

Effects of tubers of the Jerusalem artichoke (*Helianthus tuberosus*) and potatoes (*Solanum tuberosum*) on the intestinal microbiota of pigs and evaluation of a procedure for quantification of microbial mass in pig faeces



Charlotte Marien

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for quantification of microbial mass in pig faeces

Charlotte Marien

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Erster Gutachter: Prof. Dr. Albert Sundrum

Zweiter Gutachter: Prof. Dr. Monika Krüger

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1st Supervisor: Prof. Dr. Albert Sundrum, University of Kassel

2nd Supervisor: Prof. Dr. Monika Krüger, University of Leizig

Examiner: Prof. Dr. Prof. Dr. Eva Schlecht, University of Kassel

Examiner: Prof. Dr. Rainer Georg Jörgensen, University of Kassel

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Table of abbreviations

ADF	Acid detergent fibre	<i>I_s</i>	Quantity of the supplement
ADL	Acid detergent lignin	<i>IT</i>	Daily intake
BEDN	Bacterial and endogenous debris nitrogen	<i>IT_{JA}</i>	Daily intake of the JA tubers in the experimental treatment
C	Carbon	JA	Jerusalem artichoke
Ca	Calcium	kg	Kilogramme
CA	Crude ash	lg	Logarithm to the base of 10
CF	Crude fibre	LI	Large intestine
CFU	Colony-forming units	LMC	Lean meat content
CH ₄	Methane	LPS	Lipopolysaccharides
CL	Crude fat	µg	Microgram
CO ₂	Carbon dioxide	µl	Microlitre
CP	Crude protein	µm	Micrometre
CRP	C-reactive protein	mg	Milligramme
CT	Control treatment	ml	Millilitre
<i>D</i>	Intake of the external marker	M	Molar
DA	D-alanine	Man	Mannosamine
DAPA	Diaminopimelic acid	ME	Metabolisable energy
DM	Dry matter	MJ	Megajoule
DP	Degree of polymerisation	MP	Microbial pellet
DWG	Daily weight gain	MP-N	Bacterial nitrogen in the faeces
<i>DWG_{CT}</i>	Daily weight gain of the control treatment	Mur	Muramic acid
<i>DWG_{ET}</i>	Daily weight gain of the experimental treatment	nm	Nanometre
<i>EC_{JA}</i>	Energy content of the JA tubers	N	Nitrogen
EDOM	Enzyme digestible organic matter	N ₂ O	Nitrous oxide
<i>EI</i>	Energy intake	NaCl	Sodium chloride
<i>EI_{CT-Con}</i>	Energy intake of the control treatment by the concentrate	NDF	Neutral detergent fibre
<i>EI_{ET-Con}</i>	Energy intake of the experimental treatment by the concentrate	NH ₃	Ammonia
<i>EI_{JA}</i>	Energy intake of the experimental treatment by the JA tubers	NH ₄	Ammonium
ET	Experimental treatment	NH ₄ -N	Ammonium bound nitrogen
<i>F</i>	Faecal concentration of the external marker	OM	Organic matter
FM	Fresh matter	<i>OMD_h</i>	Organic matter digestibility of the herbage
FOS	Fructo-oligosaccharides	<i>OMD_s</i>	Organic matter digestibility of the supplement
g	Gramme	OR	Organic rest
<i>g</i>	Gravity	P	Phosphorus
Gal	Galactosamine	PT	Potato treatment
GIT	Gastrointestinal tract	RS	Resistant starch
Glu	Glucosamine	RU	Relative units
HCl	Hydrochloric acid	SAB	Solid associated bacteria
<i>HI</i>	Intake of the herbage	SCFA	Short chain fatty acids
Hp	Haptoglobin	ST	Silage treatment
Ig	Immunoglobulin	TiO ₂	Titanium dioxide
		Trm	Treatment
		UDN	Undigested dietary nitrogen
		WSN	Water soluble nitrogen

My parents

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1 General introduction

In the pig, the whole gastrointestinal tract is settled by microorganisms, which utilise the ingested feeds as nutrient substrate for reproduction and the release of metabolisable products into the gut⁽¹⁾. There is a sensitive balance between populations of beneficial and potential pathogenic bacteria in the gastrointestinal tract (GIT) with appearance of many symbiotic and competitive interactions between them⁽¹⁾. Composition and activity of the intestinal microbiota is eminently dependent on the source of nutrients and thus on the feeding regime⁽²⁾. In addition, the microbiota in the GIT of pigs is an important factor influencing health of the gut and the whole host animal⁽³⁾. The comprehensive impact of the microbiota has led to various attempts to modify composition and activity of the gut flora, especially to stimulate selectively the growth of beneficial populations of bacteria as lactobacilli and bifidobacteria. A common approach constitutes the supplementation of the diet with pro- and prebiotics as food additives⁽⁴⁾.

Utilization of probiotics implies the administration of living bacteria which are expected to improve the balance of the intestinal microbiota in the host and to provide a temporal health benefit using antagonistic metabolites produced by microorganisms as lactic acid of lactobacilli and bifidobacteria⁽⁵⁾. Prebiotics are nutritional components, which are at least partly indigestible by endogenous enzymes of mammalian. They escape the digestion in the small intestine more or less unaffected and are utilised selectively by a restricted group of microorganisms expected to provide health promoting properties^(3,6).

In various studies, inulin and oligofructose has been identified as effective prebiotics^(3,1,4,6). Inulin is a naturally occurring mixture of fructose polymers serving as storage carbohydrates in plants. Oligofructose, which is also called fructo-oligosaccharides (FOS), constitutes a subgroup with polymers of a degree of polymerization (DP) below twenty^(1,7,8). Inulin and FOS are supposed to decrease pathogenic bacteria in the intestine of humans and animals and stimulate the immune system^(4,8,9). They are associated with a reduced risk of many diseases such as colon cancer, osteoporosis and atherosclerosis^(8,9). Indeed, research in promoting animal

health by inulin and FOS has focused on the application of prebiotic functional food additives in the form of industrial purified inulin or meal of dried inulin-rich crops to the diet of pigs⁽¹⁰⁻¹³⁾.

In organic agriculture, the provision of roughages to pig's diet is required by the EC-regulation for organic production⁽¹⁴⁾, with a primary intention to enhance pigs satiety and allow them to satisfy natural behaviours as foraging, rooting and exploring⁽¹⁵⁾. Tubers of Jerusalem artichoke (JA) (*Helianthus tuberosus*) are considered as root vegetables and would likewise fulfil the EC-regulation⁽¹⁴⁾, by having a high energy content of up to 15 megajoule metabolisable energy (MJ ME) per kg in the dry matter (DM)^(16,17). Despite of their high potential for pig nutrition, through expensive and laborious cultivation, harvesting, storage and provision to the pigs JA tubers are seldom utilised so far. Indeed, JA tubers are also known to be a rich source of inulin and FOS. Unlike to the supplementation of purified inulin or meal of dried inulin-rich crops, the first study aimed to determine the prebiotic effect of high amounts of inulin and FOS derived from fresh JA tubers on the intestinal microbiota of pigs.

Potatoes (*Solanum tuberosum*) are also a high appropriate root vegetable for pigs and constitute a well known energy source in animal nutrition due to their high starch content^(18,19). In recent decades, they have been included in huge amounts into the daily ration of pigs⁽²⁰⁾. To improve digestibility of potato starch and nutrient availability, the starch structure of potatoes has been generally modified by thermal processing⁽²¹⁾. But the influence of thermal treated potatoes on the intestinal microbiota has not been investigated so far. Since JA tubers had been approved to be effective providing beneficial microbiota while inhibiting potential pathogenic bacteria, the second study aimed to determine the effect of high amounts of steamed and steamed-ensiled potatoes on the intestinal microbiota of pigs.

Beside the qualitative composition of the intestinal microbiota, which were examined in the first two trails, the quantity of bacteria harboured in the gut is also an important parameter. The bacterial mass detectable in the faeces of pigs can give information on activity and growth of gut bacteria and allow the assessment of nutritional influences from different feeding regimes^(22,23,24). In addition, the

proportion of the microbial biomass in faeces is associated with lower N emissions from the manure and a better nutrient availability in plant growth^(25,26). But methods for a direct quantification of faecal bacteria are insufficient, since they are based on indirect measurement or necessitate complex conversion calculations. So far only few attempts have been conducted to develop ways for direct assessment of bacterial biomass in faecal samples. Therefore the third study aimed to develop procedure for direct quantification of the microbial biomass in faeces of pigs.

2 Chapter 1.

Effects of feeding tubers of Jerusalem artichoke on the
intestinal microbiota in fattening pigs

2.1 Abstract

Inulin and fructo-oligosaccharides (FOS) have shown promising results as prebiotics in human and animal nutrition. While in previous studies inulin has been provided in small quantities as feed additive the aim of this study was to determine the effects of inulin-rich tubers of the Jerusalem artichoke (JA) offered *ad libitum* to finishing pigs on intestinal microbiota in pigs' gastrointestinal tract (GIT). A total of 72 finishing pigs in a free-range system were allocated to a control treatment (CT), fed demand-actuated with a wheat and grain legume-based concentrate mixture, and an experimental treatment (ET), receiving 70% of the concentrate amount apportioned to the CT, but with access to an allocated area with cultivated JA tubers. Voluntary intake of JA tubers under free-range conditions was estimated to 1.24 kg dry matter (DM)/day, corresponding to a mean inulin ingestion of approximately 800 g/day. While growth performance of the pigs in the CT averaged 0.642 ± 0.014 kg/day, it increased in the ET to the extent of 0.765 ± 0.015 kg/day ($P=0.000$), emphasizing the tuber's suitability as a nutrient source for free-ranged pigs. Abundant availability of inulin and FOS in pigs GIT significantly increased total anaerobes ($P=0.000$), lactobacilli ($P=0.046$) and yeasts ($P=0.000$) and drastically reduced *Clostridium perfringens* from $\lg 5.24 \pm 0.17$ colony-forming units (CFU)/g wet faeces in the CT to $\lg 0.96 \pm 0.20$ lg CFU/g wet faeces in ET ($P=0.000$) in pig faeces. C-reactive protein (CRP) and antibodies versus lipopolysaccharides (LPS) showed no differences between treatments. The differences in the microbiota between the treatments indicate the capacities of inulin and FOS to improve gut health.

2.2 Introduction

The microbiota of the gastrointestinal tract (GIT) plays an important role in the gut health of host animals⁽¹⁾. The large intestine (LI) of pigs is colonized by up to 10^{12} bacteria per g ingesta, representing the highest number of microorganisms found in the different species of farm animals⁽²⁾. The microbiota constitute an extremely complex and dynamic ecosystem, which is eminently dependent on the food composition⁽³⁾. To improve gut health, the application of functional food additives, which are considered as prebiotics, has gained increased interest⁽⁴⁾. In human and animal nutrition, inulin and fructo-oligosaccharides (FOS) have been detected as effective prebiotics^(5,6). They resist the decomposition by endogenous enzymes and serve as substrates for microbial fermentation⁽⁷⁾. Short chain fatty acids (SCFA) are major products of this fermentation process and can contribute up to a level of 30% to the energy supply of the host animal⁽¹⁾. A positive side-effect of the SCFA production is the lowering of the pH-value and in succession the proliferation of beneficial lactic acid producing bacteria and a competitive exclusion and inhibition of potential pathogenic bacteria^(4,7). Therefore, inulin and FOS are supposed to increase density of bifidobacteria and lactobacilli^(8,9), to reduce the number of pathogenic clostridia^(4,10), and strengthen the barrier function of the gut and hence reducing the burden for the immune system leading to an enhanced disease resistance^(11,12).

In pigs, however, the effects of inulin and FOS on intestinal microbiota are described inconsistently. Most studies supplementing inulin and FOS to pigs found no alterations of intestinal microbiota^(8,13-15), whereas others detected a growth stimulation of selected bacterial populations in single segments of the GIT^(9,16-19). The studies have in common that they refer to the application of inulin and FOS as feed additive in small amounts to piglets or growing pigs. In contrast, the effects of high amounts of inulin and FOS on the intestinal microbiota in finishing pigs have so far not been investigated. As the tubers of the Jerusalem artichoke (JA) (*Helianthus tuberosus*) are known as a rich source of inulin, of FOS and are also an energy-rich feedstuff for pigs, the present study aimed to determine the effects of inulin and FOS

offered as field feed on the intestinal microbiota and on selected parameters of the immune system in finishing pigs.

2.3 Materials and methods

2.3.1 Animals, diets and experimental design

A total of 72 crossbred pigs (German Landrace x Large White x Pietrain) were allocated to a control treatment (CT) and an experimental treatment (ET) in a free-range system. The CT enclosed 24 pigs with both genera equally distributed. In the ET, 48 pigs were subdivided in two groups of 24 castrated males and 24 females. In CT the diet was formulated to meet the requirements of the pigs in relation to nutrients and energy to a performance level of 700 g daily weight gain (DWG) according to the German Society of Nutrition Physiology⁽²⁰⁾. The calculated values agreed with results of the nutrient analysis. The concentrate was composed of 410 g/kg wheat, 205 g/kg barley, 200 g/kg faba bean, 100 g/kg lupine, 35 g/kg potato protein, 25 g/kg mineral mix and 25 g/kg oil, resulting in an energy content of 15.6 megajoule (MJ)/kg metabolisable energy (ME) in the dry matter (DM) and 168 g/kg crude protein (CP) in the DM (Table 2.1). In both treatments, the concentrate was offered in individually segregated troughs to assure the same intake of concentrate within the treatments. Pigs in the ET received only 70% of the concentrate apportioned to the CT, but had weekly access to an allocated area with cultivated JA tubers. The JA tubers in average contained 15.0 MJ/kg ME and 69 g/kg CP in the DM (Table 2.1). Inulin content of the JA tubers was estimated via the analysis of the sugar content of 803.4 g/kg in the DM, which according to Stolzenburg⁽²¹⁾ approximately equates to 80% of the inulin concentration of the tuber. Therefore inulin content in the DM was estimated to the level of 650 g/kg.

Table 2.1. Chemical composition and metabolisable energy of the concentrate and the Jerusalem artichoke tubers

Analysed composition	Concentrate	JA tubers
	g/kg DM	
DM (g/kg FM)	853.2	228.9
CA	48	63
CP	168	69
CL	60.0	7.8
CF	43.3	41.2
Starch	504	0.0 ^a
Sugar	40.1	803.4
Energy (MJ ME/kg)	15.6	15.0

^aafter Hydrolysis starch fraction is ascribed to the sugar fraction

JA, jerusalem artichoke; CA, crude ash; CP, crude protein; CL, crude fat; CF, crude fibre; DM, dry matter; FM, fresh matter; ME, metabolisable energy; MJ, megajoule

2.3.2 Data collection and sampling

In the finishing period, starting with an average live weight of 69.9 ± 1.0 kg, six castrated male and six female pigs in both treatments were randomly chosen as donors of faecal and blood serum samples. In the course of the experiment, DWG of each animal and daily intake (*IT*) of the concentrate per group were recorded. The daily intake of the JA tubers in the experimental treatment (IT_{JA}) was estimated on the premises of a similar energy/protein ratio in the diet and a similar carcass composition (protein/fat accretion) in the CT and the ET. Assuming a similar relation between energy intake and DWG in both treatments, the unknown energy intake by the JA tubers (EI_{JA}) of the experimental treatment was considered as

$$(EI_{JA} + EI_{ET-Con})/DWG_{ET} \approx EI_{CT-Con}/DWG_{CT}$$

with EI_{JA} = energy intake of the experimental treatment by the JA tubers, EI_{ET-Con} = energy intake of the experimental treatment by the concentrate, EI_{CT-Con} = energy intake of the control treatment by the concentrate, DWG_{ET} = daily weight gain of the experimental treatment and DWG_{CT} = daily weight gain of the control treatment. Subsequently, the EI_{JA} was estimated by the following calculation:

$$EI_{JA} = (EI_{CT-Con} * DWG_{ET} / DWG_{CT}) - EI_{ET-Con}$$

Since the energy intake by the JA tubers is closely related to the energy content of the tubers, IT_{JA} of the experimental treatment was estimated as

$$IT_{JA} = EI_{JA} / EC_{JA}$$

with IT_{JA} = daily intake of JA tubers of the experimental treatment and EC_{JA} = energy content of the JA tubers.

Faecal samples were collected from the rectum on day 20, 24, 41 and 45 after the beginning of the finishing period. For bacteriological investigation samples were immediately filled in faeces tubes (Sarstedt, Nümbrecht, Germany) and kept frozen below -20°C until analyzed. For procedural reasons it was not possible to conduct microbial analysis of fresh faecal samples. Although it can not be excluded that freezing and defrosting affect microbial counts, it is expected that the impact concerns the faecal samples in both treatments to the same degree⁽²²⁻²⁴⁾.

Pigs were slaughtered with an average body weight of 120.1 ± 1.24 kg. Blood samples were taken during the slaughter process and filled into blood collection tubes with a clot activator (Sarstedt, Nümbrecht, Germany). After centrifugation (10 min, 5000g) the serum was filled in micro tubes (Sarstedt, Nümbrecht, Germany) and frozen at -20°C until analyzing.

