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Chemical Markers for the Evaluation of Raw Material Hygienic Quality in Egg Products

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The aim of this research was to study uracil and lactic and acetic acids as chemical markers for hygienic quality evaluation of raw material in liquid pasteurized egg products. Uracil, absent in sound whole eggs, was formed in raw and pasteurized egg products as a consequence of high microbial contamination ($>10^6$ cfu/g) after a sufficient lag time, remaining stable at 4 °C but disappearing after 7 days of storage at 25 °C. Both lactic and acetic acids, starting from initial values of 1–7 mg/kg dry matter, presented trends similar to that of uracil; however, acetic acid never decreased during the storage of raw egg products. With few exceptions, all three metabolites were produced by *Enterobacter cloacae*, *Escherichia coli*, *Morganella morganii*, *Serratia liquefaciens*, *Aeromonas hydrophyla*, *Pseudomonas fluorescens*, *Enterococcus avium*, and *Enterococcus faecalis*, separately inoculated in whole egg samples. Uracil seems to be the most sensible marker, with a suggested limit corresponding to the detectable level.

KEYWORDS: Acetic acid; lactic acid; microbial contamination; uracil; uridine; egg products

INTRODUCTION

Seventy percent of the worldwide production of egg products is concentrated in the United States and the European Union (*1*). In the USA, the most marketed typology is the pasteurized liquid egg product (81.4% of the total production), followed by frozen (13.3%) and dried (5.3%) product (*2*), while the most commonly processed fraction is whole egg (64.0%), followed by egg white (23.5%) and yolk (12.5%) (*3*). A similar distribution might be hypothesized for Europe, although reliable statistics are lacking.

The manufacture of pasteurized liquid egg products includes washing and disinfection of dirty eggs, egg breaking, homogenization, and pasteurization treatment. Special attention throughout all of the production process is given to its microbiological aspects, following well defined (*4, 5*), good hygienic practices. For example, only clean and dry eggs should be broken, and the breaking operation should minimize contamination. In the USA, egg centrifugation is allowed only for clean, sanitized, and intact eggs when the egg product is for cooked or baked foods; however, if the end product involves the use of uncooked eggs, a different breaking system must be used (*5*). After shelling, liquid egg products must be kept at a maximum temperature of 4 °C and must be processed as quickly as possible, to minimize microbial hazards. In the European Union, centrifugation and egg crushing are forbidden, and the 4 °C-storage period before heat treatment must not exceed 48 h. Minimal conditions, that is, whole egg pasteurization treatment at 60 °C for 3.5 min (as mandatory in the USA) or at 64 °C for 2.5 min (as used in the UK), should provide a 5–9 log cycle reduction in the number of the most frequent *Salmonella* serotypes (*6*). The shelf life of pasteurized liquid egg products (contained in tanks) is determined by the manufacturer and is generally limited to 5–6 days at a maximum temperature of 4 °C. Pasteurized egg products can reach a shelf life of 28 days if more severe heat treatment conditions, coupled to aseptic packaging, are performed. The addition of ingredients (i.e., salt or sugar) significantly extends shelf life.

The current European legislation (*7*) classifies shell eggs in two quality categories: grade A or fresh eggs and grade B or eggs for food or nonfood industry. In order to satisfy the quality standards required by the food industry, egg products manufacturers in Europe frequently process grade A eggs. Similarly, the US manufacturers use grade B or higher quality eggs. However, the industry some times fraudulently recycles production rejects, such as broken eggs discarded from the shell egg packaging industry, highly contaminated eggs, or incubator rejected eggs.

Pasteurization conditions cause a reduction of microbial population to acceptable levels only if the starting material has good hygienic quality and is adequately handled during breaking. In fact, it is important to avoid any contact between the internal egg content (almost sterile when coming from healthy laying hens) and the dirty shell egg surface, which can reach 10^7–10^8 cfu/egg microbial count (*8*). In the case of highly spoiled liquid raw egg, slightly more severe heat treatment conditions or repasteurization and/or the addition of antimicrobial substances could significantly reduce the microbial population. Contamination and growth of bacteria in egg products before pasteurization jeopardizes consumer safety and promotes

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functional property decay, besides causing alterations of the sensory and/or nutritional characteristics of the egg product (*9, 10*).

The US legislation regarding egg product hygiene, besides the well-known *Salmonella enteritidis* Action Plan, mandates the evaluation of the acceptability level of egg products by the egg-smelling procedure followed by trained personnel. The European legislation limits the presence of *Salmonella* (absent in 25 g) and Enterobacteriaceae (max. 100 cfu/g) (*11*) and defines limits for 3-hydroxybutyric acid (10 mg/kg dry matter), a chemical index of incubator-rejected eggs in egg products, and for lactic acid (1000 mg/kg dry matter), a chemical index of hygienic quality of raw material (*4*). However, the reference limit for lactic acid is exclusively referred to the untreated product (neither pasteurized nor fermented). Thus, at the moment, both legislations do not provide an index for the control of raw material hygienic quality, as measured in the pasteurized egg product. The availability of chemical indices that, unlike microbial analysis, permit the evaluation of raw material quality in the pasteurized egg product is of primary importance. These indices must be thermostable since they should not be influenced by heat treatments carried out during egg product and food manufacturing as well as quantifiable without analytical interferences of the food matrix.

