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Uracil evolution in tomato pulp inoculated with different microbial strains during a long incubation time

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Abstract

Uracil has been previously proposed as a successful index of lactic acid bacteria (LAB) contamination in tomato products. The uracil produced in tomato pulp, as a consequence of uridine hydrolysis by different LAB strains (*Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, and *Pediococcus* spp.), remained constant over the entire incubation time (180 days), confirming uracil as a good index of LAB contamination. An anaerobic spore-forming microorganism (*Clostridium pasteurianum*) was also able to produce uracil. In order to deepen the understanding of uracil formation mechanisms, uracil and uridine were determined in De Man Rogosa Sharpe (MRS) uridine-enriched broth inoculated with some LAB strains: the maximum level of uracil formation was followed by a total or partial uracil decrease. Only under stressful conditions, such as reduced glucose concentrations (0.2–0.02%) in culture media, did LAB strains not degrade or use the uracil produced, as observed in tomato pulp.

Keywords: *Clostridium pasteurianum*; Lactic acid bacteria; Hygienic quality; Tomato products; Uracil

1. Introduction

The hygienic quality of tomato products before sterilization is a consequence of raw material quality, depending not only on sanitary conditions of both the tomato berries and the pre-treatment operations, but also on whether deteriorated tomato products are remanufactured. Microbial spoilage of the tomato products is mainly caused by under-processing and leakage. Lactic acid bacteria (LAB) and, secondarily, some spore-forming organisms, such as *Bacillus coagulans* and *Clostridium pasteurianum* (Gould, 1992; Lund & Snawdon, 2000), are the organisms usually responsible for such deterioration. Like yeasts and molds, LAB too can spoil tomato berries (ICMSF, 1998; Mossel, Corry, Struijk, & Baird, 1995).

Recently, uracil, a highly thermostable base, has been proposed as a successful index of LAB contamination of tomato products (Hidalgo, Pompei, Galli, & Cazzola, 2005). This research demonstrated that, unlike yeasts and molds, LAB inoculated in tomato pulp produce high quantities of uracil, coupled with the disappearance or the reduction of uridine, which is naturally present in tomato fruit. The level of the uracil formed by the hydrolytic activity of LAB strains did not decrease over a 30-day incubation time of spoiled tomato pulp. Hidalgo et al. (2005) concluded that uracil, together with the legal index Howard Mold Count (EEC, 1986; FDA, 2006; Howard, 1911), is able to provide more complete information about the microbial contamination history of tomato products, especially because it could allow the detection of the reprocessing of deteriorated tomato products. Since these industrial (bag-in-drum package) tomato products undergo long storage times, it is necessary to verify uracil stability over long incubation times.

The aim of this study was to verify uracil stability in tomato pulp inoculated with different LAB strains and *Cl. pasteurianum*, during a long incubation time (six

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months) and to improve the understanding of uracil formation mechanisms.

2. Materials and methods

2.1. Uracil and uridine evolution in tomato pulp inoculated with microbial strains

Nine 700 g bottles of tomato pulp of the same brand and batch were purchased from a supermarket. From each bottle, 40 ml aliquots of tomato pulp were aseptically transferred into 16 sterile containers or large tubes. Each of the nine series of samples was then inoculated with one microbial strain. Five LAB strains from the DISTAM collection were used: *Lactobacillus brevis* isolated from two different sources (Lb1 and Lb2), *Lactobacillus fermentum* and *Leuconostoc mesenteroides* (Lm1, used for two different assays: a and b; and Lm2). Two strains from the DSMZ collection (Braunschweig, Germany): *Lactobacillus buchneri* (cod. 20057), and *Cl. pasteurianum* (cod. 525, used for two different trials: a and b) were also tested. All samples were incubated at 30 °C, with the exception of *L. mesenteroides*, which was kept at room temperature. For *Cl. pasteurianum*, gas-pack anaerobic conditions were adopted. Microbial cell count, uracil, uridine, and dry matter determinations were performed at different intervals of time, up to 180 days of incubation. At time 0, chemical analyses were performed on the tomato pulp, whereas the microbial count was carried out on the freshly inoculated samples.

