RESEARCH ARTICLE

Establishment of intestinal microbiota with focus on yeasts of unweaned and weaned piglets kept under different farm conditions

Vladimir Urubschurov[1,3], Pawel Janczyk[1,3], Wolfgang-Bernhard Souffrant[1,3], Gertraude Freyer[2] & Annette Zeyner[3]

[1]Leibniz Institute for Farm Animal Biology (FBN), RU 'Oskar Kellner', Dummerstorf, Germany; and [2]FBN, Unit Genetics and Biometry, Dummerstorf, Germany; and [3]Chair for Nutrition Physiology and Animal Nutrition, University of Rostock, Rostock, Germany

Correspondence: Annette Zeyner, Chair for Nutrition Physiology and Animal Nutrition, University of Rostock, Justus-von-Liebig-Weg 8, 18059 Rostock, Germany. Tel.: +49 381 498 3320; fax: +49 381 498 3322; e-mail: annette.zeyner@uni-rostock.de

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Abstract

This study aimed to characterize the intestinal yeasts in weaning piglets and to establish their possible relationships with main bacterial groups. German Landrace piglets were weaned (WP, $n = 32$) at 28 days of age or kept with the dams until day 39 without creep feed (UP, $n = 32$). The experiment was performed at an experimental and a commercial farm (CF). Faeces were collected from the piglets, sows and pen floors on days 28, 33 and 39 for isolation of DNA and cultivation for enumeration of yeasts, enterobacteria, enterococci and lactobacilli. Fragments of the D1 domain of 26S rRNA gene were amplified and separated by denaturing gradient gel electrophoresis (DGGE). No yeasts could be cultured from water and feed samples. No or only low numbers of yeasts were detected among all UP. In WP at CF, yeasts correlated with lactobacilli ($r = 0.456$; $P = 0.009$) and enterobacteria ($r = -0.407$; $P = 0.021$). *Kazachstania slooffiae* dominated among the cultured yeasts. It was the only yeast species detected by PCR-DGGE. Yeasts, especially *K. slooffiae*, established in the porcine gastrointestinal tract after consumption of grain-based feed and may interrelate with the intestinal microbiota. The study provides data indicating importance of *K. slooffiae* for the development of balanced porcine gut microbiota.

Introduction

The gastrointestinal tract (GIT) of pigs harbours a highly complex and diverse microbiota (Leser *et al.*, 2002) that plays an important role in the development and activation of the mucosal and systemic immune system, metabolism of nutrients and helps to maintain the health of the host, specifically in young animals (e.g. Lallès *et al.*, 2004; Bauer *et al.*, 2006). Bacterial microbiota of the GIT of weaning piglets has been extensively investigated (e.g. Konstantinov *et al.*, 2006; Castillo *et al.*, 2007; Janczyk *et al.*, 2007a, 2010; Pieper *et al.*, 2008, 2009). However, not much attention has been focused on yeasts inhabiting this environment. Recently, a wide variety of yeasts was found in the intestine of insects (Boekhout, 2005; Suh *et al.*, 2005; Molnar *et al.*, 2008) and fish (Gatesoupe, 2007). Current knowledge about yeasts colonizing the porcine gut is still predominantly based on the culture-dependent investigations of the late 1950s to 1960s (Van Uden *et al.*, 1958; Van Uden & Do Carmo-Sousa, 1962; Mehnert & Koch, 1963; Gedek, 1968). Therefore, the authors started to investigate the intestinal yeasts of piglets around weaning. In a previous study, the on-farm conditions were shown to be an important factor affecting the diversity of yeasts in the porcine gut (Urubschurov *et al.*, 2008). In total, 17 yeast species were found, and *Kazachstania slooffiae*, *Galactomyces geotrichum*, *Candida catenulata* and *Candida glabrata* were the most often isolated species from the GIT of piglets (Urubschurov *et al.*, 2008).

Anaerobic fungi support the degradation of fibre in ruminants as they produce cellulolytic enzymes (Akin & Borneman, 1990; Gordon & Phillips, 1998). Application of probiotic strains of *Saccharomyces cerevisiae* or *Saccharomyces boulardii* to animals and humans was shown to affect the intestinal microbiota, to increase feed digestion and to improve host immune defence (Buts & De Keyser, 2006; Fonty & Chaucheyras-Durand, 2006; Jouany *et al.*, 2008; Buts, 2009). By contrast commensal yeasts (mainly *Candida*

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spp.) harboured in the gut of humans and animals are rather considered as opportunistic pathogens that can cause diseases in immune-suppressed individuals (Spencer & Spencer, 1997; Fidel *et al.*, 1999; Schulze & Sonnenborn, 2009). Nevertheless, it remains unclear whether the yeast population, and particularly if single yeast species play any role in the GIT of pigs.

Previous studies showed that yeasts occur in the porcine GIT in low numbers. Hence, culturing on selective agar media, allowing the detection of even a small amount of microorganisms, was chosen in the present study. The intestinal yeasts and main bacterial groups were cultured. In addition, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) was performed to investigate the changes in the yeast population present in the faeces, without prior culture. PCR-DGGE was applied as an established method for research on intestinal microbial ecology (Simpson *et al.*, 2000; Konstantinov *et al.*, 2004; Janczyk *et al.*, 2007a, 2010), as well as on yeast populations in different environments (Cocolin *et al.*, 2002; Prakitchaiwattana *et al.*, 2004; Fleet, 2007). To authors' knowledge, there are no published data concerning yeasts in the GIT of pigs based on the PCR-DGGE approach.

Current opinion is that yeasts harboured in the GIT are of only minor importance for the microbial community and the host. Nevertheless, they may be of physiological relevance, even though they are present to a much lesser extent than bacteria. They have a cell volume 30–100-fold higher than bacteria, providing a relevant biomass (Gatesoupe, 2007). Therefore, from the nutritional, medical and veterinary points of view, additional knowledge on the intestinal yeasts is important for better understanding of the GIT microbial ecology in pigs. For that reason, the present study was performed to: (1) determine the origin and time of appearance of yeasts in the GIT of weaned and suckling piglets, (2) investigate possible relationships between yeasts and selected bacterial groups inhabiting the porcine GIT at the time around weaning and (3) study whether yeast establishment in the GIT of piglets varies depending on different sanitary farm conditions.

