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Microbiological Characterization of Wet Wheat Distillers' Grain, with Focus on Isolation of Lactobacilli with Potential as Probiotics

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Wet wheat distillers' grain (WWDG), a residue from ethanol fermentation, was examined from a microbiological perspective. After storage, WWDG was characterized by a high content of lactobacilli, nondetectable levels of other bacteria, occasional occurrence of yeasts, and a pH of about 3.6 and contained a mixture of lactic acid, acetic acid, and ethanol. The composition of lactobacilli in WWDG was simple, including primarily the species *Lactobacillus amylolyticus*, *Lactobacillus panis*, and *Lactobacillus pontis*, as determined by 16S rRNA gene sequencing. Since the use of WWDG as pig feed has indicated a health-promoting function, some relevant characteristics of three strains of each of these species were examined together with basal physiological parameters, such as carbohydrate utilization and growth temperature. Seven of the strains were isolated from WWDG, and two strains from pig feces were included for comparison. It was clear that all three species could grow at temperatures of 45 to 50°C, with *L. amylolyticus* being able to grow at temperatures as high as 54°C. This finding could be the explanation for the simple microflora of WWDG, where a low pH together with a high temperature during storage would select for these organisms. Some strains of *L. panis* and *L. pontis* showed prolonged survival at pH 2.5 in synthetic stomach juice and good growth in the presence of porcine bile salt. In addition, members of all three species were able to bind to immobilized mucus material in vitro. Especially the isolates from pig feces but, interestingly, some isolates from WWDG as well possessed properties that might be of importance for colonization of the gastrointestinal tracts of pigs.

Distillers' grain is the fermentation residue of ethanol production from cereal grains and is extensively used in the wet or the dried form as an animal feed worldwide. The bulk of the production is dried in order to facilitate transport and trade. The current annual production in North America (http://ddgs.umn.edu) amounts to 3.2 to 3.5 million metric tons of dried distillers' grain (DDG). Based on data on the use of cereal grains for ethanol production in Europe (http://europeanspirits.org), it can be estimated that the annual production of DDG there corresponds to 0.5 million metric tons. In Sweden, the annual production can be estimated to be 38,000 metric tons of DDG and 300,000 metric tons of wet distillers' grain (9 to 10% dry matter). By tradition, the major part of the DDG is used for ruminants, but recent research suggests that DDG with soluble agents produced from new ethanol plants has nutritional properties that would allow more extensive use in pig production (21).

The wet wheat distillers' grain (WWDG) used in the present study was produced by The Absolut Company (Åhus, Sweden). After storage, WWDG is characterized by a low pH, high numbers of lactobacilli, high concentrations of organic acids, a high fiber content, and a dry matter content of about 9.5%. WWDG has three times as much ash, nitrogen, and fiber as wheat, while the starch content is almost zero (17). Because of the high fiber content (neutral detergent fiber content of 35%), WWDG has been fed primarily to ruminants. In addition, as the low dry matter content results in high transport costs, it is most often used locally. Furthermore, there has been a gradual decrease in the number of ruminants in a radius of 100 km from the ethanol factory, a factor which has led to an increase in the use of the feed by pig producers in southern Sweden.

In recent years, due to the risk of the development of antibiotic resistance among pathogenic microorganisms, the use of antibiotics as growth promoters has been banned by the European Union (1). Alternatives such as probiotics (primarily lactic acid bacteria), enzymes, and organic acids (e.g., formic, fumaric, and citric acids) have been suggested (22). In the search for alternative growth promoters, WWDG has been identified as an interesting candidate, since it contains both lactobacilli and organic acids. In order to investigate the potential of WWDG as a growth promoter for pig production, Pedersen and Lindberg recently performed a feeding trial with weaned piglets and this product. An interesting finding in that study was a significant reduction in the frequency of diarrhea, without any negative effects on feed intake or daily weight gain (C. Pedersen and J. E. Lindberg, submitted for publication).

The term "probiotics" has been defined as "living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition" (7). The characteristics that are currently used for the identification of probiotic bacteria have not been clearly established. However, tests for bile tolerance, gastric acid resistance, and adherence to host mucosal surfaces are reasonable screening parameters for the selection of probiotic strains for nonruminant livestock. Eventually, of course, the clinical effects of the administration of probiotics must be studied.

