Eco Process Assistance

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TN 22.4

Breakdown of human faeces by co-cultures and the characterization of the microbial strains present in human faeces by means of a literature study.

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1. GENERAL INTRODUCTION

Previous experiments proved that no efficient biodegradation of artificial human faeces and pig manure by axenic strains of *Clostridium thermocellum, Clostridium thermosaccharolyticum* and *Coprothermobacter proteolyticus* I8 nor by a co-culture of these strains occurred (see TN 22.1, TN 22.2, TN 22.3).

The next step was to investigate whether a co-culture of *Clostridium thermocellum, Clostridium thermosaccharolyticum* and *Coprothermobacter proteolyticus* I8 could biodegrade human faeces.

A literature study on the microbiota present in human faeces was carried out to prepare a future experiment (TN 22.5) in which the autochtonous strains in human faeces will be used as inoculum.

2. LITERATURE STUDY: THE MICROBIOLOGY OF THE HUMAN INTESTINAL TRACT

2.1. COMPOSITION OF THE MICROBIAL POPULATION

2.1.1. General

The anaerobic decomposition of food components is a very complex process and follows different catabolic pathways, eventually resulting in the formation of relatively simple substances.

Also the microbial populations, which are responsible for decomposition, are very diverse. Detailed studies are mainly available for human beings and are therefore used as reference data.

The human gastro-intestinal (GI) tract contains about 400 to 500 different species, among which 30 to 40 are important. Parts of the GI tract, such as the human colon, have a low oxidation-reduction potential and the ratio of obligate anaerobes to facultative anaerobes is equal to 1000 over 1 (Tannock, 1988).

The most important characteristics of some microbial groups in the human intestinal tract are given in Table 2.1.The optimal temperature for most GI-bacteria is the body-temperature (36 to 38 ° C).Table 2.2 gives some real data for some of these bacteria (l groups) based on the characterization of the faeces of seven healthy adults (Ikeda et al., 1994).

Table 2.2. Occurrence of bacteria in faeces of seven healthy adults over 7 months (Ikeda et al., 1994)

Bacteria (l group)	Mean (log bacterial counts/g wet faeces)
Enterobacteriaceae	7.4
Streptococcus	7.3
Staphylococcus	2.6
Lactobacillus	4.4
Bifidobacterium	10.1
Eubacterium	10.0
Bacteroidaceae	10.5
Clostridium	9.1
Megasphaera	5.2
Total aerobes	8.4
Total anaerobes	10.8
Total bacteria	10.8

Other important species are *Coprococcus* (anaerobic, G^+), *Fusobacterium* (anaerobic, G^-), *Ruminococcus* (anaerobic, G^+) and *Staphylococcus* (facultative anaerobic, G^+) (Benno et al., 1989a).

For *Lactobacillus* and *Bifidobacterium* spp. it is known that they especially influence the partner strains and not so much the host. For other genera no information about this is available (Norris et al., 1991).

Table 2.1. Important characteristics of some microbial groups in the human intestinal tract

Morfological characteristics	Substrates	Fermentation products
Bacteroides anaerobic G^-; straight, curved and spiral rods	cellulose, cellobiose, hemicellulose, pectin, starch	succinate, acetate, ethanol, H_2, formiate, butyrate, lactate
Bifidobacterium anaerobic G^+; Y-shaped non spore forming rods	lactate, malate, proteins, amino acids	acetate
Lactobacillus facultative anaerobic G^+; regular non spore forming rods	different sugars, starch, maltose	lactate
Clostridium anaerobic G^+; endospore forming rods	cellulose, cellobiose, hemicellulose, pectin, starch, fatty acids, amino acids	succinate, acetate, ethanol, H_2, formiate, butyrate, lactate
Escherichia coli facultative anaerobic G^-; straight rods	acetate, glucose, lactose	pyruvate
Eubacterium anaerobic G^+; irregular non spore forming rods	glucose, lactate, pyruvate	butyrate, acetate, CO_2, H_2, lactate
Peptococcus anaerobic G^+; coccoids	proteins, amino acids fatty acids	volatile fatty acids
Streptococcus facultative anaerobic G^+; coccoids	starch, maltose, sugars	lactate, acetate
Peptostreptococcus anaerobic G^-; coccoids	lactate, malate, proteins, amino acids	volatile fatty acids
Megasphaera anaerobic G^-; coccoids	lactate, glucose, other sugars	volatile fatty acids, H_2, lactate, propionic acid
Enterobacter facultative anaerobic G^-; rods	different sugars	acetate, formiate

