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Microscopic study of migration of microbes in food-packaging paper and board

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The microbiological barrier properties of food-packaging paperboards, coated with polyethylene, mineral pigment or a biodegradable polymer and of high-density paper were examined with confocal laser scanning microscopy. The results show that the spatial distribution of microscopically observable bacterial cells was uneven inside the paperboard. The concentration in the interface between the polyethylene coating and the cellulose fibers was 100–200 times higher than inside the cellulose matrix. The bacteria in the interface and the mineral coating layer grew in response to access to food and moisture, whereas no growth was observed inside the fiber web, not even after extended exposure for up to 90 days. The paper and paperboards studied contained soluble nutrients (C:N:P 54:9:1 to 309:3:1) and no measurable antimicrobial activity. The factor limiting growth and migration of bacteria inside the fiber web was most likely limited access to free water, even under conditions of extensive wetting. The studied paperboards functioned as efficient barriers against translocation of microbes. The microbes residing between the paperboard and its polymer coating facing food, was the only potential site from which microbes could leak into food. This emphasizes the need for high hygienic quality of surface-sizing chemicals. Mineral-coating pigments were a source of microbes and their application behind the PE coating facing food is contraindicated.

Keywords: barrier; confocal laser scanning microscopy (CLSM); food packaging; microbes; migration; paper and paperboard

Introduction

Paper and paperboard are renewable, recyclable materials with potential for sustainable development. For food packaging 2.5 million tons of paper and board are used world wide [13]. Paper is made of natural materials which are biodegradable. Paper machines provide conditions favourable for growth of microbes. It is known that the main contaminants in food-packaging paper and board belong to the genera *Bacillus* and *Paenibacillus* [9,12,18] many of which produce enzymes like amylase and caseinase that are food spoilage-related [12]. Good quality food-packaging paper and board contains orders of magnitude less bacteria [12,18] than the packed food in most cases. But even a low number of bacteria in the packaging material can be of concern for aseptic food, if bacteria migrate across the package when stored at room temperature for a long period (eg 5 months for baby food). Little is known about the microbiological barrier properties of paperboard and of the different materials used for coating or as barriers in food packages. Adequate methods for assessing the migration of microbes in paper and board and in the coating or barrier layers have been lacking.

The aim of this study was to develop experimental tools to study migration of bacteria in paperboard and to gain knowledge on the microbiological barrier properties of paperboard and of coating materials. An experimental set-up to simulate a worst case situation was designed and the spread of the micro-organisms contained inside the paperboard for extended periods of time was followed by non-destructive microscopic techniques (CLSM).

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Materials and methods

Materials studied

(1) Polyethylene (PE)-coated liquid packaging board: virgin fiber paperboard with PE coating on both sides; (2) mineral-coated paperboard: recycled fiber paperboard with mineral coating on one side; (3) biopol-coated paperboard: virgin fiber paperboard with a coating of HB/HV (poly-β-hydroxybutyrate and -β-OH-valerate) on one side; (4) high-density paper (grease resistance achieved by refining the paper).

Microbiological analyses

The total viable heterotrophic counts in paper and board were measured from samples homogenised in distilled water, on plate count agar (PCA, Biokar Diagnostics, Beauvais, France), 10 times diluted PCA (1/10 PCA) or starch agar (SA: soluble starch 10.0 g L^{-1}, beef extract 3.0 g L^{-1}, agar 12.0 g L^{-1} [5]) at 28°C as described in TAPPI Standard T 449 om-90 [2] and nutrient agar (Oxoid CM 3, Basingstoke, Hants, UK). Yeasts and moulds were detected on Sabouraud agar (Sab, Merck 7662, Darmstadt, Germany) after incubation at 25°C for 3–14 days. For the detection of actinomycetes, Actinomycete isolation agar (AIA, Difco 0957, Detroit, MI, USA) was used. The numbers of thermophilic and mesophilic actinomycetes were recorded after incubation at 45°C for 7 days and at 30°C for 14 days, respectively [16].

The most abundant colony types were isolated and pur-

ified on the same media and identified by whole cell fatty acid analysis (FAME). Methyl esters were prepared for FAME analysis from 24-h cultures (48 h for slow-growing isolates) grown at 28°C on tryptic soy broth agar (TSBA, BBL, Becton Dickinson, Cockeysville, MD, USA) as described elsewhere [19]. The aerobic TSBA library version 3.9 (MIDI Inc, Newark, DE, USA) was used. Names are given in the Results section when the similarity index exceeded 0.3. Physiological properties of the bacterial isolates were recorded using Biolog GP MicroPlate™ test panels containing 95 different substrates for Gram-positive bacteria (Biolog Inc, Hayward, CA, USA) or API-50 CHB test strips (bioMérieux sa, Marcy l-Etoile, France). Partial 16srDNA sequences (*ca* 450 bp) of 15 strains were determined as described previously [12].

Antimicrobial activity of the paper and the boards was assayed by the inhibition zone method on nutrient medium inoculated with *Bacillus subtilis* DSM 347 as the test organism as described in European Standard EN 1104 [3], read after 3 days at 30°C. Calibration antimicrobials were discs of penicillin (5 μg), chloramphenicol (60 μg) and tetracycline (80 μg) (Neosensitabs; Rosco AS, Taastrup, Denmark). Calibration biocides were a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one (0.13, 0.033 and 0.013 kg active compound per ton paper), 2,2,-dibromo-3-nitrilopropionamide (0.7, 0.18 and 0.07 kg active compound per ton paper), methylene bisthiocyanate (0.6, 0.15 and 0.06 kg active compound per ton paper) (Betz-Dearborn, Helsinki, Finland) and glutaraldehyde (0.55, 0.13 and 0.06 kg active compound per ton paper) (Aspokem Oy, Helsinki, Finland).