The aims of the present study were to describe WWDG from a microbiological point of view, to identify the *Lactobacillus* species present, and to investigate some characteristics that

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could be important for their potential as probiotics. Furthermore, strains isolated from WWDG were compared with lactobacilli of the same species isolated from pig feces to determine whether they shared these characteristics.

MATERIALS AND METHODS

Sampling of WWDG. WWDG is the fermentation residue from ethanol production. In the process, whole wheat is milled and, after the addition of water, enzymes, and yeasts, fermentation takes place. The fermented product is distilled, and the residue (WWDG) is stored in open outdoor containers. WWDG continually fills one storage container, and occasionally some WWDG fills a second storage container. Samples were taken from the pipe leading to the first storage container (samples 1a and 1b), from the first (sample 2a) and the second (sample 2b) storage containers, and from three local farms that had been using WWDG as a feed supplement for pigs for at least 15 years (samples 3a to 3d). Most local farmers receive new WWDG once per week. At one of the farms, two samples were taken from two different storage tanks. Samples 1a, 1b, 2a, 2b, and 3a to 3d were analyzed within 4 h after sampling. Finally, samples were collected from WWDG that was being used in a pig feeding trial at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, where WWDG was delivered in a 1-m^3 sealed container once for the whole trial. The trial lasted 5 weeks, and WWDG was stored at 15°C. Samples were taken at the beginning (sample 4a) and at the end (sample 4b) of this trial. The volume of each sample was 1 liter.

Microbial composition of WWDG. After the samples were blended, 1 g was serially diluted in phosphate-buffered saline (PBS) (pH 7.3) (8.0 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4 \cdot 2H_2O$, and 0.2 g of KH_2PO_4 per 1,000 ml of distilled water), and 100-μl portions were spread on different agar plates. Lactobacilli were quantified on Rogosa agar (E. Merck AG, Darmstadt, Germany) plates incubated anaerobically at 37°C for 48 h. Yeasts were enumerated on malt extract agar (Merck) plates supplemented with 100 μg of chloramphenicol ml^{-1} and incubated at 25°C for 2 to 4 days. Molds were enumerated on malt extract agar plates supplemented with 100 μg of chloramphenicol ml^{-1} and 10 μg of cycloheximide ml^{-1} and incubated at 25°C for 3 to 5 days. Enterobacteria were quantified on pour plates of violet-red bile-glucose agar (Oxoid, Basingstoke, England) incubated at 37°C for 48 h. Clostridia were quantified on reinforced clostridium agar (Merck) plates incubated anaerobically at 37°C for 72 h. Propioni bacteria were quantified in modified sodium lactate broth (containing, per 1,000 ml, 5 g of KH_2PO_4, 5 g of yeast extract, 10 g of tryptone, 16 ml of sodium lactate [60% syrup], and 14.4 g of agar) incubated anaerobically at 30°C for 7 days. A GasPak system (Becton Dickinson, Sparks, Md.) was used throughout the study in order to obtain an anaerobic environment.

Other characteristics of WWDG. WWDG samples 1 and 2 were analyzed for acids by high-performance liquid chromatography with an H^+ column as described by Andersson and Hedlund (2) with some modifications. Fresh 50-ml samples were centrifuged (2,000 × *g*, 4°C, 10 min), and the supernatant was filtered (0.2-μm-pore size) in a sterile manner and stored at −20°C until analysis. Freeze-dried samples (samples 1 and 2) of WWDG were also analyzed for carbohydrates by high-performance liquid chromatography with a Pb^{2+} column (2). Carbohydrates were determined directly (water soluble) and after hydrolysis with 1 M H_2SO_4 at 103°C for 1 h. Starch and nonstarch polysaccharides (NSPs) were analyzed as described by Lindberg et al. (12).

Isolation of lactobacilli from WWDG. A sample was taken from homogenized WWDG, and 10 μl was spread on Rogosa agar plates, which were incubated anaerobically at 37°C for 24 h. The plates were scraped, and the total mixture of bacteria was frozen at −70°C in glycerol-salt solution (0.82 g of K_2HPO_4, 0.18 g of KH_2PO_4, 0.59 g of $NaC_6O_7H_7$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 172 ml of glycerol [87%], 828 ml of distilled H_2O). At 1 to 2 months later, material was taken from the frozen vials with inoculation loops and streaked on Rogosa agar plates, which were incubated for 24 h at 37°C. Ten colonies were selected from each plate in such a way that the diversity of colony types selected reflected the diversity of the types present on the agar plate. The bacteria were grown in MRS broth (Oxoid). In parallel, fresh samples (nonfrozen) were analyzed by streaking on Rogosa agar plates and selecting colonies by using the same procedure.