2.2. Uracil and uridine evolution in De Man Rogosa Sharpe (MRS) broth inoculated with LAB

In a first test, uridine and uracil contents were evaluated in MRS broth (Merck, Darmstadt, Germany), and in MRS broth supplemented with 100 mg/l of uridine (Sigma Chemical, Company, St. Louis, MO), separately inoculated with the following lactic acid bacteria strains from the DISTAM collection: *L. brevis* (Lb1), *L. fermentum*, *Lactobacillus plantarum*, *L. mesenteroides* (Lm1) and *Pediococcus* spp. Analyses were performed after 72 h of incubation at 30 °C, with the exception of *L. mesenteroides*, which was kept at room temperature. In a second test, the evolution of uridine and uracil contents was evaluated in MRS broth, as well as in MRS broths enriched with 100 mg/l of uridine and with different glucose concentrations (2%, 0.2% and 0.02%), separately inoculated with *L. plantarum* and *L. fermentum*. Incubation temperature was 30 °C; the tests lasted 80–144 h.

2.3. Chemical analyses

Dry matter content (g/100 g of product) was determined, following the AOAC official gravimetric method no. 964.22 (AOAC, 1995). Uracil and uridine analyses were performed using the HPLC analytical method reported by Hidalgo et al. (2005).

For peak quantification, calibration curves were constructed using 19 different concentrations (from 0.2 to 50 mg/l) of the uracil standard (Merck, Darmstadt, Germany), and 12 different concentrations (from 5 to 85 mg/l) of the uridine standard (Sigma Chemical Company, St. Louis, MO). On the basis of the calibration curves, the detection limits were calculated as the intercept value of the regression line plus three times the standard error of the estimate (Miller & Miller, 1988). The results are expressed as mg/kg dm. In the concentration ranges considered, uracil and uridine calibration curves were linear ($r^2 = 1.0$; $p \leqslant 0.001$) and showed detection limits of 0.70 and 1.2 mg/l, respectively, corresponding to 33.3 and 57.5 mg/kg dm in tomato pulp. The tomato pulp values were calculated by considering a mean dry matter content of 8 g/100 g.

2.4. Microbiological analyses

The microbial cells for tomato pulp inoculation, MRS broth, and modified MRS broths were obtained by centrifugation from an overnight broth culture of each microorganism and then put into a pH 7 tryptone salt solution (NaCl, 8.5 g; tryptone, 1 g; distilled water, 1000 ml). To obtain a proper inoculum concentration, a cell count in the stock suspensions was performed with a Burker chamber in the optical microscope.

Each microorganism was inoculated in different series of containers with 30 ml of MRS broth or modified MRS broths or 40 ml of sterile tomato pulp. The concentration of the cells inoculated was 10^3–10^4 cfu/ml.

Microbial growth was monitored by plate counting, using the serial decimal dilution technique. Microbial count was performed by pour plates in MRS Agar (Merck, Darmstadt, Germany), with incubation at 30 °C for 48–72 h under anaerobic conditions (De Man, Rogosa, & Sharpe, 1960) in gas pack (Oxoid, Basingstoke, England); *L. mesenteroides* plate incubation was carried out at room temperature.

Only plates with 30–300 colonies were considered for computing the colony forming units (cfu), using the equation of Peeler and Maturin (1992). The results were expressed as total number of microbial cells per gramme of analysed product (cfu/g).

All chemical and microbiological determinations were performed in duplicate.

3. Results and discussion

3.1. Uracil and uridine evolution in tomato pulp inoculated with microbial strains

Fig. 1 shows the uracil and uridine content evolutions in tomato pulp during the growth of *L. buchneri*, *L. fermentum* and two different strains of *L. brevis* (Lb1, Lb2). The total disappearance of the uridine initially present (*ca.* 3.69 mmol) led to the formation of an almost equimolar

