DIGESTIVE TRACT MICROFLORA IN HEALTHY AND DIARRHEIC YOUNG HARES BORN IN CAPTIVITY. EFFECT OF INTAKE OF DIFFERENT ANTIBIOTICS

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SUMMARY

Quantitative differential analysis of the intestinal microflora of young hares before weaning shows the predominance of strictly anaerobic bacteria.

Our findings show a correlation between early mortality at 8 days and the simultaneous presence of *Welchia Perfringens*, *Clostridium* sp. and *Plectridium* sp. in the faeces of the animals.

Treatments based on the intake of antibiotic mixtures lead to an almost total suppression of this early mortality, but a high late mortality rate then appears at the age of 25-30 days, even if the intakes of antibiotics are limited to the first 3 days of life.

Vaccination of the young hares by mean of the 3 bacteria involved and the ingestion by the youngs of rabbit immunosera against the same bacteria appears to be without any effect on early mortality.

INTRODUCTION

The rearing of hares is limited at present by a high death rate among young hares before weaning. Average mortality among hares raised at the National Research Center for Animal Production, where the present study was carried out, was 50 p. 100 for 300 young hares born between 1969 and 1973 ; a mortality peak occurred on day 7 after birth. Death was usually preceded by diarrhea, as is the case in neonatal diseases common to other young mammals, such as piglet or calf. In the latter animals, neonatal diarrheas are accompanied by proliferation of pathogenic bacteria in the digestive tract. Although it has not always been proved, these bacteria are considered as the cause of the disease.

In this study, our aim was to determine if the appearance of mortal diarrhea in young hares is accompanied by extensive changes in digestive tract microflora, and if administering antibiotics would permit the animals to survive or not.

MATERIAL AND METHODS

1. — Animals

Adult animals were placed in pairs in wire cages situated in a closed building. They were given 300 to 400 g daily of concentrated rabbit feed (I. N. R. A. n⁰ 0521). The young were isolated at birth, and placed with the mother to suckle once a day. They were weaned at 30 days.

2. — Administration of antibiotic solutions

The types and concentrations of antibiotics used varied with the experiment, and are given in the text. All antibiotic solutions were sterilized by filtration on millipore filter 0.45 μ. A syringe was used to put them into the mouths of young hares less than 20 days old. The older animals received antibiotics incorporated into the drinking water which was available at all times.

3. — Sampling

Young hares were placed on a sheet of filter paper in a small cage and their feces were recovered in a sterile tube as soon as they fell on the paper. For organ sampling, the animals were sacrificed with ether, autopsied immediately, and the stomach, small intestine and caecum sampled separately.

Bacteriological techniques.

As soon as sampling was completed, samples were diluted in 10 to 100 times their weight of sterile water (according to their weight), homogenized for 20 to 30 seconds with an ultra-turrax, diluted in dilution medium 1 (RAIBAUD et al., 1966) using a range of decimal dilutions, and inoculated into different selective or non-selective media, according to techniques described elsewhere (RAIBAUD et al., 1966).

Dominant facultative anaerobic bacteria were counted in a Petri dish in medium GAPTs 1 (RAIBAUD et al., 1961) and, in some cases, in medium C (RAIBAUD et al., 1966). Enterobacteria were counted in deoxycholate agar medium (Difco). *Actinobacillus* was also counted in this medium. Dominant strictly anaerobic bacteria were counted in an 8×400 mm tube (RAIBAUD et al., 1966) in medium A (RAIBAUD et al., 1966) and medium B' (SACQUET, RAIBAUD, GARNIER, 1971).

Other bacteria counted anaerobically were : *Bacteroides* and *Welchia* in medium B' with an admixture of 0.013 p. 100 neomycin (NBC), *Plectridium* in medium B' with an admixture of either 0.09 p. 100 streptomycin (Specia) or 0.013 p. 100 neomycin, *Clostridium* in medium B' with an admixture of 0.0075 p. 100 bacitracin (NBC) or 0.09 p. 100 streptomycin and in media D_1 and G_1 (RAIBAUD et al., 1966), sporulated fusiforms in media A, B' with or without an admixture of 0.09 p. 100 streptomycin, D_1, E (RAIBAUD et al., 1966) and G_1. *Bacillus* was also counted in medium B' with an admixture of 0.0075 p. 100 bacitracin. In some samples, *Veillonella* was also counted in medium E, *Acuformis* in media B', D_1, *Catenabacterium*, *Bifidobacterium* and *Eubacterium* in media B' and E, *Butyribacterium* in medium B' with an admixture of 0.0075 p. 100 bacitracin or 0.09 p. 100 streptomycin, and *Peptostreptococcus* in media B', D_1 and G_1.