2.1.2. Special organisms

a. *Bacteroides succinogenes*

B. succinogenes is an anaerobic G^- coccoid bacterium, sensitive to vegetable phenols. It is the most important decomposer of intact plant cell walls (crystalline cellulose). This organism solubilizes cellulose and hemicellulose. It ferments however only cellulose via glucose mainly to succinate. It leaves the pentoses to the commensals and in this way propagates the microbial diversity.

b. Spirochetes (Cowley & Hill, 1989)

The spiral bacteria especially live in the caecum. They appear in densities of about 10^6/g faeces. They penetrate into the cytoplasm of epithelial cells but they seem to cause no damage or infection. Till now, their role is unknown and they cannot be grown in pure cultures.

c. Lactic acid bacteria (LAB)

Four genera are distinguished : *Lactobacillus* sp., *Streptococcus* sp., *Pediococcus* sp. and *Leuconostoc* sp.

Lactic acid bacteria ferment lactose and produce several enzymes:

- Proteinases by which the amino acids are used for rapid growth;
- Bile salt hydrolases (bile salt = steroid + amino acid) by means of which the emulsifying action of the bile salts is inactivated;
- Antimicrobial factors such as H_2O_2, bacteriocins,...

According to Mitchnikoff (Nobel price 1908) lactic acid bacteria (LAB) are essential for a good health and a long life (Bibel, 1989). This is actually put in question, because LF-animals (Lactic acid bacteria Free) and control animals grow equally well (Tannock et al., 1989).

2.2. MICROBIAL CONVERSIONS

2.2.1. General scheme

The microbial association uses a big part of the uptaken food components to make own cell dry weight (CDW). Another important part of the microbial fermentation products however is resorbed by the host.

2.2.2. Conversion of products

a. Carbohydrates

Carbohydrates are hydrolysed with the help of enzymes (cellulases, amylases, xylanases, ...) to monosaccharides (Schlegel, 1981).

b. N-containing compounds

The decomposition of proteins via peptides to amino acids comes about by the proteolytic activity of diverse micro-organisms by means of proteases and peptidases. During the degradation of proteins in the stomach-colon tract a few potentially toxic metabolites are formed such as NH_3, phenols, indols and amines (Gibson et al., 1989).
The decomposition of amino acids happens via a decarboxylation or a desamination reaction (Schlegel, 1981).

Decarboxylation gives rise to the formation of primary amines among which are some very troublesome compounds such as cadaverin, putrescin and agmatin. They appear during the normal digestion and anaerobic degradation processes (Schink, 1988).

In case of desamination, ammonia is set free. The desamination can be oxidative, desaturative or hydrolytic. An example of this last mechanism is the hydrolysis of urea with the help of urease.

In animals, other N-containing compounds such as purines and pyrimidines, build-stones of nucleic acids, are degraded to N-containing by-products. Urinic acid, N-source in human urine (Roche Lexikon Medizin, 1984), is an intermediary product in the degradation of purines and is degraded to CO_2, acetic acid and ammonia (Vogels & Van Der Drift, 1976).

