3 Material and Methods

Each experiment was approved by the Ethical Committee of the Ministry for Nutrition, Agriculture, Forestry and Fishery Mecklenburg-Vorpommern / Germany.

3.1 Slaughter trial

3.1.1 Animals and feeding

To characterize the gut content of piglets around weaning a slaughter trial was conducted. The trial was conducted under constant conditions (26-28 °C ambient temperature, natural light system) in the institute's experimental station with purebred German Landrace piglets of both genders. Piglets were raised conventionally from the sow (farrowing crate) with sow milk (SM) as sole diet and no access to creep feed. At 28 days of age pigs were weaned, weighed and randomly allotted to group pens, each containing 8 animals. In four consecutive periods four different starter diets (fine-ground) were offered *ad libitum*: 2 reference diets (Table 7), one supplemented with the in-feed antibiotic avilamycin (+AB), one without in-feed antibiotic (-AB) and 2 home-produced diets (Table 8) with 3% crude fibre content (LF) and 8% crude fibre content (HF), respectively. Diets were formulated to meet requirements of weaning piglets as recommended by the NRC (1998) and were approximately isoproteinogenic. The two reference diets were manufactured and delivered to our institute by Dr. Bernard Sève, INRA, Centre de Recherches de Rennes, Unité Mixte de Recherches sur le Veau et le Porc (UMRVP), 35590 St. Gilles, France.
Representative samples were taken from each of the 4 starter diets and analysed in the laboratory. Nitrogen content was determined applying the DUMAS combustion method, using a LECO CNS-2000 (Leco Instrumente GmbH, Möchengladbach, Germany, UST-ID: no. DE 129401155). Dry matter (DM), crude fibre (CF) and ash were determined by Weender standard procedure (Naumann & Basler, 1993). Furthermore amino acid content was measured by means of ion-exchange column chromatography in basic, acid and oxidised hydrolysate on a Biochrom 20 Plus Amino Acid Analyser (Biochrom Ltd., Cambridge, UK). D-alanine was determined according to Garret et al. (1987). The chemical composition is given in Table 9. Detailed information about the techniques used for the determination of amino acids and D-alanine is given in point 3.1.3.1.

Table 7. Physical composition of reference diets

Ingredients (%)	+AB[1]	-AB[2]
Wheat seeds, ground	24.66	24.66
Barley seeds, ground	24.70	24.70
Soybean meal	16.00	16.00
Maltodextrin	4.20	4.00
Whey, dehydrated	15.00	15.00
Soluble fish protein concentrate	8.25	8.25
Sunflower oil	2.80	2.80
Calcium Carbonate	1.52	1.52
Monocalcium Phosphate, 2 H_2O	2.00	2.00
Trace elements & Vitamin Premix	0.50	0.50
L-Lysine HCl	0.13	0.13
L-Threonine	0.13	0.13
DL-Methionine	0.09	0.09
L-Tryptophan	0.03	0.03
Antibiotic Premix [4]	0.20	0.00

[1] +AB: reference diet supplemented with avilamycin

[2] -AB: reference diet non-supplemented

[3] Trace Elements & Vitamine Premix; content per kg: 25.70 g zinc oxide; 16.00 g copper sulphate; 11.70 g manganese oxide; 50.00 g iron carbonate; 0.32 calcium iodate; 1.905 g cobalt sulphate; 6.00 g sodium selenite premix (1% Se); 2.4 Mio IU vitamine A; 600.000/120.000 IU vitamine A/D_3; 480.000 IU vitamine D_3; 8000 IU vitamine E; 1.76 g vitamine K_3 (menadione); 0.40 g vitamine B_1; 2.50 g vitamine B_2; 6.00 g niacin; 3.00 g pantothenate; 2.00 g vitamine B_6; 2.00 g biotine; 0.40 g folic acid; 10.00 g vitamine B_{12}; 20.00 g vitamine C; 266.67 g choline chloride

[4] Antibiotic Premix; content per kg: 200.00 g avilamycin; 800 g excipient (calcium carbonate)

diets manufactured by Dr. Bernard Sève, INRA, Centre de Recherches de Rennes, Unité Mixte de Recherches sur le Veau et le Porc (UMRVP), 35590 St. Gilles, France

Table 8. Physical composition of home-produced diets

Ingredients (%)	LF[1]	HF[2]
Triticale seeds, ground	24.00	17.00
Barley seeds, ground	31.00	18.00
Wheat seeds, ground	24.00	12.00
Sweet Lupine (Lupinus angustifolius L.)	1.00	20.00
Horse Bean (Vicia faba L.)	1.14	4.50
Peas (Pisum sativum L.)	4.00	0.00
Wheat gluten meal	5.00	2.00
Wheat bran	2.20	18.52
Fish meal	4.00	3.00
Soya oil	0.50	1.50
Trace elements & Vitamin Premix [3]	2.50	3.00
L-Lysine HCI	0.50	0.36
I-Threonine	0.15	0.09
L-Tryptophan	0.01	0.00
DL-Methionine	0.00	0.03

[1] LF: home-produced diet, low fibre (3% crude fibre)

[2] HF: home-produced diet, high fibre (8% crude fibre)

[3] Trace Elements & Vitamin Premix; content per kg: 1.250 mg niacin; 3.000 µg biotin; 12.5 mg folic acid; 625 mg pantothenate; 5.000 mg choline; 5.000 mg iron; 4.000 mg copper; 5.000 mg zinc; 3.000 mg manganese; 20 mg cobalt; 30 mg iodine; 10 mg selenium; 500.000 IU vitamine A; 50.000 IU vitamine D_3; 1.500 mg vitamine E; 75 mg vitamine K_3 (menadione); 100 mg vitamine B_1; 250 mg vitamine B_2; 150 mg vitamine B_6; 1000 µg vitamine B_{12}; 23.74 % calcium; 6.5 % phosphate; 6.48 % IVP; 5 % sodium; 1 % magnesium

Table 9. Chemical composition of starter diets

Parameter	+AB	-AB	LF	HF
Dry matter (%)	89.80	90.13	88.95	88.82
Nitrogen (% DM)	3.78	3.79	3.41	3.75
Crude protein (% DM)	23.75	24.06	21.32	23.47
Ash (% DM)	8.62	8.36	4.58	5.95
Fibre (% DM)	3.13	3.05	4.11	7.59
Fat (% DM)	4.92	5.18	3.22	4.81
Total amino acids (g/16g N)	93.28	88.64	95.20	88.57
Asparagine (ASP)	8.45	7.87	5.76	7.14
* Threonine (THR)	4.28	3.93	3.87	3.50
Serine (SER)	4.50	4.41	4.33	4.24
Glutamine (GLU)	17.74	16.26	24.77	20.62
Glycine (GLY)	5.62	5.10	4.09	4.06
Alanine (ALA)	4.74	4.31	3.76	3.57
* Valine (VAL)	4.54	3.91	4.55	4.18
* Isoleucine (ILE)	4.04	3.37	3.63	3.52
* Leucine (LEU)	6.92	6.30	6.77	6.36
Tyrosine (TYR)	2.55	2.42	2.03	2.59
* Phenylalanine (PHE)	4.45	3.88	4.66	4.01
* Histidine (HIS)	2.34	2.17	2.58	2.66
* Lysine (LYS)	6.19	5.72	5.51	5.23
* Arginine (ARG)	5.58	4.97	4.56	6.18
Proline (PRO)	6.09	5.69	9.52	6.66
Cysteine (CYS)	1.34	1.49	2.10	1.77
* Methione (MET)	1.98	1.92	1.58	1.41
* Tryptophane (TRP)	0.95	1.11	1.12	0.89
D-alanine (g/16g N)	0.28	0.27	0.26	0.16
Putrescine (mg/100 g DM)	1.63	1.75	1.15	1.15
Histamine (mg/100 g DM)	0.00	0.00	0.00	0.00
Cadaverine (mg/100 g DM)	2.62	2.67	0.78	0.41
Spermidine (mg/100 g DM)	2.42	2.48	2.48	3.27
Spermine (mg/100 g DM)	1.09	1.09	1.28	1.28

* essential amino acids

3.1.2 Experimental Design

A slaughter trial was conducted with defined time points before and during weaning transition. Three time points before weaning, weaning day itself and five time points after weaning were defined as slaughter days. At each of these days 8 animals per dietary treatment were slaughtered in 2 cycles (Table 10).