Bacterial DNA was isolated by using a DNeasy tissue kit (Qiagen, Hilden, Germany). The almost complete 16S rRNA gene was amplified by PCR with the following primers specific for the domain *Bacteria*: 16SS (5′-AGAGTTTGATC CTGGCTC-3′) and 16SR (5′-CGGGAACGTATTCACCG-3′). The resulting PCR products were purified by using a Qiagen PCR purification kit. One strand of the first part (approximately 500 bp) of the purified fragment was sequenced by standard methods with primer 16SS. The sequences obtained were compared with the sequences in the GenBank database (http://www.ncbi.nih.gov) by using the BLASTN program. More than 98% similarity to a known species was considered a positive match.

In addition, lactobacilli were isolated in a similar manner from the feces of pigs which had been fed either dry feed or liquid feed with WWDG.

Characteristics of different isolates. Lactobacilli isolated from WWDG and pig feces were further characterized according to the descriptions below.

(i) Sugar fermentation. Sugar fermentation patterns were determined by using an API 50CHL system (BioMérieux, Marcy l'Etoile, France). The analyses were performed according to the manufacturer's instructions but with the modification that the incubations were performed in anaerobic jars at 37°C.

(ii) Growth at different temperatures. One milliliter of MRS broth culture grown at 37°C overnight was added to 9 ml of MRS broth, and the bacteria were incubated at 15°C for 48 h or at 37, 45, 50, 52, 54, and 56°C for 24 h. Growth was characterized as no growth, weak growth, or strong growth. Strong growth represented a culture density comparable to that at 37°C, while weak growth was at least a doubling of the amount of inoculated bacteria. Furthermore, strains DAF 3, DAF 353, and DAF 355 were grown in MRS broth at 37 and 45°C, and growth was determined by measuring the optical density at 600 nm (OD_{600}) after 0, 3, 6, 9, 12, and 24 h.

(iii) Survival in synthetic stomach juice. Survival studies were performed with synthetic stomach juice (8.3 g of Proteose Peptone, 3.5 g of glucose, 2.05 g of NaCl, 0.6 g of KH_2PO_4, 0.11 g of $CaCl_2$, 0.37 g of KCl, 0.05 g of bile, 0.1 g of lysozyme, and 13.3 mg of pepsin dissolved in 1 liter of distilled water) (5) adjusted to pH 2.5 with 1 M HCl. The juice was heated to 37°C for 30 min and filtered in a sterile manner before use. To test tubes containing 10 ml of juice, 10 μl of *Lactobacillus* culture was added (giving approximately 10^6 CFU ml^{-1}). Samples were taken at 0, 30, and 180 min, and the survival rate was measured by spreading 100 μl of different dilutions on MRS agar plates, which were incubated anaerobically at 37°C for 48 h.

(iv) Bile salt tolerance. The bacteria were examined for their ability to grow in the presence of porcine bile extract. One milliliter of an overnight MRS broth culture was added to 9 ml of MRS broth supplemented with 0.1, 0.3, 0.5, 1.0, or 2.0% porcine bile extract (B8631; Sigma, St. Louis, Mo.), and the bacteria were incubated at 37°C for 24 h. Growth was characterized as no growth, weak growth, or strong growth. Strong growth represented an OD comparable to that of a nonsupplemented culture, while weak growth was at least a doubling of the OD.

(v) Mucus binding assay. The bacteria were grown at 37°C in MRS broth for 24 h. This medium was supplemented with 0.1% pig gastric mucin (M1778; Sigma) to test for the induction of binding (10). Microtiter wells were coated with mucus from pig small intestine as previously described (19). Wells coated with bovine serum albumin were used as a control. The bacterial strains were grown as described above, washed once in PBS supplemented with 0.05% Tween 20, and diluted to an OD_{600} of 0.5 in the same buffer. One hundred microliters of bacterial suspension was added to each well and incubated overnight at 2°C. The wells were washed with PBS–0.05% Tween 20, and binding was examined with an inverted microscope. The buffer was poured off and, after the wells had dried, the OD_{405} was measured with an enzyme-linked immunosorbent assay plate reader. All measurements were obtained in triplicate.