Genera were determined by critera described elsewhere (RAIBAUD et al., 1966), and the nomenclature adopted is that of PREVOT (PREVOT, 1961), except for non-sporulated strictly anaerobic gram negative bacilli or gram positive streptococci which we will call here either *Bacteroides* or *Peptostreptococcus* according to BERGEY. In this study sporulated fusiforms have not been identified with a determined genus.

Immunological techniques.

Preparation of vaccines. Strains of *Plectridium* sp., *Clostridium* sp. and *Welchia perfringens* described in the text were used. Single cultures were grown for 24 h in 1 liter of medium A at

37° under nitrogen flow. Following centrifugation at 4 000 rpm for 20 min, the pellet of bacteria is harvested and suspended in 40 ml of the supernatant of the same culture. The suspension thus obtained was heated for 30 min at 60°, then neutralized formol was added at the rate of 4 g per 1 000 ml, and the suspension was placed at 37° for 5 days. After checking spores and vegetative cells destruction, the mixture of suspensions obtained was used to immunize the animals.

Vaccination of doe-hares.

Each animal was first given two sub-cutaneous injections of 1 ml of vaccine one week apart. One ml booster injections were given afterwards every four weeks during the periods of gestation and lactation.

Preparation of rabbit immune-serums.

Each animal was first given three sub-cutaneous 0.5 ml injections of vaccine mixed with 0.5 ml of adjuvant (FREUND or floculated ammonium alun). One week later they were given a second series of the same three injections intravenously. Two weeks later the animals were bled.

Determination of blood agglutinins.

This was done by plaque agglutination. The antigen was obtained from a 24-hour culture of bacterial strains, rinsed and concentrated 10 times in a phosphate buffer at pH 7.2. Determination was effected on the serum sampled one month after the second injection in the doe-hares, and at the time of bleeding in the rabbits.
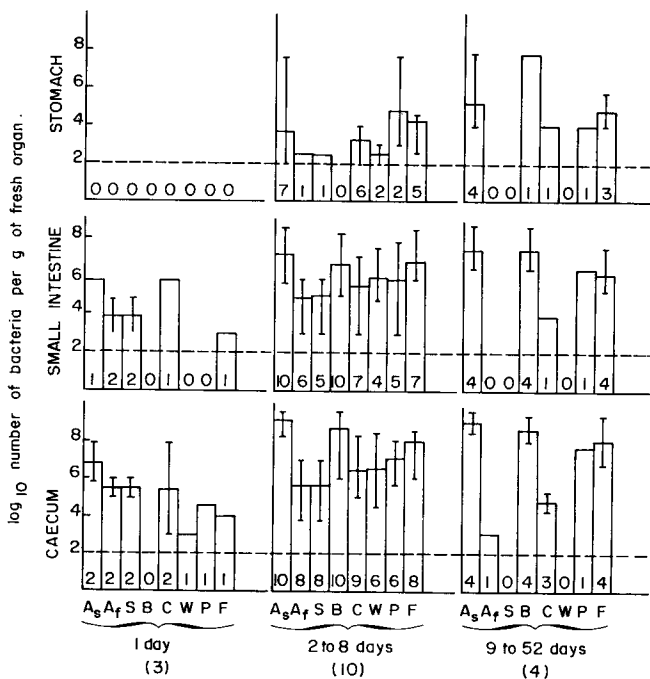

FIG. 1. — The most frequent genera counted in the digestive tract of young hares

A s : Dominant strictly anaerobic bacteria ; A f : Dominant facultative anaerobic bacteria ; S : *Streptococcus* (facultative anaerobic bacteria) ; B : *Bacteroides* ; P : *Plectridium* ; C : *Clostridium* ; F : Sporulated fusiforms ; W : *Welchia*.

Numbers in parentheses show total number of animals analyzed for each age group. Numbers in graph bars indicate the number of animals harboring the bacterial group under consideration in the 1/100 sample dilution. Dotted line shows the level below which the bacterial group is considered as absent. Vertical lines in bars indicate the differences between the extreme values observed.