Table 10. Animals and dietary treatment

Slaughter days	-6	-4	-2	0	1	2	5	8	15
Age (d)	22	24	26	28	29	30	33	36	43
Diet	SM[1]				+AB[2] / -AB[3] / LF[4] / HF[5]				
Animal number	16 per slaughter day				16 per slaughter day & diet				
In total: 384 piglets									

[1] SM: sow milk, no creep feed
[2] +AB: reference diet supplemented with avilamycin
[3] -AB: reference diet non-supplemented
[4] LF: home-produced diet, low fibre (3% crude fibre)
[5] HF: home-produced diet, high fibre (8% crude fibre)

At slaughter day animals were denied access to feed two hours prior to slaughtering. Piglets were weighed, euthanised by intracardial injection of 2 mL T61® (Intervet Germany) per animal. The abdominal cavity was opened by a midline incision and the entire GIT removed. Stomach, small intestine and large intestine were closed by surgical clamps, separated from each other and laid out on a stainless steel table. The small intestine was subdivided into three equal sections, which were defined as duodenum, jejunum and ileum (from proximal to distal). Sections were clamped and separated from each other. Entire contents of ileum were collected and pooled for 4 animals, i.e. per slaughter day and diet 4 samples were collected in total, representing 16 animals. Immediately after collection samples were transferred to the laboratory for analysis.

3.1.3 Laboratory analysis

3.1.3.1 Chemical parameters

Samples were homogenised and divided to carry out a range of laboratory analysis. The pH of each sample was measured directly by means of a glass electrode. DM and ash were determined by Weender standard procedure (Naumann & Basler, 1993) and nitrogen content by the DUMAS method on a LECO CNS-2000 (Leco Instrumente GmbH, Möchengladbach, Germany, UST-ID: no. DE 129401155). Ammonia was measured by use of microdiffusion method (Voigt & Steger, 1967) and LA by colorimetric procedure (Haacker et al., 1983).
Concentrations of VFA were determined by gaschromatography with *i*-caproic acid as internal standard (Geissler et al., 1976), using a SHIMADZU GC-17A with a FFAP 25m x 0.25 mm i.d. column (Machery-Nagel GmbH & Ko.KG, Düren, Germany). Total VFA and following individual fatty acids were measured: acetic acid, propionic acid, i-butyric acid, n-butyric acid, i-valeric acid, n-valeric acid and caproic acid.
Amino acids were determined according to the specifications of Kreienbring & Wünsche (1974) and Kreienbring (1987) by means of ion-exchange column chromatography (200 x 4.6 mm column) in basic, acid and oxidised hydrolysate on a Biochrom 20 Plus Amino Acid Analyser (Biochrom Ltd., Cambridge, UK).
For the determination of biogenic amines samples were prepared according to the specifications of § 35 LMBG (Amtliche Sammlung für Lebensmitteluntersuchungen) and measured on a Biochrom 20 Plus Amino Acid Analyser (Biochrom Ltd., Cambridge, UK), using a 50 x 4.6 mm column.

For information about microbial mass D-alanine was determined according to the specifications of Garret et al. (1987). Slight modifications were made for the hydrolysing step. Digesta samples were hydrolysed in 6 N HCl at 110 °C for 22 h under CO_2, using screw-cap culture tubes. Required HCl amount was calculated on the basis of nitrogen content in the sample, i.e. 1 mg N equalled 1 mL 6 N HCl. After adding 0.2 g activated charcoal to each tube, hydrolysates were filtered, evaporated to dryness, and diluted twice with 15 mL hot distilled water and evaporated. Residues were made up to a final volume with 10 mL n/10 HCl. Subsequent analytical steps were identical with the method of Garrett et al. (1987).

3.1.3.2 Classical Microbiology

Fresh digesta samples were homogenised, serially diluted and plated on selective agar plates (SIFIN®, Berlin, Germany) to cultivate *Enterobacteriacea,e Enterococcus spp.* and *Lactobacillus spp.* at 37 °C and yeast at 30 °C. *Enterobacteriaceae* were cultivated on VRBD (Crystal-Violet Neutral-Red Bile Glucose agar acc. to MOSSEL) agar for 24 h, *Enterococcus spp.* on SB (Enterococcus agar acc. to SLANETZ-BARTLEY) agar for 72 h and yeast on SGA (Sabouraud-Glucose-Agar) for 7 days under aerobic conditions. *Lactobacillus spp.* was grown on MRS agar (Lactobacillus agar acc. to De Man, ROGOSA and SHARPE) under anaerobic conditions for 72 h. To create an anaerobic environment agar plates were enclosed in an anaerobic jar with Anaerocult® A (Merck®, Germany) and Anaerotest® test-strip (Merck®, Germany). Colony-forming units (cfu) were counted manually and numbers given as log cfu/g.

3.1.3.3 Molecular Microbiology

For extended investigations different techniques of molecular microbiology were applied: DGGE to obtain a genetic fingerprint of the microbial community, cloning and 16S rDNA-sequence analysis for identification and for quantification FISH.
These analyses were performed in collaboration with the group of Prof. A.D.L. Akkermans/Dr H. Smidt at the Laboratory of Microbiology, Molecular Ecology Group, Hesselink van Suchtelenweg 4, 6703 CT Wageningen /The Netherlands.

3.1.3.3.1 Sample Preparation

Two batches of each digesta sample were prepared for DNA-isolation and FISH. For DNA isolation 0.5 g fresh sample material were mixed in a beaker with 8.33 mL ice-cold K-phosphate-buffer pH 7.01 (CertiPur®, Merck, Germany). Beakers were placed in a basin containing crushed ice and transferred onto a magnetic stirrer to homogenise samples for 30 min. Afterwards samples were divided into 1 mL aliquots in Eppendorf tubes (1.5 mL size, Merck, Germany) and stored at –20 °C until usage. The second batch was processed in preparation for FISH. In a 10 mL centrifuge tube 0.5 g sample were suspended in 4.5 mL PBS-buffer (1 x concentrated) and homogenised on a horizontal shaker for 3 min (3-5 glass beads included per sample). To remove large particles samples were centrifuged at low speed (700 g for 1 min). Then 1 mL supernatant was removed (1-2 cm below surface), mixed with freshly prepared 4% paraformaldehyde

solution and incubated at 4 °C overnight. Samples were then mixed well, divided into 6 aliquots (2 mL cryo-vials, Merck, Germany) and stored at –75°C until usage.

3.1.3.3.2 DNA Isolation

DNA was isolated from the prepared samples, using the Fast DNA®SpinKit® for soil (Q Bio Gene®, Carlsbad, USA) according to the manufacturer's protocol. To check for DNA presence 5 µl of isolate were mixed with 1 µl loading buffer, loaded on a 1% agarose gel (w/v) containing ethidium bromide and electrophoresed at 135 mV for 15 min. Subsequently gels were checked under UV-light.