Nucleotide sequence accession numbers. The sequences of the 16S rRNA genes were deposited in the GenBank database under the following accession numbers (strains): AY323493 (DAF 1), AY323494 (DAG 76), AY323495 (DAF 355), AY323496 (DAF 3), AY323497 (DAF 18), AY323498 (DAG 139), AY323499 (DAF 262), AY323500 (DAF 285), and AY323501 (DAF 353).

RESULTS AND DISCUSSION

Microbial composition of WWDG. The microflora of WWDG was composed mainly of lactobacilli and occasionally contained yeasts (Table 1). Notably, no other bacteria could be detected in the various samples. Samples taken directly from the pipe leading from the distillation container to the storage container did not contain a detectable level of microorganisms. The heat process used during distillation, together with the low pH in the product, would have been restrictive to most microorganisms. The high numbers of lactobacilli led to a reduction of the pH from 4.2 in the newly distilled product to about 3.6 in the stored product (Table 1). Lactic acid was primarily detected, together with acetic acid and ethanol in lower concentrations (Table 2). In parallel with the increase in the con-

TABLE 1. Concentrations of lactobacilli, yeasts, clostridia, molds, enterobacteria, and propionibacteria and pH in samples of WWDG

Sample[a]	CFU of the following organisms ml^{-1}						pH
	Lactobacilli	Yeasts	Clostridia	Molds	Enterobacteria	Propionibacteria	
1a	<1[b]	<1[b]	<1[b]	<1[b]	<0[b]	<1[b]	4.27
1b	<1	<1	<1	<1	<0	<1	4.22
2a	8.2	<1	<1	<1	<0	<1	3.59
2b	8.3	4.0	<1	<1	<0	<1	3.68
3a	8.0	3.2	<1	<1	<0	<1	3.63
3b	7.7	6.2	<1	<1	<0	<1	3.92
3c	8.1	<1	<1	<1	<0	<1	3.60
3d	8.1	6.0	<1	<1	<0	<1	3.78
4a	8.8	NT[c]	NT	NT	NT	NT	3.57
4b	8.4	NT	NT	NT	NT	NT	3.72

[a] Samples 1a and 1b, WWDG directly from the distillation; samples 2a and 2b, from storage container at factory; samples 3a to 3d, from different farms; samples 4a and 4b, from pig trial at SLU.
[b] Lowest detection level.
[c] NT, not tested.

centrations of acids, a decrease in the concentrations of free sugars, especially water-soluble fructose and glucose after acid hydrolysis, could be seen. Only a small decrease in the concentration of starch, from 17 to 13 g per kg of dry matter (DM), was recorded, and the concentrations of NSPs were not reduced at all (Table 3). Obviously, the sterile WWDG coming from the distillation container had been spontaneously inoculated with lactobacilli from the environment during storage. A relationship was observed between the concentration (log CFU per milliliter) of yeasts and pH ($y = 0.08x + 3.37$; $R^2 = 0.84$; four observations) in WWDG. This finding can be explained by the fact that some yeasts can metabolize lactic acid (14).

It was evident that the method of isolating the bacteria affected the results (Table 4). For most samples, lactobacilli were cultured and stored in a freezer before isolation of single colonies. This procedure was disadvantageous, especially for *Lactobacillus amylolyticus*, which was found essentially when individual bacteria were isolated directly from the samples. The bacterium was replaced mainly by *Lactobacillus pontis* after the mixture of lactobacilli was cultured and frozen. A similar observation was previously made in an analysis of lactobacilli in pig feces (C. Pedersen, S. Roos, J. E. Lindberg, and H. Jonsson, submitted for publication). We suggest that one explanation for this effect might be that *L. amylolyticus* shows weaker growth than most of the other lactobacilli present and thus will be outcompeted during the isolation procedure. Analysis of bacteria isolated directly from the samples revealed that *L. amylolyticus* and then *Lactobacillus panis* were the most common lactobacilli in WWDG. However, the isolation procedure involving culturing and freezing of the bacteria showed that especially *L. pontis*, but also some other species of *Lactobacillus*, was present in the product (Table 4). *L. amylolyticus* was first isolated from beer malt and wort, while *L. panis* and *L. pontis* were first found in sourdough with a long fermentation time (23, 25). These are milieus with many similarities to WWDG.