2.3.3 Parameters determined in faeces

DM content was estimated by measuring weight loss after drying the samples at 105°C . For ash weights, samples were heated in a muffle furnace at 550°C for 8 h. Faecal samples were analyzed for total aerobes, *Escherichia coli*, total anaerobes, lactobacilli, *Clostridium perfringens*, yeasts and fungi. From a suspension of 0.5 g of wet faeces and 4.5 ml of a sterile phosphate buffered saline solution (ph 7.4), dilution series (10^{-1} to 10^{-7}) were produced. 10 μl of each dilution were spread onto agar plates with selective media (blood, Gassner agar, Sabouraud, MacConkey,

SIFIN, Berlin). For anaerobe bacteria, plates were cultivated under anaerobic conditions. After cultivation the last two covered agar plates were used for counting the colony-forming units (CFU) per g wet faeces and counts are expressed as logarithm to the base of 10 (lg) CFU/g of wet faeces. Yeast strains were additionally typed. For purposes of statistical analysis in samples that had no colonies at the detection limit of lg 3 CFU/g, which conforms to 1000 CFU/g, a value of either 0 or 2.99 was assigned. As the results obtained from the calculation with the different values for samples below the detection limit did not change ascertained significances, calculations were conducted with 0 for the corresponding samples.

2.3.4 Immunological investigations

C-reactive protein (CRP) and the specific antibodies to lipopolysaccharide (LPS) (IgA-anti-LPS, IgM-anti-LPS and IgG-anti-LPS) in the serum samples were determined quantitatively via enzyme immunoassay. CRP was detected with polyclonal antibodies from rabbit anti-CRP (DAKO, Hamburg, Germany). Antibodies against LPS were determined using purified LPS from *E. coli* J5 (Sigma, Taufkirchen, Germany). For a more detailed description of the method see Krüger *et al.*⁽²²⁾.

2.3.5 Statistical analysis

Statistical analysis was conducted using the statistical program SPSS 15.0 of Windows (Version 15.0.1.1). Data were tested for normal distribution by Shapiro-Wilk-test and for homogeneity of variance by Levene's test. Repeated samplings of the microbial parameters were averaged for each animal. The effect of the feeding regime on microbial and serological parameters as well as performance and carcass data was assessed by one-way ANOVA. Differences were considered to be significant at $P < 0.05$.

2.4 Results

2.4.1 Production data

Within the experimental period, neither the pigs in CT nor the ones in ET showed signs of disease. The apportioned concentrate was consumed completely by the pigs in both treatments and resulted in a daily intake of 37.9 MJ ME and 408 g CP for pigs in the CT. Growth performance data and carcass traits are presented in Table 2.2. Despite the restricted supply of concentrate, pigs in ET showed a significantly higher DWG (0.765 kg/day) in comparison to pigs in CT with 0.642 kg/day. Within the ET, female pigs achieved a higher DWG compared to the castrated pigs ($P=0.020$). Feed conversion ratio of the apportioned concentrate amounted to 3.78 kg per kg body weight gain in the CT and 3.17 kg concentrate per kg body weight gain in the ET. Lean meat content was not affected significantly by the treatment. Intake of the JA tubers by the ET was estimated to be on average 1.24 kg DM/day, corresponding to an inulin ingestion of approximately 800 g/day. Taking into account these assumptions, pigs of ET consumed about 45 MJ ME and 370 g CP per day with about 41% of the energy intake and 23% of the protein intake resulting from the ingested JA tubers.

Table 2.2. Means of daily weight gain in the finishing period and lean mean content distinguished by treatment and gender

	CT*	ET	P-values	Gender of pigs					
				CT		P-values	ET		P-values
				Barrows	Gilts		Barrows	Gilts	
DWG (kg)	0.642 ± 0.014	0.765 ± 0.015	0.000	0.661 ± 0.022	0.625 ± 0.019	0.221	0.731 ± 0.021	0.802 ± 0.020	0.020
LMC (%)	58.0 ± 0.5	57.1 ± 0.3	0.126	57.8 ± 0.8	58.2 ± 0.8	0.725	56.7 ± 0.4	57.4 ± 0.5	0.290

DWG, daily weight gain; LMC, lean meat content

*CT, control treatment; ET, experimental treatment

2.4.2 Microbiota in faeces

DM content of the faeces in CT showed a mean value of 309 ± 7 g/kg and tended to result in higher values than in faeces of pigs from ET with 266 ± 16 g/kg ($P=0.087$). Ash content of the faeces was on a high level in both treatments with 388 ± 25 g/kg DM in CT and 376 ± 15 g/kg DM in ET without being significantly different.

Numbers of total aerobes, *E. coli*, total anaerobes, lactobacilli, *C. perfringens*, yeasts and fungi determined in the faeces of pigs from the CT and ET are presented in Table 2.3. Bacterial counts showed a high variation among the four sampling dates as well as within and between treatments. Significant increases in the faeces of pigs fed JA tubers were detected for anaerobic bacteria ($P=0.000$), lactobacilli ($P=0.046$) and yeasts ($P=0.000$). Moreover, the counts of *C. perfringens* showed a drastic reduction with $\lg 5.24 \pm 0.17$ CFU/g wet faeces in CT and $\lg 0.96 \pm 0.20$ CFU/g wet faeces in ET ($P=0.000$). The total numbers of aerobes and *E. coli* were not affected by the treatments. Fungi were countable only in single cases and tended to be higher in faeces of the CT than of the ET. Isolated yeasts were solely strains of facultative anaerobic thermophilic yeast such as *Candida slooffiae*, which was detected most frequently and being without known pathogenic potential.

Table 2.3. Counts of bacterial populations, yeasts and fungi in the control treatment and the experimental treatment

	Total aerobes	<i>Escherichia coli</i>	Total anaerobes	Lactobacilli	<i>Clostridium perfringens</i>	Yeasts	Fungi
	lg CFU/g wet faeces						
CT*	6.91	2.44	7.90	7.62	5.24	0.99	1.01
	± 0.19	± 0.25	± 0.07	± 0.08	± 0.17	± 0.25	± 0.18
ET	7.13	2.11	8.60	7.88	0.96	2.82	0.48
	± 0.14	± 0.41	± 0.09	± 0.10	± 0.20	± 0.20	± 0.22
P values							
Trm	0.351	0.504	0.000	0.046	0.000	0.000	0.080

CFU, colony forming units; lg, logarithm to the base of 10; Trm, treatment

*CT, control treatment; ET, experimental treatment

2.4.3 Serological parameters

Results of the serological parameters quantified in the serum samples of CT and ET pigs are shown in Table 2.4. For CRP, a large variation was detected, ranging from 2.7 to 37.5 µg/ml. The mean CRP value of 17.7 ± 3.6 µg/ml for the pigs in ET and 9.9 ± 2.2 µg/ml for pigs in CT did not differ significantly ($P=0.101$). Antibodies against LPS showed a tendency towards a higher level in CT in comparison to ET pigs.

Table 2.4. Concentration of C-reactive protein and antibodies against lipopolysaccharides in the serum in the control treatment and the experimental treatment

	CRP µg/ml	IgA-a-LPS <i>E. coli</i> J5	IgM-a-LPS <i>E. coli</i> J5 RU/ml	IgG-a-LPS <i>E. coli</i> J5
CT*	9.9 ± 2.2	138.7 ± 40.5	483.7 ± 63.3	393.0 ± 52.4
ET	17.7 ± 3.6	118.6 ± 14.2	432.6 ± 35.9	370.8 ± 38.2
P values				
Trm	0.101	0.620	0.472	0.730

CRP, C-reactive protein; IgA(M, G)-a-LPS, antibody A (M, G) against lipopolysaccharide *Escherichia coli* J5; RU, relative units; Trm, treatment

*CT, control treatment; ET, experimental treatment

2.5 Discussion

2.5.1 Production data

Assuming a similar ratio of energy and protein in the nutrient supply for both treatments, the higher growth rate of the ET pigs could be attributed to a higher nutrient supply due to the intake by JA tubers, estimated to a mean dry matter intake of about 1.2 kg/day. Correspondingly, about 41% of the energy and 23% of the protein the ET pigs consumed were derived from the JA tubers. The high intake of JA tubers might be associated with pig's natural rooting behaviour⁽²⁵⁾ and indicates the high potential of JA tubers as nutrient source for pigs under free-range conditions. Similar results with respect to the growth rate of pigs when provided free access to JA tubers on the field were obtained by Farke & Sundrum⁽²⁶⁾. A high intake

of JA tubers by weaned pigs has been reported by Ly *et al.*⁽²⁷⁾, Ly *et al.*⁽²⁸⁾ and Piloto *et al.*⁽²⁹⁾. Significant differences in growth performance between pigs gender were observed only in the ET with 0.731 kg/day for castrated and 0.802 kg/day for female pigs. As the carcass composition was without gender-specific distinctions, the difference in DWG is attributable to a higher intake of JA tubers by the female pigs in comparison to the castrated pigs. This observation contradicts with the generally higher feed consumption of castrated pigs^(30,31). The results, however, provide no hints for further explanations. Feed conversion ratio of 3.78 kg concentrate/kg body weight gain in the CT is comparable with results obtained by finishing pigs housed indoors⁽³²⁾. Certainly, the feed conversion ratio must be considered in the context of the free-range system. In general, pigs in outdoor systems require more energy to compensate for an increased activity and the higher exposure to ambient temperatures^(33,34). Because of the free access to JA tubers, a conclusive feed conversion ratio could not be quantified for the ET.

Due to the high acceptance of JA tubers, the ET pigs' ingestion of inulin and FOS came to a level of up to 800 g/day. Since inulin and FOS naturally occur in plants as a means of storing energy and some grains are known as sources of short-chain fructans⁽³⁵⁾, an additional ingestion of prebiotic ingredients was caused by the concentrate the pigs consumed. In barley fructan contents amount from 50 to 100 g/kg DM and in wheat from 7 to 29 g/kg DM^(36,37). Lupines contain up to 120 g/kg DM galacto-oligosaccharides, which are reported to have prebiotic properties comparable to those of inulin and FOS^(38,39). Therefore, the concentrate with barley, wheat and lupines (in all 70% of the diet) affected an ingestion of prebiotic effective ingredients estimated to 30 g/kg DM of the diet. In studies, investigating the effect of inulin and FOS in pigs as a prebiotic feed additive, supplement quantities range from 3 to 10 g/day or from 15 to 40 g/kg of the total diet^(7,8,40).

With almost 400 g/kg mineral components in the DM, the mean content of ash in the faeces of the pigs was remarkably high in both treatments, noting that concentration in faeces of pigs accommodated indoors ranged from 120 to 240 g/kg in the DM (own observation, unpublished). These results can be reasonably explained by the consumption of soil particles in large quantities. Information

regarding soil ingestion of free-ranged pigs is scarce⁽⁴¹⁾. In faeces of sows kept on pasture Rivera Ferre *et al.*⁽⁴²⁾ found ash contents rising from 200 g/kg to 400 g/kg DM when animals did not wear nose rings and rooting occurred. The high earth consumption in this study impeded the initial designated determination of JA tubers intake with titanium dioxide (TiO₂), which has proven to be a suitable inert marker for digestibility studies in indoor housed pigs^(43,44). As the consumed soil already contained titanium oxide, the analysed TiO₂ content in pigs faeces was to a high degree forged by soil intake. Therefore, TiO₂ has shown to be inappropriate as an inert marker in pigs under conditions of free-range systems.

2.5.2 Changes within microbiota

Providing free access to JA tubers resulted in a significant increase in counts of anaerobe bacteria, lactobacilli and yeasts and in a drastic reduction of *C. perfringens* in the faeces. The results contradict with the results of other studies examining microbiota of the GIT when inulin and FOS are supplemented to pigs. In investigations of Loh *et al.*⁽⁸⁾, Branner *et al.*⁽¹³⁾, Mountzouris *et al.*⁽¹⁴⁾ and Eberhard *et al.*⁽¹⁵⁾, the application of 10 to 30 g inulin/kg to the concentrate did not alter microbiota in pigs in all examined sections of the GIT. Also, the supplementation of a piglet's diet with 40 g FOS/kg had no effect on anaerobic bacteria, lactobacilli and enterobacteria in pigs faeces⁽⁶⁾. Only in the case of yeasts, a significant increase has been detected^(5,6).

Significant impacts on the caecal or colonic microbiota in pigs via the application of inulin or FOS were found only for single bacterial populations or by the synergistic effect of pro- and prebiotica respectively two prebiotic substances^(9,16-19). Tako *et al.*⁽¹⁸⁾ found increased counts of *Lactobacillus* and *Bifidobacterium* populations by providing pigs with inulin at a level of 40 g/kg of the concentrate, whereas in amounts of *E.coli* and *Clostridium* no differences were detected. At Nemcová *et al.*⁽¹⁶⁾ pigs were supplied with 3 g FOS/day and 2 g *Lactobacillus paracasei*/day. This led to a significant increase of total aerobes and anaerobes, bifidobacteria and lactobacilli in relation to the untreated treatment and to the

treatment with only 2 g *L. paracasei*/day. At the same time, counts of clostridia and Enterobacteriaceae were significantly reduced towards the untreated group. Similar results were obtained by Bomba *et al.*⁽¹⁷⁾ combining probiotics and FOS. At Lynch *et al.*⁽⁹⁾ the additional administration of 15 g inulin/kg to a diet supplemented with 230 g lactose/kg led to significant increased counts of lactobacilli and reduced counts of enterobacteria in piglets.

Possible reasons for an unaffected colonic microbiota in the studies mentioned above are considered with respect to the praecaecal digestibility of inulin and FOS in pigs or prebiotic substances derived from the concentrate. Praecaecal degradation of inulin and FOS in pigs has been approved in several studies and attributed to acid hydrolysis in the stomach and to the microbiota in the upper gut^(13,28,45,46). While Mikkelsen *et al.*⁽⁴⁷⁾ found a praecaecal degradation of 0.20 for FOS in piglets, Branner *et al.*⁽¹³⁾ and Böhmer *et al.*⁽⁴⁶⁾ determined values of 0.98 resp. 0.57 for the praecaecal digestion of inulin in growing pigs. In the current study, JA has proved to be an effective prebiotic in the porcine LI, demonstrating its effectiveness on faecal microbiota when being administrated in large quantities.

Considering that the crude ash levels in pig's faeces were almost 400g/kg DM, the bacterial counts, which are generally referred to g of wet faeces, have probably been downscaled in both treatments. Comparisons of the magnitude in CFU counts with other observations are difficult, because up to now only few studies have examined the colonic microbiota in finishing pigs while data of faecal ash contents are not specified^(23,24,48).

Changes in the counts of *C. perfringens* and yeasts between CT and ET are remarkable. *C. perfringens* is known to be potentially harmful pathogenic through its proteolytic capabilities and toxin production⁽⁴⁹⁾. Therefore, the obvious reduction of *C. perfringens* by intake of JA represents a clear improvement in the status of eubiosis. This may be a result of the inability of oligofructose to act as nutrient source for *C. perfringens* and could be probably attributed to inulin and FOS related stimulation of beneficial bacteria⁽⁵⁰⁾. Several *Lactobacillus* strains of the porcine and poultry GIT show a high effectiveness against growth of *C. perfringens* and *E. coli*^(51,52). Furthermore, Wang & Gibson⁽¹⁰⁾ demonstrated in batch culture

experiments, particularly when oligofructose was used as carbon and energy source, that acidification via excretion of acetate and lactate by *Bifidobacterium infants* inhibits the growth of *C. perfringens* and *E. coli*.

Increase of yeasts in ET pigs in the current study illustrates their ability to produce inulinase and therefore, utilize inulin as nutrient substrate. Mikkelsen & Jensen⁽⁶⁾ and Mikkelsen *et al.*⁽⁵³⁾ presented in their studies a strong effect of FOS on the number of yeast throughout the GIT in faecal samples from piglets. An increase in the gastrointestinal population of yeast has been observed in piglets fed a fermented liquid diet containing a high concentration of lactic acid⁽⁵⁴⁾. Little is currently known concerning the potential effects of yeasts on criteria of animal health and performance. Some strains are suspected to cause disorders of the digestive tract in humans⁽⁵⁵⁾. Other yeasts are able to resist against invading pathogens by binding toxins and pathogenic bacteria and help stimulate the immune system^(56,57). The most frequently isolated species in this study, *C. slooffiae*, is commonly occurring in the GIT of pigs and possesses no pathogenic potential⁽⁵⁸⁾.

2.5.3 Immunological parameters

Assessment of CRP, IgA-anti-LPS, IgM-anti-LPS and IgG-anti-LPS in the serum of pigs did not show significant differences between treatments. Measured CRP values of the CT and the ET with a maximum of 37.5 µg/ml were in the range of healthy pigs reaching up to 50 µg/ml⁽⁵⁹⁻⁶²⁾. Tendency to a slightly higher CRP value in ET pigs might be related to the occurrence of yeasts on a high level (W Schrödl, personal communication, 2009). Carbohydrates of inulin and FOS can stimulate the immune system through interaction with immune cells, exhibiting the complement carbohydrates receptor. Soluble beta-glucans, derived from cell walls of yeasts, are also particularly potent stimulators of these receptors^(11,12).

Levels of antibodies against LPS, especially of IgA-anti-LPS, do not imply increased occurrence of pathogenic bacteria with their pathogen-associated molecular pattern like LPS, lipoteichoic acid and special motifs of deoxyribonucleic acid in the CT (W Schrödl, personal communication, 2009)⁽⁶³⁾.

