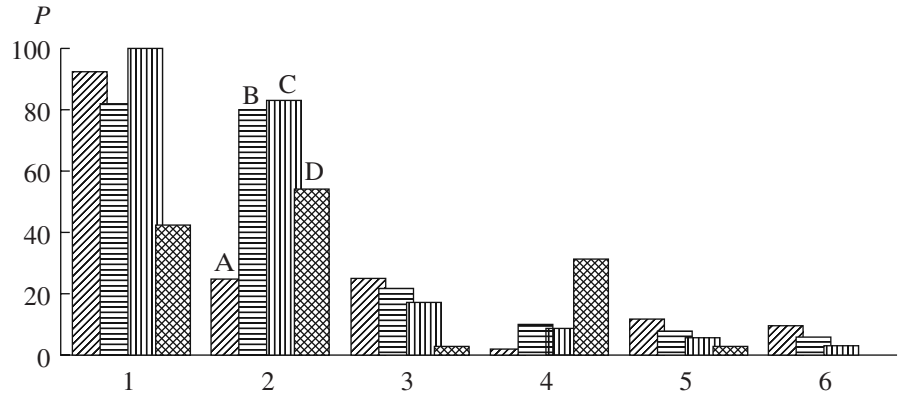

Fig. 2. Dynamics of occurrence of the major species of enterobacteria in the bees of a hibernating family. *K. oxytoca*, 1; *Providencia rettgeri*, 2; *M. morganii*, 3; *Proteus vulgaris*, 4; *H. alvei*, 5; *Citrobacter* sp., 6. A, November 10, 2004; B, February 7, 2005; C, March 28, 2005; D, April 6, 2005.

P. rettgery were revealed in 43 and 54% of the bees, respectively (Fig. 2).

In the bee family under study, the most numerous intestinal enterobacteria belonged to two groups of *Enterobacteriaceae* species differing in their biochemical characteristics, namely, *Klebsiella* spp. fermenting a broad spectrum of sugars and *Providencia, Morganella*, and *Proteus*, which are relatively less versatile in respect to carbohydrates (Table 3). In the course of investigation, *M. morganii* (fermenting only three of the 20 carbohydrates used for initial identification) was replaced by *P. vulgaris* utilizing five carbohydrates out of 20. Both species are known as components of the normal microflora of bees. However, while *P. vulgaris*, first found in bees by Maasen and Borcher in 1919 [19], has since been detected by other researchers [5, 20], *M. morganii* has been found only in a few samples of autumn hive bees [6].

The frequency of occurrence of *Hafnia alvei* and *Citrobacter* sp., agents of infectious diseases in bees decreased from 12 and 10% at the beginning of hibernation to 3 and <3% (absence), respectively. Occurrence of *Hafnia alvei* in normal bee microflora has been reported in its original description as a bee pathogen

Table 3. Reaction of enterobacteria isolated from intestines of healthy bees to the carbohydrates and amino acids used for identification

Characteristics	*Klebsiella oxytoca*	*Providencia rettgeri*	*Morganella morganii*	*Proteus vulgaris*
Phenylalanine deaminase	–	+	+	+
Lysine decarboxylase	+	–	–	–
Arginine dehydrolase	–	–	–	–
Ornithine decarboxylase	–	–	+	–
Acid produced from:				
D-glucose	+	+	+	+
mannose	+	+	+	–
D-adonite, m-inositol, L-rhamnose, melibiose	+	+	–	–
L-arabinose, dulcitol, lactose, D-mannitol, raffinose, D-sorbitol, sorbose, cellobiose	+	–	–	–
D-xylose, maltose, methyl-D-glucoside, sucrose	+	–	–	+
salicin	+	*	–	–
trehalose	+	–	[+]	–

Note: "+" indicates positive results in over 90% of all cases; [+] indicates positive results in 75–90% of the cases; "*" indicates positive results in 25–72% of the cases; "–" indicates positive results in less than 25% of the cases.

