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Scanning Electron and Light Microscopic Study of Microbial Succession on Bethlehem St. Nectaire Cheese

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St. Nectaire cheese is a semisoft cheese of French origin that, along with Brie and Camembert cheeses, belongs to the class of surface mold-ripened cheese. The surface microorganisms that develop on the cheese rind during ripening impart a distinctive aroma and flavor to this class of cheese. We have documented the sequential appearance of microorganisms on the cheese rind and in the curd over a 60-day ripening period. Scanning electron microscopy was used to visualize the development of surface fungi and bacteria. Light microscopy of stained paraffin sections was used to study cross sections through the rind. We also monitored the development of bacterial and yeast populations in and the pH of the curd and rind. The earliest stage of ripening (0 to 2 days) is dominated by the lactic acid bacterium *Streptococcus cremoris* and multilateral budding yeasts, primarily *Debaryomyces* and *Torulopsis* species. *Geotrichum candidum* follows closely, and then zygomycetes of the genus *Mucor* develop at day 4 of ripening. At day 20, the deuteromycete *Trichothecium roseum* appears. From day 20 until the end of the ripening process, coryneforms of the genera *Brevibacterium* and *Arthrobacter* can be seen near the surface of the cheese rind among fungal hyphae and yeast cells.

St. Nectaire cheese is a pressed, uncooked, semisoft cheese that belongs to the class of surface mold-ripened cheese that includes Brie and Camembert. St. Nectaire cheese has been made for centuries in the Auvergne region of France and is considered among the best cheeses of France (16). The production of mold-ripened cheeses, formerly restricted to limited geographical areas, has spread to many countries because of the increasing consumer demand for specialty cheeses (8).

Texture and flavor development in mold-ripened cheeses depend on the biochemical activity of microbial populations that develop in and on the cheese during the ripening process (8). The yeasts associated with the ripening of St. Nectaire cheese have been identified as *Debaryomyces hansenii*, *Torulopsis sphaerica*, *T. candida*, *Kluyveromyces lactis*, *Candida sake*, *C. intermedia*, and *Yarrowia lipolytica*, with *D. hansenii* predominating (5, 6, 14, 17). The yeast-like fungus *Geotrichum candidum* has been credited with giving St. Nectaire cheese its grayish white crust while contributing lipolytic and proteolytic enzymes to the ripening process (5, 7–9). Other fungi that have been reported as associated with the surface ripening of St. Nectaire cheese are *Penicillium*, *Mucor*, *Cladosporium*, and *Aspergillus* spp. (6, 8).

Previous studies on microbial populations associated with the ripening of St. Nectaire cheese have relied on the isolation of individual microorganisms and identification by conventional diagnostic testing (1, 6, 9, 14, 17). Such an approach does not reveal the positions of populations on or in the cheese. Scanning electron microscopy (SEM) has been used to study microbial development in Camembert cheese and microstructural changes occurring in St. Paulin and Emmental cheeses during manufacture and ripening (11–13). Here we report on the use of SEM to study surface changes of Bethlehem St. Nectaire cheese and paraffin sectioning to study the subsurface portions of the cheese. These results, combined with the microbiological results, provide a comprehensive view of the development of microbial populations in the ripening of St. Nectaire cheese.

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MATERIALS AND METHODS

Cheese source. The Bethlehem St. Nectaire cheese used for this study was manufactured at the Benedictine Abbey of Regina Laudis in Bethlehem, Conn., using traditional techniques developed in the Auvergne. The cheese was made with unpasteurized bovine milk without the addition of a starter culture. The curd was pressed by hand into a wooden form and ripened naturally in a cheese cellar. At no point during the ripening process was the cheese inoculated with cultures of bacteria or fungi. Cheese produced in this manner resembles that produced in the Auvergne in the surface appearance, flavor, color, and texture of the finished product.

Collection and preparation of samples. Samples were obtained from cheeses at various stages of ripening. The study using light microscopy and SEM was done twice with different cheeses at each time point and yielded essentially the same results. Zero time is defined as the point at which the cheese curd was removed from the cheese press before being placed in the cellar. Samples (about 3 mm thick) of the rind were cut with a sterile scalpel. A sterile cork borer was used to cut samples (about 4 cm thick) from the interior of the cheese. The samples were transported on ice to the laboratory. Cheese samples were weighed, and each was homogenized in a 40-ml Dounce homogenizer. Dilutions made in sterile deionized water were plated in duplicate on peptonized-milk agar (pH 6.5), wort agar (pH 4.8), Sabouraud dextrose agar (pH 5.6), and tryptic soy agar-glucose (pH 7.3) (Difco, Detroit, Mich.) and incubated at 20°C. The pH of each diluted sample was determined, and a surface electrode was used to record the pH of the cheese surface at each time point.

