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Age Effect on In vitro Fermentation Pattern and Methane Production in the Caeca of Chickens

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Summary

Age-dependent changes of the caecal fermentation pattern were studied in female chickens using *in vitro* batch incubation technique. Chickens were sequentially killed at the age of 1, 2, 3 and 4 months, their caecal contents added to a broth with starch and incubated at 39 °C for 20 h. Net productions of short-chain fatty acids (SCFA), succinate, ethanol, lactate, methane, hydrogen and ammonia were determined. Methanogenesis was absent in caeca of 1-month-old chickens. Production of methane started in the second month and doubled in the third month of age. The start of methanogenesis was accompanied by changes of the fermentation stoichiometry. The production of succinate ceased and that of ethanol decreased to less than one tenth. There were no major changes of the caecal fermentation pattern in the fourth month of age. The ammonia production increased in the second month, indicating increased deamination activity. No major shifts in SCFA molar composition dependent on age were found. Calculated hydrogen recoveries suggest a decrease of reductive acetogenesis until 3 months of age. It can thus be concluded that age and the onset of methane production affect the fermentation pattern in the caeca of chickens.

Key words
Chicken – Caeca – Fermentation – Ontogenesis

Introduction

Microorganisms in anaerobic parts of the animal digestive tract ferment a range of substrates which would not be otherwise available for the host. Short-chain fatty acids (SCFA), the main fermentation end-product, are absorbed into the blood and utilized as energy and carbon sources in animal tissues. The process of fermentation inevitably produces metabolic hydrogen, which must be disposed of in order to reoxidate coezymes reduced during glycolysis. The regeneration of reduced coenzymes can only proceed at low H_2 partial pressure. This can be obtained by an association between H_2-producing and H_2-utilizing microorganisms. Animal species differ in partitioning of metabolic hydrogen among major hydrogen-utilizing reactions and in postnatal development of hydrogenotrophic microflora (methanogenic archaea, sulphate-reducing and acetogenic bacteria). Several authors demonstrated a competition for H_2 between methanogenic archaea and hydrogenotrophic acetogenic bacteria in the human and porcine large intestine (Bernalier *et al.* 1993, De Graeve *et al.* 1994, Doré *et al.* 1995). In energetic terms, competition for H_2 should favour methanogens rather than acetogens ($\Delta G_o' = -131$ and -95 kJ.mol^{-1}, respectively).

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Indeed, the hydrogenotrophic reductive acetogenesis in the human colon appears to be quantitatively important only in the presence of very low numbers of methanogens (Bernalier *et al.* 1996). In the rumen, the major part of H_2 is used for production of methane (Demeyer *et al.* 1989). The production of acetate from CO_2 and H_2 is relatively unimportant, except for a short period after birth. When methanogens appeared in the rumen of newborn lambs, there was a proportional decrease in the numbers of acetogenic bacteria, indicating a competition for hydrogen between these two groups (Morvan *et al.* 1994). In the caecum of young rabbits, the principal hydrogen-utilizing reaction is the reductive acetogenesis, to be taken over later by methanogenesis (Piattoni *et al.* 1996). The reduction of sulphate to sulphide is the most important H_2-disposal system in the pig caecum (Ushida *et al.* 1995). In poultry, the primary site of microbial fermentation are the caeca. It has been suggested that the caeca are a site for digestion of fibre, absorption of water, SCFA and amino acids synthesized by microorganisms, and the breakdown of uric acid (Barnes 1972). The caecal metabolism of hydrogen is not well understood, in spite of the fact, that the production of methane in poultry caeca was detected many years ago (Shrimpton 1966). We found in a preliminary experiment that the methanogenesis was absent in one-month-old chickens, but present in one-year-old hens. The aim of this study was to specify the time of the methanogens appearance and related changes of the fermentation stoichiometry in caeca of chickens 1–4 months old. A simple *in vitro* batch incubation technique suitable for studies of hindgut metabolism was used.