Nitrate-nitrogen is of big importance in the colon tract. Nitrate concentrations in drinking water may not exceed 50 ppm. Most of the nitrate however is consumed via vegetables. Furthermore, it is noticed that daily about as much $NH4^+$ is oxidised to $NO3^-$ as is taken up via the food. This oxidation seems to occur via a radical mechanism (Dull & Hotchkiss, 1984).

c. Bile salts

Bile salts are of importance for the digestion of fats. Man e.g. has a reserve of 3 to 4 g bile salts. Bile salts pass through the so-called enterohepatic cycle with an efficiency of ca. 95 %. About 5 % of the bile salt reserve is not recuperated because of bacterial transformations. The formed modified bile salts are less efficiently resorbed in the last part of the small intestine and are lost with the faeces. The cycle proceeds at a rate of ca. 3 times per meal.

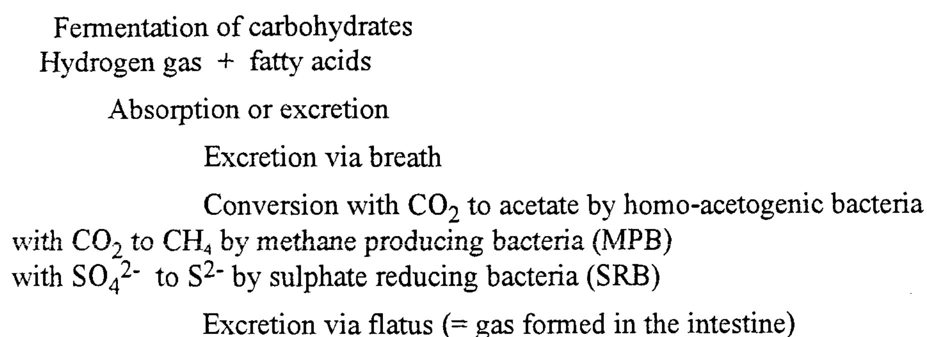
Bile salts can be hydrolysed by autochtonous lactobacilli resulting in the formation of deconjugated bile salts. Bacterial bile salt hydrolysis takes place in the GI tract of both man and animal.

d. Gases

During the fermentation of carbohydrates, hydrogen gas is produced. Out of 40 to 50 g of carbohydrates more than 1 liter of hydrogen gas can theoretically be produced. This hydrogen gas is digested in different ways (Figure 2.1).

The flatus of most people amounts to ca. 0,5 l gas/day and consists of N_2 (70 %), O_2 (1 %), CO_2 (10 %), CH_4 (6 %) and H_2 (13 %). H_2S, NH_3 and mercaptanes are also present in lower concentrations. Gastro-enterologists are since long fascinated by the meaning of hydrogen metabolism in the colon.

Figure 2.1 Mechanisms for hydrogen gas conversion in the colon (after Gibson, 1993)



It is remarkable that :
- few or no people convert H_2 to acetate;
- SRB and MPB exclude each other; ca. 60 % of the European people produce only S^{2-}, 30 % produce only CH_4 and 10 % produce both gases.

There are no hard data on a direct relation between H_2S/CH_4 production and intestinal diseases (Gibson, 1993). However it is considered that patients with ulcerative colitis usually produce more sulphide. The accumulation of this gas in the colon can result in cellular damage resulting in breaking of the protective intestinal epithelium.

e. End products

Table 2.3 gives some real data of faecal putrefactive products, ammonia, short chain fatty acids, moisture and pH in the faeces of seven healthy adults (Ikeda et al., 1994).