Materials and methods

Animals, housing and diet

The study was conducted in two facilities with different hygienic conditions: at a modern experimental farm (EF) of the Leibniz Institute for Farm Animal Biology (FBN, Dummerstorf, Germany) and at a commercial farm (CF). The description of the farms has been reported in detail by Janczyk *et al.* (2010). No antibiotics were administered to all sows and piglets neither as prophylaxis nor as therapeutics at either farm to avoid any late changes in the GIT microbiota (Janczyk *et al.*, 2007b). The pigs were exposed to natural light regime and humidity (50–60%) at both farms. A total of 64 German Landrace piglets were used in this study. At each farm, four litters were randomly chosen, from six to 11 piglets. All piglets received only the sow's milk until weaning. Piglets from half of each litter were weaned at 28 days of age (WP), and were allocated to one pen per litter, without mixing the piglets from foreign litters. After weaning, a commercial diet (Porcibig, Trede & von Pein, Itzehoe, Germany) and water were offered to the piglets *ad libitum*. The composition of the piglets' diet is shown in Table 1. Other siblings remained with the sows until 39 days of age without creep feeding (UP). All procedures involving animal handling were performed in compliance with the German Law of Animal Protection.

Sample collection

At d28, d33 and d39, samples of faeces were collected directly from the anus of UP and WP and of sows, as well as from the pen floors of UP. They were placed on ice and transported immediately to the laboratory. In addition, samples of water ($n = 5$; 100 mL) and the feed ($n = 5$; 100 g) were collected at different times for cultivation analysis of the yeast presence, considering these could be possible sources of yeasts for the piglets.

Cultivation analysis of yeasts and bacteria

The collected samples were homogenized in a BagMixer® 400 (Interscience, St-Nom, France), serially diluted (10-

Table 1. Composition of the piglets' diet in the study according to manufacturer*

Ingredients	%	Chemical composition	g kg^{-1}
Wheat	30.0	Dry matter	884.3
Barley	20.2	Starch+sucrose	461.4
Soybean meal (43% protein)	18.0	Crude protein	176.1
Corn flakes	12.0	Ash	62.5
Cow's milk powder	4.5	Crude fat	51.6
Premix†	3.5	Crude fibre	36.3
Soy protein isolate	3.0	Lysine	12.2
Dried beet pulp	2.0	Ca	8.7
Soybean oil	1.3	K	8.0
Sucrose	1.0	Threonine	7.6
Fish oil	0.5	P	6.5
Vegetable fat†	0.4	Methionine	4.1
Vitamins and minerals†	to 100.0	Cystine	3.2
		Na	2.5
		Tryptophan	2.0
		Mg	1.9
		Cl	0.5

*The diet was produced as a powder without addition of acid complexes, digestive enzymes and probiotics.
†Provided by producer without further details.

fold) in NaCl solution (0.9%) and plated in duplicates onto selective agar plates: crystal-violet neutral-red bile glucose agar according to Mosel for enumeration of enterobacteria; enterococcus agar according to Slanetz–Bartley for enterococci; lactobacilli agar according to De Man, Rogosa and Sharpe for lactobacilli; and Sabouraud glucose agar, containing 50 μg mL^{-1} chloramphenicol for yeasts, as described by Pieper *et al.* (2006). The CFUs of enterobacteria, enterococci and yeasts were quantified manually after aerobic incubation at 37 °C, for 24 h, 72 h and 5 days, respectively. Enumeration of lactobacilli was performed after cultivation for 72 h at 37 °C in anaerobic jars using Anaerocult A (Merck, Darmstadt, Germany). After quantitation, the yeast colonies found on the agar plates were analysed visually and microscopically for their morphology. The mean numbers of CFU of the duplicates were transformed to log CFU g^{-1} faeces for further statistics.

Molecular analysis of intestinal yeasts

DNA extraction

Total genomic DNA was extracted from faeces using the FastDNA® Spin Kit (MP Biomedicals, Heidelberg, Germany) according to the manufacturer's instructions. The purity and concentration of eluted DNA was measured on Nanodrop® ND–1000 spectrophotometer (PEQLAB Biotechnology, Germany). DNA extracts were then diluted to set up a working solution (WS) containing 15 ng DNA μL^{-1}. Equal amounts of four individual WS were combined to obtain four DNA-mixes per group, day and farm. The DNA-mixes were then used as templates for PCR-DGGE.

PCR-DGGE

To investigate the intestinal yeast population, fragments of the D1/D2 domain of 26S rRNA gene from the collected samples were amplified by PCR in a MJ Mini™ Personal Thermal Cycler (Bio-Rad, Munich, Germany). Furthermore, the DNA extracted from different cultivable yeasts obtained in a previous study (Urubschurov *et al.*, 2008) and several yeast strains from Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands) (Table 2) were also amplified. Direct (one-step) PCR (sPCR) and nested two-step PCR (nPCR) were performed in order to prove whether lower numbers of yeast could be detected by nPCR. Moreover, preliminary tests of PCR with different concentrations of extracted DNA (1, 10 and 15 ng μL^{-1}) showed an unsuccessful outcome with the lower DNA concentrations. Therefore, 4 μL (60 ng) of extracted DNA was added as a template for sPCR and the first step of nPCR. PCR products obtained in the first step of nPCR (1 μL) were used as templates for the second step of nPCR. Taq DNA Polymerase, dNTP's Mix and incubation buffer used for the PCR were obtained from MP Biomedicals in Q-BioTaq Core Kit. The first step of nPCR was carried out with primer set NL1-f (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4-r (5′-GGT CCG TGT TTC AAG ACG G-3′) (Kurtzman & Robnett, 1998), applying the following program: 3 min at 98 °C, followed by 35 cycles of 95 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 7 min. For the second step of nPCR and for sPCR the primer set NL1-GC-f (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA TAT CAA TAA GCG GAG GAA AAG-3′) (GC clamp is underlined) and LS2-r (5′-ATT CCC AAA CAA CTC GAC TC-3′) (Cocolin *et al.*, 2000) was used. The above PCR program was modified only for the annealing temperature that was set to 56 °C. The obtained amplicons were separated on 8%-polyacrylamide gels with a denaturing gradient of 30–60% using DGGE as described by Janczyk *et al.* (2007a). The amplicons obtained for the isolates were used as references. The electrophoresis was conducted at 85 V for 16 h in 0.5 × TAE buffer at a constant temperature of 60 °C in a DCode Universal Mutation Detection System (Bio-Rad). Gels were stained with SYBR Gold (Invitrogen, Eugene, OR) and photographed in an AlphaDigiDoc RT gel documentation system (Alpha Innotech Corporation, San Leandro, CA) as described elsewhere (Janczyk *et al.*, 2007a). All visible bands present in the gels were picked with a sterile needle, resuspended in 100 μL Millipore water, vortexed shortly before incubation for 4 h at 4 °C and reamplified using the primers NL1 and LS2 as described previously. The amplicons obtained were purified and sequenced as described by Urubschurov *et al.* (2008). The retrieved sequences were aligned to the GenBank database entries applying the MEGABLAST tool available at the NCBI website (Altschul *et al.*, 1997).