3.1.3.3.3 Polymerase Chain Reaction (PCR)

The primers 968GC and L1401r were used to amplify the whole V6 – V8 region of 16S rDNA as this pair is specific for bacterial 16S rDNA and yields PCR products (amplicons) of 470-bp length. PCR was performed using the *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, MD, USA). For one reaction (49 µl total volume) a PCR mastermix was used:

10 x PCR buffer (10 x buffer)	5 µl
$MgCl_2$ (50 mM)	3 µl
dNTP mix (10mM)	1 µl
forward primer (10 µM)	1 µl
reverse primer (10 µM)	1 µl
Taq DNA polymerase (5 U/µl)	0.25 µl
MilliQ	37.75 µl

Finally 1 µl of template DNA was added for each reaction, tubes placed in a thermocycler T1 Whatman Biometra (Göttingen, Germany) and following program run: pre-denaturation 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 56 °C for 20 sec, 68 °C for 40 sec, and 68 °C for 7 min final extension. Size and amounts of amplicons were checked by electrophoresis (135 mV, 15 min) on a 1% agarose gel (w/v) containing ethidium bromide. To investigate the Lactobacillus-specific community of the porcine GIT the approach of a nested PCR was chosen. For the initial amplification the primers +8f and Lab-677 were applied using the following cycling conditions: pre-denaturation at 94°C for 5 min; 35

cycles at 94°C for 30 sec, 66°C for 20 sec, 68°C for 40 sec and the final extension at 68°C for 7 min. PCR-products were subsequently used as templates in nested PCR-reactions, applying the primers Lab-159f and Lab-515GC. The PCR-programme was identical with the one used for amplification of the V6-V8 region of 16S rDNA (mentioned above). All primers applied in this study are given in Table 11.

Table 11. List of oligonucleotide primers used in this study

Primer name	Primer sequence (5'–3')	Reference
S-D-Bact-0968-a-S-GC	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC	Konstantinov, 2002
S-D-Bact-1401-a-A-17	CGG TGT GTA CAA GAC CC	Konstantinov, 2002
S-D-Bact-0008-a-S-20	AGA GTT TGA TCM TGG CTC AG	Leser, 2001
S-G-Lab-0677-a-A-17	CAC CGC TAC ACA TGG AG	Heilig, 2002
S-G-Lab-0159-a-S-20	GGA AAC AG(A/G) TGC TAA TAC CG	Heilig, 2002
S-*-Univ-0515-a-A-24	ATC GTA TTA CCG CGGCTG CTG GCA	Heilig, 2002
* GC-clamp	CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G	Heilig, 2002
S-D-Bact-1492-a-A-19	GGT TAC CTT GTT ACG ACT T	Leser, 2001
S-Univ-1100-a-A-15 with IRD800	GGG TTG CGC TCG TTG	Konstantinov, 2002
T7	TAA TAC GAC TCA CTA TAG G	Heilig, 2002
SP6	GAT TTA GGT GAC ACT ATA G	Heilig, 2002

3.1.3.3.4 Denaturing Gradient Gel Electrophoresis (DGGE)

To separate PCR-amplicons DGGE was applied according to the guidelines of Muyzer *et al.* (1993). A Dcode TM system (Bio-Rad Laboratories, Hercules, CA, USA) was used and electrophoresis performed in 8% polyacrylamide gel 37.5:1 acrylamide-bisacrylamide using a 30-60% denaturing gradient. Gels were electrophoresed for 10 min at 200 V and subsequently for 16 h at 85 V in 0.5 x TAE buffer at a constant temperature of 60 °C. Gels were silver-stained according to Sanguinetti *et al.* (1994) and for preservation covered with cellophane and dried at 60 °C overnight.

Analysis of the DGGE gel

The gel was scanned at 400 dpi and analysed with the software Bionumerics (Applied Maths Europe, Sint-Martens-Latem, Belgium). At first bands were assessed per lane by the programme's bands searching algorithm and checked manually. DGGE fragments constituting less than 1% of the total band area were omitted from further analysis. The intensity of bands was judged as peak heights in densitometric curves and dendograms were generated from the assessed bands on the gel tracks. Based on these bands and its peak heights the Shannon-index of general diversity H' was calculated as a parameter for structural diversity of the community, using equation 1:

Equation 1.

$$H' = - \sum P_i \log P_i$$

P_i – importance probability of the bands in a lane

P_i was calculated as $P_i = n_i / n$, where n_i is the height of an individual band and n the sum of all band heights in the densitometric curve. H' and the number of bands were statistically analysed with the GLM procedure of SAS system, version 8.

3.1.3.3.5 Cloning

To identify the bands on the DGGE gels, a cloning experiment with subsequent sequence analysis was accomplished. A PCR mastermix was made up, using primers +8f and 1492n, and amplification was performed under same conditions as described earlier. Amplicons were purified with QIAquick PCR Purification kit (Westburg, Leusden, The Netherlands) and cloned into a pGEM-T vector system (Promega, Madison, WI, USA). Ligation was done at 4 °C overnight followed by transformation into competent *E.coli* JM109 on LB agar plates with ampicillin added to a concentration of 100 µg/mL. Colonies of ampicillin-resistant transforms, appearing white on plates, were transferred to eppendorf tubes containing 20 µl TE buffer, boiled at 95 °C for 10 min and subsequently spun down at 13.000 rpm for 2 min. To check the size of the inserts, PCR was performed immediately with pGEM-T-specific primers T7 and SP6 (table 11) and the cell lysate as a template. Plasmids containing an insert of approximately 1.6 kb were further used for a restriction enzyme reaction. A 4-cutter enzyme (MSP1) was used and the restriction enzyme reaction performed according to following protocol: Selected plasmids were

purified with QIAquick PCR Purification kit (Westburg, Leusden, The Netherlands) and 10 µl of it added to 7 µl MilliQ, 1 µl MSP1 and 2 µl of 10 x MSP1-buffer. Tubes were shortly centrifuged at quick run and placed into an incubator at 37 °C for 1.5 h. After incubation 20 µl of each sample were checked by electrophoresis on a 2% agarose gel (w/v). Those differing in their pattern were used to amplify V6-V8 regions of 16S rDNA (universal PCR) and loaded on a DGGE gel for comparison with bands of digesta samples in question. Clones matching bands of samples were picked for sequence analysis and purified with QIAquick PCR Purification kit (Westburg, Leusden, The Netherlands) according to manufacturer's manual.

3.1.3.3.6 Sequence Analysis

For sequence analysis Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, UK) was used according to the manufacturer's specifications. 5 µl of purified clones were added to 6 µl MilliQ and 2 µl 1100r sequence primer (table 5), the 5'-end labelled with infra-red IRD-800. In each of four microtubes (labelled with A, C, G, T) 3 µl of this mix were added and a PCR cycle run with following criteria: 93 °C for 3 min, 30 cycles of 93 °C for 30 sec, 44 °C for 30 sec, 70 °C for 15 sec, and 4 °C to hold. 1.8 µl loading buffer were added to stop the reaction and tubes were stored at –20 °C until analysis. Sequences were automatically analysed on a LI-COR DNA Sequencer 4000L (LiCor, Lincoln, NE, USA) and corrected manually. They were compared to sequences available in public databases by using BLAST analysis of sequences from the Ribosomal Database Project.

