Zb. Bioteh. Fak. Univ. Ljubl., Kmet. Zooteh., 80(avgust 2002)1, 51–59.

*http://www.bfro.uni-lj.si/zoo/publikacije/zbornik*

Original scientific article
Izvirni znanstveni prispevek

# THE INFLUENCE OF VARIOUS FIBER SUPPLEMENTS ON QUANTITY OF MICROBIAL BIOMASS IN DIGESTIVE TRACT OF PIGLETS DETERMINED BY ATP-LUCIFERASE ASSAY *

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Received February 15, 2002, accepted July 05, 2002.
Delo je prispelo 15. februarja 2002, sprejeto 05. julija 2002.

## ABSTRACT

Dietary fibre has positive effects on digestion and on gastrointestinal tract of pigs. Pigs do not possess indigenous enzymes for its digestion. Fibre is fermented by gastrointestinal microflora. The influence of different fibre sources on quantity of microbial biomass in gastrointestinal tract (GIT) of weaned piglets was studied in our experiment. First we introduced ATP-luciferase assay with pure *E. coli* culture, mixture of *E. coli* and somatic cells and samples from small and large intestine. The proper protocol for preparation of samples from GIT for ATP-luciferase assay was established. Twenty four weaned piglets, divided into four groups, were fed the same feed except the included fibre: carboxymethylcellulose (group CMC), lignified cellulose (group LIG) and pure cellulose (group CEL). The control group received starch instead of fibre (group KON). Piglets were sacrificed after 13 days of feeding and digesta samples were taken from: stomach, proximal part of small intestine, distal part of small intestine, caecum, colon and rectum together. The quantity of microorganisms in different parts of GIT was estimated by ATP-luciferase assay. The highest concentration (conc.) of microbial ATP was found in large intestine in all groups. Statistically significant effect of different fibres on conc. of microbial ATP in different parts of GIT was not demonstrated. The experiment did not provide evidence that the soluble dietary fibre (carboxymethylcellulose) would enable more intensive microbial fermentation in weaned pigs than insoluble fiber. The reason was probably in big variability and nonhomogenity of samples from GIT.

Key words: pigs / piglets / animal nutrition / dietary fibre / gastrointestinal tract / microbial biomass / ATP-luciferase assay

* The article is a part of graduation thesis (justification September 13th, 2001), supervisor ass.prof. Romana Marinšek Logar, Ph.D.

# VPLIV DODATKA RAZLIČNIH VRST VLAKNINE NA KOLIČINO MIKROBNE BIOMASE V PREBAVILIH PUJSKOV DOLOČENE Z ATP-LUCIFERAZNIM TESTOM *


## IZVLEČEK

Prehranska vlaknina pozitivno vpliva na prebavo in prebavila pri prašičih. Prašiči nimajo endogenih encimov za razgradnjo vlaknine, zato je ta odvisna od mikrobov, ki naseljujejo prebavila. Proučevali smo vpliv različnih vrst vlaknine na količino mikrobne biomase v prebavilih odstavljenih pujskov. Najprej smo preizkusili ATP-luciferazni test za določanje količine bakterijske biomase s čisto kulturo *E. coli*, z mešanico *E. coli* in somatskih celic ter z vzorci tankega in debelega črevesa. Našli smo ustrezen postopek priprave vzorcev za določanje bakterijskega ATP v vsebini prebavil. V prehranski poskus smo vključili 24 odstavljenih pujskov, razdeljenih v štiri skupine. Dobivali so krmo, ki se je razlikovala le po viru vlaknine: karboksimetilceluloza (skupina CMC), lignificirana celuloza (skupina LIG) in čista celuloza (skupina CEL). Kontrolni skupini (skupina KON) smo namesto vlaknine v krmo dodali škrob. Po 13 dneh krmljenja smo pujske žrtvovali in odvzeli vzorce vsebine prebavil: želodec, prvi del tankega črevesa, drugi del tankega črevesa, slepo črevo ter kolon in rektum skupaj. V vzorcih smo določali koncentracije ATP z ATP-luciferaznim testom. Največje koncentracije mikrobnega ATP smo v vseh skupinah zasledili v debelem črevesu. Statistično značilnega vpliva vrste vlaknine na koncentracijo mikrobnega ATP v različnih delih prebavil večinoma nismo uspeli dokazati. V raziskavi nismo uspeli dokazati, da bi topna vlaknina (karboksimetilceluloza) pri odstavljenih pujskih omogočala boljšo mikrobno fermentacijo kot netopna vlaknina. Vzrok temu je bila verjetno velika variabilnost in nehomogenost vzorcev prebavil.


