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MICROBIAL ASPECTS OF THE FORMATION OF MALODOROUS COMPOUNDS IN ANAEROBICALLY STORED PIGGERY WASTES

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op vrijdag 8 december 1978
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

STELLINGEN

1. Veranderingen in concentraties van *p*-cresol en vluchtige vetzuren in varkensdrijfmest kunnen een indruk geven van de werkzaamheid van aan drijfmest toegevoegde stankbestrijdingsmiddelen.

2. Het toevoegen van bacteriemengsels aan drijfmest ter bestrijding van stank is zinloos.

3. Het schema voor de anaërobe afbraak van tyrosine zoals voorgesteld door Drasar en Hill berust in belangrijke mate op foutieve veronderstellingen van Baumann.

   Drasa, B.S. en Hill, M.J. Human intestinal flora. Academic Press. London. 1974.
   Baumann, E. Ber. dtschn. chem. Ges. 12, 1450 (1879).

4. De opmerking van Yasuhara en Fuwa dat fenolen in varkensdrijfmest afkomstig zijn van ontsmettingsmiddelen getuigt van lichtvaardig concluderen.

   Yasuhara, A. en Fuwa, K.
   Bull. Chem. Soc. Japan 50, 731 (1977).

5. *Acetobacterium woodii* in combinatie met een acetaat-benuttende gist biedt betere vooruitzichten voor de productie van "single cell protein" uit celluloserijk afval dan het kweken van schimmels hierop.

6. De betekenis van strict anaërobe niet spore-vormende bacteriën bij de fermentatie van kuilvoer wordt onderschat.

7. Onderzoek naar de rol van kuilvoer als infectiebron van *Listeriosis* zoals uitgevoerd door Gouet et al. geeft geen relevante informatie.

   Gouet, P., Girardeau, J.P. en Riou, J.
   Anim. Feed Sci. Techn. 2, 297 (1977).

8. Het aantal sporen van lactaat-vergistende clostridia in voordroogkuil kan worden beperkt door het in te kuilen gras te hakselen.

9. De gebruikelijke lange middagpauzes bij het kleuter- en basisonderwijs zullen binnenkort niet meer in onze samenleving passen.

10. Fouten in referentielijsten kunnen gedeeltelijk worden voorkomen door van de namen van auteurs van wetenschappelijke publicaties steeds eerst het hoofdwoord en vervolgens, gescheiden door een komma, voornamen en dergelijke te vermelden.

Sierk F. Spoelstra
Microbial aspects of the formation of malodorous compounds in anaerobically stored piggery wastes.
Wageningen, 8 december 1978.

Ter herinnering aan

Kirsten Sophie Didrikke Kaurel

This study was carried out at the Laboratory of Microbiology, Agricultural University, Wageningen, The Netherlands. Financial support came from the Commissie Hinderpreventie Veeteeltbedrijven, The Hague, The Netherlands.

VOORWOORD

Bij het gereedkomen van dit proefschrift realiseer ik mij dat velen, direkt of indirekt, hebben bijgedragen tot het uiteindelijke resultaat. Al deze mensen ben ik zeer erkentelijk voor hun inbreng.

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De mensen van de dienstverlenende sector ben ik erkentelijk voor hun medewerking; graag memoreer ik de hulpvaardigheid (onder andere bij het maken van de tekeningen) van Van Velzen, en de snelle reparaties van apparatuur door Frits Lap. Van de Kolk en Chris Schouten zorgden voor het steriliseren van de voedingsmedia en Wessels maakte de foto's. Vaak heb ik versteld gestaan over de snelle manier waarop Mevr. Möller en Willy van Rooyen onooglijk schrijfwerk omtoverden tot net getypte manuscripten.

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De heren Huisman en Muilwijk van het ILOB te Wageningen dank ik voor hun medewerking bij het verkrijgen van mest en urine van varkens. Ook de heer Jochemsen ben ik erkentelijk voor de geboden gelegenheid om steeds drijfmestmonsters van zijn bedrijf te halen.

De prettige contacten met medewerkers van het IMAG te Wageningen wil ik hier niet onvermeld laten.

Bijzondere dank gaat uit naar de Commissie Hinderpreventie Veeteeltbedrijven te Den Haag die dit onderzoek heeft gefinancierd.

* Jonathan Swift. Gullivers reizen.

CONTENTS

INTRODUCTION

In the past decennia important changes in the agricultural practices of intensive livestock farming for meat production have occurred. Larger production units with less labour available resulted in highly efficient systems for feeding and waste handling. In the Netherlands the slatted floor method has become the predominant waste handling system for piggeries. The pigs are kept on floors with slots, through which the droppings fall into the underlying pits, where the wastes are collected and stored until discharge.

This intensification has some negative, mainly environmental, sides. The area available for landspreading wastes diminishes, especially in districts with high densities of animal confinement units. As a consequence greater amounts of farm slurries are often applied than justified for fertilizing. Pollution of water courses with animal manures is the result and the necessity of applying untraditional methods for the disposal of animal manures has become apparent.

The large emission of obnoxious odours has become another main problem associated with modern intensive animal farming.

The labour-saving system of anaerobic storage of swine wastes under slatted floors in pig fattening houses is thought to be the main source of malodours which are emitted upon ventilation. The ventilation air is enriched with the malodorous components produced in stored slurry (Skarp, 1975).

The goal of the present investigation was to provide information about the microbial processes in anaerobically stored piggery wastes which lead to the accumulation of malodorous constituents.

Odour

Odours can be defined objectively (instrumentally) only by giving the complete spectrum of the composing volatile chemicals, including their respective concentrations. For nearly all odorous systems this is impossible because of the great number of components involved, often present in very low concentrations. In case the odour can be reproducibly defined, no information is givem about the nature of the odorous sensation. To include the sensorial sensation in an odour

description, instrumental methods fail and sensorial methods must be applied. Accurate characterization of an odour includes reference to (a) the odour strength (intensity) and to (b) the quality (nature) of the odour. Odour strength as perceived by observers does not increase proportionally with the concentration of the odorant, but with a fractional power of the concentration (Dravnieks, 1972). Odour strength is usually measured by estimating the dilution of odorous air with odour-free air until a mixture is obtained which is just undistinguishable from odour-free air, or by indicating the strength on a scale of intensity. For reliable and reproducible results, rather large panels are needed. Estimation of threshold dilution of air from piggeries with an olfactometer and a panel has been reported by Lindvall (1974). One man olfactometers have also been used for comparative measurements (Wolfermann et al., 1977). But the results obtained depend too much on the response of one individual. Differences in sensitivity between individuals are known to be large (Moncrieff, 1967).

To describe odour quality, odour classification systems are used (Harper et al., 1968), whereby not only the subjectivity of the human nose (as with the measurement of odour intensity) but also the subjectivity of the human language (as to the description of the observed sensation) is introduced (compare e.g. Bart, 1973).

For the odour from piggeries no attempts have been made to classify the odour quality in more precise terms than offensive, objectional etc. The unique unchangeable quality of the smell has never been proven. It is likely, indeed, that changes in the quality of the smell from piggeries do occur just as well as changes in the intensity. Nevertheless the odour always seems to be identifiable as originating from swine wastes. That these odours do not possess a constant chemical composition can be derived from the occurrence of different concentrations of some main volatiles, and of differences in pH value of the wastes. Volatility of short chain carboxylic acids and basic components (NH_3 and amines) are markedly influenced by pH value. Furthermore, changes in observed sensation will occur upon dilution of the odorous air.

Investigators have tried to find correlations between odour strength and instrumentally measured concentrations of odorous components. Barth et al. (1974) found high correlations between odour intensity and the concentrations of volatile fatty acids, NH_4^+ and H_2S in a limited number of samples of dairy manure with different pH values due to aeration. However, it is theoretically difficult to understand how a direct relationship can exist beween odour

intensity and concentration of volatile acid and basic components in the wastes independent of the pH level of the wastes.

Schaefer (1977) correlated odour intensity with the concentrations of volatile fatty acids (C_2- C_5), phenol, *p*-cresol, indole, skatole and NH_3 in the ventilation air at about twenty piggeries. The highest correlation coefficient was obtained with *p*-cresol. Correlations with the other components were non-significant.

Schaefer et al. (1974) tried to characterize odour quality of swine wastes by comparing the odour of the wastes with those of synthetic mixtures of malodorous components. A mixture of phenol, *p*-cresol, indole, skatole, butyric acid and diacetyl was selected by a panel as having similarity with the odour from swine wastes.

Volatiles identified in livestock confinement units

In order to characterize the smell of animal wastes in terms of concentrations of the malodorous components, a great number of volatiles has been identified in the air of animal confinement units. Most work has been directed to the identification of components in the air of piggeries. Some workers in addition have identified volatiles in the liquid manure. Table 1 contains a compilement of components identified by different authors. It shows that largely the same constituents have been identified in the air and in the mixed wastes. This confirms the general assumption that malodours which are emitted from piggeries originate from the wastes. In addition to the components listed in Table 1, about 25 different hydrocarbons, 7 chlorinated hydrocarbons and 2 terpenes have been identified by Velghe (pers. comm.) in the head space of piggery wastes. Schaefer et al. (1974) analysed also barn dust for volatiles. In a dust extract they identified 32 components. Seventeen of these have been demonstrated in the wastes or air by other workers. The remaining components were terpenes which probably originated from feed particles.

That some volatile components have been found in the wastes and not in the air may have two explanations.

1) Volatiles present in the wastes are undetectable in the air because of chemical reaction in the atmosphere, consequently compounds are formed which do not originate from the wastes. Notably reduced sulphur compounds are very reactive in air.

2) Discrepancies because of the use of different analytical techniques by workers analysing air and wastes.

Table 1. Literature review of volatiles identified in the air of swine confinement units and in anaerobically stored piggery wastes

Component	Air	Waste
Methanol	2	12
Ethanol	2	12
1-Propanol	2	12
2-Propanol	2	12
1-Butanol	2, 10	12
2-Butanol	10	12
2-Methyl-1-propanol	2	12
3-Methyl-1-butanol	10	12
2-Ethoxy-1-propanol	10	
2-Methyl-2-pentanol	16	
2,3-Butanediol	10	
3-Hydroxy-2-butanone	10	12
Propanone	8	
2-Butanone	16	
3-Pentanone	8, 16	12
Cyclopentanone	16	
2-Octanone	10, 16	12
2,3-Butanedione	7, 10, 11, 16	
Methanal	2	
Ethanal	2, 8	
Propanal	2, 8	
Butanal	2	
Pentanal	2	12
Hexanal	2	12
Heptanal	2	12
Octanal	2	12
Decanal	2	12
2-Methyl-1-propanal	2	
Methanoic acid		12
Ethanoic acid	10, 11, 16[a]	12
Propanoic acid	10, 11, 16	12
Butanoic acid	7, 10, 11, 16	12
2-Methylpropanoic acid	10, 11, 16	12
Pentanoic acid	10, 11, 16	12
2-Methylbutanoic acid		12
3-Methylbutanoic acid	11	12
Hexanoic acid	11	12
2-Methylpentanoic acid		12
4-Methylpentanoic acid	11	12
Heptanoic acid	10, 11	12
Octanoic acid	11	12
Nonanoic acid	11	12
Ethylformate		12
Methylacetate		12
Ethylacetate	16	12
Propylacetate		
Butylacetate		12
i-Propylacetate		12
i-Butylacetate		12
i-Propylpropionate		

Phenol	10, 11, 16	12, 17
3-Methylphenol		12, 17
4-Methylphenol	7, 10, 11, 16	12, 17
4-Ethylphenol	10, 16	12
Toluene	10, 16	12
Xylene	10, 16	
Indane	10	
Benzaldehyde	10, 16	12
Benzoic acid	10	12, 17
Methylphtalene	10	12
Indole	11	12
Skatole	11, 16	12
Acetophenone	10, 16	12
Phenylacetic acid		12, 17
3-Phenylpropionic acid		12, 17
Ammonia	11	
Methylamine	4	
Ethylamine	4	
Trimethylamine	10	
Triethylamine	4	
Carbonylsulphide	13	
Hydrogen sulphide	9, 11, 13	12, 15
Metnanethiol	13	15
Dimethylsulphide	13	12
Diethylsulphide		12
Dimethyldisulphide	13, 10	12
Dimethyltrisulphide	10	12
Ethanethiol		12, 15
Diethyldisulphide	16	12
Propanethiol	16	15
Butanethiol	16	15
Dipropyldisulphide	16	
2-Methylthiophene	16	
Propylprop-1-enyldisulphide	16	
2,4-Dimethylthiophene	16	
2-Methylfuran	16	

[a] Components with underlined references are considered by the respective author as main constituents responsible for the offensive odours from piggery wastes.

1 Bethea and Narayan (1972)
2 Merkel et al. (1969)
3 White et al. (1971)
4 Miner and Hazen (1969)
5 Burnett (1969)
6 Mosier et al. (1973)
7 Hammond et al. (1974)
8 Hartung et al. (1971)
9 Day et al. (1965)
10 Miner et al. (1975)
11 Schaefer et al. (1974)
12 Schreier (pers. comm.) In part published: Schreier (1975)
13 Banwart and Bremner (1975)
14 Elliott and Travis (1973)
15 Janowski et al. (1975)
16 Velghe (pers. comm.)
17 Yasuhara and Fuwa (1977)

As data on volatiles in livestock wastes from other animals than pigs are scarce, no statement can be made concerning the effect of kind of animal on composition of volatiles in the wastes. Schreier (pers. comm.) found no quantitative differences in the volatile components present in liquid swine manure and in liquid cattle manure.

If highly advanced analytical techniques would be applied, other volatile components than those reported in Table 1 would undoubtedly be identified in pig wastes. But these additional components are most likely present in very low concentrations. Even if these compounds would contribute significantly to the bad smell, further research can not be recommended because methods to be applied in routine analyses of such constituents are lacking.

During anaerobic storage of the wastes microbial degradation takes place which leads to the accumulation of the various volatile products among which various malodorous components. These microbial processes in animal wastes have been described in general terms only. Few attempts have been made to describe the formation of malodorous compounds from precursor to smelling substances.

In the following section the important groups of volatiles in piggery wastes will be discussed in connection with literature data on their formation in model systems and by pure cultures of bacteria.

S-containing volatiles. Many sulphur-containing compounds have been detected in the head space of pig wastes (Table 1). Most of these compounds are present in trace amounts only (Banwart and Bremner, 1975). Hydrogen sulphide and methanethiol are most frequently reported as constituents of piggery wastes and are quantitatively the most important S-containing volatile constituents. In the ventilation air traces only of these compounds have been reported (Schaefer et al., 1974; Avery et al., 1975). This is probably due to the oxidation of mercaptans to the less volatile disulphides by air (Kadota and Ishida, 1972) and possibly by adsorption. Hydrogen sulphide is likely to originate mainly from the microbial reduction of sulphate. Urine contains about 1100 mg/l of sulphur mainly as sulphate which originates from animal metabolism (Loehr, 1974). Sulphate-reducing organisms have been found in pig wastes in numbers of 10^3- 10^4 per ml (Rivière et al., 1974). Sulphate-reducing bacteria have been shown to produce trace amounts of carbon disulphide, carbonyl sulphide, methanethiol, ethanethiol and propanethiol (Hatchikian et al., 1976). In addition hydrogen sulphide can be produced by microbial degradation of cysteine and cystine (Freney, 1967; Rivière et al., 1974). Carbon disulphide and diethyl sulphide have been reported as products from

cysteine (Table 2). Methionine is decomposed mainly to methanethiol and dimethyl sulphide (Freney, 1967; Kadota and Ishida, 1972). Most of the other identified S-containing volatiles seem to be derived from more seldomly occurring amino acids like substituted cysteins, which occur in plants (Freney, 1967; Meister, 1965).

Addition of plant residues to soils lead to volatilization of methanethiol, dimethyl sulphide, dimethyl disulphide, carbonyl sulphide and carbon disulphide (Banwart and Bremner, 1976). Hydrogen sulphide does not evolve because this gas is strongly adsorbed by soils (Bremner and Banwart, 1976). From most of the minor S-containing constituents of plants (Freney, 1967) the products of anaerobic decomposition are not known, however, these products may contribute to the smell. Likewise no precursors can be indicated for some of the identified S-containing compounds.

Table 2. Sulphur-containing volatiles and their possible precursors

Products	Possible precursors
H_2S	Cysteine, cystine (4), sulphate
Methylmercaptan	Methionine, methioninesulphoxide, methioninesulphone, S-methylcysteine (1)
Ethylmercaptan	Ethionine, S-ethylcysteine (1)
Dimethyl sulphide	Methionine, methioninesulphoxide, methioninesulphone, S-methylcysteine, homocysteine (1)
Dimethyl disulphide	Methionine, methioninesulphoxide, methioninesulphone, S-methylcysteine (1)
Diethyl disulphide	Ethionine, S-ethylcysteine (1)
Methylethyl sulphide	Ethionine, S-ethylcysteine (1)
Carbon disulphide	Cysteine, cystine, lanthionine, djenkolic acid, homocysteine (1)
Carbon sulphide	Lanthionine, djenkolic acid (1)
Methylthioacetate	Methionine + glucose (2)
Diethyl sulphide	Cysteine, cystine (3)
Dipropyl disulphide	Propylcysteine (5)
Diallyl disulphide	Allylcysteine (5)

1 Banwart and Bremner (1975a)
2 Francis et al. (1975)
3 Kondi (1923)
4 Freney (1967)
5 King and Coley-Smith (1969)

Volatile amines. Anaerobic incubation of protein-containing products with bacteria is often leading to the production of volatile amines. Inoculation of meat with *Clostridium sporogenes* gave rise to the production of dimethylamine, ethylamine and 1,4-diaminobutane; after inoculation with *Bacillus licheniformis*, propyl-, butyl-, i-butyl-, amyl-, and isoamylamine and diaminoethane could be identified (Curda et al., 1972).

Golovnya et al. (1969) detected 8 primary, 9 secondary and 4 tertiary amines in a culture of *Streptococcus lactis* in skim milk. Various authors reported the presence of volatile amines in the spent culture media of clostridia (Prévot and Sarrat, 1960; Billy, 1962b; Prévot and Thouvenot, 1962; Brooks and Moore, 1969). Among the non-sporeforming bacteria which have been studied, volatile amines have been demonstrated to be produced by bacteria belonging to the genera *Bacteroides*, *Streptococcus*, *Staphylococcus*, *Eubacterium* (Persky and Billy, 1962), *Aerobacter*, *Escherichia*, *Micrococcus*, *Pseudomonas*, *Sarcina*, *Mycobacterium*, *Corynebacterium* (Bast, 1971) and *Proteus* (Proom and Woiwod, 1951). The principal volatile amines produced by these organisms include methyl-, ethyl-, propyl-, butyl-, amyl-, iso-butyl-, iso-amyl-, hexyl-, dipropyl-, and dibutylamine.

Two mechanisms of the microbial formation of amines have been proposed. Ekladius et al. (1957) and Bast et al. (1971) demonstrated the presence of a neutral amino acid decarboxylase in *Proteus vulgaris* and *Bacillus sphaericus*. This enzyme catalyses the formation of ethylamine, propylamine, butylamine, iso-butylamine, iso-amylamine, 3-methylbutylamine, and 2-phenylethylamine from the corresponding amino acids.

Bast (1971) obtained experimental indication that the formation of hexylamine and ethylamine by *Sarcina lutea*, hexylamine by *Escherichia coli*, and iso-butylamine by *Aerobacter aerogenes* were performed by amination of the corresponding aldehydes.

Another source of the amines in wastes is found in urine. Daily excretion of dimethylamine is estimated at 20 mg in humans of which about 50% originates from choline by the activity of the gut flora (Asatoor and Simenhoff, 1965). Choline is degraded to either ethylamine plus ethanolamine or to trimethylamine which is easily demethylated (Drasar and Hill, 1974).

Carbonaceous volatiles. A great number of volatiles built up of C, H and O only have been identified in piggery wastes (Table 1). Among these are volatile fatty acids, aldehydes, ketones, esters and alcohols. The microbial aspects of the short-chain carboxylic acids will be discussed separately in Chapter 2 because of their abundant occurrence in piggery wastes.

The carbonaceous volatiles detected in piggery wastes are formed mainly by microbial action, though it cannot be excluded that some of the compounds reported in Table 1 originate directly from the feed and have passed unchanged the alimentary tract.

A number of alcohols, ketones and acids may be produced as a result of deamination of amino acids, with subsequent reduction or oxidation of the formed ketones. Similar products may, however, also arise from concerted action of bacteria on simple carbohydrates. Adamson et al. (1975) and Francis et al. (1975) could identify a great number of volatile products after the addition of glucose to soils under anaerobiosis. The identified products in the head space consisted of 2 aldehydes, 4 ketones, 9 alcohols and 15 esters.

Studies with pure cultures of anaerobic bacteria have shown that volatile fermentation products accumulate in the spent media. However, usually the used media are analysed only for the most abundant products like alcohols, volatile fatty acids, acetoin, and 2,3-butandiol, which are of interest for taxonomic purposes (Doelle, 1975). Few reports deal with the formation of volatiles in minor or trace quantities by pure cultures.

Aromatic compounds. The formation of aromatic volatile compounds will be discussed in detail in Chapters 3 and 4. In Chapter 3 the formation of indole and skatole is also given attention.

Properties of swine excreta

Farm slurry is composed of urine and faeces. In additon, depending on farm management water, bedding material and other wastes of undefined origin may enter the pit. The production ratio of faeces to urine is a main factor governing the dry matter content of the mixed excreta. For this production ratio different figures have been published. An average ratio of faeces to urine of 1:1.5 is mentioned by Tietjen et al. (1977) for fattening pigs. For animals of 40 kg they give a ratio of 1:2.6 and for heavy pigs (130 kg) 1:1.3. The production ratio depends also on the feeding and drinking water regime. O'Callaghan et al. (1971) found a linear relationship between water intake of pigs and urine production. The same authors concluded that there is also a linear relationship between the production of feaces and total excreta, and meal and meal-plus-water intake. Literature data on properties of urine and faeces are summarized in Tables 3 and 4. The presented data are averages of experiments reported by different authors and can be considered as approximate values only. Considerable variation in composition of urine and faeces may arise from differences in feed. The data on composition of mixtures of freshly voided excreta can be calculated from Tables 3 and 4, but they have also been published by several authors (Dale, 1972; Loehr, 1974; Rivière et al., 1974; Miner and Smith, 1975; Sutton et al., 1976).

Table 3. Literature data of chemical and physical properties of pig urine[a]

Component	g/kg	Reference
Dry matter	20	Baader et al. (1972)
Ash	10	Loehr (1974)
BOD_5	9	Baader et al. (1972)
N-total	11.6	Tietjen and Vetter (1972)
Urea	4.1-10.7	Vogel (1969)
P	0.87	Tietjen and Vetter (1972)
S	1.1	Loehr (1974)
Ca	0.14	Loehr (1974)
Mg	0.12	Loehr (1974)
Cu	0.25	Robinson et al. (1971)
Density	1022 kg/m^3	Backhurst and Harker (1974)
Viscosity	1.106 Ns/m	Backhurst and Harker (1974)

[a] Production/day.100 kg 3.0-4.5 kg (Baader et al., 1972; Tietjen and Vetter, 1972).