Analysis of the paper materials

The pH of the paper and board homogenates (in distilled water) was recorded 1 and 2 h after preparing the homogenate. To measure the soluble carbon in the paper and board, the homogenate was filtered through a 0.45-μm Supor®-450 membrane filter (Gelman Sciences, Ann Arbor, MI, USA) and the filtrate analysed for total organic carbon with a carbon analyser (TOC-5000 Shimadzu, Kyoto, Japan). Total carbon was measured with a SSM-5000A solid sample module (Shimadzu). Total nitrogen was determined by application of the SFS standard 5505 [1]. In the combustion step a double amount of H_2SO_4 was used, deviating from the standard which was not developed to be used for analysis of paperboard.

For the determination of total phosphorus and metal cations the samples (1 g) were decomposed using a 1200-mega microwave combuster (Milestone mls, Sorisole, Italy) with concentrated HNO_3 (6 ml) and concentrated H_2O_2 (2 ml). Phosphorus was measured from the homogenate by the ascorbic acid method [4] and iron, manganese, copper, magnesium, calcium and potassium by flame atomic absorption spectrometry (AAS-flame Perkin Elmer 4100, Überlingen, Germany; AAS-furnace Perkin Elmer THGA 5100, Norwalk, CT, USA). For determining the water sorption capacity of the paperboard, paperboard void of any coating was incubated at 28°C in presence of the same amount of water as was done with the coated materials, weighed and weighed again after drying at 105°C.

Microscopic methods

A coupon of paper or board (16 cm^2) with a perforated (200-μm) surface was placed in a sterile petri dish with the barrier material facing up. PCA was poured on the barrier material to simulate food and to establish a gradient of feed towards the paper. Distilled sterile water (1 ml) was added to the dish to maintain moisture and the dish was sealed with parafilm to prevent evaporation. After incubation at 28°C for the indicated times the agar was removed, the coupons stained for 5–10 min with acridine orange (1% in water; Merck AG, Darmstadt, Germany) and examined with a Leitz Diaplan confocal laser scanning microscope (CLSM; Leica Lasertechnik GmbH, Heidelberg, Germany) using an excitation wavelength of 488 nm. Composite pictures were made using a combination of simulated fluorescent process and stereo image.

The paperboards were sliced for polarised light micrography after embedding the paperboard into Epon/Ladd LX-112 resin using a rotating microtome and glass knife. The slices were examined with a Leitz Diaplan light microscope using two polarising filters.

Results

Properties of the paper and paperboards studied are summarised in Tables 1 and 2. The total viable count ranged from $<10^2$ CFU g^{-1} for the PE (polyethylene)-coated food packaging board to 10^6 for the mineral pigment-coated paperboard (Table 1). The paper boards contained carbon, nitrogen and minerals necessary for bacterial growth. The ratio of soluble C:N:P ranged from 54:9:1 to 309:3:1. Soluble carbon represented 0.2–2.2% of the total carbon contents. All isolates identified to species level from the paper(boards) belonged to the genera *Bacillus* and *Paenibacillus* (see Table 2). The mineral pigment-coated paperboard contained 10^3 CFU g^{-1} of moulds and 2×10^2 CFU g^{-1} thermophilic actinomycetes. Yeasts or mesophilic actinomycetes were not detected.

Figure 1A shows a transverse section of the PE-coated paperboard widely used for packaging liquid foods (milk, juice). The paperboard is coated on both sides with PE, a thicker layer of PE meant to face the packed food and a thinner layer of PE to the outside of the package. The PE layer on the paperboard surface was uneven in thickness, 16–36 μm, reflecting the uneven surface of the fiber web (Figure 1A). An optical section in *z*-direction of the PE-coated liquid packaging board was taken using a CLSM and is shown in Figure 1B. In the PE layer fluorescent spots with a diameter from 1 to 3 μm are visible. PE is a hydrophobic material and a short staining time will not allow for extensive diffusion inside the PE. The stain must have penetrated into the PE via pre-existing pores. Penetration indicates that the PE layer is not completely impermeable towards aqueous solutions of lipophilic solutes like acridine orange (log $K_{ow} = 1.24$) [7].

During the transportation of packaged food the barrier layer (PE) may suffer damage and the paperboard may then sorb water. The consequences of this situation were studied with the following experimental set-up: the PE layer of the paperboard was perforated, the paperboard subsequently exposed to food (= PCA) contact in moist environment for

Table 1 Properties of the food packaging paper and paperboards studied

Material		Coating		Measurements of homogenized paper and paperboard			Elementary composition								
Type	Weight ($g\ m^{-2}$)	Type	Thickness	Bacteria ($CFU\ g^{-1}$)	pH	DOC ($mg\ g^{-1}$ d.w.)	C	N	P	Fe	Mn ($mg\ kg^{-1}$ d.w.)	Cu	Mg	Ca	K
PE-coated food packaging board	332 ± 12	polyethylene	29 ± 0.9 μm	$<10^2$	8.2–9.1	7.8 ± 2.0%	494 ± 1	300 ± 9.0	33 ± 1.0	10 ± 0.3	1.6 ± 0.16	1.9 ± 0.19	47 ± 1.4	160 ± 4.8	24 ± 0.7
Mineral-coated paper board	414 ± 17	mineral pigment	50 ± 10 μm	8×10^4 to 2×10^6	7.4–7.7	8.5 ± 2.0%	383 ± 3	1400 ± 42	156 ± 4.7	470 ± 14.1	30 ± 0.9	24 ± 0.72	680 ± 20.4	44000 ± 1320	360 ± 10.8
High-density paper	50 ± 2		50 ± 10 μm	5×10^2	5.2–5.9	0.6 ± 2.4%	427 ± 3	1100 ± 33	6 ± 0.6	35 ± 1.1	0.4 ± 0.04	5.3 ± 0.53	170 ± 5.1	18 ± 0.5	34 ± 1.0
Biopol-coated paperboard	214 ± 9	Biopol	27 $g\ m^{-2}$	$<10^2$ to 2×10^3	5.8–6.1	6.8 ± 0.8%	449 ± 5	700 ± 21	22 ± 0.7	13 ± 0.4	0.8 ± 0.08	1.0 ± 0.1	8.0 ± 0.8	80 ± 2.4	46 ± 1.4