Phylogenetic analysis

The sequences of the D1/D2 domain of 26S rRNA gene of the cultured yeasts (Table 2) were lopped off the size of the fragments amplified with the primers NL1 and LS2 and then used for the construction of the phylogenetic tree. The tree was built using the neighbour-joining method based on the Tamura–Nei distance model. The branches were supported in more than 50% of 1000 bootstrap replicates. The whole procedure was performed using GENEIOUS PRO 5.1.7 software (Biomatters Ltd, Auckland, New Zealand).

Statistical analysis

The data from the piglets were analysed for the log of CFU of the traits yeasts, lactobacilli, enterobacteria, enterococci by adapting the MIXED procedure using the SAS package version 9.1 (SAS Institute Inc., Cary, NC). The general

Table 2. Strains of the cultivable yeasts applied to the PCR-DGGE fingerprinting and included in the phylogenetic analysis

NCBI accession numbers*	Species	G/C content (%)†	Strain origin
AY545579	*Kazachstania slooffiae*	52.3	Caecum, pig, Portugal, CBS 9733
–‡	*Kazachstania slooffiae*	52.3	Caecum, pig, Portugal, CBS 4068
EU445375	*Kazachstania slooffiae*	52.4	Colon, pig, obtained in Urubschurov *et al*. (2008)
EU445377	*Candida glabrata*	48.8	
EU445378	*Candida oleophila*	45.8	
EU445379	*Candida parapsilosis*	46.5	
EU445381	*Candida catenulata*	50.2	
EU445382	*Candida tropicalis*	45.7	
EU445384	*Issatchenkia orientalis*	56.9	
EU445385	*Pichia anomala*	45.6	
EU445386	*Pichia farinosa*	46.0	
EU445388	*Rhodotorula mucilaginosa*	49.8	
EU445392	*Trichosporon montevideense*	45.5	
EU445444	*Galactomyces geotrichum*	44.5	

*Sequences registering on the accession numbers represent D1/D2 domain of the 26S rRNA gene.

†GC content correspond to D1 domain of the 26S rRNA gene.

‡The fragment of the strain revealed 99% similarity with AY545579, therefore it was not deposited to GenBank.

statistical model for analysing the data followed the design of the study and included the fixed effects of farm (EF and CF), weaning group (UP and WP), litter (one litter of four sows each) and age (three groups of age: days 28, 33 and 39, d28, d33 and d39, respectively). First, all four traits of interest were analysed including these factors into the model. Further, we tested pair-wise interaction effects between the given factors (age, weaning group, litter and farm). The effect of the interaction effects on each trait was considered to be significant and it was used trait specifically in further analyses if $P < 0.05$.

The trait relationship was targeted next. In a first step, all possible combinations of covariables were examined. The Pearson correlation coefficients were calculated between all traits. A selection of them was of interest for a more detailed consideration. For this reason lactobacilli were taken as a covariable for yeasts, and yeasts were taken as a covariable for analysing the other three traits. The differences between the trait-specific log CFU, within single groups, ages and farms ('classes' for comparison of two-factorial interaction effects each) in the piglet data were marked if those effects were significant (according to an *F*-test based on MIXED procedure, $P < 0.05$). Interactions affecting a trait significantly were included in the trait-specific model.

Results

All animals remained in good condition throughout the whole study. No diarrhoea or animal losses were recorded.

Microbiological culture analyses

Related to the rather small number of piglets, quite a large number of factors affecting the CFU in piglets had to be considered. The hypothesis was that the management system in a farm has an effect on these traits, besides age and weaning status. As a result (Supporting Information, Table S1), the effects of group and age were always significant in analysing piglets ($P < 0.0001$), except for lactobacilli (factor weaning group). As expected, significant interaction effects of factors in all traits were obtained only for weaning group × age (Table S1). But yeasts showed also sensitivity to interactions: group × age, farm × age, farm × group and group × litter; lactobacilli to age × litter and enterococci to farm × litter. Therefore, the differences between single groups were tested separately, as shown in Table 3. For lactobacilli, enterococci and enterobacteria, results of involving yeasts as a covariable were significant ($P < 0.0001$, $P = 0.04$ and $P = 0.01$) if considering it to be linear. Apart from yeast, no significance was obtained from the quadratic model (Table S2).