3.1.3.3.7 Fluorescence *in situ* Hybridisation (FISH)

For FISH we applied the oligonucleotide probes EUB-0338, EC 1531, LAB-0158 and LAB-0722 (Table 12). Gelatine-coated slides (each with 8 wells), prepared according to the specifications of Wildeboer-Veloo were used. Prepared samples (present in a 40 x dilution) were already used directly for FISH. To apply EUB-0338 following hybridisation protocol was used: 10 µl of fixed sample were spotted on wells of the gelatine-coated slide and dried in a hybridisation oven at 50°C for 2min. After drying specimen were dehydrated in an increasing ethanol series (3 min each in 50, 80 and 96% (v/v) ethanol) and slides dried with compressed air. The oligonucleotide probe was thawed and 8.5 µl mixed with 100 µl hybridisation buffer (18 µl 5M NaCl, 2 µl 1M Tris/HCl pH 8.0, 80 µl MilliQ). 11 µl of this mix was placed on each well (containing sample) and spread without scratching the slide surface (caution: scratches are visible under fluorescence microscope). Immediately

slides were transferred to a hybridisation box (moisture chamber) and incubated in a hybridisation oven (50°C) for 3 h. Subsequently slides were rinsed for 8 to 10 min in washing buffer (1 mL 1M Tris/HCl pH 8.0, 9 mL 5M NaCl, 40 mL MilliQ), that was preheated to 37°C (water bath) prior to use. 100 µl 0.1% DAPI stain were added to washing buffer and slides incubated for another 2 min at 37°C. Washing buffer was removed with distilled water at 4 °C and slides dried quickly under compressed air. Slides were then embedded with vectashield (1 medium-sized drop per well), a cover slip placed on top and attached with nail polish. Slides prepared according to these specifications could be stored in the dark at –20 °C. The hybridisation protocol for LAB-0158 and LAB-0722 was basically the same, save for a lysis step inserted after spotting samples on the slide and hybridisation time (16 h at 50°C). For this following enzyme solution was prepared: 100mM Tris, 5 mM EDTA and 10 mg/mL lysozyme, adjusted to pH 7.5 with HCl. On each well 10 µl of enzyme solution were added and slides incubated at 50 °C for 10 min. Thereafter we resumed to given protocol. By epifluorescence microscopy slides were inspected and obtained images analysed with Q550FW (Leica Microsystems, The Netherlands). Pictures were taken using a DCC camera connected to an epifluorescence microscope.

Table 12. List of oligonucleotide probes used for FISH in this study

Probe	Sequence (5' – 3')	Target genera / species	Reference
EUB-0338	GCT GCC TCC CGT AGG AGT	Domain bacteria	Amman, 1990
LAB-0158	GGT ATT AGC A(C/T)C TGT TTC CA	*Lactobacillus-Enterococcus* group	Harmsen, 1999
LAB-0722	(C/T)CA CCG CTA CAC ATG (A/G)AG TTC CAC T	*Lactobacillus-Leuconostoc-Pediococcus*	Sghir, 1998
EC 1531	CAC CGT AGT GCC TCG TCA T	*Escherichia coli*	Poulsen, 1995

3.1.4 Statistical Analysis

For each combination of slaughter day and dietary treatment two experimental cycles were conducted, using 2 x 8 animals. The cycles differed with respect to time during the year and with respects to animal's parents. That means for each combination of slaughter day and dietary treatment we had two groups of 8 animals. Therefore the effect of groups was considered as random. Within every group 2 samples (each sample representing 4

animals) of ileal digesta were obtained, i.e. for every characteristic there are only 2 observations per group. For the analysis a mixed model with following equation and factors was applied:

Equation 2.

$y_{ptdgs} = W_p + T_{t(p)} + D_{d(p)} + TD_{td(p)} + \underline{G}_{g(tdp)} + \underline{\varepsilon}_{s(gtdp)}$

y – Characteristic, e.g. lactic acid

W – pre/postweaning

T –time (slaughter day, nested in W)

for W = pre: -6, -4, -2, 0

for W = post: 1, 2, 5, 8, 15

D – diet (nested in W)

for W = pre: D = SM

for W = post: D = +AB, -AB, LF, HF

TD – interaction between D and T within W = post

$\underline{G}$ – group (nested in T and D, random)

$\underline{\varepsilon}$ – random residual effect = effect of the sample within group. The variance of $\underline{\varepsilon}$ (residual variance) were estimated for W = pre and W = post separately.

Analysis was performed using the procedure MIXED of the SAS-System, version 8. For comparison between the effects of pre- and postweaning period the t-Test was applied. Comparison of all postweaning dietary treatments with sow milk (preweaning dietary treatment) was accomplished using the Dunnett-Test. The Dunnett-Test is a multiple test procedure for the simultaneous comparison of the mean of a "standard" treatment with the means of k further treatments, i.e., the following k null hypotheses.

$H_{0,i} : \mu_0 = \mu_i$, $i = 1,...,k$ are to be tested simultaneously.

The aim of this procedure is to control the multiple significance level, i.e. the probability, that at least one out of all true null hypotheses will be rejected and will be less than a given α . We used $\alpha = 0.05$.

If all pair-wise null hypotheses $H_{o,ij} : \mu_i = \mu_j$ $i, j = 1,...,k; i \neq j$

are meant to be tested simultaneously, controlling the corresponding multiple significance level, then the Tukey-test must be applied.

3.2 Balance Trial Postweaning

To investigate the nitrogen flow and endogenous nitrogen losses in weaning piglets fed different starter diets we applied the ^{15}N tracer technique techniques. Animals were surgically fitted with an ileorectal anastomosis (IRA) according to the specifications of Redlich (1997), which we modified to meet our requirements.

3.2.1 Animals and Feeding

Male castrated German Landrace piglets, provided by the institute's experimental swine station, were used in this trial. Before weaning animals were exposed to the same environmental conditions mentioned under 3.1.1. Piglets were raised conventionally from the sow and provided with creep feed from 7th day of age until weaning with 28 days (8.45 ± 0.84 kg BW). Creep feed was identical with the respective starter diet used in the trial. The trial was set up in four consecutive periods, equalling four dietary treatments. Diets applied were the same as in the slaughter trial: in period 1 reference diet +AB, period 2 reference diet -AB, period 3 home-produced LF and period 4 home-produced diet HF. For physical and chemical composition see 3.1.1.

3.2.2 Preparation of ^{15}N-labelled Yeast

Prof. Klappach from the Technical University Köthen / Germany, Institute of Biotechnology was responsible for the production of ^{15}N-labelled yeast. For the production a non-pathogenic, non-prototrophic yeast, *Pichia jadinii*, was choosen. This yeast is capable to utilize mineral nitrogen sources and its metabolism doesn't switch to fermentation at oxygen deficiency. Cultivation of *Pichia jadinii* occurred as discontinuous aerobic fermentation at 30 °C, pH 4.8 – 5.0 and >20 % soluble oxygen concentration for 10 hours. The growing medium contained glucose, mineral salts and ^{15}N-labelled ammonium sulphate (> 95 % atom percent) as sole nitrogen source. During the whole fermentation process concentrations of carbon (glucose) and nitrogen source (ammonium sulphate) were analysed and microscopic pictures of the yeast population evaluated. After complete nitrogen conversion, the growing medium was cooled down and centrifuged at 12.000 rpm (Biofuge 28 RS, Hereus, Germany) to separate yeast cells. The obtained highly concentrated yeast substance (25 – 26 % dry matter content) was stored at 4 °C for 8 hours and then re-diluted with sterile water (16 – 18 % dry matter) to gain a suspension capable of flow. Yeast cells were disintegrated in two steps using a high-pressure

homogenizer (homogenizer 040/10009, APV-Homogenizer Group, Wilmington, USA): first cycle at 1800 bar and second at 1500 bar. Between the two cycles the cell suspension was cooled in an ice bath to ensure that temperature didn't exceed 25 °C during the whole process. The disintegration process was evaluated by microscopy after each step. More than 85 % of yeast cells were destroyed after then second cycle. The cell homogenate was freeze-dried and inactivated by radiation (cobalt, 15 kGy). The so derived ^{15}N-yeast was used for oral ^{15}N-labelling of piglets in the balance trials pre- and postweaning.

3.2.3 Experimental Design

In four consecutive periods, each involving 6 barrows, four different starter diets were tested. At weaning day piglets were weighed and transferred to the experimental station in Rostock (22 °C ambient temperature, natural light system). They were allotted to individual metabolism crates, made of stainless steel with slatted floor, feeding trough, and 2 trays for separate collection of urine and ileal digesta. Space allowance for each pig was 0.33 m^2, excluding trough and trays. Experimental design per dietary period is depicted in figure 4.