Diagnostic tests. Identification of bacteria, yeasts, and fungi was done by standard methods (2, 10, 15, 18, 19). Representative isolated bacteria were Gram stained and

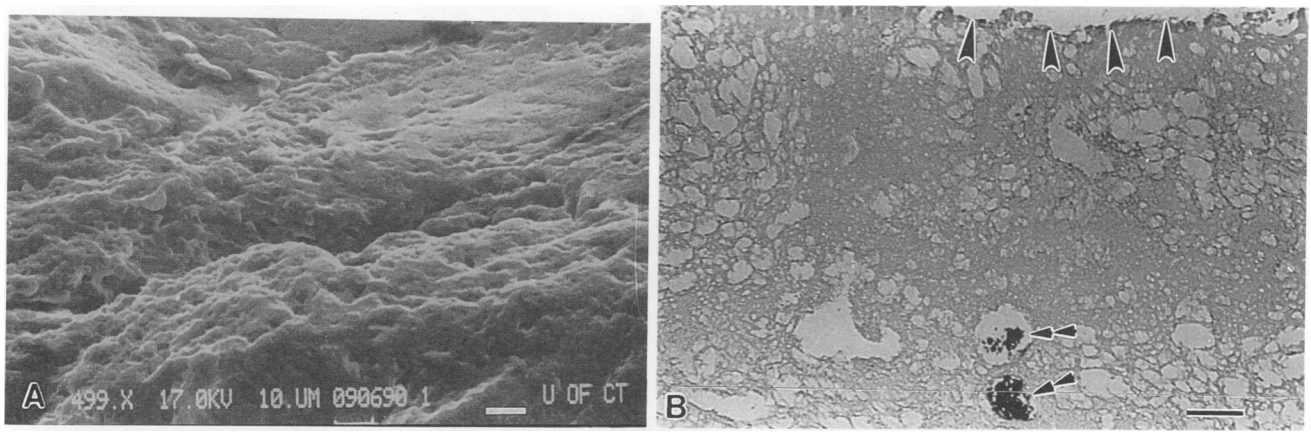

FIG. 1. SEM and light microscopy micrographs of Bethlehem St. Nectaire cheese at day 0 of ripening time. (A) SEM of cheese showing crevices in the surface, with no obvious microbial growth. Bar, 10 μm. (B) Light microscopy of a stained paraffin section. There is no microbial growth on the cheese surface (single arrowheads), and a rind has not yet formed on the cheese. Two isolated pockets (double arrowheads) of lactic acid bacteria are visible within the cheese curd. Bar, 50 μm.

submitted to the following tests: oxidase; nitrate reduction; carbohydrate fermentations; catalase; and casein, starch, and gelatin hydrolysis. An arginine dihydrolase test was also done on the lactic acid bacteria. Yeasts and filamentous fungi were identified primarily by morphological criteria after isolation from cheeses.

SEM. Rind samples (1 to 2 cm by 3 mm) were prepared for SEM by being treated with 2% OsO_4 vapors in a sealed chamber for 24 h and then trimmed to a 1-mm thickness. The samples were washed briefly in toluene to dissolve surface lipids and then dried in a desiccating chamber overnight over P_2O_5. The dried samples were fastened to aluminum stubs, brushed with conductive paint on the sides and bottoms, and coated with gold in a sputter coater (Polaron sputter coater, model E5100, series II). The coated samples were stored in a vacuum desiccator until viewed by SEM (Coates and Welter field-emission scanning microscope, model HPS 50B).

Paraffin sectioning. For light microscopy, samples were cut into 5-mm-thick sections, immersed in alcoholic formalin for 4 h, and placed in an automatic tissue processor (model LX120; Fisher Scientific Co., Springfield, N.J.). In the tissue processor, the samples were washed with an alcohol substitute (Prosoft) to remove water and Propar (Analtech Ltd., Battle Creek, Mich.), a toluene substitute, to dissolve lipids. The samples were then immersed in three changes of Paraplast X-tra tissue embedding medium (Oxford LabWare, Div. of Sherwood Medical, St. Louis, Mo.), alternated with vacuum treatments to infiltrate paraffin into the samples. The samples were removed from the tissue processor and embedded in Paraplast X-tra. Five-micrometer-thick sections were cut, floated on a sodium silicate solution, and picked up on slides. The slides were dried, paraffin was removed in three changes of xylene, and the slides were washed in three changes of isopropyl alcohol. The slides were then washed with deionized water and stained with Brown-Hopps stain, which yields results similar to those provided by the Gram stain (3).

RESULTS AND DISCUSSION

Macroscopic appearance. The appearance and taste of St. Nectaire cheese change dramatically during ripening, especially within the first few days. At zero time, when the cheese is removed from the cheese press, the surface appears moist and pale yellow, with no apparent microbial growth. At this point, a well-defined rind has not yet formed. The curd of the cheese looks white and has a uniform rubbery consistency. By day 4 of ripening, a white crust can be seen on the cheese surface. By day 6, the crust is covered by a fungal coat of "white hair." The surface takes on a grayish brown velvety appearance by day 9, and a visible rind has formed beneath the fungal coat. In the interior, the curd is pale yellow and has a softer consistency than at zero time. At about days 25 to 30, patches of pink powder can be seen on top of the grayish brown coat. From this point on until the end of the 60-day ripening period, the surface of the cheese becomes increasingly drier and the hardened rind underneath turns deep yellow and orange. The curd becomes softer and creamier, turning deeper yellow from the outside in, as ripening continues.