Ključne besede: prašiči / pujski / prehrana živali / prehranska vlaknina / prebavila / mikrobna biomasa / ATP-luciferazni test

## INTRODUCTION

A key feature of the alimentary tract is ability to digest foodstuffs before absorption. Digestion occurs through hydrolysis (endogenous enzymes) and microbial fermentation. In pig, microorganisms are most numerous in the caecum and large intestine, where they play an important role in the fermentation process. Most of these organisms are anaerobic with many of them being strictly obligate anaerobes (Ewing and Cole, 1994).

Dietary fiber is important in pig's nutrition, because of its benefits on digestion. Passage of feeds is faster through stomach and small intestine (Salobir, 1999), it makes faeces softer (Bach-Knudsen and Hansen, 1991) and facilitates laxation (Davidson and McDonald, 1998), it is source of energy and essential nutrients for colonocytes (Sakata and Iganaki, 2001), it reduces synthesis of amines and nitrogen absorption. Faster passage of feeds through the gastrointestinal tract (GIT) means also faster elimination of potentially pathogenic bacteria and their toxic products (Salobir, 1999). Feeds with more fiber causes suitable digesta-passage into the large intestine, what means better conditions for continuous fermentation (Drochner, 1993).

Intensity of large intestine fermentation depends on total number of microorganisms and type of microbial population (Drochner, 1993). Most common bacteria in stomach and small intestine of pigs are species from genera *Streptococcus*, *Lactobacillus* and *E. coli* (Ewing and Cole, 1994; McCracken and Lorenz, 2001), whereas *Bacteroides ruminicola, Selenomonas ruminantium, Butyrivibrio fibrisolvens* and other anaerobic genera are predominant in caecum and large intestine (Robinson *et al.*, 1981).

* Prispevek je del diplomskega dela (zagovor 13. september, 2001), mentorica doc. dr. Romana Marinšek Logar

There are many ways for microbial biomass determination. One method, gaining widespread use, is firefly luciferase assay of ATP (ATP-luciferase assay). Concentration of ATP remains a fairly constant per microbial cell of a certain type. When the cell membrane opens, ATP will be degraded rapidly by the high concentration of ATP-degrading enzymes of the cell (Stanley, 1986; Lundin and Thore, 1975; Lundin, 2000). So, with ATP-luciferase assay we can count only live cells. The ATP-luciferase assay offers a high degree of sensitivity, rapidity, accuracy and reproducibility in comparison with classical techniques for determination of microbial biomass, that are usually slow and require rather complex media (Chappelle and Levin, 1968; Cheer *et al.*, 1974).

The most complex systems for relevant ATP determination are somatic and microbial cells mixed together with nonliving material (soil, meat, milk, rumen contents, fruit juices, clinical samples, etc). In these systems we have to selectively extract ATP from microbial or somatic cells. When we wish to determine microbial ATP in one of the above samples, usually ATP from somatic cells is selectively extracted using Triton X-100. Somatic ATP together with any free ATP can be hydrolysed by ATPase (EC 3.6.1.3) or apyrase (EC 3.6.1.5) (Stanley, 1986; Lundin, 2000; Thore *et al.*, 1975) or by concomitant use of two enzymes, adenosine phosphate deaminase (EC 3.5.4.17) and apyrase (Sakakibara *et al.*, 1997).

Here we report on introductory experiments using ATP-luciferase assay for the determination of microbial biomass in GIT of domestic animals in our laboratory. The main goal was to find the suitable experimental conditions for successful determination of bacterial biomass in different parts of piglets' GIT in order to finally test the influence of various fiber supplements on quantity of bacterial biomass in GIT of weaned piglets. The main question we wanted to answer was: could we expect any differences in the quantity of the microbial biomass in GIT of weaned piglets as a response to different fiber sources fed.