2.5.4 Further benefits

A desirable side effect of JA tubers in pig nutrition arises from their influence on boar taint. According to Hansen *et al.*⁽⁶⁴⁾, supplementing finishing pigs with inulin-rich chicory roots or inulin extracted from the roots implying an inulin intake up to 450 g/day reduced scatole and androsterone levels to almost zero. This effect is supposed to be mainly based on microbial changes in GIT associated with inulin and FOS and the suppression of bacteria producing putrefactive products like skatole, indole and p-cresol in the caecum and colon⁽⁶⁵⁾. Therefore, influence of JA tubers on meat quality might be a promising research topic.

2.6 Conclusions

Abundant availability of inulin and FOS in pigs GIT led to a significant increase in the counts of total anaerobes, lactobacilli and yeasts and drastically reduces the counts of *C. perfringens* in pig's faeces. Changes in intestinal microbiota imply a clear improvement in status of eubiosis. Large consumption of soil particles in both treatments requires soil analyses before free-ranged pigs are placed in an area to protect them from soilborne health burdens such as heavy metals and from risks concerning food safety. On the other hand, increased growth rates of pigs with *ad libitum* access to JA tubers indicate the tuber's suitability as a valuable nutrient source for free-ranged pigs.

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3 Chapter 2.

Feeding steamed potatoes and steamed-ensiled potatoes to finishing pigs alter the faecal microbiota and faeces composition

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3.1 Abstract

Heat treated potatoes (*Solanum tuberosum*) appear to be particularly appropriate for pig nutrition. Since implications on the intestinal microbiota had only been investigated for potato starch as a constituent of the concentrate, the present study aimed to determine effects of freshly steamed and steamed-ensiled potatoes offered in high amounts to finishing pigs on intestinal microbiota in pigs' gastrointestinal tract (GIT). A total of 58 finishing pigs were assigned to a control treatment (CT), fed with a concentrate mixture formulated to meet animals requirements, and two experimental treatments consisting of a potato treatment (PT) and a silage treatment (ST). The PT and ST received only 46% and 43% respectively of the concentrate amount apportioned to the CT, but were additionally provided with 1.2 kg dry matter (DM)/day steamed potatoes and potato silage respectively. Criteria of performance and carcass showed no significant differences between treatments. In the PT and ST, pigs' faeces were significantly lower in pH value and contents of DM, neutral detergent fibre (NDF), undigested dietary nitrogen (UDN) and partially acid detergent fibre (ADF) ($P=0.000$) and higher in contents of ammonium (NH_4) and ammonium bound nitrogen ($\text{NH}_4\text{-N}$) ($P=0.000$). High provision of steamed potatoes and potato silage respectively reduced *Escherichia coli* ($P=0.000$), *Clostridium perfringens* ($P=0.000$) and antibodies against lipopolysaccharides (LPS) of *E. coli* J5 ($P=0.001$) remarkably. Further, in the ST total aerobes, total anaerobes, lactobacilli and yeasts in the first sampling period were significantly increased towards the PT. The changes in the intestinal microbiota and presence of antibodies imply beneficial effect of thermal processed potatoes on intestinal microbiota in pigs.

3.2 Introduction

Potatoes (*Solanum tuberosum*) appear to be particularly appropriate for pig nutrition. The tubers are of a low fibre content and consist mainly of starch, which constitutes a good energy source for pigs^(1,2). In addition, the protein of potatoes possesses a high biological value and contributes to the supply of total nitrogen (N) and of essential amino acids^(1,2). The use of potatoes in pig nutrition has declined through the past decades, even though cull potatoes or those from excess production are available on the local market, frequently affordable. As a consequence of the increasing costs for cereals in the past years, potatoes could gain renewed interest as an alternative for cereal based diets in pig nutrition.

To improve gut health, the application of prebiotic food additives has gained increased interest in human and animal nutrition. Resistant starch (RS) is known to be an effective prebioticum and behaves as a growth substrate for probiotic microorganisms, since it escapes the endogenous digestion of mammals and is fermented by the intestinal microbiota^(3,4). In various studies on pig and humans a time-dependent shift in faecal and large bowel short chain fatty acids (SCFA) profiles has been revealed, suggesting the possible interaction of RS with the ingested bacteria^(3,5). High amounts of RS are contained in raw potatoes^(5,6). Supplementing raw potato starch to pigs' diets has been proved to enhance microbial fermentation in the large intestine (LI) with the proliferation of nonproteolytic bacteria and rise in SCFA production, to reduce colonocytes apoptosis by increased butyrate production and to prevent postweaning diarrhoea⁽⁷⁻¹²⁾.

However, in pig nutrition raw potatoes are associated with a particularly poor nutrient digestibility and reduced performances^(1,2). By heat treatment the composition of the starch can be modified through the process of gelatinisation and becomes well digestible⁽¹³⁾. The starch and N of heat treated potatoes can contribute considerably to the energy and protein supply of pigs⁽¹⁾. In most studies, the use of potatoes is referred to the application of raw or pregelatinised potato starch as feed additive in small amounts to piglets or growing pigs' diets^(8,9,11,14,15). In contrast, the provision of high amounts of heat treated potatoes and its effects on intestinal

microbiota in pigs have so far not been investigated. As ensiling of steamed potatoes is a favourable possibility to make potatoes storable and to ensure availability throughout the year⁽¹⁾, the present study aimed to investigate the effects of steamed potatoes and steamed and ensiled potatoes respectively on the intestinal microbiota and on selected parameters of the immune system, when being fed in large quantities to finishing pigs.

3.3 Materials and methods

3.3.1 Animals, diets and experimental design

A total of 58 crossbred pigs (Danish Landrace x Hampshire x Duroc) were assigned to a control treatment (CT) and two experimental treatments. The CT enclosed 20 pigs (10 males and 10 females) and was fed with a concentrate mixture. The experimental treatments consisted of a potato treatment (PT) of 19 pigs (8 males and 11 females), fed with concentrate and steamed potatoes, and of a silage treatment (ST) of 20 pigs (of 12 males and 8 females), fed with concentrate and potato silage. In the CT the concentrate was formulated to meet the requirements of the pigs in relation to nutrients and energy to a performance level of 700 g daily weight gain (DWG)⁽¹⁶⁾. The concentrate was composed of 488 g/kg barley, 195 g/kg peas, 97 g/kg soybean cake, 97 g/kg oats, 68 g/kg rapeseed cake, 24 g/kg mineral mix, 20 g/kg monocalcium phosphate, 10 g/kg calcium carbonate and 1 g/kg oil, resulting in 14.3 megajoule metabolisable energy (MJ ME)/kg and 164 g/kg crude protein (CP) in the dry matter (DM). Pigs in the PT and the ST received only 46% and 43% of the concentrate apportioned to the CT respectively, but were additionally provided with 1.2 kg DM/day steamed potatoes or steamed ensiled potatoes per animal. Steamed potatoes contained 14.0 MJ/kg ME and 92 g/kg CP in the DM (Table 3.1). Potato silage amounted to 13.9 MJ/kg ME and 116 g/kg CP in the DM. To predict pigs individual intake of steamed potatoes and potato silage, the concentrate was supplemented with the inert marker titanium dioxide (TiO₂) in the amount of 1 g/kg and offered once daily in individually segregated troughs, while the steamed potatoes and potato silage were fed twice daily to the entire group. Enzyme digestible organic

matter (EDOM) in the concentrate, the steamed potatoes and the potato silage were ascertained by the method of Boisen & Fernandez⁽¹⁷⁾ and resulted on a DM basis in a degradability of 809 ± 3 g EDOM/kg organic matter (OM) for the concentrate, 893 ± 14 g EDOM/kg OM for the steamed potatoes and 854 ± 1 g EDOM/kg OM for the potato silage.

Table 3.1. Ingredients, digestibility and pH value of the concentrate, the steamed potatoes and the potato silage

Analysed composition	Concentrate	g/kg DM	
		Steamed potatoes	Potato silage
DM (g/kg FM)	890	191	193
CA	77	72	64
CP	164	92	116
CL	49	6	8
CF	53	38	41
NDF	328	417	231
ADF	102	99	129
ADL	23	13	26
Starch	459	646	436
Sugar	39	6	51
OM	923	928	936
EDOM (g/kg OM)	809	893	854
OR	186	146	335
pH value	-	5.80	4.06
Energy (MJ ME/kg)	14.3	14.0	13.9

ADF, acid detergent fibre; ADL, acid detergent lignin; CA, crude ash; CF, crude fibre; CL, crude fat; CP, crude protein; EDOM, enzyme digestible organic matter; ME, metabolisable energy; MJ, megajoule; NDF, neutral detergent fibre; OM, organic matter; OR, organic rest

3.3.2 Data collection and sampling

In the finishing period, starting with an average live weight of 79.2 ± 15.1 kg, daily weight gains were quantified for each pig. On day 11, 13 and 15 (first sampling period) and on day 38, 40 and 42 (second sampling period) after beginning of the finishing period faecal samples were collected from the rectum of each animal. Samples were filled in disposable containers with screw cap (Sarstedt, Nümbrecht, Germany) and kept frozen below -20°C until analysed. For bacteriological investigation faecal sub-samples were taken on day 11 and 42 and frozen in faeces

tubes (Sarstedt, Nümbrecht, Germany). For procedural reasons it was not possible to conduct microbial analysis of fresh faecal samples. Although it cannot be excluded that freezing and defrosting affect microbial counts, it is expected that the impact concerns the faecal samples in both treatments to the same degree⁽¹⁸⁻²⁰⁾. Pigs were slaughtered with an average body weight of 131.2 ± 14.2 kg. Dressing percentage was calculated on the basis of a pig's live weight one day before slaughter and the carcass weight. Blood samples were taken during the slaughter process and filled into blood collection tubes with a clot activator (Sarstedt, Nümbrecht, Germany). After centrifugation (10 min, 5000g) the serum was pipetted in micro tubes (Sarstedt, Nümbrecht, Germany) and frozen at -20°C until analysing.

3.3.3 Parameters determined in faeces

PH was measured in defrosted and carefully homogenised samples at room temperature, using a digital pH-meter type 640 (Knick, Berlin, Germany) with a combination pH puncture electrode (SE 104, Knick, Berlin, Germany). For contents of DM, neutral detergent fibre (NDF), undigested dietary nitrogen (UDN), acid detergent fibre (ADF), total carbon (C), total N, ammonium (NH_4) and ammonium-bound nitrogen ($\text{NH}_4\text{-N}$), samples were dried at 60°C for 72 hours, milled through an 1 mm sieve and scanned by near infrared reflectance spectroscopy. Calculation values of NDF, ADF and ADL were developed by the method of Van Soest *et al.*⁽²¹⁾. Ash weights were determined by incinerating the dried samples in a muffle furnace at 550°C for 5 h. TiO_2 concentration in the faeces and the concentrate was determined photometrically in 1 g Kjeldahl digested samples by the modified method of Brandt & Allam⁽²²⁾. Intake of steamed potatoes and potato silage by the pigs of the two experimental treatments was estimated by the equation of Piasentier *et al.*⁽²³⁾:

$$HI = [D / F - Is(1 - OMD_s)] / (1 - OMD_h)$$

with HI = intake of the herbage (kg OM/day), D = intake of the external marker (mg/day), F = faecal concentration of the external marker (mg/kg OM), Is = quantity

of the supplement (kg OM/day), OMD_s = organic matter digestibility of the supplement and OMD_h = organic matter digestibility of the herbage. Subsequent intake of steamed potatoes and potato silage were converted into kg DM/day.

3.3.4 Bacteriological investigations

Faecal samples were analysed for total aerobes, *Escherichia coli*, total anaerobes, *Clostridium perfringens*, lactobacilli, bifidobacteria and yeasts. From a suspension of 0.5 g of wet faeces and 4.5 ml of a sterile phosphate buffered saline solution (pH 7.4), dilution series (10^{-1} to 10^{-7}) were produced. Subsequently, 10 μ l of each dilution were spread onto agar plates with special media (blood, Gassner agar, Sabouraud, MacConkey, SIFIN, Berlin). For anaerobe bacteria, plates were cultivated under anaerobic conditions. After cultivation the last two covered agar plates were used for counting the colony-forming units (CFU) per g wet faeces and counts are expressed as logarithm to the base of 10 (lg) CFU/g of wet faeces. Yeast strains were additionally counted. For purposes of statistical analysis in samples that had no colonies at the detection limit of lg 3 CFU/g, which conforms to 1000 CFU/g, a value of either 0.00 or 2.99 was assigned. As the results obtained from the calculation with the different values for samples below the detection limit did not change ascertained significances, calculations were conducted with 0 for the corresponding samples.

3.3.5 Immunological investigations

Haptoglobin (Hp), C-reactive protein (CRP) and the specific immunoglobulin A (IgA) to lipopolysaccharides (LPS) of *E. coli* strain J5 (IgA-anti-LPS) in the serum samples were determined quantitatively with enzyme-linked-immunosorbent assay (ELISA) as described previously⁽²⁴⁾. The porcine Hp standard for the ELISA was isolated by affinity chromatography after covalent immobilisation of the rabbit anti-human Hp antibodies on Sepharose 4 (Pharmacia, Sweden). The porcine CRP standard was purified to the method described by Sarikaputi *et al.*⁽²⁵⁾. The porcine IgA in the anti-LPS-IgA-ELISA was detected with affinity purified IgG (sheep) anti-

porcine IgA conjugated with horseradish peroxidase (Bethyl Laboratory, Inc., USA). The intra- and interassay coefficients of variance were <10% and <15%.

3.3.6 Statistical analysis

Statistical analysis was conducted using the statistical program SPSS 17.0 of Windows (Version 17.0.1.1). Data were tested for normal distribution by the Kolmogorov-Smirnov test and for homogeneity of variance by Levene's test. Repeated samplings within a week were averaged for each animal. To determine differences between the groups with regard to the repeated sampling periods and animals' sex, data were analysed with repeated measures ANOVA with the effects for the treatment, sex and their joint effect. As significance of sex and the interaction of treatment and sex were only detectable for growth performance data and carcass composition, presentation of statistical significances for the remaining parameters were conducted only for the effect treatment. P values between groups within a week were determined by one-way ANOVAs *post hoc* test. Data with inhomogeneities in variance were analysed by nonparametric tests for independent samples. Differences were considered to be significant at $P < 0.05$.

3.4 Results

3.4.1 Production data

The apportioned quantity of the concentrate, the steamed potatoes and the potato silage were consumed completely within the experimental phase. In CT, the nutrient and energy consumption by the concentrate amounted to 36.2 MJ ME and 415 g CP/day (Table 3.2). In the experimental treatments, nutrient and energy intake by the concentrate and the preserved potatoes was assessed to be on average 33.8 MJ ME and 303 g CP/day in the PT and 31.3 MJ ME and 310 g CP/day in the ST.

Table 3.2. Nutrient and energy intake by the apportioned concentrate, the steamed potatoes and the potato silage distinguished by the treatment

Feed intake	CT*	PT		ST	
	Concentrate	Concentrate	Steamed potatoes (g/day)	Concentrate	Potato silage
Intake (kg DM/day)	2.5	1.2	1.2	1.1	1.2
CA	194		177		156
CP	415		303		310
CL	124		64		62
CF	135		109		104
NDF	819		894		616
ADF	254		240		257
ADL	56		42		58
Starch	1161		1329		992
Sugar	99		53		101
OM	2336		2213		2064
OR	470		395		585
Energy (MJ ME/kg)	36.2		33.8		31.3

ADF, acid detergent fibre; ADL, acid detergent lignin; CA, crude ash; CF, crude fibre; CL, crude fat; CP, crude protein; DM, dry matter; EDOM, enzyme digestible organic matter; ME, metabolisable energy; MJ, megajoule; NDF, neutral detergent fibre; OM, organic matter; OR, organic rest

*CT, control treatment; PT, potato treatment; ST, silage treatment

Growth performance data and carcass traits, presented in Table 3.3, were not significantly influenced by feeding regime. Despite the lower energy and nutrient supply, pigs of the experimental treatments showed with 0.734 ± 0.113 kg/day in the PT and 0.610 ± 0.103 kg/day in the ST higher DWGs in comparison to CT pigs with 0.564 ± 0.121 kg/day. A significant effect on DWG could be observed for the interaction of treatment and sex ($P=0.003$). LMC was significantly higher in female pigs (55.4 ± 3.7) compared to the castrated pigs (52.2 ± 3.1 ; $P=0.034$).

Table 3.3. Means of growth performance data in the finishing period, lean meat content and dressing percentage distinguished by the treatment

	Initial body weight kg	Final body weight kg	DWG kg/day	LMC	Dressing percentage %
CT*	78.4 ± 11.1	123.6 ± 8.0	0.564 ± 0.121	55.8 ± 3.7	77.2 ± 4.5
PT	77.7 ± 15.8	140.4 ± 15.9	0.734 ± 0.113	53.5 ± 4.4	75.3 ± 4.1
ST	81.0 ± 18.1	132.2 ± 13.7	0.610 ± 0.103	53.1 ± 2.6	75.9 ± 2.8
P values					
Trm			0.294	0.092	0.241
Sex			0.806	0.034	0.333
Trm*sex			0.003	0.741	0.674

DWG, daily weight gain; LMC, lean meat content, Trm, treatment

*CT, control treatment; PT, potato treatment; ST, silage treatment

3.4.2 Parameters determined in the faeces

The composition of the faeces was highly influenced by the provision of potatoes and led to significantly lower values of pH, DM, NDF and UDN and increased amounts of NH_4 and $\text{NH}_4\text{-N}$ in the faeces of pigs from the PT and the ST in comparison to the CT (Table 3.4). A significantly increased ash content was observed in the ST in the first sampling period. In the second sampling period ash content was significantly lower in the PT and the ST towards the CT. ADF concentration in pigs' faeces tended to be lower in the experimental treatments, but a significant decrease towards the CT appeared only in the first sampling period for the ST and in the second sampling period for the PT. The significantly highest C concentrations occurred in the PT and in the second sampling period a significantly higher amount was detected in the PT towards the CT.