SEM and microtome cross sectioning. Figures 1 to 4 show that the microbial populations develop very early in the ripening process, with changes occurring at a rapid pace during the first week.

Figure 1 shows the surface of the cheese and a cross section through the surface at day 0 of ripening. At this point, the pressed, salted, and washed curd has been in the cheese press for about 24 h. The surface appears barren, showing only crevices within the otherwise smooth surface, with no evidence of microbial growth. Below the surface (Fig. 1B), isolated pockets containing gram-positive cocci can be seen; their presence indicates that bacterial fermentation has already begun within the curd. Each pocket containing microorganisms was probably derived from the growth of a single cell, since more than one morphological type was not seen in a pocket. Throughout the ripening process, bacteria and some yeasts were seen in discrete pockets within the curd, while filamentous fungi were not observed within the curd.

By day 2 (Fig. 2), the surface is covered with a lawn of multilateral budding yeasts embedded within the cheese rind. We have identified the yeasts as primarily *Debaryomyces* and *Torulopsis* species on the basis of cell and spore morphology and fermentation and nitrate reduction tests. This identification of yeasts on Bethlehem St. Nectaire

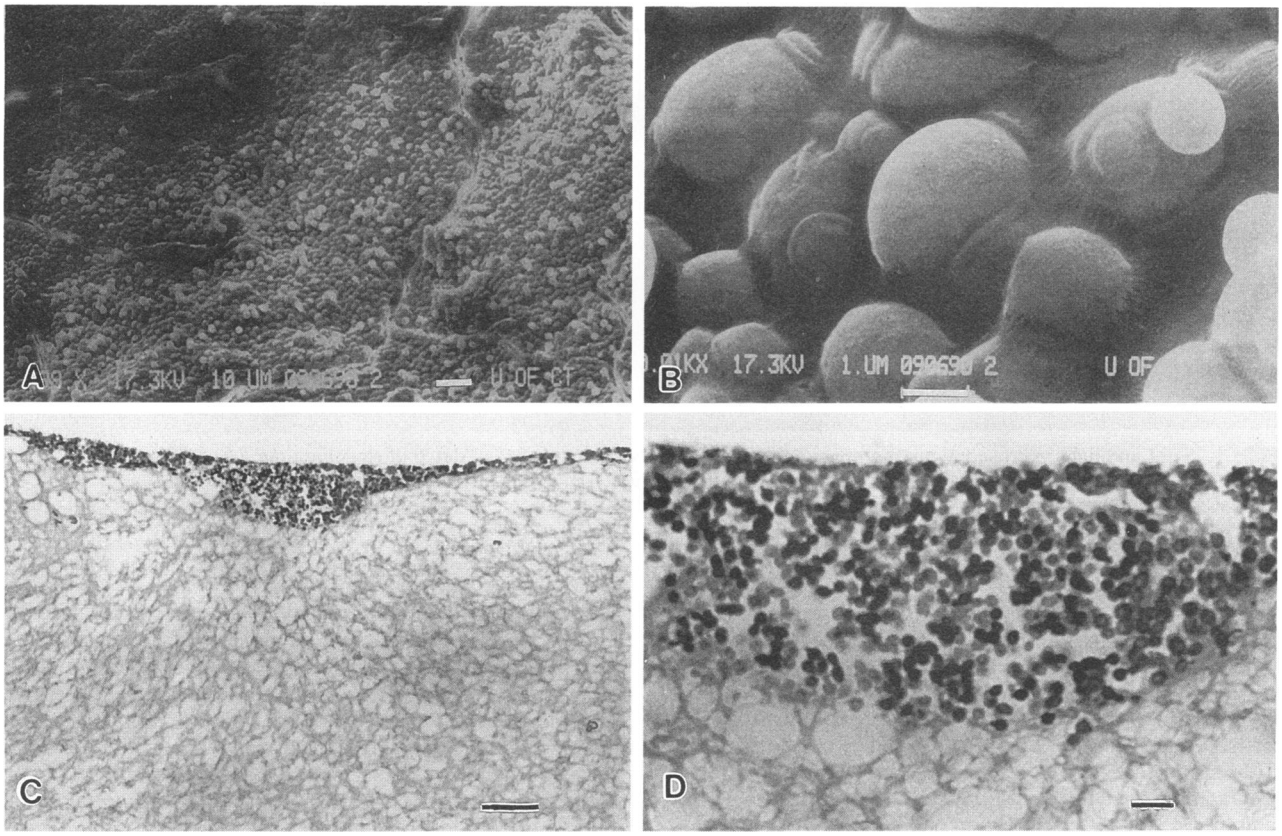

FIG. 2. SEM and light microscopy micrographs of cheese after 1 to 2 days of ripening in the cheese cellar. (A) SEM micrograph of cheese at day 2 of ripening showing a lawn of yeasts covering the surface. Bar, 10 μm. (B) Higher-magnification SEM micrograph showing multilateral budding yeasts embedded within the cheese surface. Buds and bud scars are clearly visible. Bar, 1 μm. (C) Light microscopy micrograph of a stained paraffin section showing yeasts near the cheese surface. No microbial growth can be seen in the cheese curd. Bar, 50 μm. (D) Higher-power light microscopy micrograph of yeast cells forming the nascent cheese rind. Bar, 50 μm.

cheese is consistent with previous work (6, 17). The *Torulopsis* strain predominating in the interior is a lactose fermenter. The action of lactose removal by lactose-fermenting strains has been described in cheeses, such as St. Nectaire, that are not very acidic (7).

At day 2 of ripening, the number and size of the pockets of microbial colonies within the curd have increased since day 0 of ripening (Fig. 3A). Pockets of yeasts (Fig. 3B) are scattered sporadically among pockets of lactic acid bacteria, which predominate (Fig. 3C). *Streptococcus cremoris* was consistently identified as the major lactic acid bacterium within Bethlehem St. Nectaire cheese.