## MATERIAL AND METHODS

### Feed experiment

We included 24 weaned piglets in our experiment. Animals were divided into four groups, which were fed the same feed except the included fiber. Group CMC received 3% carboxymethylcellulose, group CEL received 3% pure cellulose, group LIG 3% lignified cellulose and group KON 3% starch included in feed. Group KON was the control group. Piglets were fed twice a day, they had water *ad libitum*. Feed was limited per day. Piglets were sacrificed after 13 days of feeding, samples were taken from stomach, proximal part of small intestine, distal part of small intestine, caecum, colon and rectum together and kept at –70 °C until analysed. Concentration of microbes in different parts of GIT was determined by ATP concentration of samples by ATP-luciferase assay.

### ATP – luciferase assay with pure *E. coli* culture

Fifty ml of pure *E.coli* culture, strain JM109 was cultivated in liquid LB medium (Sambrook *et al.*, 1989). The culture was incubated overnight at 37 °C in shaking incubator (150 rpm). It was frozen in liquid nitrogen (–196 °C) and kept at –70 °C until analysed. ATP was extracted with boiling buffer (Zrimec, 2001, Thore *et al.* 1975): 0.9 ml 0.1M Tris-HCl with 2 mM EDTA, pH 7.8 was heated for three minutes at 100 °C, 0.1 ml sample was added and again heated for three minutes at 100 °C. Samples were cooled on ice. Following the instructions of the manufacturer of FL-AA kit (Sigma) samples with extracted ATP or ATP standard solutions (made from FL-AAS) and reagent FL-AAM were displaced onto ice. 100 µl FL-AAM was then displaced onto room temperature for three minutes. Measurement of luminiscence started with

quick injection of 100 µl of sample or ATP standard to diluted FL-AAM reagent. The mixture was displaced to luminometer (Biofotonika, Ljubljana). Measurements were done in relative luminescent units (RLU). From RLU of ATP standard solutions and their known concentrations a standard measuring curve was made. Concentrations of ATP in samples were calculated by the standard curve equation and RLU of samples.

**Reproducibility of the method for ATP extraction from *E. coli* and ATP standard solutions with boiling buffer**

ATP was extracted with boiling buffer 10-times from the same *E. coli* culture, strain JM109 and the luminescence measured. ATP standards were treated with boiling buffer and then after measuring the luminescence compared with untreated ATP standards. The possible influence of boiling buffer on molecules of ATP was tested by this experiment.

**Concentration of ATP in bacterial cells**

ATP was extracted from *E. coli* culture and the concentration of ATP was determined. Number of bacterial cells in the same culture was determined by flow citometry (FACScan, Becton Dickinson). Bacterial cells were diluted 1000-times in PBS buffer, pH 7.4 and stained with SYTO13. As internal standard Polyfluor 511 balls (diameter 1 µm, Polysciences) were used.

**ATP – luciferase assay with the mixture of *E. coli* and somatic cells**

For extraction and degradation of somatic ATP, the protocol from Thore *et al.* (1975) was used with exception of an enzyme. We used ATPase (Sigma) not apyrase. The concentration of somatic cells (mieloma cells) in the mixture with E. coli was $5.6 \times 10^6$ cell/ml of mixture. 0.1% of Triton X-100 /ml and 0.04 ATPase /ml was added to the mixture. The procedure took place on ice. The mixture was incubated for 10 min at 37 °C. Bacterial ATP was extracted with boiling buffer. Samples with extracted ATP were centrifuged (10600 X g) at 4 °C and the concentration of ATP determined. We trivially named this procedure the whole protocol (WP).

**The influence of Triton X-100 and ATPase on bacterial cells**

The following samples were prepared:
- *E. coli*: ATP extraction with boiling buffer
- *E. coli*: WP
- somatic cells: ATP extraction with boiling buffer
- somatic cells: WP
- mixed culture: ATP extraction with boiling buffer
- mixture of *E. coli* and somatic cells: WP

**ATP – luciferase assay in samples from GIT**

Samples from GIT were diluted with sterile 0.9% NaCl and homogenised by glass homogeniser. The concentrations of ATP in GIT samples were determined following the WP procedure.

**Determination of total solids (TS) in samples from GIT**

2.5 ml of diluted samples from GIT were dried overnight at 105 °C to constant weight (Eaton *et al.*, 1995).