Table 2.3. Mean data of faecal putrefactive products ($mmol.kg^{-1}$ wet weight), ammonia ($mmol.kg^{-1}$ ww), short chain fatty acids (SCFAs)($mmol.kg^{-1}$ ww), moisture (%) and pH in the faeces of seven healthy adults (after Ikeda et al., 1994)

Parameter	Mean ± standard deviation
Phenol	0,02 ± 0,02
p-Cresol	0,56 ± 0,11
4-Ethylphenol	0,06 ± 0,11
Indole	0,38 ± 0,12
Skatole	0,11 ± 0,09
Ammonia	43,6 ± 10,1
Succinic acid	3,10 ± 9,53
Lactic acid	11,6 ± 7,22
Formic acid	9,00 ± 3,17
Acetic acid	9,20 ± 22,12
Propionic acid	31,80 ± 10,58
i-Butyric acid	4,80 ± 6,32
Butyric acid	23,6 ± 9,33
i-valeric acid	3,2 ± 2,3
Valeric acid	4,20 ± 2,63
Total SCFAs	170,4 ± 36,25
Moisture	76,2 ± 5,98
pH	6,55 ± 0,72

3. EXPERIMENTS

3.1. MATERIALS AND METHODS

3.1.1. Human faeces

The human faeces were collected from five healthy persons (age 20-30 years: four persons and 50 years: one person). The faeces had a dry matter content of 20 to 35%.The faeces were immediately stored at a temperature of minus 18°C.

At the start of the experiments, the faeces were defrosted and diluted with demineralised water. The dilution was necessary to determine the composition in an accurate way and to obtain a liquid medium for the biodegradation tests.

3.1.2. Inoculum

Table 3.1 shows the three strains used in the degradation test. The strains were grown in the cultivation media during five days.

Table 3.1. Medium and substrate used to grow the strains

Strain	Reference	Medium	Substrate	Activity
Clostridium thermocellum	ATCC 27405	MS-medium	Cellobiose (3 g/l)	Cellulolytic
Clostridium thermosaccharolyticum	LMG 2811	MS-medium	Cellobiose (3 g/l)	Cellulolytic
Coprothermobacter proteolyticus I8	DRANCO-isolate	MS-medium	Gelatine (3 g/l)	Proteolytic

MS-medium (see addendum 1)
DRANCO-isolate (Kersters, 1992)

3.1.3. Analytical techniques

The *dry matter (DM)* of the sample was determined after 24 hours drying at 105° C. The *ash content* was determined after incineration at 450°C for 3 hours.

A sample was filtered and the residue was dried for 24 hours at 105°C to determine the *suspended solids* (SS). The *volatile suspended solids (VSS)* were determined by incineration of the dried residue at 450°C for 3 hours.

Volatile fatty acids (VFA) were extracted with diethyl ether from acidified samples and determined by gas chromatography using a flame ionization detector coupled to a glass column containing chromosorb 101.

Total protein concentrations were determined by acid hydrolysis (decomposition into amino acids) and a colorimetric measurement (Hattingh et al., 1967).

The NH_4-N content was determined by steam distillation in a Kjeltec 1002 apparatus under alkaline conditions. *(NO_3 + NO_2)-N* was determined by steam distillation in a Kjeltec 1002 after reduction to NH_3 by the addition of Devarda alloy. *Kjeldahl nitrogen* was determined similarly after complete destruction of the sample in strong acid.

The *chemical oxygen demand (COD)* corresponds to the amount of oxygen necessary for complete oxidation of all organic matter present in a given volume of sample. The organic content of the sample was subjected to oxidation by potassium dichromate, in a strong acid medium (sulphuric acid plus silver sulphate) at a temperature of 150° C for two hours. The excess dichromate was then measured by back titration with ferrous ammonium sulphate. The *total COD* (COD_{tot}) was determined on the total sample, whereas *soluble COD* (COD_{sol}) was determined on a centrifuged sample.

3.1.4. Experimental set-up

3.1.4.1. Experiment 1: Autoclaving during 20 minutes

Bottles of 250 ml were filled with 120 ml of human faeces and flushed with nitrogen gas. The pH was set at 7.2 and the bottles were autoclaved during 20 minutes at 121° C. Afterwards 0.8 ml of a 2,5% Na_2S - solution was added to ensure anaerobic conditions.