By day 6, the rind has developed a white hairy appearance that is conferred by the hyphae of zygomycetes of the genus *Mucor* (Fig. 4A). By day 9, the *Mucor* sporangiospores have been released and can be seen dispersed among the collapsed hyphae of the fungus (Fig. 4B). The mass of released spores and hyphae covers the cheese with a grayish brown velvety coat. The identification of *Mucor* organisms was based on sporangial morphology and the appearance of sporangial columella, as viewed in cross sections in light micrographs (data not shown).

Also at day 9, among the *Mucor* hyphae, larger collapsed angular hyphae measuring about 10 μm in diameter can be seen, suggesting that *G. candidum* develops at about the same time as but beneath the *Mucor* hyphae (Fig. 4B). This observation is supported by Fig. 4C, which shows at day 7 vertical channels in the rind created by the broad vegetative hyphae of *G. candidum* developing beneath the *Mucor* aerial hyphae. By day 7, the lawn of yeasts that was seen on the surface of the cheese at day 2 has grown thicker, as the *Mucor* and *G. candidum* hyphae have "tilled" the surface. Yeasts can be seen surrounding the channels created by the vegetative hyphae of *G. candidum* (Fig. 4C). The identification of *G. candidum* on Bethlehem St. Nectaire cheese was confirmed on the basis of direct isolation of the fungus from the cheese rind on day 4 and viewing of arthrospore development by light microscopy.

G. candidum is typically found on pressed cheeses and is known to impart a white crusty appearance to the surface (5, 9). Specifying the times of appearance of *G. candidum* is difficult because of the complexity of fungal growth on surface-ripened cheeses after only 1 week of ripening. Rousseau cites a similar phenomenon in specifying the time of appearance of *G. candidum* during the ripening of Camembert cheese (11). The fungus was first seen in Camembert cheese on days 5 to 6 of ripening but was not visible on the cheese surface during week 3 of ripening. However, cross sections of Camembert cheese rind observed by SEM during week 4 showed superimposed layers of microorganisms. *Geotrichum* arthrospores were observed interdispersed with degenerating *Penicillium* conidiophores just above a yeast layer, and *Penicillium* hyphae were closest to the outer surface of the cheese. *G. candidum* reappeared at the

