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Potential microbiological hazards in the production of refined paper products for food applications

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This study sought to investigate the significance of raw materials (starch-based glues, raw material papers) at different microbiologically critical stages in the manufacturing process of refined paper products. The study examined the occurrence of microorganisms in the process and in end-product samples. Microbiological surveys verified that the production and use of pasteurized starch-based glue was the most important factor threatening the process hygiene and product safety. Subsequently, the production and use of starch-based glue was changed, and a follow-up programme targeting the microbiological quality of glue was developed as part of a hygiene and safety management system. A total of 33 spore-forming bacterial and 15 enterobacterial isolates were ribotyped, and 22 and 10 different ribogroups (ribotypes), respectively, were generated. These isolates from starch-based glue, raw material paper and end products were atypical and, thus, in many cases physiological, chemotaxonomic (FAME) and molecular (partial 16S rDNA) results did not correspond. The most common spore-forming bacteria (55% of the isolates) were *Paenibacillus* sp. and within this genus several new species were also proposed. The most common enterobacteria (87%) were *Enterobacter cloacae* and *Citrobacter freundii* belonging to bacteria in hazard group 2, or species closely related to them. It was demonstrated that the same spore-forming bacteria (ribotypes) were present in both the glue samples and the end products (45% of isolates). All RiboPrint® patterns were saved at the VTT identification library for future use.
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Introduction

The importance of hygiene in the paper and packaging industry has increased considerably as a result of changes in legislation, stronger international competition and tougher customer requirements. European Union food hygiene legislation [5] has led producers and retailers of food to demand higher standards from their suppliers.

Harmful microorganisms may enter the manufacturing process and reach the end product in several ways, for example, via raw materials, air in the manufacturing area, chemicals employed, process surfaces or factory personnel [6,12–14]. Problems caused by microorganisms include spoiling of raw materials (e.g., *Enterobacter cloacae*, *Bacillus subtilis*), indirect defects related to microbial growth, such as the volatile, malodorous compounds produced during microbial metabolism (e.g., *Clostridium* spp., *Desulfovibrio* spp., actinomycetes), slime production (e.g., *Burkholderia cepacia*, *Klebsiella* spp., *Bacillus* spp.) and the occurrence of microorganisms detrimental to product hygiene and human health (e.g., *Bacillus cereus*, coliforms, *Staphylococcus* spp.) [7,13,14,21,26]. However, to cause problems, microorganisms possibly present in paper packaging materials must come in contact with the food, and growth and migration of organisms into the product need to occur [23].

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Starch is an important raw material commonly used in the paper industry and in the further refining of paper or board. It is used in different phases of paper or board manufacturing as a strengthening agent, as a surface sizing agent, as an adhesive agent in coating colours and as a spray. In the further refining of paper or board, starch-based glues are commonly used. Starch offers favourable growing conditions for microorganisms and is a particularly important source of organic carbohydrates and other nutrients. If starch is contaminated with certain microorganisms producing acidic by-products, the pH decreases and amylase enzymes are able to break amylose or amylopectin molecule bonds resulting in a loss of viscosity. Loss of viscosity normally causes rejection of the starch batch. However, the microbial load in the starch solution at the time viscosity is lost is already very high (e.g., 10^8 cfu/ml). Microbial growth in starch can be difficult to detect; for that reason the overall problem may easily be overlooked [3,13,14,16].

The aim of this study was to carry out detailed quantification of microorganisms in refined paper manufacturing processes. In addition to a quantitative determination of different microbial groups, another goal was to identify the bacterial isolates and to create a RiboPrint® identification database for these bacteria.

Materials and methods

Samples and sampling

Microbial surveys were carried out for process surfaces (including those in storage and refining areas), process samples (starch-based glues), air in manufacturing areas, raw material paper and end products. The process surfaces were analysed twice and process samples and end products three times.

Table 1 Sample type, time of sampling and number of samples taken in microbiological surveys

Sample type	No. of samples taken			Total
	June 1998	October 1998	November 1999	
Surface samples	71	19	–	100
Air samples	27	–	–	27
Glue samples	8	5	6	19
Raw material paper samples	–	2	–	2
End product samples	5	2	2	9

Microbial samples were taken in June and in October 1998 and in November 1999. The samples are listed in Table 1. The microbial surveys were carried out by determining total aerobic bacteria, enterobacteria, yeasts and moulds from 100 surface samples, and by determining aerobic bacteria, aerobic spore-forming bacteria, enterobacteria, yeasts and moulds from 19 glue samples, 2 raw material paper and 9 end-product samples. In addition, aerobic bacteria, yeasts and moulds were determined from 27 ambient air samples.