Table 4. Literature date of chemical and physical properties of pig faeces[a]

Component	g/kg	Reference
Dry matter	170	Baader et al. (1972)
Ash	30	Smith (1973)
BOD_5	55	Baader et al. (1972)
Crude protein	30	Smith (1973)
Crude fibre	70	Smith (1973)
Fat	8	Hennig and Poppe (1975)
N	5.4	Tietjen and Vetter (1972)
P	5.9	Tietjen and Vetter (1972)
Ca	8.3	Tietjen and Vetter (1972)
Mg	1.3	Tietjen and Vetter (1972)
K	0.7	Robinson (1971)
Mn	0.04	Robinson (1971)
Zn	0.31	Robinson (1971)
Cu	0.24	Robinson (1971)
S	0.5	Loehr (1974)
Density	1164 kg/m^3	Backhurst and Harker (1974)
Calorific value of dried faeces	16.3 MJ/kg	Backhurst and Harker (1974)

[a] Production/day.100 kg 2.0-3.2 kg (Baader et al., 1972; Tietjen and Vetter, 1972).

In common farming practice the mixed excreta are stored in pits. Under conditions of anaerobiosis the components of the slurry are subject to microbial degradation. Notably the organic fraction (about 75-80% of the dry weight) is converted from polymerous products to components with a low molecular weight. This results in a changed composition of farm slurries as compared with mixtures of faeces and urine. Characteristic analyses of slurries from different pig

fattening units with anaerobic storage of the wastes are given in Table 5. They show that there is a large variation in composition of slurries. Though there is considerable difficulty in drawing representative samples from farm pits, sampling errors probably account for a minor part of the observed variability. Experimental evidence from Sutton et al. (1976) and Rivière et al. (1974) support this assumption. These workers sampled one farm pit for periods up to two years and found relatively narrow ranges of concentrations of the various components analysed.

Table 5. Composition of anaerobically stored piggery wastes

Component	Units	Mean	Standard deviation	Range	Reference
Dry matter	%	8	5.9	1-21	1
Dry matter	%	8.2	3.2	3.3-14.9	2
Ash	% d.m.	31.1	4.6	24-40	2
Total N	g/kg wet weight	4.3	1.9	1.2-7	1
NH_4^+-N	"	-	-	2.3-9.1	4
K	"	2.0	0.8	0.6-3.4	1
P	"	1.8	1.1	0.1-4.5	1
Mg	"	0.7	0.4	0.1-1.5	1
Phenol	mg/kg wet weight	23.2	14.2	7-55	2
p-Cresol	"	235	71	140-340	2
4-Ethylphenol	"	27.8	19.7	6-72	2
Indole	"	2.9	2.1	0-11	2
Skatole	"	40	16	9-54	2
Total volatile fatty acids	g/kg wet weight	11.8	5.6	4-27.6	3
Acetic acid	"	6.8	3.5	2-15.7	3
Propionic acid	"	2.8	1.3	1.2-6.6	3
iso-Butyric acid	"	0.4	0.3	0.2-1.0	3
Butyric acid	"	0.9	0.7	0.4-3.1	3
branched-Valeric acids	"	0.4	0.4	0.2-1.0	3
n-Valeric acid	"	0.4	0.6	0.1-1.0	3

1 Tunney and Molloy (1975)
2 Spoelstra (1977)
3 Spoelstra (see Chapter 2)
4 Loehr (1974)

It is not possible with the available information to quantify the importance of the various factors of farm management involved in pig fattening on the composition of the anaerobically stored wastes. Drinking water regime of the animals, drinking water system installed and composition of the feed have been shown to have great influence (O'Callaghan et al., 1971a). Storing conditions are expected to be important as well, but as long as no treatment techniques

(aeration, methane fermentation, treatment with chemicals) are applied the temperature of the wastes will be the ruling factor, which is climate-dependent. The policy of pit emptying is also management-dependent, emptying the pit completely or withdrawing by preference the more liquid or more sedimented fraction of the heterogeneous pit contents, and the frequency of pit emptying are of great importance on waste composition. The decision to empty pits depends on favourable weather conditions for landspreading, kind of crop and demand for fertilizer. In the case of a too small storage capacity pit emptying may be forced (O'Callaghan et al., 1971b, 1973).

Generally, the storage of swine wastes is characterized by continuous input with a discontinuous discharge with often large intervals between two successive discharges. Thus the decomposition of the wastes is more comparable with batchwise kinetics than with a continuous flow system. In the waste channels an uneven distribution of solids over the depth occurs. The ash content of the dry matter decreases with depth indicating that organic material settles. The decrease of pH with depth suggests a poor transport of even soluble components in the wastes (Figure 1).

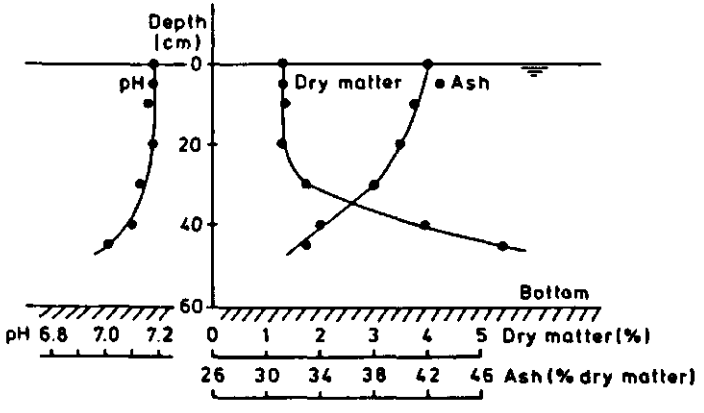

Fig. 1. Change of pH, dry matter and ash with the depth of waste contents in a waste channel.

Microbiology

Little work has been performed on the microbial composition of piggery wastes. Some reports have appeared which deal with hygienic aspects (Elliott et al., 1976) and occurrence of organisms used as indicators for faecal contamination (Rivière et al., 1974; Tamâsi, 1977). The last-mentioned authors estimated also the number of bacteria possessing different physiological properties. After 77 days of anaerobic storage of mixed swine excreta they found 1.1×10^3 indole-producing, 1.1×10^5 H_2S-producing, 1.5×10^4 urealytique, 4.5×10^5 ammonifying, 1.1×10^5 gelatine-degrading and 4.5×10^4 sulphate-reducing bacteria per ml of waste. Because they did not apply anaerobic incubation techniques it must be assumed that these data refer to facultative anaerobic organisms only. Hobson and Shaw (1973, 1974) studied the microbiology of swine wastes in relation with methane fermentation. They tried to demonstrate the presence of cellulolytic bacteria but did not succeed. For starting methane fermentation they found it advisable to dilute the influent wastes. This indicates that the growth of methanogenic and/or acetogenic bacteria is inhibited by high concentrations of some constituents of the wastes.

Odour abatement

In the literature many attempts have been described to abate odours from intensive animal farming. Most research has been directed to waste treatment but treatment of the ventilation air has also been investigated. The main difficulty in judging the effectiveness of odour-abating methods is found in the measurement of the odour intensity. Good results have been obtained by the use of olfactometers with sniffing panels (Lindvall, 1974). But for reliable results rather large panels are needed which makes this method expensive. In an other approach, concentrations of one or more constituents of the odour are monitored as an indicator for the odour intensity. Compounds which have been used as such indicators are ammonia, hydrogen sulphide (Avery et al., 1975; Cole et al., 1976; Miner and Stroh, 1976) and volatile fatty acids (Avery et al., 1975).

Aeration of the wastes is the most approved technique of odour abatement (e.g. Loehr, 1969; Converse et al., 1971; Hashimoto, 1974). Aeration can be applied to diminish obnoxious odours or as part of a more advanced treatment system. The goal of waste treatment may be the production of an effluent which can be discharged safely onto watercourses (Robinson, 1974) or recycling as feed (Day and Harmon, 1974). Aeration can be performed in indoor or outdoor basins (Day et al., 1975) with mechanical aerators (Baader et al., 1977).

Addition to the wastes of other hydrogen acceptors than air oxygen has been investigated. Cole et al., (1976) found that nitrate at the rate of initially 18.3 kg/m^3 and subsequently weekly 5.3 kg/m^3 changed the odour and moderately removed sulphide. Also H_2O_2 was found to diminish odours (Cole et al., 1976). This product acts as an oxygen donor for microbial processes and as a chemical oxidation agent of some malodorous constituents of the wastes. Notably reduced sulphur compounds are oxidized (Kibbel et al., 1972).

Strongly oxidizing chemicals like NaOCl, ClO_2 and $KMnO_4$ have been tested but were found to have effects for short periods only (Cole et al., 1976).

Many other chemicals or commercial mixtures of chemicals have been considered for their odour-diminishing effect upon addition to wastes (Burnett and Dondero, 1970; Ritter et al., 1975; Cole et al., 1976). These chemicals can be divided into different groups dependent on their presumed way of action. Masking agents cover the obnoxious odour with a stronger pleasant smell. Deodorants destroy the odour by chemical reaction. Counteractants add an odour to the malodour in such a way that an odourless mixture results. Though some of these products had a positive influence on the odour of animal wastes according to panel members, they have not found wide application.

Enzymes and dried mixtures of bacterial cultures have been tested for their odour abating value by Hofman (1975), Cole et al. (1976) and Miner and Stroh (1976). No significant influence on the bad smell of farm wastes could be detected by these authors.

Another class of odour-abating chemicals has been tested to poison the microflora of the wastes and thus preventing the formation of malodorous substances. Formaldehyde (Seltzer et al., 1969), cyanamide (Meyer and Hugenroth, 1973) and heavy doses of a strong acid or a base (Sweeten et al., 1977) have been reported to be more or less successful. However, costs are considerable and health hazards for the applicant are not excluded.

Methane fermentation of animal wastes also removes the offensive odours, though an ammoniacal smell remains (Hobson et al., 1974; Van Velsen, 1977; Welsh et al., 1977). The same applies for the aerobic process of liquid composting (Grabbe et al., 1975).

OUTLINE OF THE INVESTIGATION

Because odour is not directly accessible to measurement, the work has been restricted to general microbial aspects of piggery wastes and to the microbial

production of a number of volatile components shown by Schaefer et al. (1974) to contribute to the bad smell.

In Chapter 2 overall processes of the degradation of plant fibre material and protein to volatile fatty acids are described.

Aspects of the microflora were studied because it was expected that information about the predominant bacteria in the wastes would contribute to the understanding of the processes involved.

Results of experiments on the enumeration and isolation of the strict anaerobic microflora are given in Chapter 3.

In Chapter 4 attention is given to the formation of indoles and simple phenols in piggery wastes. The conversion of tyrosine to simple phenols in freshly voided faeces and in anaerobically stored farm slurry was studied in more detail and is discussed in Chapter 5.

In Chapter 6 tyrosine catabolism of *Clostridium sporogenes* NCIB 10696 and of a strain of *Clostridium ghoni* is described. This study was needed to perform some of the experiments mentioned in the preceding chapter.

Chapter 7 contains a general discussion section on the possible causes of the accumulation of volatile components in piggery wastes. The obtained information is suggested to be helpful in judging the effectiveness of odour-abating methods applied to such wastes.

VOLATILE FATTY ACIDS IN ANAEROBICALLY STORED PIGGERY WASTES

Volatile fatty acids (VFA) were estimated in 30 samples of farm slurries. The total concentration of VFA in these samples ranged from 4.0 to 27.6 g/l (average 11.8 g/l with standard deviation of 5.6 g/l). From laboratory experiments it was derived that VFA are produced from faeces whilst only nonsignificant amounts originate from urine. During 70 days incubation of a mixture of freshly voided faeces and urine, 43% of the protein and 24% of the plant-fibre residues were degraded. The resulting products were predominately VFA and carbon dioxide.

INTRODUCTION

During the storage of mixed wastes from piggeries in pits under slatted floors, anaerobic microbial decomposition of the wastes takes place. Under the prevailing conditions, numerous organic compounds of low molecular weight are accumulated. Among these, the volatile fatty acids (VFA) are quantitatively the most important group. The VFA in piggery wastes have received some attention in literature. They have been demonstrated to contribute to the bad smell of the wastes (Schaefer et al.,1974). Removal of VFA from the wastes by methane fermentation has been studied (Hobson and Shaw, 1974; Van Velsen, 1977). In addition attempts have been made to utilize the VFA of the wastes as substrates for microorganisms, with the object to produce single cell protein (Henry et al., 1976; Ensign, 1977; McGill and Jackson, 1977). The processes leading to the accumulation of VFA in the wastes have received little attention. In the work reported here, information is given about concentrations of VFA in piggery wastes and about their precursors.

MATERIALS AND METHODS

Samples

Faeces and urine were collected separately from pigs held in metabolism cages. Samples of slurries from farms with storage of wastes under slatted floors were composed from grab samples. The sampling procedures have previously been described in more detail (Spoelstra, 1977).

Experiments

Urea in the urine used in the experiments was hydrolysed by incubation with urease and the liberated ammonia neutralized with 2 N HCl previously to mixing the urine with faeces. Incubation mixtures were seeded with a drop of farm slurry.

Experiment 1. A mixture was prepared of freshly voided faeces and urine (mixing ratio 1:1 w/w) and diluted with water to a dry matter content of 6%. This suspension and a mixture of faeces with water but without urine were incubated at 15°C and 25°C in closed 1-l flasks. The pH value of the suspension was maintained at 7.0. Samples to be analysed for VFA were taken periodically.

Experiment 2. In another experiment 8 l of a mixture of faeces and urine (mixing ratio 1:1.1 w/w, dry matter content 9%) was stored anaerobically for 150 days at about 18°C. The initial pH value was brought to 7.0 and was not adjusted during the experiment. Samples were taken and analysed for VFA, dry matter, ash, pH, NH_4^+-N, Kjeldahl-N and fibre content. In addition gas production and the composition of gas formed were analysed.

Estimation of VFA.

Waste samples were centrifuged at 24,000 x g for 20 minutes. The supernatant was subjected to gas chromatographic analysis without further treatment. It was introduced into a glass column (1 m x 4 mm) packed with 20% Tween 80 on Chromosorb W-AW (80-100 mesh). The column was installed in a Packard model 409 gas chromatograph equipped with a flame ionization detector. The temperature setting of the column oven was 115°C and that of the injector and the detector 170°C. As carrier gas nitrogen (80 ml/min) saturated with formic acid was applied (Fohr, 1974). Formic acid is not detected by this sytem. Isovaleric and α-methylbutyric acids are not separated by the column used. Both isomers are probably present in the wastes being decomposition products of amino acids.

The concentration of isovaleric acid plus α-methylbutyric acid will be referred to as branched valeric (b-valeric) acids.

Gases

Gas production was measured by replacement of acidified water by the gas formed. The gas composition was determined in samples drawn from the head space of the container in which the slurry was stored. The samples were analysed gas-chromatographically as described by Van Kessel (1978).

Nitrogenous compounds

Protein content was calculated as the difference between total Kjehldahl-N and NH_4^+-N multiplied by 6.25. NH_4^+-N was determined by distillation of NH_3 from a sample to which borate buffer (pH 8.6) had been added. The ammonia was collected in a 4% solution of boric acid which was subsequently titrated with HCl.

Fibre content

Samples were washed with an equal volume of a 0.5 M EDTA solution (pH 7.0) and centrifuged for 30 min at 39,000 x g. Washings with 0.5 M EDTA were repeated twice and the residues were subsequently subjected to three washings with each of the solvents 5% n-butanol in water, 96% ethanol, and acetone. The acetone-washed residues were dried and weighed.

RESULTS

VFA in farm slurries.

Concentrations of VFA in samples of farm slurries are presented in Table 1. The samples from piggery number 1 were taken at weekly intervals from December 1976 to April 1977. The temperature of the waste during this period fluctuated from 10°C to 15°C. In this farm, pigs have free access to water which is supplied by drinking nipples. A considerable amount of the drinking water is spoiled and consequently the wastes are diluted. This is in contrast with piggery 3. Here liquid feeding is practised and very high waste concentrations are reached. The concentrations of VFA in samples from this piggery were also found to be high.

Table 1. Concentrations (g/kg wet weight) of volatile fatty acids in slurry samples from 6 piggeries.

Piggery number	1	2	3	4	5	6
No. of samples	19	3	3	1	1	1
Sampling period	Dec. 76 - April 77	July 75	Febr. - July 77	Jan. 77	Nov. 75	July 73
Dry matter	45.4 ± 18.5[a]	63 ± 24	123 ± 33	26	14	71
pH	7.19 ± 0.17	N.D.[b]	7.6 ± 0.14	7.5	7.4	7.2
Formic acid	N.D.	N.D.	N.D.	N.D.	N.D.	0.07
Acetic acid	6.0 ± 2.5	8.1 ± 1.8	12.3 ± 3.9	4.7	6.9	8.1
Propionic acid	2.8 ± 1.3	3.4 ± 1.0	4.2 ± 0.8	1.27	1.24	2.85
Isobutyric acid	0.33 ± 0.12	0.56 ± 0.17	0.90 ± 0.49	0.32	0.27	0.69
Butyric acid	0.71 ± 0.29	1.26 ± 0.67	1.72 ± 1.24	0.79	0.30	0.60
b-Valeric acids	0.36 ± 0.13	0.92 ± 0.29	1.59 ± 0.31	0.47	0.20	1.17
Valeric acid	0.20 ± 0.05	0.24 ± 0.06	0.27 ± 0.07	0.37	0.04	0.21

a Mean and standard deviation of the mean
b Not determined

About 30 samples of slurry from 6 farms were analysed for VFA. The total amounts ranged from 4.0 to 27.6 g/l (mean and standard deviation of the mean were 11.8 ± 5.6 g/l). Generally, the lower VFA concentrations were found in the more diluted wastes. In the samples tested, acetic and propionic acid represented 53.5 ± 12.2% and 24.4 ± 8.4% (mean and standard deviation of the mean), respectively, of the total amount of VFA present in the wastes. These figures amounted to 3.5 ± 1.5, 6.8 ± 5.9, 4.2 ± 2.3 and 2.4 ± 2.9 for isobutyric, butyric, b-valeric and valeric acids, respectively. Formic acid was determined in one sample only (piggery 6). The concentration found corresponds with 0.6% of the total amount of VFA in this sample.

Experiment 1.

No important differences in the accumulation of VFA were observed between mixtures with and without urine added to the faeces. In the presence of urine, VFA tended to be slightly higher at the end of the incubation period. The mixtures incubated at 25°C showed a much larger increase in the content of VFA than those incubated at 15°C (Figure 1).

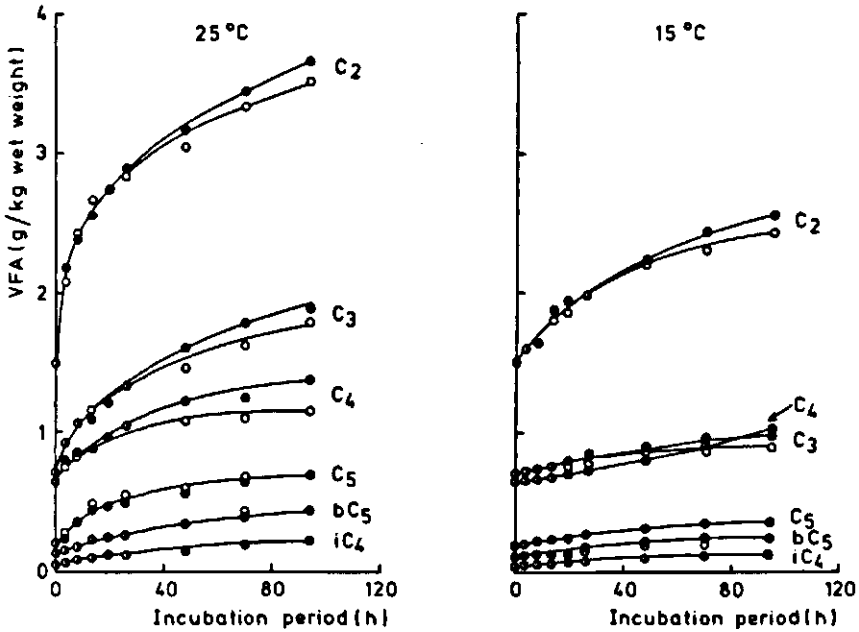

Fig. 1. Production of VFA in mixtures of faeces plus water (o—o) and faeces plus urine (•—•) incubated at 25°C and 15°C, respectively. C_2, C_3, i C_4, b C_5 and C_5 denote acetic, propionic, isobutyric, butyric, branched valeric and valeric acids, respectively.

Experiment 2.

Data on the production of VFA during an incubation period of 150 days of a mixture of faeces and urine are presented in Figure 2. The total amount of VFA increased from 5 g/l to 20 g/l. Acetic, propionic and butyric acids were in this order the most important acids. The other acids accumulated from about 0.1 to 0.8 g/l. The pH value declined during the storage period from 7.0 to 6.4. The NH_4^+-N concentration increased from 2.63 to 4.11 g/l during the same period.

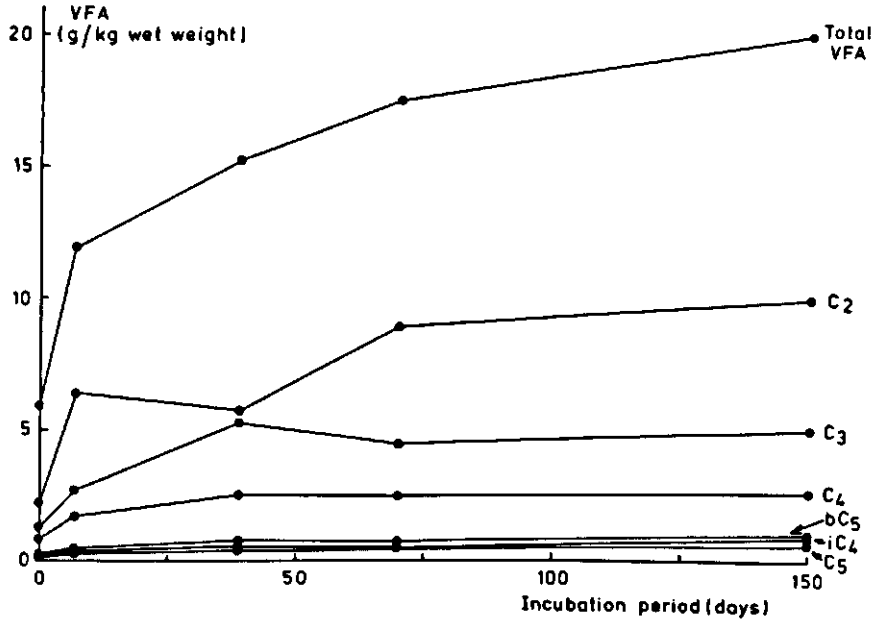

Fig. 2. Production of VFA in a mixture of faeces and urine incubated at 18°-20°C. C_2, C_3, i C_4, C_4, b C_5 and C_5 denote acetic, propionic, isobutyric, butyric, branched valeric and valeric acids, respectively.

The results on decomposition of fibre and protein and the production of gas and VFA during the first 70 days of the experiment are given in Figure 3. Gas was produced during the first 40 days of the experiment, afterwards no gas production was measurable. The average composition of the gas produced was 80% CO_2 and 20% methane. Hydrogen was always present in low amounts (about 0.02%). During the 70 days period about 24% of the fibre and 43% of the protein was degraded.

From the available information a mass-balance was calculated which is presented in Figure 3[b]. An attempt was made to differentiate the fibre fraction in cellulose, hemicellulose and lignin. The analytical results were, however, not accurate enough to allow conclusions about the extent to which the cellulose and hemicellulose fractions were degraded.