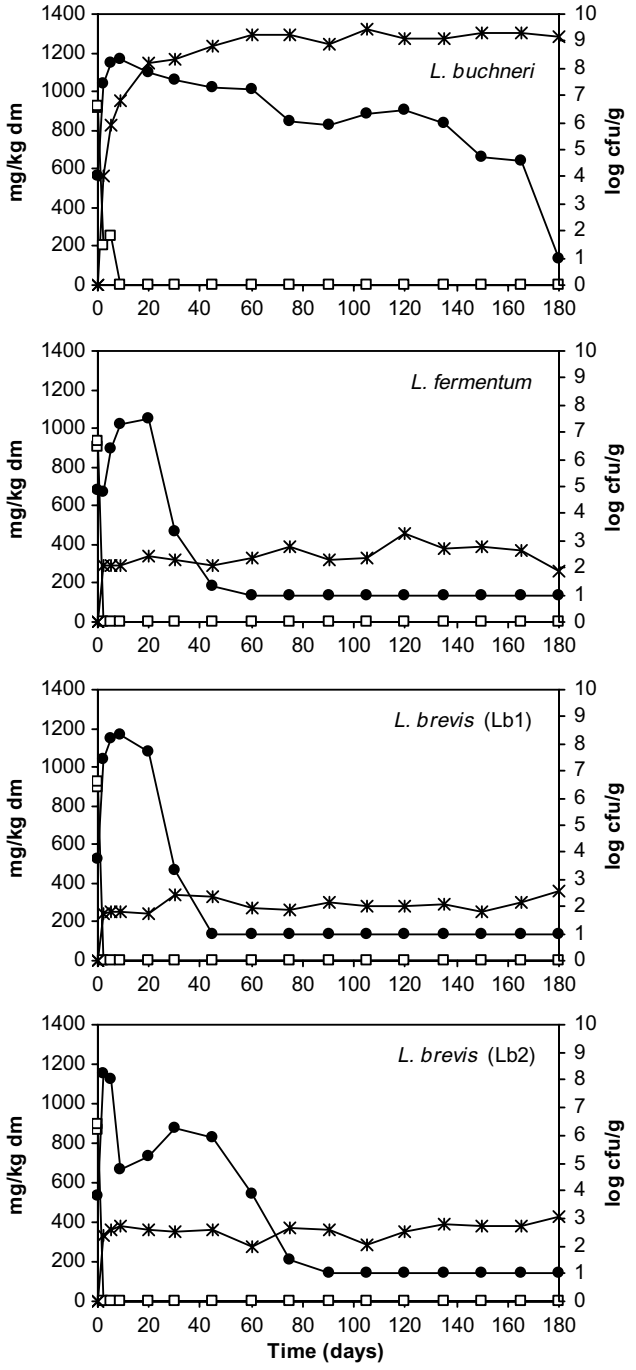

Fig. 1. Uracil (✱), uridine (□), and microbial count (●) evolution during incubation of tomato pulp inoculated with different LAB species. Lb1 and Lb2 represent two different strains of the same species, as indicated in Section 2.

quantity of uracil (*ca.* 3.57 mmol) for all the microorganisms, apart from *L. buchneri*. In this last case, the high quantity of uracil formed does not seem to be produced only from the uridine naturally present, but it also seems to be synthesized in other ways. In fact, the ability of some microorganisms to deaminate cytidine to uridine by cytidine deaminase (Sakai, Tochikura, & Ogata, 1965) has been reported. It is important to note that the quantity of uracil formed during the long incubation period considered (180 days) did not decrease for any microorganism. The uracil values of *L. fermentum* were exactly the same as those reported by Hidalgo et al. (2005), whereas those formed by *L. brevis* proved to be lower in both incubation tests conducted.

Fig. 2 shows the evolution of uridine and uracil contents during the growth of *L. mesenteroides* in tomato pulp. The first trial (Lm1-a) presented a different uridine evolution and higher uracil levels than those reported by Hidalgo et al. (2005) for the same microorganism. To verify the behaviour of different strains of the same specie, two trials were conducted, inoculating, respectively, the strains Lm1 and Lm2 of the DISTAM collection. In both assays (Lm1-b and Lm2) uridine never disappeared and remained at levels similar to those reported by Hidalgo et al. (2005). Uracil content, instead, in the first trial, reached values of 65 mg/kg dm while, in the other test, it never reached significantly detectable levels. These results indicate that

