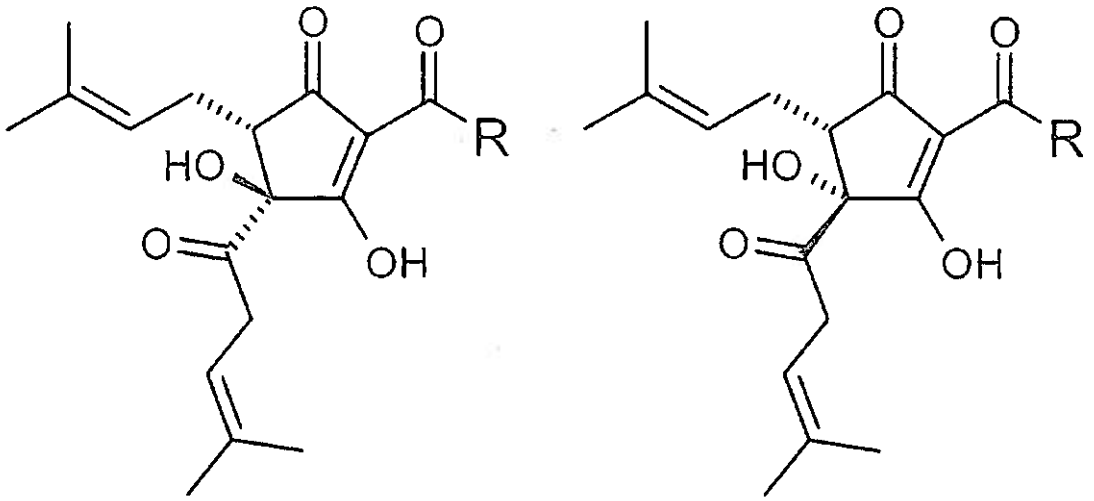
A **B**



R	Name
$-(CH)_2CH(CH_3)_2$	isohumulone
$-CH(CH_3)_2$	cohumulone
$-CH(CH_3)CH_2CH_3$	adhumulone

Fig. 1-1 Chemical structure of iso-α-acid.
The bittering compounds of beer were found to comprise iso-α-acids, which inhibit growth of Gram-positive bacteria but not of Gram-negative bacteria. A: trans-isohumulones, B: cis-isohumlones.

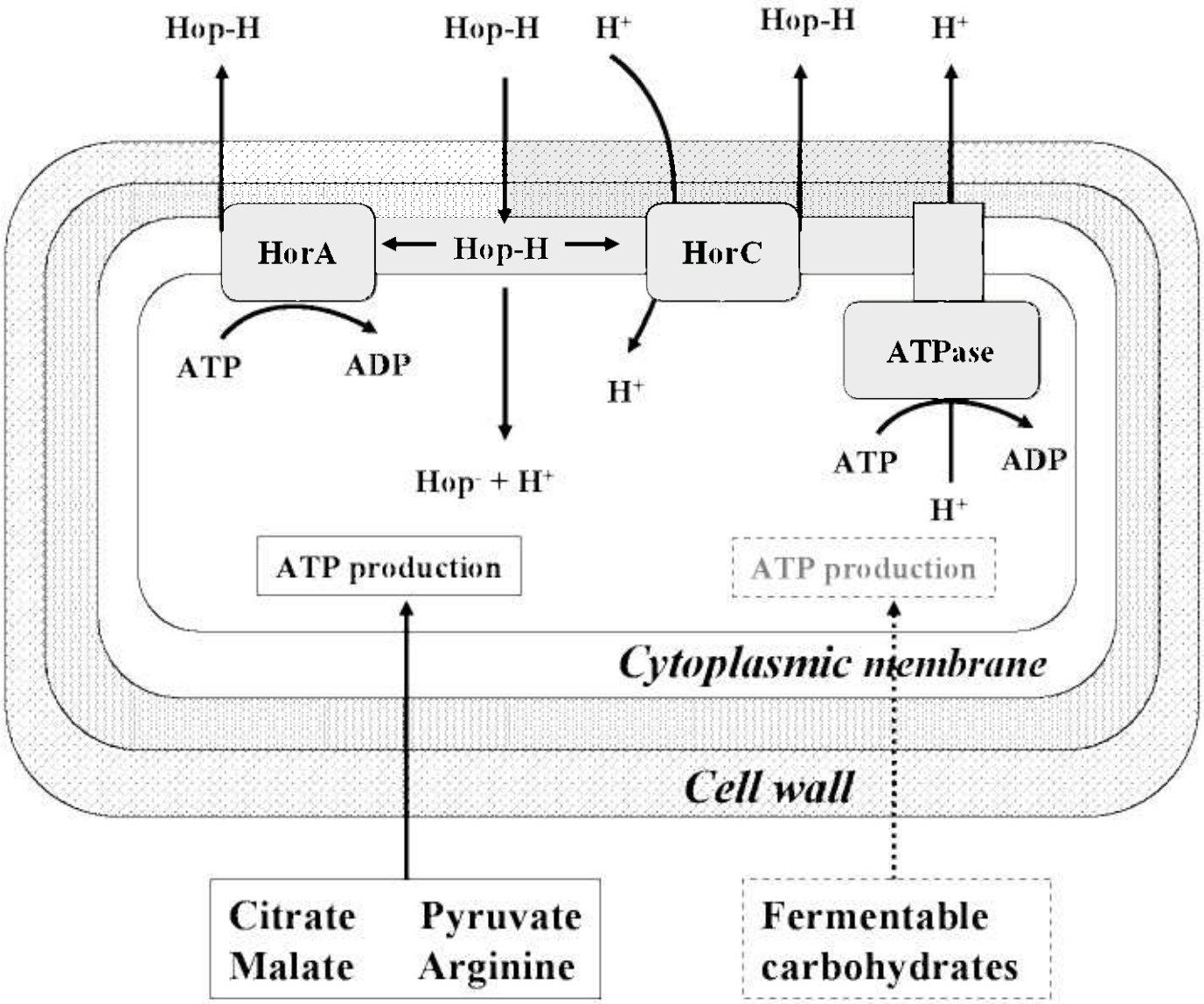


Fig. 1-2 The proposed mechanisms of hop resistance in beer spoilage LAB
HorA and HorC are multidrug transporters that are driven by ATP and PMF respectively. The undissociated hop compounds (Hop-H) intercalate into the cytoplasmic membrane and are pumped out by the multidrug transporters. Some portions of Hop-H escape the pumping activities of the transporters and enter the cytoplasm. In the cytoplasm, Hop-H dissociates into the anionic form (Hop^-) and H^+ due to the higher internal pH. H^+ also enters the cytoplasm in antiport with Hop-H by the action of HorC. To prevent the acidification of the cytoplasm and maintain the transmembrane pH gradient, proton translocating ATPase excretes H^+ across the membrane. The energy sources for these hop-resistance mechanisms are supplied from the metabolisms of citrate, pyruvate, malate and arginine. In some cases, fermentable carbohydrates, such as maltotriose, may be utilized to produce ATP (Suzuki 2008)

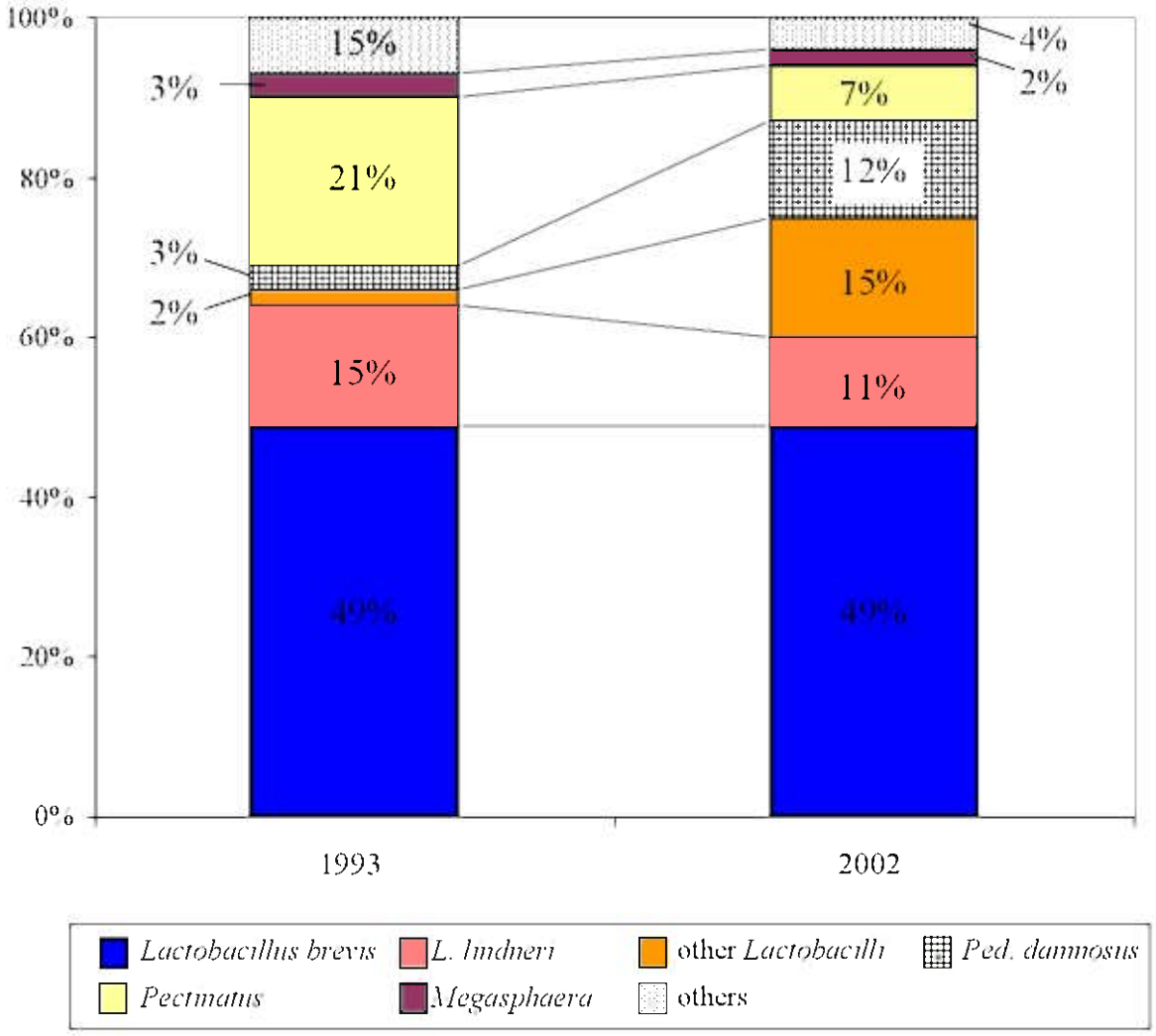


Fig. 1-3 Percentages of beer-spoilage microorganisms in incidents reported in 1993 and 2002 (Back 1994, 2003).