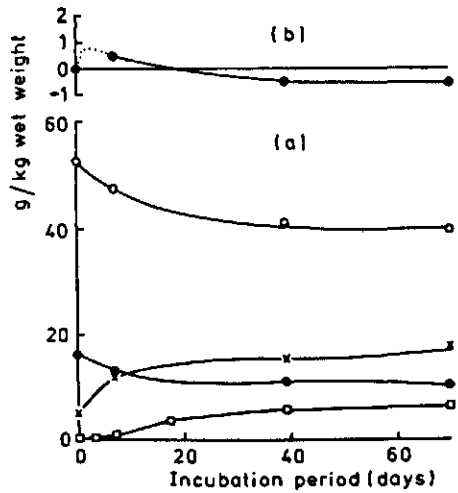

Fig. 3. a) Decrease of fibre (o--o) and protein (•—•) content, increase of VFA (x—x) and gas production(□—□) in an anaerobically stored mixture of faeces and urine.
b) Mass balance calculated from the data in figure 3[a]. Note different scale.

The percentage of fibre that was hydrolysed after incubation for 30 min with 72% H_2SO_4 at ambient temperature and subsequently 6 h at 100°C decreased during the 70 days incubation period from 78 to 67.

DISCUSSION

The amounts of VFA in farm slurries (Table 1) show large variations which must mainly be attributed to the different amounts of water entering the pits. However, variations in microbial activity due to storage conditions (temperature) also influence the content of VFA. Generally, the mutual ratios of the VFA were found to be rather constant. Acetic acid, propionic acid and butyric acid showed in this order the highest concentrations. The remaining acids are

usually present in concentrations of about 5-10% of the acetic acid concentration. Formic acid is probably present in even lower concentrations. These results are consistent with those of Cooper and Cornforth (1978).

From Figure 1 it is concluded that VFA are mainly produced from constituents of the faeces. No significant increase of the VFA content was obtained when urine was added to the faeces. The production of VFA proceeds more rapidly at higher storage temperatures.

The results presented in Figure 3 suggest that the main overall processes which take place in anaerobically stored piggery wastes are 1) degradation of plant fibre residues to VFA 2) degradation of protein to VFA and ammonium. The decomposition of fibre and protein is accompanied with the formation of CO_2. Besides some methane is formed in the slurry. Production of CO_2 and CH_4 in piggery waste has also been observed by Stevens and Cornforth (1974).

As is shown in Figure 3^b the production of gas and VFA does not balance completely with the degradation of fibre and protein. After an incubation period of 7 days more VFA and gas is formed than polymers are degraded. This is possibly the result of the formation of VFA and gas from easily degradable compounds that have not been included in the analyses. A further factor which may be responsible for this surplus is the incorporation of water during hydrolysis of polymers like various soluble carbonaceous components and possibly fat. At the end of the experiment about 0.5 g more fibre and protein were degraded than gas and VFA formed. This may be explained by the formation of soluble products other than VFA e.g. alcohols, phenols (Spoelstra, 1977), phenolic acids.

Plant fibre consists of cellulose, hemicellulose and lignin which is probably not degraded under anaerobic conditions. Cellulose and hemicellulose are first decomposed to oligomers or monomers, which subsequently are converted to mainly acetic, propionic and butyric acids. Most amino acids - the monomers of protein - are also decomposed to one or more of these VFA. However, valine, leucine and isoleucine are degraded to isobutyric, isovaleric and α-methyl-butyric acids, respectively (Allison, 1978). Acetic acid may also be synthesized from H_2 and CO_2 as has been demonstrated to occur in the intestine of some rodents (Prins and Lankhorst, 1977). The VFA content of the wastes may be lowered by methane fermentation, in which formic or acetic acid may be used as substrate. Methane fermentation in stored farm wastes is usually not a quantitatively important process. This has also been observed for slurry

of cattle and poultry (Ensign, 1977).

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Enumeration and Isolation of Anaerobic Microbiota of Piggery Wastes

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Media for enumeration of the microbiota of anaerobically stored piggery wastes were tested. Highest colony counts were obtained with 80 to 100% farm slurry supernatant included in the anaerobic roll tube media. Colony counts with these media numbered 2×10^9 to 12×10^9/g (wet weight), which represents about 20% of the microscopic counts. Lower percentages of slurry supernatant in the media gave lower colony counts. Addition of glucose, cellobiose, and starch or of Trypticase to media with 20% slurry supernatant did not increase colony counts. Higher values were obtained when hemicellulose preparations were added to these media. Incubation at 25°C gave the highest numbers. Incubation at 15 to 37°C gave counts of about 70 and 10%, respectively, of those at 25°C. Of the colonies picked for isolation, about 20% were obtained in pure culture. The isolates apparently belonged to the genera *Peptococcus, Ruminococcus, Peptostreptococcus,* and *Bacteroides.*

In intensive pig-fattening units, the animals are usually kept on slatted floors. The droppings are collected and stored underneath the floors, where the mixture of feces and urine is decomposed under the prevailing anaerobic conditions. This leads to accumulation of malodorous compounds, which may cause a serious odor nuisance to the surroundings when emitted with the ventilation air. Odor problems also arise when the slurry is spread on the land.

Wastes as stored in farm pits differ in composition. Notably, the dry matter contents show large variations (18). Variation in composition is a result of farm management; it is influenced by such factors as the rations fed, drinking water regimen, frequency and way of pit emptying, and storage conditions. In spite of all these variations, slurries from different piggeries may be considered as a group of wastes that are characterized by the anaerobic degradation of fibrous and proteinaceous matter leading to the accumulation of volatile fatty acids. These processes proceed in the presence of high NH_3 levels, derived from the decomposition of urea and protein. Table 1 contains measurements of the main features of farm slurry and of freshly voided pig feces.

The microbiota of farm slurry was studied as part of a research program on the origin of the obnoxious odors emitted from farm slurries. Because little microbiological work has been performed on pig slurries, no special techniques and media were available.

Anaerobic culturing techniques developed for the study of the microbiota of the rumen and of the intestinal tract have been adapted and used throughout this study. Typical rumen media, like M10 (4) and a 40% rumen fluid medium, were used as a starting point for the experiments.

MATERIALS AND METHODS

Slurry samples. Farm slurry samples were taken as grab samples from farm pits with storage of wastes under slatted floors. The sampling procedure has been described in detail elsewhere (16). Weights of the pigs ranged from 20 to 100 kg. The animals received commercial rations. The average age of the wastes cannot be established, but must be assumed to have ranged from several weeks to several months.

Anaerobic techniques. Media were prepared according to the methods of Hungate (10). However, Na_2CO_3, cysteine-hydrochloride, and Na_2S were added after boiling the media but before tubing and autoclaving. Tubes were gassed with an oxygen-free mixture of 96% CO_2 and 4% H_2. This gas mixture was used for all counting experiments. The tubes were provided with a screw cap to prevent blowing-out of the stoppers during autoclaving. Serial dilutions of farm slurry were made in the medium described as "dilution blanks" by Holdeman and Moore (9). For the first dilution, containing 10^{-2} g of slurry per ml, a preweighed sample of about 1 g of slurry, added to 99 ml of dilution medium, was mixed for 1 min under a stream of CO_2 in an electric blender. Subsequent dilutions were made by injecting 0.5 ml of liquid with a 1-ml disposable syringe into a stoppered tube (28 by 140 mm, Kontes) containing 49.5 ml of dilution medium. Tubes (22 by 140 mm, Kontes) containing 6 ml of molten agar medium were inoculated with 1 ml containing 10^{-8} g of slurry. Inoculation was done in

TABLE 1. *Characteristics of freshly voided feces and of anaerobically stored wastes from piggeries*

Characteristic	Feces (ref.)	Anaerobically stored wastes (ref.)
DM[a] (%)	17–25	2–14 (16)
Ash (% of DM)	17 (15)	20–40 (16)
Crude protein (% of DM)	19 (15)	
Fiber (% of DM)	40 (15)	
pH		6.7–7.8 (16)
Ammonia (g/liter)		2.3–9.1 (11)
Volatile fatty acids (g/liter)		4–20 (12)
Cresol (mg/liter)		100–350 (16)
Storage temperature (°C)		10–20

[a] DM, Dry matter.

eight replicate tubes. Roll tubes were prepared by spinning the tubes in a refrigerator until solidification of the agar and then were incubated in upright position at 25°C.

Media. Medium 10 (M10) of Caldwell and Bryant (4) was used, but the fatty acid mixture was replaced by the mixture of Bryant (2). The 40% rumen fluid medium (R40) was similar to medium 98-5 of Bryant and Robinson (3). Rumen fluid was obtained from a fistulated grazing cow with free access to water.

Farm slurry supernatant (SS) was prepared from the same batch of slurry in which bacteria were counted. For each experiment, a new batch of farm slurry was used. An almost clear SS was obtained by centrifugation of the slurry at 25,000 × *g* for 30 min. It was found to be convenient to strain the slurry through cheesecloth before centrifugation to eliminate the coarsest particles.

Media containing SS were supplied with minerals, $Na_2S \cdot 9H_2O$, cysteine-hydrochloride, resazurin, Na_2CO_3, and agar in the same concentrations as in medium 98-5 (3). Rumen fluid was replaced by either 10, 20, 40, 60, or 80% SS. These media are referred to as SS10, SS20, SS40, SS60, and SS80. To the medium with 100% SS (SS100) no minerals were added. Carbohydrates, when included in the media, were supplied at a level of 1 g of each per liter.

The formulations of Sweet E broth and peptone-yeast extract-glucose (PYG) broth are given by Holdeman and Moore (9). The pH value of the media after autoclaving was 6.9. The hemicellulose preparation added to the media was a mixture of: xylan (Fluka), gum xanthan, locust bean gum, and gum arabic (the latter three purchased from Sigma). Incorporation of a mixture of 1 g of each of these polymers per liter gave the following composition on a monomer base (milligrams per liter): mannose (1,580), galactose (610), arabinose (355), xylose (350), glucuronic acid (285), glucose (260), rhamnose (155), and pyruvic acid (70). Hemicellulose (gum mixture), sometimes difficult to dissolve, was mixed with water, boiled while stirred, left overnight at room temperature, and added to the media, which were then boiled to expel oxygen.

Bacterial counts. Colony counts were performed after 5 or 6 weeks of incubation time unless otherwise stated. Only colonies visible with the unaided eye were counted. For the microscopic count, a Burker-Turk counting chamber with a depth of 0.01 mm was used. In 20 to 40 squares of 0.0025 mm^2, all bacterial cells and clumps were counted in a dilution containing 10^{-2} g of slurry per ml.

Means were statistically compared by *t* tests (17).

Isolation and identification of strains. For the isolation of bacteria, roll tubes were inoculated with 10^{-9} g (wet weight) of slurry. Inoculation with highly diluted suspensions was chosen to increase the possibility of picking pure cultures. Colonies were transferred after 4 and 7 weeks to either Sweet E broth or SS100 medium enriched with hemicellulose but without agar. Media and methods for identification of strains were similar to those of Holdeman and Moore (9). Minor modifications were the following. Media to test acid formation from carbohydrates were tubed into 10-ml Vacutainer tubes (Becton, Dickinson & Co.) and inoculated with a syringe. Incubation was done at 25°C, and identification tests were performed after 7 to 10 days. The composition of volatile fatty acids in spent media was determined gas chromatographically. One microliter of culture fluid supernatant (centrifuged for 20 min at 25,000 × *g*) was introduced on a glass column (1 m by 4 mm [ID]) packed with 20% Tween 80 on Chromosorb W-AW (80 to 100 mesh). Carrier gas (N_2; flow, 80 ml/min) was saturated with formic acid. The column oven temperature was 115°C.

From nonvolatile acids, methyl esters were prepared as described by Holdeman and Moore (9). Gas-chromatographic separation was achieved by a glass column (2 m by 2 mm [ID]) packed with 5% Carbowax 20M on Chromosorb W-HP (100 to 120 mesh). Operational temperature was 140°C. Nitrogen was applied as carrier gas (30 ml/min). The gas chromatographs used were equipped with flame ionization detectors.

Individual isolates were tested in PYG for growth at 37°C. Controls were incubated at 25°C. Cultures were observed for 5 weeks.

Isolated strains were maintained in Sweet E broth stored at 25°C. Transfer to fresh medium once per 3 months was sufficient to maintain viability.

RESULTS

Preliminary experiments. In preliminary experiments, colony counts on rumen media M10 and R40 were compared with those on media including SS (Table 2). The media with 20 and 40% SS were found to be superior to both M10 and the rumen fluid medium (R40). Colony counts on M10 and R40 gave mutually comparable results. To find an optimum level of SS, media containing different percentages of SS were tested (Table 3). Colony counts were nearly doubled when the percentage of SS was increased from 20 to 80% or higher.

Incubation temperature. Counts at 25°C with SS100 increased by about 35% when the incubation period was extended from 2 to 3 weeks. Longer incubation gave additional small increases of colony counts. With incubation at 15°C, colony numbers were nearly doubled when the incubation period was increased from 5 to 10 weeks.

Highest colony numbers were obtained when

TABLE 2. *Comparison of colony counts of farm slurry on media M10 and R40 with media including SS*

Expt[a]	Medium[b]	Colony count[c]
1	M10	3.47 ± 1.11[x]
	R40	3.90 ± 0.45[x]
2	M10	1.20 ± 0.86[x]
	SS40	2.96 ± 0.98[y]
3	M10	2.95 ± 0.29[x]
	SS20	5.83 ± 0.79[y]
4	R40	1.44 ± 0.36[x]
	SS20	2.03 ± 0.48[y]

[a] Only counts of the same experiment are comparable.

[b] Cellobiose, glucose, and starch were included with all media except the SS20 of experiment 4, which contained no carbohydrates.

[c] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols (x, y) denote significance at $P < 0.01$.

TABLE 3. *Effects of amounts of SS contained in media on colony counts*

Expt[a]	% SS	Carbohydrates[b] included	Colony count[c]
1	10	C, G, S	3.32 ± 0.67[x]
	20	C, G, S	3.72 ± 1.59[y]
	40	C, G, S	6.60 ± 0.89[y]
2	20	C, G, S	5.83 ± 0.79[x]
	40	C, G, S	8.17 ± 1.54[y,p]
	60	C, G, S	9.40 ± 1.20[q]
	80	C, G, S	12.0 ± 2.30[r]
3	20	None	1.48 ± 0.44[x]
	100	None	2.62 ± 0.47[y]
4	20	None	2.02 ± 0.40[x]
	100	None	3.92 ± 0.63[y]

[a] Only counts of the same experiment are comparable.

[b] C, Cellobiose; G, glucose; S, starch.

[c] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols denote significance at $P < 0.01$ (x, y) or $P < 0.05$ (p, q, r).

incubation was at 25°C (Table 4). Counts at 15°C were about 70% of the counts obtained at 25°C. Incubation at 37°C gave numbers amounting to less than 10% of the counts at 25°C.

Additions to media. The results of experiments with different carbohydrates added to SS20 and SS100 are presented in Table 5. The most frequently used additions to rumen media (cellobiose, glucose, and starch) tended to decrease colony counts when added to SS20. No significant effect (at $P < 0.05$) was obtained when starch or cellobiose was deleted from M10 (data not recorded). Addition of Trypticase to SS20 (Table 6) gave no general effect on numbers when counted after 10 weeks. Only the addition of 30 g of Trypticase per liter gave higher colony numbers than that of 2 g/liter ($P < 0.05$). However, counted after 17 days, 10 and 30 g of added Trypticase per liter gave higher numbers of bacteria than 2 g/liter ($P < 0.01$ and $P < 0.05$, respectively), whereas 10 g/liter showed increased counts compared with those with no Trypticase added ($P < 0.01$). Here, as in the case with the addition of cellobiose, glucose, and starch, the higher substrate levels tended to give some large colonies that might overgrow others.

Colony counts were higher when the hemicellulose preparation was included in the SS20

TABLE 4. *Effect of incubation temperature on colony counts*

Expt[a]	Medium	Carbohydrates[b] included	Incubation temp (°C)	Colony count[c]
1	SS80	C, G, S	15	4.82 ± 0.55[x]
	SS80	C, G, S	25	7.10 ± 0.80[y]
	SS80	C, G, S	37	0.31 ± 0.18[z]
2	SS100	None	15	3.84 ± 0.20[x]
	SS100	None	25	5.17 ± 0.63[y]
	SS100	None	37	0.63 ± 0.15[z]

[a] Only counts of the same experiment are comparable.

[b] C, Cellobiose; G, glucose; S, starch.

[c] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols (x, y, z) denote significance at $P < 0.01$.

TABLE 5. *Effect on colony counts of carbohydrates added to media with SS*

Expt[a]	Medium	Carbohydrates[b] included	Colony count[c]
1	SS100	None	6.06 ± 1.26[x]
	SS100	C, G, S	5.86 ± 0.31[x]
2	SS80	C, G, S	7.10 ± 0.80[x,p]
	SS80	G	6.23 ± 0.58[x,q]
	SS80	C	6.30 ± 1.04[x]
3	SS20	None	2.02 ± 0.40[x]
	SS20	C, G, S	1.21 ± 0.39[y]
4	SS20	None	2.03 ± 0.48[x]
	SS20	S	1.81 ± 0.67[x]

[a] Only counts of the same experiment are comparable.

[b] C, Cellobiose; G, glucose; S, starch.

[c] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols denote significance at $P < 0.01$ (x, y) or $P < 0.05$ (p, q).

medium (Table 7). When hemicellulose was added to the SS100 medium, no significant increase in colony numbers was obtained in most experiments. An additional indication that the added mixture of gums was utilized as substrate is derived from the greater average colony size on the roll tubes containing this mixture.

In Table 8, microscopic counts and colony counts are compared. With the SS80 and SS100 media, colony counts reached about 20% of the microscopic counts.

Isolation and identification. In two experiments, bacteria were isolated from farm slurry and subsequently identified. In the first experiment, 42 colonies were transferred to Sweet E broth. Only 22 tubes showed good growth after a 3-week incubation period. On transfer to PYG broth, 7 isolates developed, and another 5 strains, all of them cocci, grew when 0.2% Tween 80 had been included in the PYG broth. In the second experiment, 100 colonies were inoculated into SS100 with the hemicellulose preparation but without agar. Growth in this medium was difficult to observe because of undissolved hemicellulose particles. From all of the latter tubes, 0.1 ml was transferred to PYG broth enriched with 0.2% Tween 80. Twenty-one tubes showed good growth. Inoculating 0.1 ml of liquid from the 79 hemicellulose-containing tubes that did not give growth in PYG without Tween 80 did not give positive results as contrasted with PYG with 2% oxgall, which enabled 5 more strains to grow. These 5 bacteria (4 cocci and 1 small rod) were not further identified. The strains growing in PYG or PYG with Tween 80 were checked for purity by microscopic examination and subsequently used for identification tests. The 33 isolates could be divided into three groups.

Group I consisted of 10 strains of non-saccharolytic gram-positive cocci. Only 2 of these grew at 37°C. The bacteria occurred in pairs or chains, and 1 organism showed tetrads. They fermented carbohydrates weakly or not at all. These bacteria resemble *Peptococcus* species or the non-saccharolytic species of the genus *Peptostreptococcus*.

Group II comprised 13 cocci that fermented carbohydrates. Four of these (1 grew at 37°C) were gram-positive to gram-variable cocci that fermented cellobiose, glucose, fructose, lactose, and mannose. The main acids produced were acetic and succinic acids. They were tentatively identified as *Ruminococcus* spp. Two other gram-positive cocci (1 grew at 37°C) were present in chains only. The cells had tapered ends and produced mainly acetic and succinic acids from all carbohydrates tested. These 2 strains were identified as *Peptostreptococcus productus*. Seven isolates (5 grew at 37°C) of gram-positive cocci occurred in pairs or chains and fermented glucose, fructose, maltose, and starch. The main acids formed were acetic, succinic, and (less) lactic. These isolates belong, probably, to the genus *Peptostreptococcus*.

Group III comprised gram-negative rods that

TABLE 6. *Effect on colony counts of Trypticase added to SS20*

Expt[a]	Trypticase added (g/liter)	Colony count[b]
1	0	1.24 ± 0.44[x]
	10	1.48 ± 0.37[x]
2	0	2.04 ± 0.34[x]
	2	1.77 ± 0.27[x,p]
	10	2.06 ± 0.39[x]
	30	2.32 ± 0.28[x,q]

[a] Only counts of the same experiment are comparable.

[b] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols denote significance at $P < 0.01$ (x, y) or $P < 0.05$ (p, q).

TABLE 7. *Effect on colony counts of addition of hemicellulose preparation to media including SS*

Expt[a]	Medium	Hemicellulose prepn	Colony count[b]
1	SS100	−	2.62 ± 0.47[x]
	SS100	+	3.64 ± 0.31[y]
2	SS100	−	5.17 ± 0.63[x]
	SS100	+	4.96 ± 0.39[x]
3	SS100	−	4.17 ± 0.93[x]
	SS100	+	4.44 ± 0.77[x]
	SS20	−	1.90 ± 0.34[y]
	SS20	+	3.64 ± 0.75[z]
4	SS20	−	2.03 ± 0.48[x]
	SS20	+	3.30 ± 0.39[y]

[a] Only counts of the same experiment are comparable.

[b] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols (x, y, z) denote significance at $P < 0.01$.

TABLE 8. *Comparison of microscopic counts with colony counts of farm slurry*

Medium	Microscopic count[a]	Colony count[a]	%[b]
SS40	36	2.96	8
SS80	62	12.0	19
SS80	30	7.10	24
SS100	20	5.17	26
SS100	17	3.92	23

[a] Count per 10^{-9} g (wet weight).

[b] Colony count as percentage of the microscopic count.

often occurred in long pleomorphic cells. Of the 10 isolates in this group, 5 were able to grow at 37°C. The acids produced were acetic and succinic, and 7 isolates also formed propionic acid as a major product. The bacteria in this group can be described as saccharolytic *Bacteroides*.

DISCUSSION

Colony counts on SS-containing media. The data of Tables 2 and 3 show that it is advisable to incorporate about 80% SS in media for colony counts of the microbiota of farm slurries. The optimum quantity may vary with the degree of dilution and with the composition of the slurry under investigation. Lower amounts of SS in the media result in lower colony counts, indicating that the main substrates for many organisms are diluted too much to allow development to visible colonies. It may be expected that addition of the proper substrates to SS20 will give colony numbers of the same magnitude as obtained with media with high levels of SS. Addition of the compounds most frequently used as substrates in rumen media (cellobiose, glucose, and starch) to SS20 media did not increase colony counts (Table 5). The negative results of enrichment of the media with Trypticase (Table 6) suggest that protein degradation products are not limiting.

Of the products tested as added substrate, only the hemicellulose preparations increased colony numbers, though counts remained lower than those obtained with SS80 and SS100 (Table 7). Probably, only a limited fraction of the hemicellulose in the slurry is present in water-soluble form. The remaining insoluble fraction, including the residues of plant cell walls from the forage, is removed by centrifugation and, therefore, does not occur in the SS fraction that is used in the nutrient media used for counting. The relatively low concentration in the SS fraction of soluble components derived from hemicellulose apparently is sufficient for giving optimum growth of the microbiota. However, upon fivefold dilution, the concentration of the nutrients for many bacteria is too low to give visible colonies. Enrichment of the diluted SS medium with the hemicellulose preparation restored growth of these bacteria to a large extent. However, restoration is not complete, presumably because the added hemicellulose preparation does not contain the required components in optimum concentration.