Table 2 Identity of 79 strains isolated from the food-packaging paper and paperboards studied

Material analysed	Strains analysed	Strains identified		
		by FAME[a] (similarity index) and 16srDNA[b,c,d]	by API-50 CHB (%-id)	by Biolog (similarity index)
PE-coated, mineral pigment-coated	4	*Bacillus amyloliquefaciens*[c] (0.523–0.565)	nd	*B. licheniformis* (0.614)/no identification
PE-coated, mineral pigment-coated	6	*B. subtilis*[c] (0.759–0.925)	*B. amyloliquefaciens* (63%)	*B. subtilis* (0.795)/*B. brevis* (0.385)
PE-coated, mineral pigment-coated	5	*B. pumilus*[c] (0.563–0.937) (1 strain = *B. halodurans* by 16srDNA)	*B. lentus* (98.9–99.9) *B. stearothermophilus* (95.8–99.7)/ *B. brevis* (86.5–98.0)	*B. pumilus* (0.853)
PE-coated	7	*Paenibacillus macerans*[d] (0.254–0.364)	*B. circulans* (93.0–99.9)	nd
PE-coated	2	*B. cereus*-group[b] (0.723–0.811)	*B. cereus* (99)	nd
PE-coated	1	*B. coagulans* (0.690)	nd	*B. licheniformis* (0.545)
high-density paper	1	*B. sphaericus* (0.586)	nd	*B. brevis* (0.790)
high-density paper	1	*Bacillus* sp	nd	*B. coagulans* (0.730)
high-density paper	1	*B. laterosporus* (0.610)	nd	*B. brevis* (0.810)
mineral-coated	8	*P. macerans*[c] (0.370–0.641) (2 strains = *B. flexus* by partial 16srDNA)	nd	*B. pasteurii* (0.338–0.698)/ *B. subtilis* (0.878)/*B. brevis* (0.684)/ *B.amyloliquefaciens* (0.597[a])
mineral-coated	18	*B. megaterium* (0.305–0.906)	nd	no identification/*B. brevis* (0.540–0.889)/*B. megaterium* (0.710–0.829)/*B. pasteurii* (0.724)
mineral-coated	13	*B. licheniformis* (0.465–0.914)	nd	*B. licheniformis* (0.581–0.887)/ *B. amyloliquefaciens* (0.706–0.777)/ *B. subtilis* (0.770–0.777)/*B. brevis* (0.464)/*B. megaterium* (0.624)/ *B. polymyxa* (0.886)
mineral-coated	4	*P. pabuli* (0.382–0.535)	nd	*B. amyloliquefaciens* (0.396–0.524)/*B. megaterium* (0.679)/ *B. cereus*-group (0.332)/*B. pasteurii* (0.577)
mineral-coated	2	*B. chitinosporus* (0.599–0.662)	nd	*B. cereus*-group (0.237)/*B. pasteurii* (0.415)
Biopol-coated	4	*B. circulans*[c] (0.340–0.589[e])	nd	nd
Biopol-coated	1	*Paenibacillus* sp[b]	nd	nd
High-density paper	1	no match	nd	nd

[a]Results read after 24 h.
[b,c,d]Indicates the closest relative (>95% similarity) as determined by partial 16srDNA sequencing carried out on 1, 2 or 3 strains in this group, respectively.
[e]FAME/CLIN library, but cultivated as for TSBA.
nd = not determined.

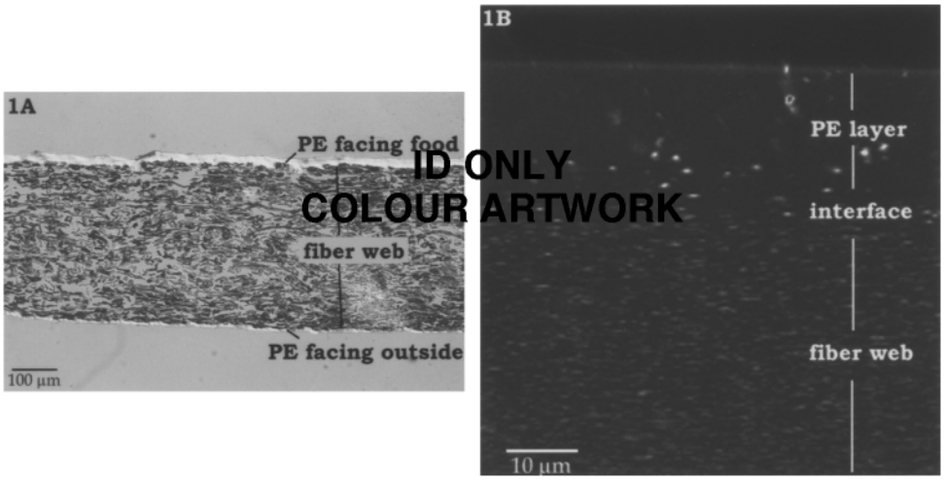

Figure 1 Vertical profile of PE-coated liquid packaging board. (A) polarised light micrograph. The colourless zones flanking the fiber web are PE; (B) optical CLSM section in *z*-direction of intact paperboard, stained with aqueous acridine orange (5–10 min). Inside the PE layer fluorescent spots, diameter 1–3 μm, are visible, indicating the presence of pores and channels through which the aqueous solution of the fluorescent stain had penetrated into the PE layer.