Next, 5 ml of an inoculum of *Clostridium thermocellum*, *Clostridium thermosaccharolyticum* and *Coprothermobacter proteolyticus* I8 was injected in the bottles. To the blank 15 ml MS-medium was added. Each application was carried out in treble. The bottles were incubated at 60°C and shaken manually several times per day.

At the end of the experiment (after 21 days) the volatile fatty acids, ammonia, Kjeldahl nitrogen, the COD_{tot}, the COD_{sol}, the dry matter, the ash content, the suspended solids and the volatile suspended solids were determined.

3.1.4.2. Experiment 2: Autoclaving during 40 minutes

In the second experiment the faeces were autoclaved during 40 minutes at 121°C. The experimental set-up was exactly the same as in the first set-up (see 3.1.4.1), with the exception that 10 ml of inoculum of each strain was added instead of 5 ml.

After 21 days the concentration of ammonia and volatile fatty acids and the pH were measured.

3.2. RESULTS

3.2.1. Composition of the human faeces

The collected human faeces had a dry matter content of 20 tot 35%. The faeces were diluted ten times with demineralised water. This was necessary to guarantee accurate determinations.

Table 3.2 shows the characteristics of the human faeces. The dry matter consisted for 80% of organic material. About 80% of the organic material was suspended. Based on the COD_{tot} and COD_{sol} determinations, it can be concluded that the main part of the organic material was present under an insoluble form.

The protein content was calculated by multiplying the organic nitrogen content by a factor 6.25. The results showed that one third of the dry matter of the human faeces was present under the form of proteins.

Table 3.2. Composition of the diluted human faeces used in experiments 1 & 2 (mean value and standard error$^{n=4}$)

Parameter	Value mg/l	Standard error$^{n=4}$ mg/l	Value mg/g DM	Standard error$^{n=4}$ mg/g DM
Dry matter	32399	3000	-	-
Organic matter	25879	3010	798	118
Ash	6520	250	202	19
SS	21225	2303	652	93
VSS	19229	2113	591	85
COD_{tot}	45625	1702	1305	303
COD_{sol}	14931	586	404	50
NH_4^+-N	221	16	7	1
NO_3^--N	0	0	0	0
Kjeldahl N	1736	152	54	7
Organic N	1515	153	47	6
Protein content*	9470	957	292	40
VFA	1114	79	36	4
pH**	7.2	0	-	-

* calculated

** no unit

3.2.2. Experiment 1 : Autoclaving during 20 minutes

Table 3.3 gives an overview of the results obtained after 21 days. At the end of the test, there was a significant amount (0.05 level) of ammonia and volatile fatty acids produced in the blank and the application 3S (20 m).

The evolution of the volatile fatty acid concentration is presented in Figure 3.1. It can be concluded that after 14 days, a maximum concentration of VFA is already present. The same can be concluded for the evolution of the ammonia concentration (Figure 3.2.).

In application 3S (20 m) and the blank, the protein content and the organic matter decreased with about 20%. Due to the high standard error however, this decrease is statistically not significant.
The production of volatile fatty acids and ammonia in the blank indicates that after autoclaving the human faeces during 20 minutes there was probably no complete die off of the autochtonous strains.