Between the experimental treatments, there was a higher pH value, with being significant only in the first sampling period, and a significantly increased NH_4 and $\text{NH}_4\text{-N}$ concentrations in faeces of pigs from the ST compared to the PT.

Table 3.4. PH values and contents of dry matter, ash, neutral detergent fibre, acid detergent fibre, carbon and nitrogen, ammonium and ammonium-bound nitrogen in pigs faeces distinguished by the treatment and sampling period

	pH	DM g/kg FM	Ash	NDF	ADF	C	N	UDN	NH ₄	NH ₄ -N
			g/kg DM							
First sampling period										
CT*	6.40 ^a	305 ^a	220 ^a	476 ^a	283 ^a	391 ^a	27.0 ^a	5.0 ^a	3.1 ^a	2.4 ^a
PT	5.74 ^b	204 ^b	207 ^a	443 ^b	285 ^a	416 ^b	25.7 ^b	4.7 ^b	4.2 ^b	3.3 ^b
ST	5.95 ^c	193 ^b	246 ^b	368 ^c	261 ^b	384 ^a	27.9 ^a	4.4 ^c	6.1 ^c	4.7 ^c
Second sampling period										
CT	6.41 ^a	316 ^a	242 ^a	446 ^a	280 ^a	373 ^a	28.2	5.1 ^a	2.6 ^a	2.0 ^a
PT	6.02 ^b	194 ^b	219 ^b	409 ^b	268 ^b	408 ^b	27.9	4.5 ^b	4.4 ^b	3.4 ^b
ST	6.19 ^b	198 ^b	227 ^b	395 ^c	275 ^{ab}	389 ^c	28.0	4.5 ^b	5.6 ^c	4.4 ^c
P values										
Trm	0.000	0.000	0.000	0.000	0.011	0.000	0.000	0.000	0.000	0.000

ADF, acid detergent fibre; C, carbon; DM, dry matter; FM, fresh matter; N, nitrogen; NDF, neutral detergent fibre; NH₄, ammonium; NH₄-N, ammonium-bound nitrogen; Trm, treatment; UDN, undigested dietary nitrogen

*CT, control treatment; PT, potato treatment; ST, silage treatment

3.4.3 Microbiota in faeces

Numbers of individual bacterial populations determined in the faeces of pigs in the control and experimental treatments are presented in Table 3.5. Bacterial counts were highly influenced by the provision of either steamed potatoes or potato silage, which caused a significant reduction in counts of *C. perfringens* and *E. coli* towards the CT. Further, in the faeces of pigs of the PT, numbers of total anaerobes and lactobacilli were significantly lower in comparison to the ST and to the CT in the first sampling period. Significantly highest counts of total aerobes and total anaerobes were ascertained in the ST. For yeasts, there was a significant increase detectable in the ST in the first sampling period towards the other treatments.

Table 3.5. Counts of bacterial populations and yeasts in pigs faeces distinguished by the treatment and sampling period

	Total aerobes	<i>Escherichia coli</i>	Total anaerobes	<i>Clostridium perfringens</i>	Lactobacilli	Yeasts
	lg CFU/g wet faeces					
First sampling period						
CT*	5.81 ^a	3.61 ^a	7.97 ^a	2.48 ^a	8.12 ^a	0.99 ^a
PT	5.64 ^a	0.16 ^b	7.16 ^b	0.00 ^b	7.20 ^b	0.16 ^a
ST	6.58 ^b	3.13 ^a	8.43 ^c	0.15 ^b	8.14 ^a	2.51 ^b
Second sampling period						
CT	7.01 ^a	3.74 ^a	7.90 ^a	3.61 ^a	7.81 ^a ^b	0.50
PT	6.89 ^a	0.83 ^b	7.74 ^a	1.28 ^b	7.57 ^a	0.32
ST	8.03 ^b	0.83 ^b	8.24 ^b	0.15 ^c	8.06 ^b	0.89
P values						
Trm	0.000	0.000	0.000	0.000	0.000	0.000

CFU, colony forming units; lg, logarithm to the base of 10; Trm, treatment

*CT, control treatment; PT, potato treatment; ST, silage treatment

3.4.4 Serological parameters

Results of the serological parameters quantified in the serum samples of pigs from the control and the experimental treatments are demonstrated in Table 3.6. Hp and CRP concentrations showed a large variation, ranging from 0.01 to 4.10 mg/ml for Hp and from 2.5 to 73.8 µg/ml for CRP. For both parameters, a tendency towards a higher level in the experimental treatments was identifiable, but values did not differ significantly. The concentration of IgA against LPS of *E. coli* J5 in the serum of pigs from the CT was significantly higher than in the PT.

Table 3.6. Concentration of Haptoglobin, C-reactive protein and immunoglobulin A against lipopolysaccharides of *Escherichia coli* J5 in the serum of the control treatment and the treatments with steamed potatoes and potato silage

	Hp mg/ml	CRP µg/ml	IgA-a-LPS <i>E. coli</i> J5 RU/ml
CT*	0.55	7.31	257.0 ^a
PT	0.66	14.52	116.6 ^b
ST	0.69	8.77	119.6 ^b
P values			
Trm	0.715	0.107	0.001

Hp, Haptoglobin; CRP, C-reactive protein; IgA-a-LPS, antibodies A against lipopolysaccharide of *E. coli* J5; RU, relative units; Trm, treatment

*CT, control treatment; PT, potato treatment; ST, silage treatment

3.4.5 Estimation of potato intake

Distinct lower TiO₂ concentrations were found in faeces of pigs from the experimental treatments in both sampling periods (P=0.000; Table 3.7). Based on the mean TiO₂ content in the CT, the faecal recovery of TiO₂ was estimated to be on average 86.0%. The calculation of potato intake in the experimental treatments by the formula of Piasentier *et al.*⁽²³⁾ for the individual pigs resulted the huge variation of -0.48 to 21.12 kg DM/day. On average, the intake in the PT amounted to the high quantity of 1.55 kg the first and 1.58 kg DM/day in the second sampling period and in the ST in to the low level of 0.64 kg in the first and 0.81 kg DM/day in the second sampling period. Regarding the observed faecal titanium dioxide recovery of 86.0% in the CT, the calculated ingestion of potatoes would further decrease. In the PT, the intake of steamed potatoes would average to 1.06 kg DM/day in both sampling periods and in the ST the intake of potato silage to 0.35 kg DM in the first and 0.49 kg DM/day in the second sampling period (values not included in Table 3.7).

Table 3.7. Titanium concentration in the faeces and intake of steamed potatoes and potato silage distinguished by the treatment and sampling period

	TiO ₂ g/kg DM	Intake potato kg DM/day	Intake potato kg FM/day
First sampling period			
CT*	4.12 ^a	0.62 ^a	3.23 ^a
PT	3.12 ^b	1.55 ^b	8.06 ^b
ST	3.28 ^b	0.64 ^a	3.33 ^a
Second sampling period			
CT	4.40 ^a	0.68 ^a	3.56 ^a
PT	3.05 ^b	1.58 ^b	8.01 ^b
ST	3.26 ^b	0.81 ^a	4.27 ^a
P values			
Trm	0.000	0.000	0.000

DM, dry matter; FM, fresh matter; TiO₂, titanium dioxide; Trm, treatment

*CT, control treatment; PT, potato treatment; ST, silage treatment

3.5 Discussion

3.5.1 Performance data

Diets were calculated for an isoenergetic nutrient supply and a comparable quantity of potatoes (on FM basis) in both experimental treatments. Nevertheless, it has to be considered that disparities in the energy supply between treatments cannot be excluded completely due to variations in DM, and energy concentrations in the applied feeding stuffs, as concentrate, steamed potatoes, and potato silage had to be proportioned in the stable on fresh matter basis. Furthermore, potatoes were offered in group feeding while the concentrate was offered individually in segregated troughs. The apportioned diet had been completely consumed and the low pH level of the potato silage apparently had no adverse effect on the acceptance by the pigs. The latter has been described by Partanen & Morz⁽²⁶⁾ when diets high in acids with a strong odour and flavour were fed to growing pigs.

Criteria of performance and carcass showed no significant differences between treatments. However, the standard deviation indicated a high variance between pigs, possibly due to the restricted group feeding in the first place⁽²⁷⁾. Nevertheless, the low growth performance of the pigs in the CT was unexpected. In PT and ST, about

51% of the consumed energy and 38% and 43% respectively of the consumed protein resulted from the provided potatoes. While the addition of organic acid to pigs' diet can lead to a higher growth performance^(26,28), the highest DWG were achieved by the pigs in PT. The lower dressing percentage of pigs in the experimental treatments might be due to the fact that pigs were weighed one day before slaughtering without an emptied digestive tract and hence with the ingested potatoes. On the other hand, it is well known that the use of voluminous and succulent feeds has the potential to increase the weight of the digestive tract in pigs^(29,30).

3.5.2 Faecal composition

Analytical composition of pigs' faeces was influenced significantly by the feeding regime. The lower DM content and pH value in pigs from the experimental treatments appear to result from a higher SCFA production in the LI, since a direct influence by acidity of the potato silage on faecal pH value can be excluded^(26,31). Moreover, it seems likely that fermentation of a considerable part of the diet in the hindgut declined colonic pH value in comparison to the CT. Due to a higher osmotic gradient in the colon of PT and ST pigs, it is expected that more water was released into the colon lumen and led to a reduction in faecal DM content⁽³²⁾. Indeed, it is well known that relevant amounts of RS, a fraction which resists the endogenous digestion and constitutes a valuable energy source for the bacteria in the lower gut, are only contained in raw potatoes⁽⁵⁾. In heat treated potatoes the starch granules become gelatinised and the RS content decreases to a scale of 1.2 to 3.4% of the potato DM and might increase by subsequent cooling under the process of starch retrogradation again to a range from 4.6 to 7.4% in the DM^(6,33). The amount of retrograded RS in the processed potatoes of this study were scarcely to assess, as retrogradation is a dynamic process, which is dependent on factors such as cooking temperature and time^(6,33). Since RS is to some degree part of all starch containing feed^(34,35), ingredients of the concentrate effected an additional ingestion of RS. On the other hand, the amounts of RS in the single compounds of the concentrate are also expected to diverge wildly. While Englyst *et al.*⁽³⁶⁾ declared RS amounts in barley, triticale and oat to be negligible, other studies ascertained RS content in

barley to 130 g/kg DM, in field peas to a range from 21 to 220 g/kg DM and in soybeans to 12 g/kg DM^(37,38).

In pigs fed solely with potatoes subjected to different heat treatments an amount of 8 to 15% of the ingested starch has reached the caecum undigested and was subsequently completely degraded by the bacteria in the LI⁽²⁾. Regarding the considerable amounts of potatoes provided to the pigs in the experimental treatments and the reduction in pH value and DM content in these treatments, it seems likely that a certain proportion of the starch from the ingested potatoes escaped ileal digestion and was utilised by the intestinal microbiota.

The lower NDF, UDN and in parts lower ADF contents in the faeces from the experimental treatments presumable reflect the high degradability of heat treated potatoes, as the cell wall structure highly influences OM degradability and the UDN fraction consists solely of indigestible food residues⁽³⁹⁾. In the ST, the lower NDF ingestion certainly contributed to the significant reduced faecal NDF concentration.

The N concentration in pigs' faeces was almost unaffected by the dietary treatment, except for the PT in the first sampling period. An increased fermentation activity in the LI usually causes a shift in N excretion from urine to faeces and raises the faecal N output^(39,40). However, the alteration in N containing fractions towards a higher NH₄ and NH₄-N content in the faeces of pigs from the experimental treatments indicate an enhanced N mineralisation process due to activity of the intestinal microbiota. The shift in N excretion patterns in the experimental treatments might have been overlaid by the higher protein supply in the CT.

3.5.3 Bacterial counts

The bacterial populations in pigs' faeces were significantly influenced by the feeding treatment, effectuating a remarkable reduction in counts of *E. coli* and *C. perfringens* with the provision of steamed potatoes or potato silage. Moreover, in the ST numbers of total aerobes, total anaerobes, lactobacilli and the counts of yeasts in the first sampling period were increased in comparison to the PT.

C. perfringens and *Clostridium difficile* are the principal pathogenic clostridia of swine⁽⁴¹⁾. *C. perfringens* is known to be potentially harmful through its proteolytic capabilities and the production of toxins. The *C. perfringens* types A and C are most common causes of swine diseases^(41,42). Small numbers of *C. perfringens* type C can occur in the gut of healthy animals, but they possess the ability to proliferate and induce diseases under appropriate conditions. The site of the highest occurrence of *C. perfringens* type A is the colon⁽⁴¹⁾. The reduction in counts of *C. perfringens* in the faeces of pigs from the experimental treatments represents a clear improvement in the status of eubiosis. Moreover, the inhibition of *E. coli* implies an additional health benefit for the PT and ST pigs, since specific strains of *E. coli* are known to be a harmful pathogen involved in intestinal diseases of pigs⁽⁴³⁾.

Support of beneficial bacteria by prebiotic substrates and a competitive exclusion of potentially pathogenic bacteria might be the first and foremost reason for the reduction of *E. coli* and *C. perfringens* in this study. Feeding prebiotic inulin by tubers of the Jerusalem artichoke resulted in a reduction of *E. coli* (not significant) and a remarkable decline of *C. perfringens* in pigs' faeces with simultaneously promoted anaerobe bacteria and lactobacilli (chapter 2.4.2). Furthermore, *in vitro* experiments of May *et al.*⁽⁴⁴⁾ showed that supporting beneficial lactobacilli and bifidobacteria by a high fermentable fibre diet led to a decline in the counts of *C. difficile*. In addition, alone reduction in intestinal pH level, as ascertained in the faeces of the experimental treatments, can impair pathogen bacteria^(45,46). In a study of Wrigley⁽⁴⁵⁾, a lowered pH level in a sporulation broth of *C. perfringens* due to the addition of SCFAs acetate, isobutyrate, isovalerate, and succinate, which were produced by the common faecal bacterium *Bacteroides fragilis*, was effective decreasing the germination of *C. perfringens* spores. In the current study, however, an enhancement of lactobacilli was only detectable in the ST compared to the PT.

Another cause for the reduction of *E. coli* and *C. perfringens* could appear from phenolic acids, substances which possess antimicrobial potential and are present in peelings of potatoes serving as a natural defence mechanism against plant phytopathogens⁽⁴⁷⁻⁴⁹⁾. Rodriguez de Sotillo *et al.*⁽⁵⁰⁾ proved specific antibacterial activity of extracts from potato peelings containing chlorogenic, caffeic, gallic and

protocatechuic acids. Applying the extract in a concentration of 10^5 µg/ml to agar plates prevented the growth of gram-negative *E. coli*, *Salmonella typhimurium* and the gram-positive *Bacillus cereus*. In an *in vitro* study of Rauha *et al.*⁽⁵¹⁾ a potato-peel extract was effective in inhibiting the growth of *Staphylococcus aureus*.

Increase of lactobacilli in the ST in comparison to the PT in this study is most likely attributable to the low pH value and the lactobacilli counts of the potato silage. Even though acidification of pigs' diets does not affect intestinal pH value, ensiled feeding stuffs contain a high number of lactic acid bacteria by itself⁽⁵²⁾. Moreover, acid conditions of the potato silage in the initial sections of the digestive tract are advantageous to lactobacilli, while inhibiting the growth of gram-negative bacteria as *E. coli*.

Bifidobacteria have not been detected in faecal samples, neither in the CT nor in the experimental treatments. The absence of bifidobacteria in pigs is not uncommon since they are not a dominant germ in the porcine intestine as noted in several studies⁽⁵³⁻⁵⁶⁾ and a diet induced stimulation of bifidobacteria seems to be strongly dependent on their initial number^(55,57,58).

3.5.4 Immunological parameters

Assessment of Hp and CRP in the serum of pigs did not show significant differences between treatments and the measured values were within the range of healthy pigs⁽⁵⁹⁻⁶²⁾. The significant decrease in the concentration of IgA antibodies against LPS of *E. coli* J5 in pigs of the PT and the ST can be related to the reduction of *E. coli* in these treatments and implies beneficial effect of thermal processed potatoes on intestinal gut flora in the pigs.

