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What is the Effect and Fate of Orally Administered Living or Heat Killed Bacteria and Endotoxin?

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ABSTRACT

The authors studied the fate of different living bacteria given orally to mice or adult volunteers in the years of late 60th and early 70th. Furthermore, consequences of consuming of heat killed bacteria and lyophilized Boivin's extract/endotoxin prepared from different intestinal bacteria and Salmonella cells excreted by patients suffering from food-borne infection were also examined. It was observed that neither the consumption of high dose Boivin's extract nor a great amount of heat killed bacteria caused any visible enteric or general symptoms in men. When great amount of non-pathogenic living bacteria were administered orally to mice or such bacteria were engulfed by the volunteers the germs disappeared from their faces after some days seemingly without eliciting any symptoms. But when non-virulent Salmonella bacteria were used in case of mice a long excretion was observed. If infant specific *Escherichia coli* bacteria were consumed by adult volunteers then diarrhoe and cramps appeared in some cases. On the basis of the data it can be stated that the non-pathogenic bacteria administered by oral route disappear from the bowel of mice and men after some days but the pathogenic ones are present in the faecal samples for longer time in accordance with their invasive and multiplying capacity.

Key words: Administration, bacteria, endotoxin, mice, volunteers, excretion

INTRODUCTION

Research of enteric bacteria began earlier than 1880 in our country [1]. In 1888 Klug [2] wrote in his book that *in the intestine of newborns no putrefaction, decomposed substances and gas could be detected*. *In consequence of swallowing of air and fluids Schizomycetes got into the gut of babiesand fermentation startedthere.* He observed that intestinal microorganisms belonged to fungi and spherical, short or long rod-shaped and spiral bacteria, namely he alreadyknew that a lot of different microorganisms lived and metabolized substances and produced gas in the enteric canal. He also mentioned that some kinds of the enteric bacteria might be pathogenic causing toxicosis or enteric infection with death. In 1908 Metchnikoff [3] proposed to consume fermented milk products containing lactic acid bacteria as their beneficial effect was supposed. In the 1920th Preisz and his co-workers [4] studied the presence of phages in faecal samples. At the Pécs University in 1939 Duzár [5] and Gagyi [6] analysed the enteric Lactobacillus flora of babies

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artificially fed. In the early 1960th Kétyi [7] examined composition and stability of the gut flora and later we started to workon this field (Ralovich et al., [8], Ralovich & Rauss, [9], Ralovich et al., [10,11], Emődy et al., [12], Emődy & Ralovich, [13]). Since the proposal of Metchnikoff huge amount of therapeutic and/or prophylactic experiments have been performed with living bacterial cells all over the world [14].

In our study we demonstrate consequences of the consumption of living or heat killed bacteria or lyophilized Boivin's extract/endotoxin [15] in volunteers as well as that of the orally administered living bacteria in mice under controlled conditions.

MATERIALS AND METHODS

In our experiments faecal samples and internal organs of orally injected mice and faeces of healthy adult volunteers who drank different living enteric bacteria as well as that of patients who consumed creamy cake containing *S*. *enteritidis* in a food-born epidemic were studied. Also we controlled clinical and subjective condition of volunteers after oral challenge with different quantity of lyophilized Boivin's extract/endotoxin [15] and heat killed – at 56 Cº for 30 minutes – bacteria the number of which was determined by opacity standard. Our strains (*E*. *coli* O111:K58 (B4), *E*. *coli* O126:K71 (B16), *E*. *coli* O83, *S*. *typhi* O901, *S*. *enteritidis* "268", *S*. *hvittingfoss, S*. *paratyphi B* "6441", *Proteus* (now *Morganella*) *morganii* "558" and "8662/64", virulent *L*. *monocytogenes* and non-virulent Listeria), the cultivation and counting methods and that of the injection were published earlier [8-13]. Our *E. coli* strains were streptomycin and *P. morganii* strains polymixin B resistant. Quantity of serum components – number of w.b.c., sugar, iron and phosphorus – was determined on the basis of the usual clinical laboratory methods. As to the healthy volunteers, who lived their usual life, offered to participate in these experiments without any compensation and they belonged to three groups:

- The first group contained some members of the Microbiological Institute of Pécs University with the age of 30, 33, 47 and 49 years.
- Into the second one university students of 19, 22 and 23 years old were classed.
- The third group consisted of members of hotel for old persons with the age of 62, 62 and 66 years.

The volunteers drank the bacterial suspension after breakfast in the late morning.