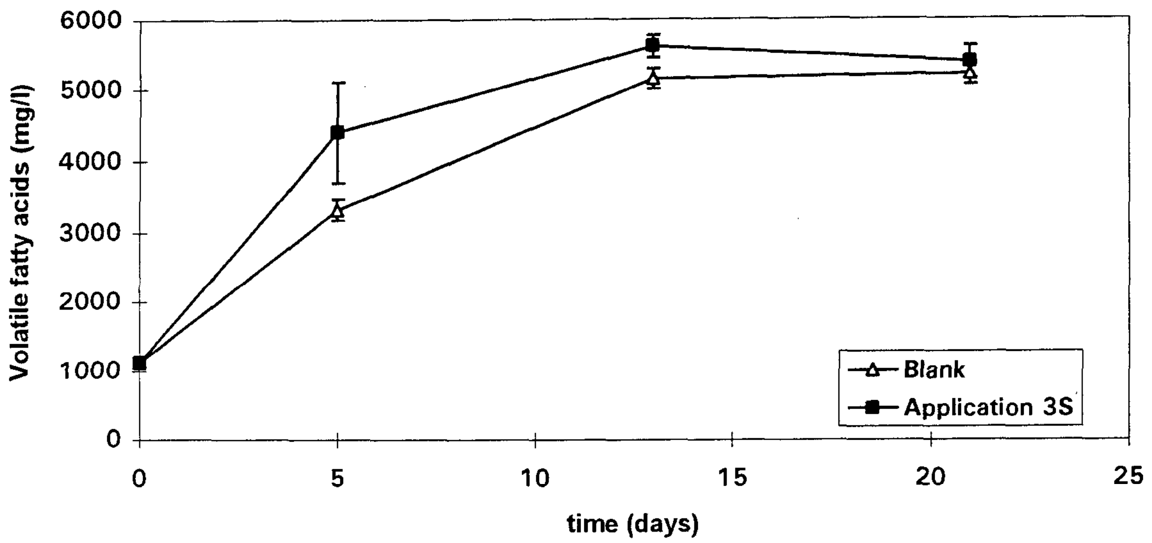


Figure 3.1. Evolution of the volatile fatty acids concentration during the experiment (mean value and standard errors)

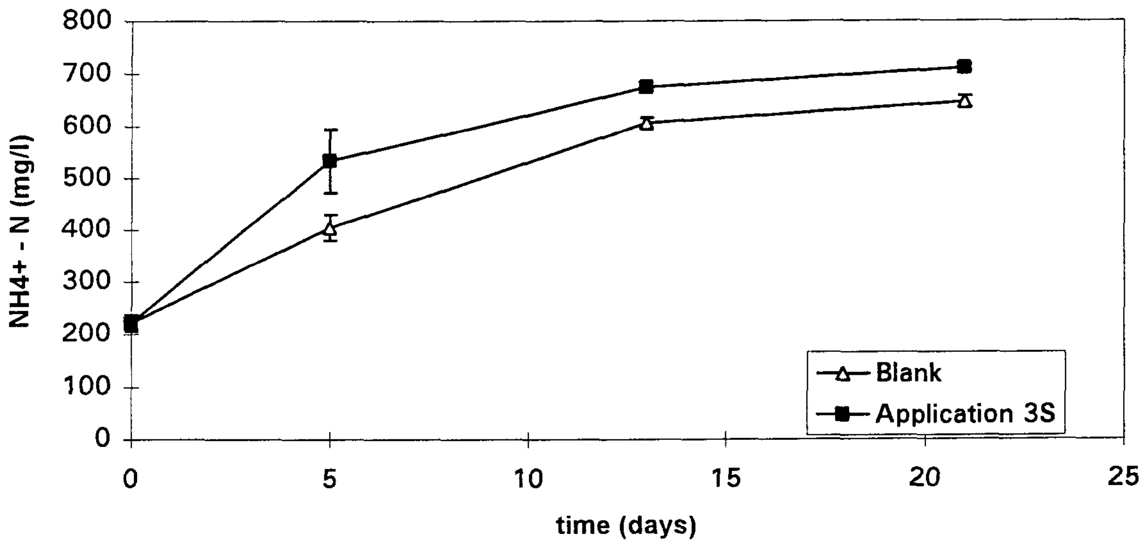


Figure 3.2. Evolution of the ammonia concentration during the experiment (mean value and standard errors)

Table 3.3. Results of the first biodegradation test with human faeces autoclaved during 20 minutes (mean value and standard error$^{n=3}$)