3.5.5 Estimation of potato intake

Titanium dioxide is an established external marker in pig digestibility trials showing high faecal recovery⁽⁶³⁻⁶⁶⁾. Even though the decline in faecal titanium concentration in PT and CT indicate a dilutional effect from the ingestion of the

potatoes, the calculated amounts were not in a line with the expected values, since allowance of the steamed potatoes and the potato silage in the experimental treatments were known. According to Dove *et al.*⁽⁶⁷⁾, Penning⁽⁶⁸⁾ and Peters⁽⁶⁹⁾, a miscalculation of the estimated *in vitro* digestibilities of the applied feeding stuffs can cause equivalent mistakes. Further, since concentrate was fed once a day while the steamed potatoes and potato silage were offered in the morning and again in the afternoon, TiO₂ in faeces of pigs from the PT and the ST might have fluctuated during the course of the day.

3.6 Conclusions

High amounts of steamed potatoes and potato silage led to significant decreased counts of *E. coli* and *C. perfringens*, representing a clear improvement of pigs' gut health. The provision of the potato silage further increased numbers of total aerobes, total anaerobes, lactobacilli and the counts of yeasts in the first sampling period in comparison to the treatment with steamed potatoes. Promoting animal health by the inclusion of large amounts of potatoes in to the daily ration of pigs might be a good opportunity to improve animal health without the use of medications or antibiotics. Benefits of the improved animal health have to be regarded when considering additional labour and costs.

3.7 References

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4 Chapter 3.

Quantification of microbial mass in pig faeces by direct
isolation with high-speed centrifugation

4.1 Abstract

The composition of faeces provides relevant information about the previous fermentation processes in the hindgut and about the starting conditions for the subsequent degradation processes in the manure, making faeces either a nutrient source for plant growth or for losses via emission. While numerous analyses have been carried out regarding the total and the mineral nitrogen (N) and carbon content of pig faeces, the knowledge about faecal carbon (C) and N fractions is scarce. Especially little is known about the microbial biomass in faeces. To assess the microbial biomass in the faeces, a microbial pellet (MP) was isolated from a liquid solution using high-speed centrifugation steps. In addition, the amount of bacterial and endogenous debris nitrogen (BEDN) and the amino sugars galactosamine, glucosamine, mannosamine and muramic acid, a substantial part of cell walls of both bacteria and fungi, were determined in the faeces and the MP. Faecal samples were obtained from ten male castrated pigs, housed individually in metabolism cages and with a average live weight of 51.1 ± 8.5 kg during the whole experiment. Pigs were allotted to five dietary treatments, formulated to meet their nutrient and energy requirements, while daily phosphorus (P) and phytase supply differed between treatments.

Faecal N averaged to 45.6 ± 4.2 g/kg dry matter (DM). The microbial pellet (MP) derived from pigs' faeces amounted to a mean value of 468 ± 52 g/kg DM while the N proportion incorporated into the bacterial mass (MP-N) averaged to 27.0 ± 3.4 g/kg DM or 58% of the total faecal N. Low standard deviations confirmed that the isolation procedure by means of high-speed centrifugation provided a high repeatability and is appropriate for quantification of faecal microbial biomass. The calculation of the BEDN fraction resulted in a mean value of 73% of the total faecal N. Faecal N and P concentrations ranged between 43.5 ± 4.3 and 47.2 ± 3.2 g/kg DM and between 13.7 ± 1.6 and 8.0 ± 1.6 g/kg DM. Values were in agreement with results described in the literature. N concentration in the MP showed a variation from 55.8 ± 3.2 to 60.6 ± 4.5 g/kg DM. P concentration in the MP ranged between 1.8 ± 0.4 and 4.8 ± 0.5 g/kg DM and significantly decreased with increasing dietary

phytase ($P=0.000$). Correlation coefficient of N and P between the faeces and MP amounted to 0.525 ($P=0.003$) and 0.656 ($P=0.000$), respectively. The amino sugars galactosamine, glucosamine, mannosamine and muramic acid did not differ significantly between treatments and were in the range of values from cattle faeces. Proportion of the amino sugars muramic acid to glucosamine in faeces and MP imply that the pellet was almost free of fungi. Decreasing dietary P and increasing phytase supplementation diminished faecal ash content ($P=0.024$). Simultaneously, DM ($P=0.017$) and P concentration ($P=0.000$) decreased in the MP.

4.2 Introduction

Emission from manure contributes considerably to environmental pollution. Reducing the proportion of easy emittable urea and ammonia respectively in swine manure is important to decrease emission potentials from pig husbandry. Since the largest quantity of ammonia is emitted from the urine^(1,2), efforts to reduce urinary nitrogen (N) excretion have been in the focus of science and advisory service, while the composition of solid manure is often neglected. Indeed, inorganic bound N from faeces is very mobile through the soil layers⁽³⁾ and might contribute substantially to N eutrophication of the environment. N incorporated into the microbial biomass is less exposed to emission^(4,5), that is why the proportion of bacterial N in the faeces constitutes an important parameter for the estimation of potential nutrient losses from the manure.

So far only few investigations have been conducted to assess faecal N fractions and microbial biomass in faeces of pigs. Mason⁽⁶⁾ subdivided the faecal N fractions by its chemical and physical properties into the so-called water soluble nitrogen (WSN), undigested dietary nitrogen (UDN) and bacterial and endogenous debris nitrogen (BEDN). The BEDN fraction is calculated by subtracting the amount of WSN and UDN from the amount of total faecal N. While this approach provides a rough estimation of soluble and insoluble fractions, it does not allow a distinction between N incorporated in the microbial biomass and N derived from endogenous secretions and desquamated epithelial cells of the gut mucosa.

In other studies, the assessment of microbiological growth was based on internal and external marker substances in the bacterial cells⁽⁷⁻¹¹⁾. This approach, however, is only feasible if the marker to N ratio in the bacteria is well known and representative for the bacterial population⁽¹²⁾. Furthermore, natural occurrence of internal marker substances in the feeds and variability between individual animals in marker uptake has to be considered and might affect accuracy in the calculation of microbial biomass⁽¹³⁾.

While quantification of the microbial biomass was previously relying on indirect quantifying of bacterial marker substances, no attempts have been described so far to

quantify bacterial mass by directly weighing the isolated microbial pellet. The aim of this study was to determine the microbial biomass and bacterial N in pigs' faeces by isolation of the bacteria with high-speed centrifugation. Furthermore, the amount of the amino sugars galactosamine, glucosamine, mannosamine and muramic acid, representing a substantial part of cell walls of both bacteria and fungi^(14,15), was analysed as a specific bacterial component.

Since phosphorus (P) is considered to be indispensable for bacterial growth as being a constituent of the main cell metabolites⁽¹⁶⁾, additionally, the effect of different P availabilities by the level of dietary phytase onto intestinal microbiota was assessed.

4.3 Materials and methods

4.3.1 Animals, diets and sampling

The investigation was performed in accordance with the technical regulation of the Committee for Requirement Standards of the Society of Nutrition Physiology⁽¹⁷⁾ and local institutional regulations of the Friedrich Loeffler Institute, Brunswick with a certificate of exemption according the German Animal Protection Law. A total of 10 male castrated pigs (German Landrace x Pietrain) were housed individually in metabolism cages with variable dimensions according to the animals' body mass. Animals were adapted to five dietary treatments (Table 4.1). The diets were formulated to meet the requirements in relation to nutrients and energy considering a performance level of approximately 700 g daily weight gain (DWG) according to the German Society of Nutrition Physiology⁽¹⁸⁾, except for phosphorus supply. In the treatments 2 to 5, daily P supply was below the specific requirements of the animals. The dietary treatments 3 to 5 were supplemented, graduated with 50, 100 and 200 mg/kg respectively of an experimental phytase. Average live weight during the whole experiment was 51.1 ± 8.5 kg. Faeces were collected quantitatively on three consecutive days and freeze immediately after excretion in fluent nitrogen. Afterwards samples were stored at -20°C until analysis. Subsequently, pigs were assigned to a P metabolism trial. Faeces and urine excretion and urine N

concentration were recorded as mean values for a seven-day period and daily weight gains were determined for a successive eleven-day period. Both periods immediately followed the three days of faeces collection for this study.

Table 4.1. Composition and ingredients of the experimental diets

Diets	1	2	3	4	5
Ingredients			%		
Maize	45	45	45	45	45
Maize starch	14	14	14	14	14
Peas	5	5	5	5	5
Soybean extraction meal	21	21	21	21	21
Sugar beet pulp	9.2	10.4	10.4	10.4	10.4
Mineral mix	2	2	2	2	2
Soybean oil	2	2	2	2	2
Lysin HCl	0.25	0.25	0.25	0.25	0.25
DL-Methionin	0.15	0.15	0.15	0.15	0.15
L-Threonin	0.05	0.05	0.05	0.05	0.05
Salt	0.15	0.15	0.15	0.15	0.15
Dicalciumphosphat	1.20	-	-	-	-
Phytase	-	-	0.005	0.01	0.02
Analysed composition			g/kg DM		
DM (g/kg FM)	884	884	882	883	883
CA	54	45	44	43	43
CP	182	185	183	184	183
CL	49	55	57	60	56
CF	45	45	46	44	47
NDF	147	155	145	144	146
ADF	52	57	58	57	56
Starch	514	521	524	523	523
Sugar	40	41	40	41	40
P	5.4	3.5	3.2	3.4	3.3
Ca	11.3	8.6	8.0	8.0	7.4
Energy (MJ ME/kg)	15.2	15.5	15.5	15.6	15.5

ADF, acid detergent fibre; Ca, calcium; CA, crude ash; CP, crude protein; CL, crude fat; CF, crude fibre; DM, dry matter; FM, fresh matter; HCl, hydrochloric acid; ME, metabolisable energy; MJ, megajoule; NDF, neutral detergent fibre; P, phosphorus

4.3.2 Parameters determined in faeces

Analytical procedures

Faeces were thawed, homogenised and apportioned to three aliquots. A moist sample of 100 g was dried at 60°C until constant weight and ground through a 1-mm pore size sieve for dry matter (DM), organic matter (OM), neutral detergent fibre (NDF) and acid detergent fibre (ADF). For ash weights, samples were heated in a muffle furnace at 550°C for 8 hours. N concentration in the faeces was analysed using a Macro N auto-analyser (Elementar Analysensysteme, Hanau, Germany). P content was determined spectrally photometric by the Vanadat-Molybdat-method according to the VDLUFA procedure⁽¹⁹⁾ modified by Krutzinna⁽²⁰⁾ and adapted to pig faeces. The amino sugars galactosamine, glucosamine, mannosamine and muramic acid were determined according to the method described by Appuhn *et al.*⁽²¹⁾, which was modified by Jost *et al.*⁽²²⁾ to faecal samples. Moist samples of 2 g were weighted into 20 ml test tubes, mixed with 10 ml 6 molar (M) hydrochloric acid (HCl), and heated for 2 hours at 105°C. After HCL removal from the filtered hydrolysates in a vacuum rotary evaporator at 40°C and centrifugation, the samples were transferred to vials and stored at -18°C until measurements took place with high-performance liquid chromatography. Following the derivatisation with ortho-phthaldialdehyde, the fluorometric emission of amino sugars was measured at a wavelength of 445 nm after excitation at a wavelength of 330 nm.

Nitrogen fractioning

The faecal nitrogen fractions UDN, WSN and BEDN were determined according to the procedure of Mason⁽⁶⁾, which has been adapted by Kreuzer *et al.*⁽²³⁾ for the analysis of pig faeces. For quantification of UDN, a moist faeces sample of 90 g was homogenised in 250 ml distilled water with Ultra-Turrax (Ultra-Turrax T25, IKA-Labortechnik, Germany) (40 sec, 20500 rpm) and incubated in a cooled shaker (24 hours, 160 rpm, 5°C). Subsequently, 90 g of the homogenate was cooked with 100 ml hot neutral detergent solution, 2 ml decahydronaphthalene and 0.5g of sodium sulphite for 1 h. After aspiration of the supernatant through a 22 µm sieve, the residuent was dried at 105°C to a constant weight. N concentration was analysed

using a Macro N auto-analyser (Elementar Analysensysteme, Hanau, Germany). WSN was calculated by subtracting bacterial and undigested nitrogen (BUN) from total faecal N. For the determination of BUN, a moist faecal sample of 10 g was suspended into 50 ml saline solution (1 g methycellulose + 9 g sodium chloride (NaCl)/litre distilled water) and thoroughly mixed with a magnetic stirrer for 2 min. Following a renewed centrifugation (22000g, 40 min, 4°C), the supernatant was discarded. The residuent was freeze-dried and weighted. Nitrogen concentration in the bacterial pellet was analysed by a Macro N auto-analyser (Elementar Analysensysteme, Hanau, Germany). An estimate of BEDN was attained by subtracting WSN and UDN from total faecal N.

Quantitative isolation of microbes

For quantitative separation of the faecal microbes, the procedures described by Carro & Miller⁽⁷⁾ was implemented, following the recommendations of Minato & Suto⁽²⁴⁾ with respect to the isolation of solid associated bacteria (SAB), which adhered to feed particles. A faecal sample of 10 g was suspended into 50 ml saline solution, thoroughly mixed with a magnetic stirrer for 2 min and filtered through a nylon tissue with 60 µm wide mesh wide to retain feed particles. The supernatant fraction containing the faecal bacteria was retained. The residuent was collected from the nylon tissue and repeatedly submitted to the washing procedure, pooling the supernatant fractions. Subsequently, the residuent from the nylon tissue was incubated with 50 ml of the saline solution in a shaking water bath (39°C, 30 min) and filtered through the nylon tissue. The supernatant fractions were pooled again. The residuent from the nylon tissue was incubated in a beaker with 50 ml of saline solution (at least 6 hours, 4°C). After stirring for 2 min, the solution was filtratered through the nylon tissue, the supernatant fractions were pooled and the residuent was discarded. The pooled supernatant fractions were centrifuged (22000g, 40 min, 4°C). The supernatant was discarded and the residual microbial pellet (MP) was freeze-dried and weighted.

The nitrogen concentration in the pellet (MP-N) was analysed using a Macro N auto-analyser (Elementar Analysensysteme, Hanau, Germany). For determination of amino sugar concentration, the method of Appuhn *et al.*⁽²¹⁾, modified by Jost *et al.*⁽²²⁾

was implemented by using an reduced initial amount of 0.5 g of the moist pellet, weighted within the test tubes and mixed with only 5 ml 6 M HCl. Apart from that the procedure agreed with the method applied to faecal samples. The P content in the pellet was determined via spectral photometric by the Vanadat-Molybdat-method in the ashed substrate according to VDLUFA procedure⁽¹⁹⁾ with adaption to pig faeces.

Statistical analysis

Statistical analysis was conducted using the statistical program SPSS 18.0 of Windows (Version 18.0.1). Data were tested for normal distribution by Kolmogorov-Smirnov test and for homogeneity of variance by Levenes test and Mauchly test, respectively. Correlations were calculated using Pearson's bivariate correlation coefficient. To determine the effect of the treatment (Trm), data were analysed with univariate ANOVA, repeated measures ANOVA, and subsequent post hoc test. Data with inhomogeneities in variance were analysed by nonparametric test for independent samples. Differences were considered to be significant at $P < 0.05$.

4.4 Results

4.4.1 Production data

Feed was consumed almost completely by the pigs during the total experimental period. Only for the first day, minor leftovers were accounted for three pigs, resulting in a slight variance in nutrient intake (Table 4.2). Ingestion of the concentrate revealed an almost equal intake of energy ranging from 19.7 to 20.0 megajoule (MJ) metabolisable energy (ME)/kg DM and protein from 232 to 237 g/kg DM.

Table 4.2. Feed, nutrient and energy intake by the diet in relation to the different treatments

Dietary treatment	1	2	3	4	5
Feed intake	g DM/day				
Intake (g DM/day)	1296	1274	1272	1273	1295
CA	70	58	56	55	56
CP	236	236	232	234	237
CL	64	70	72	77	72
CF	58	57	59	56	61
NDF	191	198	184	183	189
ADF	67	73	74	73	73
Starch	667	664	667	667	677
Sugar	51	52	51	53	52
P	6.9	4.4	4.1	4.3	4.2
Ca	14.7	10.9	10.2	10.2	9.6
Phytase	-	-	6.4	12.7	25.9
Energy (MJ ME/day)	19.7	19.7	19.7	19.9	20.0

ADF, acid detergent fibre; Ca, calcium; CA, crude ash; CP, crude protein; CL, crude fat; CF, crude fibre; DM, dry matter; ME, metabolisable energy; MJ, megajoule; NDF, neutral detergent fibre; P, phosphorus; Trm, treatment

DWG averaged to 0.564 ± 0.057 kg/day and was not significantly affected by the feeding regimes ($P=0.478$).

4.4.2 Parameters determined in faeces

Composition of pigs' faeces is presented in Table 4.3. DM, ADF, NDF, P and the amino sugars galactosamine, glucosamine, mannosamine and muramic acid in pigs' faeces did not differ significantly between treatments. Only for faecal ash content, a significant decline with increasing phytase supplementation was observed ($P=0.024$). Faecal ADF and NDF content amounted from 246 ± 21 to 283 ± 18 and 345 ± 21 to 362 ± 32 g/kg DM. P concentration in the faeces ranged from 8.0 ± 1.7 to 13.7 ± 1.6 g/kg DM. The levels of the different amino sugars showed a wide variation with galactosamine 0.39 ± 0.16 mg/kg DM, glucosamine 2.97 ± 0.73 mg/kg DM, mannosamine 0.04 ± 0.01 mg/kg DM and muramic acid 0.24 ± 0.06 mg/kg DM, respectively.