Stimulatory effects of hemicellulose preparations on bacteria have been reported from rumen isolates by Dehority (6). Some workers found increased counts of rumen bacteria when fibrous material of the forage fed to ruminants was included in the media (5, 7). Salyers et al. (14) reported the fermentation of gums by *Bacteroides* spp. The use of commercial gums as a substitute for the hemicellulose of plant fiber in nutrient media has the advantage that a more homogeneous medium is obtained and that rather well-defined products are used (19). The combination of gums used in the present work was chosen as a trial mixture. The monomer composition does not reflect the composition of the hemicellulose fraction of plant material left in piggery wastes. It may be that other combinations of the gums used or including other gums in SS20 medium would have further increased colony counts.

Temperature. Highest colony counts were obtained at an incubation temperature of 25°C, with a small decline for counts at 15°C. These incubation temperatures do not deviate much from temperature generally found in stored farm slurries. Counts at 37°C, the expected optimum temperature of the microbiota of the incoming feces, show a sharp decrease. These results indicate that the intestinal microbiota of the pig does survive poorly in the wastes and is not likely to play a role in the decomposition of the slurry during storage. This conclusion is supported by the work of Allen and Brock (1), who found that only 1% of the aerobically growing intestinal microbiota of the rat was cultivable at 25°C.

When different slurries were inoculated into the respective SS100 media, considerable differences in colony counts were found. These differences were, probably, largely due to differences in dry matter content of the wastes. With the media SS80 and SS100, colony counts ranged from 2.6×10^9 to 12.0×10^9/g (wet weight). These numbers are considerably higher than the 6.5×10^8/ml reported by Hobson and Shaw (8), who used a medium with centrifuged fluid from an anaerobic digester with cellobiose, maltose, glucose, and lactate as substrates. But their counts have probably been negatively influenced by the incubation temperature of 38°C.

The colony counts with the media SS80 and SS100 amount to 20% of the microscopic counts. Comparable figures have been reported for the rumen microbiota (5). Recoveries of the intestinal microbiota are usually higher (13). However, farm slurries differ from both these habitats by not being a continuous system but a system without discharge, thus more approaching a batch culture, in which higher percentages of dead organisms are expected.

Isolates. The few samples from which organisms were isolated and the low number of isolates obtained for identification give a limited

impression of the microbiota of piggery wastes. Isolates that could be grown in PYG represent about 20% of the colonies picked for isolation and only 4% of the microscopic count. Colonies were picked after 4 and 7 weeks, so part of the picked colonies may have been dead. Recoveries increased somewhat when isolates growing only in PYG with 2% oxgall were included.

All organisms isolated were strict anaerobes. Earlier experiments had shown that counts on aerobically incubated media yielded 1×10^8 to 5×10^8 colonies per g (Spoelstra, unpublished results).

The tentatively identified anaerobic organisms consisted of gram-positive cocci (70%) and gram-negative pleomorphic rods (30%). Hobson and Shaw (8) also reported gram-positive cocci as the most important group of bacteria in piggery wastes. They also found clostridia to be an important group. However, in the present study no clostridia were isolated.

The identified organisms seem to belong to the genera whose representatives usually are isolated from the gastrointestinal tract of humans and animals and consequently have optimum temperatures around 37°C. However, the majority of the present strains did not grow at 37°C. This discrepancy causes some doubt about the identity of the isolated organisms, but it is also a reflection of the little attention that has been paid to the microflora of anaerobic ecosystems at lower temperatures.

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Simple Phenols and Indoles in Anaerobically Stored Piggery Wastes

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Phenol, *p*-cresol, 4-ethylphenol, indole and skatole, which contribute to a large extent to the bad smell of piggeries, were estimated in anaerobically stored piggery wastes. *p*-Cresol, indole and skatole were also found in freshly voided faeces. Phenol, *p*-cresol and 4-ethylphenol are present in the urine as their glucuronides. It was shown that upon contact of urine and faeces, the urinary glucuronides are hydrolysed almost instantly liberating phenols. This hydrolysis is caused by the high β-glucuronidase activity of faeces. Protein degradation followed by transformation of tyrosine and tryptophane were found to be additional processes contributing to the accumulation of phenol, *p*-cresol, indole and skatole during the anaerobic storage of piggery wastes.

1. Introduction

In a recent report, Schaefer *et al.*[1] using organoleptic techniques, identified phenol, *p*-cresol, indole, skatole and volatile fatty acids as the main components causing the smell of the ventilation air of piggeries. They also identified these components in the stored piggery wastes.

Phenol, *p*-cresol and 4-ethylphenol occur as conjugated compounds in the urine of mammals.[2] These phenolic compounds originate from the microbial degradation of tyrosine in the intestinal tract.[3–5] They are absorbed by the body and subsequently detoxicated by conjugation with glucuronic acid or sulphuric acid to glucuronides and sulphates, respectively.[2,6] However, the sulphate conjugation is of minor importance in pigs.[7,8]

Intestinal microbial degradation of tryptophane can lead to indole and skatole. Indole can be absorbed and hydroxylated to 3-hydroxyindole. As possible detoxication products of skatole, various hydroxyskatoles and indole-3-carboxylic acid are mentioned.[2] The detoxication products are mainly excreted with the urine.

In the intestinal contents of pigs, a high level of β-glucuronidase of bacterial origin occurs.[9,10] This enzyme hydrolyses glucuronides. Therefore, it was expected that mixing of faeces with urine would cause a rise in the contents of free phenols due to enzymatic hydrolysis of the glucuronides present in the urine.

Faeces contain a considerable amount of protein, which was expected to be an additional source of phenols and indoles in the slurry.

Possible pathways for the microbial formation of simple phenols from tyrosine have recently been discussed by Drasar and Hill.[11] Tryptophane decomposition to indole and skatole by the rumen microflora has been described by Yokoyama and Carlson.[12]

The present work deals with the origin and formation of phenol, *p*-cresol, 4-ethylphenol, indole and skatole in anaerobically stored mixed wastes of piggeries. The presence of these compounds in the slurry will be related to the malodours in the ventilation air of piggeries.

2. Materials and methods

2.1. Samples

Faeces and urine were collected separately overnight from castrated male pigs (Dutch Landrace or Yorkshire) held in metabolism cages. The urine was collected in buckets containing 5 ml 6% HCl

to prevent microbial growth. All animals from which samples were taken were fed with fodders comparable with commercial forage. This means that 350 parts/10^6 copper as $CuSO_4$ and 10 parts/10^6 chlortetracycline or virginiamycine were used as food additives.

Samples of slurry were taken from farms with storage of the wastes under slatted floors. Generally the wastes were accessible only through the provision used for emptying the pit. The sampling device consisted of a 100-ml container mounted on a 1.8 m long stick. The container could be opened and closed by a rubber stopper which was manipulated by an attached lath. Several samples at different depths were taken and mixed.

2.2. Experimental design

A number of laboratory experiments were performed in which fresh faeces and urine were mixed. The mixing ratio of faeces and urine was 1:1.1 (w/w) approximating production ratios. To get a manageable fluid, the mixed wastes were diluted to a dry matter content of about 6%. The mixtures were inoculated with a few drops of a farm slurry and incubated anaerobically in closed 1–1 flasks. The pH value was measured twice a day and if necessary adjusted to pH 7.0. This pH value was chosen arbitrarily from the pH range 6.7–7.8 generally found in farm slurries.

To avoid a rise in pH value immediately after incubation due to urea hydrolysis, the urine used in these experiments was incubated with 50 mg/litre urease (Merck, 250 000 E/g) for 3 h at 50°C and subsequently neutralised with 2 N-HCl. The formation of phenols and indoles was followed by sampling periodically and analysing as outlined in section 2.3.

To demonstrate the importance of faecal β-glucuronidase in liberating phenols from urinary glucuronides, mixtures of faeces and urine (1:1.1) were incubated both in the absence and presence of glucaro-1,4-lactone, which is a specific competitive inhibitor of β-glucuronidase. To be sure of complete inhibition, the initial inhibitor concentration was 20 mM, and because of the instability of this compound at pH values higher than 6.1,[13] glucaro-1,4-lactone was added again after 8 and 13 h incubation time in amounts corresponding to 6.7 and 8.7 mmol, respectively. The formation of phenols and indoles by bacterial metabolism during the incubation period was prevented by shaking the mixtures with 4% (v/v) toluene for 30 min prior to incubation. Quantities of 100 ml were incubated in conical flasks of 300 ml capacity under an argon atmosphere at 20°C. The pH value was maintained at 7.0.

Separate experiments were performed to test the occurrence of intermediates in the anaerobic degradation of tyrosine and tryptophane by adding these compounds to faeces as well as to farm slurries. The formation of phenols and indoles was determined semiquantitatively after overnight incubation under an atmosphere of nitrogen. The tested compounds were added in concentrations of about 1 mg/ml. The pH value was buffered with 0.05 M sodium phosphate at pH 7.0.

2.3. Estimation of phenols and indoles

Two ml of a freshly prepared 6% $NaHCO_3$ solution and 1.00 ml of a standard solution of 5-decanol in ether were added to a sample of slurry of about 5 g. 5-Decanol was used as an internal standard in the gas-chromatographical analysis. Subsequently, the sample was extracted four times with 20 ml of ether. The emulsion, which was usually formed during the ether extraction, was broken by gentle centrifugation. The combined ethereal fractions were evaporated under reduced pressure at a maximum temperature of 25°C to a volume of a few millilitres. The residual ether was dried by adding anhydrous $CaSO_4$, and transferred to a 10 ml test tube. The volume was further reduced to 0.5–1 ml by holding the test tube in a waterbath of 30°C. Approximately 1 microlitre of the final ethereal solution was injected for gas-chromatographical analysis (Figure 1).

The gas chromatograph used was a Varian 2240 model equipped with a flame ionisation detector. A column of stainless steel was used of 3 m × 3 mm o.d. packed with 10% SE 30 on chromosorb W/AW, 80/100 mesh. The operational data were: temperature, oven 140°C; injectionport, 150°C; detectorblock, 190°C; flow of carrier gas (N_2), 40 ml/min; air, 300 ml/min and H_2, 30 ml/min.

The peak heights obtained were measured relative to the peak height of the internal standard and compared with a calibration curve, which had been obtained by the same procedure as described

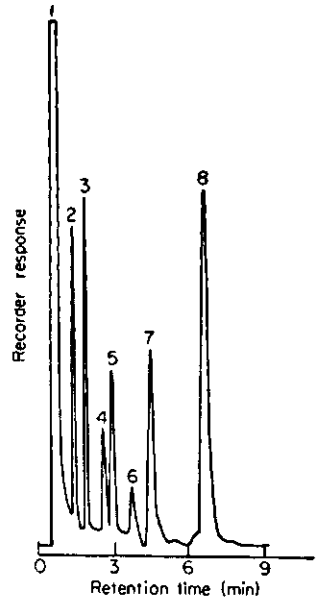

Figure 1. Gas chromatogram of the analysis of a slurry sample. Peak number with attenuation in brackets: 1, solvent; 2, phenol (16); 3, *p*-cresol (32); 4, 4-ethylphenol (32); 5, internal standard (32); 6, unknown (2); 7, indole (2); 8, skatole (4).

above with mixtures of known concentrations of phenol, *p*-cresol, 4-ethylphenol, indole and skatole, dissolved in water.

To get information more quickly, a semiquantitative modification was also used. To about 3 g of a sample in a 10 ml centrifugation tube 1.5 ml 6% $NaHCO_3$ and 2.0 ml of a solution of 5-decanol in ether were added. The tubes were closed and shaken. The layers were separated by centrifugation and about 1 microlitre of the ethereal layer injected for gas-chromatographical analysis.

2.4. Hydrolysis of urinary conjugates

Both acidic and enzymatic hydrolysis of conjugates in the urine were carried out as described by Duran *et al.*[14] Acidic hydrolysis was performed with 2 N hydrochloric acid. The enzyme preparation used for enzymatic hydrolysis was a mixture of arylsulphatase and glucuronidase (glusulase, Boehringer).

2.5. Glucuronidase assay

Slurry samples were diluted 10–20-fold and shaken with 4% (v/v) toluene for 30 min. Toluene-treated samples were incubated at 30° C with 0.05 M sodium phosphate buffer (pH 7.0) and 1.0 mM *p*-nitrophenylglucuronide (Merck). Periodically, 2 ml samples were taken from the assay mixture and the reaction stopped by placing the sample for 1 min in a boiling water bath. Subsequently, the sample was centrifuged for 15 min at 10 000 *g*. The supernatant was diluted to a suitable volume and supplied with 1.0 ml of 1 N-NaOH. The extinction was measured at 400 nm with a spectrophotometer. From the slope of the obtained line and a calibration curve of *p*-nitrophenol, the amount of *p*-nitrophenol liberated per unit time was calculated. The β-glucuronidase activity is expressed in units: one unit corresponds with 1 μg *p*-nitrophenol liberated per minute and per gram of sample (wet weight).

3. Results

3.1. Phenols and indoles in slurries

Slurry samples from 13 piggeries with animals on slatted floors were analysed (Table 1). The sample of piggery number 1 was taken when the pit had recently been emptied and cleaned. The samples of piggeries 2–6 were taken at different times of the year.

Table 1. Analytical data for slurry samples from piggeries

Piggery number	Date of sampling	Dry matter (%)	Ash (% d.m.)	Phenol	p-Cresol	4-Ethylphenol	Indole	Skatole
				(mg/kg wet weight)				
1	Oct. 1975	14.9	24	12	140	8	3	10
2	Aug. 1975	4.5	36	55	200	13	3	49
3	Aug. 1975	5.4	31	20	150	6	1	42
4	Aug. 1975	9.1	32	40	170	18	2	53
5	March 1976	8.5	27	20	170	19	5	50
6	March 1976	8.3	31	21	200	15	5	50
7	Oct. 1975	10.9	32	43	300	72	11	54
8	Oct. 1975	4.4	35	17	280	25	2	29
9	Oct. 1975	7.6	27	18	290	32	3	41
10	Oct. 1975	10.1	26	19	340	48	6	56
11	Oct. 1975	9.0	28	19	320	38	5	38
12	Oct. 1975	3.3	35	7	180	15	0	9
13	Oct. 1975	10.8	40	8	320	52	6	38

The remaining samples were taken in the autumn of 1975 just before landspreading of the slurries, which had been stored during the preceding summer. The monthly mean temperatures for June, July, August and September 1975 were 15.1, 17.8, 19.9 and 15.2°C, respectively. The monthly mean temperatures for these months departed −0.4, +0.8, +3.1 and +0.9°C from normal (measured at the Dutch Meteorological Institute in De Bilt).

The concentration of *p*-cresol in the samples tested varied between 140 and 340 mg/kg of slurry wet weight; it was generally higher in the slurry samples taken in the autumn of 1975 than in the other samples. The concentrations of phenol, 4-ethylphenol and skatole amounted to about 10% and indole to 1% of the concentrations of *p*-cresol.

3.2. Phenols and indoles in freshly voided excreta

Part of the phenols and indoles in farm slurries were already present in the freshly voided excreta (Table 2). Fresh faeces were found to contain large amounts of *p*-cresol, moderate quantities of indole and skatole, while phenol and 4-ethylphenol could not be detected. Urine contained considerable amounts of conjugated phenols, whereas free phenols were found in minor or trace quantities only.

Table 2. Example of an analysis of freshly voided faeces and urine of pigs (mg/kg wet weight)

	Phenol	p-Cresol	4-Ethylphenol	Indole	Skatole
Faeces (undiluted; dry matter 21%)	0	137	0	5	34
Urine, free phenols	0	4	trace	0	0
Urine, conjugated phenols[a]	1	78	21	0	0
Urine, conjugated phenols[b]	2	78	22	—	—

[a] Enzymatically hydrolysed.
[b] Acid-hydrolysed.

3.3. Phenols and indoles in faeces and in a mixture of faeces and urine upon incubation (Figure 2)

Faeces incubated anaerobically without urine at 25°C produced considerable amounts of *p*-cresol and skatole, but at 15°C only small quantities were formed. Indole production occurred in small quantities at both temperatures, while phenol and 4-ethylphenol were formed neither at 15 nor at 25°C.

Addition of urine to faeces gave a fast initial increase in the concentrations of phenol, *p*-cresol and 4-ethylphenol. The initial increase of *p*-cresol at 25°C was slightly higher and that of phenol about four times higher than that at 15°C.

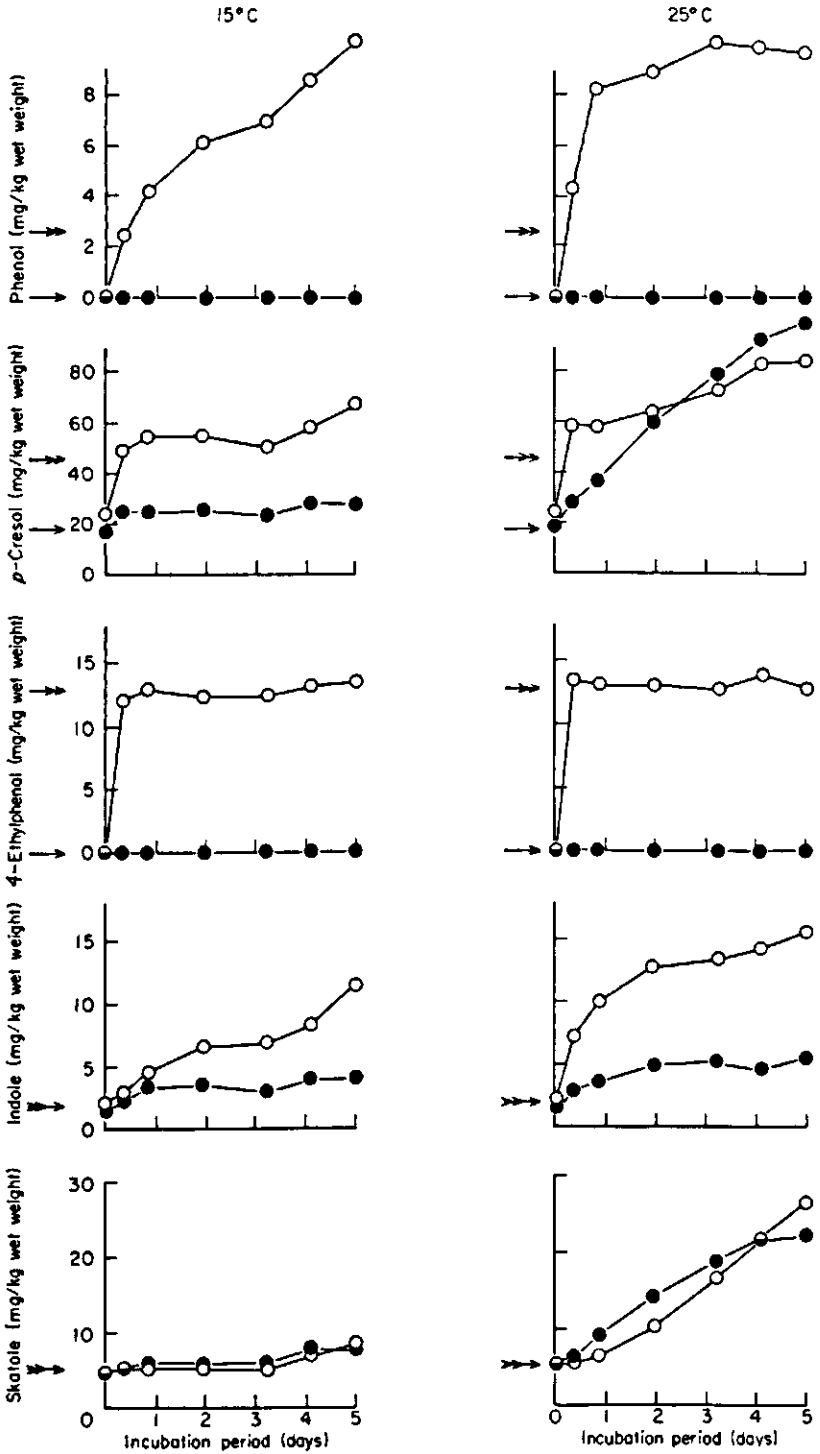

Figure 2. Phenol, *p*-cresol, 4-ethylphenol, indole and skatole in faeces and in a mixture of faeces and urine upon incubation at 15 and 25°C. ●——●, Faeces; ○——○, faeces + urine (1 : 1.1). Dry matter content of the mixtures was about 6%. Arrow → indicates the level of the compound in the mixture which originated from fresh faeces. Arrow →→ indicates calculated level in the mixture of faeces and urine originating from fresh faeces and urinary glucuronide.

No temperature influence was observed with 4-ethylphenol. The production of indole was also stimulated by added urine, while no stimulating effect of urine on the formation of skatole was observed.

In a similar experiment, a mixture of faeces and urine was incubated for 150 days. Phenol, *p*-cresol and 4-ethylphenol accumulated during this period, while the content of indole and skatole declined after an increase during the first 2 months (Figure 3).

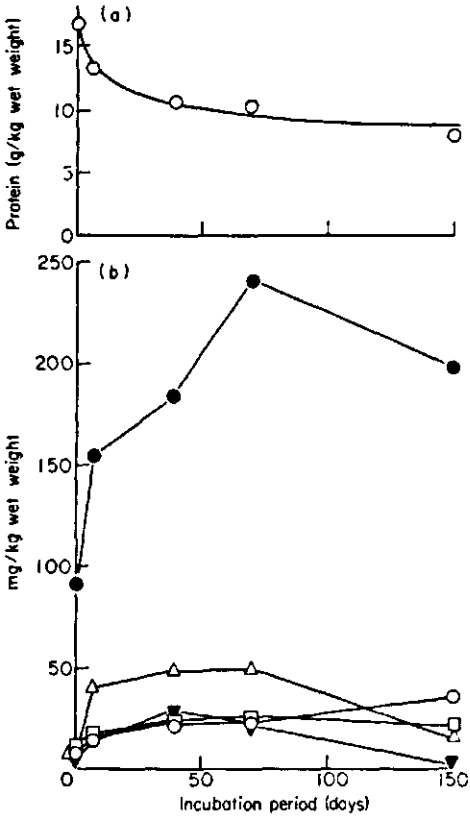

Figure 3. (a) Protein, (b) Phenol, ○——○; *p*-cresol, ●——●; 4-ethylphenol, □——□; indole, ▼——▼ and skatole, △——△ in a mixture of faeces and urine (dry matter content 9%; mixing ratio 1:1) during anaerobic storage at 18–20°C. The first sample was taken 2 h after mixing faeces and urine when the conjugated phenols of the urine had been completely hydrolysed by faecal glucuronidase.

3.4. Activity of β-glucuronidase

Twelve samples of slurries were analysed for activity of β-glucuronidase. The average activity found in these samples was 12.9 units. The lowest and highest activities amounted to 3.2 and 21.6 units, respectively.

The addition of glucaro-1,4-lactone to a mixture of faeces and urine prevented the primary increase of phenols. This effect is shown for *p*-cresol in Figure 4. At the beginning of the incubation period the mixture contained 27 mg/litre free and 18 mg/litre conjugated *p*-cresol, which originated from the faeces and the urine used, respectively. The rise in the content of *p*-cresol in the presence of glucaro-1,4-lactone after 25 h incubation time must be attributed to the instability of the inhibitor at the pH value of the mixture.

Similar results were obtained for 4-ethylphenol. The results for phenol were unclear due to the low content of phenol in the mixture and the inaccuracy of the analytical method in that range.

No effect of the addition of glucaro-1,4-lactone on the formation of indole and skatole was observed.