Material and Methods

Fourteen female chickens (Dominant D-102 breed) were obtained from a local hen producer at the age of 3 weeks. Chickens were kept together in a pen with wooden shavings and fed commercial concentrates without antimicrobials *ad libitum*. Standard feed mixtures for rearing of pullets were used. The first feed mixture (0–9th week of age) contained (%, w/w): wheat 52, maize 20, soya-bean meal 20, meat and bone meal 4 and a vitamin-mineral supplement 4. The second feed mixture (10–18th week of age) contained (%, w/w): wheat 78, maize 10, soya-bean meal 5, meat and bone meal 3 and a vitamin-mineral supplement 4. Three chickens were killed by cervical dislocation at the age of 1 and 2 months. Four chickens were killed at the age of 3 and 4 months. Chicken caeca were emptied by gentle squeezing and caecal contents added to sterile VL broth (Barnes and Impey 1971) with starch as the substrate. The VL broth contained (g/l): trypton 10, yeast extract 5, meat extract 2, $NaHCO_3$ 10, NaCl 5, starch 6, cysteine. HCl 0.5. One gram of the caecal contents was added to 50 ml of the broth (pH = 7.0) in serum bottles. Bottles were flushed with CO_2, hermetically closed by rubber stoppers (headspace volume of 75 ml) and incubated at 39 °C for 20 h. Starch was chosen as the substrate with respect to its presence (0.93–3.78 % of dry matter) in the caecal digesta (Marounek *et al.* 1997). Samples of the headspace gas were taken by means of a gas-tight syringe and analysed by gas chromatography, as described previously (Marounek *et al.* 1996). Short-chain fatty acids were estimated by gas-liquid chromatography on a column of Chromosorb WAW with 15 % SP 1220 and 1 % H_3PO_4 (Supelco, Bellefonte, USA). Lactate and succinate were esterified with methanol and estimated on a column of Supelcoport with 3 % SP 2340. Formate was determined colorimetrically (Sleat and Mah 1984).

All findings were corrected for end-products present in the non-incubated blanks. Metabolic hydrogen recovery was calculated according to Demeyer and Van Nevel (1975) except that productions of succinate and ethanol were also incorporated into the calculations. The former compound is the propionate precursor (Barnett and Reid 1961), the latter one represents the redox equivalent of lactate

$$2H_{released} = 2A + P + S + 4B + 3V + E$$

$$2H_{accepted} = 2P + 2S + 2B + 4V + E + 4M$$

$$2H_{recovery} = (2H_{accepted} / 2H_{released}) . 100$$

where A, P, B, V, S, E and M represent molar productions of acetate, propionate, butyrate, valerate, succinate, ethanol and methane, respectively. Such calculations test the hypothesis that the amount of metabolic hydrogen liberated in pyruvate and acetate formation equals that deposited in production of reduced metabolites.

Data were analysed statistically by one-way analysis of variance (ANOVA) with a model including the main effect of age (GraphPAD Software, version 1.14, Birthe Avery, Md). The Bonferroni test was used for means comparison, where appropriate.

Results

Caecal microorganisms converted substrate to SCFA, ethanol, succinate and methane (Table 1). Small amounts of hydrogen (<5 μmol/flask) and lactate (<20 μmol/flask) were produced. No formate was detected in culture fluid, the detection limit of the method, however was about 0.5 mM, i.e. 25 μmol/flask. Methanogenesis was absent in caeca of one-month-old chickens. Production of methane started in the caecal contents of two-month-old chickens. The start of

methanogenesis was accompanied by changes of fermentation stoichiometry: succinate production ceased and the average production of ethanol decreased from 227 to 22 μmol/flask. The methane production increased twofold in the third month of age. There were no major changes of the fermentation pattern of caecal microbes in the fourth month of age. Ammonia production increased slightly in the second month of age, indicating an increase of the deaminative activity. Hydrogen recovery varied from 59.3±5.4 to 75.6±4.3 %.