Table 4.3. Faecal dry matter, ash, acid and neutral detergent fibre, phosphorus and amino sugar concentrations in relation to the different treatments

Dietary treatment	DM	Ash	ADF	NDF	P	Gal	Glu	Man	Mur
	g/kg FM	g/kg DM				mg/g DM			
1	275	208 ^a	246	362	13.7	0.3	2.7	0.04	0.23
	± 21	± 13	± 21	± 32	± 1.6	± 0.2	± 0.6	± 0.01	± 0.04
2	295	180 ^{ab}	259	362	10.5	0.3	2.8	0.03	0.21
	± 14	± 12	± 25	± 20	± 2.4	± 0.1	± 0.9	± 0.01	± 0.08
3	270	173 ^b	277	362	11.7	0.4	2.9	0.04	0.22
	± 25	± 13	± 9	± 13	± 1.3	± 0.1	± 0.8	± 0.01	± 0.06
4	290	156 ^b	283	357	9.8	0.5	3.3	0.03	0.23
	± 30	± 6	± 18	± 17	± 1.9	± 0.2	± 1.4	± 0.01	± 0.04
5	267	156 ^b	282	345	8.0	0.5	3.3	0.04	0.28
	± 32	± 8	± 11	± 21	± 1.7	± 0.2	± 0.8	± 0.01	± 0.08
P value									
Trm	0.672	0.024	0.318	0.634	0.209	0.273	0.189	0.225	0.457

ADF, acid detergent fibre; DM, dry matter; FM, fresh matter; Gal, Galactosamine; Glu, glucosamine; Man, mannosamine; Mur, muramic acid; NDF, neutral detergent fibre; P, phosphorus; Trm, treatment

The N content and N fractions of pigs' faeces are presented in Table 4.4. Neither faecal N, the weight of the MP nor the faecal N fractions MP-N, UDN, WSN and BEDN were significantly affected by the feeding regime. The weight of the MP ranged from 451 ± 65 to 476 ± 47 g/kg DM. The content of microbial N showed a variation from 26.0 ± 3.2 to 27.6 ± 5.0 g/kg DM. UDN amounted to the smallest proportion of faecal N with a range of 3.0 ± 0.4 to 3.3 ± 0.3 g/kg DM. The second largest fraction was embodied by WSN with values from 7.7 ± 2.5 to 11.5 ± 4.3 g/kg DM. BEDN represented the largest faecal N proportion, varying from 32.0 ± 2.8 to 35.2 ± 4.2 g/kg DM. The correlation coefficient between MP-N and BEDN amounted to 0.398 ($P=0.030$). On average over the feeding regimes, MP-N amounted to 58% of the faecal N content, while BEDN, UDN and WSN came to 73%, 7% and 20%. N fractions and results for MP and MP-N showed low standard deviations and a good repeatability in double measurements with correlation coefficients of 0.737 for MP, 0.751 for MP-N and 0.862 for WSN.

Table 4.4. Nitrogen concentration, nitrogen fractions, microbial pellet and nitrogen concentration in the pellet in pig's faeces in relation to the treatments

Dietary treatment	N	MP	MP-N g/kg DM	UDN	WSN	BEDN
1	44.5 ± 2.0	465 ± 47	26.0 ± 3.2	3.3 ± 0.3	9.3 ± 1.5	32.0 ± 2.8
2	47.1 ± 5.9	451 ± 65	27.6 ± 5.0	3.0 ± 0.4	8.9 ± 6.7	35.2 ± 4.2
3	43.5 ± 4.3	476 ± 47	27.0 ± 1.3	3.2 ± 0.2	7.7 ± 2.5	32.6 ± 3.0
4	47.2 ± 3.2	475 ± 38	27.6 ± 3.7	3.3 ± 0.3	11.5 ± 4.3	32.3 ± 3.9
5	45.5 ± 4.2	469 ± 70	27.1 ± 3.9	3.3 ± 0.1	7.9 ± 4.2	34.3 ± 3.1
P value						
Trm	0.440	0.243	>0.05	0.772	0.765	0.721

BEDN, bacterial and endogenous debris nitrogen; DM, dry matter; MP, microbial pellet; MP-N, microbial N in the faeces; N, nitrogen; Trm, treatment; UDN, undigested dietary nitrogen; WSN, water soluble nitrogen

4.4.3 Composition of the microbial pellet

The composition of the MP is presented in Table 4.5. DM and P content of the MP significantly declined with decreasing dietary P and increasing phytase supplementation. P concentration in the MP ranged from 1.8 ± 0.4 to 4.8 ± 0.5 g/kg DM. The correlation coefficient of P in the faeces and the MP amounted to 0.656 ($P=0.000$). The concentration of N and the amino sugars galactosamine, glucosamine, mannosamine and muramic acid in the MP were not different between treatments. N concentration in the pellet ranged from 55.8 ± 3.2 to 60.6 ± 5.4 g/kg DM and the correlation coefficient of N in the faeces and the MP amounted to 0.525 ($P=0.003$). The level of the amino sugars differed widely with galactosamine 0.44 ± 0.16 mg/kg DM, glucosamine 0.79 ± 0.18 mg/kg DM, mannosamine 0.07 ± 0.04 mg/kg DM and muramic acid 0.42 ± 0.14 mg/kg DM. Correlation coefficients of the several amino sugars between their amounts in the faeces and the MP were 0.151 ($P=0.426$), 0.092 ($P=0.630$), -0.087 ($P=0.654$) and 0.041 ($P=0.828$) respectively, without being significant.

Table 4.5. Composition of the microbial pellet derived from pig's faeces in relation to the treatments

Dietary treatment	DM	N	P	Gal	Glu	Man	Mur
	g/kg FM	g/kg DM		mg/g DM			
1	212 ^a ± 16	55.8 ± 3.2	4.8 ^a ± 0.5	0.37 ± 0.08	0.86 ± 0.12	0.06 ± 0.02	0.42 ± 0.07
2	211 ^a ± 29	60.6 ± 5.4	3.6 ^b ± 0.3	0.45 ± 0.20	0.77 ± 0.23	0.08 ± 0.04	0.42 ± 0.16
3	203 ^{ab} ± 16	57.2 ± 4.9	2.5 ^c ± 0.4	0.43 ± 0.19	0.72 ± 0.28	0.05 ± 0.03	0.37 ± 0.18
4	181 ^{ab} ± 13	58.0 ± 4.5	1.8 ^d ± 0.4	0.51 ± 0.12	0.84 ± 0.23	0.08 ± 0.06	0.47 ± 0.15
5	174 ^b ± 22	58.0 ± 3.9	1.9 ^d ± 0.3	0.50 ± 0.20	0.79 ± 0.17	0.08 ± 0.05	0.42 ± 0.19
P value							
Trm	0.017	>0.05	0.000	0.598	0.158	0.130	0.331

DM, dry matter; FM, fresh matter; Gal, Galactosamine; Glu, glucosamine; Man, mannosamine; Mur, muramic acid; N, nitrogen; P, phosphorus; Trm, treatment

4.4.4 Nitrogen excretion parameters

Patterns of N excretion in the seven-day period followed by the three days of faeces collection are presented in Table 4.6. The daily ingestion of N amounted from 37.1 to 37.9 g/day. With a DM content from 303 to 349 g/kg in the FM, the daily faeces excretion ranged from 0.11 to 0.14 kg DM/day while faecal N excretion amounted from 4.4 to 5.7 g N/day. A quantity of 17 to 24 kg FM of urine was collected daily per animal and N excretion via urine showed a range between 12 to 14 g N/day. On average over the feeding regimes, 13.8% of the ingested dietary N was excreted with the faeces and 34.5% with the urine.

Table 4.6. Mean nitrogen intake, faecal dry matter content, faeces and urine excretion and nitrogen excretion via faeces and urine in relation to the treatments

Dietary treatment	N intake g/day	Faeces DM g/kg FM	Faeces excretion kg DM/day	Faecal N excretion g DM/day	Urine excretion kg FM	Urinary N excretion g DM/day
1	37.8	311	0.14	5.6	19	12
2	37.8	349	0.13	5.7	23	12
3	37.1	316	0.11	4.4	17	14
4	37.4	305	0.12	5.2	24	12
5	37.9	319	0.12	5.0	22	13

DM, dry matter; FM, fresh matter; N, nitrogen; Trm, treatment

4.5 Discussion

4.5.1 Isolation of faecal microbes

The methodology used for the isolation of the microbial pellet derived from techniques applied predominately to separate a pure sample of rumen bacteria, for instance for the determination of bacterial patterns of amino acids or the quantification of bacterial protein synthesis using marker techniques⁽¹⁰⁾. This bacterial sample can be produced by high-speed centrifugation (22000g), where bacteria in solution sediment on the bottom of a centrifuge tube forming the so-called microbial pellet^(24,25). Even though the production of a microbial pellet had been recently adapted to faeces of fattening pigs by Metzler *et al.*^(26,27), no attempts have been described in literature to quantify microbial biomass directly by isolation via high-speed centrifugation.

The determination of a marker to N relationship in bacteria requires the microbial sample to be free of undigested feed residues and representative proportions of the present bacterial populations, since the marker to N ratio may vary between the individual species of bacteria and is only constant when bacterial growth is balanced⁽¹²⁾. As microbes are supposed to be adherent to undigested feed particles⁽²⁸⁾, the detachment of SAB is an important step for the separation of a microbial pellet. Indeed, procedures for detachment of SAB vary between studies. The application of a saline solution in combination with incubation at about 39°C, followed by cooling

treatment at 4°C is frequently used^(8,10,29-31). The appropriateness of a solution containing methylcellulose, a water soluble derivate of cellulose, in combination with different incubation steps has been approved by Minato & Suto⁽²⁴⁾. Removal of a major proportion of SAB from solid rumen content was achieved by Ranilla & Carro⁽¹⁰⁾ with saline solution, containing 0.1% methylcellulose and 0.9% NaCl, combined with incubation at 38°C for 15 min and chilling for 24 hours at 4°C. In connection with these procedures, no adverse effect on microbial cell integrity was detectable⁽¹⁰⁾. This conclusion was based on the concentration of purine bases in the MP. If any treatment had caused microbial lysis, this would have lowered the concentration of purine bases in the MP compared to other treatments, because purine bases appear exclusively in the microbial cytoplasm⁽¹⁰⁾. Boguhn *et al.*^(30,31) submitted SAB mixed with a methylcellulose containing saline solution for 30 minutes into a beaker at 39°C and subsequent applied cooling treatment to a minimum of 6 hours. However, other studies only use a saline solution to detach bacteria from feed particles^(9,26,27,32).

For the separation of microbes from undigested feed particles in the faeces commonly a low-speed centrifugation step is applied, whereby feed particles sediment on the bottom of the tubes while the bacteria remain in the supernatant^(7,27,30,31). In this study, this step was omitted, since microscopic examination of the sedimented feed particles showed repeatedly a high occurrence of bacteria. Instead, retention of feed particles was obtained by the filtration through the nylon tissue with a pore size of 60 µm. By a size of bacteria from 0.5 to 5 µm with *Escherichia coli* as a typical sized bacterium with 0.5 x 2.0 µm⁽³³⁾, faecal bacteria are supposed to easily pass the nylon tissue while feed particles are retained. The almost pure isolation of the faecal bacteria from undigested feed particles could be confirmed by microscopic examination of the MP from the high-speed centrifugation, revealing exclusively densely packed microbes.

For procedural reasons it was not possible to submit the MP to a repeated resuspension in the saline solution and centrifugation to remove residual water soluble N as applied in the study of Minato & Suto⁽²⁴⁾, while Carro & Miller^(7,8) and Boguhn *et al.*^(30,31) actually repeated this washing process twice. Thus, the application

of a single high-speed centrifugation step in this study might cause in a slight overestimation of the bacterial-bound N. On the other hand, this practise is in accordance with Metzler *et al.*⁽²⁷⁾ and might preserve from weight losses of the MP. In addition, bacteria remaining in the gauze tissue after the filtration steps are not attainable by the MP-N method and might constitute a method immanent sink term.

4.5.2 Faecal nitrogen fractions and composition of the microbial pellet

A significant influence of the differences in P supply on faecal composition was only detected for ash; the values decreased with an ascending supply of phytase.

According to the weight of the freeze-dried MP (Table 4.4), the proportion of microbial mass in the faeces came to a high level of almost 50%. There is little information on the proportion of bacteria in the faeces of pigs. Metzler *et al.*⁽²⁷⁾ quantified N and P concentration in faecal bacteria of pigs, but did not assess their quantity. In human faeces, the proportion of bacteria on DM basis is reported to amount between 47 to 55%⁽³⁴⁾.

The N concentration in the MP was slightly higher than the N content in the faeces, reflecting the accumulation of N-rich bacteria in the MP. The N concentration in the MP was in agreement with those found by Metzler *et al.*^(26,27), who ascertained values between 50.8 to 62.3 g N/kg DM for faecal bacterial mass from pigs using differential centrifugation. Indeed, the N concentration of bacteria can be highly variable. Schoenhusen *et al.*⁽¹²⁾ reported for cultivated bacterial populations of *Lactobacillus* sp., *Enterococcus* sp. and Enterobacteriaceae, which are predominantly harboured in the porcine gastrointestinal tract, N concentrations ranging from 77.5 to 137.1 g/kg DM of the bacterial mass. In rumen bacteria, the N concentration may vary from 42.0 to 105.8 g/kg DM of the separated bacterial mass^(10,35). Differences are supposed to result from different growth stages and the nutrition of particular bacterial populations as well as from varying bacterial species within the populations^(9,12,36). For example, liquid associated bacteria are reported to show higher N concentrations than SAB^(9,36). In a culture of *Enterococcus* sp., the N content amounted to 106.3 g/kg DM, while *Enterococcus faecium* contained

77.5 g N/kg DM⁽¹²⁾. Since bacterial N content of the new procedure is not dependent on the bacterial species, it may serve as reference method for indicator based procedures, which are eminently dependent on the bacterial species.

The proportion of N incorporated in the faecal microbes amounted to 58% of the total faecal N. In comparison, the BEDN value, determined in this study as reference for MP-N, resulted in the high level of 73% of faecal N. BEDN values reported in literature show a variation from 42% to 76%^(26,27,37-39). However, the BEDN fraction is always calculated indirectly, based on the assessed amounts of WSN, UDN and faecal N. While WSN values in this study were found within the reported range, the amount of UDN (7.0% of the faecal N) was lower than those in comparative studies, ranging between 9.6 to 14.8%^(23,37,39,40,41). As UDN consists exclusively of indigestible feed residues, the low value in this study possibly resulted from the absence of fibre-rich components in pig's concentrate like barley, oats or wheat bran. The husks of these cereals contain indigestible substances and were present in the concentrate mixtures of the prior mentioned studies. On the other hand, N which would escape the determination of WSN or UDN will be ascribed to the BEDN fraction and will consequently result in an overestimation of this fraction. Moreover, BEDN is composed of N originating from bacteria, endogenous secretions into the gut and desquamated epithelial cells, while MP-N solely comprises the N which is incorporated into the bacteria, explaining the difference between MP-N and BEDN and unexpected low correlation coefficient of 0.398 in this study.

While a proportion of 58% from the faecal N was incorporated into the bacteria, 6.9% of the ingested N was excreted with the faeces as microbial biomass. This amount was distinctively lower than values determined in other studies. In an experiment of Hanneken⁽⁴²⁾, bacterial proportion to the faecal N related to the N intake amounted from 13.9 to 17.7% for pigs fed different types of fermentable substrates. Laurinen *et al.*⁽⁴⁰⁾ ascertained values from 9.8 to 10.8% supplementing wheat bran of different processing conditions to a diet of pigs. Indeed, both studies determined faecal bacteria by the method of Mason⁽⁶⁾, which might have affected a slight overestimation of the truly bacterial bound amount of N.

4.5.3 Advantage of the new procedure

Even though procedures for estimation of bacterial bound N by marker substances and the N fractions of Mason⁽⁶⁾ are well established⁽²³⁾, the significance of results can provide difficulties. Kreuzer *et al.*⁽²³⁾ reported the determination of bacterial bound N with the marker diaminopimelic acid (DAPA), a constitutive component in bacteria⁽¹²⁾, to be not sufficiently precise. High variability in results with DAPA as marker substance has been also reported by von Heimendahl *et al.*⁽⁴¹⁾. Schoenhusen *et al.*⁽¹²⁾ found the ratio of N to the marker substance D-alanine (DAL), a peptidoglycan-polysaccharide complex in bacterial cell walls, to be highly variant and recommended not to use a general DAL to N ratio for bacterial N calculation, requiring labour intensive determinations of present marker to N ratios. Moreover, the natural occurrence of internal marker substances in the feeds can disturb the calculation⁽¹³⁾. Disadvantages of the N fractioning by Mason⁽⁶⁾ rely mainly on the indirect assessment of the bacterial N by other N fractions as explained above (chapter 4.5.2). Additionally, since the procedure only determines N fractions, there is no information available on the quantity of microbial biomass in the faeces. In contrast, the new procedure applied in this study enables by the direct measurement of the faecal microbial biomass further analyses within the MP for assessment of bacterial bound N or other specific bacterial compounds. Moreover, the new procedure allows to investigate the effect of feeding treatments on the faecal microbial biomass.