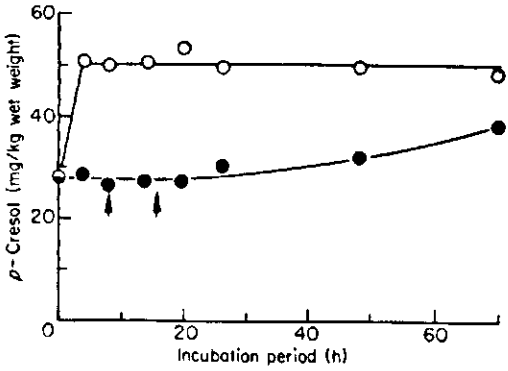

Figure 4. Inhibition of the initial increase of *p*-cresol in a mixture of faeces and urine by glucaro-1,4-lactone. ●——●, Mixture of faeces and urine treated with toluene and glucaro-1,4-lactone added. ○——○, Same mixture without glucaro-1,4-lactone. Arrows indicate time of addition of inhibitor.

3.5. Phenols and indoles formed from tyrosine and tryptophane and possibly occurring intermediates

Addition of possible intermediates of the anaerobic degradation of tyrosine and tryptophane to faeces gave approximately the same pattern of phenols and indoles formed as when these intermediates were added to slurries (Table 3). However, a few remarkable exceptions were found. When tyrosine was added to slurry, a rise in the content of phenol was noticed, which did not occur when tyrosine was added to faeces. Ethylphenol was formed when *p*-coumaric acid was added to faeces but not when it was added to slurry.

Table 3. Formation of phenols and indoles from tyrosine and tryptophane and from possibly occurring intermediates of the degradation of these two amino acids upon incubation of faeces and slurry

	Faeces (37°C)					Slurry (25°C)					Slurry (37°C)				
Compound added	P	C	EP	I	S	P	C	EP	I	S	P	C	EP	I	S
Tyrosine		+ +				+ +	+				+ +	+			
4-Hydroxypyruvic acid		+				+	+				+	+			
trans-p-Coumaric acid			+ +												
4-Hydroxyphenyl-acetic acid		+ + +					+ +					+ +			
4-Hydroxyphenyl-propionic acid															
4-Hydroxybenzoic acid	+ + +					+ +					+ +				
Phenylalanine															
Tryptophane				+ + +	+ +				+ +	+				+ + +	+
Tryptamine															
Indole-3-pyruvic acid				+	+				+	+				+ +	+
Indole-3-acrylic acid															
Indole-3-propionic acid															
Indole-3-acetic acid					+ + +					+					
Indoxylsulphate															
Indole-3-carboxylic acid				+					+ +					+ +	

Formed in small (+), moderate (+ +) and large (+ + +) quantities.

P, phenol; C, *p*-cresol; EP, 4-ethylphenol; I, indole; S, skatole.

Compounds were added in concentrations of about 1 mg/ml. pH was buffered with 0.05 M phosphate buffer at pH 7.0. Incubation was done overnight at the indicated temperature under nitrogen.

4. Discussion

Phenol, *p*-cresol, indole and skatole were mentioned by Schaefer *et al.*[1] as important components of the ventilation air causing the smell of piggeries. These compounds, and in addition 4-ethylphenol, which also contributes to the phenolic components in the odour, were shown to be constituents of slurry.

The *p*-cresol contents found in the samples of slurries taken in the autumn of 1975, which were generally higher than those in the other samples, may have been influenced by the long storage time at relatively high temperatures (Table 1). The age of the farm slurries sampled was difficult to establish. In general the pits were emptied in relation with land spreading wastes, which occurred by preference in the spring and in the autumn. The dry matter contents as reported in Table 1 probably give an inaccurate estimation of the dry matter content of the sampled slurries. This was caused by the difficulty of taking representative samples of the heterogeneous slurry at farm sites.

The fast initial increase of phenols, upon the addition of urine to faeces (Figure 2), the inhibitory effect of glucaro-1,4-lactone (Figure 4) and the high β-glucuronidase activity in faeces[9,10] indicate that the increased concentrations of phenols were, at least partially, due to the hydrolysis of the corresponding urinary glucuronides by faeces β-glucuronidase. From the concentrations of glucuronides in the urine used in these experiments it was calculated that the initial increase of *p*-cresol was largely explained and that of 4-ethylphenol completely explained by hydrolysis of their urinary glucuronides. In the case of phenol the primary increase was higher than can be explained by hydrolysis of conjugated phenol alone. So other compounds may be present in the urine which are readily degraded to phenol. 4-Hydroxybenzoic acid is probably such a compound, because it is normal constituent of urine,[2] and it can be decarboxylated by faeces.[15] Data from Table 4 confirm that 4-hydroxybenzoic acid is decarboxylated by faeces as well as by farm slurry. The same table indicates that urinary tyrosine was not likely to play a role in phenol production in the experiments described, but that it might do so in slurries.

It is likely that urinary glucuronides were hydrolysed nearly instantaneously upon contact with faeces at both 15 and 25°C (Figure 2). Other urinary precursors of phenol, *p*-cresol, and indole may be transformed slower or transformed in relation with bacterial growth. The high β-glucuronidase activities in slurries reported in section 3.4 suggest that fast hydrolysis of urinary glucuronides also occurs under farm conditions.

p-Cresol, skatole and small quantities of indole and phenol formed in faeces incubated at 25°C were probably derived from degradation of the proteinaceous fraction of the faeces. Incubated at 15°C no or only small amounts of these compounds were formed from faeces indicating that their formation is related with bacterial growth. In faeces mixed with urine the formation of skatole and the secondary increase of *p*-cresol is also attributed to bacterial protein degradation (Figures 2 and 3).

Indole is present in fresh faeces (Table 2) and it is also produced from both urinary precursors and probably from protein degradation (Figures 2 and 3). Possible urinary precursors are tryptophane and indole-3-carboxylic acid (Table 3). Indole and skatole are the only compounds of which a decrease in concentration has been observed during incubation (Figure 3). Disappearance of indole in an anaerobic environment has also been described in relation to the indole test as a determinative factor for *Enterobacteriaceae*.[16]

Skatole is present in fresh faeces. The production of this compound did not increase upon addition of urine. The observed accumulation of skatole is due to protein putrefaction (Figures 2 and 3).

The results of the laboratory experiments given in Figures 2 and 3 demonstrate that in addition to glucuronide hydrolysis, phenols and indoles are also derived from protein degradation. It is likely that the same processes take place in slurries stored under farm conditions. This view is supported by the data of Tables 1 and 2 giving the levels of phenols and indoles in piggery wastes and those in urine and freshly voided faeces, respectively. Comparison of the concentration of *p*-cresol in slurries (200–300 mg/kg wet weight) with that in mixed freshly voided excreta (about 100 mg/kg wet weight) suggests that 100–200 mg/kg wet weight is derived from protein degradation.

The contribution of protein degradation to the amount of phenols and indoles in slurry is dependent on temperature and consequently may show seasonal variations. This is in contrast with the contribution of freshly voided excreta and that of urinary precursors which are more constant.

The information obtained may be helpful in predicting the effect of odour-abating measures. This concerns especially methods aiming to diminish the formation of odorous substances by alternative waste handling systems or addition of chemical or biochemical products to the slurry.

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DEGRADATION OF TYROSINE IN ANAEROBICALLY STORED PIGGERY WASTES AND IN FAECES OF PIGS

Radioactively labeled compounds that might be intermediates in the anaerobic degradation of tyrosine were added to pig faeces and to stored piggery wastes. Changes in the compounds were followed by using thin-layer and gas chromatography. In faeces, *p*-cresol and 3-phenylpropionic acid were the end products of tyrosine metabolism; in anaerobically stored mixed wastes, phenol, *p*-cresol and minor quantities of phenylpropionic acid were formed. Schemes were proposed for the degradation of tyrosine in pig faeces and in mixed wastes.

INTRODUCTION

Research on the chemical composition of the malodour emitted by piggeries revealed that *p*-cresol, phenol, and 4-ethylphenol are important constituents of the bad smell (32). Because the origin of these simple phenols was poorly understood a research program was started on the formation of simple phenols in piggery wastes.

In a previous investigation (37) high levels of *p*-cresol and moderate amounts of phenol and 4-ethylphenol were detected in anaerobically stored piggery wastes; the origin of these compounds was studied. Some of the simple phenols in these wastes were present at the time that excreta were voided. Upon anaerobic storage of the mixture of faeces and urine, which generally occurs in pits under slatted floors, protein degradation proceeds and concentrations of phenol and *p*-cresol increase (37). The simple phenols present in freshly voided faeces and urine are believed to originate from microbial degradation of tyrosine in the intestinal tract (13).

Formation of phenol from tyrosine has been reported for many bacteria possessing the enzyme tyrosine phenol lyase (EC 4.1.99.2) (9,11,15,16,26,31). An alternate pathway leading to phenol, with decarboxylation of 4-hydroxybenzoic acid as the final step, has also been proposed (6,13).

p-Cresol is produced from tyrosine via decarboxylation of (4-hydroxy)-phenylacetic acid (22,34,39). Organisms reported to produce *p*-cresol belong mainly to the clostridia (15,31), but non-spore-forming organisms have also been mentioned (39).

It has been suggested that 4-ethylphenol, present in the urine of mammals would also be derived from tyrosine (28). But Bakke (2,3) concluded from experiments with rats that *p*-coumaric acid in plant material of fodders is the precursor of 4-ethylphenol. The conversion of *p*-coumaric acid to 4-ethylphenol is performed by the intestinal microflora of rats (4,34). No organisms have been described which produce 4-ethylphenol from tyrosine.

The microbial degradation of tyrosine as part of an anaerobic ecosystem has been studied in the faeces of man (12), the caecal contents of rats (3-5) and the rumen of sheep (36). For these different ecosystems the available information shows a complex pattern of possible degradation routes. End products of tyrosine degradation in the intestine seem to be simple phenols, whereas in the rumen aromatic acids are accumulating. In the work reported here tyrosine degradation was studied in anaerobically stored piggery wastes and in freshly voided faeces of pigs.

MATERIALS AND METHODS

Samples

Faeces, separated from urine, were collected overnight from castrated male pigs (Dutch Landrace or Yorkshire), held in metabolism cages.

Farm slurry was taken as a grab sample from farms with storage of mixed wastes under slatted floors.

Extraction procedure

For the estimation of phenols and aromatic acids in slurry or faeces, samples (usually 3 g) were adjusted to pH 8.5 and extracted three times with double volumes of ether. The ethereal layers were combined, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to a volume of about 1 ml (neutral extract). The pH of the extracted sample was adjusted to a value of 2.0 and the sample again extracted twice with double volumes of both ether and ethylacetate. The combined organic layers were dried over Na_2SO_4, evaporated under reduced pressure to dryness, and dissolved in about 1 ml of acetone (acid extract).

Gas chromatography

The neutral extracts were subjected to gas-chromatographical analysis for the qualitative determination of phenol, cresol, ethylphenol and tyrosol. The operational data have been described before (37).

Aromatic acids were analysed as their trimethylsilyl derivatives or as methyl esters. The dry acid extract was supplied with 0.2 ml of a mixture of B.S.A. (N,O-bis-(trimethylsilyl)acetamide, Pierce) and chloroform (1:2 v/v). The reaction mixture was allowed to stand for 20 min at room temperature and was subjected to gas chromatographical analysis. Methyl esters were prepared with boron trichloride-methanol reagent (Sigma). The gas chromatograph used was a dual column Varian 2440 model equipped with flame ionization detectors. For the analysis of trimethylsilyl- and methyl derivatives of aromatic acids, a 3 m stainless steel column (outer diameter 1/8") packed with 10% OV-17 on Chromosorb W/AW was used. The column oven temperature was 200°C, temperature settings of injection port and detector block were set 30°C higher than the oven temperature. As carrier gas nitrogen (15 ml/min) was applied. Hydrogen and air flows were adjusted at 30 ml/min and 300 ml/min, respectively. Table 1 gives retention times of phenolic acids.

Table 1. Gas chromatography of some phenolic acids

Compound	Relative retention time[a]	
	Trimethylsilyl derivatives	Methyl derivatives
Phenylacetic acid	0.36	0.31
4-Hydroxybenzoic acid	1.00	1.00
3-Phenylpropionic acid	0.57	0.45
(4-Hydroxyphenyl)acetic acid	1.12	1.16
p-Coumaric acid	3.53	3.05
3-(4-Hydroxyphenyl)propionic acid	1.75	1.51
(4-Hydroxyphenyl)lactic acid	2.50	-
(4-Hydroxyphenyl)pyruvic acid	4.48	-

[a] 4-Hydroxybenzoic acid = 1.00

Thin-layer chromatography

Neutral and acid extracts were applied to 0.25-mm layers of cellulose (MN 300, Macherey Nagel) or of silicagel G 1500 Ls 254 with an ultraviolet indicator (Schleicher-Schüll). For the two-dimensial development solvent 1 (1.5% formic acid) and solvent 2 (upper layer of benzene:acetic acid:water = 10:7:3) were used in that order. Silica gel layers were developed usually

in one direction only with solvent 2. Cellulose chromatograms were sprayed with either diazotised sulfanilamide or with Fast blue B salt followed by a 10% Na_2CO_3 solution (38). Rf values are given in Table 2. Colours of the various compounds produced by the sprays were reported by Scheline (33).

Table 2. Names and abbreviations used, and Rf values, of phenolic compounds studied by thin-layer chromatography

			Rf, cellulose		Rf, silica gel
Chemical name	Trivial name	Abbreviation	Solvent 1	Solvent 2	Solvent 3
Phenol	-	-	0.81	1	0.41
4-Methylphenol	*p*-Cresol	-	0.82	1	0.43
4-Hydroxybenzyl alcohol	-	-	0.87	0.19	0.06
4-Hydroxybenzaldehyde	-	-	0.79	0.58	0.17
4-Hydroxybenzoic acid	-	-	0.60	0.41	0.19
4-Hydroxystyrene	*p*-Vinylphenol	-	-	-	-
4-Ethylphenol	-	-	0.83	1	0.44
2-(4-Hydroxyphenyl) ethanol	Tyrosol	-	0.84	0.35	0.08
4-(2-Aminoethyl) phenol	Tyramine	-	-	-	-
Phenylacetic acid	-	PAA	-	-	0.51
4-(Hydroxyphenyl) acetic acid	-	HPAA	0.84	0.37	0.15
trans-3-(4-Hydroxy-phenyl)-acrylic acid	*trans*-*p*-coumaric acid	PCA	0.35	0.49	0.18
3-Phenylpropionic acid	Hydrocinnamic acid	PPA	-	-	0.53
3-(4-Hydroxyphenyl)-propionic acid	Phloretic acid	HPPA	0.80	0.56	0.20
DL-(4-Hydroxyphenyl)-lactic acid	-	HPLA	0.88	0.04	0.03
(4-Hydroxyphenyl)-pyruvic acid	-	HPPyrA	-	-	-
(4-Hydroxyphenyl) alanine	Tyrosine	-	-	-	-

Media

Clostridia were grown in closed 1-l serum bottles with either medium A (peptone 5 g/l, yeast extract 2 g/l, tyrosine, 0.2 g/l) or one quarter strength reinforced clostridial medium (RCM; Oxoid) enriched with 0.2 g/l tyrosine.

Radiochemical experiments

Radioactive compounds that were possible intermediates in the degradation of tyrosine were added to tubes of 14 ml capacity containing 3 g of either slurry

or faeces diluted with 2 parts of water. The tubes were supplied with about 0.30 µmol (approximately 0.05 µCi) of the test substances. The added tyrosine and tyramine contained 0.1 µCi and 0.25 µCi, respectively. The tubes were flushed with oxygen-free nitrogen, closed with Suba-seal stoppers and incubated at 20 (farm slurry) or 37°C (faeces). At the end of the incubation period the samples were extracted as outlined under extraction procedures. About 10 µl of the extract was supplied to thin layers of silica gel, which were developed with solvent 2. Distribution of radioactivity was determined by scraping the spots into scintillation vials. Non-radioactive reference compounds were run together with the samples.

Because phenol and *p*-cresol could not be separated by thin-layer chromatography, the ratios of phenol and *p*-cresol formed from added radioactive test substances were estimated by gas chromatography. For this reason 5-10 µl of the neutral extract was injected on a gas chromatograph equipped with an effluent splitter, thus offering the possibility of collecting 90% of a compound after separation on the OV-17 column. The compounds were collected in a glass capillary which could be attached to the outlet of the stream splitter. The capillary was cooled with a mixture of solid carbon dioxide and acetone. The collected fractions were washed with ether into scintillation vials.

Total activity in the neutral and acid extracts was determined by using an internal standard ([^{14}C]-toluene) as correction for quenching. The liquid scintillation counter used was the Mark I model of Nuclear Chicago.

Chemicals

Tyrosol was prepared microbiologically by incubating *Saccharomyces cerevisiae* with tyrosine and sucrose according to Ehrlich (14). The Laboratory strain S_3 was used.

4-Hydroxystyrene was isolated from the culture medium of a *Clostridium* sp., strain A, which had been incubated with *p*-coumaric acid at pH 5.7. The strain had been isolated from pig faeces and was identified as *Cl. ghoni*. The product was isolated by extraction with ether of the culture medium brought to pH 8.5, and after reduction of the ethereal layer purified by gas chromatography. The nature of the product was confirmed by mass spectrometry.

4-Hydroxybenzylalcohol, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, (4-hydroxyphenyl)acetic acid, 3-(4-hydroxyphenyl)propionic acid, *p*-coumaric acid, tyramine, (4-hydroxyphenyl)pyruvic acid, 3-phenylpropionic acid and phenylacetic acid were purchased from Fluka. DL-(4-hydroxyphenyl)lactic acid

was obtained from Sigma. Abbreviations used for chemical compounds are given in Table 2.

Radiochemicals

L-[U-^{14}C] tyrosine and side chain labeled [2-^{14}C] tyramine were purchased from the Radiochemical Centre, Amersham.

[U-^{14}C] tyrosol, [U-^{14}C] HPAA, [U-^{14}C] HPLA and [U-^{14}C] HPPA were prepared microbiologically as described below.

Cells of an overnight culture of *Clostridium* strain A, grown in 1 l medium A at 37°C, were harvested and aseptically transferred to a 25-ml tube. The cells were incubated under a nitrogen atmosphere at 37°C for one week with 0.5 mmol L-[U-^{14}C] tyrosine (20 µCi), 0.25 mmol α-ketoglutaric acid and 1.0 mmol glycerine in 10 ml 0.1 M sodium phosphate buffer (pH 7.0). The only products formed from tyrosine under these conditions were tyrosol, HPAA and HPLA. The results of these experiments will be dealt with in a separate paper.

[U-^{14}C] HPPA was isolated from the spent culture medium of *Cl. sporogenes* NCIB 10696. This organism was shown to accumulate HPPA by Elsden et al. (15). The organism was grown in 50 ml one quarter strength RCM supplemented with 0.5 mmol L-[U-^{14}C] tyrosine (5 µCi) at 37°C.

Tyrosol was extracted from the reaction mixture at pH 8.5 with ether. The aromatic acids were extracted at pH 2 with ether and ethylacetate. The residues of the dried organic layers were taken into a little acetone and applied to thin layers of cellulose which were developed with solvent 2. The areas with the desired products were scraped from the plates and the scrapings reextracted with acetone. The nature of the products was confirmed by gas chromatography of trimethylsilyl derivatives and by two-dimensiol thin-layer chromatography. Ultraviolet light was used to detect spots on the silica gel layers with fluorescence indicator. The thin-layer chromatograms of HPPA when observed under UV light showed impurities which, however, did not carry label.

The labeled substances were dissolved in water and their concentrations were determined gas-chromatographically in a subsample as trimethylsilyl derivatives.

RESULTS

Preliminary experiments (Table 3)

Experiments were set up to get an impression of the pathways involved in tyrosine degradation leading to simple phenols. Possibly occurring intermediates

Table 3. Production of phenol, *p*-cresol and 4-ethylphenol from tyrosine and from possibly occurring intermediates of the anaerobic degradation of tyrosine upon incubation of these compounds with farm slurries and faeces [a]

Substrates	Products					
	Phenol		*p*-Cresol		4-Ethylphenol	
	Farm slurry	Faeces	Farm slurry	Faeces	Farm slurry	Faeces
Tyrosine	++[b]	-	++	+++	-	-
4-Hydroxystyrene	-	-	-	-	++	+++
4-Hydroxybenzaldehyde	+	+	++	++	-	-
4-Hydroxybenzyl alcohol	+	+	++	++	-	-
Tyrosol	-	-	[+]	[+]	-	-
4-Hydroxybenzoic acid	+++	+++	-	-	-	-
Tyramine	-	-	[+]	[+]	-	-
(4-Hydroxyphenyl)-acetic acid	-	-	++	+++	-	-
p-Coumaric acid	-	-	-	-	-	+++
(4-Hydroxyphenyl)-propionic acid	-	-	-	-	-	-
(4-Hydroxyphenyl)-lactic acid	-	-	+	+	-	-
(4-Hydroxyphenyl)-pyruvic acid	+	-	++	+++	-	-
None	-	-	-	-	-	-

[a] Test substances were added in concentrations of about 1 mg/ml. The pH value was buffered with 0.05 M phosphate buffer at pH 7.0. Stoppered tubes were incubated under a nitrogen atmosphere at 25 (farm slurry) or 37°C (faeces) and analysed qualitatively after 20 h.

[b] Formed in small (+), moderate (++) and large quantities (+++). Symbol in brackets denotes that no *p*-cresol had been formed after normal incubation time, but that it was formed on long-term incubation.

were added to the wastes which were analysed qualitatively for simple phenols after overnight incubation. No formation of phenol or *p*-cresol was detected with the qualitative method of analysis used when no substrate had been added to farm slurry or faeces. To approach the temperatures of the ecosystems farm slurry was incubated at 25 and faeces at 37°C. Previous experiments (37) had shown that no substantially different results were obtained when farm slurry was incubated at 20 or at 37°C. Phenol was formed in farm slurry from tyrosine and in minor quantities from HPPyrA, whereas no phenol was formed when these substances were added to faeces. In farm slurry as well as in faeces 4-hydroxybenzoic acid was decarboxylated to phenol. Small quantities of phenol

were also formed from 4-hydroxybenzyl alcohol and from 4-hydroxybenzaldehyde. *p*-Cresol was produced from a greater number of substances than phenol; the types of compounds which were converted to *p*-cresol were similar with farm slurry and faeces. Moderate to large amounts of *p*-cresol were produced from tyrosine, HPPyrA, HPAA, 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde. Minor quantities were found when HPLA was added. Tyramine and tyrosol gave no detectable quantities of *p*-cresol after overnight incubation, but large amounts were found when analysed after one week or more.

4-Ethylphenol was detected when PCA had been added to faeces, but not when it was added to farm slurry. 4-Hydroxystyrene was totally reduced to 4-ethylphenol.

The influence of the pH value of farm slurry and faeces on the formation of simple phenols was studied by adding the above-mentioned precursors to the wastes mixed with sodium phosphate buffer of the desired pH value (final concentration 0.1 M) and incubating the mixture overnight. The pH was controlled and adjusted, if necessary, after the addition of the solution with the test substances. After fermentation the pH value never deviated more than 0.1 unit from the initial value. In farm slurry phenol was produced from added tyrosine in the pH range 7.0 - 8.0, whereas the decarboxylation of 4-hydroxybenzoic acid occurred over the whole range studied (pH 6 - 8.5), with slightly higher activities at the lower pH values (Fig. 1). The optimum pH for the conversion of tyrosine and HPAA to *p*-cresol was about pH 7.0 in farm slurry. In faeces no optimum was found and *p*-cresol was produced over the whole pH range studied (Fig. 2). The formation of 4-ethylphenol from PCA in faeces declined with rising pH values. At pH 5.5, 4-hydroxystyrene was also accumulated (Fig. 3).