Parameter	Set-up		
	t=0* mg/l	Blank 3S (20 m) mg/l	3S (20 m) mg/l
Dry matter	32399±3000	29177±185	28845±240
Organic matter	25879±3010	21515±188	20554±862
Ash	6520± 250	7662±378	8291±828
SS	21225±2303	19085±414	19016±276
VSS	19229±2113	17052±350	16990±10
COD_{tot}	45625±1702	46269±1035	44030±3110
COD_{sol}	14931± 586	15533±176	15475±165
NH_4^+-N	221±16	645±27	710±7
NO_3^--N	0	0	0
Kjeldahl N	1736±152	1811±22	1821±21
Organic N	1515±153	1166±30	1110±23
Protein content**	9470±957	7288±189	6941±142
VFA	1114±79	4573±558	5384±233
pH***	7.2	6.4	6.4

* n=4
** calculated
*** no unit

3.2.3. Experiment 2 : Autoclaving during 40 minutes

Table 3.4 gives an overview of the results of the second biodegradation test. The ammonia concentration in the set-up 3S (40 m) was 3 times higher compared to the blank.There was also a significant production (significance level 0.05) of volatile fatty acids noticed in set-up 3S (40 m). The concentration increased with about 80%. The pH of set-up 3S (40 m) decreased with 0.3 units compared to the two blanks. This is due to the formation of fatty acids.

The ammonia concentration and pH of the blank at the start of the experiment were not different from the blank at the end of the experiment. Autoclaving during 40 minutes was sufficient for a complete die off of the autochtonous strains.

Table 3.4. Results of the second biodegradation test with human faeces autoclaved during 40 minutes (mean value and standard error$^{n=3}$)

Parameter	Set-up		
	Blank t=0 mg/l	Blank 3S (40 m) mg/l	3S(40 m) mg/l
NH_4^+-N	149 ± 19	141*	395*
VFA	1951±193	1825*	3345*
pH	7.1	7.1*	6.8*

* : determined on a mixed sample

4. CONCLUSIONS

The composition of the human faeces used in the experiment was in accordance with literature data (see Figure 4.1). This was not the case for the artificial human faeces and the pig manure. Therefore, it seems advisable to use real human faeces as substrate for further experiments because of the representative composition.