RESULTS AND DISCUSSION

Experiments on mice: On the Figure 1 you can see the presence of bacteria in the faeces of mice injected per os with *E*. *coli* O111:K58 (B4), *E*. *coli* O126:K71 (B16), *S*. *typhi* O901 or *Proteus morganii* "558" strain. These strains were non-pathogenic for mice. The germs could be detected in the faecal samples for only few – less than 10 – days.

On the Figure 2 bacterial excretion by animals injected orally with non-mouse-virulent *S*. *enteritidis "268"*, *S*. *hvittingfoss* or *S*. *paratyphi B* "6441" strain can be seen. It seems quite clearly that these microorganisms could be found for more than two weeks in the faeces of the mice.

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Excretion in %

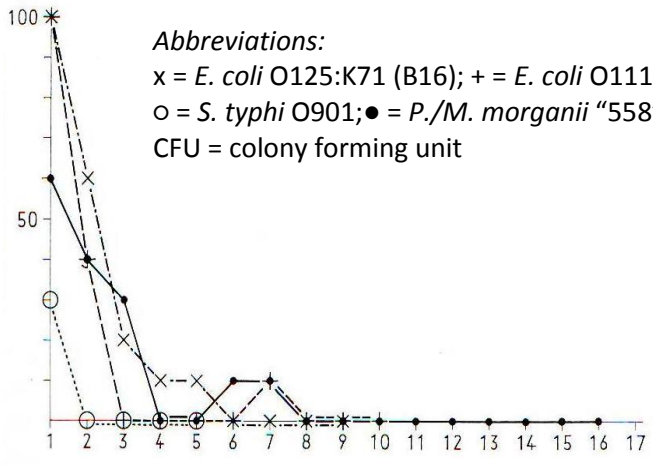
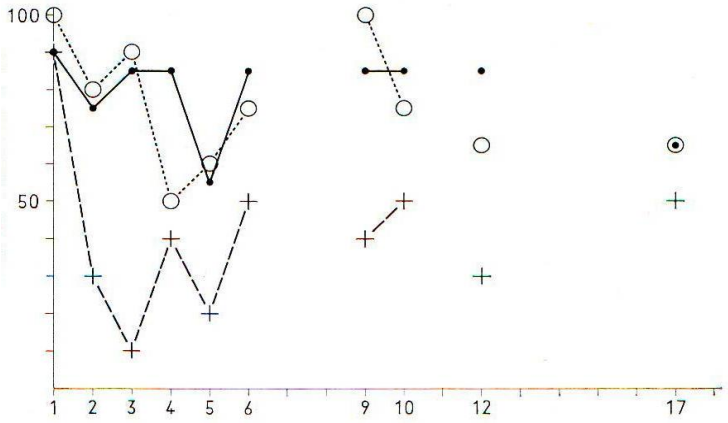


Figure1: Excretion of different non-pathogenic bacteria by mice after oral route injection (500 million germs CFU/mouse)

Excretion in %



Abbreviations:● = *S. enteritidis* "269"; ○ = *S. hvittingfoss;* + = *S. paratyphi B* "6441"
CFU = colony forming unit

Figure 2: Excretion of non-virulent Salmonella bacteria by mice after oral route injection (500 million germs CFU/mouse)

In our next experiment 80 mice were injected with human virulent *L*. *monocytogenes* and 80 animals with non-haemolytic/non-virulent Listeria bacteria and the presence of these germs was detected in the

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faecal samples of the mice for days. Both strains could be found in the faeces for about 10 days but the character of their excretion was somewhat different. The number of positive mice injected with non-virulent Listeria decreased faster.

Table 1: Persistence of non-virulent *S. enteritidis* "269" bacteria in mice after oral injection (500 million germs CFU/mouse)

Sampling time after injection	Heart blood	Liver	Spleen	Small intestine	Cecum	Large intestine	Faeces
10thminute	-	+		+		+	
20thmin.	-	-			+		
30th min.	+	+					
35th min.	-	-	-	+	+	-	
40th min.	-	-					
45th min.	-	-			-		-
60th min.	+	-		+	+	-	-
70th min.	+	+	+			+	
90th min.	-			+	+	+	-
90th min.	-		-		+	+	-
120th min.	-	-	-			+	+
160th min.	-	-	-	+	+		-
170th min.	-	-	-	-	+		+
first day	-	-	-	-	+		+
2ndd.	-	-	-	-	+		+
3rd d.	-	-	-	-	-		-
4thd.	-	-	-	-	-		-
5thd.	-	+	+		+		+
6th d.	-	+	+	+	+		+
9thd.	-	+	+	-	-		+
12thd.	-	+	+	+	+		+
13thd.	-	+	+	-		+	+
13thd.	-	+	+	-	-	+	
15thd.	-	+	+	-	-		-
16thd.	-	+	+	+	+		+
19thd.	-	+	+	-	+		+
20thd.	-	+	+	-	+		
24thd.	-	+	+	-	-	-	-
25thd.	-	-	+		-		-
26thd.	-	-	+	-	+		-
27thd.	-	+	+		+	-	-
31std.	-	-	+		+		