Figure 4. Experimental design of balance trial postweaning

<table>
<tr><td>age (d)</td><td>28</td><td>29 to 34</td><td>35</td><td>36</td><td>37</td><td>38</td><td>39 to 49</td><td>50 to 53</td><td>54</td><td>55</td><td>56</td></tr>
<tr><td rowspan="3"></td><td rowspan="3">arrival</td><td rowspan="3">adaption</td><td rowspan="3" colspan="2">operation</td><td colspan="2">recovery</td><td>^{15}N-labelling (1g yeast, 2 x daily)</td><td rowspan="2"></td><td rowspan="2" colspan="2">digesta collection</td><td rowspan="3">slaughter</td></tr>
<tr><td rowspan="2"></td><td colspan="2">blood (0-sample)</td></tr>
<tr><td colspan="5">blood (8 am.,6 pm.)</td></tr>
<tr><td>day</td><td>1</td><td>2 to 7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12 to 22</td><td>23 to 26</td><td>27</td><td>28</td><td>29</td></tr>
</table>

For 7 days animals were adapted to housing conditions and feeding regime (twice daily at 8.30 am and 6.30 pm). At day 8 and 9 pigs underwent surgery. They were fitted with an end-to-end IRA and permanent blood catheters in the *V.jugularis* (for surgical procedure see below). Pigs were allowed to recover for two days with a gradual increase in feed. At day 11 (6 pm.) and 12 (8 am) basal blood samples were taken to measure natural enrichment of ^{15}N in plasma. During the entire trial, starting at day 12, blood was collected twice daily from the venous catheters just prior to feeding (8 am & 6 pm), using blood collection tubes coated with Li-heparine (Monovette®, Sarstedt, Nümbrecht, Germany). Plasma was removed by centrifugation (2500 rpm for 10 minutes), mixed with 10 % trichloroacetic acid (TCA) and centrifuged again (4000 rpm for 10 minutes). The derived

protein-free fraction was stored at 4 °C until laboratory use. Starting at day 12 we labelled the animals with ^{15}N-yeast. For a 10-day duration twice daily 1 g yeast was administered orally just prior to feeding. Thereafter yeast administration was stopped for 4 days and after that ileal digesta collected quantitatively for 2 x 24h in trays supplemented with 500mL methanole. By this we collected two samples per piglet. At the end of trial piglets were euthanised by intravenous application of 2 mL T61® (Intervet Germany).

3.2.4 Ileorectal Anastomosis (IRA)

3.2.4.1 Instrument preparation

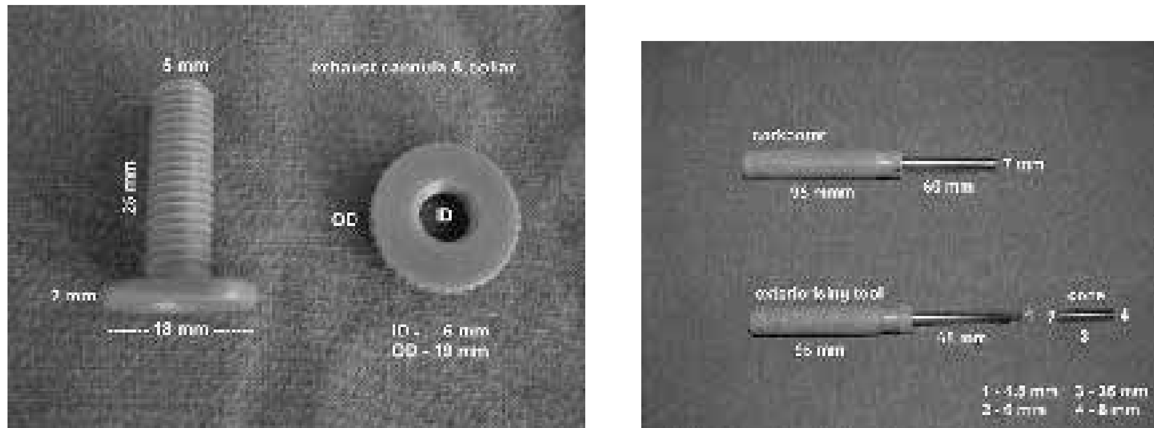
Prior to surgery surgical instruments, drapes, swabs and clothes were sterilised by hot-air sterilisation at 120 °C for 90 min. Exhaust cannulas and fittings for the large intestine, corkborer, exteriorising tool (Figure 5) and venous catheters were soaked in 70 % ethanol for sterilisation.

3.2.4.2 Pre-operative Preparation

Animals were starved for 12 h prior to surgery, but were given *ad libitum* access to water. Approximately 30 minutes prior to surgery piglets received 4 mg/kg BW azaperone (Stresnil®, Janssen-Cilag, Germany) intramuscularly (im.) for sedation and to prevent excessive salivation 0.04 mg/kg BW atropine im. (Atropinum sulfuricum solutum 1%, WDT, Garbsen, Germany). As soon as sedation had taken effect, pigs were transferred to the operation theatre and inhalation anaesthesia was introduced. A closed circuit anaesthetic machine (Komesaroff MARK 5V1G, Melbourne, Australia) operated with isoflurane (IsoFlo®) was used. All animals maintained spontaneous ventilation throughout anaesthesia. For induction piglets received 4 % isoflurane (setting ON at vaporizer) in a gaseous stream of 2 L/min oxygene via a facemask. When anaesthesia reached an adequate depth (on average after 2 minutes), piglets were intubated with an endotracheal tube (Rüschelit®Super Safety Clear, Aut.Rep.Rüsch AG, Kernen, Germany) with an OD of 3.5 to 4.0 mm, using a fibreoptic laryngoscope acc. to MILLER with a 205 mm blade (Lothar Scharras, Kolbingen, Germany). To prevent laryngospasm, 1% lidocain-spray (Xylocain®Pumpspray, AstraZeneca GmbH, Wedel, Germany) was applied to the larynx. The pig was re-connected with the anaesthetic machine and anaesthesia was maintained at about 1.5 % isoflurane (vaporizer setting 1, 2 or 3, depending on animal's respiration) at 0.2 L/min oxygene. Throughout the whole surgical procedure respiration and heart rate

were monitored closely. The surgical areas (neck, abdomen and flank) were scrubbed thoroughly, shaved and disinfected with iodine solution (Braunoderm®, B. Braun Melsungen AG, Melsungen, Germany). Piglets were then placed onto the surgical table, equipped with a heater to maintain body temperature, tied in place with soft bandages and draped with a surgical cloth of appropriate size.

Figure 5. Exhaust cannula, collar, corkborer and exteriorising tool



3.2.4.3 Surgery

At first animals were laid on their right side to fit them with a venous catheter. After an approximately 5 cm incision on the left side of the neck (line between A*ngulus mandibulae* and *Manubrium sterni*) the layers of fasciae, fat and muscles were carefully dissected and the *V. jugularis externa* located. Removing the vein carefully from the attaching tissue it was exteriorised to the wound edges using a blunt hook. A purse-string suture (2 metric Dexon, HR•17, B. Braun-Dexon GmbH, Spangenberg, Germany) was set on the vein and the vessel incised in the centre of the suture. A silicone tube (Silastic®, SEDAT, Irigny, France) of 0.90 m length, 3.175 mm outer and 1.5748 mm inner diameter was fitted into the vein up to a length of approximately 10 cm, which was marked with a silicone ring around the tube. The purse-string was tied up securely and fitted over the silicone ring. After testing the catheter's permeability with warm 0.9 % sterile saline solution, the catheter was closed with a stopper and directed subcutaneously to the mid-dorsal neck. Before closing the incision with a continuous subcutaneous suture (3.5 metric Syrgicryl®, SMI AG, Hünningen, Belgium) 1mL antibiotic (TNV Jungtiersuspension®, Veyx-Pharma GmbH, Schwarzenborn, Germany) was administered into the wound. The wound was cleaned and dressed with self-adhesive gauze (Fixomull®Stretch, Beiersdorf AG, Hamburg, Germany). For catheter protection an elastic bandage (Elastoplast®, Beiersdorf