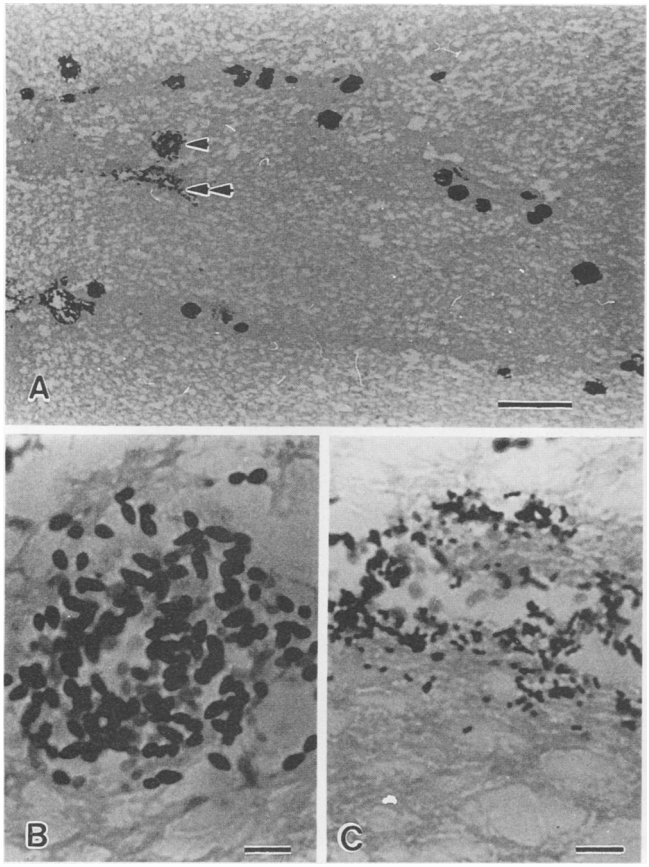

FIG. 3. Light micrographs of the cheese curd at day 2 of ripening. (A) Pockets of microorganisms can be seen within the curd. Only one morphological type can be found within each isolated pocket. Bar, 50 μm. (B) Higher magnification of a pocket (arrowhead) showing yeasts. Bar, 5 μm. (C) Higher magnification of a pocket (double arrowheads) showing cocci. Bar, 5 μm.

surface during later stages of ripening. In like manner, cross sections of Bethlehem St. Nectaire cheese show that *Mucor* hyphae on the outer edge of the rind have grown over a layer of yeast and *G. candidum* during week 1 of ripening (Fig. 4C).

At day 31 of ripening, two-celled fungal conidiospores are observed on the cheese surface (Fig. 5A). The spores measure 10 to 15 μm in length and about 5 μm in width, in contrast to the *Mucor* spores, which measure about 5 μm in diameter. The conidiospores develop from the tip of a conidiophore and are diagnostic of the deuteromycete *Trichothecium roseum*. The identification of the fungus as *T. roseum* was confirmed by isolation of the fungus and observation of condiospore development as short chains in basipetal succession from the conidiophore. Patches of *T. roseum* can be seen as early as day 13, appearing as a pink powder on top of the grayish brown coat of *Mucor* spores and collapsed hyphae. The combination of *T. roseum* and *Mucor* organisms in the surface microbiota may be responsible for the violet-colored rind of St. Nectaire cheese made in the Auvergne. We have isolated *G. candidum* and *Mucor* strains and have microscopically observed *Trichothecium* conidiospores on the surface of authentic St. Nectaire cheese imported from France.

Final stages of ripening. From days 30 to 62, few macroscopic changes occur on the cheese rind. The rind is covered by a mass of fungal debris that imparts a dry, dusty appearance. However, a complex population of bacteria has developed near the cheese surface. Figure 5B shows coryneform bacteria, with their distinctive V shapes. *Brevibacterium* and *Arthrobacter* spp., identified mainly on the basis of cell morphology and colony color, have been isolated from Bethlehem St. Nectaire cheese and generally appear at about 20 days of ripening. Cocci present in the rind, other than the dominant streptococci, have been identified as two *Micrococcus* strains.

Figure 6 shows the orientations of the major microbial populations within 37-day-old ripened cheese. A *Mucor* sporangium and aerial mycelia can be seen on the outer surface. The spores and collapsed *Mucor* hyphae overlay a layer of yeast and bacterial colonies that have entered deeper into the cheese rind because of the tilling action of the fungi. The vegetative hyphae of the fungi extend into the rind as far as the division between the rind and the curd but not into the curd. The identities of the fungi that participate in the formation of the dense mycelia associated with the deeper regions of the rind (region c of Fig. 6) are not known because of a lack of obvious reproductive structures. Isolated pockets of bacteria and occasionally yeasts can be seen throughout the rind and the curd. The border between the cheese rind and curd is now well defined (between regions c and d of Fig. 6). The demarcation of the rind is probably a consequence of the aerobic nature of the organisms that participate in rind formation. Thus, rind formation is primarily due to the slow but continuous penetration of fungal hyphae into the cheese curd over time.

Enumeration of lactic acid bacteria and yeasts. Figure 7 shows that the number of lactic acid bacteria remains relatively constant throughout the entire ripening process in both the curd and the rind. The main lactic acid bacterium isolated from Bethlehem St. Nectaire cheese is *S. cremoris* which, with *S. lactis* and *S. lactis* subsp. *diacetylactis*, is known to dominate populations of cheese-ripening microorganisms in soft and uncooked hard cheeses (5). The source of the streptococci is the unpasteurized milk used to make the cheese.