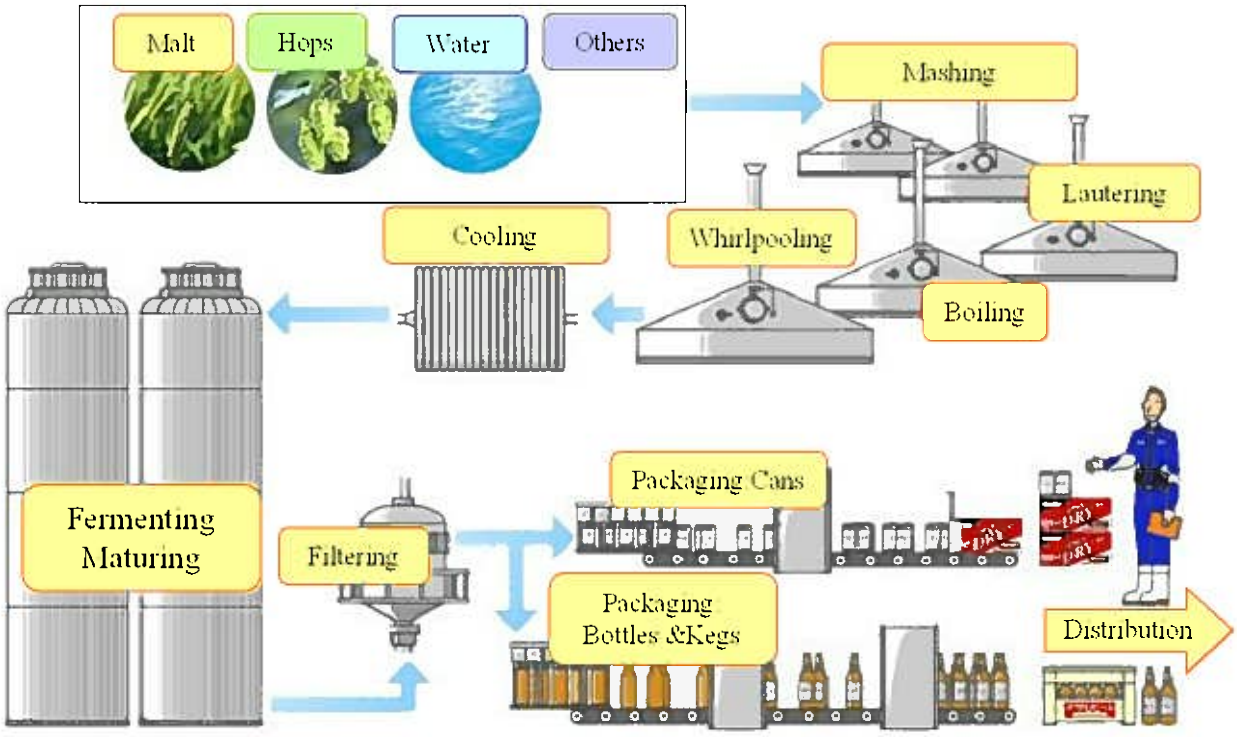


Fig. 1-4 Overview of the brewing process of beer.
(edited from http://www.asahibeer.co.jp)

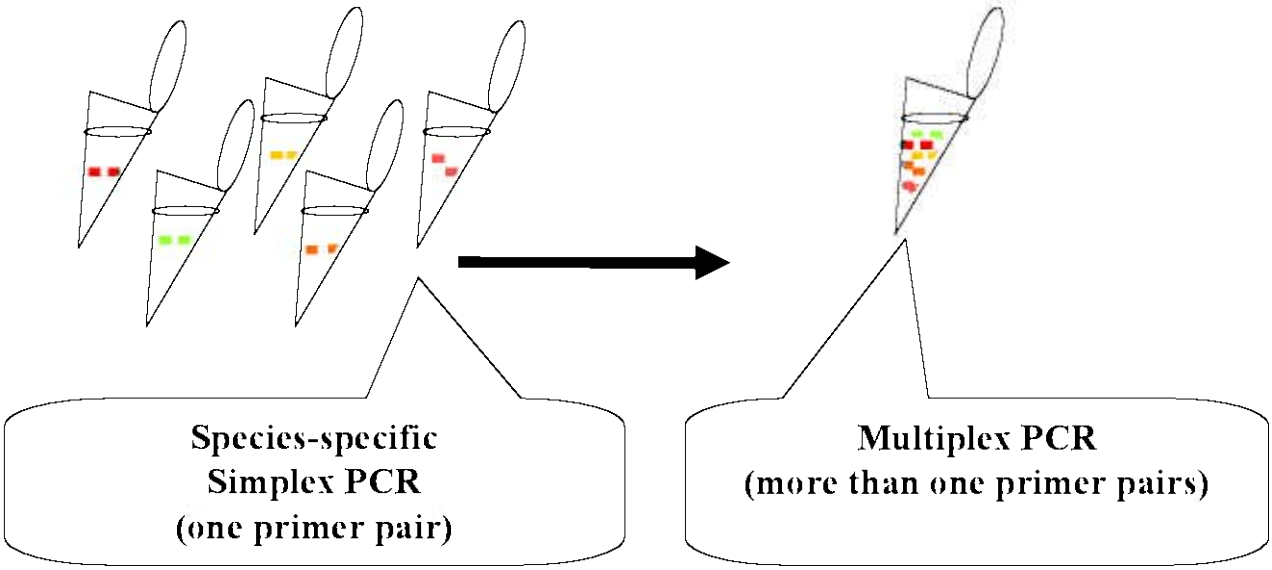


Fig. 2-1 Multiplex PCR method
A species-specific simplex PCR usually contains one pair of primers: a forward primer and a reverse primer. In multiplex PCR more than one pair of primers are included and multiple target sequences can be amplified

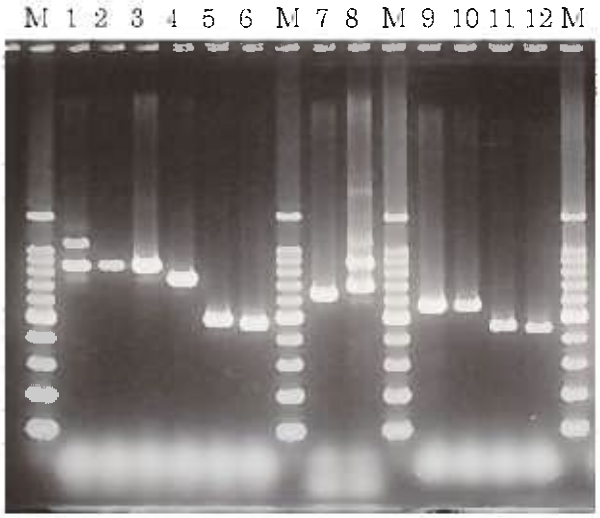


Fig. 2-2 PCR bands of multiplex PCR methods with corresponding target species as positive controls
Three multiplex methods cover 12 beer-spoilage species of bacteria. Extracted DNAs from reference strains belonging to each target species were subjected to L multiplex (lane 1-6), P multiplex (lane 7and 8) and C multiplex (lane 9-12). Lane 1 *Lactobacillus lindneri*, lane 2 *L. brevis*, lane 3 *L. paracollinoides*, lane 4 *L. casei*, lane 5 *L. plantarum*, lane 6 *L. coryniformis*, lane 7 *Pectinatus cerevisiiphilus*, lane 8 *Pect. frisingensis*, lane 9 *Pediococcus damnosus*, lane 10 *Ped. inopinatus*, lane 11 *Ped. claussenii*, lane 12 *Megasphaera cerevisiae*

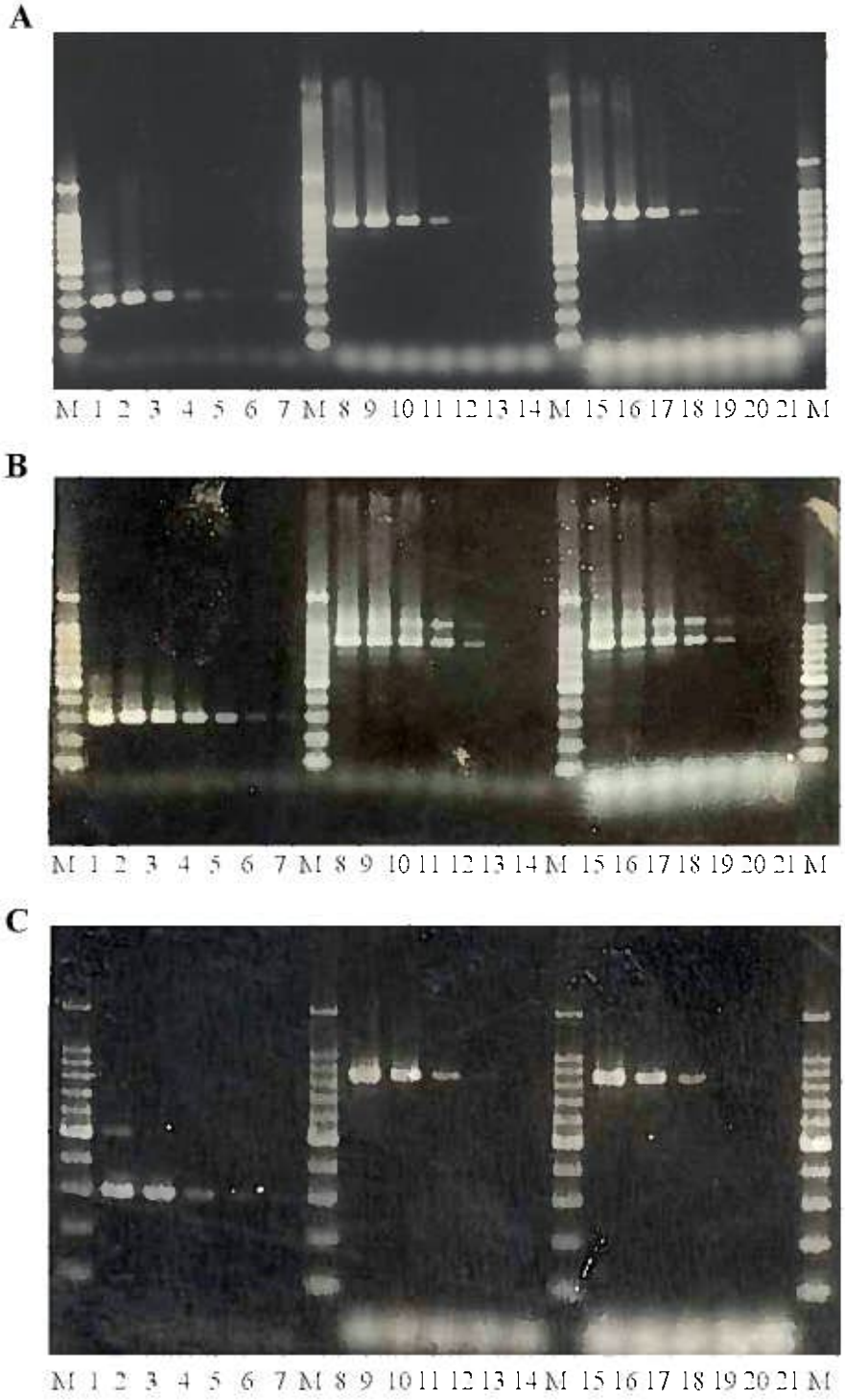


Fig. 2-3. Comparison of sensitivity between L. multiplex and simplex PCR with *Lactobacillus brevis* (A), *L. lindneri* (B) and *L. paracollinoides* (C).
A series of ten-fold dilutions of DNA extraction from relevent species were tested for detection limits, using universal primer pair (lane 1-7), relevent species-specific simplex primer pair (lane 8-14) and L. multiplex primer mixes (15-21). Lane 1-6, lane 8-13 and lane 15-20 represent DNA extracts containing from 10^6 cells to 10^1 cells of each strain in decimally decreasing orders, lane 7, 14 and 21 sterile distilled water (negative controls), lane M 100 bp DNA ladder.