AG, Hamburg, Germany) was wrapped securely around the pig's neck with an outlet for the catheter.
Having accomplished catheterisation, the animal was turned into dorsal recumbency and draped with a new sterile cloth. The abdominal cavity was opened with a paramedian incision approximately 1 cm left from the penis in the *regio abdominis caudalis* and fat, muscles and peritoneum were dissected. The urinary bladder was placed caudal to gain sufficient overview on the surgical area. When exceedingly filled, the urinary bladder was emptied by manual pressure on its corpus. Caecum and ileum were located and exteriorised. The wound edges were covered with sterile gauze, soaked with warm saline and the exteriorised viscera placed on it. Approximately 1 cm cranial of the ileocaecal valve the ileum was grasped by two soft intestinal forceps, dissected with a scalpel and the mesenterium split up to loosen the two parts sufficiently, taking care to preserve mesenterial vessels as complete as possible. The cranial ileum part was wrapped in saline soaken gauze swabs (forceps closing dissected end) and placed aside. The caudal part was closed with a purse-string suture using 3.5 metric Dexon (Dexon, HR•26, B. Braun-Dexon GmbH, Spangenberg, Germany), inverting the gut mucosa and placed back into the abdominal cavity. Now, the rectum was located, its mesenterium loosened as much as possible to allow exteriorisation and intestinal forceps were attached. It was dissected and its caudal part, wrapped in saline soaked swabs, placed aside. In the cranial section of the rectum a small longitudinal incision was made to enlarge the lumen and a purse-string suture (3.5 metric Dexon, HR•26, B. Braun-Dexon GmbH, Spangenberg, Germany) set around the edges. The base of the exhaust cannula was fitted into the gut lumen and the purse-string tied up securely, taking care to invert the rectal mucosa. Now, by means of a sharpened corkborer, a hole was made through the skin and muscles, just behind the costal arc and as dorsal as possible. The peritoneum was teased with a scalpel and the exteriorising tool inserted into the cavity. Connecting cannula and threaded cone to the tool, the cannula was carefully pulled through the body wall until its base fitted snugly to the inner abdominal wall. Ensuring that neither gut loops were trapped behind the cannula nor the cannula-holding part twisted, the collar was attached to the outer cannula part. To prevent leakage, iodine soaked gauze was fixed between body wall and collar.
The final step was the IRA. Therefore, from the two remaining intestinal sections – cranial ileum and caudal rectum – the swabs were removed and both parts brought together closely, making sure that the connection wasn't twisted (antimesenterial to antimesenterial side). Ileum and rectum were attached with an end-to-end anastomosis, applying a continuous suture according to LEMBERT (3 metric Dexon, HR•22, B. Braun-Dexon GmbH, Spangenberg, Germany). Prior to closing the gut, a soft glycerol suppository

(Nene-Lax®0.5, Dentinox KG, Berlin, Germany) was inserted into the ileal part (cranial of anastomosis). After complete closure permeability was checked by gently massaging the suppository towards the rectum. The anastomosed gut was placed back into the abdominal cavity and the proper setting of all involved viscera checked again. Throughout the entire surgical procedure the abdominal cavity was flushed frequently with warm saline to prevent serosal drying and adhesions, always ensuring the saline flow to be outward. Peritoneum and muscle layers were closed with a continuous suture according to REVERDIN (3.5 metric Dexon, HR•26, B. Braun-Dexon GmbH, Spangenberg, Germany) and 2 mL antibiotic (TNV Jungtiersuspension®, Veyx-Pharma GmbH, Schwarzenborn, Germany) were administered intraabdominally prior to final closure. The skin was closed with an intracutaneous continuous suture and dressed with self-adhesive plaster after cleaning. Anaesthesia was switched back to setting 1 and OFF, respectively, as the skin closure took place and oxygen was set to 1 mL/min for a few minutes after surgery. Finally the endotracheal tube was disconnected and removed from the trachea.

3.2.4.4 Post-operative care

Piglets received 0.2 mL/ kg BW antibiotic im. (TNV Jungtiersuspension®, Veyx-Pharma GmbH, Schwarzenborn, Germany) and 4 mg/kg BW carprofen im. as a long-lasting painkiller just after surgery (Rimadyl®, Pfizer, Karlsruhe, Germany). They were reallocated to the metabolism crates, each provided with a red heating lamp above and monitored closely until they regained full conscience. Venous catheters were connected to a continuous saline drip, set to 6 mL/h by an automatic pump (IPC-12, ISOMATIC SA, Labortechnik-Analytik, Glattbrugg-Zürich, Switzerland) and maintained over the entire trial. Animals were allowed to recover for two days with free access to sweetened black tea infusion at the first post-operative days and gradual increase of feed. Exhaust cannulas were flushed twice daily with warm saline to remove remaining contents of the large intestine and therefore prevent bloating due to bacterial fermentation in the large bowel.

3.2.5 Laboratory Analysis

Following samples underwent analysis: ileal digesta, blood samples and urine samples.
In ileal digesta DM, nitrogen content, aminoc acids and D-alanine were determined according to the specifications given in point 3.1.3.1.

^{15}N-enrichment in ileal digesta and plasma (TCA-soluble fraction) was determined by means of isotope-ratio-mass-spectrometer (IRMS, DELTA S, Finnigan, USA). For this, organic nitrogen was converted into ammoniumchloride and subsequently ^{15}N was measured.

3.2.6 Statistical Analysis

Values were obtained individually for each piglet and combined (LSM ± SE) per dietary period. Subsequently statistical analysis was performed using the General Linear Model of SAS (version 8) and equation 3. Data were compared with respect to diet using the Tukey-test.

Equation 3.

$$y_d = D_d + \underline{\varepsilon}_{s(gd)}$$

y – characteristic, e.g. lactic acid

D_d – factor diet, index d: diets +AB, -AB, LF, HF

$\underline{\varepsilon}_{s(gd)}$ – random residual effect, index s: samples

3.3 Balance Trial Preweaning

To investigate the ileal nitrogen flow and nitrogen digestibility by means of ^{15}N tracer technique and insoluble marker in suckling piglets, an experiment was conducted in collaboration with Dr. BG Miller at the University of Bristol, Division of Animal Husbandry, Langford House, Langford, Bristol BS18 7DT, United Kingdom. The Ethical Committee of the University of Bristol approved all experimental techniques.

3.3.1 Animals and feeding

10 Piglets (Landrace x Large White) were raised on a farm until 7 to 9 days of age (2.52 ± 0.29 kg BW) and moved subsequently to the isolator unit at the department of animal husbandry, clinical veterinary science in Langford / Bristol. Animals were housed individually in cages. To mimic feeding conditions at the sow, piglets were fed a

commercial porcine milk replacer (PiggiMilk, Trouw Ltd., Cheshire, UK) dispensed by an automated liquid feeding system at an hourly interval (Figure). A fresh batch of milk replacer (Table 13) was prepared on a daily basis (125 g PiggiMilk powder / L water) and kept at 4°C during the entire day. Daily the entire system was flushed with disinfectant solution and subsequent hot water to ensure high hygiene standard and prevent bacterial growth in the system due to feed residues. Furthermore milk replacer consumption per pig was measured daily and adjusted to their individual needs.