### Testing the presence of somatic cells in samples from GIT

0.1% Triton X-100 /ml was added to few samples from GIT, but not ATPase. This mixture was incubated for 10 min at 37 °C, centrifuged (10600 X g, 4 °C) and filtrated through 0.22 μm filter. ATP belonging to somatic cells was determined from the filtrate. Concentrations of ATP from these samples were compared to concentrations of ATP in samples that were treated with Triton and ATPase and were not filtrated.

### Statistical analysis

For statistical evaluation of experimental data, the computer program SAS/STAT (SAS User's guide, 1995) was used. Basic statistical parameters were calculated by MEAS procedure. Data were tested for normal distribution and analysed by the GLM (General Linear Models) procedures. The influence of the group was included as a systematic influence in the statistical model. Statistically significant differences among groups were evaluated by linear contrasts method.

## RESULTS AND DISCUSSION

The purpose of this experiment was to test the influence of different fiber sources (carboxymethylcellulose, lignocellulose and pure cellulose) in comparison with starch, on microorganisms in GIT of weaned piglets.

First we had to find out the proper protocol for bacterial ATP determination in samples from GIT of piglets. We used the boiling buffer method for ATP extraction from microbial cells. It is very important to have the buffer really at its boiling point before addition of sample. If this condition is not achieved, ATPases and other enzymes would change the ATP content even if they are active just for few seconds (Cheer *et al.*, 1974; Stanley, 1986). First we introduced ATP-luciferase assay with pure *E. coli* culture, strain JM109. Concentration of extracted ATP was in linear relation with dilutions of culture. Measurements were reproducible. Standard deviation for 10 repeats was $0.23 \times 10^{-3}$ mmol ATP $g^{-1}$ TS at average $4.87 \times 10^{-3}$ mmol ATP $g^{-1}$ TS for *E. coli* culture.

The concentration of ATP remains a fairly constant per bacterial cell. Chappelle and Levin (1986) determined ATP concentrations in 19 different bacterial species. They measured 0.03–$0.89 \times 10^{-15}$g ATP per one cell. Data of Stanley (1986) showed $1 \times 10^{-15}$g ATP and of Thore *et al.* (1975) $1.8 \times 10^{-15}$g ATP per cell. In our experiment we determined $0.53 \times 10^{-15}$g ATP per one cell of *E. coli* (number of cells was measured by flow cytometry). This is similar to data mentioned before, so the used method should be the proper one.

We determined the effect of Tris-HCl buffer on ATP molecules on ATP standard solutions. Boiling buffer Tris-HCl reduced the concentration of ATP for 15–22%. Prioli *et al.* (1985) also introduced the loss of ATP, but lower (6–15%). Otherwise molecules of ATP should be stabile after their extraction (Prioli *et al.*, 1985). We used the same procedure for determination of bacterial biomass in GIT of weaned piglets, so the loss of ATP in such extent is not so important.

Some somatic cells in samples from GIT could be expected, for example: immune cells and mucous cells that peeled off mucosa of GIT (McCracken and Lorenz, 2001). We had to remove somatic ATP and measure only the concentration of bacterial ATP. For that purpose we used the combination of Triton X-100, that selectively extracted ATP from somatic cells (but not from bacterial cells) and enzyme ATPase, which degraded released somatic ATP and any free ATP present in samples (Stanley, 1986; Lundin, 2000; Thore *et al.*, 1975). We introduced the ATP-luciferase method with mixture of *E. coli,* strain JM109 and somatic cells (mieloma cells NNS0). Used concentrations of Triton X-100 (0.1%) and ATPase (0.004 U $ml^{-1}$) removed somatic ATP,

but did not have much effect on bacterial cells. The whole procedure (WP) for measuring ATP concentration completely removed somatic ATP and lowered bacterial ATP only for 3.6%.

We checked the presence of somatic ATP in samples from stomach, caecum and colon and rectum together. One series of samples was filtrated and the other not, so that we could determine the portion of somatic ATP. From the results of measuring of ATP in filtrated and unfiltrated samples which are shown in Table 1, we concluded, that there was 1.09% or less somatic ATP in comparison to total ATP in samples from GIT of piglets.

Measuring of microbial ATP with ATP-luciferase assay showed that there were different concentrations of ATP present in different parts of GIT of piglets (Fig. 1).