Glue samples were taken aseptically into sterile tubes with the aid of sterile injectors and the raw material paper and end-product samples were taken aseptically into a sterile bag with the aid of a sterile knife. Sterile gloves were used during the sampling.

Samples were shipped (3 h) in a chilled container and the quantitative determination of aerobic bacteria, spore-forming aerobic bacteria, enterobacteria, yeasts and moulds from glue samples was performed immediately after arrival at the laboratory.

Microbial enumeration

Surface samples: Surface samples were taken aseptically from selected process surfaces using the commercial rapid cultivation methods Petrifilm™ and Hygicult®. Aerobic bacteria were detected using a Petrifilm™ Aerobic Count Plate (3M Health Care, Loughborough, Leicestershire, UK). Enterobacteria, and yeasts and moulds were determined using Hygicult®-E and Hygicult®-Y&F contact slides, respectively (Orion Diagnostica, Espoo, Finland). For surface samples, Petrifilms (Petrifilm™ Aerobic Count Plate, 3M Health Care) were moisturized with 1 ml Lethen solution (Difco, Detroit, MI) prior to use. Incubation for the determination of aerobic bacteria was at 30°C for 3 days and for enterobacteria at 37°C for 1 day. Incubation for detecting yeasts and moulds was at 25°C for 5 days. Results were calculated according to the instructions and model charts of the methods as recommended by the suppliers.

Air samples: Air samples were taken by sediment plates. Open Petri dishes were placed at different process sampling sites and exposed for 1 h. Aerobic bacteria were determined on plate count agar (Difco) supplemented with 100 μl of 2.5% Pimafucin® (Yamanouchi, Leiderdorp, The Netherlands) to inhibit the growth of moulds and yeasts. Moulds and yeasts were detected on yeast extract glucose chloramphenicol agar (Merck, Darmstadt, Germany). Incubation for the determination of aerobic bacteria was carried out at 30 °C for 3 days and for moulds and yeasts at 25°C for 5 days.

Glue, raw material paper and end-product samples: The glue, raw material paper and end-product samples were analysed using the pour-plate method from logarithmic dilutions. Three grams of raw material paper or end-product sample was weighed aseptically in 297 ml of Ringer solution. The sample (1% solution) was homogenized with a Waring Blender-homogenizer (Waring Products Division, New Hartford, CT) for 120 s under low power prior to microbiological analysis. Alternatively, 1 g of end-product sample was weighed aseptically and homogenized in 99 ml of Ringer solution using a "Virtis 23"-homogenizer (VirTis, Gardiner, NY) for 120 s under medium power prior to microbial analysis.

Plate count agar (Difco) was used to detect aerobic bacteria, Nutrient agar (Oxoid CM3, Basingstoke, Hampshire, England) for aerobic spore-forming bacteria after heat-shock treatment (10 min at 80°C) and violet red bile glucose agar (Merck) for enterobacteria. Yeasts and moulds were detected on yeast extract glucose chloramphenicol agar (Merck) and anaerobic sulphate reducing bacteria were determined by the rapid commercial cultivation method Easicult®-S (Orion Diagnostica). Incubation for determination of aerobic bacteria was carried out at 30°C for 3 days and for enterobacteria at 37°C for 1 day. Incubation parameters for yeasts and moulds were 25°C for 5 days and for anaerobic bacteria 37°C for 4 days. The method's detection limit (threshold level) for aerobic bacteria, aerobic spore-forming bacteria, enterobacteria, and yeast and moulds was 10 cfu/g.