A promising index is uracil, a thermostable base produced by microbial metabolism. Morris et al. (*12*) correlated the presence of uracil to the acceptability of the pasteurized egg product odor, evidencing uracil levels higher than 7 mg/kg dry matter (dm) in samples with a nasty smell. Hidalgo et al. (*13*) suggested uracil as a promising chemical index for hygienic quality evaluation of egg products; however, they did not determine the hygienic quality of the raw material in most of the industrial samples nor identify the microorganisms responsible for uracil production. Uracil represents a marker of the microbial spoilage of raw material even when contaminant microorganisms are destroyed by thermal treatments; besides, it is absent in fresh eggs (*14*) and is formed from uridine naturally present in egg (*13*) as a consequence of the hydrolytic action of the nucleoside phosphorylase produced by microorganisms (*15, 16*). Uracil presence in egg-based foods could also represent a hygienic quality index of the egg products used as ingredients, as demonstrated for fresh egg pasta (*17*).

The purpose of this research was to evaluate the potential of some microbial metabolites as hygienic quality indices of raw material to be measured in the final pasteurized whole egg products. To this aim, the concentrations of uracil, uridine, lactic acid, acetic acid, and microbial development were determined during storage at 4 and 25 °C of raw and pasteurized liquid whole egg products and during incubation of whole egg samples separately inoculated with eight different microbial strains.

MATERIALS AND METHODS

Sample Preparation. *Spontaneous Microbial Growth.* The following samples were prepared: (a) laboratory raw whole egg contaminated by direct contact with unwashed shells. It was obtained by manual breaking and mixing of brown grade A fresh eggs of large size (*7*), bought from a supermarket 10 days after laying. The mix containing broken shells was stored at 25 °C, and samples without shell fragments were collected for analysis at different time periods up to 7 days. (b) Industrial raw and pasteurized whole egg samples of the same lot, but collected just before (raw) and after heat treatment (pasteurized) in an industrial production line, were aseptically stored in sterile 150 mL plastic containers. The sampling was repeated on two lots processed in two different days. The samples collected from one lot were stored at 4 °C and those from the other one at 25 °C. The two series of samples were analyzed at different time periods up to 7 days of storage, with the exception of the pasteurized whole egg kept at 4 °C, which was analyzed up to 45 days. At time 0, analyses of the laboratory raw whole egg sample were performed immediately after sample preparation, while analyses of industrial samples were carried out upon arrival in our laboratories the same day of production.

Total bacterial count (TBC), Gram-negative bacteria, *Salmonella* spp. and Enterococci, dry matter, pH, and the contents of uridine, uracil, lactic acid and acetic acid were evaluated in all samples. Furthermore, in samples stored at 25 °C, total lactic acid bacteria and Enterobacteriaceae were quantified, while in samples stored at 4 °C total coliforms were evaluated. All of the measurements were carried out in duplicate.

Selected Microbial Strains Growth. Eight series of 12 whole egg samples were aseptically prepared from 96 fresh brown grade A eggs of large size (*7*), purchased in a supermarket about 15 days after laying. The albumen and the yolk of each egg were separated and collected aseptically. To this end, the eggs were immersed in a water/sodium hypochloride (1:2) solution for 15 min, then they were individually extracted from the solution using sterile gloves and their surface dried with autoclave-sterilized paper napkins. Subsequently, they were rinsed by immersion in sterile water at room temperature, dried as previously outlined, and placed in sterile bags. All these steps were done under a sterile laminar flow hood. Each egg was then broken with a sterile metal rod, its content was poured into a sterile bag, and homogenization was carried out with STOMACHER 400 Circulator (PBI International, Milan, Italy) at 230 rpm for 1 min.

Each series of samples was inoculated with one of the eight microbial strains isolated from the samples analyzed in the spontaneous microbial growth trial: *Enterobacter cloacae*, *Escherichia coli*, *Morganella morganii*, and *Serratia liquefaciens* (all Gram-negative, facultative anaerobes from the Enterobacteriaceae family); *Aeromonas hydrophyla* (Gram-negative, facultative anaerobe); *Pseudomonas fluorescens* (Gram-negative, aerobe); *Enterococcus avium* and *Enterococcus faecalis* (Gram-positive, lactic acid bacteria of intestinal origin). The inoculated samples were stored at 25 °C for 48 h, and every 3–10 h, the following determinations were conducted: TBC, dry matter, pH, uridine, uracil, lactic acid, and acetic acid contents. The samplings for the microbiological analyses were always performed before those for the chemical analyses. All of the measurements were carried out in duplicate.

Microbial Analyses. *Spontaneous Microbial Growth.* TBC, Gram-negative bacteria, Enterococci, Enterobacteriaceae, lactic acid bacteria, and coliforms were monitored by plate counting, using the serial decimal dilution technique in tryptone salt (NaCl, 8.5 g; tryptone, 1 g; distilled water, 1000 mL). TBC was performed by pour plates on Plate Count Agar (PCA; VWR, Germany) (*18*) incubation at 30 °C for 48 h; Gram-negative bacteria by spread technique on Plate Count Monensin-KCl agar Mug (PMK Mug, made up of 23.5 g plate count agar, 35 mg monensin, 7.5 g KCl and 75 mg 4-methylumbelliferyl-β-D-glucuronide (Mug)) with incubation at 30 °C for 48 h; Enterococci by spread technique on Kanamicin Azide Esculine Agar (KEA; VWR, Germany) (*19*) incubation at 37 °C for 48 h; lactic acid bacteria by pour plates of Man Rogosa Sharpe Agar (MRS; Merck, Darmstadt, Germany) (*20*) incubation at 30 °C for 48–72 h under anaerobic conditions (gas-pack) (*20*); Enterobacteriaceae and coliforms by double layer pour plates on Violet Red Bile Destrose (VRBD) (*21*) and Violet Red Bile (VRB) agars (VWR, Germany) (*22*), respectively, incubation at 37 °C for 24 h. Manual counting was performed.