Aromatic acids

After overnight incubation of both farm slurry and faeces with tyrosine, the gas-chromatographical analysis of the acid extracts showed minor peaks with the same retention time as HPAA, which were absent when no tyrosine had been added. But interpretation of the chromatograms was difficult because of the presence of many unknown components. Inoculation of media A and RCM with 10^{-4} g of farm slurry per litre of medium did show accumulation of mainly HPPA. Small amounts of HPAA and tyrosol were also present in both media. A minor peak with the same retention time as PCA appeared in the gas chromatogram when both trimethylsilyl- and methyl derivatives were prepared. This peak could not with certainty be identified as PCA. Simple phenols were not formed.

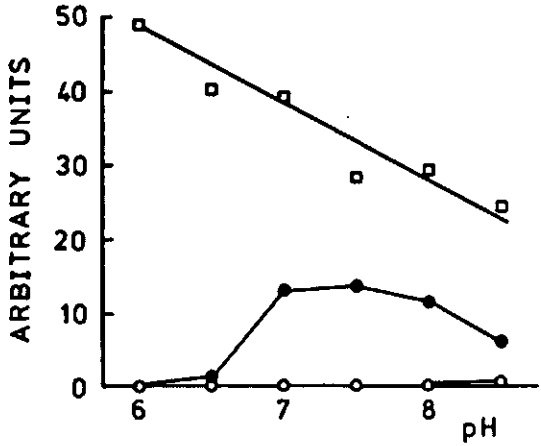

Fig. 1. Effect of pH value on the formation of phenol from tyrosine (●——●) and 4-hydroxybenzoic acid (□——□) added to farm slurry.
o——o No addition.

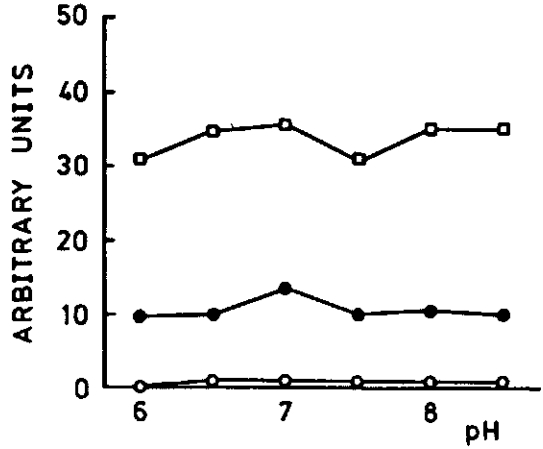

Fig. 2. Effect of pH value on the formation of *p*-cresol from tyrosine (●——●) and (4-hydroxyphenyl)acetic acid (□——□) added to pig faeces.
o——o No addition.

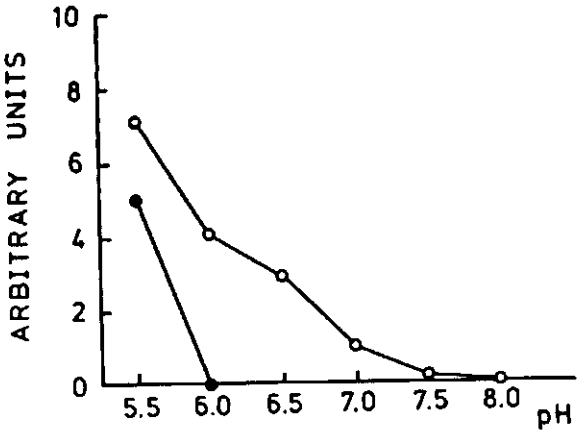

Fig. 3. Effect of pH value on the formation of 4-hydroxystyrene (●———●) and 4-ethylphenol (o- - - - -o) from *p*-coumaric acid added to pig faeces.

Radiochemical experiments with farm slurry

In Table 4 the distribution of radioactivity over the neutral and acid extracts are given after incubation of [U-^{14}C] tyrosine with farm slurry. Thin-layer chromatograms of the neutral extract showed that radioactivity was present only in the spot with the same Rf values as that of phenol and *p*-cresol. Gas chromatography with the use of the effluent splitter confirmed that no labeled neutral compounds other than phenol and *p*-cresol were present. In two slurry samples the ratio of phenol to *p*-cresol formed was found to approach 1. Aromatic acids present in slurry sample I (Table 4) after the incubation period consisted mainly of HPAA (53%) and PPA (25%), with minor quantities of HPPA and HPLA. In a similar experiment labeled HPAA and HPPA were incubated with farm

Table 4. Distribution of radioactivity (%) after addition of L-[U-^{14}C]-tyrosine to farm slurry. Incubated for 20 h at 20°C.

Waste sample	pH	Phenol + *p*-cresol	Ratio $\frac{\text{phenol}}{p\text{-cresol}}$	Aromatic acids
I	7.5	51	0.79	12
II	7.8	56	-	11
III	7.7	55	0.84	10
IV	7.3	55	-	-

slurry (Table 5). *p*-Cresol was formed from HPAA, and no PAA was present, whereas 25% from the added HPPA was recovered dehydroxylated as PPA. About 3% of the added label was found in the neutral extract.

Table 5. Distribution of radioactivity (%) after addition of [U-^{14}C] (4-hydroxyphenyl)acetic acid or [U-^{14}C] 3-(4-hydroxyphenyl)propionic acid to farm slurry. Incubated for 20 h at 20°C

Compound added	*p*-Cresol	Aromatic acids			
		HPAA	HPPA	PAA	PPA
(4-Hydroxyphenyl)-acetic acid	75	9	0	0	0
3-(4-Hydroxyphenyl)-propionic acid	3	0	71	0	25

Table 6 gives the results of a similar experiment in which various labeled possible intermediates of the anaerobic degradation of tyrosine were added to farm slurry. In this experiment the incubation period was 3 hours. No transformation products of tyrosol and tyramine were detected. The transformation pattern of both HPAA and HPPA corresponded with those of Table 5. Tyrosine and HPLA were obviously readily fermented, tyrosine to mainly phenol and *p*-cresol, with the intermediate compound HPAA accumulating in this short-term experiment. A minor percentage of the label was located in HPLA, HPPA and PPA. The main products formed from HPLA were HPPA and HPAA, though *p*-cresol and PPA also carried some label.

Table 6. Distribution of radioactivity (%) after incubation under anaerobic conditions of farm slurry with labeled tyrosine and with labeled possible intermediates of tyrosine degradation. Incubated for 3 h at 20°C

Compound added	Phenol + *p*-cresol	Tyrosol	Aromatic acids			
			HPLA	HPAA	HPPA	PPA
Tyrosine	31	0	9	38	6	2
Tyrosol	0	100	0	0	0	0
Tyramine	0	0	0	trace	0	0
(4-Hydroxyphenyl)-acetic acid	17	0	0	72	0	0
3-(4-Hydroxyphenyl)-propionic acid	3	0	0	0	95	5
(4-Hydroxyphenyl)-lactic acid	2	0	75	6	11	1

Radiochemical experiments with faeces

Labeled tyrosine was added to five samples of freshly voided faeces from different pigs. The mixtures were incubated at 37°C for 20 hours. Analysis of the neutral extract by thin-layer and gas chromatography revealed that it contained no other labeled product than *p*-cresol. No label was recovered in phenol or 4-ethylphenol. *p*-Cresol comprised 19-35% (average 28%) of the label added initially. The acid extract contained 35% (range 31-46%) of the added radioactivity of which 95% (range 82-99%) was present in PPA, and 4% (1.5-13%) in HPLA; the other aromatic acids were absent or present in traces only. In these experiments on the average 63% (71% if including CO_2 lost in decarboxylation reactions) of the added label was recovered.

The results of the experiments with added labeled tyrosine and intermediates to faeces are given in Table 7. Here, as was the case with farm slurry, no *p*-cresol or aromatic acids were produced from either tyrosol or tyramine. The decarboxylation of HPAA to *p*-cresol was nearly completed within the three hours of the experimental period. Dehydroxylation of HPAA was not obvious but might have occurred in trace amounts. This is in contrast with HPPA which was dehydroxylated almost quantitatively to PPA. *p*-Cresol and PPA were accumulated in about equal amounts from the breakdown of tyrosine. The products formed from HPLA were PPA and *p*-cresol.

Table 7. Distribution of radioactivity (%) after incubation under anaerobic conditions of pig faeces with labeled tyrosine and labeled possible intermediates of tyrosine degradation. Incubation for 3 h at 37°C

Compound added	*p*-Cresol	Tyrosol	Aromatic acids			
			HPLA	HPAA	HPPA	PPA
Tyrosine	38	0	1	2	0	32
Tyrosol	trace	97	0	0	0	0
Tyramine	0	0	0	0	0	0
(4-Hydroxyphenyl)-acetic acid	73	0	0	9	0	0
3-(4-Hydroxyphenyl)-propionic acid	5	0	0	0	7	91
(4-Hydroxyphenyl)-lactic acid	14	0	40	3	trace	21

DISCUSSION

Labeled versus unlabeled test compounds

In preliminary experiments 1 mg of test compound was added per ml of sample. Addition of compounds in these concentrations might lead to erroneous results, due to enzyme induction or selection of bacteria. These problems could be overcome by the use of radioactive substances in combination with short incubation periods. Comparison of the results obtained with labeled and non-labeled test compounds revealed that they showed the same tendencies. So overnight incubation with 1 mg/ml of test substance gives good indications as to the processes occurring in the systems studied.

Phenol

In farm slurry nearly half of the tyrosine is converted to phenol. The involved bacteria probably possess the enzyme tyrosine phenol lyase, which degrades tyrosine to phenol, pyruvate and ammonia (Fig. 4). This conclusion

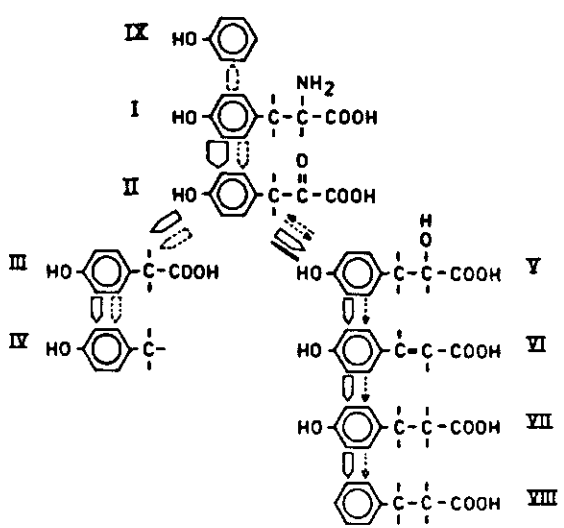

Fig. 4. Proposed schemes for the degradation pathways of tyrosine in faeces of pigs (solid arrows) and in anaerobically stored mixed wastes (dashed arrows) I, tyrosine; II, (4-hydroxyphenyl)pyruvic acid; III, (4-hydroxyphenyl)acetic acid; IV, *p*-cresol; V, (4-hydroxyphenyl)lactic acid; VI, *p*-coumaric acid; VII, 3-(4-hydroxyphenyl)propionic acid; VIII, 3-phenylpropionic acid; and IX, phenol.

is supported by several observations. Phenol was readily produced from tyrosine but only in a minor degree from HPPyrA (Table 3). This observation is consistent with the results obtained by Ichihara et al. (23) with tyrosine phenol lyase from *Escherichia coli*. The only other compound from which phenol was formed was 4-hydroxybenzoic acid. In farm slurry this compound was decarboxylated over the complete range studied (pH 6.0 - 8.5), whereas phenol was produced from tyrosine at the higher pH values only (pH 7.0 - 8.5). This corresponds with pH ranges reported for tyrosine phenol lyase from different bacteria (9,25,27).

A possible explanation for the complete absence of phenol production in faeces may be found in the repression of the enzyme tyrosine phenol lyase by simple carbohydrates which are liberated by the degradation of the cellulose-hemicellulose fraction of plant cell wall constituents in faeces. In stored farm slurry the more easily degradable fraction of plant cell wall material has already been consumed.

p-Cresol

The degradation of tyrosine to *p*-cresol via HPPyrA and HPAA is a major pathway in the breakdown of tyrosine in both farm slurry and in faeces. In experiments with tyrosine added to the samples, HPAA accumulated (Tables 6 and 7). Pure-culture studies have shown that many tyrosine-degrading bacteria accumulate HPAA in their medium, without being able to decarboxylate this compound (15,22,39,40). From the foregoing it is concluded that the conversion of tyrosine to *p*-cresol in piggery wastes probably occurs in at least two steps, by different bacteria, the first to HPAA and the second to *p*-cresol.

4-Hydroxybenzyl alcohol and 4-hydroxybenzaldehyde were found to be mainly reduced to *p*-cresol in faeces and in slurry with minor amounts of phenol being formed (Table 3). Phenol is obviously formed via oxidation to 4-hydroxybenzoic acid followed by decarboxylation. Similar results have been obtained by Scheline (33) with intestinal contents of rats. Whether these transformations are part of the tyrosine metabolism is not clear. Japanese workers found accumulation of 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid when *Proteus vulgaris* was incubated with tyrosine (21,40). However, the methods applied by these authors to isolate *p*-hydroxybenzaldehyde from the incubation mixture may not have excluded the formation of this compound by chemical decomposition of possibly accumulated HPPyrA (8).

When faeces or slurry were incubated with radioactive HPPA, 2-5% of the label was recovered in the neutral extract. Theoretically phenol may be produced

from HPPA by β-oxidation to *p*-hydroxybenzoic acid and successive decarboxylation. However, longer incubation periods with labeled HPPA did not result in higher recoveries of label in the neutral extract indicating that artefacts may have been involved. The results reported in Table 7 indicate that the conversion of HPPA to PPA in faeces proceeds much more readily than in farm slurry. In contrast to faeces the pathway of reductive tyrosine degradation (from HPPyrA → HPPA; Fig. 4) is of minor importance in farm slurry.

Tyrosine degradation with decarboxylation to tyramine as the first step and continuing according to the scheme tyramine → tyrosol → HPAA → *p*-cresol could not be demonstrated in either farm slurry or faeces (Tables 6 and 7). That this is a possible route is concluded from experiments with long incubation periods with tyramine and tyrosol (Table 3). In these long-term experiments large amounts of *p*-cresol were produced from both substances. Tyramine has been reported to be produced in small amounts by the microflora of human faeces. Asatoor (1) recovered 0.6% of the labeled tyrosine (1.8 μmol) added to human faecal bacteria as tyramine after 25 hours at 35°C.

Reductive degradation of tyrosine

PCA is probably the intermediate in the reduction of HPLA to HPPA. However, this compound has not been shown to be present in the wastes. Tanaka (41) described this pathway for *Proteus vulgaris*, and earlier Hirai (20) had noticed the accumulation of large amounts of PCA by the same organism in a medium with tyrosine. In addition, the formation of the corresponding acrylic acids from both indolelactic acid (18,30) and from phenyllactic acid (18,29) have been reported to occur in the degradation of tryptophan and phenylalanine, which are structurally related to tyrosine. The occurrence of PCA as an intermediate in the decomposition of tyrosine to HPPA makes it likely that also 4-ethylphenol can be a product of tyrosine metabolism. PCA is readily converted to 4-ethylphenol by faeces (Table 3), which agrees with findings of Scheline (34) and Bakke (4). The accumulation of 4-hydroxystyrene in faeces incubated at pH 5.5 (Fig. 3) and the observation that 4-ethylphenol is not produced from HPPA (Table 3; 4,12,34) suggest that PCA is first decarboxylated to 4-hydroxystyrene followed by hydrogenation to 4-ethylphenol. Decarboxylation of PCA in pure culture has been reported for an *Aerobacter* sp. (17), *Bacillus* sp. (24) and for a number of fungi (7,19). The conversion of PCA to 4-ethylphenol via hydrogenation to HPPA and decarboxylation as proposed first by Baumann (6) and later by other authors (13,42) is not likely to occur in the wastes studied here.

Decarboxylation reactions

A remarkable process in farm slurry as well as in faeces is the decarboxylation of various 4-hydroxyphenylcarboxylic acids (Table 3; Fig. 4). These decarboxylations did not occur when synthetic media were inoculated with farm slurry (see Results). In these experiments HPPA and HPAA accumulated. With respect to the organisms and enzymes involved in the decarboxylation reactions not much is known. Scheline (33) concluded that decarboxylation of aromatic acids occurs only when a free hydroxyl group is present in the para-position. Finkle et al. (17) reported a constitutive enzyme which decarboxylates PCA; this enzyme also decarboxylated other phenylacrylic compounds bearing a 4-hydroxy group in the ring. The same bacterium could form a different adaptive enzyme able to decarboxylate 4-hydroxybenzoic acid, but not HPAA, so probably different enzymes decarboxylate PCA, HPAA and 4-hydroxybenzoic acid.

Dehydroxylation of HPPA

In faeces PPA is a main degradation product of tyrosine, whereas in farm slurry only minor amounts of this compound are formed (Tables 6 and 7). The dehydroxylation of HPPA to PPA has been reported to occur also in the rumen of sheep (36) and in faeces of man (12); it may be a common reaction in anaerobic environments. The significance of the dehydroxylation of HPPA may lie in the linkage of the degradative pathways of tyrosine and phenylalanine.

Curtius et al. (12) reported the occurrence of rearrangement reactions of ring substituents leading to the formation of trace amounts of 3-hydroxyphenyl compounds. These reactions have not been considered in the work reported here.

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DEGRADATION OF TYROSINE BY TWO *CLOSTRIDIUM* SPP.

A study was made of the degradation of tyrosine by *Clostridium sporogenes* NCIB 10696 and by a strain of *Cl. ghoni* isolated from pig faeces.

Both organisms were found to accumulate (4-hydroxyphenyl)acetic acid, tyrosol [(4-hydroxyphenyl)ethanol] and (4-hydroxyphenyl)lactic acid in tyrosine-containing culture media. *Cl. sporogenes* produced in addition 3-(4-hydroxyphenyl)propionic acid. *p*-Coumaric acid was demonstrated to be an intermediate probably in the conversion of (4-hydroxyphenyl)lactic acid to 3-(4-hydroxyphenyl)propionic acid. *p*-Coumaric acid, when added to media of *Cl. ghoni*, was decarboxylated and hydrogenated to 4-ethylphenol. Thus the possibility of the formation of 4-ethylphenol as a product of tyrosine metabolism was shown.

INTRODUCTION

Phenol and *p*-cresol have long been known to be products of the anaerobic microbial degradation of tyrosine. Some clostridia have been described accumulating these simple phenols in their culture media (e.g. Elsden et al., 1976). Another simple phenol, *viz.* 4-ethylphenol, usually present in the urine of mammals (Lederer, 1943; Grant, 1948; Spoelstra, 1977) is presumed to originate also from the microbial degradation of tyrosine. However, no pure or mixed cultures have sofar been described that produce 4-ethylphenol from tyrosine. As precursor of 4-ethylphenol *p*-coumaric acid is well established (Finkle et al., 1962; Indahl and Scheline, 1968).

In the present communication the tyrosine catabolism of two clostridial strains is described. It was shown that under some conditions small quantities of *p*-coumaric acid accumulate. In addition the formation of aromatic acids and an aromatic alcohol by these bacteria is reported.

MATERIALS AND METHODS

Organisms

Clostridium sporogenes NCIB 10696. *Cl. ghoni* was isolated from pig faeces. This motile strain did not produce acids from carbohydrates, gelatin was liquefied completely, indole produced and esculin hydrolysed. In a peptone yeast extract medium acetic, propionic, iso-butyric, butyric, isovaleric and α-methylbutyric acids accumulated.

Media

One quarter strength reinforced clostridial medium (RCM, Oxoid) was enriched with 0.2 g/l tyrosine (medium B). Medium A had the following composition (g/l): peptone 5, yeast extract 2, and tyrosine 0.2.

The bacteria were grown in 1-l serum bottles equipped with a screw cap which held a rubber septum in place. Anaerobiosis was achieved by autoclaving the bottles with the screw cap somewhat loosened to allow air to escape. Immediately after autoclaving, the cap was tightened. Media were inoculated with a syringe and incubated at 37°C.

Extraction procedures

Tyrosol, 4-hydroxystyrene and 4-ethylphenol were extracted with diethylether from incubated culture media brought to pH 8.3.

For the extraction of phenolic acids the pH of the medium was adjusted to 2 and the media subsequently extracted with diethylether and ethyl acetate. The organic layers were combined and evaporated under reduced pressure to dryness.

Gas chromatography

Tyrosol, 4-hydroxystyrene and 4-ethylphenol were separated by transferring 1 µl of the diethylether extract, which previously had been reduced to a volume of about 1 ml, on a 10% SE-30 column. The operational data have been described before (Spoelstra, 1977).

The phenolic acids in the dried extract were either trimethylsilylated or converted into the methylesters. The thus treated samples were subjected to gas chromatography on a 10% OV-17 column. These methods have been described in detail elsewhere (Spoelstra, 1978).

Mass spectra were taken with a combined gas chromatograph-mass spectrometer equipment. The mass spectrometer was a MM 70-70 type of V.G. Mass spectra were taken at 50 eV.

Thin-layer chromatography

Extracts with phenolic acids or neutral phenolic compounds were applied to thin layers of cellulose or silica gel. For development either 1.5% formic acid or the upper layer of benzene/acetic acid/water (10/7/3) was employed. Spots were visualized by spraying with diazotised sulphanilamide followed by a 10% Na_2CO_3 solution. Rf values of the various phenolic compounds have been listed previously (Snoelstra, 1978).

To estimate the distribution of radioactivity in extracts from culture media which had been incubated with L-[U-^{14}C] tyrosine, all identified spots from thin layers were scraped into different scintillation vials and counted for radioactivity with a liquid scintillation counter.

Chemicals

Tyrosol was prepared microbiologically by incubating *Saccharomyces cerevisiae* with tyrosine and sucrose according to Ehrlich (1911). L-[U-14] tyrosine was purchased from the Radiochemical Centre, Amersham. All other chemicals were obtained commercially and were of the highest purity offered.

RESULTS

Accumulation of products derived from tyrosine by growing cultures

In preliminary experiments, gas and thin-layer-chromatographic analyses of spent culture media showed that *Cl. sporogenes* accumulated (hydroxyphenyl)-acetic acid, (hydroxyphenyl)lactic acid and (hydroxyphenyl)propionic acid. In addition a neutral product, which could be extracted at pH 8.3 with diethylether was present. Estimation of the gas-chromatographic retention times and of the Rf values of this compound and of authentic chemicals indicated that the unknown compound might be tyrosol. Its identity as tyrosol was confirmed by combined gas chromatography and mass spectrometry of the neutral extract of the culture medium of *Cl. sporogenes*. Figure 1 shows the mass spectra of tyrosol prepared as described under Chemicals and that from the tyrosine-containing culture medium of *Cl. sporogenes*. The spectrum

of the latter shows some mass peaks derived from contaminants which were not completely separated gas chromatographically from tyrosol.