RESULTS

1. — *Quantitative differential analysis of the digestive tract microflora of young hares between birth and 52 days of age*

Figure 1 shows that caecum microflora at all ages is characterized by the predominance of strictly anaerobic bacteria and especially of *Bacteroides*. The facultative anaerobic population is either completely absent from the 1/100 dilution, or, in some samples, composed of a small number of *Streptococcus, Micrococcus* or *Actinobacillus*. *Escherichia, Staphylococcus* or *Lactobacillus* were not found in any sample. Three of the young hares examined between 2 and 8 days had diarrhea at the time of sacrifice. Caecal microflora in these animals was characterized by the simultaneous presence of a high number of *Clostridium* and *Plectridium* and, in two cases, by the presence of *Welchia perfringens*. Small intestine flora was less abundant than in the caecum, but resembled it qualitatively. Bacterial populations in the stomach were low, except in the oldest animal, and in that case the dominant microflora was always strictly anaerobic.

Various other bacterial genera were counted in some animals (table 1), particularly in the oldest young hares.

TABLE I

Other bacterial genera counted in the digestive tract of young hares

Age in days	Segment	Genera counted
1 day (3) (1)	C	*Micrococcus* 4.8 (2) (1) (3), aerobic gram negative bacillus 4.8 (1)
2 days to 8 days (10)	E	*Veillonella* 4.7 (1)
	I	*Veillonella* 5.7 (1), *Acuformis* 5.0 (1) *Actinobacillus* 4.3 (1), aerobic gram negative bacillus 4.2 (4) *Butyribacterium* 5.0 (1)
	C	*Acuformis* 7.0 (3), *Veillonella* 5.5 (3), aerobic gram negative bacillus 3.7 (3) *Actinobacillus* 5.7 (1), *Micrococcus* 3.6 (1), *Bacillus* 3.0 (1) *Catenabacterium* 7.0 (1), *Butyribacterium* 6.0 (1), *Pseudomonas* 2.0 (1)
9 days to 32 days (4)	E	*Peptostreptococcus* 4.0 (1), *Micrococcus* 4.0 (1) *Butyribacterium* 5.2 (2), *Acuformis* 4.8 (1)
	I	*Eubacterium* 4.9 (1), *Butyribacterium* 4.0 (1) *Actinobacillus* 4.3 (1), *Acuformis* 6.3 (1)
	C	*Peptostreptococcus* 8.0 (1), *Butyribacterium* 6.5 (2) *Bifidobacterium* 8.0 (1), *Actinobacillus* 5.7 (1) *Acuformis* 7.9 (1), aerobic gram negative bacillus 3.2 (1)

(1) Number of animals analyzed.
(2) Arithmetic mean of $\log_{10}$ of number of bacteria per g of fresh organ.
(3) Number of animals harboring the genus under consideration.

TABLE 2

Mortality in young hares treated with antibiotics or untreated

Treatment		Number of animals treated	Percent of mortality between		Average age at death (days)
			0 and 20 days	20 and 40 days	
None		36	59	2	8.1
B		15	100	0	9.0
B + S		24	87	0	13.5
B + S + N + E	T_1	12	8	66	30.7
	T_2	39	2	47	34.0
	T_3	14	7	86	30.0
	T_4	5	20	80	22.0
	T_5	7	0	86	25.0
B + N + E		18	2	71	23.0

B : Bacitracin 10 mg/ml, intake of 1 ml daily up to death ;

B + S : Bacitracin 10 mg/ml + streptomycin 100 mg/ml, intake of 1 ml daily up to 20 days.

B + S + N + E : Bacitracin 10 mg/ml + streptomycin 100 mg/ml + neomycin 10 mg/ml + erythromycin 20 mg/ml, intake 3 times a week of :

T_1 : 1 ml from 0 to 20 days ;

T_2 : 1 ml from 0 to 40 days ;

T_3 : 1 ml from 0 to 15 days, 0.5 ml from 15 to 30 days, 0.25 ml from 30 to 40 days ;

T_4 : 0.25 ml from 0 to 15 days, 0.12 ml from 15 to 30 days ;

T_5 : 0.25 ml from 0 to 10 days + 1 ml of 10^{-2} caecal dilution of a healthy young hare ;

B + N + E : Bacitracin 10 mg/ml + neomycin 10 mg/ml + erythromycin 20 mg/ml, intake of 1 ml the day of birth and 3 days later.