Only plates with 30 to 300 colonies were considered for computing the colony forming units (cfu) using the equation of Peeler and Maturin (*23*). The results are expressed as the total number of microbial cells per milliliter of analyzed product (cfu/mL).

After two days of storage at 25 °C, of all the samples analyzed in the spontaneous microbial growth trial, 10% of the colonies from the highest countable dilution of PCA, PMK Mug, and KEA were isolated and identified. The isolates from PCA and PMK Mug were transferred on slant of Tryptic Soy Agar (TSA; VWR, Germany), while the isolates from KEA were transferred in BHI broth (VWR, Germany).

Phenotypical identification was performed by an API system (Biomerioux, France). For the isolates from KEA agar, API 20 Strep was used; for the nonfermenting Gram-negative oxidase-positive isolated from PMK Mug agar, the API 20NE system was used; and

for the fermenting and oxidase-negative Gram negatives from VRBD agar, the API 20E system was adopted.

Salmonella spp. were evaluated according to the EN/ISO 6579 procedure (*24*).

Selected Microbial Strains Growth. Each of the eight series of whole egg samples was inoculated, at a ratio of 10^5 cfu/mL, with one of the eight isolated microorganisms. The cells for the inoculation mother suspensions were obtained by centrifugation from an overnight broth culture of each microorganism and then placed into a pH 7 tryptone salt solution. To have a proper inoculum concentration, cell count in the mother suspensions was done with a Burker chamber at the optical microscope (*25*).

During samples incubation at 25 °C, the microbial growth of the selected strains was measured in duplicate by plate counting, following the decimal dilution technique. Two different media were used for each microorganism: (1) a differentially selective medium for the growth of the microorganism under examination and (2) a common standard medium (PCA), to verify the absence of contaminants. The following selective media were used: VRB agar for *Enterobacter cloacae*, *Escherichia coli*, *Morganella morganii*, and *Serratia liquefaciens*; Aeromonas Selective Medium (ASM; Biolife, Milan, Italy) for *Aeromonas hydrophyla*; King B (made with 20 g of proteose peptone, 1.5 g of K_2HPO_4, 1.5 g of $MgSO_4$, 10 g of glycerol, 15 g of agar, and 1000 mL of distilled water) for *Pseudomanas fluorescens*; and KEA agar for *Enterococcus avium* and *Enterococcus faecalis*.

Chemical Analyses. Dry matter content (g/100 g) was determined following the AOAC official gravimetric method no. 925.30 (*26*). Uracil, uridine, lactic acid, and acetic acid analyses were performed using the analytical HPLC procedure reported by Hicks et al. (*27*), following the sample preparation procedure reported by Morris (*28*), with the slight modifications suggested by Rossi and Pompei (*29*). Sample preparation consisted of the deproteinization of 2 g of whole egg product in the presence of 2.8 mL of HPLC water, 4.8 mL of 6% perchloric acid, and 0.6 mL of acetronitrile. After 1 min of stirring, 15 min of standing, and 30 s of stirring, the sample was centrifuged for 10 min at 12,000*g* (10,360 rpm) for 11 min and at 15 °C, using a Centrikon T-42K centrifuge (Kontron Instruments, Milton Keynes, Buckinghamshire, UK). The supernatant was then filtered through a 0.45 μm nylon membrane (Diana Beck Scientific, Angera, Italy). A volume of 20 μL of the filtered solution was injected in the HPLC system under the following operating conditions: column Aminex HPX87H, 300 × 7.8 mm (Bio-Rad Laboratories, Hercules, CA); column temperatures, 45 °C; guard column, Cation H cartridge (Bio-Rad Laboratories, Hercules, CA); mobile phase, 0.01 N sulfuric acid; flow rate, 0.6 mL/min; pump, Waters 510 (Millipore, Milford, MA). Uracil and uridine were detected at 260 nm, using a Millipore Waters 996 series photodiode array detector (Milford, MA) controlled by the software Millenium32 Chromatography Manager (Waters Chromatography Division, Millipore, Milford, MA). The wavelength range used was 200–290 nm. Lactic and acetic acids were detected using a refractive index detector (model 1037A, Hewlett-Packard, Geneva, Switzerland) connected to a D-2500 chromato-integrator (Merck-Hitachi, Tokyo, Japan).

For peak quantification, calibration curves were built using 19 concentrations (between 0.2 and 50 mg/L) of the uracil standard (Merck, Darmstadt, Germany), 12 concentrations (between 5 and 85 mg/L) of the uridine standard (Sigma Chemical Company, St. Louis, MO), 22 concentrations (between 4 and 4000 mg/L) of the lactic acid standard (Supelco, Bellefonte, PA), and 8 concentrations (between 28 and 497 mg/L) of the acetic acid standard (BDH Laboratory Supplies, Poole, England), all in water. On the basis of the calibration curves, the detection limits were calculated as the intercept value of the regression line plus 3 times the standard error of the estimate (*30*). The results are expressed as mg/kg dm. Uracil, uridine, lactic, and acetic acids calibration curves were linear ($r^2 = 1$; $p \leq 0.001$) in the concentration ranges considered and showed detection limits in the standard solution of 1.1, 0.3, 17.0, and 6.6 mg/L, respectively, corresponding to 4.7, 17.5, 273.2, and 106.0 mg/kg dm in whole egg product, considering mean dry matter contents of 23.7 g/100 g. Typical chromatograms for uridine and uracil (A) and lactic and acetic acids (B) are shown in **Figure 1**.