Characterization and identification of bacterial isolates

Ribotyping: Ribotyping was performed using an automated ribotyping device, RiboPrinter® Microbial Characterization System (DuPont Qualicon™, Wilmington, DE) according to the manufacturer's instructions [4]. The method involves the release of DNA from cells, digestion of DNA with a restriction enzyme (*Eco*RI), and separation of the resulting fragments by agarose gel electrophoresis followed by Southern hybridization using the *rrn*B rRNA operon from *Escherichia coli* [2] as a chemiluminescent probe. Images of the hybridization patterns were acquired with a charge-coupled device (CCD) camera and processed by software that normalizes fragment pattern data for band intensity and relative band position in relation to the molecular weight marker. To ensure reproducibility of the ribotypes, all newly generated ribogroups were analysed three times. Similarity values were calculated using the software, and automatic identification was made if similarity to an identification pattern in the DuPont (DUP) database (Qualicon release 11.2.c, 1999) was >0.85.

16S rDNA sequence analysis: DNA extraction, PCR amplification of the 16S rDNA and purification of the PCR products were carried out as described by Rainey *et al* [15]. Purified PCR products were sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Weiterstadt, Germany) as directed by the manufacturer. Sequence reactions were electrophoresed using the Applied Biosystems 373A DNA sequencer. The sequences (about 450 nucleotides) were compared with those of the type strains as well as with 16S rDNA reference sequences obtained from the Ribosomal Database Project [8].

Morphology, physiological tests and fatty acid analysis: Morphological and physiologic tests (cell size and shape, spore and pigment formation, Gram reaction, catalase, VP reaction,

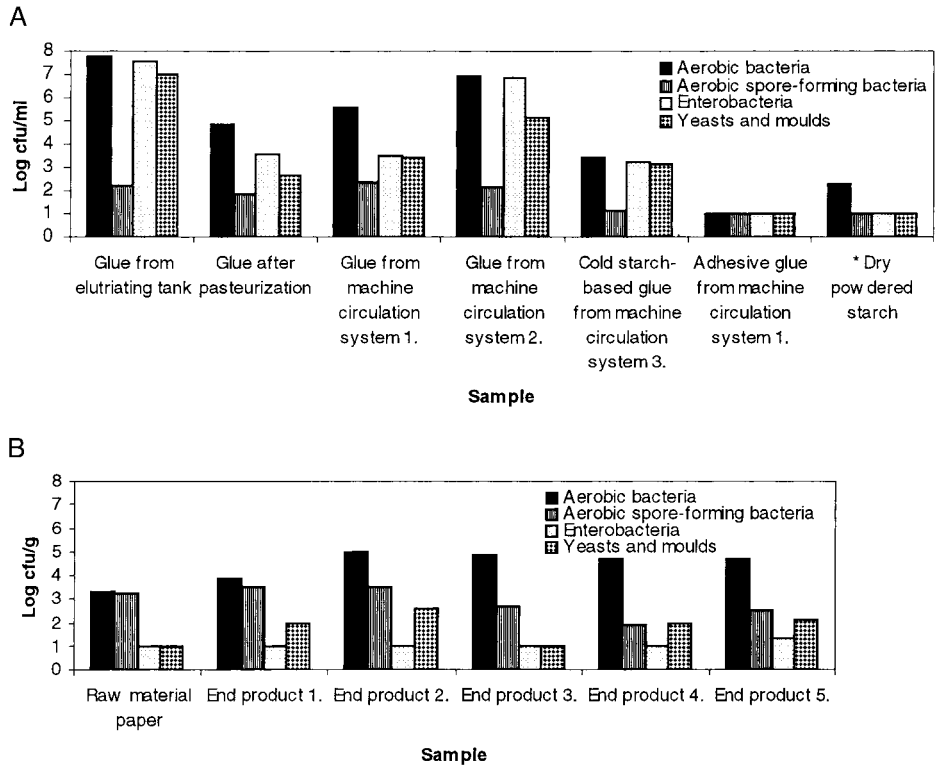

Figure 1 (A) Total bacterial count, aerobic spore-forming bacteria, enterobacteria, yeasts and moulds in starch-based glues during processing and use of pasteurized glue (*colony-forming units per gram). (B) Total bacterial count, aerobic spore-forming bacteria, enterobacteria, yeasts and moulds in raw material paper and end products.

growth at different temperatures, growth in different pH values and NaCl concentrations, acid formation from carbohydrates, hydrolysis/use/decomposition of different components) were carried out using conventional methods. Whole-cell fatty acid esters (FAMEs) were examined according to the MIDI Microbial Identification System (Microbial ID Inc., Newark, DE).