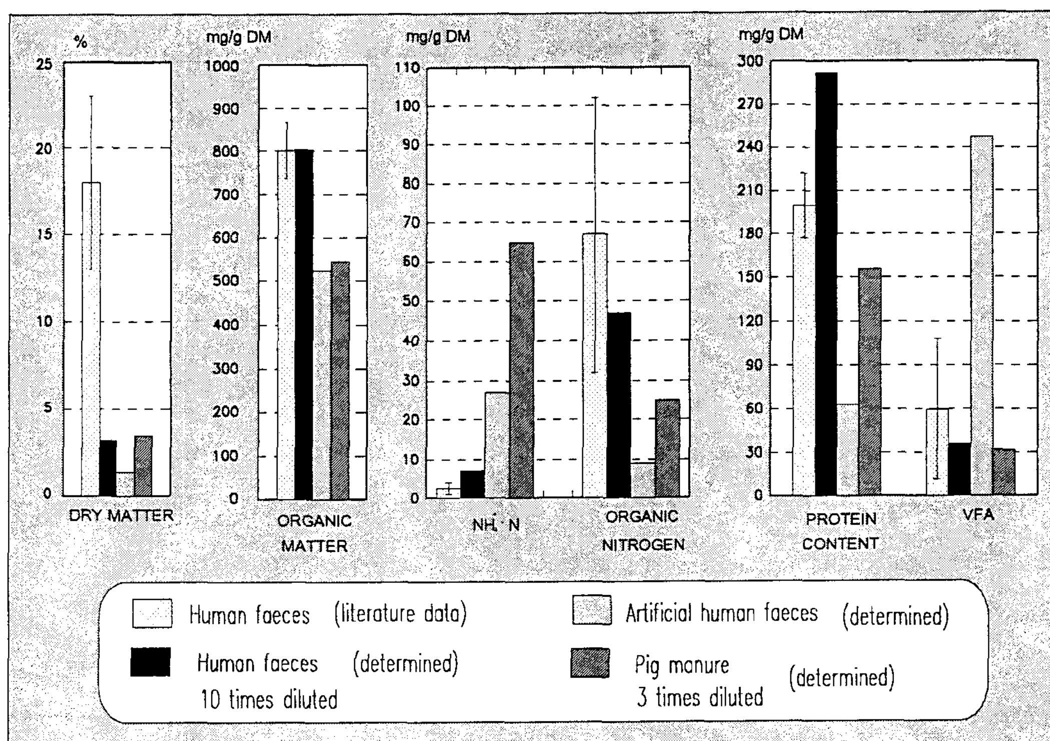


Figure 4.1. Composition of human faeces (experimental and literature data), artificial human faeces (experimental data) and pig manure (experimental data).

The first biodegradation test learned that autoclaving the human faeces during 20 minutes at 121°C was not sufficient to have a total die off of the autochtonous bacteria. The second experiment showed that autoclaving during 40 minutes is needed for a complete sterilisation.

In the first biodegradation test the faeces were broken down as well in the blank as in the set-up with the strains *Clostridium thermocellum*, *Clostridium thermosaccharolyticum* and *Coprothermobacter proteolyticus*. During 21 days of fermentation, about 3500 mg/l volatile fatty acids and 424 mg NH_4-N/l were formed in the blank and 4200 mg/l volatile fatty acids and 490 mg NH_4-N/l in the application 3S (20 m). This test confirms the ability of autochtonous strains to biodegrade the faeces, but did not allow an evaluation of the capability of the co-culture to biodegrade the faeces.

In the second biodegradation test, no autochtonous strains were present anymore. The increase of the ammonia and volatile fatty acids concentration was due to the degradation of the faeces by the added co-culture. During a fermentation period of 21 days, no volatile fatty acids and NH_4^+-N were formed in the blank. In the set-ups 3S (40 m) en 3S (40 m) respectively 1520 and 250 mg/l of NH_4-N was formed.

The production of ammonia and volatile fatty acids in the second degradation test was about two times lower compared to the production of ammonia noticed in the first biodegradation test.The second biodegradation test gave an indication of the ability of the co-culture to degrade human faeces. The efficiency was not as high as for the autochtonous strains.

In a next experiment the ability of autochtonous strains to degrade human faeces will be further investigated.

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Addendum 1: Composition of MS-medium

Component		Amount
NaOH		4 g/l
yeast extract		2 g/l
trypticase pepton		2 g/l
resazurin solution (0.2%)		0.5 ml/l
coenzyme M		0.5 g/l
Solution A		10 ml/l
NH_4Cl	100 g/l	
$MgCl_2.6H_2O$	100 g/l	
$CaCl_2.2H_2O$	40 g/l	
pH	4	
Solution B		2ml/l
$K_2HPO_4.3H_2O$	200 g/l	
Mineral solution		10ml/l
$Na_2EDTA.2H_2O$	500 mg/l	
$CoCl_2.6H_2O$	150 mg/l	
$MnCl_2.4H_2O$	100 mg/l	
$FeSO_4.7H_2O$	100 mg/l	
$ZnCl_2$	100 mg/l	
$AlCl_3.6H_2O$	40 mg/l	
$Na_2Mo_4.2H_2O$	30 mg/l	
$CuCl_2.2H_2O$	20 mg/l	
$NiSO_4.6H_2O$	20 mg/l	
H_2SeO_3	10 mg/l	
H_3BO_3	10 mg/l	
$NaMoO_4.2H_2O$	10 mg/l	
Na_2S (2.5%)		5 ml/l
final pH		7