The number of yeasts in the rind increases 40-fold by day 4 and then stabilizes from days 10 to 60 (Fig. 7A). A similar increase during early ripening is seen on other surface-ripened cheeses and may be due to the metabolism of lactic acid produced by the lactic acid bacteria in the curd (8). In the curd, the yeast colony count remains low and fairly stable throughout the ripening process (Fig. 7B). This profile is very similar to the results obtained in the study of Camembert cheese (5).

pH. Although St. Nectaire and Camembert cheeses have been noted to have similarities in the microbial populations involved in ripening, there is a contrast in the pH profiles for the two cheeses. Figure 8 shows that the pH of Bethlehem St. Nectaire cheese inside and on the rind remains relatively constant throughout the ripening process. In contrast, Camembert cheese begins with an acidic interior pH that increases gradually over 35 days and an initial acidic exterior pH that nears neutrality by day 18. The increase in pH is responsible for the softening of the curd of Camembert cheese (8). The difference in pHs for Camembert and St. Nectaire cheeses is most likely due to the contrasting techniques of production of each type of cheese, which affect the level of lactose present in the finished cheese. The early stages of production of St. Nectaire cheese involve the

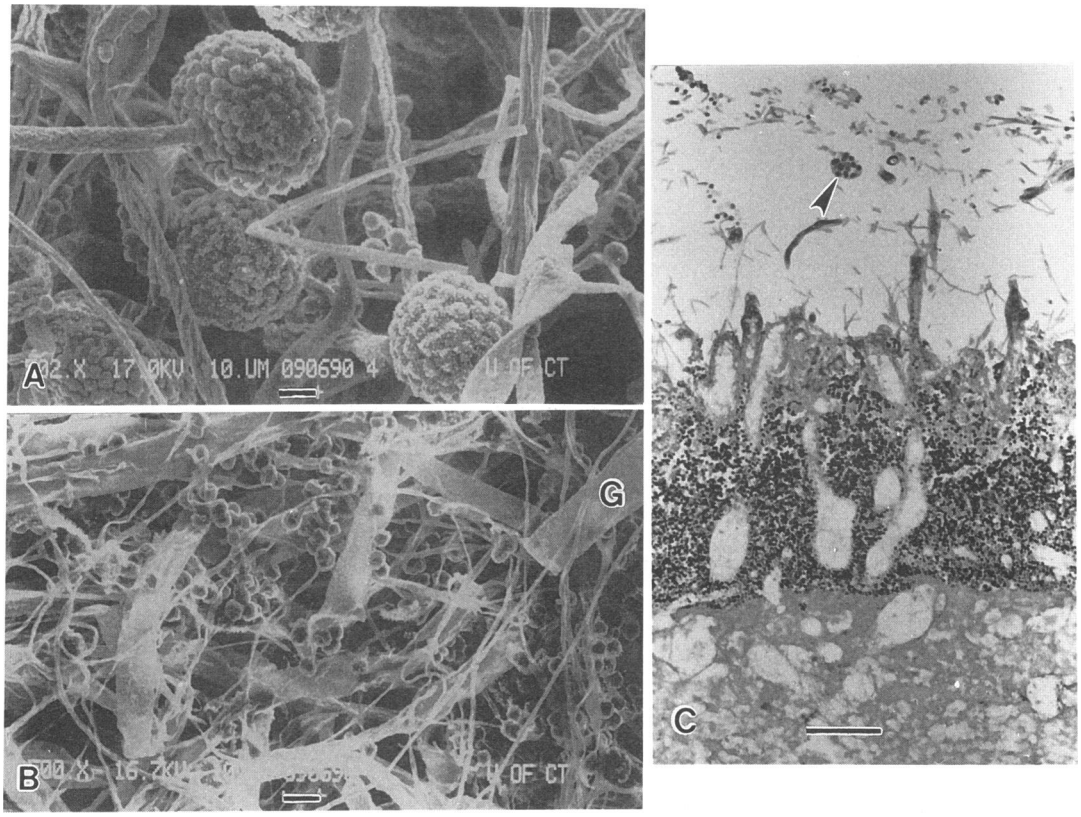

FIG. 4. SEM and light microscopy micrographs of cheese at 6 to 9 days of ripening. (A) SEM micrograph of the cheese surface at day 6 showing sporangia and hyphae of zygomycetes of the genus *Mucor*. Bar, 10 μm. (B) SEM micrograph of the cheese surface at day 9 showing released *Mucor* spores dispersed among collapsed hyphae. The *Mucor* spores measure about 5 μm in diameter. Vegetative hyphae of *G. candidum* (G), measuring 10 μm in diameter, can be seen among the thinner *Mucor* hyphae. Bar, 10 μm. (C) Light micrograph of a stained paraffin section at day 7 showing the progression of microorganisms from the cheese surface to the curd. A sporangium (arrowhead) and aerial mycelia of *Mucor* organisms can be seen on the outer surface of the cheese. The yeasts and bacteria have grown into channels of the cheese formed by *G. candidum* and *Mucor* hyphae. Below this region is a portion of the developing rind, beginning to be invaded by fungal hyphae but uninhabited by yeasts and bacteria. At this stage, the rind thickness measures 400 to 500 μm. Bar, 50 μm.

washing and forced drainage of the curd. It has been observed that after 4 to 5 h, no lactose remains in highly pressed cheeses (4). In cheeses that are not highly pressed, such as Camembert cheese, lactose remains and can cause postacidification of the curd through fermentation by lactic acid bacteria. Lactose has been found in Camembert cheese, in the rind on day 15 and in the interior on day 25 (4). The neutral exterior pH of St. Nectaire cheese (Fig. 8) is proba-