Since *L. panis* and *L. pontis* are heterofermentative (24), they probably had produced the ethanol and acetic acid found in WWDG. During heterofermentation, depending on whether external electron acceptors are used or not, either 1 mol of ethanol or 1 mol of acetic acid is produced per mol of lactic acid. Since the amount of lactic acid produced was more than

TABLE 2. Concentrations of organic acids and ethanol in sterile WWDG (sample 1a) and fermented WWDG (sample 2a)

Compound	Concn (g kg of DM^{-1}) in sample:	
	1a	2a
Succinic acid	16	18
Lactic acid	17	88
Acetic acid	1	12
Propionic acid	1	1
Butyric acid	tr[a]	tr
Ethanol	tr	5

[a] tr, trace.

TABLE 3. Concentrations of carbohydrates in sterile WWDG (sample 1a) and fermented WWDG (sample 2a)

Category	Carbohydrate	Concn (g kg of DM^{-1}) in sample:	
		1a	2a
Sugars soluble in water	Glucose	6	4
	Xylose	tr[c]	1
	Galactose	1	tr
	Arabinose	6	2
	Mannose	tr	tr
	Fructose	11	tr
Sugars soluble in water after hydrolysis[a]	Glucose	39	4
	Xylose	36	38
	Galactose	4	6
	Arabinose	15	24
	Mannose	tr	tr
	Fructose[b]	tr	2
Starch		17	13
Total sugars in NSP fraction (soluble NSP fraction)	Glucose	31 (16)	31 (11)
	Xylose	129 (83)	129 (79)
	Galactose	12 (6)	13 (6)
	Arabinose	74 (48)	75 (44)
	Mannose	20 (13)	21 (12)

[a] Hydrolyzed with 1 M H_2SO_4 at 103°C for 1 h.
[b] Values probably were underestimated due to loss during the acid hydrolysis (P. Åman, SLU, personal communication).
[c] tr, trace.

TABLE 4. *Lactobacillus* species identified in WWDG by16S rRNA gene sequencing

Species	No. of colonies in sample:								
	2a[a]	2a[b]	2b[b]	3a[b]	3b[b]	3c[a]	3c[b]	4a[b]	4b[b]
L. amylolyticus	8		1			5			
L. panis	1		2		1	3	2	8	4
L. pontis		6	5	9	6		4	2	6
L. fermentum		2							
L. mucosae					1				
L. plantarum									
Lactobacillus sp.[c]					2				

[a] Isolated directly from fresh samples.
[b] Isolated after reculturing of bacterial samples that had been cultured and frozen.
[c] Identity of <98% to the closest known species.

TABLE 5. Fermentation of carbohydrates by *L. amylolyticus*, *L. panis*, and *L. pontis*

Carbohydrate	Growth of[a]:								
	L. amylolyticus			*L. panis*			*L. pontis*		
	DAF 262	DAF 285	DAF 353	DAF 1	DAG 76	DAF 355	DAF 3	DAF 18	DAG 139
L-Arabinose	−	−	−	(+)	+	+	−	−	−
Ribose	−	−	−	+	+	(+)	−	+	+
D-Xylose	−	−	−	−	+	+	−	−	−
β-Methyl-D-xyloside	−	−	−	−	(+)	+	−	−	−
Galactose	+	−	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	(+)	+	+	+
D-Fructose	(+)	+	+	+	(+)	−	(+)	(+)	+
D-Mannose	−	+	−	−	−	−	−	−	−
Sorbitol	−	−	−	(+)	−	−	−	−	−
N-Acetyl-glucosamine	−	+	(+)	(+)	−	−	−	−	−
Amygdalin	−	−	+	−	−	−	−	−	−
Arbutin	−	−	(+)	−	−	−	−	−	−
Salicin	−	(+)	+	−	−	−	−	−	−
Cellobiose	−	−	+	−	−	−	−	−	−
Maltose	(+)	+	+	+	+	+	+	+	+
Lactose	+	−	−	+	+	+	(+)	+	+
Melibiose	+	−	−	+	+	+	+	+	+
Saccharose	+	+	+	+	+	−	(+)	(+)	+
Trehalose	−	+	+	−	−	−	−	−	−
D-Raffinose	(+)	−	(+)	+	+	+	+	(+)	+
β-Gentiobiose	−	−	(+)	−	−	−	−	−	−