7 days and then studied by non-destructive CLSM. Figures 2A, B, C show the composite images of the paperboard analysed, scanned by the laser in *x-y* direction at different depths in and below the PE coating layer. The perforation (200 μm) introduced experimentally to ensure penetration of water was left outside the microscopic frame shown in Figures 2A and 2B. Single cells, probably bacterial spores, and small colonies (10–30 cells) are visible in the interface between the layers of PE and the board in the periphery of a pre-existing, indigenous damage (20 μm $\times$ 75 μm) of the PE coating. Images taken further down towards the interior of the board display cellulosic fibers. Also particles resembling bacteria are present: 77 cells were counted in the volume of 1.6×10^{-4} mm^3 shown in Figure 2C. This is less than in the slice of similar volume just above it, nearer towards the PE layer (Figure 2B; 186 cells per 1.6×10^{-4} mm^3). When the fiber web was laser scanned further down the paperboard (Figure 2D), <1 bacterium per 1.6×10^{-4} mm^3 was encountered. Summarising, the views shown in Figures 2A–D demonstrate a declining gradient of bacteria from the PE layer towards the fiber web. This was true of all samples ($n = 60$) of the paperboard studied. The microbes were thus unevenly distributed in the paperboard. In addition, the bacterial growth rate in the interface was higher than in the fiber web: the concentration of microbes in the interface was 100–200 times higher than in the fiber web.

To analyse the colonisation of the fiber web during extended exposure, the paperboards with perforated PE coating were reanalysed after 14 days, 30 days and 90 days of exposure to food and moisture. Figure 3 shows that the situation after 14 days was the same as after 7 days. The pore in the PE layer visible in Figure 3 was an indigenous one, the experimentally made puncture was also set outside the frame of the microscopic view in this case. No increase of the bacterial density at 14 days was observable as compared to 7 days of exposure to food and moisture. Similarly to Figure 2, the bacteria concentrated in the interface of PE to cellulose in Figures 3A and 3B while much less (Figure 3C) or only few bacteria were seen in the cellulose

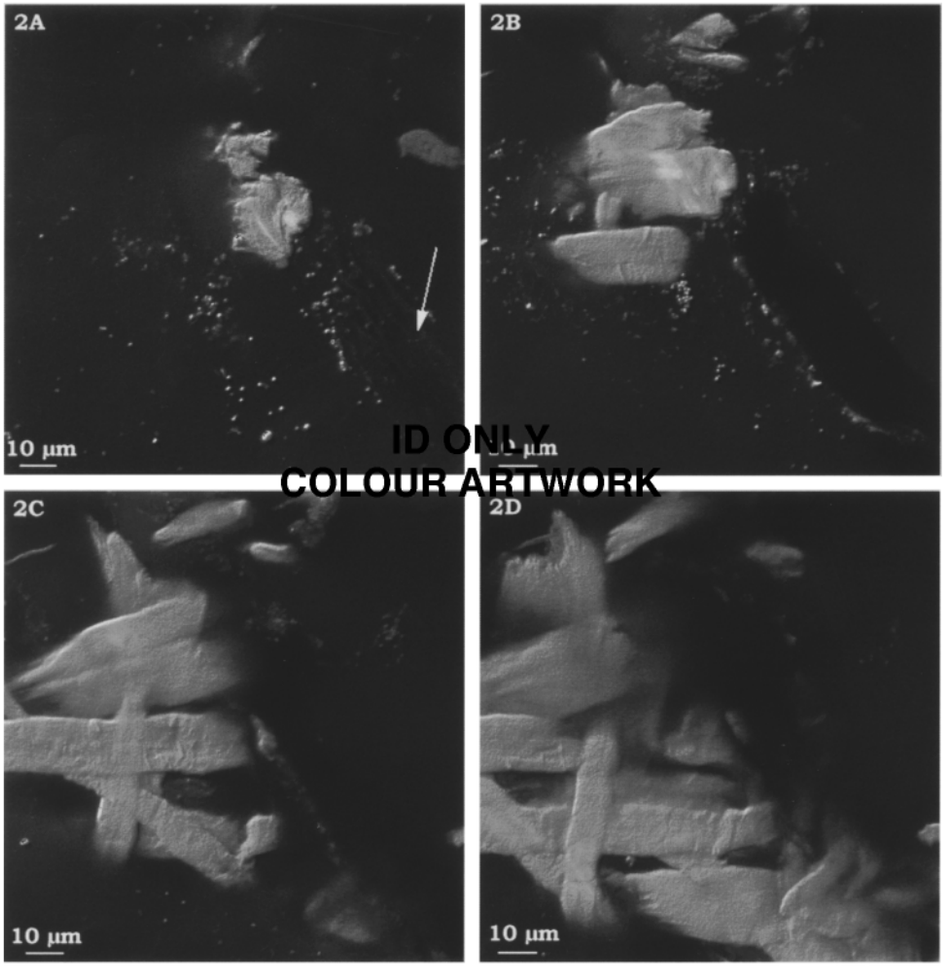

Figure 2 CLSM lateral composite images of perforated, acridine orange-stained PE-coated liquid packaging board after exposure to food and moisture for 7 days at 28°C. Panels (A)–(D) represent a pile-up of 10 lateral scans collected at 1-μm intervals, spanning a vertical distance (*z*-axis) of 10 μm through the board distal to the PE layer. Each panel represents a volume of 1.6×10^{-4} mm^3 of paperboard. Panels (A) and (B) represent the inward face of the PE coating adjacent to the fiber web. The rupture (*ca* 20 $\times$ 75 μm, arrow) visible in the PE layer pre-existed while the perforation (200 μm) induced to allow entry of water is located outside the frame of panels (A)–(D). Panels (C) and (D) represent pile-ups of 10 subsequent 1-μm scans inside the fiber web adjacent to the PE.