Table 1. Production of short-chain fatty acids, succinate, ethanol, methane and ammonia in cultures of the caecal contents of chickens of different age

Metabolite (μmol/flask)	Age (months) 1	2	3	4
Acetate	3705±372	3083±938	2674±331	3354±208
Propionate	2398±444	1801±179	1920±346	2035±106
Butyrate	1188±225	1163±259	1187±129	1332±130
Valerate	302±129	370±95	303±35	368±15
Succinate	497±163^{a}	0^{b}	0^{b}	0^{b}
Ethanol	227±49^{a}	22±22^{b}	2±2^{b}	15±10^{b}
Methane	0^{a}	290±250^{ab}	585±111^{b}	675±45^{b}
Ammonia	1787±159^{a}	2851±561^{ab}	2812±122^{ab}	3083±341^{b}
2H recovery (%)	59.3±5.4^{a}	62.3±3.5^{a}	75.6±4.3^{b}	71.9±1.7^{a}

Mean values±S.D. Three chickens were sacrificed at the age of 1 and 2 months. Four chickens were sacrificed at the age of 3 and 4 months. Caecal contents were added to a broth with starch and incubated at 39 °C for 20 h. ^{a,b} *Means within rows with different superscripts differ (P<0.05)*

Discussion

The digestive tract of newborn animals is colonized sequentially by various groups of microorganisms. Studies on the development of microflora in the digestive tract revealed both differences and similarities between various animal species. In the first period of life, the strictly anaerobic bacteria in fermentive sections of the digestive tract are accompanied by a range of facultatively anaerobic bacteria, together with some aerobic bacteria (Huhtanen and Pensack 1965, Gouet and Fonty 1979, Minato *et al.* 1992). In adult animals, microflora is characterized by a dominance of strictly anaerobic species. In the caeca of chickens, coliform bacteria and streptococci were the first organisms found in high numbers, followed by clostridia and lactobacilli. The youngest chickens, from which bacteroides were isolated, were 6-day-old (Smith 1965). Contrary to common bacterial species, information about postnatal development of hydrogenotrophic microflora is limited. Probably, the best studied digestive ecosystem in this respect is the rumen. Morvan *et al.* (1994) reported the numbers of methanogens, sulphate-reducers and acetogens in the rumen of lambs 20 h – 15 days old. Methanogens appeared in the rumen of 30 h old lambs, and as they developed, there was a proportional decrease in the numbers of acetogens. In the caecum of young rabbits, up to the age of 36 days, almost no methane was detected, although considerable amounts of SCFA were formed (Piattoni *et al.* 1996). These authors concluded, on the basis of hydrogen recovery values, that the reductive acetogenesis, which was a major characteristics of caecal fermentation in suckling rabbits, was replaced gradually and partially by methanogenesis with the increasing age. In our experiment, the caecal methane production started in the second month of life of chickens and increased with age with a concomitant increase of the hydrogen recovery. Average hydrogen recovery values (59.3–75.6) suggest the presence of the hydrogenotrophic acetogenesis. In methanogenic cultures of the rumen contents such recoveries are seldom lower than 80% (Demeyer *et al.* 1989). Other age-dependent changes included the disappearance of succinate and a decrease of ethanol production. In chickens *in vivo*, the caecal succinate concentration decreased from 33 to 6–8 mmol/g between the first and the fourth week of age (Barnes *et al.* 1979). Similarly, succinate was found in the rumens of 1-

month-old calves, but not in older ones (Barnett and Reid 1961). No clearly defined pattern of SCFA formation was observed in our study, probably because more SCFA were formed heterotrophically (*via* pyruvate) than from CO_2 and H_2. In conclusion, this study shows that in chickens, as in mammalian species, caecal fermentation is affected by age and that the appearance of methanogenesis affects fermentation stoichiometry.

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