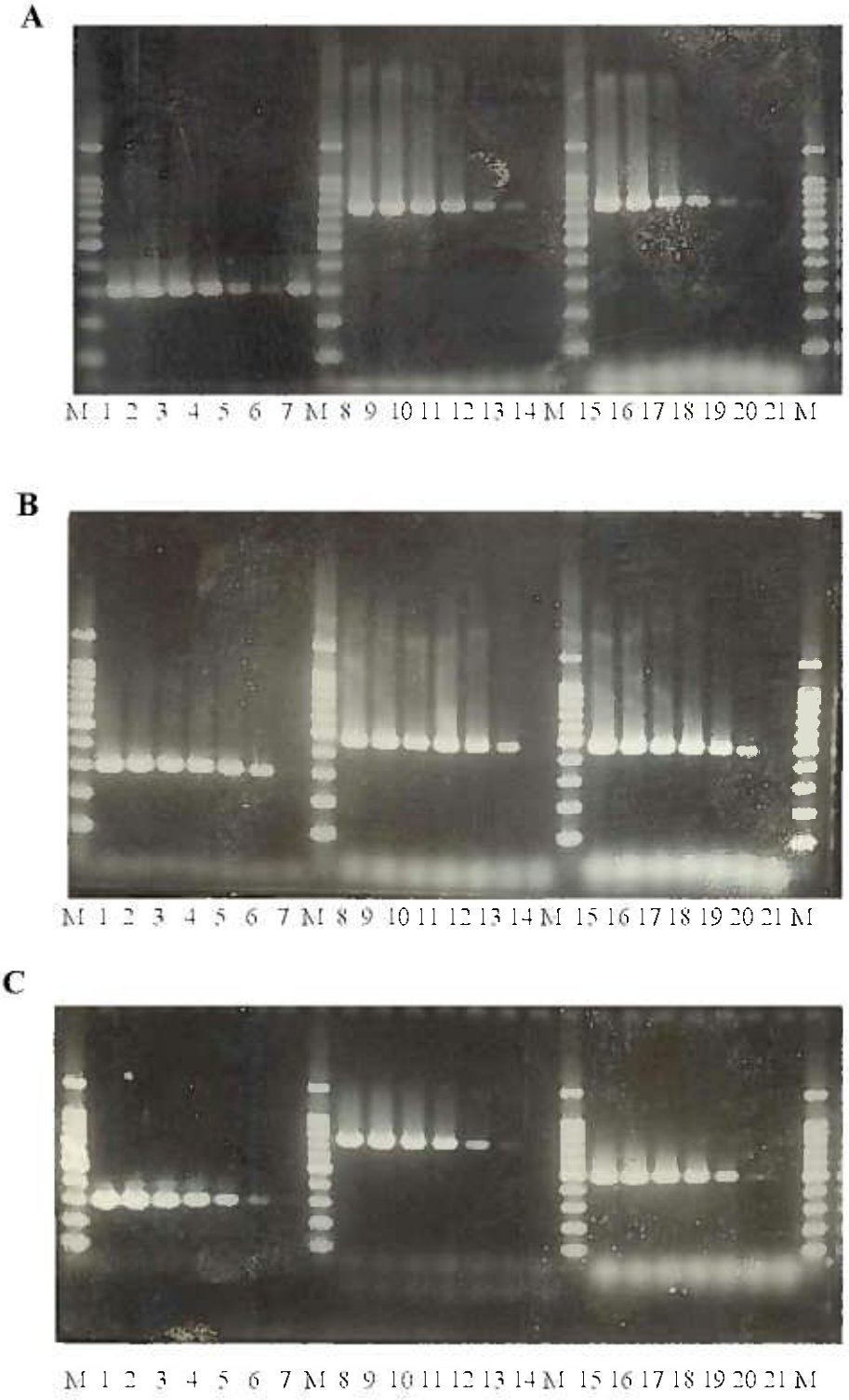


Fig. 2-4. Comparison of sensitivity between L. multiplex and simplex PCR with *Lactobacillus casei* (A), *L. coryniformis* (B) and *L. plantarum* (C).

A series of ten-fold dilutions of DNA extraction from relevant species were tested for detection limits, using universal primer pair (lane 1-7), relevant species-specific simplex primer pair (lane 8-14) and L. multiplex primer mixes (15-21). Lane 1-6, lane 8-13 and lane 15-20 represent DNA extracts containing from 10^6 cells to 10^1 cells of each strain in decimally decreasing orders, lane 7, 14 and 21 sterile distilled water (negative controls), lane M 100 bp DNA ladder

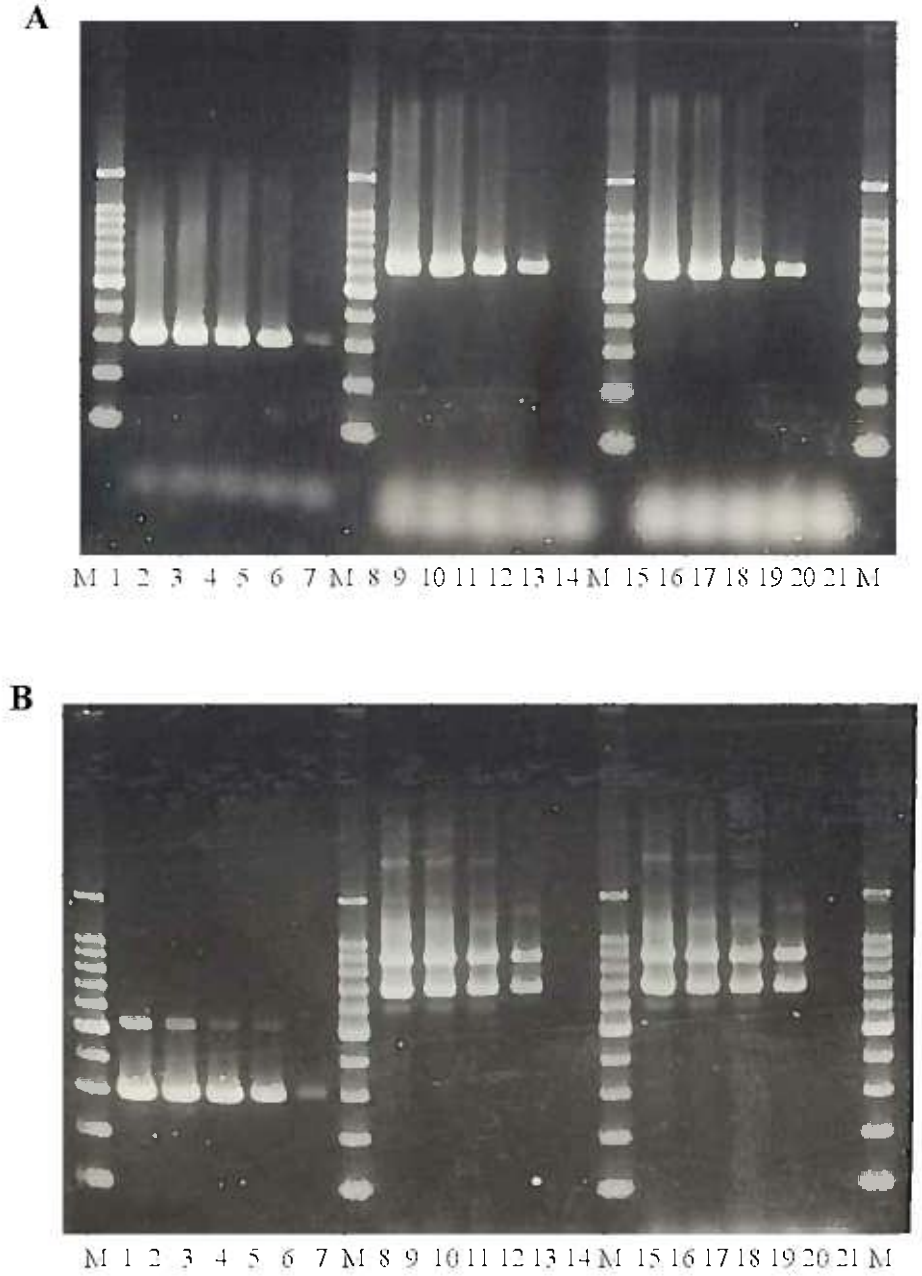


Fig. 2-5. Comparison of sensitivity between P multiplex and simplex PCR with *Pectinatus cerevisiiphilus* (A) and *Pect. frisingensis* (B)

A series of ten-fold dilutions of DNA extraction from relevant species were tested for detection limits, using universal primer pair (lane 1-7), relevant species-specific primer pair (lane 8-14) and P multiplex primer mixes (lane 15-21). Lane 1-6, lane 8-13 and lane 15-20 represent DNA extracts containing from 10^6 cells to 10^1 cells of each strains in decimally decreasing orders, lane 7, 14 and 21 sterile distilled water (negative controls), lane M 100 bp DNA ladder