No yeasts could be cultivated from the samples of water and feed. The CFU of yeasts and the selected bacterial groups are presented in Table 3. When the CFU of 28-day-old piglets were taken together within a farm, no differences in lactobacilli and enterobacteria counts were observed between farms, whereas yeasts ($P < 0.001$) and enterococci ($P < 0.0213$) counts were significantly lower in piglets at CF than at EF (Table 4). No changes in the microbial counts were observed in the faeces of UP until d39. No or only low numbers (10^1–10^2 CFU g^{-1} faeces) of yeasts were found among all UP. The yeast counts increased in WP with age, and there was a farm effect. At d39 they reached 10^4 and 10^6 CFU g^{-1} in EF and CF, respectively. Enterococci counts decreased in WP from d28 to d33 in EF and in CF ($P < 0.001$ and $P < 0.001$, respectively); enterobacteria counts decreased at both farms (EF; $P < 0.02$ and CF;

Table 3. Counts (log CFU g^{-1}, mean ± SD) of yeasts, lactobacilli, enterobacteria and enterococci cultivated from faeces of unweaned (UP) and weaned piglets (WP), collected at 28, 33 and 39 days of age

	EF			CF		
Group	d28	d33	d39	d28	d33	d39
Yeasts						
UP	0.7 ± 1.4	2.2 ± 1.6	2.0 ± 1.8	0.8 ± 1.5^{a}	1.1 ± 1.6^{B}	$2.4 \pm 1.8^{B,b}$
WP	1.6 ± 1.7^{a}	3.8 ± 1.7^{b}	3.9 ± 2.0^{b}	1.0 ± 1.5^{a}	$4.4 \pm 1.5^{A,b}$	$6.6 \pm 0.8^{A,c}$
Lactobacilli						
UP	8.6 ± 0.4	8.5 ± 0.6	8.1 ± 0.5^{B}	8.4 ± 0.7	8.3 ± 0.4^{A}	8.2 ± 0.3
WP	8.7 ± 0.6	$8.5 \pm 0.5^{\#}$	$8.7 \pm 0.9^{A,\#}$	8.4 ± 0.6^{a}	$8.1 \pm 1.4^{c,B,\#}$	$9.3 \pm 0.3^{b,\#}$
Enterobacteria						
UP	8.4 ± 0.5	8.1 ± 0.8	7.6 ± 1.0	8.3 ± 0.8	7.7 ± 0.6	7.7 ± 0.7^{A}
WP	8.5 ± 0.5^{a}	$6.8 \pm 1.0^{b,\#}$	6.8 ± 0.9^{b}	7.8 ± 0.8^{a}	$7.0 \pm 0.8^{b,\#}$	$6.0 \pm 0.6^{c,B}$
Enterococci						
UP	7.6 ± 1.3	7.4 ± 1.2^{A}	7.1 ± 1.1^{A}	6.4 ± 1.4	5.9 ± 1.1	5.9 ± 0.8
WP	7.6 ± 1.1^{a}	$4.8 \pm 1.2^{b,B,\#}$	3.8 ± 1.2^{B}	6.6 ± 1.3^{a}	$4.0 \pm 2.1^{b,\#}$	3.1 ± 2.5^{b}

The piglets were reared either at an EF or at a CF, number of piglets per farm and weaning group was 16.
Different lower case superscripts referred to significant age effects in a farm either within WP or UP. Upper case superscripts referred to significant effects of WP vs. UP within days and a farm.
#Significant effects EF vs. CF within days of WP or UP ($P < 0.05$).

Table 4. Combined counts (log CFU g^{-1}, mean ± SD) of yeasts and bacterial groups cultivated from faeces of sows ($n = 4$ per farm) and from pens, collected at 28, 33 and 39 days after farrowing, from piglets' faeces collected at 28 days of age

Source	Farm	No. of samples	Yeasts	Lactobacilli	Enterobacteria	Enterococci
Sow	EF	12	5.6 ± 0.3	8.5 ± 0.5	7.3 ± 0.4	5.6 ± 1.2
	CF	12	2.2 ± 2.0	8.4 ± 0.6	5.8 ± 1.0	3.8 ± 0.7
Piglets	EF	32	1.1 ± 1.6^{a}	8.7 ± 0.5	8.5 ± 0.5	7.6 ± 1.2^{a}
	CF	32	0.9 ± 1.5^{b}	8.4 ± 0.6	8.0 ± 0.8	6.5 ± 1.3^{b}
Faeces from pens	EF	12	5.0 ± 1.7	7.8 ± 2.4	7.5 ± 0.9	6.4 ± 1.1
	CF	12	3.3 ± 2.1	8.0 ± 0.3	6.8 ± 1.0	7.1 ± 0.7

Feed and water did not display any yeast. Bacteria were not analysed in feed and water.
Superscript letters mark significant ($P < 0.05$) effects of EF and CF on a microbial group.

$P = 0.03$). Lactobacilli counts remained constant at EF and decreased at CF ($P = 0.0243$). In WP at EF, no changes were observed until d39, while at CF, yeasts ($P = 0.001$) and lactobacilli ($P < 0.0001$) increased and enterobacteria ($P = 0.01$) decreased. As there were no differences in microbial counts between the different time points in UP, only the log CFU recorded in WP on d33 and d39 were taken for calculation of Pearson's correlation coefficients between the microbial groups, separately for each farm. In CF, yeasts correlated with lactobacilli ($r = 0.456$; $P = 0.009$) and enterobacteria ($r = -0.407$; $P = 0.021$), whereas in EF, lactobacilli correlated with enterobacteria ($r = 0.455$; $P = 0.009$) and enterococci ($r = -0.358$; $P = 0.045$). In EF, yeasts correlated with lactobacilli ($r = 0.348$; $P = 0.051$).

Because there were no differences in feeding and handling of the sows, the log CFU on d28, d33 and d39 were considered as repetition and taken jointly within a farm. The counts of enterobacteria, enterococci and yeasts were numerically (not significantly) higher at EF than at CF, with no differences in lactobacilli counts (Table 4). Furthermore, all sows from EF were colonized by yeasts, whereas no yeasts were cultured from two of four CF sows. The analysis of faeces from the pen floors revealed no differences in microbial groups between farms (Table 4).

The isolated yeast colonies were evaluated according to their morphology. Slightly glanced, oval and smooth or star-like colonies with rays were identified as typical for *K. slooffiae* (Urubschurov *et al.*, 2008). They dominated on the plates of higher dilutions (10^{-4}–10^{-5}), whereas white, farinaceous or hairy colonies resembling to other yeasts, mainly *Geotrichum*, were found solely on the plates of lower dilutions (up to 10^{-2}).