Table 13. Chemical composition of milk replacer PiggiMilk

Parameter	Values
Dry matter (%)	96.01
Nitrogen (% DM)	4.15
Crude protein (% DM)	25.94
Ash (% DM)	7.23
Fibre (% DM)	0.60
Fat (% DM)	17.73
Total amino acids (g/16g N)	103.18
ASP (g/16g N)	8.10
THR (g/16g N)	4.66
SER (g/16g N)	5.46
GLU (g/16g N)	21.22
GLY (g/16g N)	2.02
ALA (g/16g N)	3.50
* VAL (g/16g N)	6.34
* ILE (g/16g N)	5.29
* LEU (g/16g N)	9.80
TYR (g/16g N)	4.02
* PHE (g/16g N)	4.72
* HIS (g/16g N)	2.76
* LYS (g/16g N)	7.79
* ARG (g/16g N)	3.26
PRO (g/16g N)	9.34
CYS (g/16g N)	1.08
* MET (g/16g N)	2.11
* TRP (g/16g N)	1.71

* essential amino acids

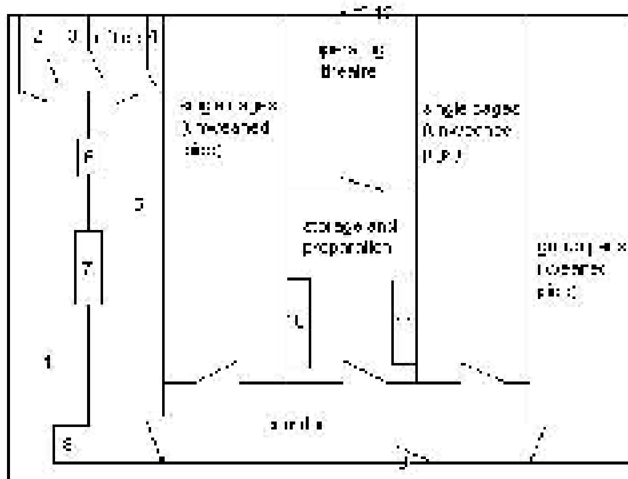
3.3.2 Experimental Design

10 piglets were moved to the isolator unit at the Department of Animal Husbandry, Clinical Veterinary Science in Langford / Bristol. The isolator unit (Figure 6) was equipped with a disinfection area for animals and staff, individual cages, group pens, an automatic feeding system for liquid diets (Figure 7, 8) and a separated operating room. An automated light system was set up to 12 h light and 12 h darkness, ambient temperature to 30°C, and the entire unit under positive pressure (Thorpe, 1999).

The experimental design is displayed in Figure 9. At arrival piglets were cleaned and disinfected thoroughly by passing them through warm disinfectant solution (Virkon®, RS Biotech, Finedon, Northants, UK) at the disinfectant area. All animals were housed in a group pen for 24 h for adaptation to the feeding regime (drinking from a bowl at hourly interval). At day 2 they were allotted to single cages, equipped with red heating lamps, blanket, stainless steel feeding trough (connected to feeding system) and a tray for urine collection. For each piglet the amount of milk replacer was set up individually and adjusted daily to the animal's requirements. Daily feed intake was measured and recorded for each pig individually. Starting at arrival all animals received a 3-day antibiotic course by injecting 0.3 mL Clamoxyl™ (Pfizer Ltd., Sandwich, Kent, UK) per pig.
To label the animals with ^{15}N, 0.5 g labelled yeast were administered orally over a period of eleven days, starting at day 4, followed by 4 days of yeast deprivation.
Also, PEG 4000 (Polyethyleneglycol, Sigma®, UK) was added as an insoluble marker to the feed, amounting to 20 g PEG 4000 /kg feed, starting at day 7.

For collection of ileal digesta, 10 piglets were surgically fitted with simple T-cannulas at the terminal ileum at day 7 and 8 (for surgical procedure see below). Digesta collection was accomplished at days 19 and 20 for 48 h via the T-cannulas by attaching plastic pouches to the cannulas. At the last experimental day (day 21) all pigs were euthanised by intracardial injection of 4.5 mL Euthatal® (Rhône Mérieux Ltd., Essex, UK) and after opening the abdominal cavity samples of gut mucosa and content have been obtained. For determination of ^{15}N enrichment, urine samples were taken over the course of the experiment, using a tray attached below single cages.

Figure 6. Layout of isolator unit (not to scale) acc. to Thorpe (1999)



1 – outside unit

2 – change room

3 – shower

4 - W.C.

5 – multipurpose room

6 – autoclave (installed through wall)

7 – animal disinfectant area (installed through wall)

8 – feeder installation

9 – door to outside unit

10, 11 – pumps for feeding system

12 - door to outside unit

Figure 7. Set-up of automatic feeding system for liquid feed in the isolator unit