Abbreviations: + = Salmonella positive; - = Salmonella negative; CFU = colony forming unit

Furthermore presence of different bacteria mentioned before was also examined in the inner organs of the orally injected mice by cultivation. When *E. coli* O111:K58 (B4) bacteria were administered into 28 mice only 1 liver sample and 1 spleen sample of the same animal and 1 omentum sample of another mouse were positive within the first hour after injection. When the intestinal samples (cecum, large

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intestine, faeces) of the animals were detected 7 mice out of all – including the above mentioned two also – were positive only for two days after the injection. Later no *E. coli* germs could be detected in the animals.

The results of the animals injected with *S. enteritidis* "268" bacteria can be seen in the Table 1. You can see that the salmonellae persisted not only in the internal organs of the mice for days but they were present in the intestinal content also. It seems that there is a connection between the ability of tissue persistence and the length of faecal excretion of this bacterium.

On the basis of these experiments it can be concluded that the character and the length of the faecal excretion of mice injected by oral route is such a phenomenon which depends on both the pathogenic property and virulence – invasive and multiplying ability – of the bacteria. It is natural that the species and the age of the host are also important but we did not extend our investigations to this area that time. It is important to mention that we did not observe permanent colonization of non-pathogenic bacteria in the gut/faeces of the mice after oral administration. That is the internal environment and peristalsis of the bowel of a healthy mouse could clear the external germs out from its own enteric canal.

Experiments on adult volunteers: First we examined the effect of heat killed bacteria and that of the so called endotoxin – 150, 200 or 250 mg-s of lyophilized Boivin's extract prepared from different pathogenic enteric bacteria – consumed by healthy volunteers. Their temperature, number of w.b.c., serum quantity of sugar, iron and phosphorus were measured. After the ingestion neither patho-physiological symptoms nor significant or regular change of the serum parameters could be observed. Therefore we can state that quite high doses of Boivin's extracts – on the basis of our calculation the applied doses were roughly equal to about 10^{13} germs – prepared from pathogenic bacteria as well as heat killed germs were harmless in the gut. After these studies we performed experiments with non-pathogenic living enteric bacteria, too.

Table 2: Excretion (a) and quantity (b) of non-pathogenic *E. coli* O83 in the faeces of volunteers who drank that bacterium suspension

(a)

Number and (age) of the person		Number of the strain ingested	Duration of excretion in days with enrichment***
1	(30)	5.4×10^{10}	11
2	(23)	5×10^{10}	5
3	(62)	1.7×10^{10}	5
4	(49)	1.7×10^{10}	e.n.p.
5	(62)	1.7×10^{10}	13****
6	(66)	1.7×10^{10}	15
7	(47)	1.7×10^{10}	7
8	(33)	1.7×10^{10}	7
9	(23)	10^6	1
10	(22)	10^6	2

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(**b**)

Number and (age) of the person		Serial number of the stool samples	Number of germs excreted in one gram of faeces
1	(33)	1	5×10^6
		2	3×10^9
		3	2.3×10^8
		4	5×10^5
		5	-
2	(49)	1	e.n.p.
		2	1.1×10^8
		3	10^6
		4	-
		5	e.n.p.

Abbreviations: *** = in streptomycin medium; e.n.p. = the examination was not possible; **** = the examination was interrupted

First the faecal excretion of non-entero-pathogenic *E*. *coli* O83 bacteria and their number in the faeces of the volunteers were controlled. The results can be seen in the Table 2.These germs were present in the faecal samples less than two weeks without any symptom and their number in the faeces gradually decreased. That means the strain was not able to stably clutch and multiply inside the intestine for longer time. The situation was almost the same when *P*. m*organiic* "558" and *P*. *morganii* "8662/64" were consumed – see Table 3.