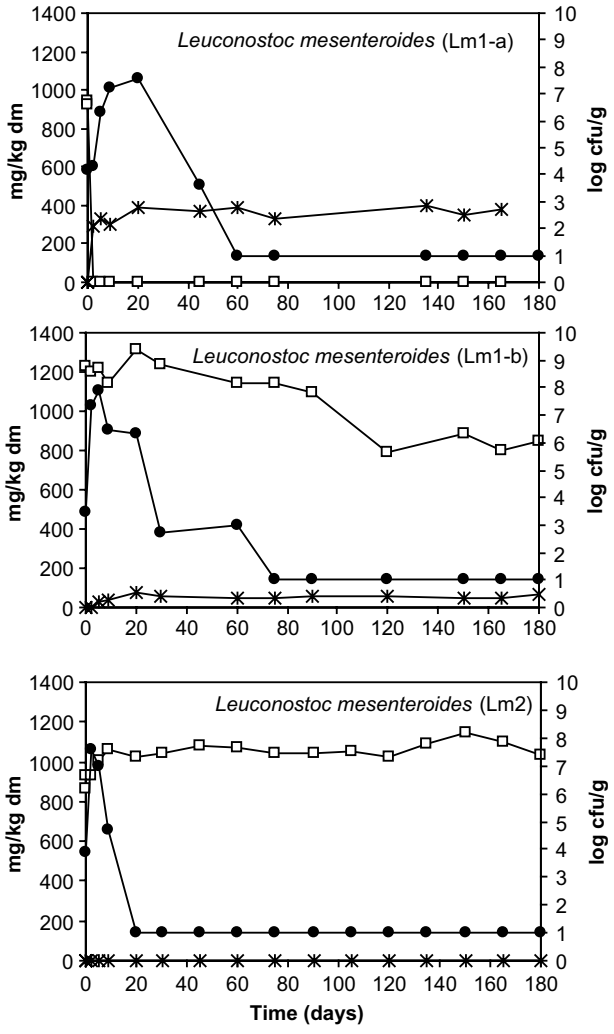

Fig. 2. Uracil (✱), uridine (□), and microbial count (●) evolution during incubation of tomato pulp inoculated with two different strains of *Leuconostoc mesenteroides* (Lm1 and Lm2), as indicated in Section 2; a and b are replicated assays of the same strain.

microbial biodiversity can also translate into different uracil production levels.

Fig. 3 shows the evolution of uridine and uracil contents during the growth of *Cl. pasteurianum* in tomato pulp. In the first test, the initial uridine content remained unchanged, and consequently no uracil formation was observed. Instead, in the second test, there was a slight variation in the level of uridine, with the production of a low quantity of uracil. Our results in these last two tests could be attributed to relatively low and variable microbial growth. According to the findings of Hidalgo et al. (2005), values of over 10^6 cfu/ml should be present in order to detect a significant uracil content in tomato pulp. It was, however, demonstrated that an anaerobic spore-forming microorganism, in this case *Cl. pasteurianum*, can also form uracil. Similarly, Pollach, Hein, Leitner, and Zoellner (2002) reported uracil production by two other clostridia strains (*Cl. thermosaccarolyticum* and *Cl. thermohydrosulfuricum*) and suggested uracil as a marker of the undesirable clostridial fermentation during extraction of sugar from sugar beet pulp.

3.2. Uracil and uridine evolution in De Man Rogosa Sharpe (MRS) broth inoculated with LAB

Uridine and uracil contents in the MRS broth were low (Table 1). In order to detect the two compounds' variations following the development of the LAB, 100 mg/l of uridine were supplemented. In the MRS broth, and in that enriched with uridine, the initial uridine content disappeared after three days of incubation for all LAB, apart

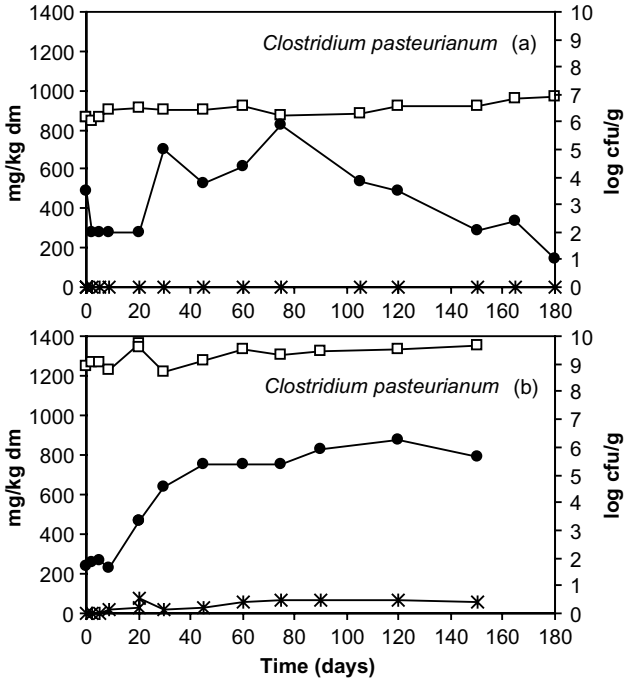

Fig. 3. Uracil (✱), uridine (□), and microbial count (●) evolution during incubation of tomato pulp inoculated with a *Cl. pasteurianum* strain; a and b are replicated assays of the same strain.