2. — *Plectridium and Welchia counts in the digestive tract
of untreated animals*

Table 2, line 1, shows that 59 p. 100 of the untreated control animals die when
they have diarrhea in the second week of life.

In this group, we tried to determine if the presence of *Plectridium* and *Welchia*
in the digestive tract could be related to the onset of mortal diarrhea. The genus
Welchia occurs only occasionally in young hares surviving after 40 days (table 3,
line 1), and is transitory. In two cases where more than 10^5 *Welchia* were counted in a
sample of feces they had disappeared in the following samples. The genus *Plectridium*
is more frequent, but is also transitory, no longer being found in young hares more
than 5 days old. *Plectridium* is always found in young hares which die before 40 days,
and *Welchia* is usually present. A high number of these bacteria often persist in
several successive fecal samples, and as the caecum analysis at the time of death or
during a diarrheic episode has shown, there is always a high number of *Plectridium*
or *Welchia*, and usually both. Only three animals among those dead before 40 days
died after 10 days. *Plectridium*, but no *Welchia*, was counted in these. Neither
Plectridium nor *Welchia perfringens* was found in the 1/100 fecal dilution of doe-hares.

TABLE 3

Plectridium *sp. and* Welchia perfringens *count in feces
and caeca of untreated animals*

Type of sample	Fate of animal	Total number of animals analyzed	Number of animals having a count per g of fresh matter in at least one sample of		
			More than 10^5 *Plectridium*	More than 10^5 *Welchia*	Simultaneously more than 10^5 *Plectridium* and 10^5 *Welchia*
Feces	Survivors at 40 days	12	8	2	2
	Dead before 40 days	16	16	10	10
Caecum	Dead or sacrificed during a diarrheic episode before 20 days	17	14	13	10

3. — *Treatment by bacitracin intake*

A group of 15 animals was given daily a 1 ml dose of a solution of bacitracin at
10 mg per ml beginning on the day of birth. All these animals died during the second
week of life (table 2, line 2).

A bacteriological examination of feces was performed on 10 of the animals and
showed the absence of *Welchia perfringens* in the digestive tract all through out their
life span, but 10^5 to 10^7 of *Plectridium* sp. were present in each of the animals.

4. — *Treatment with bacitracin and streptomycin*

In an attempt to eliminate the strain of *Plectridium* which had proved sensitive *in vitro* to the dose of streptomycin used in our culture media, we gave to another lot of animals a daily dose of 1 ml of a solution containing 10 mg of bacitracin and 100 mg of streptomycin per ml. This treatment was used as a preventive beginning on day 3 of life, or as a curative when diarrhea first appeared. It did not alter the high death rate of the animals. But the average age at death was retarded by 3 to 4 days (table 2, line 3).

Bacteriological examination showed that the before preventive treatment was started, that is before 3 days of age, many animals already harbored a high number of *Plectridium* and *Welchia*. All the animals examined at death except for one harbored a high number of streptomycin-resistant *Plectridium* in the caecum. A high number of streptomycin-resistant *Clostridium* were also present in all the dead animals, although they had never been found in the mothers' feces. Results obtained for the curative treatment were very similar.

5. — *Treatment with an antibiotic mixture*

A number of *in vitro* experiments were done in order to find a mixture of antibiotics which would inhibit the simultaneous growth of *Welchia perfringens*, *Plectridium* sp. and *Clostridium* sp. strains. We chose a mixture of bacitracin (10 mg/ml), neomycin (10 mg/ml), streptomycin (100 mg/ml) and erythromycin (20 mg/ml), all three antibiotics being little absorbed or not absorbed at all by the digestive tract.

For the preventive treatment, 1 ml of the antibiotic mixture was given on the first day after birth and continued for 20 days 3 times a week (T_1). Table 2, line 4 shows that only one animal out of the 12 treated died during the antibiotic treatment. However, almost all these animals died in the two weeks following withdrawal of the treatment, at an age when mortality is rare among surviving untreated animals. In the caeca of animals analyzed at death, there was always a high number of *Plectridium* resistant to the antibiotics used in the treatment, accompanied by equally resistant *Clostridium* in most cases.