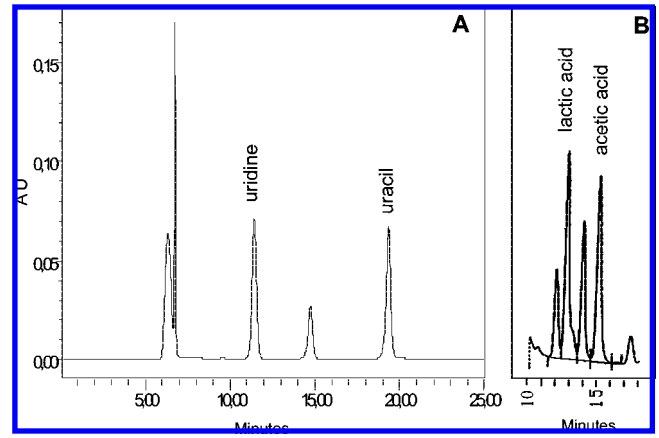

Figure 1. Typical chromatogram of uridine and uracil (**A**), obtained at 260 nm using a diode array detector, and of lactic and acetic acids (**B**), using a refractive index detector, in a spoiled liquid whole egg sample.

The repeatability of uridine, uracil, lactic, and acetic acids analytical methods was assessed by performing, in each case, 10 replicate measurements on the same whole egg product sample. The coefficients of variation (CV%) were 0.81, 1.64, 2.09, and 2.53%, respectively. The repeatability of the methods was high; thus, for simplicity only the mean values of each duplicated determination are reported.

RESULTS AND DISCUSSION

Spontaneous Microbial Growth. Figure 2 shows the evolution of total bacterial count, Gram-negative bacteria, Enterococci, lactic acid bacteria, Enterobacteriaceae, and total coliforms as a function of storage time in the laboratory raw whole sample kept at 25 °C and in the industrial raw and pasteurized whole egg samples kept at 25 and 4 °C. The laboratory-prepared raw whole egg sample contaminated by direct contact with the unwashed shells simulates the illicit industrial practice of preparing egg products with shell eggs not suitable for direct commercialization, such as broken eggs discarded by the grading and packing equipments or collected in containers placed below the packaging process critical points (points where the eggs are more frequently broken). Industrial whole egg samples, instead, were analyzed to evaluate the contamination levels observed in raw and pasteurized products treated under standard industrial good hygienic conditions. The raw and pasteurized samples were stored at 25 °C to accelerate microbial growth and reduce the time of each trial and at 4 °C, the maximum storage temperature allowed by the European legislation (*4*), commonly used during the commercialization of egg products.

The initial total bacterial counts of industrial raw egg products (about 10^6–10^7 cfu/mL; **Figure 2B** and **D**) were higher than that observed in the laboratory raw whole egg sample (10^4 cfu/mL; **Figure 2A**) prepared with grade A eggs (unwashed, but clean) because, unlike the industrial samples, the laboratory prepared sample at time 0 was analyzed immediately after shelling. Comparing the initial bacterial counts of raw (**Figure 2B** and **D**) and pasteurized (**Figure 2C** and **E**) samples, it is evident that heat treatment (65.4 °C × 3 min in both trials) efficiently reduced the TBC by about 6 log cycles.

In all of the samples stored at 25 °C, TBC exponentially increased during the first two days reaching final values around 10^8–10^9 cfu/mL. At 4 °C, instead, a lag phase was observed, lasting about two days for raw (**Figure 2D**) and eight days for pasteurized (**Figure 2E**) products. Furthermore, the lower temperature delayed reaching the 10^8−10^9 cfu/mL upper limit value.

Gram negatives, as already reported by Stoeppler (*31*), were the most abundant microbial group in all of the samples, with