Table 1
Uridine and uracil contents (mg/l) in MRS and MRS enriched with uridine, before inoculation with different strains of LAB and after three days of incubation

	MRS		MRS + 100 mg/l uridine	
	Uridine	Uracil	Uridine	Uracil
Before inoculation	14 (0.06)	8 (0.07)	114 (0.47)	9 (0.08)
L. plantarum	0	0	0	5 (0.04)
L. brevis	0	0	0	33 (0.29)
L. fermentum	0	0	0	36 (0.32)
L. mesenteroides	0	0	20 (0.08)	32 (0.28)
Pediococcus sp.	0	0	0	9 (0.08)

Concentration values, expressed in millimoles, are in brackets.

from *Leuconostoc mesenteroides*, where the concentration dropped from 114 to 20 mg/l in the uridine-enriched broth. Hypothesizing an equimolar transformation of uridine into uracil, a quantity of uracil equal to the sum of the millimoles of uridine plus those of uracil initially present in the broths should be detected. This sum gives a theoretical value of 0.13 mmol in the MRS broth and 0.55 mmol in the enriched broth. However, the uracil content measured after three days of incubation proved to be lower than expected. Whereas, for all of the strains, it was undetectable in the MRS broth, in the medium with added uridine, the quantity of uracil found proved to be variable, depending on the microorganism: for example, practically no uracil was produced by *L. plantarum* and *Pediococcus* spp. (*ca.* 0.06 *vs* an estimated 0.55 mmol), whereas the uracil produced by *L. brevis*, *L. fermentum* and *L. mesenteroides* proved to be around 40% lower than expected (approx. 0.33 instead of 0.55 mmol). This suggests that, once formed, uracil disappears at different speeds, depending on the microorganism inoculated.

In order to better ascertain whether the uridine present in the broths before inoculation turns into uracil and whether the latter undergoes a partial or total decrease, the same experiment was repeated, measuring uridine and uracil contents at various times (Fig. 4). We chose to inoculate *L. plantarum* and *L. fermentum*, as these two microorganisms produced the highest and lowest final quantities of uracil, respectively. Initial and final (after 72 h) levels of uridine and uracil, both in the MRS broth (2% glucose) and in that enriched with uridine (2% glucose + uridine), proved to be identical to those obtained in the previous experiment (Table 1). The maximum level of uracil formed corresponded to the total disappearance of the uridine initially present in the uridine-enriched broth, followed by the total disappearance of uracil for *L. plantarum* and a partial disappearance for *L. fermentum*. In both cases, the initial number of millimoles of uridine (0.48) was almost equal to the maximum amount of uracil produced (0.52).

The different microbial metabolism, with regard to uracil stability/degradation observed in tomato products and MRS broth, can be ascribed to the composition of the substrates. The uracil decrease in the ideal growth medium can be attributed to a degradation of this metabolite for the synthesis of other compounds. Uracil phosphoribosyltrans-