[a] DAF, strains isolated from WWDG; DAG, strains isolated from pig feces. +, growth; (+), weak growth; −, no growth.

twice the amounts of the other compounds produced (Table 2), it can be concluded that homofermentative lactobacilli, such as *L. amylolyticus*, dominated during the fermentation process. This conclusion supports the finding that this species is the most abundant in WWDG.

Physiological properties of the isolated lactobacilli. Characterization of properties believed to be of importance for the fermentation process was performed with seven lactobacilli representing the most common species in WWDG: *L. amylolyticus* DAF 262 (isolated from sample 2a), DAF 285 (sample 2b), and DAF 353 (sample 3c); *L. panis* DAF 1 (sample 4a) and DAF 355 (sample 3c); and *L. pontis* DAF 3 (sample 4a) and DAF 18 (sample 4b). In additon, *L. panis* DAG 76 and *L. pontis* DAG 139, isolated from pig feces, were included in the study.

Since we have not been able to isolate any *L. amylolyticus* from pigs, no such isolate could be tested. To ensure that the isolates from one species were not identical, isolates with small but significant differences in the 16S rRNA gene were chosen.

The abilities of the strains to ferment various carbohydrates are shown in Table 5. The following sugars were fermented by most strains: D-glucose, maltose, and galactose (eight of nine); D-fructose (eight of nine); saccharose (eight of nine); and D-raffinose (eight of nine). *L. panis* and *L. pontis* (two of three strains) fermented ribose. Only *L. panis* fermented L-arabinose and D-xylose (two of three strains) and β-methyl-D-xyloside (two of three strains), while two of three *L. amylolyticus* strains fermented trehalose. The strains of *L. pontis* tested by Vogel et al. (23) and *L. amylolyticus* tested by Bohak et al. (3) showed fermentation profiles similar to those of the strains tested in the present study. There was less similarity between the two strains of *L. panis* tested by Wiese et al. (25) and the *L. panis* strains tested in the present study. Based on the results of testing with the API 50CHL system, it can be concluded that all three species could use glucose and fructose in WWDG, while the decrease in the concentration of arabinose could be ascribed only to *L. panis* (Table 5).

The growth of *L. amylolyticus* DAF 353, *L. panis* DAF 355, and *L. pontis* DAF 3 in MRS broth was tested at different temperatures. At 37°C but not at 45°C, *L. panis* and *L. pontis* grew 30 to 40% faster than *L. amylolyticus*. The latter could grow well at temperatures as high as 54°C (Table 6), a finding which is in agreement with the findings of Bohak et al. (3). In addition, the other two species had high maximum growth temperatures, which varied between 45 and 52°C for the different isolates. None of the bacteria could grow at 15°C. In agreement with this finding, Wiese et al. (26) reported that *L.*

TABLE 6. Growth of *L. amylolyticus*, *L. pontis*, and *L. panis* at different temperatures in MRS broth

Isolate[a]	Growth at the following temp (°C)[b]:						
	15	37	45	50	52	54	56
L. amylolyticus							
DAF 2621	−	+	+	+	+	+	(+)
DAF 285	−	+	+	+	+	+	(+)
DAF 353	−	+	+	+	+	+	(+)
L. panis							
DAF 1	−	+	+	−	−	−	NT
DAG 76	−	+	+	+	+	(+)	NT
DAF 355	−	+	+	(+)	−	−	NT
L. pontis							NT
DAF3	−	+	+	+	−	−	NT
DAF 18	−	+	+	+	−	−	NT
DAG 139	−	+	+	(+)	−	−	NT

[a] DAF, strains isolated from WWDG; DAG, strains isolated from pig feces.
[b] +, Strong growth; (+), weak growth; −, no growth; NT, not tested.