In an attempt to abolish late mortality, treatment T_2 was given to another group of animals. The same antibiotic mixture already described was administered to the animals for 20 days by means of a probe. The probe treatment was stopped between 20 and 40 days, but the animals continued to absorb the same amount of antibiotics diluted in the drinking water. We carefully checked that the hares absorbed all the diluted antibiotic solution. In these conditions, mortality was almost completely abolished during the first 20 days of treatment. Mortality increased, however, during the following 20 days. At no time during the treatment was *Welchia perfringens* or *Clostridium* detected in the feces of living animals, or in the caeca of deal animals. *Plectridium* was found in half of the fecal samples in numbers of 10^5 to 10^6, and in the caeca of almost all animals dying between 20 and 40 days in numbers of 10^6 to 10^7.

We thought that late mortality could be due to high antibiotic intake disturbing the establishment of the microflora usually present in « holoxenic » young hares. We

TABLE 4

Count of Welchia perfringens, Clostridium *sp. and* Plectridium *sp.*
resistant to streptomycin in the caeca of animals treated
for 20 days with a mixture of 4 antibiotics (treatment T_1)

Number of caeca analyzed	Number of animals having a count of		
	10^2 *Welchia*	Between 10^6 and 10^9 *Plectridium*	Between 10^5 and 10^8 *Clostridium*
9	0	9	7

TABLE 5

Effect on mortality of young hares when mothers are immunized
and the young treated with immune-serum

Treatment	Number of animals	Percentage of mortality between		Average age at death (days)
		0 and 20 days	20 and 40 days	
Vaccination of mothers	29	59	3	8.1
Treatment (^{1}) with immune-serum	10	80	10	9.1

(^{1}) Daily intake of 1 ml of anti-*Plectridium* sp., *Clostridium* sp. and *Welchia perfringens* rabbit immune-serum from day 0 to day 15.

thus decided to study two other types of treatment in which the antibiotic dose in the beginning was either identical to (T₃) or lower than (T₄) that of treatment T₂, and which in both cases decreased with time. We observed (table 2, lines 6, 7) that these treatments always decreased early mortality but did not improve late mortality. Intake of diluted healthy young hare caecum at the same time as the antibiotics did not change the results (T₅). Finally, we noted that a mixture of three antibiotics (bacitracin, neomycin and erythromycin) given at birth and at day 3 was sufficient to decrease early mortality. However, a high late mortality still persisted and as in treatments T₃ and T₅, bacteriological analyses gave results comparable to those reported for treatment T₂.

6. — *Immunological treatments*

Seventeen mother hares were vaccinated with a mixture of three strains, *Plectridium* sp., *Clostridium* sp. *Welchia perfringens*. Agglutination rates of 1/100 to 1/2 000 were obtained in their serum for *Plectridium* and *Clostridium*, but those for *Welchia perfringens* were less than 1/10. These mothers gave birth to 29 young hares showing no improvement of death rate or of length of survival (table 5). Furthermore, during the first ten days of life, 10 young hares daily ingested 1 ml of rabbit immune-serum having agglutination rates of 1/2 000 for *Plectridium* and *Clostridium* and 1/128 for *Welchia perfringens*. No improvements of length of survival or mortality rate was observed (table 5).

DISCUSSION

Our results show that most, and sometimes all, digestive tract microflora in the young hare is composed of strictly anaerobic bacteria from birth. This clearly differentiates this microflora from that of laboratory rodents such as rats and mice, which have facultative anaerobic bacteria appearing at birth, but no strictly anaerobic bacteria appearing until weaning (RAIBAUD *et al.*, 1966 ; LEE *et al.*, 1971). However, it is comparable to the microflora of young rabbit which is mainly composed of *Bacteroides*, while *Lactobacillus* is absent (GOUET, FONTY, 1973).

We have shown in « gnotoxenic » mouse that the late establishment of strictly anaerobic bacteria is not due to antagonism of the previously established facultative anaerobic bacteria, but rather to the mother's milk (DUCLUZEAU, RAIBAUD, LADIRE, 1974 ; CHOPIN, DUCLUZEAU, RAIBAUD, 1974). It appears logical that in « holoxenic » young hares, mother's milk may also be responsible for the sequence of microflora establishment. Thus, the peculiar composition of doe-hare's milk would explain the absence of such common bacteria as *E. coli* or *Lactobacillus* in young hares.

Our results also show that early mortality of the young breeding hares studied is very likely due to bacterial action because antibiotic intake almost entirely abolishes it. The metabolic or viral causes held as responsible in neonatal diseases of other animals would, in this case, be only secondary to bacterial infection.