Results

Microbiological survey of the process

An extensive survey covering sampling from process and environment surfaces, plant air, starch-based glue and end products (Table 1) was performed in June 1998. The number of aerobic bacteria in glue samples was high except in the machine circulation system using cold glue and an adhesive glue (Figure 1A). The total count of aerobic bacteria in glue samples varied between 2.8×10^3 and 5.7×10^7 cfu/ml with the highest levels found in the elutriating tank of starch-based glue and the lowest in the machine circulation system using cold glue (Figure 1A). Enterobacteria, moulds and yeasts were detected in all samples except in adhesive glue and dry powdered starch. The number of enterobacteria varied between 1.6×10^3 and 3.9×10^7 cfu/ml and yeasts and moulds between 4.5×10^2 and 1.0×10^7 cfu/ml. The highest amounts were detected in the elutriating tank and in the machine circulation systems. Amounts of aerobic spore-forming bacteria were low, varying between 1.5×10^1 and 2.2×10^2 cfu/ml. Anaerobic bacteria were detected at moderate levels in the elutriating tank of starch-based glue and at low levels in machine circulation systems (results not shown). Anaerobic bacteria were not detected in the pasteurized glue and in the machine circulation system, which used cold glue. Furthermore, the dry powdered starch did not contain detectable aerobic spore-forming bacteria, enterobacteria or moulds and yeasts, and the total aerobic count was low (2.0×10^2 cfu/g). The microbial load was high in the elutriating tank of starch-based glue with an approximate 3–4 logarithmic decrease after pasteurization (Figure 1A). However, the microorganisms were not completely eliminated. The microbial load remained at the same level in the machine circulation system (1) where the tube line carrying starch-based glue is short. However, in the machine circulation system (2) where the tube line was long, the microbial load had increased to the same level as in the elutriating tank. The results also indicated that in newly produced, unpasteurized and unstored cold glue microbial growth was lower.

The effect of heavily colonized glue was also detected in process surface samples. High numbers of aerobic bacteria were detected on process surfaces having contact with starch-based glue such as the glue supplier and the mouth of the machine circulation system. Process surfaces having no contact were clean (results not shown).

The end product contained aerobic bacteria (5.1×10^2 to 9.7×10^4 cfu/g) and aerobic spore-forming bacteria (8.3×10^1 to 3.2×10^3 cfu/g). The glue samples contained enterobacteria; however, they could be detected only at very low amounts (2.0×10 cfu/g) in one end-product sample. Moulds were detected at moderate amounts (0 to 3.8×10^2 cfu/g) (Figure 1B). The raw material paper contained both aerobic bacteria (2.0×10^3 cfu/g) and aerobic spore-forming bacteria (1.7×10^3 cfu/g), but no enterobacteria or moulds.

Air samples from the process environment were taken simultaneously with the surface, glue, raw material paper and

Table 2 Identification of aerobic spore-forming bacteria from pasteurized starch-based glue samples

VTT code	Ribogroup (isolates), closest similarity to	Partial 16S rDNA, closest similarity (%) to	Physiological properties	FAME, closest similarity to	Final identification
E-991352	483-S-1 (1) 0.71 *P. alvei*	95.2 *P. azotofixan* 94.9 *P. amylolyticus*	Point to the genus *Paenibacillus*	0.053 *B. circulans*	*Paenibacillus* sp., probably a new species
E-991353	484-S-1 (1) 0.74 *B. circulans*	88.5 *P. curdlanoticus* 88.3% *P. kobensis*	Point to the genus *Paenibacillus*	0.184 *B. circulans*	*Paenibacillus* sp., probably a new species
E-991354	**483-S-6 (3)** 0.75 *P. macerans* 0.72 *P. alvei*	93.4 *P. azotofixans*	Point to the genus *Paenibacillus*	0.335 *B. circulans*	*Paenibacillus* sp., probably a new species
E-991355	**471-S-6 (2)** 0.80 *P. macerans*	94.5 *P. azotofixans*	Point to genus *Paenibacillus*	0.377 *P. azotofixans*	*Paenibacillus* sp., probably a new species
E-991356	714-S-7 (1) 0.68 *B. pumilus*	95.1 *B. benzoevorans*	Point to *B. benzoevorans*	Correlation to *Bacillus*	*Bacillus* sp., probably a new species
E-991357	483-S-3 (1) 0.98 *B. circulans*	98.5% *P. lautus*	nd	nd	*Paenibacillus* sp., probably a new species
E-991358	483-S-4 (2) 0.69 *B. thuringiensis*	91.5 *B. simplex* 91.5 *B. flexus*	Not typical for *B. simplex* or *B. flexus*	Correlation to *Bacillus*	*Bacillus* sp., probably a new species
E-991359	720-S-7 (1) 0.94 *B. circulans*	nd	nd	nd	*B. circulans*? see E-991357