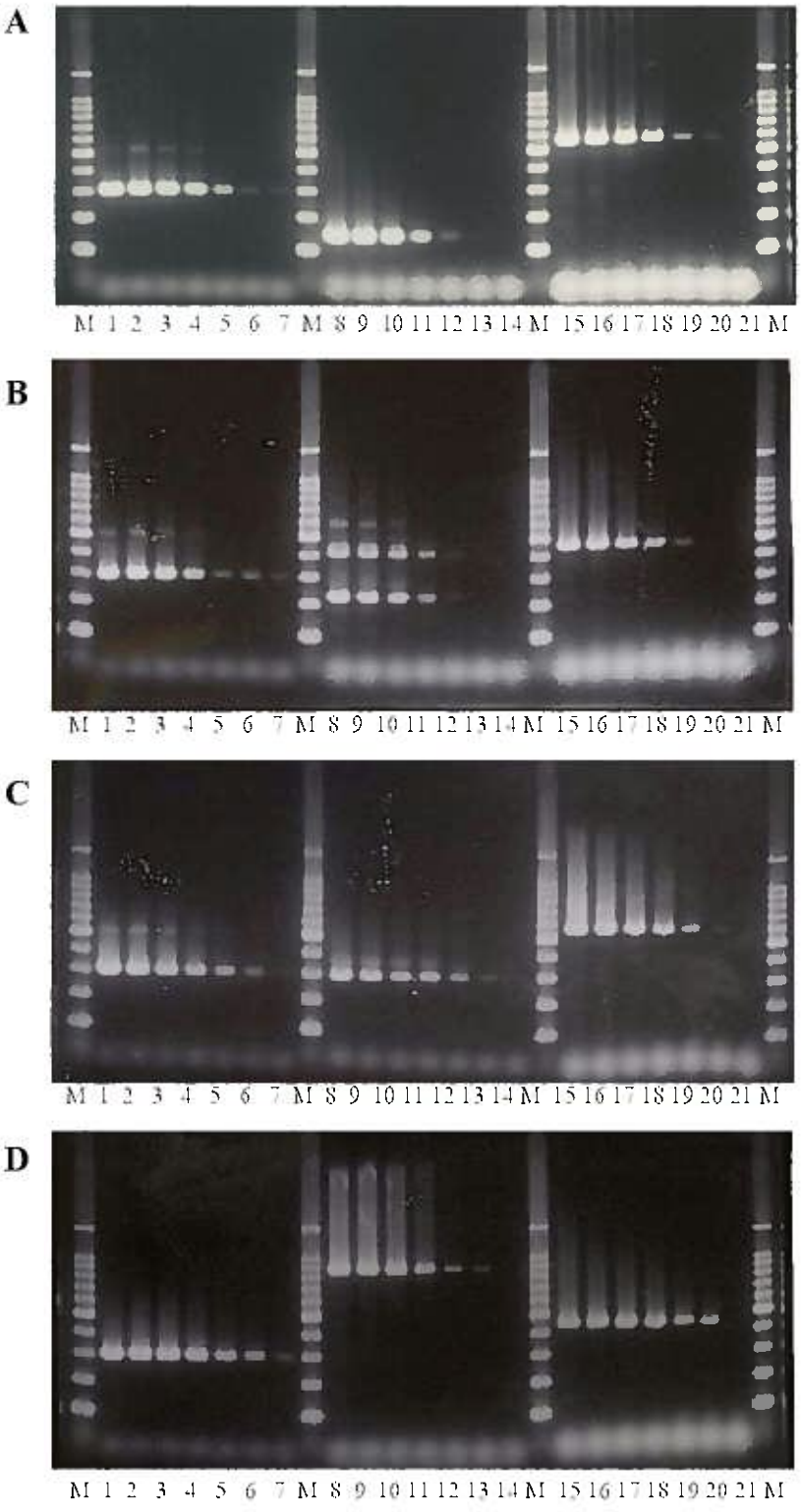


Fig. 2-6. Comparison of sensitivity between C multiplex and simplex PCR with *Pediococcus damnosus* (A), *Ped. claussenii* (B), *Ped. inopinatus* (C) and *Megasphaera cerevisiae* (D)
A series of ten-fold dilutions of DNA extraction from relevant species were tested for detection limits, using universal primer pair (lane 1-7), relevant species-specific primer pair (lane 8-14) and P multiplex primer mixes (lane 15-21). Lane 1-6, lane 8-13 and lane 15-20 represent DNA extracts containing from 10^6 cells to 10^1 cells of each strains in decimally decreasing orders, lane 7, 14 and 21 sterile distilled water (negative controls), lane M 100 bp DNA ladder

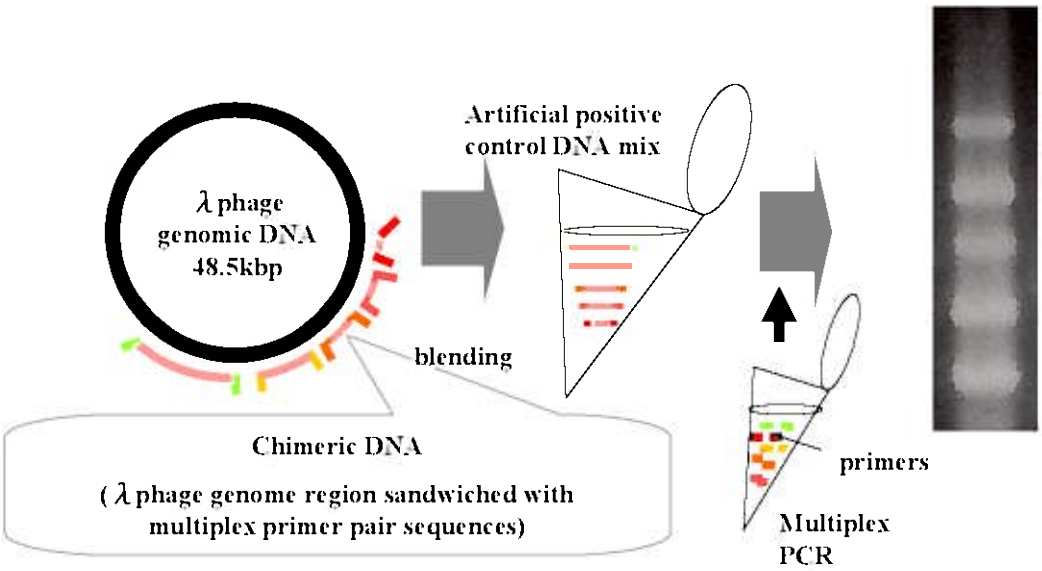


Fig. 2-7. Scheme for construction of artificial positive control DNAs
As a first step, chimeric primer pairs, containing multiplex primer sequences in 5' end and λ phage sequences in 3' end, were synthesized to amplify short DNA regions (50bp-1000bp) from λ phage genomic DNA. The amplified DNA regions thus contain the sequences of λ phage genome sandwiched with the multiplex PCR primer sequences for each beer-spoilage species. Therefore, the amplicons act as artificial positive control DNAs and are used to make sure each primer pair is functional in multiplex PCR tests. Upon designing positive controls, non-overlapped λ phage DNA regions should be selected to amplify progressively increasing sizes of artificial positive control DNAs to produce a ladder profile (agarose gel electrophoresis image on the right)

Fig. 2-8. PCR products of artificial positive control DNAs with corresponding multiplex PCR methods
Lane 1: L multiplex, lane 2: P multiplex, lane 3: C multiplex, lane M: 100 bp DNA ladder

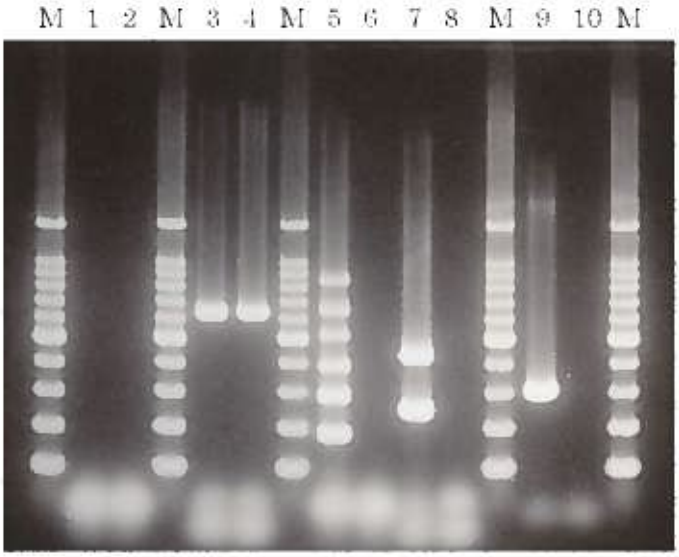


Fig. 2-9. Example of application
A Gram-variable rod-shape bacterium was tested with L multiplex and P multiplex PCR methods and identified as *Pect. cerevisiphilus*. Sample DNA was tested with L multiplex (lane 1 and 2) and with P multiplex (lane 3 and 4). Lane 5 and 7 represent artificial positive control DNAs tested with L multiplex and P multiplex, respectively. Lane 6 and 8 represent negative controls (sterile distilled water) with L multiplex and P multiplex, respectively. Lane 9 and 10 are the results of the sample DNA and negative control (sterile distilled water) with universal primers to confirm the successful DNA extraction for this sample.

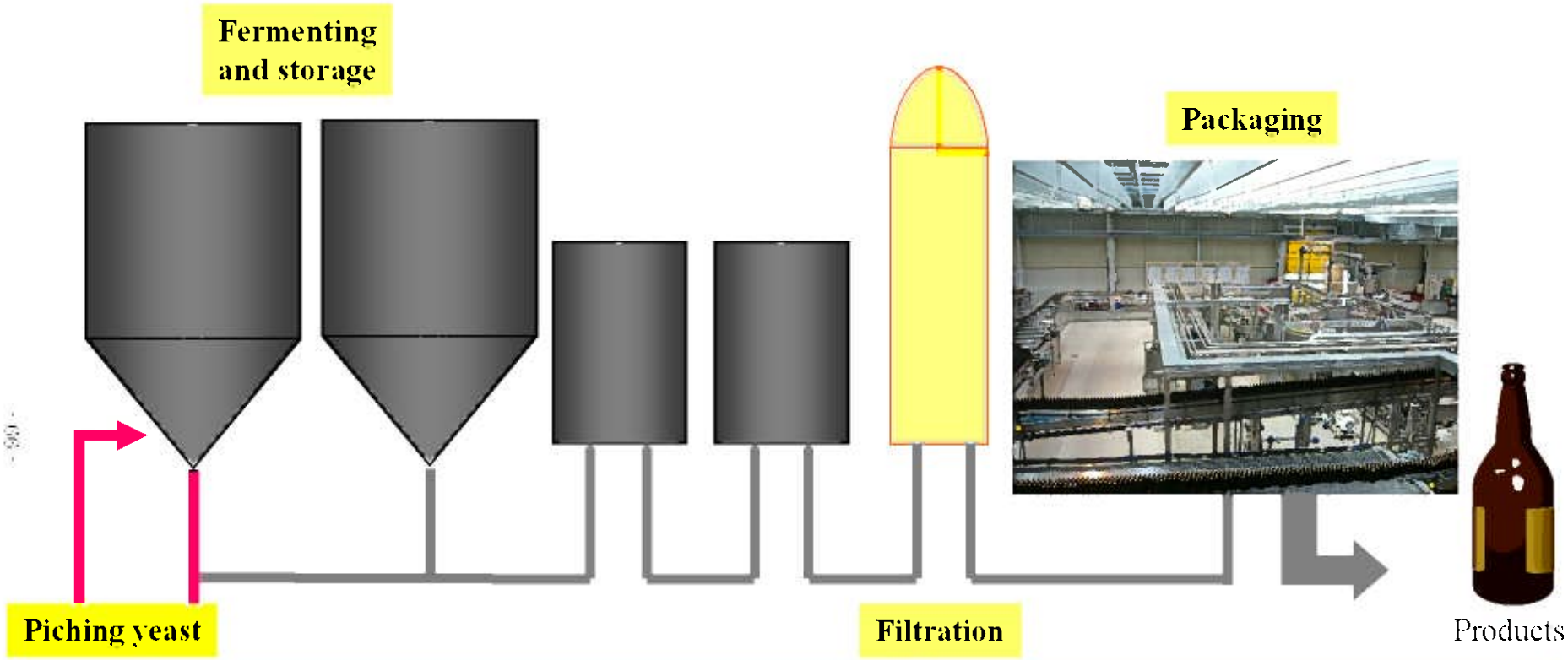


Fig. 3-1. Bacterial contamination during brewing process (Back 1994a).