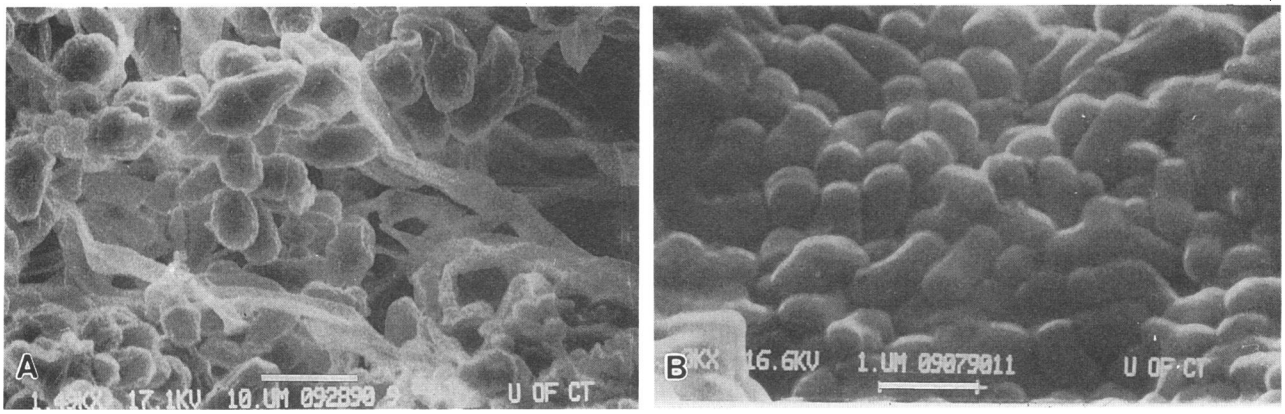

FIG. 5. SEM micrographs of the cheese surface during the later stages of ripening. (A) The two-celled fungal conidiospores of the deuteromycete *T. roseum* can be seen on the cheese surface at day 31. Bar, 10 μm. (B) At day 59, the V shapes of coryneform bacteria can be distinguished at a high magnification beneath a mass of fungal debris on the cheese rind. Bar, 1 μm.

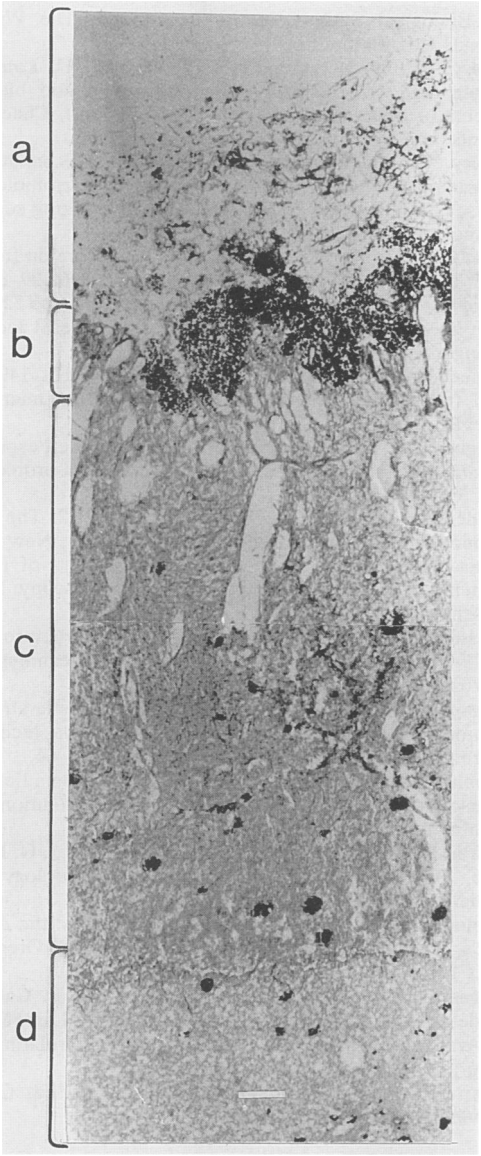

FIG. 6. Light micrograph of a stained paraffin section taken from the outer surface to the curd of 37-day-old ripened cheese. The positions of microbial populations within the cheese environment can be clearly seen. Fungal spores and collapsed hyphae (a) cover a layer of yeast and bacterial colonies (b). Dense fungal mycelia (c) can be seen throughout the rind. A clear demarcation between the rind and the curd (d) can be distinguished. The fungal hyphae and channels do not extend into the curd. Discrete pockets of lactic acid bacteria and yeasts can be seen throughout the rind and the curd. The rind measures 1.5 mm thick. Bar, 100 μm.

bly due to the metabolism of lactic acid by the fungi that develop sequentially on the rind (5, 8).