B.=*Bacillus*, *Br.*=*Brevibacillus*, *P.*=*Paenibacillus*, nd=not determined.

end-product samples (results not shown). Aerobic bacteria were detected at low quantities (0–31 cfu/plate/h). However, similar significant amounts of moulds (60 cfu/plate/h) were detected near the glue suppliers, in the packaging area and in the storage areas.

The results of this microbiological survey showed that the production and use of pasteurized glue could threaten end-product safety. The use of pasteurized glue was consequently terminated and the use of cold glue extended to cover all process lines. Furthermore, machine circulation systems were refitted with enclosed automatic glue suppliers.

Additional sampling of starch-based glue and end product was performed in October 1998 and November 1999 (Figure 2). In October 1998, the results revealed a 1–1.5 logarithmic decrease in the amount of aerobic bacteria and enterobacteria when compared with results obtained in June 1998. Aerobic spore-forming bacteria were not detected in glue samples (Figure 2). The end product contained no enterobacteria or moulds and there was more than a 1 logarithmic decrease in the number of aerobic bacteria. In November 1999 starch-based glue was found to contain aerobic bacteria (7.1×10^4 cfu/ml) but no spore-forming bacteria, enterobacteria or moulds and yeasts. Furthermore, the end product did not contain enterobacteria or moulds. Spore-forming bacteria detected in the end product most probably originated from raw material paper (Figure 1B).

Characterization and identification of bacterial isolates

Aerobic spore-forming bacteria: From pasteurized starch-based glue samples 12 isolates were ribotyped and 8 different ribogroups (ribotypes) were generated (Table 2). One isolate from each ribogroup was deposited in the VTT Culture Collection using VTT codes. The commercial database (DUP) was able to identify

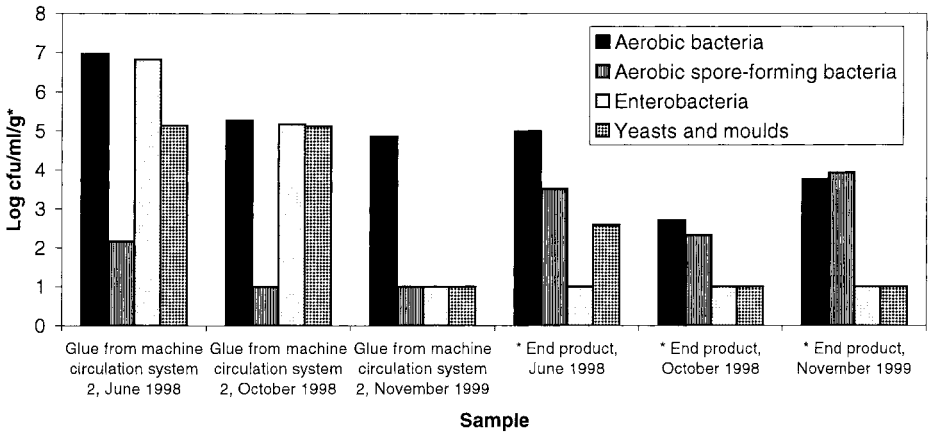

Figure 2 Total bacterial count, aerobic spore-forming bacteria, enterobacteria, yeasts and moulds in starch-based glues and end products determined in June 1998, October 1998 and November 1999 (*colony-forming units per gram).