TABLE 7. Survival of *L. amylolyticus*, *L. panis*, and *L. pontis* in synthetic stomach juice at pH 2.5

Isolate[a]	CFU of lactobacilli ml^{-1} at (% survival):		
	0 min	30 min	180 min
L. amylolyticus			
DAF 262	5.4 (100)	<2.0[b] (<0.04)	<1.0[b] (<0.004)
DAF 285	3.3 (100)	<2.0 (<5)	<1.0 (<0.5)
DAF 353	5.4 (100)	<2.0 (<0.04)	<1.0 (<0.004)
L. panis			
DAF 1	4.2 (100)	3.0 (6)	2.2 (1)
DAG 76	5.7 (100)	4.7 (10)	3.7 (1)
DAF 355	5.5 (100)	<2.0 (<0.03)	<1.0 (<0.003)
L. pontis			
DAF 3	6.0 (100)	5.3 (20)	4.8 (6)
DAF 18	6.1 (100)	4.0 (0.8)	3.8 (0.5)
DAG 139	4.8 (100)	4.5 (50)	3.5 (5)

[a] DAF, strains isolated from WWDG; DAG, strains isolated from pig feces.
[b] Lowest detection level.

panis can grow at 45°C but not at 15°C. However, Vogel et al. (23) found that their isolates of *L. pontis* could grow at 15°C. Compared to many other lactobacilli (11), the isolates found in WWDG had high maximum growth temperatures. This finding may explain why relatively few species of lactobacilli could be isolated from WWDG, where a constant high storage temperature of about 45°C is maintained at the factory.

Testing of properties considered important for probiotic function. The original idea with probiotics was to change the composition of the normal intestinal microflora from a potentially harmful composition to a microflora that would be beneficial for the host (16). In order to function as probiotics, lactobacilli first must pass through the stomach and survive its low pH and then proceed to the small intestine and tolerate the bile salt present (13). Finally, it is believed that the bacteria need to adhere to mucosal surfaces. Since WWDG previously was shown to possess health-promoting properties (Pedersen and Lindberg, submitted) that might be attributable to the resident lactobacilli, we tested the abilities of the seven strains from WWDG to survive at a low pH, grow in the presence of bile, and adhere to pig mucus components. For comparison, the same characteristics were tested for the same species of lactobacilli isolated from pig feces.

None of the three strains of *L. amylolyticus* could survive in synthetic stomach juice (pH 2.5) for 30 min, although incubation of two strains of *L. panis* and all three strains of *L. pontis* allowed the recovery of detectable numbers of bacteria (Table 7). The two strains of animal origin had the highest survival rates, a finding which is in agreement with the findings of Haller et al. (8). The five strains that survived in the stomach juice test also grew best in the bile salt test (Table 8), a finding which is in agreement with the observations of others (4, 8, 20). In particular, two strains of *L. pontis* were resistant to the porcine bile extract used to supplement the substrate. Jacobsen et al. (9) carried out a similar experiment and tested 44 different strains of lactobacilli, 29 of which survived at pH 2.5 for 4 h. All but 1 survived in 0.3% oxgall for 4 h, although only 18 could grow in this environment. All 10 strains of *Lactobacillus plantarum* and *Lactobacillus fermentum*, isolated from Ghanaian fermented maize, grew in oxgall. Thus, resistance to gall salts seems to be common for lactobacilli isolated from plant materials as well.

Several of the strains in this study possessed the ability to adhere to pig mucus (Fig. 1). Lactobacilli are commonly found to adhere to the mucus layer of the gastrointestinal tract (6, 18), and this interaction has also been studied in vitro (10, 15). Furthermore, it has been shown that the adherence of strains of *Lactobacillus reuteri* to mucus in vitro can be stimulated by growing the bacteria in the presence of mucin, the main component of mucus (10). This effect was also observed in this study, where strains DAF 285, DAF 353, DAG 76, and DAF 18, representing three species, responded to mucin in this way. Thus, the induction of mucus binding by growing the bacteria in the presence of mucin seems to be a property of various species of lactobacilli. *L. pontis* DAG 139 responded in a different manner to the addition of mucin. This strain showed strong adherence to mucus when grown in the absence of mucin, but the addition of mucin to the substrate reduced the adherence. This result might have been caused by blocking of the adhesion component on the bacterial cell surface by the added mucin (10). Both strains from pigs (DAG 76 and DAG 139) adhered to mucus, but the other three adherent strains were from WWDG. The adherence of these strains was also induced by mucin, a finding which may indicate that they are adapted to the gastrointestinal tract.