4.5.4 Phosphorus in faeces and the microbial pellet

The slight decrease in faecal P contents in this study reflects the improved P degradation and absorption by the pig when phytase was supplemented to the diet⁽⁴³⁾. The faecal P concentrations are in agreement with data reported in the literature, which show in dependence on the level of P and phytase supplementation a huge variation, reaching from 3.9 to 27.5 g/kg DM⁽⁴⁴⁾. P concentrations in the MP, however, showed a stronger and significant decline with increasing levels of phytase. The P level ranged between 1.8 to 4.8 g/kg DM and was remarkable low in

comparison to the P concentration in the faeces. Ascertained P values for bacteria in pigs' faeces, irrespective of the provision of P and phytase, range from 12.2 to 36.9 g/kg DM^(26,27). Since hydrolysis of P by dietary phytase and P resorption appear almost completely in the SI, phytase might have reduced P availability for the bacteria in the hindgut^(43,45,46). A strong relationship of P availability in the colon and P content of bacteria has been approved by Metzler *et al.*⁽²⁶⁾, which can be explained by indispensability of P for bacterial growth as being a constituent of the main cell metabolites, the cell walls of gram-positive bacteria and the cytoplasmic and outer membranes of gram-negative bacteria⁽¹⁶⁾. Under the conditions of a low P availability in the LI, the metabolism is supposed to be altered, such that P is secreted into the LI^(27,47).

A possible direct effect of the phytase on intestinal microorganisms is not likely, since in this study the bacterial mass was unaffected by dietary treatment. This might be due to the fact, that phytase is confirmed to be predominantly active in the stomach and the increase in pH level posterior the duodenum above 6 inhibits its activity^(43,45). Moreover, the intestinal microbiota produces phytase by itself for the breakdown of dietary P⁽⁴³⁾.

However, bacteria are susceptible to changes in the P availability. In studies of Metzler-Zebeli *et al.*⁽⁴⁸⁾, greater P availability in the small intestine induced by dietary phytase enhanced the growth of strictly anaerobic bacteria, while numbers of total bacteria remained unaffected. The type of fermentable substrate might also affect the P concentration of bacteria⁽²⁷⁾. In the study of Metzler *et al.*⁽²⁷⁾, the ileal infusion of pectin into pigs fitted with T-cannulas resulted in lower P concentrations in the faecal bacteria towards infusion of cellulose. Low amounts of fermentable substrates in pigs' diets, which might have been already visible in the low UDN value, possibly resulted in bacteria populations with lower P contents.

4.5.5 Amino sugars in faeces and the microbial pellet

Amino sugars represent a substantial part of cell walls of both bacteria and fungi^(14,15). The use of amino sugars has been mainly limited to the field of soil

biology, where they function as indicators for microbial residues^(16,21,49). Recently, Jost *et al.*⁽²²⁾ applied the quantification of the amino sugars to cattle faeces with the purpose of quantifying microbial biomass.

In this study, the levels of amino sugars were not affected by phytase supply, neither in the faeces nor in the MP. The amounts of muramic acid and galactosamine were slightly lower and of glucosamine slightly higher in pig faeces in comparison to cattle faeces⁽²²⁾. High similarities in repeated measured samples confirm appropriability of the method to faecal samples. Changes in amount of the single amino acids between the faeces and the MP may allow some conclusions regarding the composition of the MP. Muramic acid is known to appear almost exclusively in bacterial cell walls⁽²¹⁾. Thus, the increase in muramic acid in the MP towards the faeces might display the accumulation of bacteria, even though the rise was comparatively low. Additionally, the content of glucosamine decreased from the faeces to the MP. In soil, the major source of glucosamine is the chitin of fungal cell walls⁽⁴⁹⁾. Indeed, bacterial cell walls also contribute to the soil glucosamine content, which was determined by Engelking *et al.*⁽⁴⁹⁾ for cultured bacteria to twice the amount of their content of muramic acid. By this proportion of muramic acid to glucosamine calculated to 1:2 cultured bacteria, the main share of glucosamine in the MP would be of bacterial origin, meaning that the MP was almost free of fungi. The high correlation of 0.917 ($P=0.000$) between muramic acid and glucosamine in faecal samples as well as in the MP support the assumption, that both amino sugar were originating exclusively from bacteria. Furthermore, the removal of fungi which had been contained in pigs faeces might also explain low correlation coefficients of the several amino sugars between their amounts in the faeces and the MP.

Only little is known about Galactosamine and mannosamine and their influencing factors, even though galactosamine is reported to contribute to the soil amino sugar content up to a portion of 30% to 50%⁽⁵⁰⁾.

4.6 Conclusions

High repeatability in results of the microbial parameters, the microscopic examination and composition of the MP confirmed the hypothesis that the isolation of faecal bacteria with high-speed centrifugation and direct weighing of the MP constitutes of an appropriate procedure for quantification of the microbial biomass in pig faeces. It provides a more precise estimation of the bacteria than the BEDN procedure, is not tainted with imprecision connected with marker methods. Moreover, analyses within the MP as the assessment of bacterial bound N provide information on metabolism of the intestinal microbiota. The procedure facilitates determination of the microbial biomass and turn over rates in the hindgut and might promote research concerning the effects of dietary adjustments onto the bacterial growth.

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5 General discussion

5.1 Feeding regimes with Jerusalem artichoke tubers and potatoes

In the first tow trails the feeding strategies with JA tubers and potatoes have been approved to be suitable for nutrition of fattening pigs. By utilisation of these root vegetables it was possible to substitute up to 50% of the concentrate towards a solely on concentrate based feeding regime as with the control treatments. Suitability of JA tubers and potatoes in pig nutrition could be further shown by higher weight gains of pigs fed those tubers, while carcass traits were not influenced significantly compared to the control pigs. Also in previous studies performances of free-ranged pigs provided JA tubers had been noticeable high^(27,28). However, the appropriateness of JA tubers is limited by their elaborate and expensive requirements for harvesting and storage as well as the continued growth in the subsequent years on the field^(29,30). Thus, the production of JA tubers in the European agriculture is still uncommon. Research on the use of Jerusalem Artichokes in Europe is mainly focused on its utility for industry as an important source of fructans⁽²⁹⁻³²⁾. But in free-range systems use of JA tubers is more suitable, since pigs dig out the tubers by themselves and harvesting and storage are not needed. Furthermore, pigs are more thoroughly than harvesters, so that the continued growth in the following years is most widely suppressed⁽²⁹⁾.

Potatoes and potato products, primarily in steamed or steamed and ensiled forms, have been traditional widely used in pig feeding^(19,33). Due to the high starch content and digestibility of thermal processed potatoes, they are as energy-rich as wheat and enclose a considerable protein content with suitable amino acid pattern^(18,19). Since the improved availability of cereals, the enlargement of animal production systems and the increasingly automation of the feeding process changed the production conditions, potatoes became more unprofitable and were substituted by cereal-based diets. These days, the provision of potatoes to pig is hardly practised⁽³⁴⁾. Raw potatoes are of poor nutrient digestibility and thermal treatment, which enhances digestibility and inactivates undesired compounds, causes high energy and labour costs. In addition, storage and provision of the potatoes are further expensive and

laborious^(18,34). Thus, high labour costs impede the reutilization of potatoes as nutrient resource. In Austria, an amount of 2000 tons of feed-grade potatoes (on DM basis), which might have constitute an energy source for approximately 12300 pigs, is dissipated each year by composting⁽³⁴⁾. But considering global rise in costs for cereals and the increasing demand for field crops, the utilisation of nutrient resources such as culled potatoes might gain increased interest. In organic pig husbandry, they might be furthermore suitable, because on the one hand the provision of roughages is already required⁽¹⁴⁾ and the feeding process is less engineered. On the other hand, if the potatoes are from the own farm, purchase of feeding stuffs and imports of nutrients into the farm are lowered.

The allocation JA tubers and potatoes in high amounts up to 1.2 kg DM/day to the pigs in this study have further shown to alter the composition of the intestinal microbiota and benefit animals health. Both root vegetables obviously inhibited potential pathogenic bacteria and proliferated beneficial anaerobe bacteria and lactobacilli, even though this effect was not consistent. The counts of *Clostridium perfringens* were reduced to almost zero while numbers of *Escherichia coli* were diminished to some lesser extent. Especially *C. perfringens* is recognized as a major cause of enteric diseases in pig husbandry and considered with the production of beta2-toxin, which is strongly associated with necrotic enteritis in piglets, constituting a major problem in piglet rearing^(35,36). Lowering the shedding of *C. perfringens* by suppressing appearance in the porcine gut might be also important for food safety, since *C. perfringens* is also one of the most common foodborne illnesses in the United States⁽³⁷⁾. Spores, which are excreted with the faeces, can persist in the environment and enter food⁽³⁷⁾. With the ban of the European Union on antibiotics as a feed additive in diets of pigs, research for alternatives to maintain animal health in pig production has focused on application of pro- and prebiotics. While prebiotic effectiveness of inulin and FOS in humans has been approved in several studies^(4,9), in pig nutrition they were frequently ineffective^(11,38,39). Reasons for this have been previously considered (chapter 2.5.2). For cost reasons, pro- and prebiotica are usually supplemented cautiously. It appears reasonable that the effects of JA on the microbiota in this study largely resulted from the abundant availability of prebiotic

active inulin and FOS ingested by the pigs. By estimation of the JA tubers intake, this inulin ingestion was estimated to 800 g/day. Since heat treated potatoes are not associated with relevant amounts of prebiotic active resistant starch (RS)^(40,41), it can not be ascertained finally, if antimicrobial compounds in the peelings or properties of high amounts of bulky feeds onto the gut flora caused changes of intestinal microbiota^(42,43). But a sufficient high amount of RS, which entered the large intestine, due to the high ingestion volumes of the potatoes might be also considerable.

Because large amounts of JA tubers and potatoes have shown to improve gut health by suppression of pathogenic bacteria, feeding regimes including large amounts of those tubers may provide a better option to support animal health than the application of pro- or prebiotica or other medication. Unlike antibiotics, which have a bactericidal impact also on the beneficial bacteria, they promote the euflora and suppress pathogenic bacteria by competitive exclusion. Further utility might result from bulking properties of JA tubers and potatoes, increasing gastric filling and chewing, which leads to more satiety and satisfaction^(43,15). In addition, rooting for feeds might satisfy natural foraging behavior of pigs⁽⁴⁴⁾. Moreover, they agree to the demand of the EC-regulation⁽¹⁴⁾ on organic farming for daily provision of roughages to pigs⁽¹⁴⁾ and can enhance utilisation of on-farm growable feeds. For consideration of pro and contra all further beneficial effects resulting from the JA tubers and potatoes have to be regarded and compared to additional effort and costs for the provision.

5.2 Quantification of the microbial mass

Beside influences on animal health, the activity and growth of intestinal bacteria in response to dietary treatments is also important to the expectable properties and quality of the manure^(23,26,45). Faeces is a heterogeneous substrate and contains a mixture of inorganic and organic bound nitrogen (N)⁽⁴⁵⁾. Inorganic N is quickly available for the plant, since it is very mobile in the soil. But it can be easily be lost through gaseous losses, leaching or runoff⁽⁴⁶⁾. Thus, inorganic N forms ammonia

(NH₃), methane (CH₄) and nitrous oxide (N₂O) are considerable sources for emissions from pig faeces and contribute to a larger extent to the environmental eutrophication and disturbances of sensitive ecosystems⁽⁴⁵⁾. Further, losses of soil borne N decrease its fertility and productivity⁽⁴⁶⁾. Indeed, N which is incorporated into the protein of bacteria is less mobile and gradually available for the crops, since it first has to be mineralised^(45,47). Towards the slower breakdown of bacterial protein, taking in dependence on the temperature weeks or even months, the degradation of urinary urea to ammonia and carbon dioxide (CO₂) needs only several hours^(23,47). Thus, the proportion of bacteria in faeces is a well known size for N emissions from pig facilities. The potential of increasing the amount of faecal bacteria by the enlarged inclusion of fibre in pig's diet has been approved as an appropriate tool to shift N excretion from urea in the urine to bacterial protein in the faeces in several studies^(22,23,26,48-50). Thereby, the dietary fibre serves as an energy source on the fermentation process of the gut bacteria and stimulates their growth⁽³⁾. The increased de novo synthesis of bacterial protein enhances utilisation of intestinal available NH₃ and the secretion of urea from the blood into the colon lumen^(23,26,45). In consequence, urinary N decreases⁽⁴⁶⁾. The potential savings of N emission to the environment has been investigated by Canh *et al.*⁽²⁶⁾, Zervas & Zijlstra⁽²⁴⁾ and Bindelle *et al.*⁽²³⁾. The inclusion of fermentable dietary fibre from soybean hulls and sugar beet pulp in an investigation of Zervas & Zijlstra⁽²⁴⁾ increased faecal N output from 5.1 to 7.7 g/day and decreased urinary to faecal N excretion ratio from 2.0 to 1.3 and 1.0. Canh *et al.*⁽²⁶⁾ and Bindelle *et al.*⁽²³⁾ showed in their studies, that the inclusion of 30% sugar beet pulp reduced the urinary to faecal N ratio from 3.8 to 1.2 resp. 2.1 to 1.2, which corresponds to a decrease in urinary N excretion of the total excreted N of up to 23%. Also from slurry ammonium (NH₄) emissions could have been lowered up to 53% with including 30% of sugar beet pulp into the diet of pigs⁽⁴⁸⁾.

Even though neither JA tubers nor potatoes contain considerable amounts of fibre⁽¹⁹⁾, there are indications that feeding the JA tubers or potatoes enhanced proportion of bacteria in pigs faeces in this study. Numbers of total anaerobe and aerobe bacteria in pigs fed the JA and the potato silage were increased towards the

control groups. Since data of colony-forming units (CFU) have been expressed as logarithmic function, the increase of a single unit of CFU as for instance in counts of aerobic bacteria between the treatment with potato silage (ST) and the control treatment (CT) in the first sampling period (Table 3.5) conforms to an almost 10-fold rise in bacterial numbers. Indeed, even though growth of bacteria is commonly considered with cell division and therefore with an increase in the total numbers of bacteria⁽⁵¹⁾, the implications of counts of CFU on the bacterial mass are limited⁽⁵²⁾. The exposure to a new environment, when bacteria are excreted with the faeces and submitted to dilution series, and influences of the selective growth media might restrict their meaningfulness^(51,52). Further, the determination of counts of CFU does not regard size of the bacteria and contains no information on their mass in the faeces⁽⁵¹⁾. Here, the ability to quantify bacteria directly out of their habitat as the microbial biomass quantification with high-speed centrifugation without necessitating cultivation steps may provide a more accurate measure of the biomass present in faecal samples⁽⁵²⁾. In addition, as considered in section 4.5.3, the evaluated procedure is not connected with difficulties associated with the use of marker techniques or the back-calculation of the bacterial and endogenous debris nitrogen (BEDN) fraction of the method of Mason⁽⁵³⁾. Since further research on the effects of different feeding regimes to N emission potential, the absorption by the pigs and excretion pathways are needed⁽⁴⁶⁾, the new procedure might be a helpful tool to assess response of the intestinal microbiota to the different- diets. The more knowledge is available about the proportion of faecal bacteria in response to the diet, the better effects of feeding treatments can be adapted for the reduction of the environmental load.

6 Conclusions

Feeding strategies with large amounts of tubers of the Jerusalem artichoke and potatoes provide an appropriate option to promote beneficial bacteria and inhibit the growth of potential pathogenic bacteria in pig's. Especially the reduction of *Clostridium perfringens* represents a clear improvement in the status of eubiosis. Promoting animal health by feeds included in the daily ration might constitute a better option than medication. In development of feeding strategies implications for animal health by effects on the gut bacteria and faecal nitrogen pattern should be taken more into account. Since being a good energy source, root vegetables as the tubers of the Jerusalem artichoke and potatoes can be easily integrated into the daily ration of pigs and allow high performances. Benefits of the improved animal health have to be regarded when considering additional labour and costs. Feeding regimes cannot be changed overnight. Besides the development of farm specific strategies for the feeding technique, nutritive value of the concentrate and ingestion volumes of the dietary components have to be adapted.

The procedure for isolation of bacteria in a solution using high-speed centrifugation to obtain a microbial pellet allows the quantitative capture of the microbial biomass in pig faeces. This might be a suitable tool to predict response of the intestinal microbiota to dietary treatments concerning implications on nitrogen emissions and environmental pollution.

Summary

The microbiota of the gastrointestinal tract (GIT) plays an important role regarding the fermentation processes in the different segments of the intestine with respect to the nutrient supply and in relation to the impacts of the microbiota on health status of the gut and the whole organism. Both effects of the microbiota depend to a high degree on the food composition. Thus, attempting to modify composition and activity of the gut flora, the application of pro- and prebiotics has gained increasing interests in animal nutrition.