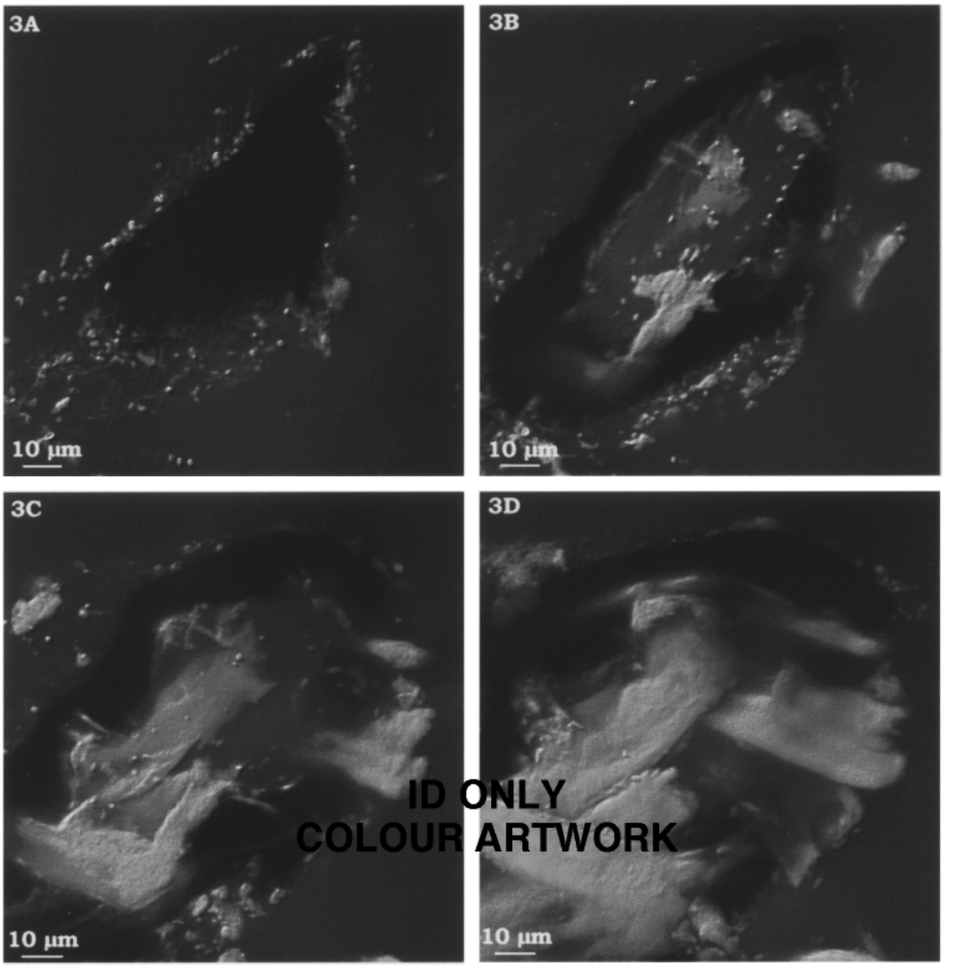

Figure 3 CLSM lateral composite images of perforated, acridine orange-stained PE-coated liquid packaging board after exposure to food and moisture for 14 days at 28°C. Panels (A)–(D) each represent a pile-up of 10 lateral scans collected at 1-μm intervals, spanning a vertical distance (*z*-axis) of 10 μm through the paperboard distal to the PE layer. Each panel represents a volume of 1.6×10^{-4} mm^3 of paperboard. Panel (A) shows the inward face of the PE layer, adjacent to the fiber web. Panels (B)–(D) show optical sections with an increasing distance distal to the PE. The rupture (*ca* 45×100 μm) visible in panels (A)–(D) was pre-existent. The perforation (200 μm) induced to allow entry of water is located outside the frame of panels (A)–(D).

layer. Some of the bacteria in Figure 3D were present as rods in contrast to after 7 days where only round cells, most likely spores, were seen. This may indicate germination and outgrowth of *Bacillus* or *Paenibacillus* present (Table 2).

After extending the exposure of the PE-coated paperboard (with perforated PE layer) to food and moisture for 30 days (Figure 4A) and 90 days (Figure 4B), still no microbes were detected inside the fiber web.

The results as presented in Figures 2 and 3 show that a relatively rich microbial community existed in liquid packaging board, located just beneath the PE layer residing adjacent to fiber web. This community grew (spores elongated to rods) on contact with water and food, but it did not penetrate into the fiber web which remained free of bacteria even after 90 days of exposure.

Some papers and paperboards are coated with mineral pigment. The impact of such a mineral pigment coating on the spread of microbes was studied by similar techniques as those shown in Figures 2 and 3. Results are shown in Figure 5. Before exposure to moisture no bacteria were seen inside the coating layer (Figure 5A) or inside the fiber web facing the coating (Figure 5B). After 2 days exposure to moisture, individual rod-shaped and coccoid cells (about 80 cells per 1.6×10^{-4} mm^3) were visible in the coating layer (Figure 5C). The presence of rod-shaped cells may indicate that some spores of *Bacilli* and *Paenibacilli* present (Table 2) had germinated. After exposure to moisture for 7 days the coating layer was heavily colonised by bacteria (Figure 5D). Approximately 450 cells per 1.6×10^{-4} mm^3 were counted, compared to 80 cells in the same volume after 2 days of exposure. Rod-shaped bacteria appeared in chains indicating germination and outgrowth of spores. The fiber web remained devoid of microbial cells (Figure 5E). The mineral-coated paperboard was richer in bacteria after 7 days of exposure than the PE-coated paperboard with no mineral coating. Most probably this was due to the 10^3–10^4 times higher number of bacteria residing in the mineral-coated paperboard as compared to the PE-coated paperboard (Table 1). In spite of this, spread of the microbes was restricted to the coating layer with no penetration into the cellulose web.