Table 3: Excretion of *P. morganii* by volunteers who drank that bacterium suspension

Number and (age) of the person	Name of the strain ingested	CFU of the germs drunk	Duration of excretion in days without enrichment*****	with
1 (22)	P.m. 558	3.5×10^{10}	-	11
2 (22)	P.m. 558	1.5×10^{10}	1	1
3 (22)	P.m. 8662/64	2×10^{10}	4	5
4 (19)	P.m. 8662/64	1.7×10^{10}	n.t.	2
5 (23)	P.m. 8662/64	1.6×10^{10}	n.t.	3
6 (22)	P.m. 8662/64	10^9	n.t.	-

Abbreviations: n.t. = not tested; ***** = in polymyxin B medium

We also followed the fate of two infant entero-pathogenic *E*. c*oli* strains – that time they were named dyspepsia coli. Our results can be read in the Table 4. Duration of the excretion in case of *E*. *coli* O111:K58 (B4) strain was similar to that of the former non-entero-pathogenic bacteria but there was an important difference also because two persons had loose stools. In case of *E*. c*oli* O126:71 (B16) strain not only loose stools but also cramps were observed and the length of the excretion was somewhat longer than in case of *E*. *coli* O111:K58 (B4) strain Table 4.

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Table 4: Excretion of *E. coli* O111:K58 (B4) by volunteers who drank that bacterium suspension

Number and (age) of the person	CFU of the germs drunk	Loose stool	Duration of excretion in days without enrichment***	with
1 (30)	3.5×10^{10}	+	4	7
2 (22)	2.8×10^{10}	−	3	4
3 (23)	1.7×10^{10}	+	e.n.p.	4
4 (23)	10^{10}	−	1	1
5 (22)	5×10^{9}	−	n.t.	−
6 (22)	10^{9}	−	n.t.	2
7 (22)	5×10^{8}	−	n.t.	−

Abbreviation: *** = in streptomycin medium

In the last Table 5 (see last page) we demonstrated the data of the hospitalized patients who consumed creamy cake contaminated with a *S. enteritidis* strain originated from duck egg. Even they were treated with antibiotic because of their severe symptoms – the treatment was effective on their fever and general condition – the presence of the salmonellae in their faecal samples was massive and lengthened for long time. Their fever and serious symptoms in the light of our above mentioned experimental results of killed bacteria or endotoxin as well as of the non-entero-pathogenic living germs verified that these *S. enteritidis* bacteria broke through the protective barrier of the wall of bowel – mucus layer with the gut bacteria and at least the surface of the cells of the mucus membrane, too – and entered into the tissue of the enteric canal.

DISCUSSION

The gut microbial flora might appear when the Coelenterates – in Hungarian: tömlőbelűek/hydrák – were developed. They had a primitive gut-bottle with only one hole. Supposedly since that point of time phylo - and ontogenesis of the gut flora has started and flowed. Gut canal with two holes was first formed in worms which appeared some 600 million years ago. The life and evolution of human enteric bacterial flora started some 30 million years ago when the first two human beings appeared. The human gut environment including the presence of the enteric flora also is one of the most complicated multifactorial living and dynamic microbiome which has enormous role in the life and development of the actual host. Formation of the normal enteric flora has a natural evolutionary process which is age and nutrition dependent as well as is in a strong connection with the hygienic environment of the baby. When the baby is fed only with mother breast milk then his/her enteric flora consists of lactobacilli. When the feeding of other foods starts then lactobacilli disappeare and *E. coli* as well as other germs settle.

In the oral cavity of a healthy person relatively many aerobic as well as anaerobic species of bacteria and fungi can be detected. In the stomach and the upper part of small intestine only few species of bacteria are rarely present. But the lower part of small intestine regularly contains bacteria less than 10^4/gram of intestinal content. When their number is over that value then they may cause discomfort [16]. In contrast with this the colon contains a lot of different microorganisms up to 10^{12} bacteria/gram faeces. These microbes may belong to more 100 different species which can be classified into Bacteroidales, Faecalibacterium, Firmicutes, Proteobacteria etc. As the Bacteroides species give about one third of the

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flora of bowel their role is especially important. Beside the mentioned bacteria fungi, phages, viruses, non-culturable bacteria and protista may also be present in the gut. Composition of the enteric flora of different individuals may be distinct but the own enterobiome of a person seems to be quite permanent during his/her own life. Different events – changes in the diet and life style or an antibiotic treatment etc. – may cause alterations in that microbiome.

It is a pity but we do not know what is/are the specific symptom/symptoms of the change of the enteric flora of a single person, because nausea, vomiting, cramp, improved peristalsis, excretion and diarrhoe as well as bed general condition are non-specific. They can be elicited by different causes – nutritional, poisoning, emotional, infection or antibiotic etc. and are parts of the natural protective reaction of a person. At present it seems that Ferran consumed first *Vibrio cholera* in 1885 [17]. He was followed by others and at present non-pathogenic living bacteria are widely used for therapeutic and prophylactic purposes [14]. It is known that different components of the intestinal tract – hydrochloric acid of stomach, peristalsis, condition of mucus membrane, pH of gut content, presence or absence of different cells, receptors, enzymes as well as members the enterobiome and their activity etc. – ensure the normal function of the bowel. It has been believed from long ago that hydrochloric acid of stomach has protective effect. We think that the role of hydrochloric acid may be limited as already 1-10 virulent Shigella, Salmonella or *Entamoeba histolytica* can infect persons.