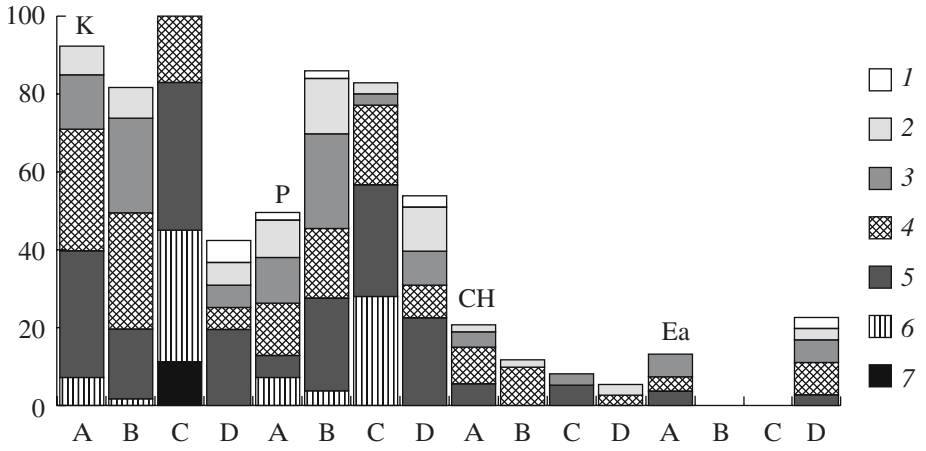

Fig. 3. Numbers and frequency of occurrence of the major groups of enterobacteria in intestines of healthy bees. K, *K. oxytoca*; P, *"Providencia rettgeri–M. morganii–Proteus vulgaris"*, CH, *"Citrobacter sp.–H. alvei"*; Ea, *"Pantoea* spp.*–Leclercia* sp.*–Enterobacter agglomerans"*. Sampling dates: A, November 10, 2004; B, February 7, 2005; C, March 28, 2005; D, April 6, 2005. *1–7*, log (CFU/bee).

[19]. Egorova reported predominance of *H. alvei* in the intestines of working bees [5]; Salimov revealed this bacterial species from the intestines of 52.5% of Far Eastern bees [21]. The presence of *Citrobacter* sp. in healthy bees in France was reported by Tysset and Duran [4].

The representatives of the "*Enterobacter agglomerans*" group were revealed in 13 (beginning of hibernation) and 23% of the bees (end of hibernation, after the first spring flight); their numbers were as high as 10^5 CFU. This group was described in 1972 as a species with extremely vague biochemical characteristics; it contains at least 12 genomic species [22]. Three of these generally coincide with the subsequently described species *Pantea agglomerans*, *P. terrae*, and *Leclercia adecarboxylata* [10]; one coincides with *Enterobacter cowanii* [23]; and the rest are still characterized as "close" to *Pantoea, Leclercia* and "other genera" (5, 1, and 2 genetic species, respectively). *P. agglomerans* is a typical endophytic microorganism [24]; it has been isolated from the pollen of anemophilous plants [25]. *P. agglomerans* and *L. adecarboxylata* were found among the root nodule microflora of three *Hedysarum* species [26].

According to our data [27], *P. agglomerans, Pantoea* sp., and *Leclercia* sp. are the most frequently isolated group of enterobacterial microflora in the pollen pellets from commercial samples. The presence of typical endophytic enterobacteria in bee intestines at the beginning of hibernation can be explained by their recent arrival with pollen consumed by bees. However, their presence after the spring flight requires another explanation since in early April the foothills of the Urals mountains are covered with snow and the primrose flowers (emerging in mid-April) are free from enterobacteria according to our data. These species were probably always present in the intestines of hibernating bees, albeit in numbers incomparable with those of the dominant winter species; therefore they were not revealed by the bacteriological techniques applied. Epiphytic and phytopathogenic microorganisms of the genera *Erwinia* and *Enterobacter* were reported by Tisset et al. [4, 17], Egorova (*Pseudomonas herbicola–E. agglomerans*) [5], and Smolska–Shimchevska [28].

Figure 3 illustrates the dynamics of numbers and frequency of the major functional groups of enterobacteria in bee intestines: "saccharolytic" (*K. oxytoca*), "proteolytic" (*P. rettgeri, M. morganii*, and *P. vulgaris*), typical agents of bee septicemia (*H. alvei* and *Citrobacter* sp.), and the group of endophytic bacteria (*P. agglomerans, Leclercia* sp., and other members of the *E. agglomerans* group).

Enterobacter aerogenes and *E. cloacae*, which have been described by other authors as typical components of this microcenosis [1, 4–6, 15, 19, 20, 28] were not revealed in the normal intestinal microflora of hibernating bees; *Escherichia coli* was isolated from a single bee.

Thus, in the family under study, enterobacteria fermenting a broad range of sugars, bacteria utilizing a limited number of carbohydrates, and endophytic species formed three major physiological groups of the enterobacterial component of intestinal microflora. Investigation of a greater number of bee families throughout the whole annual cycle of their development is required to determine the general patterns of formation of enterobacterial bee microflora, its relations to the feed microflora, and effect on the quality of the pollen load.

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