PCR-DGGE fingerprinting

To study the yeasts community of the GIT of pigs, PCR-DGGE fingerprinting of D1/D2 domain of 26S rRNA gene was performed. Two PCR approaches were applied. Application of nPCR demonstrated results comparable to sPCR, merely revealing a higher bands' intensity (data not shown).

Therefore, the sPCR was found to be sufficient for the DGGE analyses. Amplicons (approximately 250 bp) of D1 domain of 26S rRNA gene amplified using the primers NL1 GC and LS2 could be successfully separated (Fig. 1). Three *Candida* spp., namely *Candida parapsilosis*, *Candida oleophila* and *Candida tropicalis* gave the same DGGE fingerprint. Moreover, the strains of *K. slooffiae* from CBC showed presence of two bands, whereas the strain of *K. slooffiae* isolated by authors produced only one band.

No PCR products were amplified from the faecal samples collected from the pen floors and from the sows. No amplicons could be also detected in the faeces from piglets on d28 and all other UP. Hence, the application of DGGE analyses for these samples was not possible.

Only one band could be detected in the pools of DNA extracted from faeces of WP on d33 and d39 (Fig. 2), which was shown to belong to *K. slooffiae*. The band was visible in EF piglets in two of four profiles on d33 and only in one on d39, whereas it was present at all CF piglets on d39.

Phylogenetic analysis

A Neighbour-joining tree of D1 domain of the 26S rRNA gene was constructed to evaluate the relatedness among the strains of cultured yeasts used for PCR-DGGE analysis. Because the yeast strains CBS 9733 and CBS 4068 showed 99% similarity, only the sequence of CBS 9733 was introduced into the phylogenetic analysis. As shown in Fig. 3, a part of strains was affiliated into two clusters: both strains of *K. slooffiae* and *C. glabrata*, and other *Candida* spp. with *Pichia* spp. The remainder of strains represented separate branches.

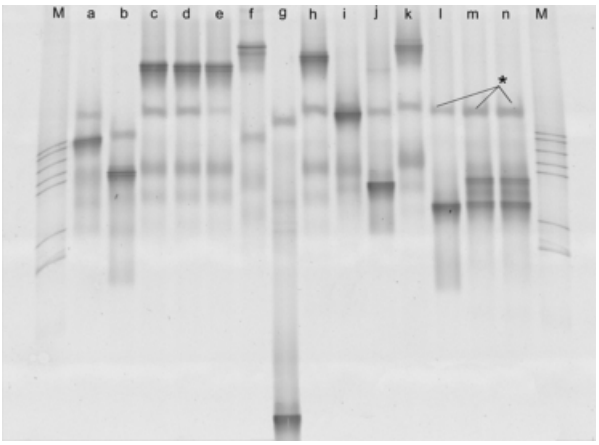

Fig. 1. DGGE profiles of PCR amplicons of D1 domain of the 26S rRNA gene from pure strains of yeasts isolated from GIT of piglets. *The common diffuse bands represent single-stranded DNA. (M) Marker, (a) *Candida glabrata*, (b) *Candida catenulata*, (c) *Candida parapsilosis*, (d) *Candida oleophila*, (e) *Candida tropicalis*, (f) *Galactomyces geotrichum*, (g) *Issatchenkia orientalis*, (h) *Pichia anomala*, (i) *Pichia farinosa*, (j) *Rhodotorula mucilaginosa*, (k) *Trichosporon montevideense*, (l) *Kazachstania slooffiae*, (m) *K. slooffiae* (CBS 4068), (n) *K. slooffiae* (CBS 9733).

Discussion

The present study recorded the time point of appearance and dynamic alterations of yeasts in the GIT of piglets, taking into account their possible relationship with selected intestinal bacterial groups.

Weaning implies complex physiological, social, environmental and dietary stresses that result in drastic changes of the microbial composition (Lallès *et al.*, 2004; Janczyk *et al.*, 2007a). These changes are not associated only with the age of the animals, as the results of the present study clearly showed. No considerable alterations in the microbial population of suckling piglets were recorded at d39, whereas the intestinal microbiota in piglets weaned at d28 showed differences at d33 and d39. The CFU of total microbiota, including lactobacilli, decrease in the GIT of piglets within 1-day postweaning (Pieper *et al.*, 2008), and drastic changes within the lactobacilli population can be observed at this time (Janczyk *et al.*, 2007a). Among other factors, this could be particularly caused by an abrupt nutritional change (from liquid milk to solid diet) and also by a drastic reduction of feed intake (Lallès *et al.*, 2004).

The intestinal microorganisms need time to adapt to solid feed, which is more complex in nutrient composition (Lallès *et al.*, 2007) considering the amount, quality and composition of protein, fat and carbohydrates in comparison to milk (Klobasa *et al.*, 1987). The dominant lactobacilli in the porcine gut, such as *Lactobacillus sobrius*, *Lactobacillus reuteri* and *Lactobacillus acidophilus* (Konstantinov *et al.*, 2006; Janczyk *et al.*, 2007a), may rapidly adapt to the new feed components (Pieper *et al.*, 2008). The present results based on cultivation analyses correspond with those obtained by FISH enumeration (Pieper *et al.*, 2008). They showed that the total number of lactobacilli in both farms after weaning (at the EF, on d33) re-established to the initial level, while at the same time enterobacteria and enterococci numbers were still 10–100-fold lower than at weaning. The number of yeast increased with the supply of solid feed (up to 4 and 6.6 $\log \mathrm{CFU\,g^{-1}}$ at EF and CF, respectively). The changes in the yeast and bacterial counts with time after weaning depended on the farm. Yeasts correlated positively with lactobacilli and negatively with enterobacteria in WP at EF; a positive correlation ($P = 0.051$) could be also observed between yeasts and lactobacilli. This is in agreement with previous observations of the authors' group (Janczyk *et al.*, 2007a, 2010; Pieper *et al.*, 2008). Such development is possibly favourable for the maintenance of intestinal equilibrium in the critical weaning period, because the lactobacilli have a beneficial effect on the host's health and may antagonistically act against pathogenic bacteria (Servin,

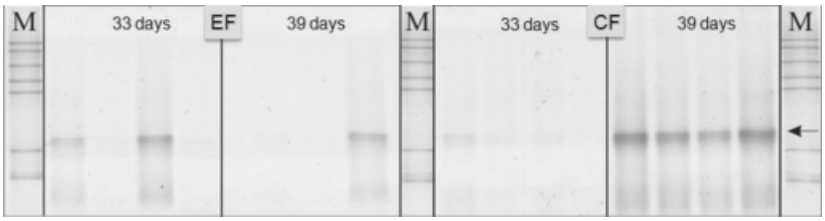

Fig. 2. DGGE profiles of PCR amplicons of D1 domain of the 26S rRNA gene of uncultured yeasts isolated from faeces of weaned piglets, collected at 33 and 39 days of age. The piglets were kept either at an EF or at a CF. (←) *Kazachstania slooffiae*, the bands were identified by sequence analysis.