Table 3 Identification of aerobic spore-forming bacteria from raw material paper samples

VTT Code	Ribogroup (isolates), closest similarity to	Partial 16S rDNA, closest similarity (%) to	Physiological properties	FAME, closest similarity to	Final identification
None	483-S-3 (2) 0.98 *B. circulans*	nd	nd	nd	same as E-991357
E-991366	516-S-8 (1) 0.56 *B. agaradhaerens*	96.5 *B. globisporus* 96.0 *B. psychrophilus*	Not typical for *B. globisporus* or *B. psychrophilus*	0.474 *B. laterosporus*	*Bacillus* sp., probably a new species
E-991367	518-S-1 (1) 0.78 *B. stearothermophilus*	95.1 *P. kobensis* 95.1 *P. curdlanolyticus*	Indicate the genus *Paenibacillus*	0.162 *B. circulans*	*Paenibacillus* sp., probably a new species
E-991368	518-S-2 (1) 0.76 *B. circulans*	88.1 *P. kobensis* 88.9 *P. curdlanolyticus*	Indicate the genus *Paenibacillus*	0.342 *B. circulans*	*Paenibacillus* sp., a new species
E-991369	563-S-5 (1) 0.38 *Br. brevis*	98.5 *Br. reuszeri* 98.2 *Br. brevi* 98.0 *Br. formosus*	Not typical for *Br. reuszeri*, *Br. brevi* or *Br. formosus*	0.159 *Br. brevis*	*Brevibacillus* sp., probably a new species
E-991370	531-S-1 (1) 0.76 *B. pumilus*	98.5 *B. pumilus*	Typical for *B. pumilus*	0.793 *B. pumilus*	*B. pumilus*
E-991371	531-S-3 (1) 0.52 *P. kobensis*	94.1 *P. kobensis* 93.1 *P. curdlanolyticus*	Indicate the genus *Paenibacillus*	0.346 *P. polymyxa*	*Paenibacillus* sp., probably a new species
E-991372	735-S-5 (1) 0.71 *B. cereus*	96.0 *B. niacini* 95.5 *B. circulans*	Not possible to identify strain at species level	0.043 *B. sphaericus*	*Bacillus* sp., probably a new species
E-991373	531-S-5 (1) 0.89 *B. cereus*	100 *B. cereus* 100 *B. anthracis*	Point to *B. cereus*	0.652 *B. cereus*/ *thuringiensis*	*B. cereus*

B.=*Bacillus*, *Br.*=*Brevibacillus, P.*=*Paenibacillus*, nd=not determined.

only two of the generated ribogroups (similarity >0.85) both as *Bacillus circulans*. The deposited isolate from each ribogroup was subjected to further analysis. According to those results, four strains (ribotypes) were proposed as new species within the genus *Paenibacillus* and two within the genus *Bacillus*. In particular, the physiological properties were not typical for the species previously described, but also the similarity of partial 16S rDNA sequences to those existing in the gene bank was often low.

From raw material paper samples 10 isolates were ribotyped and nine different ribogroups (ribotypes) were generated (Table 3). From this group one ribogroup (ribotype) was identified as *B.circulans* (the same ribotype as E-991357 in glue samples) and one as *B. cereus*. In further studies, one ribotype was identified as *Bacillus pumilus* and the rest were proposed as new species within *Bacillus* (two ribotypes), *Paenibacillus* (three ribotypes) or *Brevibacillus* (one ribotype).

From end-product samples 11 isolates were ribotyped and eight different ribogroups (ribotypes) were generated, but none was identified according to the current DUP database (Table 4). Two ribogroups (483-S-6 and 471-S-6) appeared the same as in glue

Table 4 Identification of aerobic spore-forming bacteria from end-product samples

VTT code	Ribogroup (isolates), closest similarity to	Partial 16S rDNA, closest similarity (%) to	Physiological properties	FAME, closest similarity to	Final identification
None	**483-S-6 (2)** 0.75 *P. macerans* 0.72 *P. alvei*	nd	nd	nd	same as **E-991354**
None	**471-S-6 (3)** 0.78 *P. macerans*	nd	nd	nd	same as **E-951355**
E-991360	516-S-3 (1) 0.33 *P. kobensis*	94.8 *P. kobensis* 93.9 *P. curdlanolyticus*	Indicate the genus *Paenibacillus*	0.262 *P. polymyxa*	*Paenibacillus* sp., probably a new species
E-991361	516-S-4 (1) 0.81 *B. subtilis*	99.5–100 *B. subtilis* 99.3 *B. amyloliquefaciens*	Typical for *B. subtilis*	0.713 *B. amyloliquefaciens* 0.687 *B. subtilis*	*B. subtilis*
E-991362	516-S-5 (1) 0.85 *B. circulans* 0.76 *P. glucanolyticus*	98.5 *P. lautus* 96.7 *P. glucanolyticus*	Indicate *P. lautus*	0.457 *P. pabuli*	*Paenibacillus* sp., very closely related to *P. lautus*
E-991363	563-S-4 (1) 0.74 *P. alvei*	91.4 *P. kobensis* 91.4 *P. curdlanolyticus*	Point to the genus *Paenibacillus*	0.121 *B. circulans*	*Paenibacillus* sp., probably a new species
E-991364	516-S-6 (1) 0.75 *B. pumilus*	99.6 *B. pumilus*	Typical for *B. pumilus*	0.582 *B. megaterium* 0.540 *B. pumilus*	*B. pumilus*
E-991365	516-S-7 (1) 0.81 *B. subtilis*	97.1 *B. pumilus*	Typical for *B. pumilus*	0.352 *B. megaterium* 0.262 *B. pumilus*	*B. pumilus*