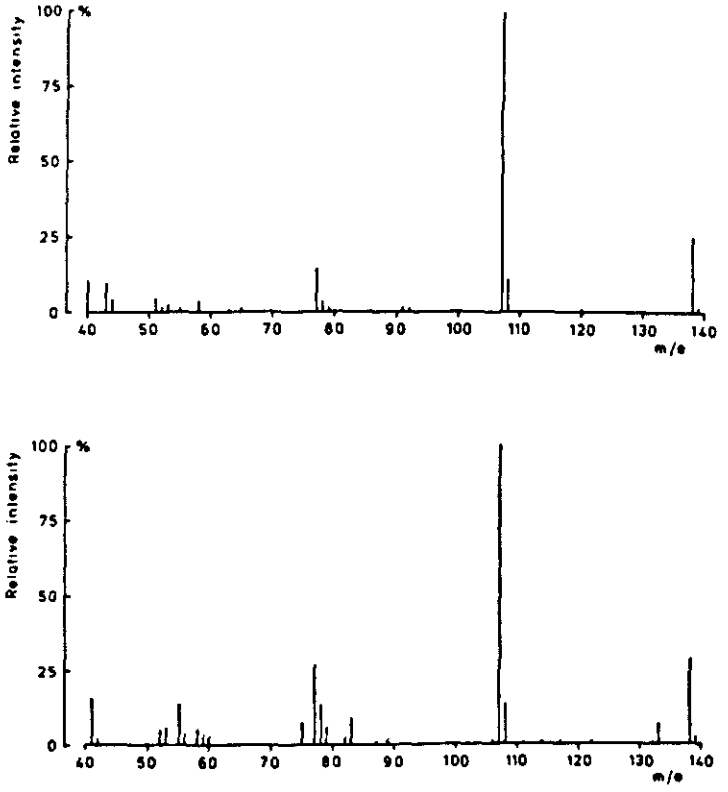

Fig. 1. Mass spectra of tyrosol obtained as described in Materials and Methods (a) and of tyrosol in the spent nutrient medium of *Cl. sporogenes* (b).

Cl. ghoni produced the same end products from tyrosine as *Cl. sporogenes*, except that (hydroxyphenyl)propionic acid was not formed by this organism. Table 1 gives the distribution of radioactivity over the accumulated

degradation products in media to which [U-^{14}C] tyrosine had been added. The data recorded show that *Cl. sporogenes* degraded 2-5 times more of the added tyrosine than *Cl. ghoni*. The main degradation product of *Cl. sporogenes* was (hydroxyphenyl)lactic acid, while smaller amounts of (hydroxyphenyl)acetic acid and (hydroxyphenyl)propionic acid and minor amounts of tyrosol were formed. The accumulated amounts of (hydroxyphenyl)propionic acid increased with the incubation period. 3-Phenylpropionic acid was formed in the media but it contained no label (see Discussion).

Cl. ghoni converted tyrosine to mainly (hydroxyphenyl)acetic acid and minor amounts of (hydroxyphenyl)lactic acid and tyrosol whereas no (hydroxyphenyl)propionic acid was formed.

Experiments with washed cells

The bacteria were grown in medium A, harvested after 18-20 hours incubation and washed twice with 0.1 M potassium phosphate buffer (pH 7.0) and incubated in the phosphate buffer under a N_2 atmosphere with tyrosine (0.025 M) and α-ketoglutaric acid (0.025 M) under sterile conditions. Separate experiments had shown that no growth occurred in this medium. After one week *Cl. ghoni* had accumulated (hydroxyphenyl)lactic acid, (hydroxyphenyl)acetic acid and tyrosol. *Cl. sporogenes* produced the same products but (hydroxyphenyl)-propionic acid which accumulated in the growing culture was not detected in the solution with washed cells. In similar experiments with short incubation periods (0.5 to 4 hours) (hydroxyphenyl)pyruvic acid was found to be accumulated by both organisms. In the case of *Cl. sporogenes* the gas chromatograms of phenolic acids both as trimethylsilyl derivatives and as methyl esters showed small peaks with retention times similar to those of the corresponding derivatives of *p*-coumaric acid.

In a similar experiment washed cells collected from 1.5 l of a culture of *Cl. sporogenes* in medium A were incubated with 5 μmol [U-^{14}C] tyrosine (4 μCi) and 5 μmol α-ketoglutaric acid (total volume 15 ml). The reaction mixture was sampled, after 2, 30 and 180 minutes, whereupon the reaction in the samples was stopped by adding HCl until a pH of 2 was reached. The extracts of the samples containing the phenolic acids, prepared as described in Materials and Methods, were applied to thin layers of cellulose. After development with 1.5% formic acid and spraying, heavily loaded layers showed in addition to spots of (hydroxyphenyl)lactic acid and (hydroxyphenyl)acetic acids faint spots with a

Table 1. Distribution of radioactivity after incubation with L- [U-^{14}C] tyrosine (1 µCi/20 ml of medium) at 37°C

Organisms	Medium	Incubation period (days)	Distribution of label (% of radioactivity added)				
			Tyrosol	(Hydroxyphenyl)-acetic acid	(Hydroxyphenyl)-lactic acid	(Hydroxyphenyl)-propionic acid	Label recovered
Cl. sporogenes	A	3	0.6	8.3	47.2	4.1	60.2
	B	3	1.9	5.7	77.7	6.3	91.6
	B	7	2.6	10.0	71.0	16.5	99.1
Cl. ghoni	A	3	1.4	25.9	0.9	0.0	28.2
	B	3	1.0	13.0	1.9	0.0	15.9

violet colour and an Rf value similar to that of *p*-coumaric acid. These spots were scraped into scintillation vials and the relative amount of these products estimated. The results of this experiment are summarized in Table 2.

Table 2. Distribution of radioactivity upon incubation of resting cells of *Cl. sporogenes* (with 5 µmol (4 µCi) of L-[U-^{14}C])tyrosine and 5 µmol of α-ketoglutaric acid at 37°C (total volume 15 ml)

Incubation period (min)	Radioactivity* (dpm)		
	p-Coumaric acid	(Hydroxyphenyl)-lactic acid	(Hydroxyphenyl)-acetic acid
2	260	9,000	3,400
30	7500	474,000	42,000
180	1800	478,000	84,000

*Label located in tyrosol and (hydroxyphenyl)pyruvic acid was not estimated

It is shown that the presumed *p*-coumaric acid is accumulated in the first phase of the experiment. After an incubation period of three hours the amount of presumed *p*-coumaric acid had decreased to 25%, when compared with the 30 min incubation period. From the reaction mixture of a similar experiment with non-labeled tyrosine which had been incubated for 30 min, the phenolic acids were extracted and trimethylsilyl derivatives prepared. Combined gas chromatography and mass spectrometry confirmed that indeed *p*-coumaric acid was present in the mixture (Fig. 2).

Incubation with p-coumaric acid

Enrichment of the nutrient media with 1 mg/ml of *p*-coumaric acid resulted in the accumulation by *Cl. ghoni* of large amounts of 4-ethylphenol at pH values ranging from 6 to 8. After incubation of this bacterium in media with pH values between 5.5 and 6.0 in the presence of *p*-coumaric acid in addition to 4-ethylphenol, a second phenol accumulated. This compound was extracted from the spent culture medium of *Cl. ghoni* grown for 10 days at pH 5.75 in RCM with 10 g/l of *p*-coumaric acid added. The unknown phenol was collected by gas chromatography by means of an effluent splitter and identified as 4-hydroxystyrene by mass spectrometry (Fig. 3).

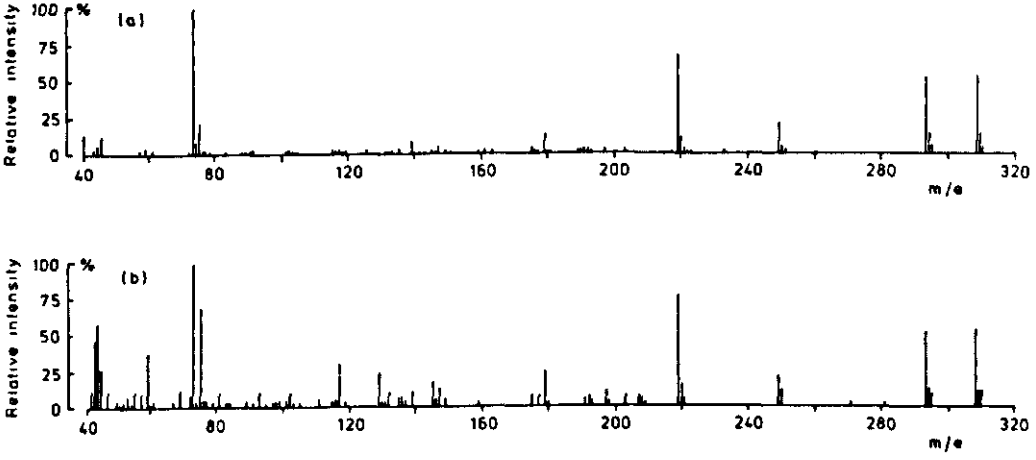

Fig. 2. Mass spectra of the ditrimethylsilyl derivatives of authentic *p*-coumaric acid (a) and of *p*-coumaric acid in the assay solution of washed cells of *Cl. sporogenes*.

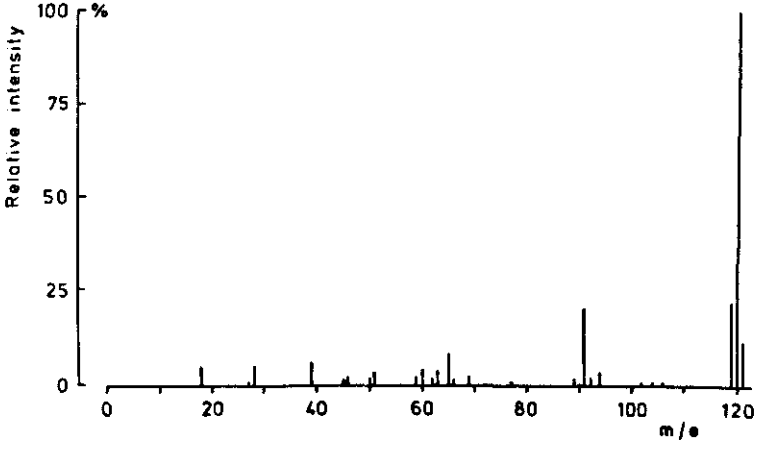

Figure 3. Mass spectrum of 4-hydroxystyrene isolated from the culture medium of *Cl. ghoni*.

DISCUSSION

In spent culture media of *Cl. ghoni* and *Cl. sporogenes* NCIB 10696 the presence of tyrosol was demonstrated. By using labeled tyrosine, it was proven that tyrosol was formed from this amino acid. As far as known, tyrosol has not earlier been demonstrated to be accumulated by clostridia.

Short-term experiments with washed cells incubated with tyrosine and α-ketoglutaric acid resulted, among other products, in the accumulation of (hydroxyphenyl)pyruvic acid, indicating that a transaminase was involved. Aromatic amino acid transaminases have been demonstrated in *Cl. sporogenes* by O'Neil and DeMoss (1968). Tyrosol and (hydroxyphenyl)acetic acid are probably formed by decarboxylation of (hydroxyphenyl)pyruvic acid and conversion of the resulting (hydroxyphenyl)acetaldehyde to mainly (hydroxyphenyl)acetic acid and less tyrosol (Table 1). In cells of yeast alcohol dehydrogenase was found by Sentheshanmuganathan and Elsden (1958) to be responsible for the reduction of (hydroxyphenyl)acetaldehyde to tyrosol.

The reduction of (hydroxyphenyl)pyruvic acid to (hydroxyphenyl)lactic acid is performed by both organisms studied. Jean and DeMoss (1968) have reported the presence of a dehydrogenase in *Cl. sporogenes* with activity towards indolepyruvic, phenylpyruvic and (hydroxyphenyl)pyruvic acids.

Cl. sporogenes NCIB 10696 was mentioned by Elsden et al. (1976) to accumulate (hydroxyphenyl)propionic acid. No other degradation products from tyrosine were detected by these authors. However, our results show that in addition to (hydroxyphenyl)propionic acid also (hydroxyphenyl)acetic acid, (hydroxyphenyl)lactic acid and tyrosol are formed by this organism.

From the present investigation it is concluded that *p*-coumaric acid probably functions as intermediate in the conversion of (hydroxyphenyl)lactic acid to (hydroxyphenyl)propionic acid. In early literature, Hirai (1921) described the accumulation of *p*-coumaric acid when cells of *Proteus vulgaris* had been incubated with tyrosine for 12 days. Tanaka (1968) obtained also indications that *p*-coumaric acid is an intermediate in the decomposition of tyrosine.

In experiments with washed cells (Table 2) *p*-coumaric acid was present in significant amounts after 30 min incubation; longer incubation periods resulted in diminished concentrations. This is consistent with observations

of Moss et al. (1970) concerning the accumulation of cinnamic acid (phenylacrylic acid) by washed cells of *Cl. sporogenes* upon incubation with phenylalanine. Microbial formation of acrylic acids from tryptophan and phenylalanine has been described by Hansen and Crawford (1967).

Cl. ghoni converts *p*-coumaric acid via 4-hydroxystyrene to 4-ethylphenol (Results). Decarboxylative activity towards (4-hydroxyphenyl)acrylic acids seems to be a common reaction among bacteria of the intestinal tract (Finkle et al., 1962; Indahl and Scheline, 1968; Scheline, 1968). The occurrence of *p*-coumaric acid as an intermediate in the degradation of tyrosine may give an explanation for the formation of 4-ethylphenol in the gut of mammals. Confusion exists about the origin of this compound which is usually present in urine (Lederer, 1943; Grant, 1948). Bakke (1969) concluded from experiments with rats receiving diets with and without plant material that *p*-coumaric acid from plant material is most likely the precursor of 4-ethylphenol. But the present results suggest that 4-ethylphenol may also arise from tyrosine via *p*-coumaric acid. Under which conditions *p*-coumaric acid can accumulate *in vivo* remains obscure; neither can the question be answered whether 4-ethylphenol is formed from tyrosine by single organisms or by mixed cultures.

In peptone-containing nutrient media Elsden et al. (1976) found that *Cl. sporogenes* NCIB 10696 formed phenylpropionic acid in addition to (hydroxyphenyl)propionic acid. The former was assumed to be derived exclusively from phenylalanine. However, phenylpropionic acid can also originate from tyrosine by dehydroxylation of (hydroxyphenyl)propionic acid as it was demonstrated in mixed cultures by Scott et al. (1964) and by Curtius et al. (1976). In the present study with labeled tyrosine added to the peptone-containing media A and B of *Cl. sporogenes* NCIB 10696, phenylpropionic acid was found to carry no label. From this result it is concluded that phenylpropionic acid was not formed from tyrosine by this bacterium.

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GENERAL DISCUSSION

Overall processes

Gross overall processes in anaerobically stored piggery wastes include the degradation of high molecular weight compounds like plant fibre and protein to volatile low molecular weight components. In Chapter 2 it was demonstrated that 24% of the plant fibre and 43% of the crude protein of swine faeces were decomposed during anaerobic storage, with volatile fatty acids being almost the sole non-gaseous products.

Degradation of plant fibre

Analyses to establish the degree to which the composing products of plant fibre (i.e. cellulose, hemicellulose and lignine) were degraded failed due to analytical errors. Lignine has never been demonstrated to be decomposable under anaerobiosis (Yamane and Sato, 1963). Consequently the demonstrated degradation of plant fibre must be attributed to the decomposition of cellulose and/or hemicellulose. Indirect evidence provided in Chapter 3 suggests that the degradation of hemicellulose is an important process in piggery wastes. Higher colony counts were obtained when hemicellulose was added to counting media, whereas cellobiose, an intermediate of cellulose degradation, did not have a positive effect on colony counts. These results indicate that many bacteria in the wastes are capable of utilizing or even need hemicellulose for their growth and that cellulolytic organisms are not predominant. In Chapter 3 isolates are mentioned which have tentatively been identified as *Ruminococcus* spp. However, these isolates have not been tested for their ability to ferment cellulose, whilst some ruminococci are known to be able to utilize hemicellulose (Dehority, 1965). Attempts to isolate cellulolytics from stored piggery wastes by Hobson and Shaw (1974) were not successful. However, these authors claim to have counted about 10^5/ml cellulolytic bacteria in a methane digestor installation with piggery wastes as influent. The limited information indicates that cellulolysis is of minor importance in stored wastes, in contrast with the degradation of hemicellulose.

Details about the anaerobic microbial decay of plant fibre and the involved bacteria are poorly understood. In pure culture the decomposition of hemicellulose can be performed by representatives of the genera *Bacteroides* (Salyers et al., 1977a, b) and *Ruminococcus* (Dehority, 1963). However, it must

be assumed that degradation in anaerobic natural ecosystems is the result of the concerted action of different kinds of bacteria.

Dehority (1965) showed that some cellulolytic bacteria (ruminococci and one strain of *Butyrivibrio*) degraded hemicellulose without utilizing the oligomeric degradation products. The same author (1973) described two-membered cultures of a cellulolytic and a non-cellulolytic bacterium which together converted hemicellulose to volatile fatty acids and alcohol. In this culture the cellulolytic organism degraded hemicellulose without utilizing the degradation products. These were converted to volatile fatty acids and alcohol by a *Bacteroides* strain, which could not attack the intact hemicellulose. Similar synergistic degradation patterns were found in various combinations of bacteria degrading hemicellulose or pectin. Latham and Wolin (1977) studied the degradation of cellulose by *Ruminococcus flavefaciens* and by a mixed culture of this bacterium and *Methanobacterium ruminantium*. In the mixed culture the electron sink products formed by the *Ruminococcus* sp. in monoculture (succinate and hydrogen) were absent or diminished and instead CO_2, CH_4 and more acetate accumulated. Similar observations were reported by Ianotti et al. (1973) of a mixed culture of *Ruminococcus albus* and *Vibrio succinogenes*. The latter bacterium utilized the hydrogen produced by the ruminococcus to reduce fumarate to succinate, thus enabling the ruminococcus to generate 4 moles of ATP instead of 3.3 from 1 mole of glucose fermented. The difference of 0.7 mole ATP was lost in the monoculture by the formation of the electron sink product ethanol from acetyl CoA. In general, no or few electron sink products accumulate if the hydrogen produced by various fermentation reactions is consumed by other organisms. Similarly the absence of considerable amounts of electron sink products in the rumen was explained by Hungate (1966) as caused by the consumption of hydrogen by methanogens. In anaerobically stored piggery wastes methane is formed in only low amounts. This means that H_2 which is intermediary formed during the anaerobic degradation of waste components (for instance the formation of acetic acid) is eliminated by the formation of other electron sink products mainly propionic acid and smaller amounts of higher fatty acids. Other hydrogen-consuming processes may be present in the wastes, like the synthesis of acetic acid from CO_2 and H_2. This reaction is performed by *Clostridium aceticum* (Wieringa, 1940, 1941) and by *Acetobacterium woodii* (Balch et al., 1977), and has been demonstrated to occur probably in the intestine of some

rodents (Prins and Lankhorst, 1977). However, no experimental data are available on the occurrence of such metabolic reactions in piggery wastes to verify these speculations.

Accumulation of low molecular weight compounds

Generally the final products of microbial degradation of carbonaceous material in an anaerobic natural ecosystem are methane and CO_2. In stored piggery wastes only some methane is formed (Chapter 2, Stevens and Cornforth, 1974). The rate of methanogenesis under storage conditions is not high enough to prevent the accumulation of products of the acid-forming fermentation. This is in contrast with digester units for methane fermentation of sewage sludge or of piggery wastes. In other words the acid-forming and methanogenic steps in the microbial degradation of the complex substrates in piggery wastes are in unbalance. This may have consequences for the degradation of other compounds. Wolfe (1971) expressed this as follows. "Under natural anaerobic conditions the methane bacteria which oxidize molecular hydrogen may be considered actually to "pull" the degradation in microbial food chains by displacing unfavourable equilibria". What actually causes the low rate of methanogenesis in stored piggery wastes is not clear, but a number of factors which are unfavourable for methane fermentation can be enumerated. Methane fermentation in digester units proceeds at temperatures around 35°C, whereas the storage temperature of wastes, dependent on the season, ranges from 10-20°C. Though methane fermentation in nature occurs below 10°C (Svensson, 1975), it is well-known that mesophilic methanogens produce methane at a lower rate and grow much slower at lower temperatures (Van den Berg, 1977; Zeikus and Winfrey, 1976). Overloading with organic degradable material causes failure of digester installations. Digestion of swine wastes was found to be inhibited at loading rates of 4-8 kg total solids/m^3.day (Schmid and Lipper, 1969). Such overloading results in diminished methane production and accumulation of VFA in the digestor. An overload situation is likely to be present in stored wastes. The presence in the wastes of substances inhibitory to methanogens is another possibility. Inhibition of the acetogenic intermediate stage is indicated by the presence of small amounts of hydrogen (Chapter 2) and by the observation of some authors that it is advisable to dilute the wastes to obtain balanced digestion (Hobson and Shaw, 1973). The high levels of NH_3 in the wastes may also cause inhibition. In the literature, several authors have reported ammonia concentrations which are inhibitory to digestion

(Schulze and Raju, 1958; McCarty, 1964). However, others obtained balanced methane fermentation of piggery waste at much higher ammonia concentrations (Kroeker et al., 1975; Van Velsen, 1977). Melbinger and Donellon (1971) stated that NH_3 becomes not toxic at high concentrations unless the rate of formation increases more rapidly than the acclimation of methanogens. Cappenberg (1974, 1975) demonstrated that methanogens were inhibited by 0.001 mM H_2S and found that addition of sulphate to mud of a freshwater lake increased the H_2S concentration which caused inhibition of methanogenesis. Zehnder and Wuhrman (1977) noticed inhibition of their strain AZ by H_2S. However, good methanogenesis in the presence of rather high H_2S concentrations was reported by Zhilina and Zavarzin (1973). The presence of H_2S in piggery waste has been reported by several authors (Stevens and Cornforth, 1974; Banwart and Bremner, 1975). A considerable contribution to the H_2S formation in the wastes originates from the reduction of sulphate which is excreted with the urine. Volatile fatty acids have in concentrations of 2000-3000 mg/l been suggested as inhibitory to anaerobic methane digestion (e.g. Schulze and Raju, 1958). But later Kugelman and Chin (1971) reported that volatile fatty acids were not toxic to methanogens in levels up to 6 g/l. Propionic acid was found to be slightly toxic at this level to acid-forming bacteria.

Toxic effects of heavy metals, notably copper, may be of importance in piggery wastes. In general, the actual concentration of the toxic cation is diminished a hundred-fold or more by complexation reactions and by precipitation as poorly soluble sulphides. Inhibition can also be caused by the presence of high concentrations of light metal cations (Van den Berg et al., 1976; Kugelman and McCarty, 1965). The toxity of these cations can partially or completely be overcome by the presence of antagonistic cations (Kugelman and Chin, 1971).

The unbalance between the processes of acid formation and methane production is the main key to understanding the accumulation of volatiles (malodorous products) in piggery wastes. Under balanced conditions the volatiles are converted to CH_4 and CO_2. With exception of formic acid, acetic acid and methanol, more than one bacterial species is needed for the conversion of volatiles to methane. The exact nature of the conversion of the end products of acid-stage fermentation towards suitable substrates (i.e. CO_2, H_2, acetic acid) for methane production is unknown. It is assumed that the so-called intermediate group of organisms depends on methanogens for maintaining a very

low hydrogen pressure. Sofar only one organism belonging to this intermediate group has been isolated. This is the S organism isolated from the former *Methanobacillus omelianskii* (Bryant et al., 1967). The S organism oxidizes ethanol to acetic acid and hydrogen and the methanogen (*Methanobacterium* M.O.H.) produces methane from H_2 and CO_2. Similar associations can be expected in the conversion of other low molecular substances to methane. This may apply also for the decomposition of aromatic substances to methane by mixed cultures (Balba and Evans, 1977). Benzoic acid (Ferry and Wolfe, 1976), phenol and catechol (Healy and Young, 1978) have been reported to be degraded to methane by mixed cultures. Van Velsen (1977) found that concentrations of phenols and indoles were reduced with 90% and more in piggery wastes during balanced methane fermentation.