CONCLUSION

Results of our own experiments support the concept of a normal clearing out effect of the enteric canal, which function may be age, life style and food dependent because the injected or consumed great quantity of external non-pathogenic germs disappeared relatively quickly from the bowel of the mice as well as from the enteric canal of the volunteers likely to thenon-living particles (for example: *Corbo activatus*) of a similar size. Therefore, it is not a surprise for us that in 2005 it was written "…*Oral delivery of free live cells, lyophilised cells and immobilised cells has been attempted, but with restricted success, chiefly because bacterial cells are unable to survive passage through the gastrointestinal tract in sufficient dosage*…" and also that "*one must presently concluded that the value of beneficial bacterial cells has not been adequately established by rigorous scientific research to support their use in all but a few cases*." [14]. Longer but not permanent excretion was only observed in cases of the pathogenic Salmonella strains in accordance with the degree of their pathogenic property and virulence – invasive and multiplying ability. We think that the virulence and quantity of a strain as well as the age and condition of a host are the most important factors in manifestation of an enteric infection and in causing long faecal excretion. Earlier it was an idea that during an enteric infection endotoxin was liberated from the bacteria disintegrated in the enteric canal causing the toxic-symptoms. On the basis of our experimental results it can be concluded that killed bacteria or endotoxin as well as non-entero-pathogenic living germs are harmless after consumption.

So we at present have doubts as to a general beneficial effect of mainly preventive but also therapeutic use of the so called "normal enteric flora" preparations because their microbes are ''foreigners'' for a given person and therefore they may be easily cleared out from the enteric canal. As there is a close mutual connection among nutrition, hygienic condition and development of babies as well as the composition of their own natural gut microbiome in their cases and also in the cases of immune-compromised patients the use of a normal enteric flora preparation may be hazardous. The physiological condition of the enteric canal including its enterobiome also is a self managing natural dynamic complex biological system with an own special evolution. Therefore it is better not to influence it by artificial living foreign microbial mass mainly for preventive aim. There is only one exception that is

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the immunization. When there is any type of enteric discomfort also better to fast diagnose its cause and to eliminate that. We think it is more natural to permit the organism to perform its own restoration which is its usual character. There may be only one exception when a ”real expert” recommends the use of living bacterium therapy. In the other cases if somebody wants to apply a living microbial culture to develope general condition of some one then the naturally fermented but not ’’further developed” – flavoured, supplemented, homogenized etc. – acid milk products like yoghourt or Caucasian kefir are available at a reasonable price.

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Table 5: Excretion of *S. enteritidis* bacteria by patients who were admitted into hospital in consequence of a food-borne infection spread with creamy cake

Number of patient	Days of																														
	June																	July									September				
	8	9	10	11	12	13	14	15	17	18	19	21	23	26	27	29	1	3	4	5	6	7	8	11	13	22	23	25			
I	a		I	+	+	-		-	-		+	+	+	-																	
	b			+	-			-	-		+	-	+	-			+		+		+	-	+	-			-	-			
			A	●	●	●									D																
II	a	I		+	-	-		+	+		+	+	-	-																	
	b			+	-			-	+		+	+	+	-			-		-		-	-	-	-			-	-			
			A●	●	+●										D																
III	a	I		+	+			+	+		+	-	-	-						+					-					
	b			+	+			+	+		+	+	+	+			+	-	-	+	-			-	-						
			A●	●	●										D																
IV	a	I		+	+	+	-	+	+		+	-	-	-				-		-		-	-	-							
	b			+	+				+		+	+	+	+									-								
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V	a		I		-	-		+	-			+	+	-									+	-			-	-	-		
	b			+	+			-	-		-	-	-	-																	
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VI”	a	I						+	+		+	+	+	+		+	-		+			-		+		-	-	-			
	b				+						+	+	+	+																	
							A		●	●					D																

Abbreviations: a = laboratorium I.; b = laboratorium II.; I = day of infection; A = admittance; ● = days of the antibiotic treatment; D = discharge; + = faecal sample was Salmonella positive; - = faecal sample was Salmonella negative; VI” = this patient was treated at home for five days