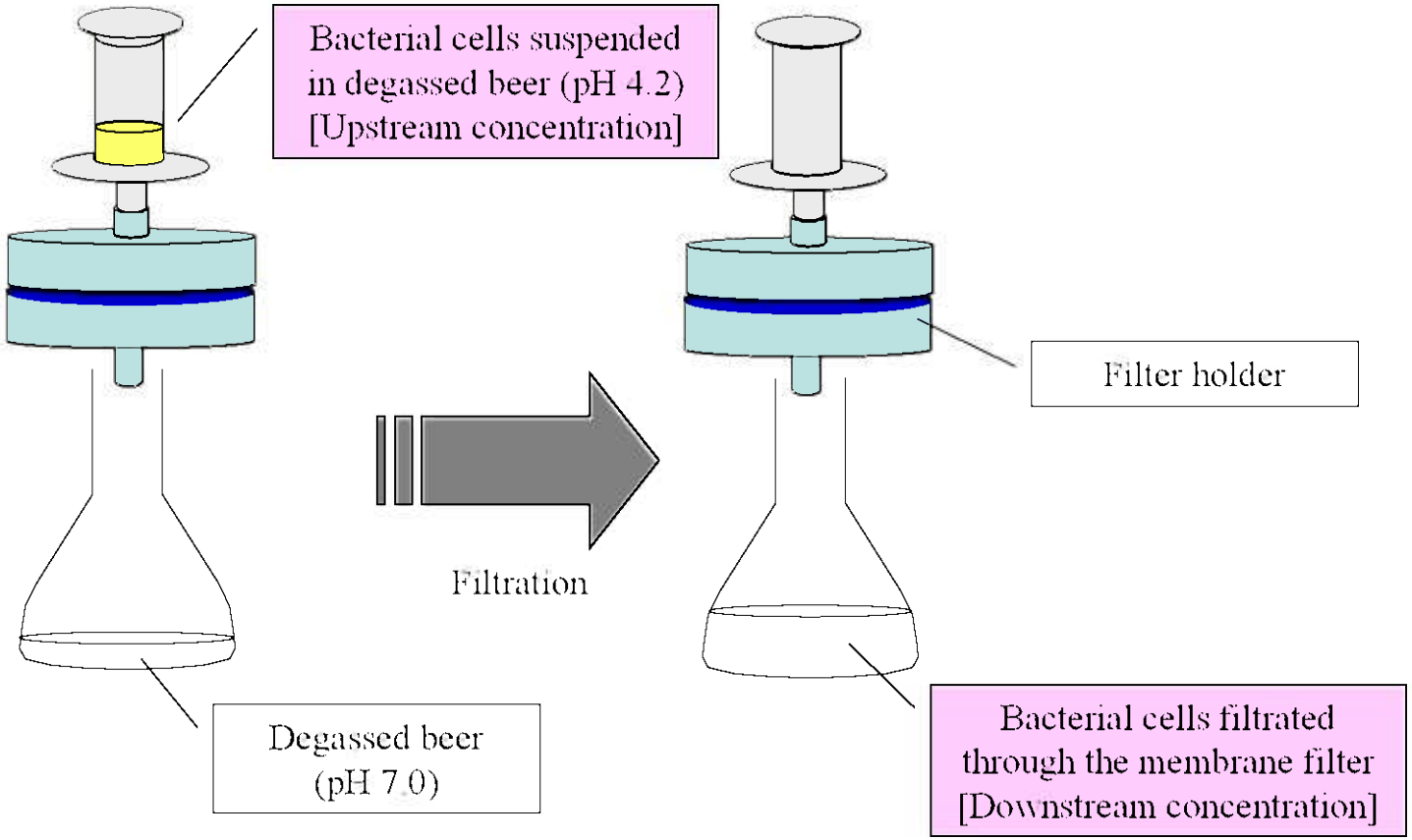


Fig. 3-2. Experimental procedure of filter evaluation.
Log reduction value (LRV) equals $\log_{10}$[upstream concentration divided by the downstream concentration].

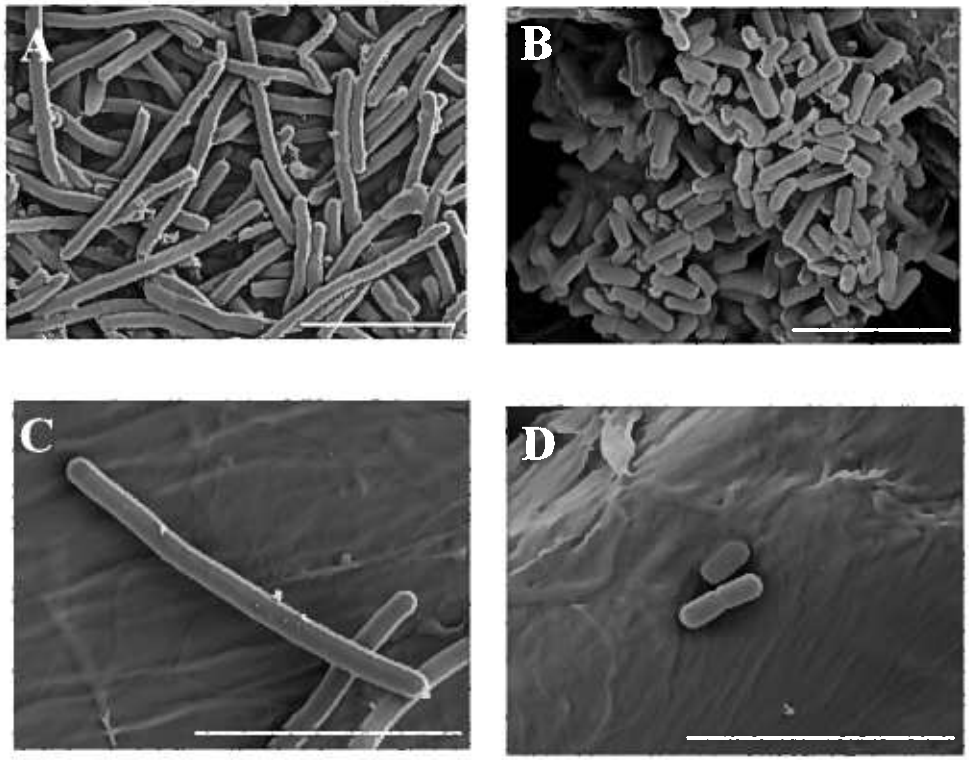


FIG. 3-3. SEM analysis of beer-adapted and non-adapted strains of LAB.
The morphological comparisons have been performed with beer-adapted and non-adapted strains of beer-spoilage LAB. (A, C) Non-adapted *Lactobacillus brevis* ABBC45; (B, D) beer-adapted *L. brevis* ABBC45. Bars: 5 µm.

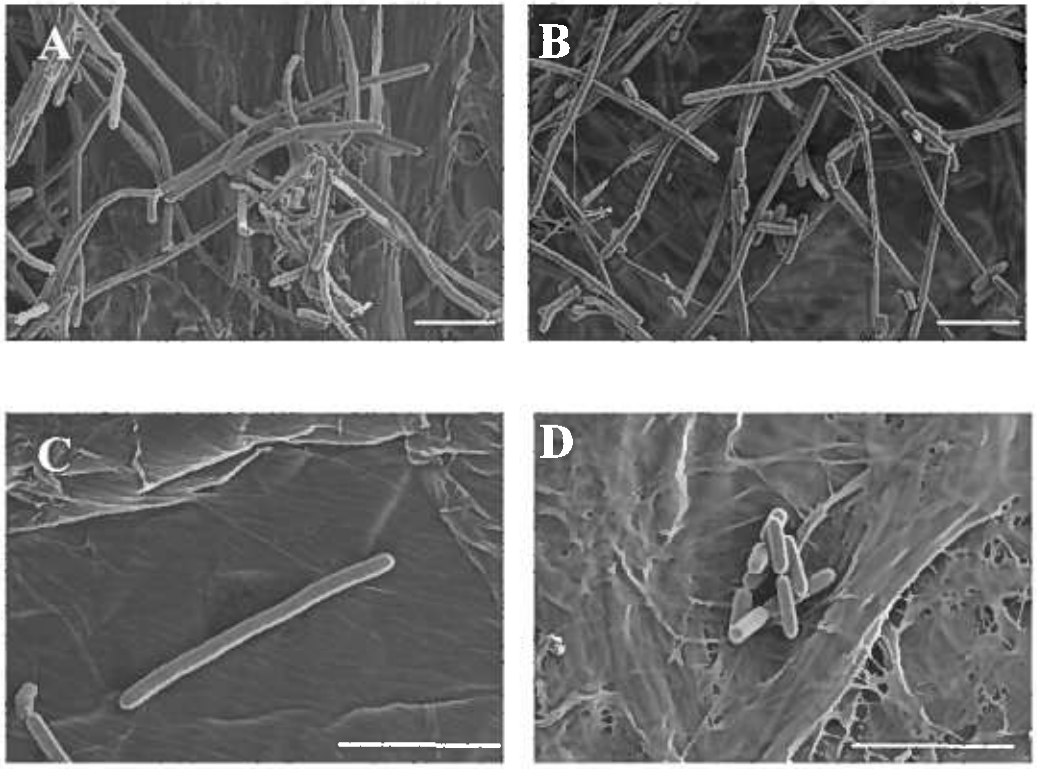


FIG. 3-4. SEM analysis of beer-adapted and non-adapted strains of LAB.
The morphological comparisons have been performed with beer-adapted and non-adapted strains of beer-spoilage LAB. (A, C) Non-adapted *Lactobacillus lindneri* DSM 20690^T; (B, D) beer-adapted *L. lindneri* DSM 20690^T. Bars: 5 µm.