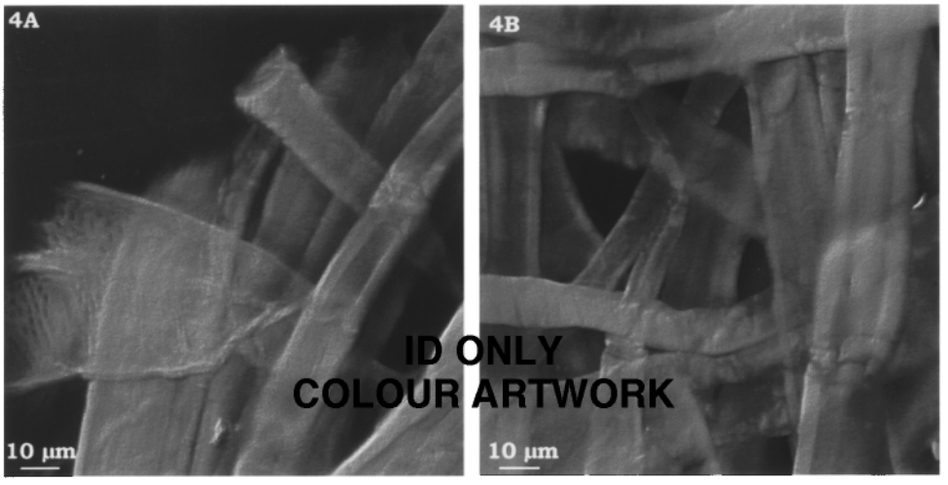

Figure 4 CLSM lateral composite images of the board layer of perforated, acridine orange-stained PE-coated liquid packaging board after long-term exposure to food and moisture. Panel (A) was taken after exposure for 30 days and panel (B) after 90 days of exposure to food and moisture at 28°C. The panels represent pile-ups of 20 (A) and 30 (B) lateral scans collected at 1-μm intervals, spanning a vertical distance (*z*-axis) of 20 μm and 30 μm respectively, through the paperboard distal to the PE layer.

High-density paper was exposed to food and moisture for 90 days. Before exposure (= day 0), no bacteria were seen inside the high-density paper (Figure 6A). After exposing the paper for 90 days, coccoid cells were detectable (Figure 6B), at a frequency of about 40 cells per 1.6×10^{-4} mm^3.

The response of a Biopol-coated paperboard to exposure of food and moisture was also studied. Before exposure (= day 0), no bacteria were seen in the interface between the Biopol coating and the fiber web (Figure 7A). After exposing the paperboard for 30 days to food and moisture, bacteria were observed beneath the Biopol layer facing the fiber web (Figure 7B), but not inside the fiber web (Figure 7C). This material thus responded similarly to the other food packaging paper(board)s.

To explain why no microbes were observed in the fiber web, the paperboards were tested for the presence of antimicrobial substances, with *Bacillus subtilis* DSM 347 as the test organism. The zone of inhibition to selected calibration antimicrobials and biocides by *B. subtilis* DSM 347 was used for quantitation. The zone for 5 μg of penicillin per disc was 14 mm, for 60 μg of chloramphenicol and for 80 μg of tetracycline 15 mm. The detection limit (inhibition zone $<$0.2 mm) for the mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one was lower than 0.0325 kg of active compound per ton paper. For 2,2-dibromo-3-nitrilopropionamide, the detection limit was between 0.07 and 0.18 kg of active compound per ton paper and for methylene bisthiocyanate between 0.06 and 0.15 kg of active compound per ton paper. The detection limit of the glutaraldehyde was close to 0.06 kg of active compound per ton paper.

Discs of the paperboards were tested under identical conditions and showed no (radius $<$0.2 mm) inhibition zone. The paperboards thus did not detectably inhibit growth of *B. subtilis* DSM 347. The mineral-coated paperboard gave a zone of growth stimulation with a radius of 1 mm. Since water is an essential element for bacterial growth, the sorbed water taken up by the paperboard was measured after exposure of 7 days. An average 0.7 g of water ($\pm$0.1 g) was found per g paperboard.

Discussion

The contents of viable bacteria in food packaging board was low ($<10^3$ CFU g^{-1}) excepting the mineral pigment-coating layer which sometimes may contain high bacterial counts ($>10^6$ g^{-1}, Table 1). In order to be able to estimate the risk of the microbes migrating into the packaged food, the spatial location and the ability of the microbes to migrate inside the paperboard has been determined. The bacteria were unevenly distributed and mainly localised in the interface area between the PE-coating layer and the cellulose fiber web. This is likely related to bacteria present in the starch used for surface sizing.

No microbial growth was observed in the fiber web when entry of food and water was offered to the bacteria present in the paper(board). The bacteria present in the interface between the fiber web and the PE-coating multiplied when given moisture and food, but did not migrate into the fiber layer. The majority of the bacteria isolated from the paper(boards) belonged to the genera *Bacillus* and *Paenibacillus*, similar to what has been described elsewhere [12,18,19]

The food-packaging paperboards and papers contained the essential growth substances in ratios similar to those of microbial cells, C: N: P of 50:10:1.5 [14]. The water-soluble carbon and the nutrients in the paper(board)s were too high to be growth-limiting. Possible carbon sources for bacteria in paper and paperboard are cellulose, starch and resins, while the fillers and mineral coating provide for minerals. *Bacilli* with cellulase and amylase activity have been found in food-packaging paper and board machines [17] and food-packaging paperboards [12,17]. This may also be the case with the paperboards studied here. Therefore, even without exposure of the perforated paper(board) to food there should have been sufficient substrate for microbial growth. The pHs of the different homogenised