Inulin and resistant starch (RS) have been identified as prebiotic active substances and purified in an industrial process to be used as feed additives. Because these substances are also available in the tubers of the Jerusalem artichoke (JA) (*Helianthus tuberosus*) and in potatoes (*Solanum tuberosum*), respectively, and since they are also known as energy-rich feedstuffs for pigs, it was the objective to assess the effects of the intake of JA tubers and potatoes on the intestinal microbiota and on selected parameters of the immune system in finishing pigs. While these studies focused primarily on qualitative effects on the microbiota, a methodological study was conducted to assess the microbial biomass quantitatively by a method for isolation of bacteria in a solution using high-speed centrifugation and comparing the amount of bacterial bound nitrogen (MP-N) with the calculated quantity of bacterial and endogenous debris nitrogen (BEDN). Additionally, the amino sugars galactosamine, glucosamine, mannosamine and muramic acid, substantial parts of cell walls of both bacteria and fungi, were determined in faeces and in the isolated bacteria.

In the first trial, a total of 72 finishing pigs were allocated in a free-range system to a control and an experimental treatment. In the control treatment (CT), pigs were fed with a wheat and grain legume-based concentrate mixture, formulated to meet animals' requirements to a performance level of 700 g daily weight gain (DWG). In the ET, pigs received 70% of the concentrate amount apportioned to the CT, but had access to an allocated area with cultivated JA tubers. Voluntary intake of JA tubers was estimated to 1.24 kg dry matter (DM)/day, corresponding to a mean inulin

ingestion of approximately 800 g/day. While growth performance of the pigs in the CT averaged 0.642 ± 0.014 kg/day, it increased in the ET to the extent of 0.765 ± 0.015 kg/day ($P=0.000$), emphasizing the tuber's suitability as a nutrient and energy source for free-ranged pigs. Abundant availability of inulin and fructo-oligosaccharides (FOS) in pigs GIT significantly increased total anaerobes ($P=0.000$), lactobacilli ($P=0.046$) and yeasts ($P=0.000$) and drastically reduced *Clostridium perfringens* from $\lg 5.24 \pm 0.17$ colony-forming units (CFU)/g wet faeces in the CT to $\lg 0.96 \pm 0.20$ CFU/g wet faeces in the ET ($P=0.000$) in pig faeces. C-reactive protein (CRP) and antibodies against lipopolysaccharides (LPS) of *Escherichia coli* J5 showed no differences between treatments. The differences in the microbiota between experimental and control treatments indicate the positive impacts of inulin on the composition of the microbiota in the hindgut.

A further study aimed to determine the effects of freshly steamed and steamed-ensiled potatoes, respectively, in comparison to a control diet offered to finishing pigs on the intestinal microbiota in pig's GIT, selected parameters of the immune system and analytical composition of the faeces. A total of 58 finishing pigs were integrated in the trial. A number of twenty pigs was assigned to a CT, fed with a concentrate mixture formulated to meet animals requirements with respect to a performance level of 700 g DWG. In the two experimental treatments, an amount of 1.2 kg DM of heat treated potatoes per day were offered additional to the concentrate. The potato supplementation consisted either of steamed potatoes (potato treatment, PT) or of steamed and ensiled potatoes (silage treatment, ST). The PT and ST, pigs were restricted in the supply with concentrate. They received only 46% resp. 43% of the amount of the same concentrate apportioned to the CT. Criteria of performance and carcass composition showed no significant differences between the treatments. In the PT and ST, pig's faeces were significant lower in pH value and contents of DM, neutral detergent fibre (NDF), undigested dietary nitrogen (UDN) and partially acid detergent fibre (ADF) ($P=0.000$) and were higher in contents of ammonium (NH_4) and ammonium bound nitrogen ($\text{NH}_4\text{-N}$) ($P=0.000$). High provision of heated potatoes, either as steamed potatoes or potato silage remarkably reduced the numbers of *E. coli* ($P=0.000$) and *C. perfringens* ($P=0.000$).

Furthermore, the concentration of immunoglobulin A versus LPS of *E. coli* J5 was clearly diminished ($P=0.001$). Moreover, in the ST treatment, total aerobes, total anaerobes, lactobacilli and yeasts were significantly increased in the first sampling period in comparison to the PT. The results indicate that thermal processed potatoes changed the intestinal microbiota and presence of antibodies and imply a beneficial effect on the gut flora of pigs.

The aim of the third experiment was to modify the procedure for isolation of bacteria in a solution using different centrifugation steps to obtain a microbial pellet (MP), which allows the quantitative isolation of bacteria in pig faeces. Additionally, the amount of BEDN and the amino sugars galactosamine, glucosamine, mannosamine and muramic acid were determined in the faeces and the MP. Faecal samples were obtained from pigs integrated in a phosphorus (P) metabolism trial. Ten male castrated pigs with a live weight of 51.1 ± 8.5 kg were housed individually in metabolism cages. Pigs were allotted to five dietary treatments, formulated to meet their requirements to a performance level of 700 g DWG, but with a daily P supply below the actual requirements of the animals in diets 2 to 5 and graduated supplementation of 50, 100 respectively 200 mg/kg of an experimental phytase in diets 3 to 5. Decreasing dietary P and increasing phytase supplementation significantly decreased ash content in the faeces ($P=0.024$) and DM ($P=0.017$) and P concentration ($P=0.000$) in the MP. The microbial biomass in pig faeces determined by the weight of the MP amounted to a mean value of 467 g/kg DM. Faecal nitrogen (N) averaged to 46.1 g/kg DM and the N proportion incorporated into the bacterial mass to 27.1 g/kg DM or 58% of the faecal N. The BEDN fraction came to a value of 73% of the faecal N. P in faeces and N in the MP amounted to mean values of 10.4 resp. 57.9 g/kg DM, which corresponded to values described in literature. P values in the MP ranged between 1.8 to 4.8 g/kg DM, depending on the level of dietary phytase ($P=0.000$). The amino sugars galactosamine, glucosamine, mannosamine and muramic acid did not differ significantly between treatments and were in the range of values from cattle faeces. Proportion of the amino sugars muramic acid to glucosamine in faeces and MP imply that the pellet was almost free

of fungi. The results indicate that the applied procedure seems to be appropriate for direct quantification of microbial biomass.

Summing up, feeding regimes which make use of inulin and starch by feeding the tubers of the Jerusalem artichoke and potatoes, respectively, provide an appropriate option to promote beneficial bacteria and inhibit growth of potential pathogenic bacteria, especially of *Clostridium perfringens*, in pig's faeces. Quantitative capture of the microbial biomass in pig faeces by producing a microbial pellet with high-speed centrifugation might be a suitable method to assess the biomass of the intestinal microbiota which might provide relevant quantitative information regarding the composition of faeces in further investigations when dealing with dietary effects.

Zusammenfassung

Die Mikrobiota im Gastrointestinaltrakt (GIT) spielt eine bedeutende Rolle beim Fermentationsprozess in den verschiedenen Darmabschnitten im Bezug auf die Nährstoffversorgung sowie die Gesundheit des Darms und des gesamten Organismus. Beide Effekte der Mikrobiota hängen in hohem Maße von der Zusammensetzung der Nahrung ab. Um die Zusammensetzung und Aktivität der Mikrobiota zu beeinflussen, hat die Nutzung von pro- und präbiotischen Futterzusätzen in der Tierernährung zunehmend Interesse erlangt.

Inulin und resistente Stärke (RS) konnten als präbiotisch wirksame Substanzen identifiziert werden und wurden in industriellen Verfahren für die Verwendung als Futterzusätze gereinigt. Da diese Substanzen jeweils auch in den Knollen der Topinamburpflanze (*Helianthus tuberosus*) und in Kartoffeln (*Solanum tuberosum*) enthalten sind und diese ebenfalls energiereiche Futtermittel für Schweine darstellen, war es die Zielstellung, die Auswirkungen der Aufnahme von Topinamburknollen und Kartoffeln auf die intestinale Mikrobiota und bestimmte Parameter des Immunsystems bei Endmastschweinen zu bestimmen. Während sich diese Untersuchungen vorrangig auf qualitative Auswirkungen auf die Mikrobiota konzentrierten, wurde eine methodische Studie durchgeführt um die mikrobielle Biomasse quantitativ mit einem Verfahrens zur Isolation von Bakterien in einer Flüssigkeit durch Hochgeschwindigkeits-Zentrifugation zu erfassen und den bakteriell gebundenen Stickstoff (MP-N) mit der Menge an bakteriellem und endogenem Kotstickstoff (BEDN) zu vergleichen. Zusätzlich wurden die Aminosucker Galactosamin, Glucosamin, Mannosamin und Muraminsäure, die substantielle Bestandteile der Zellwand von Bakterien und Pilzen darstellen, im Kot und in der isolierten Bakterienmasse bestimmt.

Im ersten Versuch wurden insgesamt 72 Endmastschweine in einem Freilandhaltungssystem in eine Kontroll- (CT) und Versuchsvariante (ET) aufgeteilt. Die Kontrollvariante wurde mit einer auf Weizen und Körnerleguminosen basierten Kraftfuttermischung gefüttert, die erstellt war, um den Bedarf der Tiere für ein Leistungsniveau von 700 g täglichem Lebendmassezuwachs zu decken. In der

Versuchsvariante erhielten die Tiere nur 70% der Kraftfuttermenge, die der Kontrollvariante zugeteilt wurde, hatten aber Zugang zu einer abgeteilten Fläche, auf der Topinamburknollen angebaut waren. Die freie Aufnahme von Topinamburknollen wurde auf 1.24 kg Trockenmasse (TM)/Tag bestimmt, entsprechend einer Inulinaufnahme von durchschnittlich 800 g/Tag. Während sich die Wachstumsleistung in der Kontrollvariante im Mittel auf 0.642 ± 0.014 kg/Tag belief, war sie in der Versuchsvariante mit 0.765 ± 0.015 kg/Tag ($P=0.000$) höher, was die Eignung der Knollen als Nährstoff- und Energiequelle für Freiland Schweine hervorhebt. Die freie Verfügbarkeit von Inulin und Fructo-oligosacchariden (FOS) im GIT der Schweine erhöhte die Keimzahlen der anaeroben Bakterien ($P=0.000$), Laktobazillen ($P=0.046$) und Hefen ($P=0.000$) signifikant und verringerte das Vorkommen von *Clostridium perfringens* im Schweinekot erheblich von $\lg 5.24 \pm 0.17$ koloniebildende Einheiten pro g Frischmasse in der Kontrollvariante auf $\lg 0.96 \pm 0.20$ koloniebildende Einheiten pro g Frischmasse in der Versuchsvariante ($P=0.000$). C-reaktives Protein (CRP) und Antikörper gegen Lipopolysaccharide (LPS) von *Escherichia coli* J5 ließen keine Unterschiede zwischen den Fütterungsvarianten erkennen. Die Unterschiede in der Mikrobiota zwischen der Kontroll- und Versuchsvariante weisen auf die positiven Auswirkungen von Inulin auf die Zusammensetzung auf die Mikrobiota im hinteren Darmabschnitt hin.

Eine weitere Untersuchung hatte das Ziel, die Auswirkungen von gedämpften sowie gedämpft-silierten Kartoffeln gegenüber einer Kontrollfütterung auf die intestinale Mikrobiota sowie bestimmte Parameter des Immunsystems und die analytische Zusammensetzung des Kotes bei Endmastschweinen zu bestimmen. Insgesamt wurden 58 Endmastschweine in den Versuch eingebunden. Zwanzig Schweine wurden einer Kontrollvariante (CT) zugeteilt, die bedarfsgerecht mit einer Kraftfuttermischung für ein Leistungsniveau von 700 g Tageszunahmen gefüttert wurde. In den zwei Kartoffelvarianten wurde zusätzlich zum Kraftfutter eine Menge von 1.2 kg TM hitzebehandelten Kartoffeln pro Tag gefüttert. Die Zufütterung der Kartoffeln bestand entweder aus gedämpften Kartoffeln (potato treatment, PT) oder gedämpften und einsilierten Kartoffeln (silage treatment, ST). Die Zufütterung von Kraftfutter war in den beiden Varianten PT und ST begrenzt. Sie erhielten je nur

46% bzw. 43% der Menge des Kraftfutters der Kontrollvariante. Die Wachstumsleistung und Schlachtkörperzusammensetzung ließen keine signifikanten Unterschiede zwischen den Fütterungsvarianten erkennen. In den Varianten PT und ST waren gegenüber der Kontrollvariante im Kot der pH-Wert sowie die Gehalte von TM, Neutral-Detergenz-Faser (NDF), unverdaulichem Futterstickstoff (UDN) und teilweise von Säure-Detergenz-Faser (ADF) signifikant niedriger ($P=0.000$) und die von Ammonium (NH_4) und Ammoniumstickstoff ($\text{NH}_4\text{-N}$) signifikant höher ($P=0.000$). Das hohe Angebot von hitzebehandelten Kartoffeln, entweder als gedämpfte oder als gedämpft-silierte Kartoffeln, führte zu einer erheblichen Verringerung von *E. coli* ($P=0.000$), *C. perfringens* ($P=0.000$) und Immunoglobulin A gegen LPS von *E. coli* J5 ($P=0.001$). Darüber hinaus waren in der ersten Versuchsperiode in der Variante ST die aeroben und anaeroben Gesamtkeimzahlen sowie die Laktobazillen und Hefen gegenüber der Variante PT signifikant erhöht. Die Ergebnisse deuten darauf hin, dass hitzebehandelte Kartoffeln die intestinale Mikrobiota und das Auftreten von Antikörpern verändert haben und einen gesundheitsförderlichen Effekt auf die Darmflora von Schweinen haben.

Das Ziel der dritten Untersuchung war die Modifizierung des Verfahrens zur Isolation von Bakterien in einer Flüssigkeit mittels verschiedener Zentrifugationsschritte, um ein mikrobielles Pellet (MP) zu erhalten, welches die quantitative Abtrennung und Erfassung der Bakterien in Schweinekot ermöglicht. Zusätzlich wurde der BEDN Anteil sowie die Gehalte der Aminosäuren Galactosamin, Glucosamin, Mannosamin und Muraminsäure im Kot und im MP bestimmt. Die untersuchten Kotproben stammten von Schweinen eines Phosphor (P) Stoffwechselversuch. Zehn männlich-kastrierte Schweine mit einem durchschnittlichen Lebendgewicht von 51.1 ± 8.5 kg wurden einzeln in Stoffwechselkäfigen gehalten. Die Tiere wurden fünf Fütterungsvarianten zugeteilt, die dem Bedarf der Tiere für ein Leistungsniveau von 700 g Tageszunahmen entsprachen, in den Rationen 2 bis 5 jedoch eine P-Gehalt unter dem Tagesbedarf der Tiere aufwiesen und in den Rationen 3 bis 5 mit abgestuften Gehalten von 50, 100 sowie 200 mg/kg einer experimentellen Phytase ergänzt waren. Die Absenkung des P Gehaltes im Futter verringerte den Asche- ($P=0.024$) und Trockenmassegehalt im Kot ($P=0.017$)

sowie die P Konzentration im MP ($P=0.000$) signifikant. Die mikrobielle Biomasse im Kot wurde durch die Wiegung des MP auf durchschnittlich 467 g/kg TM bestimmt. Der Stickstoffgehalt im Kot betrug im Mittel 46.1 g/kg TM und der in die Bakterienmasse eingebaute Stickstoffanteil 27.1 g/kg TM bzw. 58% vom Gesamtstickstoffgehalt im Kot. Die BEDN Fraktion wurde auf 73% am Kotstickstoff bestimmt. Der P-Gehalt im Kot sowie der N Gehalt im MP mit durchschnittlichen 10.4 und 57.9 g/kg TM lagen im Bereich von Literaturangaben. Die P Gehalte im MP schwankten in Abhängigkeit von der Zugabe von Phytase signifikant ($P=0.000$) von 1.8 bis 4.8 g/kg TM. Die Aminosucker Galactosamin, Glucosamin, Mannosamin und Muraminsäure wiesen keine signifikanten unterschiede zwischen Fütterungsvarianten auf und lagen im Bereich von Werten von Rinderkot. Das Verhältnis von Muraminsäure und Glucosamin im Kot und im MP lassen vermuten, dass das MP frei von Pilzen war. Ergebnisse weisen darauf hin, dass die angewandte Methode zur direkten Quantifizierung der mikrobiellen Biomasse geeignet ist.

Zusammenfassend stellen Fütterungsstrategien, die Inulin und Stärke jeweils durch die Fütterung von Topinamburknollen und Kartoffeln nutzen, eine geeignete Möglichkeit dar, gesundheitsförderliche Bakterien zu begünstigen und potentiell pathogene Bakterien, speziell *C. perfringens*, im Schweinekot zu hemmen. Die quantitative Erfassung der mikrobiellen Biomasse im Schweinekot mit der Herstellung eines mikrobiellen Pellets mit Hochgeschwindigkeits-Zentrifugation scheint eine geeignete Methode für die Bestimmung der Masse der intestinalen Mikrobiota zu sein und somit wichtige quantitative Informationen hinsichtlich der Zusammensetzung des Kotes für weiterführende Untersuchungen zu Fütterungseffekten zu liefern.

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Affidavit

I assure that this dissertation was written independently and without non-permissible help and that I used no sources other than those specified in the dissertation. All quotations that have been extracted from published or unpublished sources have been marked as such. No part has been used in other PhD processes.

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt und andere als in der Dissertation angegebenen Hilfsmittel nicht benutzt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten oder unveröffentlichten Schriften entnommen sind, habe ich als solche kenntlich gemacht. Kein Teil dieser Arbeit ist in einem anderen Promotions- oder Habilitationsverfahren verwendet worden.

Witzenhausen, 10th of February 2011

Charlotte Marien