Conclusions. The ripening of Bethlehem St. Nectaire cheese results from a complex pattern of development of bacteria and fungi leading to curd modification and rind thickening (Fig. 9) over time. In the beginning, the cheese surface is essentially devoid of microorganisms and a rind has not formed. Within the first 4 days, microorganisms grow rapidly on the surface, creating a layer of filamentous fungi and yeasts measuring from 250 to 450 μm thick, not including aerial mycelia. From 4 days to 2 months, the rind

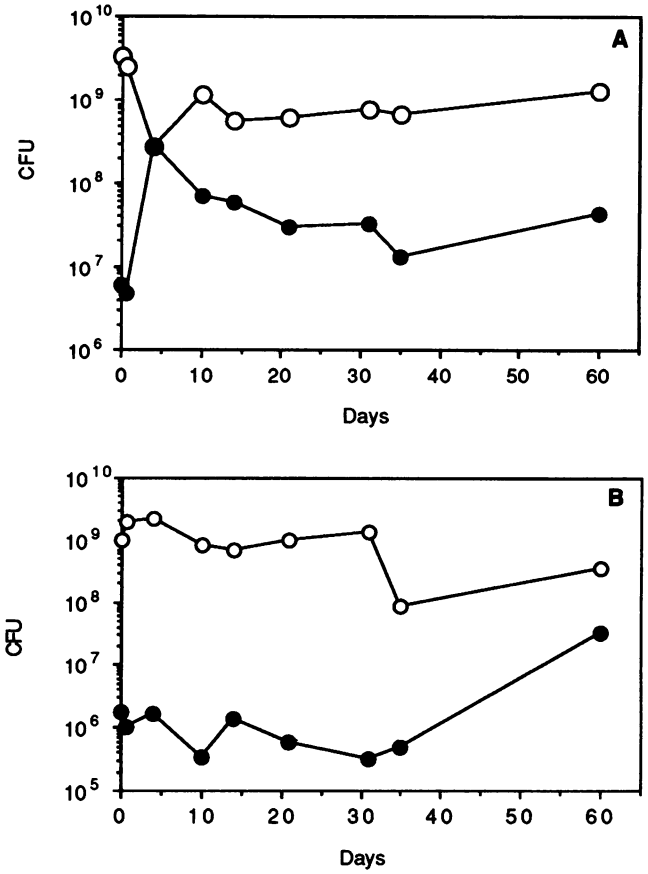

FIG. 7. Bacterial (lactic acid bacteria [○]) and yeast (●) populations in the rind (A) and the curd (B) during the ripening of Bethlehem St. Nectaire cheese.

gradually thickens to 2 to 3 mm as fungal hyphae penetrate into the curd (Fig. 9).

Since the cheese used in this study was made with raw milk and aged in the absence of inoculation, it was an unusual model for the study of a reproducible microbial

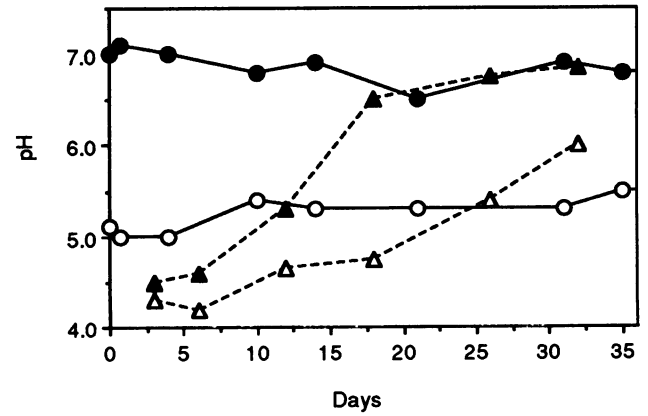

FIG. 8. Comparison of the pHs of the rind and the interior of Bethlehem St. Nectaire and Camembert cheeses over ripening time (data for Camembert cheese are from reference 4). Symbols: ●, St. Nectaire cheese rind; ○, St. Nectaire cheese interior; ▲, Camembert cheese rind; △, Camembert cheese interior.

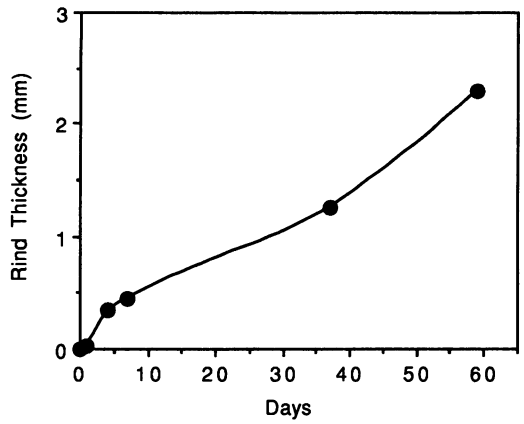

FIG. 9. Rind thickness of Bethlehem St. Nectaire cheese during ripening. The rind thickness was measured on stained sections taken at the times indicated.

succession in a natural environment. The study raises the question as to why each successive microbial population invades the previous one and invites further study of the biochemical interactions among the microorganisms within the cheese environment.

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