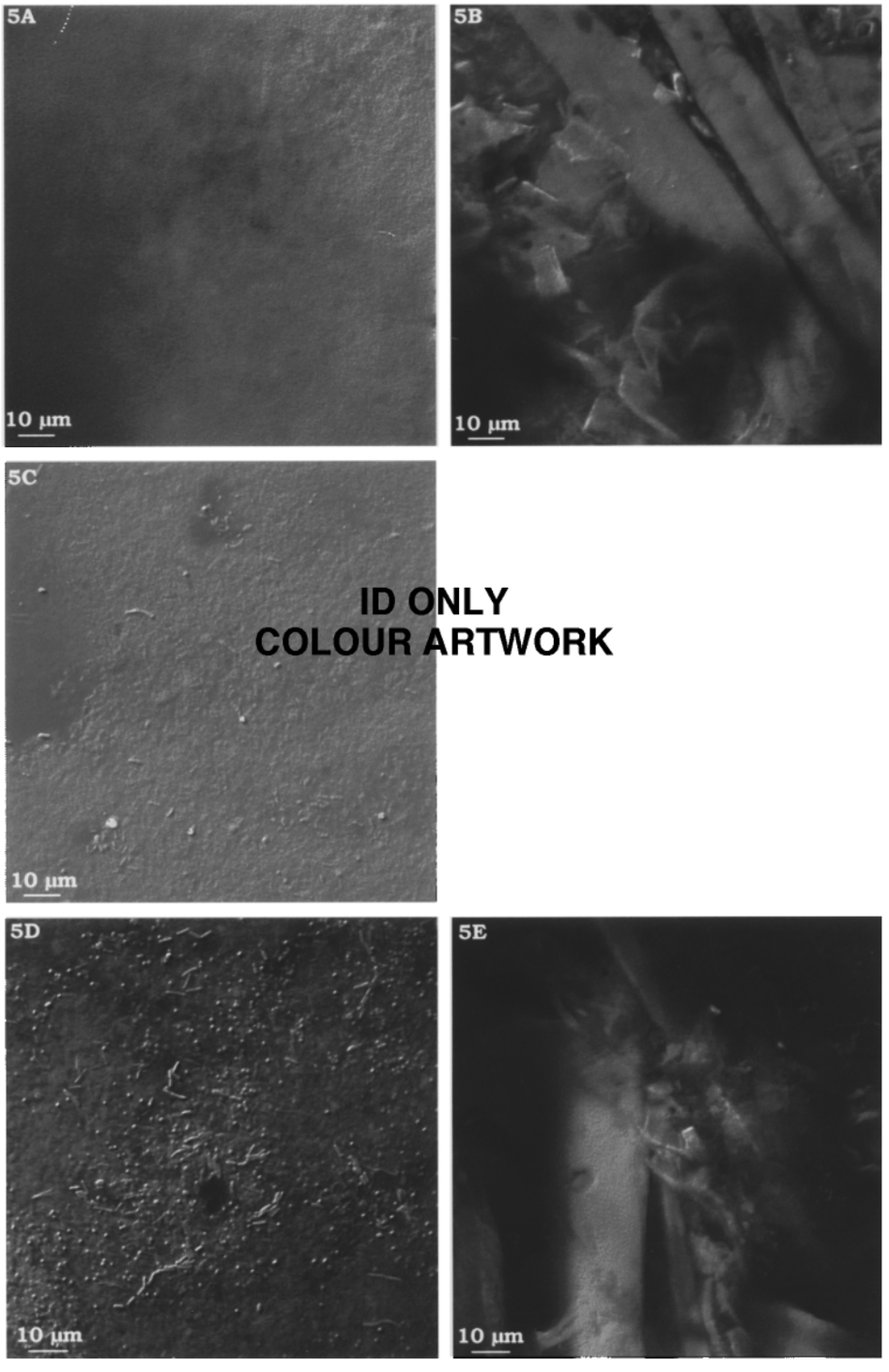

Figure 5 CLSM lateral composite images of acridine orange-stained mineral-coated paperboard. The panels show an area of 125 μm × 125 μm each. Panels (A) and (B) show the views obtained before the exposure and are composites of 20 lateral scans collected at 1-μm intervals. (A) mineral coating layer; (B) fiber layer. Panel (C) shows the coating layer after 2 days of exposure of the paperboard to food and moisture similarly to the coupons shown in Figures 2–4 and represents a pile-up of 10 lateral scans collected at 1-μm intervals. Panels (D) and (E) show the images obtained after 7 days of exposure to food and moisture and represent composite images of 10 lateral scans collected at 1-μm intervals. (D) mineral coating layer; (E) fiber web.

paper(board)s were between 5 and 9 which should be compatible with bacterial growth.

During paper production, biocides are used to control the growth of microorganisms in process water and raw materials [6]. The paperboards in this work were tested for residual antimicrobial activity with *Bacillus subtilis* DSM 347 as test organism. This standard organism for testing the presence of antimicrobial agents in food-packaging

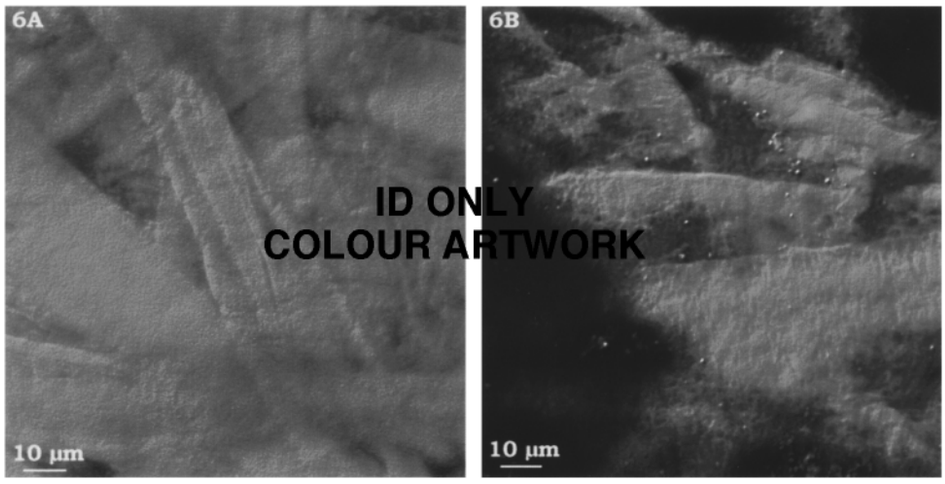

Figure 6 CLSM lateral composite images of high-density paper stained with aqueous acridine orange. Panels (A) and (B) represent a pile-up of 10 lateral scans collected at 1-μm intervals. Panel (A) shows the paper prior to any treatment and panel (B) after exposure to food and moisture for 90 days at 28°C.