Odour and microbial catabolism

From the foregoing discussion and from the data compiled in Chapter 1 Table 1 it can be derived that it is not possible to speak of the presence of specific malodour-producing microbial processes in the wastes. The accumulated volatile compounds result from the overall conversion of complex substrates. The main volatile (and consequently odour) producing microbial processes are the degradation of plant fibre and of protein. Of course partial processes can be identified which lead to one or more typical malodorous products (e.g. tyrosine degradation) but such processes do not proceed independently but are simultaneously with other partial processes leading to volatiles and are unbreakably associated with the overall process *viz.*, degradation of macromolecular components.

Likewise no specific odour-diminishing microbial processes exist. But the odour disappears together with the transformation of the accumulated volatiles when the environment is changed (e.g. aeration, methane fermentation).

Carbohydrates (plant fibre material) in the wastes are degraded to a limited number of compounds, mainly acetic, propionic and butyric acids. This is in contrast with the degradation of proteins, which in addition to straight chain carboxylic acids lead to branched chain acids, sulphur compounds, amines, phenols, and indoles among others (Chapter 1). It is not possible to quantify the contribution of volatiles derived from protein versus those from plant fibre and other miscellaneous substances to the total of smelling compounds in the wastes. But the impression arises that the most pungent and the greatest variety of obnoxious smelling compounds originate from protein. This observation

is confirmed by results of the few existing organoleptic studies on the main components of the smell from piggeries and of piggery wastes (Schaefer et al., 1974; Barth et al., 1974).

The use of p-cresol and volatile fatty acids as indicator compounds for odour

As has been pointed out in the Introduction the main difficulty in judging the effectiveness of odour-abating methods is found in the measurement of the reduction of odour intensity and the change of the quality of the odour. In the preceding section it was argued that the microbial formation and disappearance of odour are strongly related with the proceeding of overall processes, which are governed by environmental factors (temperature, oxygen input, organic loading). Instead of using sensorial methods for comparison of odour it is likely that a fair indication about odour development can be obtained by monitoring concentrations of one or more properly chosen components which reflect the degradation of the main (macromolecular) waste constituents.

Ammonia and hydrogen sulphide have been used as indicator components for the smell. However, these components seem to be less suitable. The major part of ammonia in the wastes originates from urea hydrolysis and is in addition derived from the degradation of protein. Thus the formation of ammonia does not reflect degradation kinetics of the wastes. Moreover, ammonia remains unchanged by methanogenesis and shows a retarded reaction to aerobic treatment compared with organic volatiles. Hydrogen sulphide formation does neither reflect waste degradation kinetics because a relatively large part is derived from sulphate reduction. In addition non-soluble sulphides may be formed which make concentrations measured difficult to interprete.

Some demands can be formulated to which components to be selected as indicators for smell should answer:

1°) the components must be a product of protein or eventually carbohydrate degradation.

2°) the components should be stable end products under normal (= farm practice) conditions of waste storage. Compounds which are temporarily accumulated are not suitable.

3°) the formation of the component must reflect the kinetics of waste degradation.

4°) the components must respond in a representative way to environmental changes (e.g. aeration, methane formation).

5°) the concentrations of the components must easily be measurable. Trace components can not be used.

These demands imply that detailed information must be available about the origin, formation and response to environmental changes of the component(s) to be chosen as indicator(s).

In Chapters 4 and 5 the fate of some typical fermentation products of protein in piggery wastes has been discussed. This concerns indole, skatole and in more detail degradation products of tyrosine, *viz.* phenol, *p*-cresol and possibly 4-ethylphenol. Indole and skatole are metabolized during anaerobic storage (Chapter 4) and can therefore not be recommended as indicators of microbial protein degradation. Phenol and 4-ethylphenol show also some drawbacks as indicators. Phenol is present in relatively low concentrations and is mainly formed during storage. Ethylphenol originates from the urine (Chapter 4) and was not found to be formed during anaerobic storage. This implies that with 4-ethylphenol as an indicator, proceeding microbial degradative activity would not be noticed.

p-Cresol seems to be the best choice for monitoring accumulation of volatiles produced from protein. It is present in freshly voided faeces, liberated from glucuronidesupon contact of urine and faeces and is produced during storage (Chapter 4). In Chapter 5 it was shown that in both faeces and anaerobically stored wastes about 50% of the tyrosine is converted to *p*-cresol. Apparently *p*-cresol production reflects the kinetics of microbial protein degradation in the animal as well as in the wastes, because a constant part of tyrosine is converted to this substance. Furthermore, *p*-cresol concentrations are diminished during aerobic (Spoelstra, unpubl. results) as well as anaerobic waste treatment (Van Velsen, 1977). It may be that *p*-cresol itself contributes significantly to the smell of the wastes as suggested by the work of Schaefer et al. (1974) and Schaefer (1977), but this is not a requisite for a useful indicator.

In addition to *p*-cresol, the analysis of volatile fatty acids - the most abundantly occurring volatiles in piggery wastes - will give much information about microbial metabolism in the wastes. The accumulation of volatile fatty acids under storage conditions originates mainly from carbohydrates and in addition considerable amounts are derived from proteins and other miscellaneous organic substances.

Volatile components of piggery wastes differing from *p*-cresol and volatile fatty acids might potentially be even better suitable for monitoring microbial processes in the wastes and thus might give indications about odour development during storage or waste treatment. But at present careful studies about origin, production and fate during storage and waste treatment are lacking.

Concentrations of *p*-cresol and volatile fatty acids as indicators of odour development may be applied in laboratory evaluation of chemical or biochemical additions (other than pure deodorants etc.) to wastes, as well as in testing of waste handling systems at pilot plant or farm scale experiments (e.g. channel flushing). Whether these compounds are also useful in the evaluation of air samples needs further study. Of course for judgement of odour quantity and intensity only methods which use the human nose can be applied.

SUMMARY

In modern intensive animal farming labour-saving waste handling systems are applied. As such, slatted floors have found wide application. In this system large amounts of malodorous wastes are stored in pits under the slatted floors, resulting in the emission of malodours with the ventilation air. Complaints about odour nuisance caused by piggeries do frequently occur.

In this thesis the results are reported of investigations on the microbiology of anaerobically stored piggery wastes and on the formation in such wastes of some malodorous components.

Chapter 1

In general, odour can be measured only by sensorial methods and this applies exclusively to the odour intensity. Odour quality can be approached only by comparing it with other odours as present in an odour classification system. The lack of instrumental methods for measuring odour can partially be overcome by estimating concentrations of components considered to be characteristic of odorous mixtures.

About 150 volatile compounds have been identified in piggery wastes by different workers. Based on literature data the possible origin of some groups of volatile compounds in piggery wastes has been indicated in this chapter. In addition the composition of urine, faeces and mixed wastes has been given.

Chapter 2

Anaerobically stored piggery wastes as found in farm pits contain large amounts of volatile fatty acids. The amounts measured in samples from different farms ranged from 4-27 g/l. About 20% of these acids were already present in the freshly voided faeces. During anaerobic storage large amounts of volatile fatty acids are formed by degradation of macromolecular constituents of the faeces. In an experiment with a mixture of faeces and urine 43% of the raw protein and 24% of the fibre were degraded during 70 days storage. Acetic, propionic, and butyric acids and small amounts of other lower fatty acids and carbon dioxide were the main products formed; in addition some methane was produced.

Chapter 3

Colony counts of piggery wastes on an average numbered 5.10^9 on media containing 80-100% farm slurry supernatant which were prepared and inoculated according to the strictly anaerobic methods developed by Hungate. These colony counts amounted to about 20% of the cell numbers found by direct microscopic observation. Pure cultures were obtained from only 20% of the colonies picked for isolation. The isolated bacteria apparently belonged to the genera *Ruminococcus*, *Peptococcus*, *Peptostreptococcus* and *Bacteroides*. The bacteria belonging to these genera are usually isolated from the intestinal tract of man and mammals; however, many of the isolates from piggery wastes were unable to grow at 37°C.

Addition of different carbohydrates to media with suboptimal concentrations of farm slurry supernatant revealed that the colony counts could be increased by incorporating hemicellulose preparations in the media. From these results it was concluded that the degradation of hemicellulose is an important process in piggery wastes.

Chapter 4

Piggery wastes always contain simple phenols. Notably *p*-cresol was usually present in considerable amounts (up to 350 mg/l). Phenol and 4-ethylphenol were present in concentrations from 10 to 50 mg/l. Phenol and *p*-cresol originate from the anaerobic degradation of tyrosine, a product of protein break-down. These compounds are in the pig formed by the intestinal microflora as well as during anaerobic storage of the slurry. The simple phenols formed in the gut are partially absorbed by the animal and detoxicated by conjugation with glucuronic acid. The phenylglucuronides are excreted into the urine. The phenols, and also other compounds excreted as glucuronides, can be liberated by the high activity of the enzyme β-glucuronidase which has been found in the faeces of the pig as well as in farm slurry. The phenols bound to glucuronic acid and excreted into the urine were liberated nearly instantaneously upon contact with slurry. The production of phenols from tyrosine during waste storage proceeded more slowly and was strongly influenced by storage temperature.

The concentrations of indole and skatole in farm slurry were considerably lower than those of *p*-cresol. Indole was found in amounts of 0-15 mg/l and skatole of 10-50 mg/l. Indole and skatole are produced in the intestinal tract where tryptophan is their precursor. Urine contained constituent(s) which were

readily converted to indole upon contact with faeces. Such constituents were not found in the case of skatole. During anaerobic storage of farm slurry indole and skatole were produced. However, on long-term storage the concentrations of these compounds diminished.

Chapter 5

The experiments described in this chapter showed that the pathway of tyrosine degradation in faeces differs from that in farm slurry. In faeces, tyrosine is converted to about equal quantities of *p*-cresol and phenylpropionic acid. *p*-Cresol is formed from tyrosine according to the scheme tyrosine → (hydroxyphenyl)pyruvic acid → (hydroxyphenyl)acetic acid → *p*-cresol. The pathway leading to phenylpropionic acid can be summarized as follows: tyrosine → (hydroxyphenyl)-pyruvic acid → (hydroxyphenyl)lactic acid → *p*-coumaric acid → (hydroxyphenyl)-propionic acid → phenylpropionic acid.

In farm slurry the same pathways were found to exist, however, here only about 1% of the tyrosine was converted to phenylpropionic acid, the main products of tyrosine degradation being *p*-cresol and phenol. The formation of the latter product is catalysed by the enzyme tyrosine phenol lyase. An explanation of the observed differences in tyrosine decomposition between faeces and farm slurry can possibly be found in the higher degree of decomposition of farm slurry compared with faeces. Therefore, in faeces tyrosine functions as H-acceptor (formation of phenylpropionic acid) and after decay of easily degradable carbohydrates as is the case in farm slurry, it is used as energy and carbon source (formation of phenol). A further difference between faeces and farm slurry was observed concerning the metabolism of *p*-coumaric acid. In faeces this compound was decarboxylated to 4-hydroxystyrene and this product was subsequently reduced to 4-ethylphenol. These reactions did not occur in farm slurry.

For the experiments concerning the degradation of tyrosine described in Chapter 5, ^{14}C-labeled intermediates were used. Some of these labeled intermediates were prepared microbiologically by incubating clostridial strains with ^{14}C-tyrosine and isolating the products formed.

Chapter 6

The tyrosine metabolism of two *Clostridium* strains was described. *Clostridium sporogenes* NCIB 10696 accumulated the following degradation products of tyrosine in its culture media: (hydroxyphenyl)acetic acid, (hydroxyphenyl)lactic acid, (hydroxyphenyl)propionic acid and tyrosol. *p*-Coumaric acid was demonstrated to be an intermediate product in tyrosine degradation, probably in the conversion

of (hydroxyphenyl)lactic acid to (hydroxyphenyl)propionic acid. *p*-Coumaric acid accumulated in small quantities when washed cells were incubated with tyrosine and α-ketoglutaric acid.

Cl. ghoni accumulated the same products in its culture medium as *Cl. sporogenes* did, except that (hydroxyphenyl)propionic acid was not formed. *Cl. ghoni* decarboxylated *p*-coumaric acid, when added to nutrient media and subsequently hydrogenated the product 4-hydroxystyrene to 4-ethylphenol. The combination of these two clostridial strains suggests a possible pathway for the conversion of tyrosine to 4-ethylphenol.

Chapter 7

In this chapter overall aspects of microbial conversions in piggery wastes are discussed. The accumulation of volatiles in farm slurry is attributed to the unbalance between the acid stage fermentation and methane fermentation which is nearly absent in the slurry. The methane fermentation in piggery wastes is presumably inhibited by the relatively low storage temperature and high concentrations of ammonia, H_2S, and cations. The influences of these factors were not investigated. It is concluded that the accumulation of volatile products of the acid stage fermentation (as fatty acids and phenols) is the cause of the stench of piggeries.

In addition it is stated that concentrations of *p*-cresol and volatile fatty acids can give a good indication concerning changes in odour levels in relation to the development and abatement of odour.

SAMENVATTING

In de moderne intensieve veehouderij is gezocht naar methoden van mestverwijdering die weinig arbeid vragen. Als zodanig hebben in de varkenshouderij stallen met roostervloeren een ruime toepassing gevonden. Bij deze werkwijze worden grote hoeveelheden onaangename geuren producerende drijfmest onder de roostervloeren bewaard. Een gevolg hiervan is dat met de ventilatielucht stank wordt verspreid. Klachten over stankhinder veroorzaakt door varkenshouderijen komen dan ook dikwijls voor. In dit proefschrift wordt ingegaan op de microbiologie van varkensdrijfmest en op de microbiële vorming van enkele stankkomponenten.

Hoofdstuk 1

In dit inleidende hoofdstuk worden ondermeer de algemene aspecten van het meten van stank behandeld. In het algemeen kan geur slechts met behulp van sensorische technieken worden gemeten en dit betreft dan alleen de geurintensiteit. De kwaliteit van een geur kan slechts worden omschreven door vergelijking met andere geuren. Het ontbreken van instrumentele methoden voor het meten van geur probeert men vaak te omzeilen door het meten van concentraties van verbindingen, waarvan men aanneemt dat ze een belangrijke bijdrage leveren tot de betreffende geur.

In varkensdrijfmest zijn door diverse onderzoekers in totaal ongeveer 150 verschillende vluchtige verbindingen geïdentificeerd. Voor diverse groepen van vluchtige verbindingen in drijfmest is op grond van literatuurgegevens in dit hoofdstuk aangegeven hoe deze verbindingen in drijfmest mogelijk ontstaan. Verder wordt de samenstelling van faeces, urine en drijfmest gegeven.

Hoofdstuk 2

Varkensdrijfmest heeft zeer hoge gehaltes aan vluchtige vetzuren. In monsters van praktijkbedrijven variëerden de gehaltes van 4-27 g/l. Ongeveer 20% van de vetzuren bleek reeds aanwezig in verse faeces. Gedurende anaërobe bewaring worden grote hoeveelheden vetzuren gevormd door anaërobe afbraak van de faeces. Een experiment wordt beschreven waarin tijdens een opslagperiode van 70 dagen in een mengsel van faeces en urine 43% van het ruw eiwit en 24% van de vezelfractie werden afgebroken. De produkten die hierbij ontstonden waren azijnzuur, propionzuur, boterzuur en in geringere hoeveelheden andere lagere vetzuren, CO_2, en verder enig methaan.

Hoofdstuk 3

In drijfmest konden met media die volgens de anaërobe Hungate-methode waren bereid en geënt en waaraan 80-100% mestcentrifugaat was toegevoegd, gemiddeld 5.10^9 levende bacteriën worden geteld. Deze waarden kwamen overeen met ongeveer 20% van de microscopische telling. Slechts 20% van de kolonies waarvan de bacteriën werden overgeënt, leidden tenslotte tot een reinculture. De geïsoleerde bacteriën behoorden tot de geslachten *Ruminococcus*, *Peptococcus*, *Peptostreptococcus* en *Bacteroides*. Deze bacteriën worden meestal geïsoleerd uit het maagdarmkanaal van mens en dier; het is daarom opvallend dat vele bacteriën uit drijfmest niet in staat waren te groeien bij 37°C.

Door toevoeging van verschillende koolhydraten aan tellingsmedia met suboptimale hoeveelheden mestcentrifugaat bleek dat hemicellulose het aantal kolonies sterk verhoogde. Dit leidde tot de conclusie dat de afbraak van hemicellulose een belangrijk proces is in drijfmest.

Hoofdstuk 4

Varkensdrijfmest bevat een aantal eenvoudige fenolen. Met name *p*-cresol komt in aanzienlijke concentraties voor (in dit onderzoek tot 340 mg/l). Verder zijn fenol en 4-ethylfenol in geringe concentraties steeds aanwezig. Fenol en *p*-cresol ontstaan door anaërobe microbiële afbraak van tyrosine dat vrijkomt bij de afbraak van eiwit. In drijfmest voorkomend fenol en *p*-cresol worden gevormd zowel door de darmflora in het dier als tijdens anaërobe opslag van de drijfmest. De in de darm gevormde fenolen worden gedeeltelijk door het dier opgenomen en gebonden aan glucuronzuur. Deze fenylglucuronides worden daarna in de urine uitgescheiden. Fenolen, en ook andere verbindingen die geconjugeerd met glucuronzuur worden uitgescheiden, kunnen weer vrijkomen uit hun glucuronides door de werking van het enzym β-glucuronidase. In faeces van varkens en ook in varkensdrijfmest werden hoge activiteiten van dit enzym aangetroffen. De fenolen die gebonden aan glucuronzuur in de urine werden uitgescheiden kwamen bijna onmiddellijk vrij bij contact met de mest, terwijl de vorming van fenolen uit eiwit een langzamer proces is dat sterk door de temperatuur wordt beïnvloed.

De gehaltes aan indol en skatol in varkensdrijfmest waren aanzienlijk lager dan die aan *p*-cresol. Indol werd aangetroffen in hoeveelheden van 0-15 mg/l en skatol van 40-50 mg/l. Ook indol en skatol ontstaan in de darm ten gevolge van eiwitafbraak, maar in dit geval uit het aminozuur tryptofaan. Ze worden in de urine uitgescheiden in de vorm van indolyl-3-carbonzuur en diverse hydroxyskatolen. Na menging van de urine met faeces kan uit indolyl-3-carbonzuur

indol ontstaan. Verbindingen die dienst doen als snelle precursor van skatol werden in dit onderzoek in de urine niet aangetroffen.

Bij anaërobe opslag van drijfmest werd gevonden dat bij de eiwitafbraak indol en skatol ontstaan. Bij langdurige bewaring kan het gehalte aan indol en skatol weer afnemen.

Hoofdstuk 5

De afbraak van tyrosine in drijfmest verschilde op diverse punten van die in faeces van varkens. In faeces werd tyrosine omgezet in ongeveer gelijke hoeveelheden *p*-cresol en fenylpropionzuur. *p*-Cresol onstaat via (hydroxyfenyl)-pyrodruivenzuur en (hydroxyfenyl)azijnzuur, dat gedecarboxyleerd wordt tot *p*-cresol. Bij de vorming van fenylpropionzuur traden uitgaande van tyrosine de volgende tussenprodukten op: (hydroxyfenyl)pyrodruivenzuur, (hydroxyfenyl)melk-zuur, *p*-coumaarzuur en (hydroxyfenyl)propionzuur.

In drijfmest kwamen dezelfde afbraakroutes voor maar hier werd slechts ongeveer 1% van de tyrosine omgezet in fenylpropionzuur, terwijl ongeveer gelijke hoeveelheden *p*-cresol en fenol werden gevormd. Dit laatste product ontstaat door de werking van het enzym tyrosine-fenol-lyase. Een verklaring voor dit verschil tussen drijfmest en faeces kan gezocht worden in de verdere verteringsgraadvan drijfmest t.o.v. faeces, waardoor tyrosine in faeces vooral meer als H-acceptor fungeert (vorming van fenylpropionzuur) en bij uitputting van gemakkelijk afbreekbare koolhydraten (hemicellulose), zoals in drijfmest, als energiebron en koolstofbron dienst doet (vorming van fenol). Ook t.a.v. de afbraak van *p*-coumaarzuur werd een frappant verschil geconstateerd tussen faeces en drijfmest. In faeces werd deze verbinding gedecarboxyleerd tot 4-hydroxystyreen en het product gereduceerd tot 4-ethylfenol; deze reactie vond niet plaats in drijfmest.

Voor de in dit hoofdstuk beschreven experimenten werd bij de afbraak van tyrosine gebruik gemaakt van met ^{14}C gemerkte tussenprodukten. Een aantal van deze gemerkte verbindingen werd geïsoleerd uit incubatiemedia met ^{14}C-tyrosine van een tweetal *Clostridium* spp. Het tyrosine catabolisme van deze *Clostridium* stammen is beschreven in hoofdstuk 6.

Hoofdstuk 6

Cl. sporogenes NCIB 10696 hoopte als afbraakprodukten van tyrosine de volgende verbindingen op in de cultuurvloeistof: (hydroxyfenyl)azijnzuur, (hydroxyfenyl)melkzuur, (hydroxyfenyl)propionzuur en tyrosol. *p*-Coumaarzuur

bleek een tussenprodukt bij de afbraak van tyrosine te zijn waarschijnlijk als intermediair bij de omzetting van (hydroxyfenyl)melkzuur naar (hydroxyfenyl)-propionzuur. *p*-Coumaarzuur werd in kleine hoeveelheden geaccumuleerd wanneer gewassen cellen werden geïncubeerd met tyrosine en α-ketoglutaarzuur.

Cl. ghoni, een uit varkensfaeces geïsoleerde bacterie, vormde grotendeels dezelfde produkten uit tyrosine als *Cl. sporogenes*, maar (hydroxyfenyl)propion-zuur werd niet geproduceerd. Deze bacterie bezit het vermogen om p-coumaarzuur te decarboxyleren en vervolgens het produkt, 4-hydroxystyreen, te hydrogeneren tot 4-ethylfenol. De combinatie van deze twee *Clostridium*-stammen geeft, theoretisch althans, een mogelijke route voor de vorming van 4-ethylfenol uit tyrosine.

Hoofdstuk 7

In de Discussie wordt ingegaan op diverse aspecten van de microbiële omzet-tingen die plaatsvinden in drijfmest. De ophoping van vluchtige produkten wordt toegeschreven aan het ontbreken van evenwicht tussen de zure gisting en de methaangisting, die in drijfmest vrijwel afwezig is. Waarschijnlijk wordt de methaangisting in drijfmest geremd door factoren zoals lage temperaturen, hoge concentraties van ammonia, zwavelwaterstof en diverse remmende kationen. De invloed van deze factoren werd niet nader bestudeerd. Gesteld wordt dat de ophoping van produkten van de zure gisting (zoals vetzuren en fenolen) de oorzaak is van de stank van varkensdrijfmest.

Verder wordt erop gewezen dat de concentraties van *p*-cresol en van vluchtige vetzuren een goede indicatie kunnen zijn betreffende het verloop van het stank-niveau bij het ontstaan en bestrijden van stank in drijfmest.

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