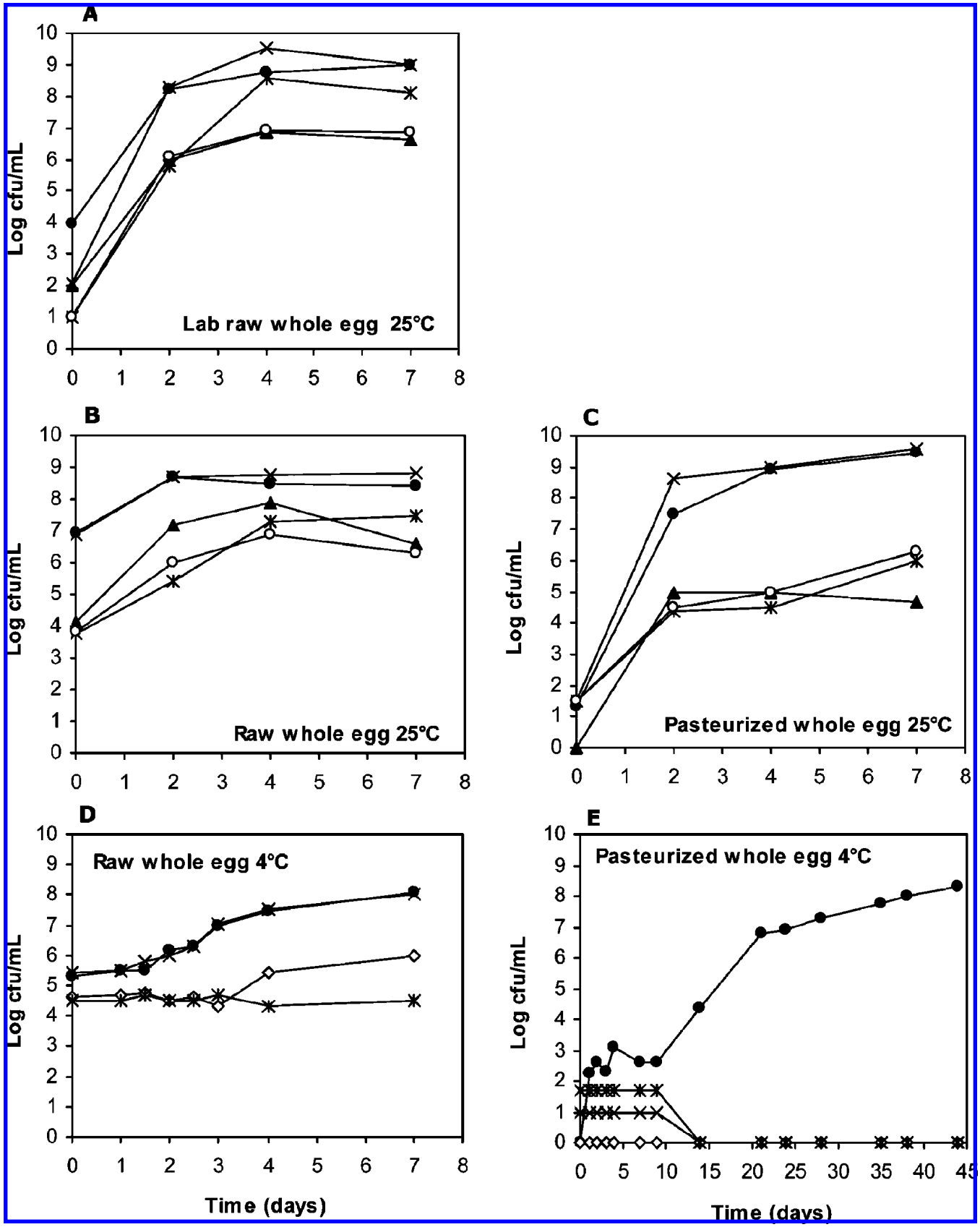

Figure 2. Evolution of total bacterial count (●), Gram-negative bacteria (×), Enterococci (*), lactic acid bacteria (○), Enterobacteriaceae (▲), and total coliforms (♦) during storage at 25 and 4 °C of laboratory prepared raw whole egg and industrial raw and pasteurized whole egg.

the exception of the pasteurized product kept at 4 °C, where their number reduction (number already negligible at time zero (10 cfu/mL)) was observed after 15 days of storage. In this case, the low storage temperature following heat treatment favored the growth of other thermoduric forms. Enterococci, lactic acid bacteria, and Enterobacteriaceae exhibited an exponential growth

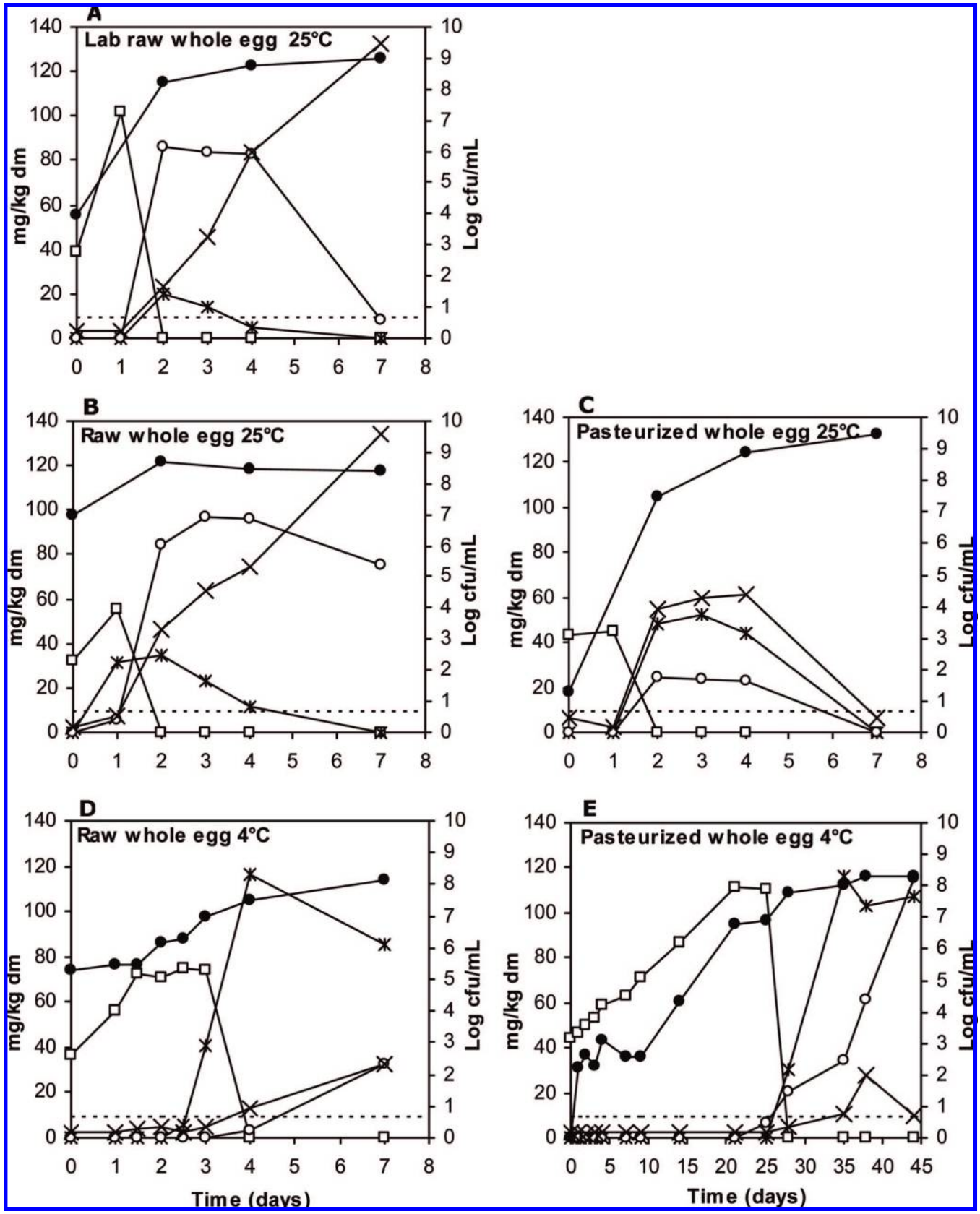

Figure 3. Evolution of total bacterial count (log cfu/mL) (●), uridine (□), uracil (*), lactic acid × 10^{-2} (○), and acetic acid × 10^{-2} (×) contents (mg/kg dm) during storage at 25 and 4 °C of laboratory prepared raw whole egg and industrial raw and pasteurized whole egg. The dotted line represents the legal limit for lactic acid (1000 mg/kg dm) (*4*).