It can be concluded that WWDG is dominated by the species *L. amylolyticus*, *L. panis*, and *L. pontis*. Although two of the *L. amylolyticus* strains adhered to mucus, all strains from the species showed very poor survival in gastric juice and did not tolerate high concentrations of bile in the substrate. These characteristics might be the reasons why we did not find any *L. amylolyticus* in the feces of pig, even when they had been fed WWDG (Pedersen et al., submitted). Both *L. panis* and *L. pontis* were found in the feces of pigs fed WWDG. Even though few strains were compared in this study, it seems that strains isolated from pigs are more adapted to the conditions in the gastrointestinal tract. Both *L. panis* DAG 76 and *L. pontis* DAG 139 survived in gastric juice, could grow in the presence

TABLE 8. Growth of *L. amylolyticus*, *L. panis*, and *L. pontis* in MRS broth supplemented with porcine bile extract

Isolate[a]	Growth in the presence of the following concn (%) of bile extract[b]:				
	0.1	0.3	0.5	1.0	2.0
L. amylolyticus					
DAF 262	(+)	−	−	−	−
DAF 285	+	(+)	(+)	(+)	(+)
DAF 353	+	(+)	(+)	(+)	(+)
L. panis					
DAF 1	+	+	+	−	−
DAG 76	+	+	+	(+)	(+)
DAF 355	+	(+)	(+)	(+)	(+)
L. pontis					
DAF 3	+	+	+	+	+
DAF 18	+	(+)	(+)	(+)	(+)
DAG 139	+	+	+	+	+

[a] DAF, strains isolated from WWDG; DAG, strains isolated from pig feces.
[b] +, Strong growth; (+), weak growth; −, no growth.

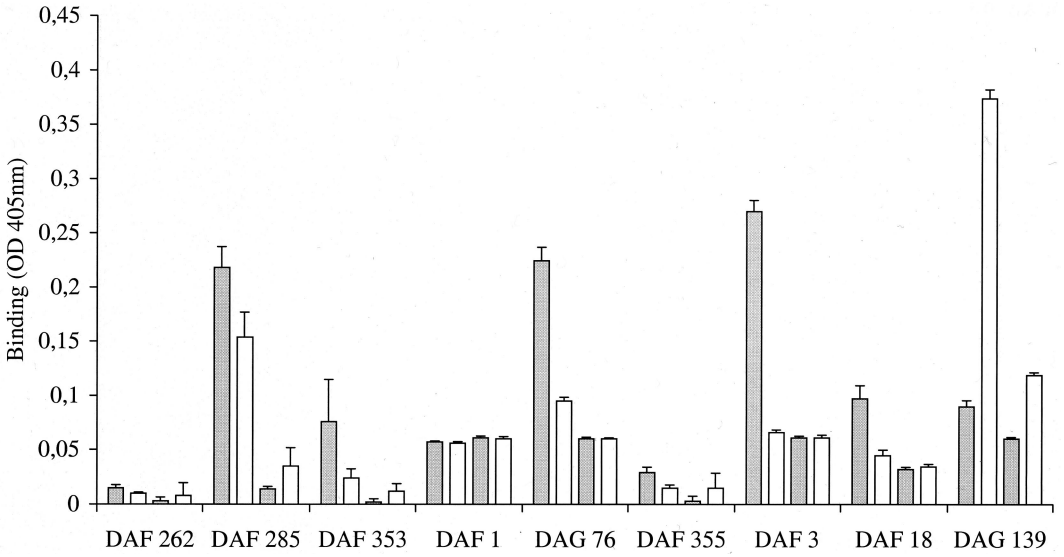

FIG. 1. Mucus binding of *L. amylolyticus*, *L. panis*, and *L. pontis* grown with (grey columns) and without (white columns) 0.1% mucin. Bovine serum albumin was used as a control. The values are based on three measurements, and errors bars show standard deviations.

of bile, and showed good adherence to mucus material. However, although *L. panis* DAF 1, *L. pontis* DAF 3, and DAF 18 did not possess all of the properties outlined as being important, it is evident that lactobacilli isolated from WWDG have characteristics that might be essential for probiotic function. The use of bacteria that can efficiently ferment feed and add health- and growth-promoting properties to the product is an attractive concept that will be further evaluated in the future.

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