B.=*Bacillus*, *Br.*=*Brevibacillus*, *P.*=*Paenibacillus*, nd=not determined.

Table 5 Identification of enterobacteria

VTT code	Ribogroup (isolates), closest similarity to	Partial 16S rDNA, closest similarity (%) to	Physiological properties	FAME, closest similarity to	Final identification
Pasteurized starch-based glue					
E-991302	693-S-1 (1) 0.73 *E. cloacae*	99.1 *E. cloacae*	Typical for *E. cloacae*	0.735 *E. taylorae*	*E. cloacae*
E-991303	501-S-2 (1) 0.73 *C. freundii*	98.9 *C. freundii*	Typical for *C. freundii*	0.888 *C. frundii*	*C. freundii*
E-991304	72-S-2 (1) 0.74 *Ph. damselae*	98.7 *P. putida*	Typical for *P. putida*	0.582 *P. putida*	*P. putida*
Cold starch-based glue					
E-991305	501-S-4 (3) 0.62 *K. pneumoniae*	98.9 *E. hormaechei* 98.2 *C. youngae* 98.0 *E. pyrinus*	Not possible to classify within *Enterobacter*/*Citrobacter*	0.785 *S. typhimurium* 0.713 *Pa. agglomerans* 0.709 *Pr. stuartii*	*Enterobacter*/*Citrobacter*
E-991306	501-S-6 (1) 0.43 *P. putida*	98.7 *P. putida* 98.5 *P. graminis* <98 *P. stutzeri*/ <98 *P. mendocina*	Indicate to *P. stutzeri* and *P. mendocina*	0.364 *P. mendocina* 0.353 *P. stutzeri*	*Pseudomonas* sp., RNA-group I
E-991309	502-S-1 (1) 0.56 *C. freundii*	100 *C. freundii*	Typical for *C. freundii*	0.812 *C. freundii*	*C. freundii*
E-991310	502-S-3 (1) 0.68 *K. pneumoniae*	98.9 *E. hormaechei* 98.2 *C. youngae* 98.0 *E. pyrinus*	Not possible to classify within *Enterobacter*/*Citrobacter*	0.791 *Pa. agglomerans* 0.735 *C. freundii* 0.668 *K. terrigena*	*Enterobacter*/*Citrobacter*
End product					
E-991307	501-S-7 (2) 0.88 *E. cloacae*	97.4 *E. hormaechei* 95.8 *E. cloacae*	Typical for *E. cloacae*	0.808 *E. taylorae* 0.512 *E. cloacae*	*Enterobacter* sp.
E-991308	502-S-4 (1) 0.74 *E. cloacae*	99.8 *E. diversus* 99.8 *E. dissolvens* 99.8 *Le. adecarboxylata*	Typical for the genus *Enterobacter*	0.735 *Kl. cryocrescens* 0.685 *E. taylorae*	*Enterobacter* sp.
E-991325	22-S-6 (3) 0.91 *E. cloacae*	nd	nd	nd	*E. cloacae*

E.=*Enterobacter*, *C.*=*Citrobacter*, *K.*=*Klebsiella*, *Kl.*=*Klyvera*, *Le.*=*Leclercia*, *Pa.*=*Pantoea*, *Ph.*=*Photobacterium*, *Pr.*=*Providencia*, *P.*=*Pseudomonas*, *S.*=*Salmonella*, nd=not determined.

samples represented by strains E-991354 and E-991355, which were proposed as new species within the genus *Paenibacillus*. One of the ribotypes was identified as *B. subtilis*, two as *B. pumilus* and three were proposed as new species within *Paenibacillus*.