during storage at 25 °C, reaching similar levels of about 10^7–10^8 cfu/mL in raw and 10^5–10^6 cfu/mL in pasteurized products. However, the storage at 4 °C did not favor Enterococci and coliforms (scarcely psycrotrophic) development. The Enterobacteriaceae count is the only microbial criterium considered by the European law for the evaluation of process

Table 1. Microbial Strains Isolated from Different Whole Egg Products after 2 Days of Storage at 25 °C

product	Gram negative	Gram positive
laboratory raw whole egg	*Enterobacter cloacae* *Escherichia coli*	*Enterococcus avium*
industrial raw whole egg	*Escherichia coli* *Morganella morganii* *Serratia liquefaciens*	*Enterococcus faecalis*
industrial pasteurized whole egg	*Aeromonas hydrophyla* *Pseudomonas fluorescens*	*Enterococcus faecalis*

hygiene, but its threshold limit (100 cfu/g) (*11*) was largely exceeded in the pasteurized product after only one day of storage at 25 °C, confirming the necessity of refrigeration for these products.

With regards to chemical parameters (**Figure 3**), at the beginning of storage, uridine concentration was between 32 and 44 mg/kg dm in all samples, higher than the values reported for the albumen (10–12 mg/kg dm) and for the yolk (2–5 mg/kg dm) of newly laid shell eggs (*29*). The higher values found might be justified not only by the high natural variability of uridine in eggs (*29*) but also by the utilization of not newly laid eggs for all laboratory and industrial products since uridine content in shell eggs increases during storage (*32–34*). However, uracil was absent, while initial concentrations of lactic and acetic acids were very low (1–7 mg/kg dm). In eggs, these compounds are metabolites produced by microbial degradation (*34*), while relevant quantities of uridine are naturally present.

Comparing the initial values of raw (**Figure 3B** and **D**) and pasteurized (**Figure 3C** and **E**) products, only uridine was slightly influenced by heat treatment (increasing from 32 to 43 and from 37 to 44 mg/kg dm, respectively). Hidalgo et al. (*34*) reported a significant rise of uridine content in tomato products as a consequence of heat treatment. However, no influence of heat treatment on the levels of uracil and lactic acid was reported by Hidalgo et al. (*13*) in egg products of differing hygienic quality. The thermostability of uracil was verified in tomato products that underwent very strong conditions (up to a treatment equivalent to 5264 min at 121 °C) (*34*).

During storage, an initial increase in uridine was observed in all samples, with the exception of the pasteurized product kept at 25 °C (**Figure 3C**). Hidalgo et al. (*13*) observed a similar increase during storage at 4 °C of pasteurized whole egg samples from fresh eggs, similar to what happens during the storage of shell eggs (*14*), as a consequence of the residual activity of native enzymes, that release this nucleoside from RNA even after egg shelling. In the laboratory raw whole egg sample (**Figure 3A**) and in the industrial raw and pasteurized whole egg samples kept at 4 °C (**Figure 3D** and **E**), the maximum concentrations of uridine were, respectively, 102, 75, and 111 mg/kg dm. Possibly, the industrial samples stored at 25 °C (**Figure 3B** and **C**) might have reached similar values, but the accelerated kinetics, given by the higher initial TBC and storage temperature, and the extended sampling intervals did not allow for the determination of the period of maximum uridine accumulation. After reaching the maximum values, uridine decreased in sync to uracil formation, rapidly (after 2 days) in samples kept at 25 °C (**Figure 3A**, **B**, and **C**) and more slowly (after 4 days for raw and after 28–35 days for pasteurized products) in samples stored at 4 °C (**Figure 3D** and **E**).

Uridine was already identified as the precursor of uracil: nucleoside phosphorylases released by different microorganisms catalyze the degradation of uridine, with the formation of ribose-1-phosphate and uracil (*15, 35–37*). Previous research suggested that uracil formation started at microbial contamination levels around 10^6 cfu/g (*34, 38*). However, the absence of uracil in the initial point of the kinetics of industrial raw samples kept at 25 °C (**Figure 3B**) suggests that such TBC levels are not sufficient to induce uracil formation but that a lag time is also necessary. In industries, this relatively high contamination can be reached even working under good hygienic conditions but does not lead to the formation of uracil if the product is maintained at low temperatures and/or if it undergoes an immediate heat treatment. In samples stored at 25 °C (**Figure 3A**, **B**, and **C**), uracil first increased up to 20–53 mg/kg dm, and then decreased to undetectable concentrations as a consequence of accelerated microbial metabolism (**Figure 2B** vs **D** and **C** vs **E**). A similar trend was reported by Hidalgo et al. (*38*) during incubation at 30 °C of uridine-enriched broth inoculated with *Lactobacillus plantarum* or *Lactobacillus fermentum*. These authors remarked that stress conditions, such as a glucose decrease in culture media, prevent uracil disappearance in the medium, as observed also in tomato pulp (*38*). In an ideal growth medium, uracil is degraded by specific enzymes, such as uracil phosphoribosyltransferase (*39*), uracil reductase, and dihydrouracil dehydrogenase (*40*), and the degradation products are used to synthesize other compounds. However, several adverse conditions (i.e., nutritional starvation or mild heat shock) can trigger the rapid degradation of uracil permeases, limiting exogenous uracil uptake (*41, 42*). Egg is an ideal substrate for microbial development and degradation of uracil; however, since egg products are currently stored at temperatures lower than 4 °C, the 25 °C kinetics reported in this study are not realistic. After only 2 days of storage at 25 °C, the raw samples (**Figure 3A** and **B**) were strongly deteriorated (from a sensory point of view) and were no longer suitable for human consumption. In contrast, in the samples stored at 4 °C (**Figure 3D** and **E**) uracil reached very high levels (116 mg/kg dm) and did not disappear even after storage times longer than normal. The legislation (*4*) imposes a maximum 48 h storage time at 4 °C for raw liquid egg. The shelf life for liquid pasteurized egg products stored in tanks is five days, but can reach 28 days in aseptically packaged samples.