Table 1. ATP concentration in unfiltrated and filtrated samples of different parts of GIT contents of piglets

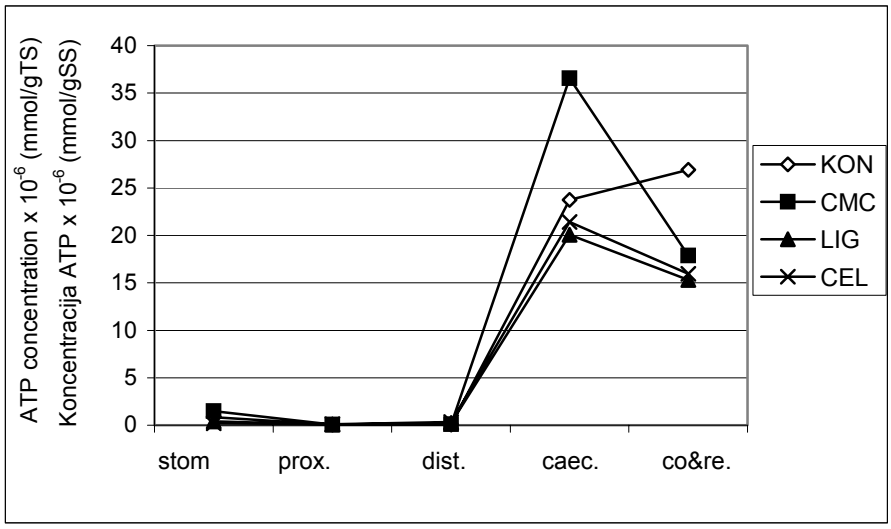
Preglednica 1. Koncentracija ATP v filtriranih in nefiltriranih vzorcih vsebine različnih delov prebavil pujskov

| Part of GIT<br>Del prebavil | ATP conc., mmol $g^{-1}$TS<br>Koncentracija ATP, mmol $g^{-1}$ SS | | The portion of somatic ATP, %<br>Delež somatskega ATP |
|---|---|---|---|
| | Unfiltrated sample (bacterial ATP)<br>Nefiltriran vzorec (bakterijski ATP) | Filtred sample (somatic ATP)<br>Filtriran vzorec (somatski ATP) | |
| Stomach<br>Želodec | $164.34 \times 10^{-8}$ | $0.88 \times 10^{-8}$ | 0.54 |
| Caecum | $46.96 \times 10^{-6}$ | $0.45 \times 10^{-8}$ | 0.96 |
| Slepo črevo | $27.34 \times 10^{-6}$ | $0.12 \times 10^{-8}$ | 0.44 |
| Colon and rectum | $56.96 \times 10^{-6}$ | $0.09 \times 10^{-8}$ | 0.15 |
| Kolon in rektum | $40.31 \times 10^{-6}$ | $0.44 \times 10^{-8}$ | 1.09 |

The concentration of ATP (microbial biomass) of all groups of piglets was the highest in large intestine. Concentrations were the highest in caecum and a bit lower in colon and rectum with exception of group KON. The concentration of bacterial biomass was low in stomach and in small intestine in comparison with the large intestine in all groups. The concentration of biomass was higher in stomach than in small intestine in all groups except the group CEL. These results are in accordance to the results of Bach-Knudsen *et al.* (1991). In stomach there was less biomass detected in groups CEL and LIG than in group KON. That was expected, because bacterial degradation of fiber (cellulose, lignocellulose) is limited in stomach (Drochner, 1993). Microbial fermentation takes place mainly in large intestine (Drochner, 1993; Davidson and McDonald, 1998; Salobir, 1999, Pluske *et al.*, 1999). There was more biomass in distal part of small intestine than in proximal part in all groups. The highest conc. of bacteria in both parts of small intestine was in group CEL, followed by group LIG. In the proximal part of small intestine there were more bacteria present in group KON, than in group CMC. In the distal part there was just the opposite. Smits *et al.* (1997) showed that very viscous carboxymethylcellulose caused in chickens the fall of starch degradation in ileum ( the last part of small intestine). That could be also the explanation for our results. In caecum we determined the highest concentration of bacteria in group CMC. The reason for that was probably a good solubility of carboxymethylcellulose (Low, 1993) and a good microbial fermentation as a consequence. The conc. of carboxymethylcellulose in colon and rectum was probably so small, that it could not enable multiplying of bacteria like starch in group KON. The high concentration of bacteria in colon and rectum in group KON indicated that piglets (their age at sacrificing was 5 weeks)

could not degrade all the starch in feed by their endogenous amylases. The starch flows into the large intestine and it is degraded there by microbial fermentation. In weaned piglets the activity of amylases is falling down for one week, then it is slowly elevated (Ewing and Cole, 1994). That means that a lot of starch could escape degradation and is degraded further on by bacterial fermentation.