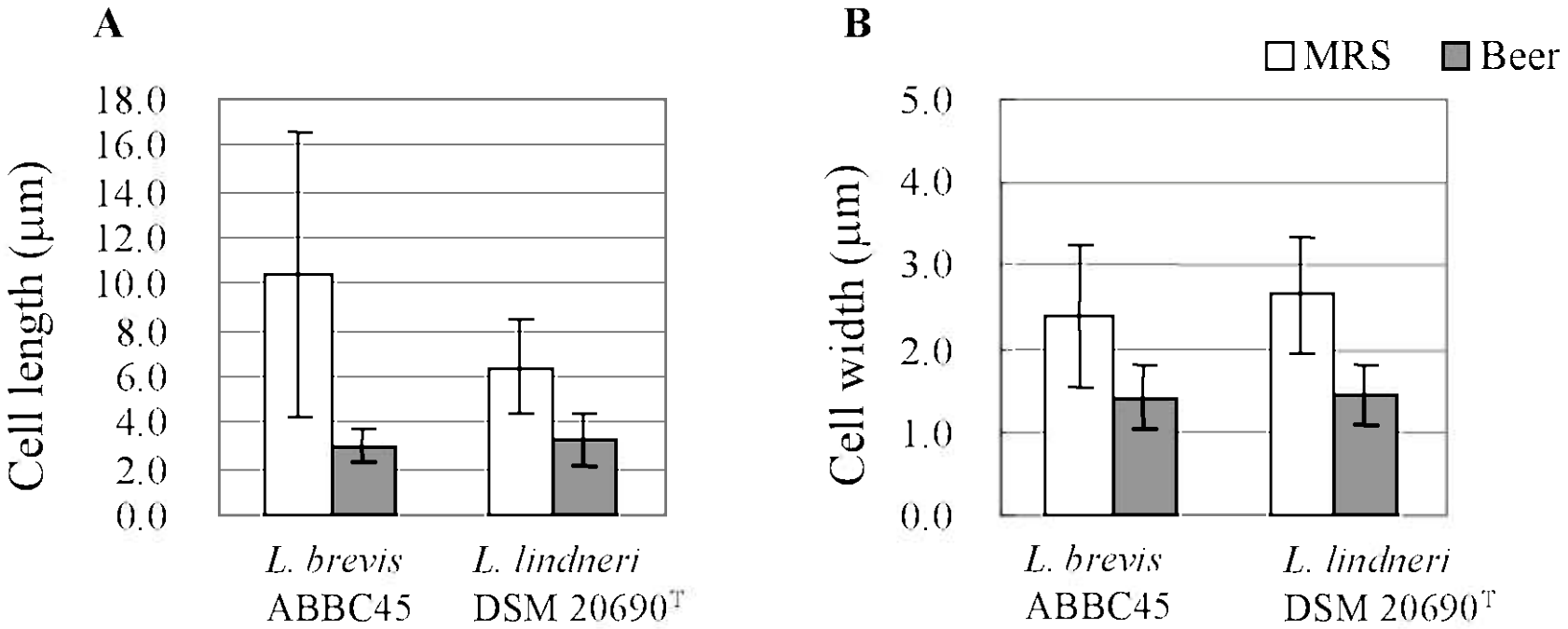


FIG. 3-5. Cell size distributions of beer-adapted and non-adapted LAB.
The cell length (A) and cell width (B) of the beer-adapted (solid bars) and non-adapted (open bars) strains of *Lactobacillus brevis* ABBC45 and *L. lindneri* DSM 20690^T were measured by μFinder with fluorescence staining. The average and standard deviation were obtained from 40 randomly selected cells.

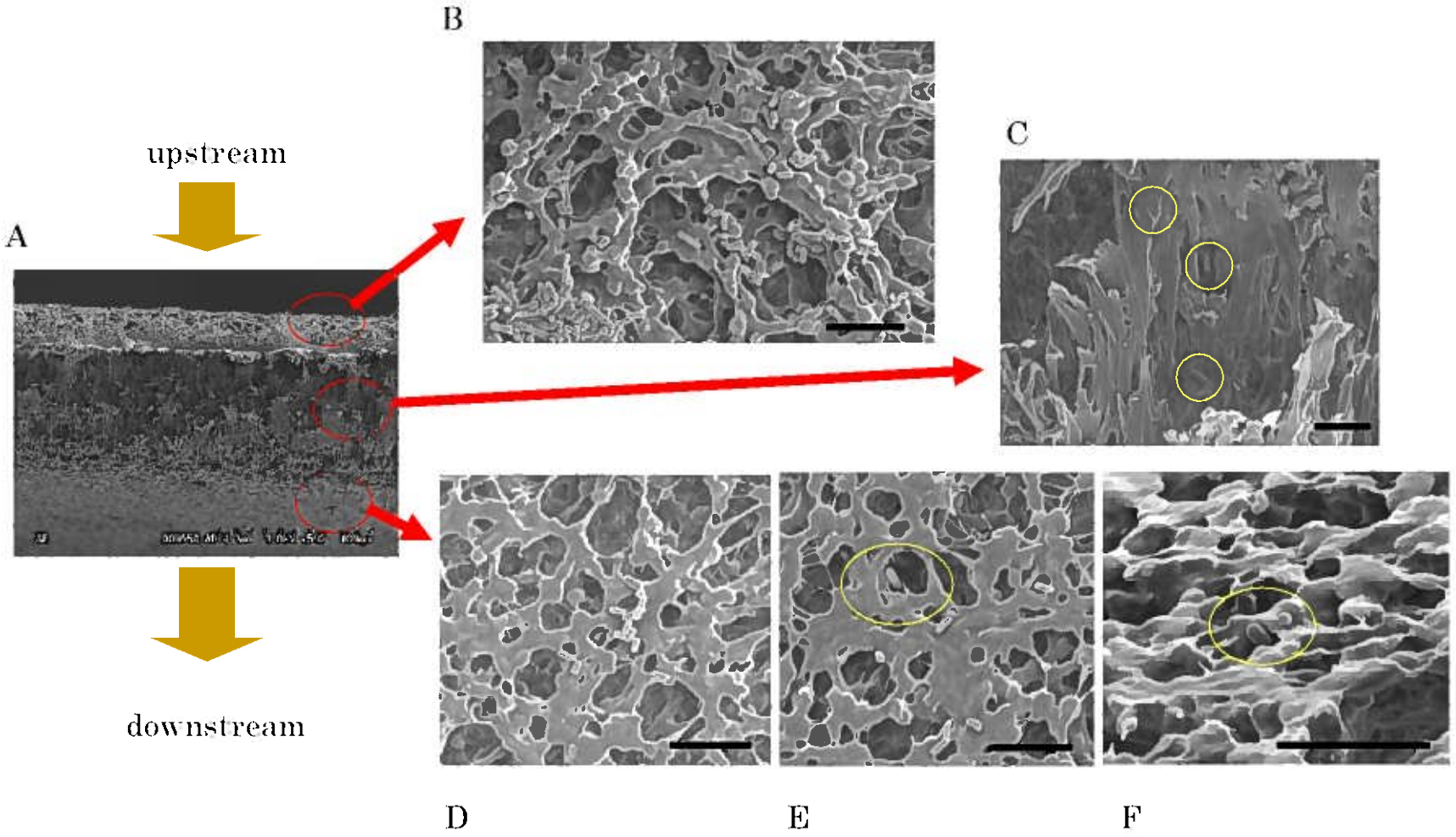


FIG. 3-6. Scanning electron micrographs of membrane filters.
(A) The cross section of a membrane filter. (B) The upstream side of the membrane filter challenged with beer-adapted *L. brevis* ABBC45. (C) Inside the dissected membrane. (D-F) The downstream side of the membrane filter with penetrated bacterial cells. Arrowheads indicate bacterial cells in the process of passing through. Pore size: 0.65 μm. Bars: (A) 100 μm and (B-F) 5 μm.

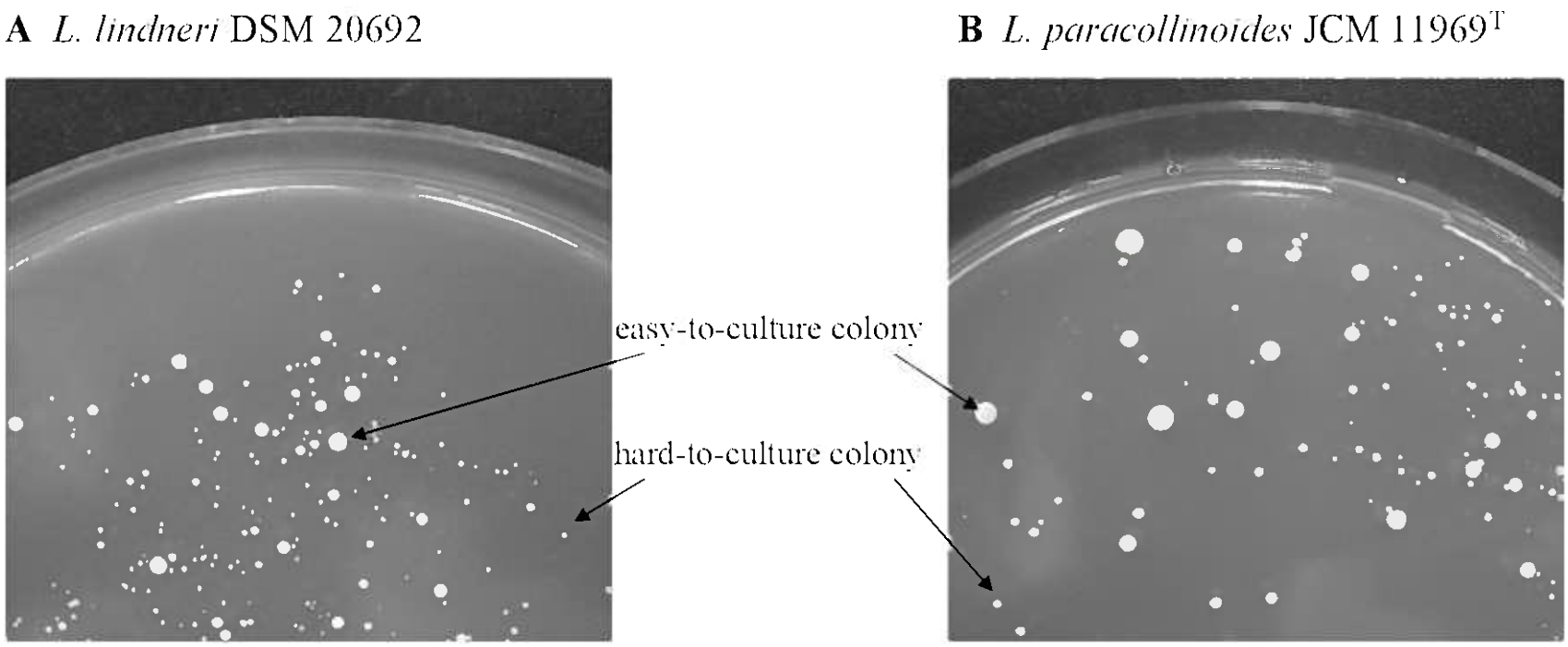


Fig. 3-7. Appearance of colonies in varying sizes after the 10th subcultures.
L. lindneri DSM 20692 (A) and *L. paracollinoides* JCM 11969^T (B) were repeatedly subcultured in degassed beers and portions of the cultures were inoculated on MRS agar. After 14 days of anaerobic incubation, the colonies were photographed. The colonies that are comparable in size with those of the wild-type strains are indicated as easy-to-culture colonies, while unusually tiny colonies are shown as hard-to-culture colonies.