The pathogenic role of *Welchia perfringens* could be the cause since this microorganism is found associated with almost all cases of early death in young hares. The

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presence of this bacteria alone, however, is not sufficient to establish the etiology of the disease because its elimination with bacitracin did not result in a lower death rate among the animals used in this study. Two complementary results lead us to believe that the etiology of the disease is related to synergy between three bacterial species, *Plectridium* sp., *Clostridium* sp. and *Welchia perfringens*. First, there is a close relationship between early mortality of young hares and the constant and simultaneous presence of these three species in the digestive tract during the first days of life. Furthermore, these same three bacterial species are simultaneously absent in all cases of treatment with antibiotics which increase the average length of life.

We used as vaccinating antigen a mixture of dead bacterial cells and detoxified supernatant of culture, because the exact mechanism of the pathogenicity of the three strains was unknown. For 2 strains the vaccination entailed the appearence of agglutinins in blood but no attempt has been done to detect the presence of antitoxin in blood and toxin in supernatant. Vaccination and immune-serum intake did not change early death rate or multiplication in the digestive tract of the three bacteria involved in this mortality. Using « gnotoxenic » models, we showed (DUCLUZEAU, RAIBAUD, 1973) that vaccinating the mothers does not alter bacterial multiplication in the digestive tract of the young. Moreover, as concerns young hares, it appears that the pathogenic activity of the strains under discussion is not altered either. Of course, it is possible that other bacteria, not revealed by our techniques, may also be involved in this etiology.

The coexistence in the same group of animals of diarrheic and healthy subject, the healthy subjects never carrying the bacteria assumed to be responsible for the diarrhea of the diseased animals, attests the complexity of the ecological relationships involved in the establishment of digestive tract microflora in the newborn. In healthy newborns animals, the barrier against pathogens is probably related to the composition of the mother's milk. The absence or presence of pathogenic bacterial species in the digestive tract of young hares would thus be explained by the irregular presence of various specific metabolites in doe-hare's milk.

At a more advanced age and especially in the adult in which *Plectridium, Welchia perfringens* or *Clostridium* were never found, the role of this barrier against pathogens could be assumed by the flora usually present in the digestive tract, as we have shown in « holoxenic » mice (DUCLUZEAU, BELLIER, RAIBAUD, 1970). Prolonged antibiotic intake could alter this microbiological barrier, and this would explain the late mortality which we have observed. That is why we tried to reduce the length of treatments and antibiotic intake. On the contrary, however, death rate tended to rise as antibiotic intake decreased (table 2), and it is difficult to understand how limiting the antibiotic intake limited to the first three days of life could affect mortality which occurs only 20 days later, at a time when the microflora has become very different from what it was at 3 days. It is also possible that late mortality of young hares may be due to a cause in which bacteria are not involved. Whatever the reason, antibiotic intake, in all cases, causes an overall increase of mortality in young hares. This emphasizes the risks run when digestive tract microflora balance is upset in an uncontrolled way.

To make a complete study of diarrhea in young hares, it would be necessary to use axenic and « gnotoxenic » subjects. The exact role played in the etiology of the disease by each of the suspected pathogenic bacteria studied here can only be deter-

mined by reassociating them in axenic subjects. Using these models, we may also be able to define the best types of nutritional and microbiological barriers which are needed for the survival of young hares.

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RÉSUMÉ

LA MICROFLORE DU TUBE DIGESTIF
CHEZ LES LEVRAUTS D'ÉLEVAGE SAINS ET DIARRHÉIQUES.
EFFET DE L'INGESTION DE DIVERS ANTIBIOTIQUES

L'analyse différentielle quantitative de la microflore du tube digestif du levraut avant sevrage montre la prédominance dès la naissance des bactéries anaérobies strictes.

Nos résultats montrent une corrélation entre la mortalité précoce à 8 jours et la présence simultanée dans les fèces des animaux de *Welchia perfringens, Clostridium* sp. et *Plectridium* sp.

Des traitements par ingestion de mélanges d'antibiotiques permettent de supprimer presque totalement cette mortalité précoce, mais il apparaît alors une mortalité tardive élevée à l'âge de 25-30 jours, même si les ingestions d'antibiotiques sont limitées aux trois premiers jours de la vie.

La vaccination des hases à l'aide des trois bactéries incriminées et l'ingestion par des jeunes d'immunsérums de lapin dirigés contre les mêmes bactéries se sont révélées sans effet sur la mortalité précoce.

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