Lactic acid exhibited similar kinetics of formation and/or degradation as that observed for uracil, achieving maximum concentrations within 2 to 3 days in the laboratory-prepared raw whole egg (**Figure 3A**) and industrial raw (**Figure 3B**) samples kept at 25 °C, and after 25 days in the pasteurized whole egg product stored at 4 °C (**Figure 3E**). Acetic acid, in contrast, continued to increase in laboratory and raw whole egg samples stored at 25 °C (**Figure 3A** and **B**) and reached maximum concentrations by day 4 in raw whole egg samples stored at 4 °C (**Figure 3D**), while in the pasteurized products, it decreased only at the end of the storage period. These results substantiate the formation of these metabolites as a consequence of microbial contamination, and their stability after formation at refrigeration temperatures, through all the shelf life period.

A different sensitivity for the chemical indices is evident for the industrial raw egg product after 1 day of storage at 25 °C (**Figure 3B**): uracil was already plentiful (30 mg/kg dm), while lactic acid was still below the legal limit (1000 mg/kg dm). This condition is more evident in the industrial raw egg product stored at 4 °C (**Figure 3D**), where uracil was already detectable after 2.5 days, while lactic acid remained below the legislation limit even after 4 days. No legislation or reference values exist for acetic acid, but its levels were similar to those of lactic acid. A lower sensitivity of lactic acid in comparison to that of uracil was already observed by Hidalgo et al. (*13*).