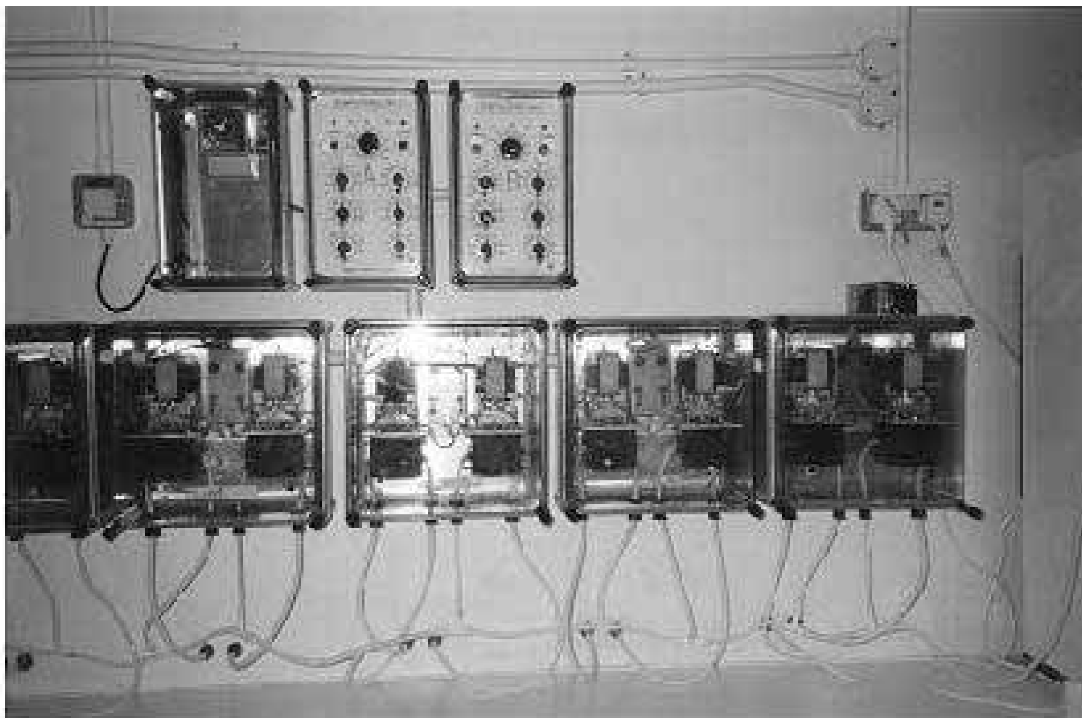


Figure 8. Set-up of individual cages in the isolator unit

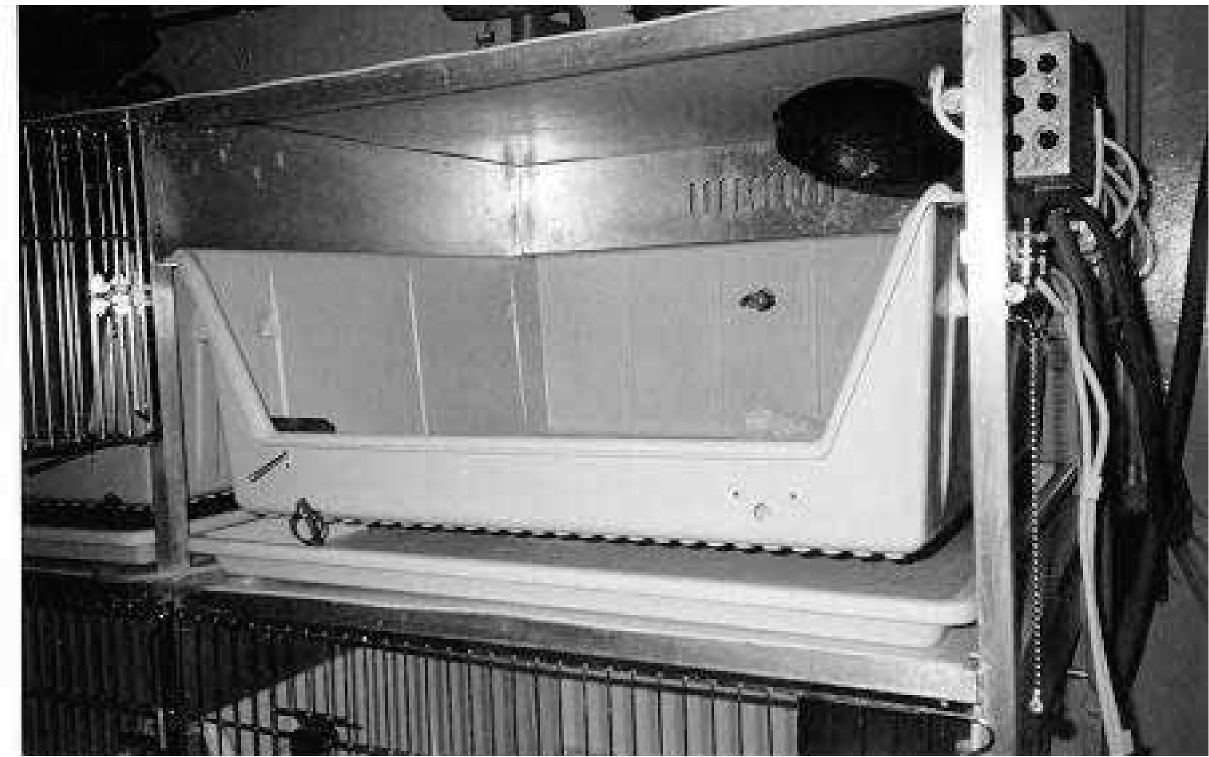


Figure 9. Experimental design of balance trial preweaning (Bristol)

age (d)	8	9	10	11 to 13	14	15	16 to 21	22	23	24	25	26	27	28
	arrival	adaption			operation							digesta collection		slaughter
				^{15}N-yeast (0.5 g orally, 2x daily)										
					PEG 4000									
			urine											
day	1	2	3	4 to 6	7	8	9 to 14	15	16	17	18	19	20	21

3.3.3 Cannulation Procedure

3.3.3.1 Cannulas & Instrument Preparation

Each T-cannula had 2 collars to hold the cannula in close contact with the pig's body wall and a cap to prevent leakage of gut content. Cannulas, collars and caps were made from PTFE material. A sharpened corkborer was used to make a hole through the abdominal wall in the right flank and a tool with a cone shaped, threaded end was used to exteriorise the cannula through this hole (Thorpe, 1999). All required surgical instruments were washed in surgical scrub (Chlorhexadine, 4%, Hibiscrub, Schering-Plough Ltd., Wellwyn Garden City, UK) and sterilised by autoclaving (120 °C, 20 min). Because cannulas consisted of PTFE, they were scrubbed and soaked in surgical spirit prior to use.

3.3.3.2 Pre-operative preparation

Prior to surgery animals were starved for 2 h. As a premedication each pig received 4 mg/kg BW azaperone im. (Stresnil®, Janssen-Cilag Ltd., Saunderton, High Wycombe, UK) and 0.04 mg/kg BW atropine im. (Atrocare®, Animalcare Ltd., Dunnington, York, UK). As soon as the pre-medication had taken effect, we applied inhalation anaesthesia using a closed circuit anaesthetic machine operated with halothane. For induction we administered 3% halothane in a gaseous stream of 1L/min oxygen and 2L/min nitrous oxide and for maintenance 1% to 1.5% halothane with 1L/min oxygen and 2L/min nitrous oxide via a facemask. Piglets were surgically prepared (scrubbed, shaved, surgical spirit applied), placed in dorsal recumbency on a covered heat pad to maintain body temperature and tied in place with soft bandages.

3.3.3.3 Surgery

Approximately 2 cm caudal from the xyphoid an incision along the *Linea alba* was performed, muscle, fat layer and peritoneum carefully dissected and the small intestine exteriorised. The ileo-caecal junction was located; the ileum followed up past the continuous Peyers Patches and a 10 cm portion above the Peyers Patches wrapped in gauze swabs soaked in warm, sterile saline. In this part a longitudinal incision (approx. 2 cm) was made and the cannula base fitted into the gut. Simple, interrupted sutures with 4-0 Vicryl® (Ethicon, Edinburgh, UK) were placed transversely across the ileum on either side of the cannula to close the gut. Furthermore a pursestring suture (4-0 Vicryl®, Ethicon and Edinburgh, UK) was set subserosal around the cannula body and tied up. Cannula and adjacent area were rinsed with saline, ensuring that none of these washings entered the abdominal cavity. Subsequently a hole was made through skin and muscle layer in the right flank by means of a sharpened corkborer and the peritoneum was teased with a small scalpel blade. Using a specially designed tool, the cannula was exteriorised through the hole. Ensuring that the cannula wasn't twisted and no gut loops were trapped behind the cannula base, one collar was attached snugly to the exterior part of the cannula. Then the peritoneum was closed with a continuous suture (4-0 Vicryl®, Ethicon, Edinburgh, UK), instilling 20 mL antibiotics (600 mg Crystapen, Britannia Pharmaceutical Ltd., Redhill, UK, dissolved in 50 mL sterile saline) in the abdominal cavity prior final closure. Muscle and fat layer were sutured with single stitches (2-0 Vicryl®, Ethicon, Edinburgh, UK) and the skin was closed by continuous, intracutaneous suture (2-0 Vicryl®, Ethicon, Edinburgh, UK). The second collar was attached to the cannula and the surgical area cleaned.

3.3.3.4 Post-operative care

Immediately after surgery, piglets were dosed with metoclopramide hydrochloride (1 mg/kg BW, Emequell, SmithKline Beecham, Surrey, UK) to prevent nausea and ileus. They received pethidine (3 mg/kg BW i.m., Pethidine, Martindale Pharmaceuticals, Romford, UK) as an immediately responding, short-acting painkiller and a non-steroidal anti-inflammatory (4 mg/kg BW carprofen i.m., Rimadyl® Large Animal Solution, Pfizer Ltd., Sandwich, UK) lasting approximately 24 h. Animals returned to their cages for recovery and were monitored closely until they appeared responsive, alert and started feed intake.

3.3.4 Laboratory Analysis

Following samples underwent analysis: ileal digesta, urine samples and milk replacer.
In ileal digesta DM, amino acids and D-alanine were determined by the same procedures as in 3.1.3.1.
Nitrogen content and ^{15}N-enrichment were determined by means of IRMS (DELTA S, Finnigan, USA). In preparation nitrogen was converted into ammoniumchloride.
In urine ^{15}N-enrichment was measured directly with IRMS.
PEG 4000 was determined photometrically in digesta and milk replacer according to the specifications of Ishikawa (1966).

3.3.5 Statistical Analysis

Statistical analysis was performed using the SAS system, version 8. Samples were taken individually for each pig. We wanted to compare common parameters in both balance trials to assess the situation before and after weaning. Least square means for each dietary treatment, i.e. for milk replacer preweaning and starter diets postweaning, were processed by means of equation 4, using a mixed model. To compare data pre- and postweaning, we used a t-test and for comparison of each starter diet with preweaning conditions we applied the Dunnett-test. For both tests $\alpha = 5$ %.

Equation 4.

$$y_{ptdgs} = W_p + D_{d(p)} + \underline{\varepsilon}_{s(gtdp)}$$

y – Characteristic, e.g. microbial nitrogen

W_p – pre/postweaning

$D_{d(p)}$ – diet

for W = pre: milk replacer (MR)

for W = post: +AB, -AB, LF, HF

$\underline{\varepsilon}_{s(gtdp)}$ – random residual effect