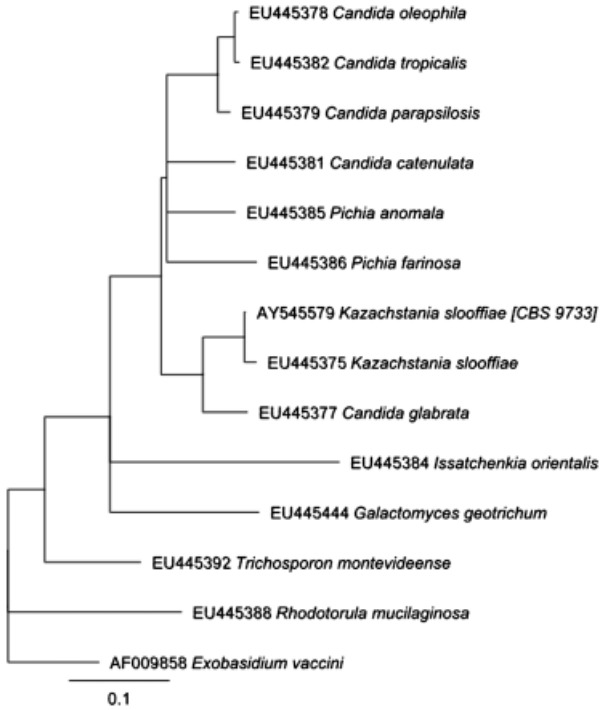

Fig. 3. Phylogenetic tree of cultivable yeasts, derived from sequence of D1 domain of the 26S rRNA gene, generated with neighbour-joining algorithm using Tamura–Nei model. *Exobasidium vaccinii* was the designated outgroup species to root the tree. The accession numbers (NCBI GenBank) provided in the figure correspond to the sequences of D1/D2 domain of the 26S rRNA gene. The sequences were truncated to the D1 domain sequence to build the tree.

2004). Besides, a reduction of the enterobacteria in the intestine, particularly the enteropathogenic strains of *Escherichia coli*, has been generally accepted to be beneficial for the host.

However, it is not known whether yeasts affect the bacterial composition or the other way around. Some yeast species and strains are characterized by having antagonistic activity against other microorganisms like producing toxins or antimicrobial compounds; others may synergistically interact with bacteria (Magliani *et al.*, 1997; Viljoen, 2006). For example, such mutualistic interrelationship between yeasts and lactic acid bacteria naturally occurs, which has been widely used in the production of fermented foods such as cheese varieties, kefir, koumiss, viili, langfil and many others (Beresford *et al.*, 2001; Wouters *et al.*, 2002). Some yeasts synthesize vitamins, amino acids or purines and thereby promote the growth of lactic acid bacteria. Consequently, this leads to a production of organic acids and thereby a pH decrease, creating favourable conditions for the development of yeasts or inhibiting proliferation of pathogenic bacteria (Viljoen, 2006). To what extent the yeast population affects directly the growth and composition of the intestinal microbiota cannot be concluded from the results obtained in this study. However, it can be hypothesized that the yeasts harbouring the GIT may also synthesize substrates affecting the growth of some bacteria.

Nevertheless, the question remains which yeast species may potentially have an impact on the intestinal microbial composition. Our previous cultivation-based study on the biodiversity of yeasts from the porcine GIT revealed *K. slooffiae*, *G. geotrichum*, *C. catenulata* and *C. glabrata* to be the most frequent isolates among the recorded 17 species (Urubschurov *et al.*, 2008). Many microorganisms remain viable but noncultivable (VBNC), and cannot be cultivated in the laboratory because of nutrient limitation or lack of optimal living conditions (Edwards, 2000). Therefore, in the present study PCR-DGGE was performed to detect the potential VBNC yeasts present in the faeces. For example, Prakitchaiwattana *et al.* (2004) investigated wine yeasts and detected more species by DGGE than by classical cultivation-based analysis and thereby confirmed the existence of VBNC yeasts.

Here, primers flanking the D1 domain were applied, which have already been proven for their suitability for DGGE analysis of yeast populations in milk (Cocolin *et al.*, 2002), oil (Hesham *et al.*, 2006) and wine (Cocolin *et al.*, 2000; Prakitchaiwattana *et al.*, 2004). In this study, the DGGE of pure yeast cultures revealed the limitations of this method, as neither phylogenetically closely related species such as *C. parapsilosis*, *C. oleophila* and *C. tropicalis* nor *G. geotrichum* and *Trichosporon montevideense* revealing 66.4% similarity could be discriminated, in agreement with previous reports (Nubel *et al.*, 1996; Cocolin *et al.*, 2000, 2001). CBS strains of *K. slooffiae* produced two DGGE bands, whereas the strain isolated by Urubschurov *et al.* (2008) resulted in only one band, that could also be determined as the solitary DGGE band in the faeces of WP

on d33 and d39. Furthermore, this band was occasionally present at low intensity in the EF piglets, whereas it was found in high intensity in all profiles from CF piglets on d39. These data indirectly confirm the cultivation results, suggesting that CF pigs were colonized by a higher yeast quantity than EF pigs. Moreover, the present DGGE findings revealed that despite the fact that many species of yeasts may be harboured the porcine GIT, only *K. slooffiae* could reach the detection limit of DGGE, which remains at 1% of the total microbial community (Muyzer & Smalla, 1998).