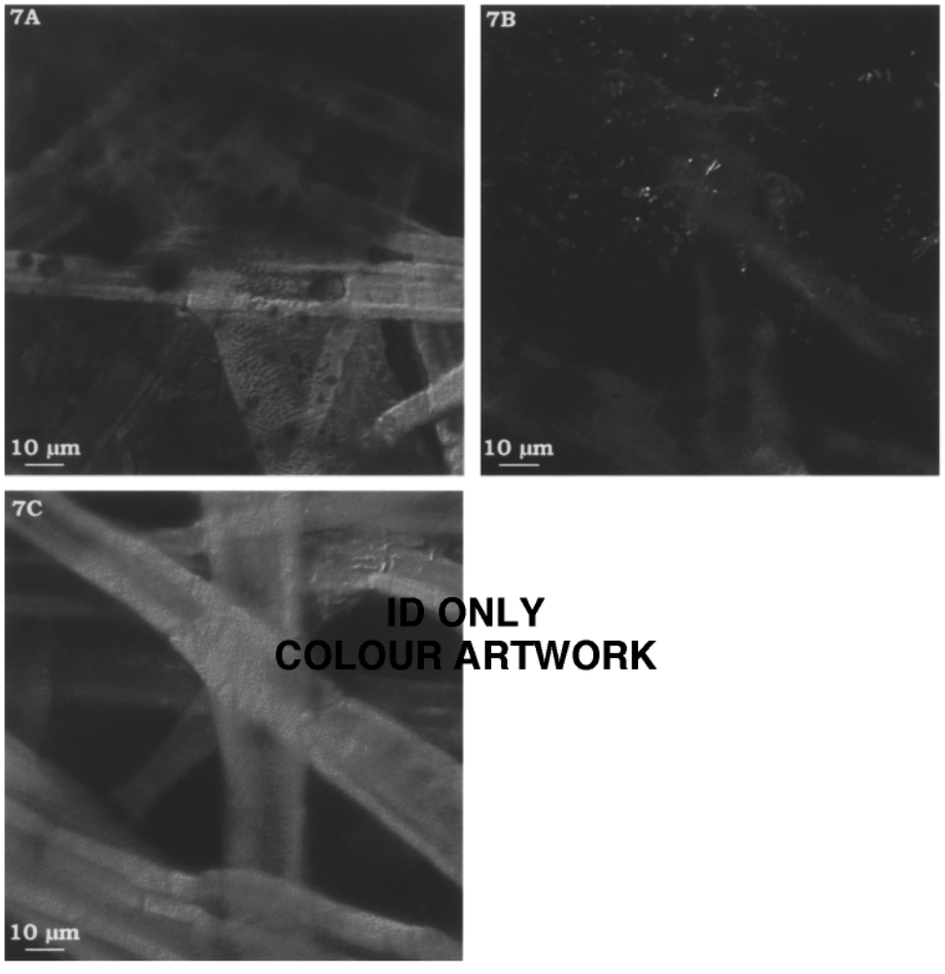

Figure 7 CLSM lateral composite images of Biopol-coated paperboard stained with aqueous acridine orange. Panels (A)–(C) represent a pile-up of 10 lateral scans each, collected at 1-μm intervals. Panel (A) shows the interface between the Biopol coating adjacent to the fiber web before exposure, panels (B) and (C) present images taken after exposure of the paperboard to food and moisture for 30 days at 28°C. (B) Biopol coating layer; (C) fiber web.

paper [3] is a relevant species because bacteria in paper(board) belong mainly to the genera *Bacilli* and *Paenibacilli* (Table 2) [12]. No antimicrobial effect was observed in the paperboards, indicating that the reason for limitation of microbial growth in the fiber web must be sought elsewhere.

Minimum water activity value for growth of bacteria is close to 0.9 [8]. The equilibrium of uptake of water by the paperboards after 7 days of exposure to water was about 0.7 g per g of paperboard. This is equivalent to 10% free intrafiber water and 0% interfiber water with the types of fiber studied (softwood and hardwood, bleached kraft pulp); >50% of free interfiber water has been shown to require water uptake of >2.5 g per g of paperboard [10]. In the experiments described in this paper the concentration of water accessible for bacteria inside the fiber web may have been limiting to bacterial growth. Inorganic materials such as the components of mineral-coating layer [15] bind less water than cellulose, leaving more of the water accessible for bacteria. This may explain the massive growth of bacteria in the mineral coating (Figure 5D) while no growth was observed at a distance of 10 μm inside the fiber web.

In pasteurised milk the level of psychrotrophic, spore-forming bacteria typically ranges from <0.5 to 300 spores per ml [11], 1.5×10^5 spores per litre of milk. A carton for 1 L of milk, weighing 30 g, would add ≤3000 CFU to the heterotrophic total viable count of the packaged food, if the paperboard content was 10^2 CFU per gram of paperboard. Therefore, with paperboard contents $<10^3$ CFU g^{-1}, the spore content of the milk is likely to exceed that of the package. Taking into account the localisation of most of the microbes in the paperboard, in the interface between coating material and the fiber web and the low penetration of microbes through the cellulose layer, the likely direction of migration of microbes may be from the coating layer towards the food. It is thus important that the coating layer facing food is intact. During transport of the food container the damage will occur on the outside of the food package, which is unlikely to have any impact on the food because of the high barrier capacity of the paperboard, ie the cellulose layer, demonstrated in this study, even during extended periods of storage (90 days studied here) and nonrefrigerated temperature, conditions which are of relevance for packaged UHT-treated products.

Our study shows that the only package-related threat to the hygiene of the paperboard-packaged food is presented by microbially contaminated starches used as surface sizers and mineral pigment coatings. The PE layers separating the food from the fiber layer and the starch or pigment, displayed indigenous damage and pores, allowing for penetration of micelles of lipophilic substances. In this paper we showed that an aqueous solution of acridine orange penetrated across the apparently intact PE-layer through micropores (1–3 μm).

Mineral pigment coating is sometimes applied also under the PE-covered surface facing food, but for food hygiene this is not recommendable. The use of Biopol, a biodegradable polymer, did not lead to increased microbial growth or migration as compared to polyethylene.

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