In colon and rectum the highest concentration of bacterial biomass was found in group CMC, followed by group CEL and then group LIG. This was expected from the point of view of accessibility of the substrate for bacterial fermentation. The differences in ATP concentration between groups were not statistically significant. The reason for such results was probably the nonhomogenity of samples from GIT at different groups of piglets, the age of experimental animals and maybe some other experimental conditions.




stom. = stomach / želodec, prox. = proximal part of small intestine / zgornji del tankega črevesa, dist. = distal part of small intestine / spodnji del tankega črevesa, caec. = caecum / slepo črevo, co&re. = colon and rectum / kolon in rectum; KON = contol / kontrola, CMC = carboxymethyl cellulose / karboksimetil celuloza, LIG = lignified cellulose / lignificirana celuloza, CEL = pure cellulose / čista celuloza

Figure 1. Average concentrations of ATP in different parts of GIT in different groups of piglets.
Slika 1. Povprečne koncentracije ATP v različnih delih prebavil pri različnih poskusnih skupinah pujskov.

## CONCLUSIONS

With the designed protocol of ATP-luciferase assay we can distinguish the quantities of bacterial biomass in different parts of GIT in weaned piglets.

Soluble fiber (carboxymethylcellulose) enhanced the growth of bacterial biomass in caecum of piglets immediately after weaning more intensively than the supplement of pure cellulose or lignified cellulose. Differences between the experimental groups, detected by the designed ATP-luciferase assay protocol, were not statistically significant. The microflora of weaned piglets might react differently to various fiber sources as the microflora of fattening pigs does.

## POVZETEK

V raziskavi smo proučevali vpliv treh različnih vrst vlaknine (karboksimetilceluloza, lignificirana celuloza, čista celuloza) na koncentracijo mikrobov v prebavilih odstavljenih

pujskov. V poskus smo vključili 24 odstavljenih pujskov in jih razdelili v 4 skupine. Skupine so dobivale krmo, ki se je razlikovala le po viru vlaknine. Kontrolni skupini pujskov smo namesto vlaknine v krmo dodali škrob. Po trinajstih dneh krmljenja s poskusno krmo smo pujske žrtvovali in odvzeli vzorce iz vsebine prebavil: želodec, prvi del tankega črevesa, drugi del tankega črevesa, slepo črevo ter kolon in rektum skupaj. Količino mikrobne biomase in intenzivnost fermentacije v posameznih delih prebavil smo spremljali z ATP-luciferaznim testom. ATP-luciferazni test smo najprej preizkusili s čisto kulturo *E. coli*. Koncentracije ATP so se linearno zmanjševale glede na večanje redčitve kulture, rezultati pa so bili tudi ponovljivi. Test smo preizkusili še z mešanico *E. coli* in somatskih celic. ATP somatskih celic smo uspešno odstranili s kombinacijo Triton X-100 in ATPaze. Test smo nato uporabili za določanje mikrobne biomase v prebavilih pujskov. Rezultati analiz so pokazali, da je fermentacija najbolj intenzivno potekala v debelem črevesu. Največ mikrobne biomase oz. največjo koncentracijo ATP in HMK smo dokazali v slepem črevesu. Največjo množino bakterijske biomase smo našli v slepem črevesu pri skupini, ki je kot dodatek vlaknine dobivala karboksimetilcelulozo, vendar razlike niso bile statistično značilne. Variabilnost rezultatov je bila namreč velika. V raziskavi nismo uspeli dokazati, da bi topna vlaknina (karboksimetilceluloza) pri odstavljenih pujskih statistično značilno povečala mikrobno fermentacijo oz. količino mikrobne biomase v prebavilih pujskov (v primerjavi s čisto celulozo in lignificirano celulozo). Morda je bil vzrok v veliki variabilnosti vzorcev vsebine prebavil, ali pa mlade poskusne živali (odstavljeni pujski), pri katerih se ustrezna mikroflora še ni uspela utrditi, reagirajo drugače kot starejše živali.

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