Enterobacteria: From pasteurized and cold starch-based glue samples nine isolates were ribotyped and seven different ribogroups (ribotypes) were generated, but none was identified according to the current DUP database (Table 5). Of the isolates, seven belonged to the genera *Enterobacter* or *Citrobacter* and only two to the genus *Pseudomonas*. The isolates from cold starch-based glue were very atypical and thus physiological, chemotaxonomic (FAME) and molecular (partial 16S rDNA) results did not correspond in all cases.

From end-product samples six isolates were ribotyped and three ribogroups (ribotypes) were generated (Table 5). The system was able to identify three isolates as *E. cloacae* with good similarity (0.91–0.97). The other three isolates were atypical members of the genus *Enterobacter*.

Discussion

The economic cost of microbial damage to industry could be reduced significantly if the species, characteristics, life cycles and proliferation patterns of harmful bacteria were known. Such information could be used to target prevention measures more effectively. Information concerning harmful microorganisms, however, is usually deficient or missing altogether.

The present paper focused on process hygiene and product safety in the production of refined paper products intended for contact with food. The microbial surveys provided information concerning the quantity and identity of microbes evident on or in process surfaces, raw materials and final products. The microbe determination process clearly indicated points that were not microbiologically clean. As a consequence, a monitoring system for microbiologically critical process phases was developed, which included systematic sampling of starch-based glue.

Starch is the third most important raw material for papermaking, being one of the most common additives. Starch is a mixture of two different glycopyranose polymers — amylose and amylopectin. A wide variety of microorganisms, mainly moulds and aerobic endospore-forming bacteria, produce and excrete exocellular starch-depolymerizing enzymes. Starch-spoiling bacteria include *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus stearothermophilus*. Enzymatic degradation of starch quickly impairs its technical and hygienic quality. According to earlier studies [3,16] and our experience [20] starch hygiene is a very important aspect of the manufacturing process though often neglected. Short storage times for starch slurries and solutions,

proper design of starch-handling equipment and regular checks on solutions used can prevent problems and result in products with more consistent quality [3,9,13,14,17,26,27].

Most identification systems and databases are first created uniquely for clinical isolates. This is also true for the DUP identification database of the RiboPrint® system. It was first used for subtyping human pathogens, e.g., *Listeria monocytogenes* [1,4,24,25], but is now also used for beer-spoilage bacteria including *Lactobacillus* [10,22], *Pectinatus* [11] and *Pediococcus* [19]. In this study, we applied it to isolates from the paper and packaging industry and, as expected, the isolates were mainly unknown to the system, or the identification was not reliable. The DUP database identified some strains as *B. circulans* with very high similarity; however, the partial 16S rDNA sequence pointed to a new species within the genus *Paenibacillus*. Consequently, VTT has been building its own identification database for bacteria present in the paper and packaging industry to augment that provided by the manufacturer. This work was part of that effort. It will later enable rapid and easy identification of these harmful contaminants. The number of isolates analyzed from different samples were rather low, but we were able to demonstrate the ability of the system to characterize isolates below the species level and trace the contamination source. The same ribotypes of spore-forming bacteria were detected from glue and final product samples. The isolates were highly adapted to their specific environments and were difficult to identify reliably. In many cases the results of physiological, chemotaxonomical (FAME) and biomolecular tests were not in good agreement or typical for any species previously described. Thus, the reliable identification of most isolates by the methods used could be done only at the genus level. The identification of these isolates needs to be continued and if they really are new species, as proposed according to these results, the new strains need to be validly described. Among the isolates identified, *E. cloacae*, *Citrobacter freundii* and *B. cereus* belong to the hazard group two of bacteria [18] and may cause some human diseases. In addition, the pathogenicity of the potential new species is unknown.

During this work the use of cold starch-based glue and automatic feeding systems clearly improved process hygiene and the safety of end products. The implementation of systematic microbial analysis of starch-based glue provided a sufficient control system for glue hygiene. The identification of contaminants in starch-based glue made it possible to focus control measures more precisely and to choose the most effective biocide to deploy against the harmful contaminants.

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