As concluded in earlier studies (Van Uden & Do Carmo-Sousa, 1962; Mehnert & Koch, 1963), *K. slooffiae* is typical for the porcine GIT. *Kazachstania slooffiae* was shed by all four sows at EF, and by two of the four sows examined at CF. We suppose, as no yeasts were detected in the feed samples, that the sow faeces were the source of yeast for young piglets. The farm employees could have possibly brought the faeces from other pigs, which resulted in colonization of the piglets' GIT. However, it cannot be excluded that, at least in part, the feed could be a source of the yeasts, as for cultivation only small samples were taken, providing a detection limit of 1000 colonies kg^{-1} feed.

This study also determined the time of appearance of yeast in the GIT of piglets. In a previous study, *K. slooffiae* was isolated in equal abundances from both WP and UP at EF (Urubschurov *et al.*, 2008). It was hypothesized that this was a result of the access of the piglets to sows' feed at EF. In order to solve this problem, in the present study the sows were fed in separate pens, so that the piglets could not access the feed. Indeed, no *K. slooffiae*-like colonies were isolated from UP. In contrast, low numbers (10^1–10^2 CFU g^{-1}) of yeasts, chiefly *Geotrichum*-like colonies, were found among all UP, up to d39. By contrast colonies characteristic for *K. slooffiae* dominated at this time among the yeasts cultivated from weaned piglets. A possible explanation of this phenomenon could be the fact that *K. slooffiae*, similar to the majority of yeast species isolated from the GIT of piglets, is unable to metabolize lactose, according to experience in our laboratory. Thus, the yeast population established in the porcine gut after consumption of grain-based solid feed, and *K. slooffiae* was confirmed to be the predominant yeast in the porcine intestine.

Kazachstania slooffiae could not be cultured from the gut of pigs depressed due to feed changes and digestive disturbances (Gedek, 1968), and no data exists on the occurrence of *K. slooffiae* in diseased pigs. This species has been isolated only from healthy animals. Therefore, it can be assumed that *K. slooffiae* commensally settles the pig intestine and probably interacts with intestinal bacteria to establish a balanced ecosystem. Unfortunately, apart from the knowledge about the morphological characterization and that under laboratory conditions *K. slooffiae* metabolizes only glucose (Kurtzman *et al.*, 2005), it remains unclear whether this yeast could produce any bacteriocins or substances beneficial to the host and/or some bacteria. Hence, further research is needed to characterize this species, its physiology and its role in the intestinal ecosystem.

To summarize, yeast colonize the porcine intestinal tract after introduction of solid feed. *Kazachstania slooffiae* dominates the intestinal yeast population. Development of the yeast community may affect the bacterial composition, and two different farm conditions have a significant impact in the establishment of the intestinal microbiota. The importance of yeasts, and in particular *K. slooffiae*, needs further research based on physiological and molecular investigations, different from PCR-DGGE.

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References

Akin DE & Borneman WS (1990) Role of rumen fungi in fiber degradation. *J Dairy Sci* **73**: 3023–3032.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Bauer E, Williams BA, Smidt H, Verstegen MW & Mosenthin R (2006) Influence of the gastrointestinal microbiota on development of the immune system in young animals. *Curr Issues Intest Microbiol* **7**: 35–51.

Beresford TP, Fitzsimons NA, Brennan NL & Cogan TM (2001) Recent advances in cheese microbiology. *Int Dairy J* **11**: 259–274.

Boekhout T (2005) Biodiversity: gut feeling for yeasts. *Nature* **434**: 449–451.

Buts JP (2009) Twenty-five years of research on *Saccharomyces boulardii* trophic effects: updates and perspectives. *Dig Dis Sci* **54**: 15–18.

Buts JP & De Keyser N (2006) Effects of *Saccharomyces boulardii* on intestinal mucosa. *Dig Dis Sci* **51**: 1485–1492.

Castillo M, Martin-Orue SM, Nofrarias M, Manzanilla EG & Gasa J (2007) Changes in caecal microbiota and mucosal morphology of weaned pigs. *Vet Microbiol* **124**: 239–247.

Cocolin L, Bisson LF & Mills DA (2000) Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiol Lett* **189**: 81–87.

Cocolin L, Manzano M, Cantoni C & Comi G (2001) Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. *Appl Environ Microb* **67**: 5113–5121.

Cocolin L, Aggio D, Manzano M, Cantoni C & Comi G (2002) An application of PCR-DGGE analysis to profile the yeast populations in raw milk. *Int Dairy J* **12**: 407–411.

Edwards C (2000) Problems posed by natural environments for monitoring microorganisms. *Mol Biotechnol* **15**: 211–223.

Fidel PL Jr, Vazquez JA & Sobel JD (1999) *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin Microbiol Rev* **12**: 80–96.

Fonty G & Chaucheyras-Durand F (2006) Effects and modes of action of live yeasts in the rumen. *Biologia* **61**: 741–750.

Fleet GH (2007) Yeasts in foods and beverages: impact on product quality and safety. *Curr Opin Biotech* **18**: 170–175.

Gatesoupe FJ (2007) Live yeasts in the gut: natural occurrence, dietary introduction, and their effects on fish health and development. *Aquaculture* **267**: 20–30.

Gedek B (1968) *Hefen als Krankheitserreger bei Tieren*. Gustav Fischer Verlag, Jena.

Gordon GLR & Phillips MW (1998) The role of anaerobic gut fungi in ruminants. *Nutr Res Rev* **11**: 133–168.

Hesham AE, Khan S, Liu XC, Zhang Y, Wang ZY & Yang M (2006) Application of PCR-DGGE to analyse the yeast population dynamics in slurry reactors during degradation of polycyclic aromatic hydrocarbons in weathered oil. *Yeast* **23**: 879–887.

Janczyk P, Pieper R, Smidt H & Souffrant WB (2007a) Changes in the diversity of pig ileal lactobacilli around weaning determined by means of 16S rRNA gene amplification and denaturing gradient gel electrophoresis. *FEMS Microbiol Ecol* **61**: 132–140.

Janczyk P, Pieper R, Souffrant WB, Bimczok D, Rothkotter HJ & Smidt H (2007b) Parenteral long-acting amoxicillin reduces intestinal bacterial community diversity in piglets even 5 weeks after the administration. *ISME J* **1**: 180–183.