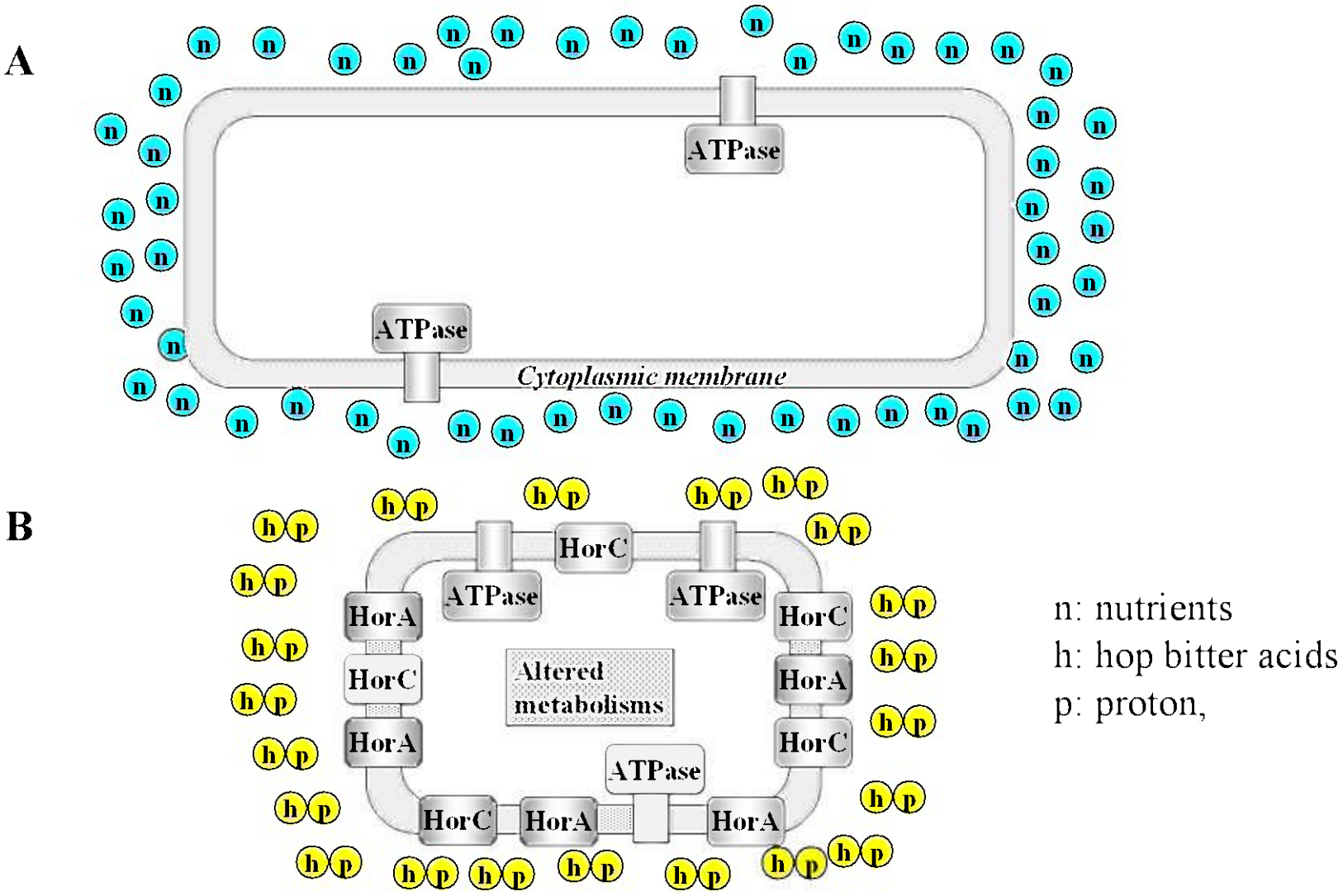


Fig. 3-8. Postulated physical and morphological changes occurring in beer spoilage lactic acid bacteria with beer adaptation. Non-beer-adapted cells grown in abundant nutrients (A) and beer-adapted cells with anti-bacterial hop bitter acids and scarce nutrients in beer-related environment (B).

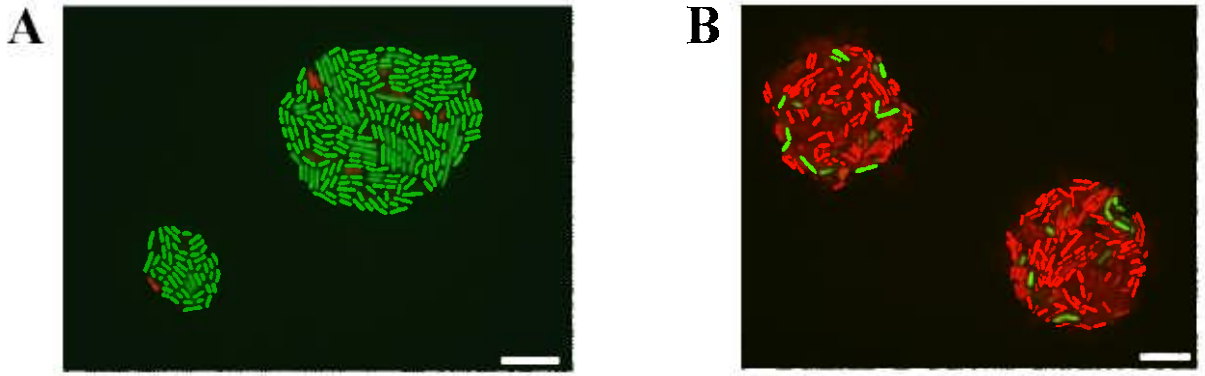
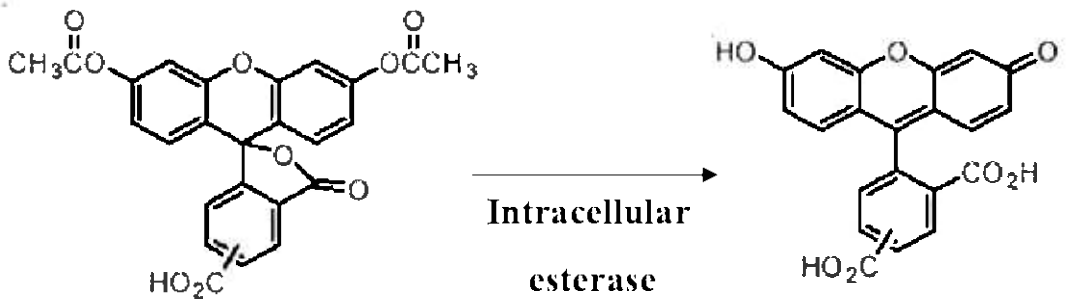


Fig. 3-9. *Lactobacillus lindneri* DSM 20692^{VN} lost viability after transition from ABD medium to MRS medium.
Cells of *Lactobacillus lindneri* DSM 20692^{VN} were filtrated onto the polycarbonate membrane filter and incubated on ABD medium at 25°C for 2 days, then transferred to another ABD plate (A) or MRS medium for 1 day (B). Both cells were double stained with 5-(and 6-) carboxyfluorescein diacetate and propidium iodide. Viable cells exhibited green, while inviable cells were stained red. (Cited from Suzuki and Asano, 2008)



carboxyfluorescein diacetate

CFDA

carboxyfluorescein

Fig. 4-1 Mechanism of the fluorescence of CFDA.

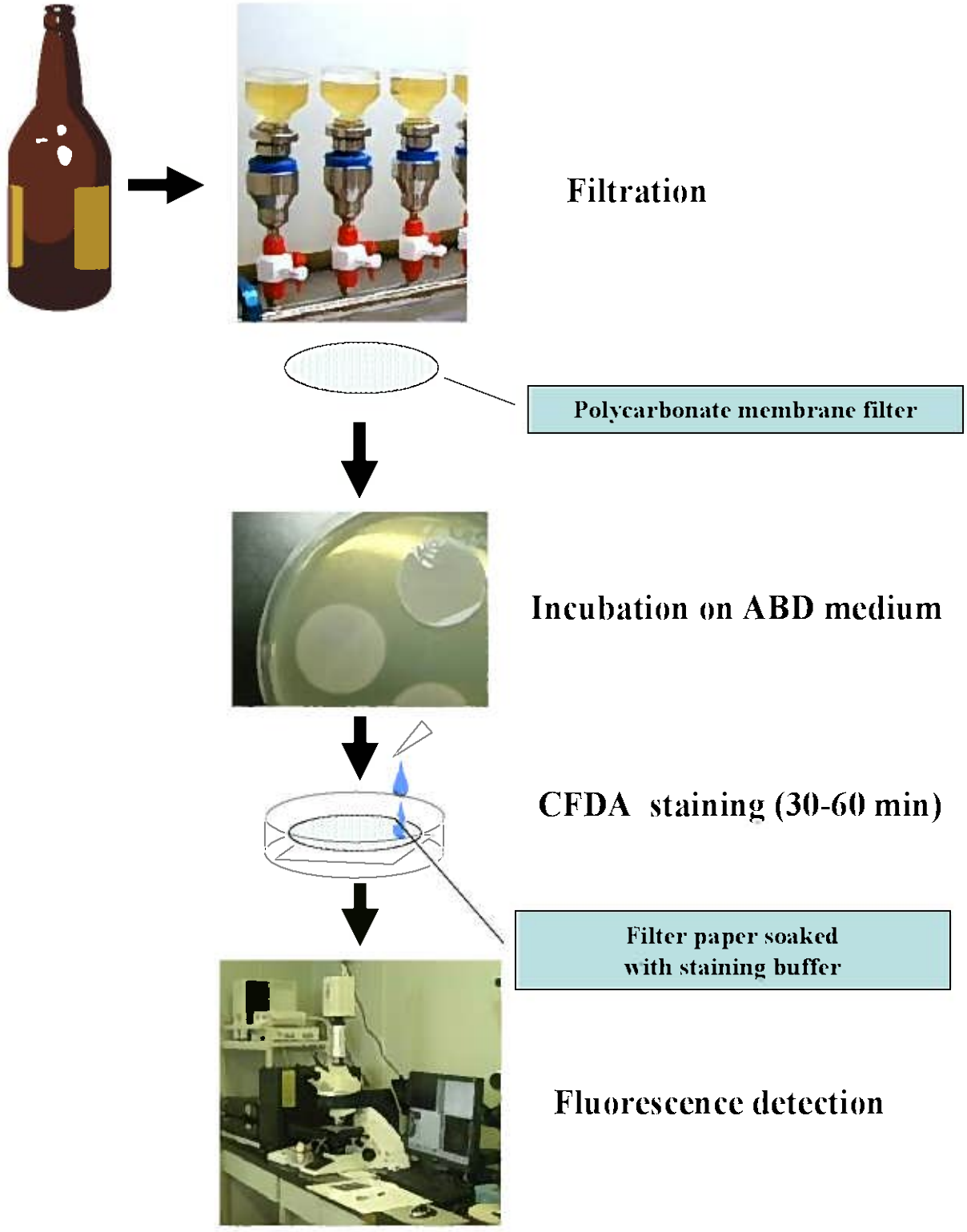


Fig. 4-2. Experimental procedure of Microcolony-CFDA method.