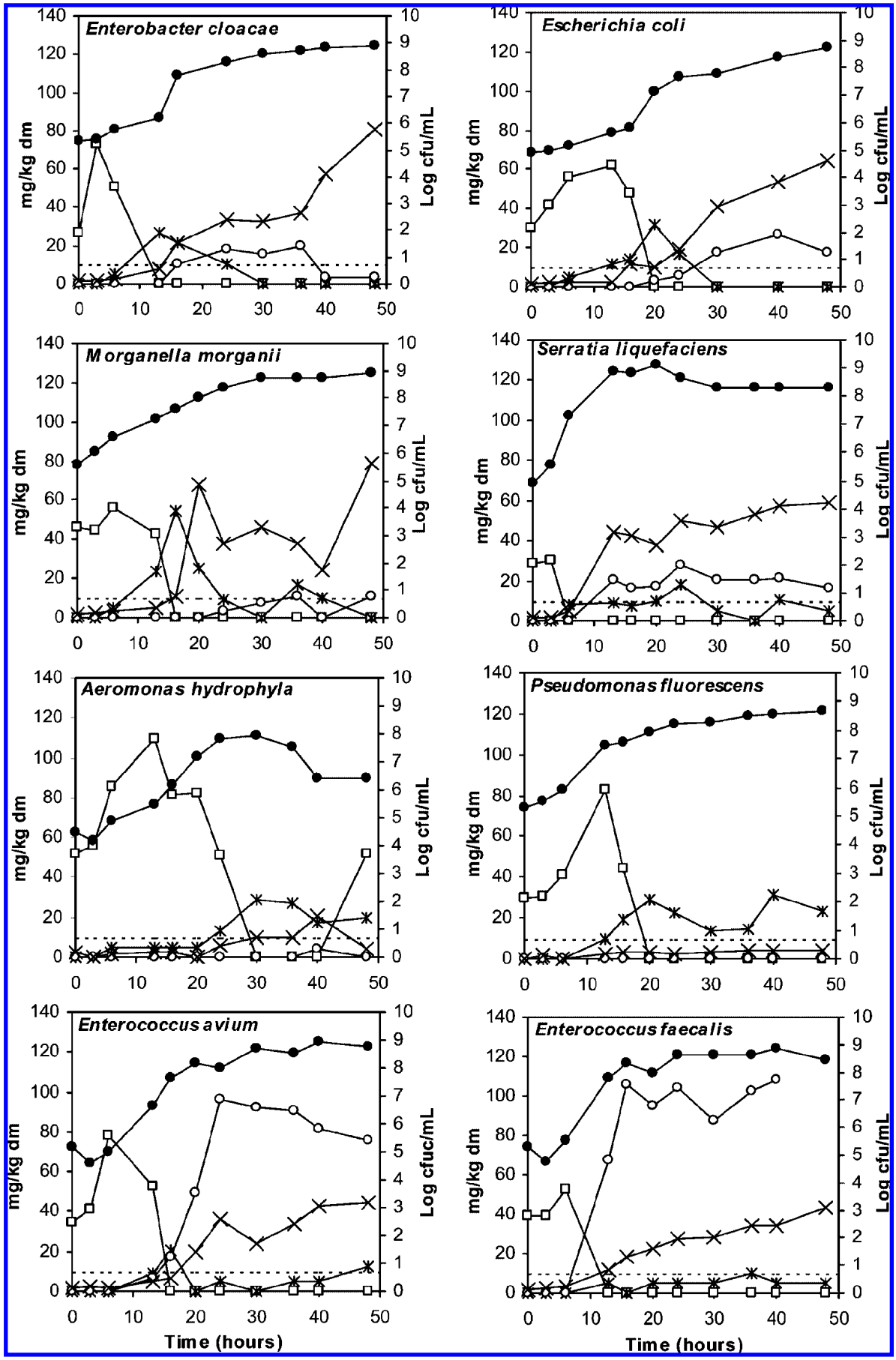

Figure 4. Evolution of total bacterial count (log cfu/mL; ●), uridine (□), uracil (*), lactic acid × 10^{-2} (○), and acetic acid × 10^{-2} (×) contents (mg/kg dm) during incubation at 25 °C of raw whole egg aseptically sampled from fresh egg inoculated with different microbial strains. The dotted line represents the legal limit for lactic acid (1000 mg/kg dm) (*4*).

Selected Microbial Strains Growth. The eight microbial strains reported in **Table 1** were isolated and identified from the different egg products analyzed during the spontaneous growth trial after 2 days of storage at 25 °C. *Salmonella* spp. were always absent. The trial conditions strongly favored the development of microbial population that commonly spoil the raw material used in the manufacturing of egg products.

To evaluate the microbial strains synthesizing capability for the studied metabolites, the isolated microorganisms were separately inoculated in aseptically prepared samples of whole egg. **Figure 4** shows microbial growth as well as uridine, uracil, lactic, and acetic acids contents during the incubation of samples at 25 °C. This temperature was chosen to accelerate microbial metabolism and to reduce the length of the trials.

As already observed in the spontaneous microbial growth, uridine was present (36 ± 9 mg/kg dm) at time zero in all of the samples analyzed. During incubation, uridine increased, reaching a maximum of 52–110 mg/kg dm, an amount that does not seem to depend on the initial uridine value. Afterward, uridine decreased until total degradation, while at the same time, uracil was formed. For almost all microorganisms, the point of uridine disappearance is linked to the maximum value reached by uracil (20−55 mg/kg dm), with the exception of *Serratia liquefaciens* and *Enterococcus faecalis*.

Uracil was subsequently degraded by *Enterobacter cloacae*, *Escherichia coli*, *Morganella morganii*, and *Enterococcus avium*; however, the two last strains formed it again, although in lesser quantities. The formation/degradation of uracil by these microorganisms was anticipated with respect to the behavior observed in the spontaneous microbial growth trials, notwithstanding the identical temperature of storage/incubation. This phenomenon might be attributed to the lack of interspecies competition or of stress by the technological industrial operations. A faster deterioration of the product's sensorial traits was also perceived during the preparation of the samples.

With regard to *Aeromonas hydrophyla*, *Pseudomonas fluorescens*, *Serratia liquefaciens*, and *Enterococcus faecalis*, uracil levels varied during storage but never completely disappeared.

Uracil, therefore, was formed (in different quantities) by all microorganisms. Lactic acid, instead, was produced at levels below the legal limit by *Morganella morganii* and was not formed by *Aeromonas hydrophyla* and *Pseudomonas fluorescens*. The other microorganisms produced quantities significantly higher than the law threshold: *Enterococcus faecalis* (12316 mg/kg dm), *Enterococcus avium* (9648 mg/kg dm), *Serratia liquefaciens* (2781 mg/kg dm), *Escherichia coli* (2676 mg/kg dm), and *Enterobacter cloacae* (1980 mg/kg dm). For this last strain, a certain degradation was registered after 40 h.

Acetic acid, however, was produced by all strains (with the exception of *Pseudomonas fluorescens*) and was never degraded.

The early and significant uracil formation suggest a major sensibility of this marker in comparison to that of lactic and acetic acid. However, the nondegradation of acetic acids observed in all of the trials could help to detect the use of extremely altered raw materials, diluted with hygienically acceptable products to hide sensorial alteration.

Conclusions. The formation of uracil, lactic acid, and acetic acid happened mainly after microbial spoilage. Early and widespread formation suggests uracil as the most appropriate marker for assessing the hygienic quality of raw materials used in pasteurized egg products. Since uracil is naturally absent in eggs and is formed from uridine by high microbial contamination ($>10^6$ cfu/g) after a lag time, detectable uracil levels evidenced the use of highly contaminated raw material. Thus, the limit for uracil could be just its detection limit, corresponding to 4.7 mg/kg dm in whole egg product, according to the analytical procedure applied in the present work.

The microbial metabolites, lactic and acetic acids, are also interesting indices of hygienic quality. However, both substances are naturally present in shell eggs, even if in reduced quantities, therefore requiring the establishment of limits. The legislation limit for lactic acid should be revised; furthermore, because of its thermostability (*13*), this parameter should be utilized also on pasteurized egg products and not, as imposed by the current legislation (*4*), only on raw products. Before establishing limits for acetic acid, its natural variability and stability during egg product manufacturing (i.e., thermal stability) should be determined.

ABBREVIATIONS USED

cfu, colony forming units; dm, dry matter; HPLC, high-performance liquid chromatography; KEA, kanamicin azide esculine agar; MRS, Man Rogosa Sharpe; Mug, methylumbelliferyl-beta-D-glucoronide; PCA, plate count agar; PMK Mug, plate count monensin-KCl agar Mug; TBC, total bacterial count; VRB, violet red bile; VRBD, violet red bile destrose.

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