Janczyk P, Pieper R, Smidt H & Souffrant WB (2010) Effect of alginate and inulin on intestinal microbial ecology of weanling pigs reared under different husbandry conditions. *FEMS Microbiol Ecol* **72**: 132–142.

Jouany JP, Gobert J, Medina B, Bertin G & Julliand V (2008) Effect of live yeast culture supplementation on apparent digestibility and rate of passage in horses fed a high-fiber or high-starch diet. *J Anim Sci* **86**: 339–347.

Klobasa F, Werhahn E & Butler JE (1987) Composition of sow milk during lactation. *J Anim Sci* **64**: 1458–1466.

Konstantinov SR, Awati A, Smidt H, Williams BA, Akkermans AD & de Vos WM (2004) Specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotype to dietary prebiotics in the guts of weaning piglets. *Appl Environ Microb* **70**: 3821–3830.

Konstantinov SR, Awati AA, Williams BA, Miller BG, Jones P, Stokes CR, Akkermans AD, Smidt H & de Vos WM (2006) Post-natal development of the porcine microbiota composition and activities. *Environ Microbiol* **8**: 1191–1199.

Kurtzman CP & Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* **73**: 331–371.

Kurtzman CP, Robnett CJ, Ward JM, Brayton C, Gorelick P & Walsh TJ (2005) Multigene phylogenetic analysis of pathogenic *Candida* species in the *Kazachstania* (*Arxiozyma*) *telluris* complex and description of their ascosporic states as *Kazachstania bovina* sp. nov., *K. heterogenica* sp. nov., *K. pintolopesii* sp. nov., and *K. slooffiae* sp. nov. *J Clin Microbiol* **43**: 101–111.

Lallès JP, Boudry G, Favier C, Le Floc'h N, Lurona I, Montagne L, Oswald IP, Pie S, Piel C & Seve B (2004) Gut function and dysfunction in young pigs: physiology. *Anim Res* **53**: 301–316.

Lallès JP, Bosi P, Smidt H & Stokes CR (2007) Nutritional management of gut health in pigs around weaning. *P Nutr Soc* **66**: 260–268.

Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M & Moller K (2002) Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Appl Environ Microb* **68**: 673–690.

Magliani W, Conti S, Gerloni M, Bertolotti D & Polonelli L (1997) Yeast killer systems. *Clin Microbiol Rev* **10**: 369–400.

Mehnert B & Koch U (1963) Über das Vorkommen von im Verdauungstrakt des Schweines vermehrungsfähiger Hefen. *Zbl Bakter I Orig* **188**: 103–120.

Molnar O, Wuczkowski M & Prillinger H (2008) Yeast biodiversity in the guts of several pests on maize; comparison of three methods: classical isolation, cloning and DGGE. *Mycol Prog* **7**: 111–123.

Muyzer G & Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* **73**: 127–141.

Nubel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W & Backhaus H (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J Bacteriol* **178**: 5636–5643.

Pieper R, Janczyk P, Schumann R & Souffrant WB (2006) The intestinal microflora of piglets around weaning with emphasis on lactobacilli. *Arch Zootech* **9**: 28–40.

Pieper R, Janczyk P, Zeyner A, Smidt H, Guiard V & Souffrant WB (2008) Ecophysiology of the developing total bacterial and *Lactobacillus* communities in the terminal small intestine of weaning piglets. *Microb Ecol* **56**: 474–483.

Pieper R, Janczyk P, Urubschurov V, Korn U, Pieper B & Souffrant WB (2009) Effect of a single oral administration of *Lactobacillus plantarum* DSMZ 8862/8866 before and at the time point of weaning on intestinal microbial communities in piglets. *Int J Food Microbiol* **130**: 227–232.

Prakitchaiwattana CJ, Fleet GH & Heard GM (2004) Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. *FEMS Yeast Res* **4**: 865–877.

Schulze J & Sonnenborn U (2009) Yeasts in the gut: from commensals to infectious agents. *Dtsch Arztebl Int* **106**: 837–842.

Servin AL (2004) Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiol Rev* **28**: 405–440.

Simpson JM, McCracken VJ, Gaskins HR & Mackie RI (2000) Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. *Appl Environ Microb* **66**: 4705–4714.

Spencer JFT & Spencer DM (1997) Ecology: the bad guys: pathogens of humans and other animals. *Yeasts in Natural and Artificial Habitats* (Spencer JFT & Spencer DM, eds), pp. 59–60. Springer-Verlag, Berlin.

Suh SO, Mchugh JV, Pollock DD & Blackwell M (2005) The beetle gut: a hyperdiverse source of novel yeasts. *Mycol Res* **109**: 261–265.

Urubschurov V, Janczyk P, Pieper R & Souffrant WB (2008) Biological diversity of yeasts in the gastrointestinal tract of weaned piglets kept under different farm conditions. *FEMS Yeast Res* **8**: 1349–1356.

Van Uden N & Do Carmo-Sousa L (1962) Quantitative aspects of the intestinal yeast flora of swine. *J Gen Microbiol* **27**: 35–40.

Van Uden N, Do Carmo-Sousa L & Farinha M (1958) On the intestinal yeast flora of horses, sheep, goats and swine. *J Gen Microbiol* **19**: 435–445.

Viljoen BC (2006) Yeast ecological interactions: yeast–yeast, yeast–bacteria, yeast–fungi interactions and yeast as biocontrol agents. *The Yeast Handbook: Yeasts in Food and Beverages*, *Vol. 2* (Querol A & Fleet G, eds), pp. 83–110. Springer-Verlag, Berlin.

Wouters JTM, Ayad EHE, Hugenholtz J & Smit G (2002) Microbes from raw milk for fermented dairy products. *Int Dairy J* **12**: 91–109.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. *P*-values of trait influencing factors obtained from analysing the data by means of MIXED procedure (for included covariable see Table S2).

Table S2. Effect of a covariable on log scores of CFU of the traits, SE and *P*-value according to the specified model with a covariable (resulting from linear and quadratic modelling for comparison).

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