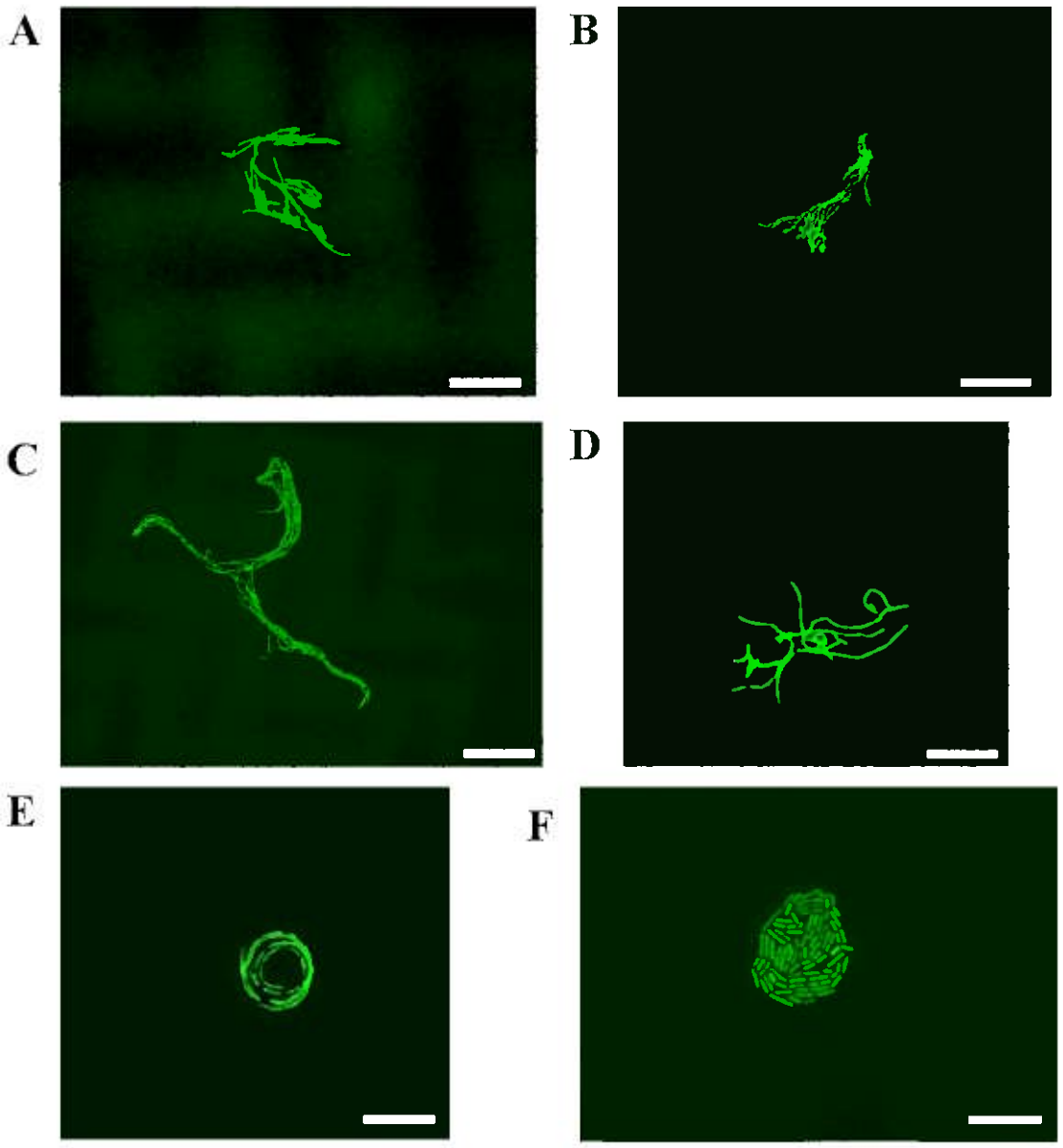


Fig. 4-3. Microcolony-CFDA method examining microcolonies.
(A, B) *Lactobacillus brevis* ABBC45, (C-E) *L. paracollinoides* JCM 11969^{vn}, (F) *L. lindneri* DSM 20692^{vn}. Bars: 50 μm (A-D), 10 μm (E, F)
"vn" indicates the deeply beer adapted variants that lost the culturability on conventional MRS medium.

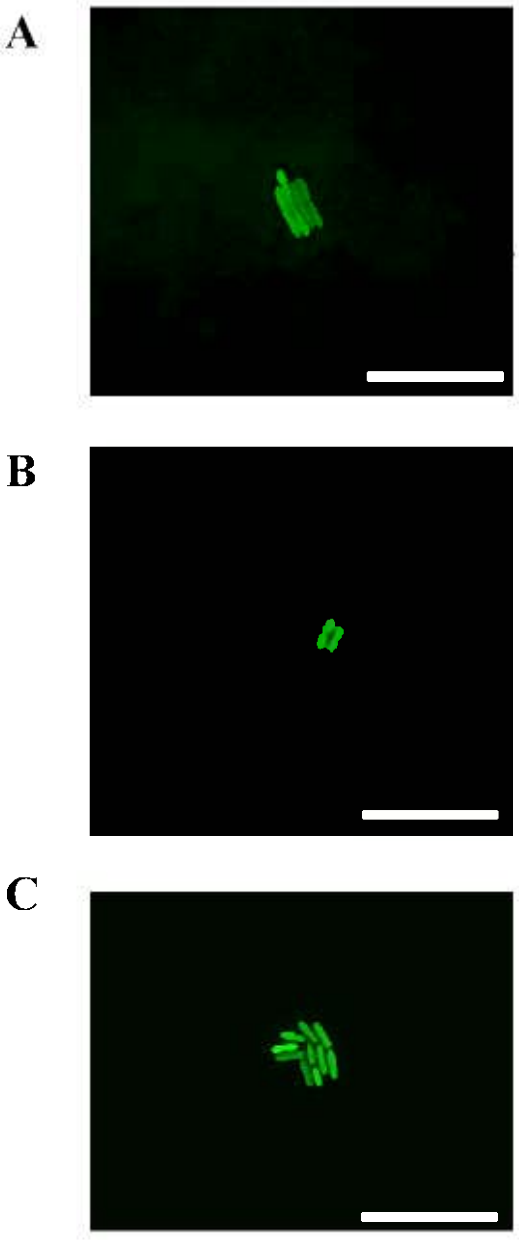


Fig. 4-4. Microcolony-CFDA method examining microcolonies, consisting of low numbers of cells. (A) *Lactobacillus brevis* ABBC45, (B) *L. paracollinoides* JCM 11969^{vn}, (C) *L. lindneri* DSM 20692^{vn}. Bars, 10μm. vn indicates the deeply beer adapted variants that lost the culturability on conventional MRS medium.

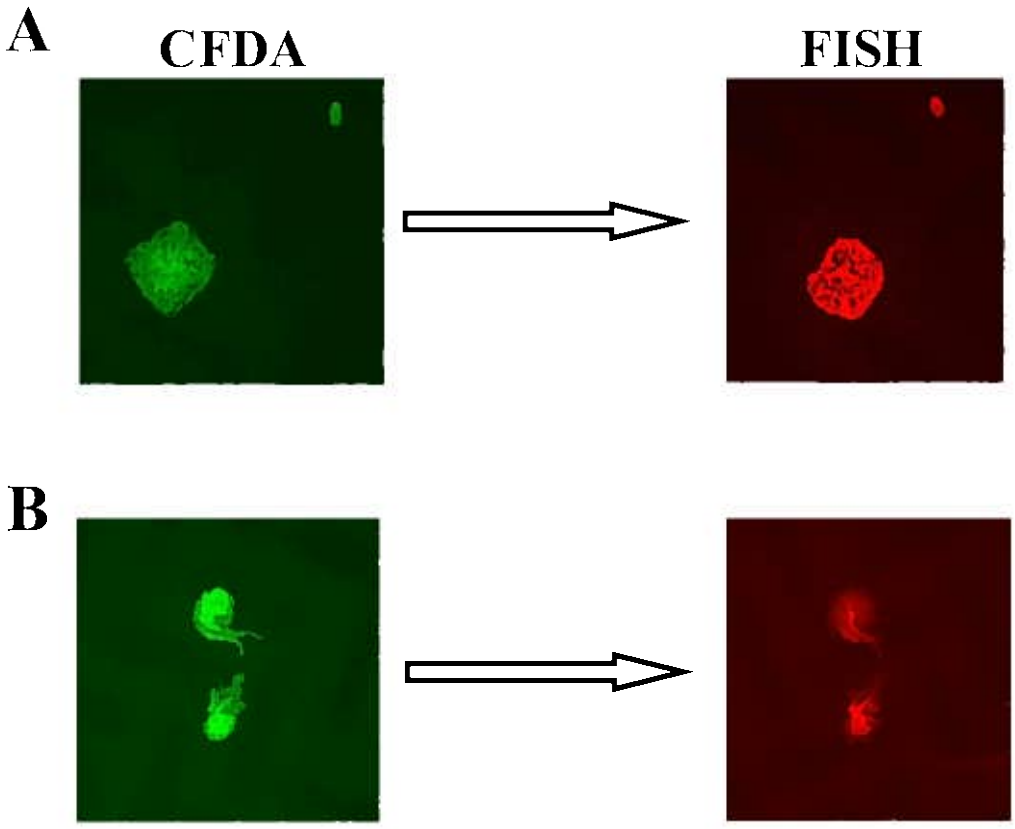


Fig. 4-5. Applications of microcolony-FISH method using μFinder Inspection System. (A) CFDA-stained microcolonies of *L. brevis* ABBC45 were subjected to FISH with probes specific to *L. brevis*. (B) CFDA-stained microcolonies of *L. paracollinoides* JCM 15728 were subjected to FISH with probes specific to *L. paracollinoides*.