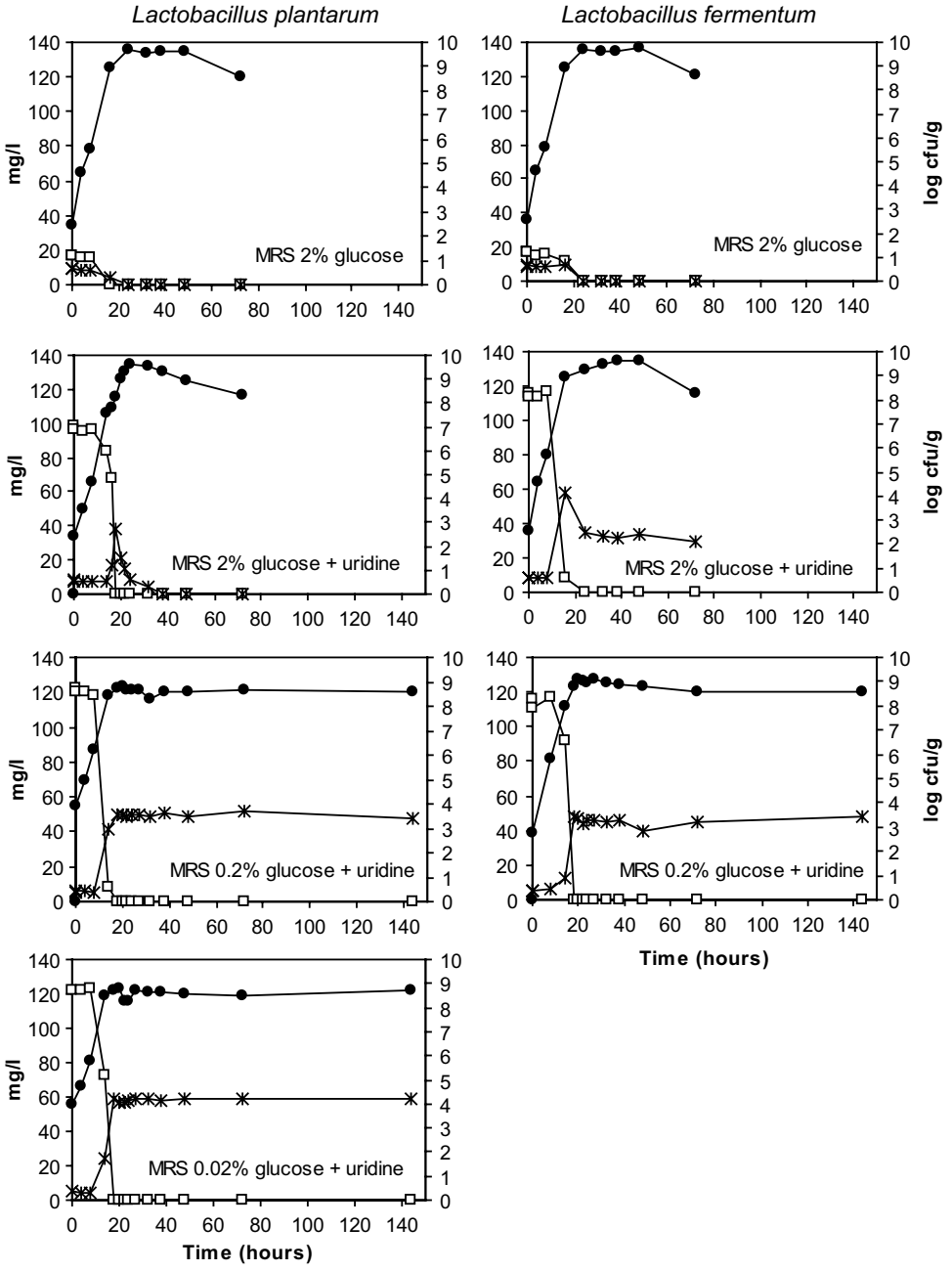

Fig. 4. Uracil (✱) and uridine (□) evolution during *L. plantarum* and *L. fermentum* growth (●) in MRS (2% glucose), and in MRS supplemented with 100 mg/l of uridine and different glucose concentrations.

ferase (Ashihara, Stasolla, Loukanina, & Thorpe, 2000), uracil reductase and dihydrouracil dehydrogenase (Katahira & Ashihara, 2002) are enzymes able to transform uracil into other compounds. Cells can convert free pyrimidine bases or nucleosides to nucleotides, but the process differs in different organisms. The free bases which originate from the environment, or from the catabolic breakdown of RNA, can undergo the pyrimidine salvage pathway (Séron, Blondel, Haguenauer-Tsapis, & Volland, 1999). For example, in the yeast *Saccharomyces cereviseae*, the highly efficient salvage pathway involves the uptake of uracil, cytosine and uridine, mediated by specific permeases. Several adverse conditions (nutritional starvation and mild heat shock) can trigger the rapid degradation of uracil permease and thus a loss of uracil uptake (Séron et al., 1999; Volland, Urban-Grimal, Geraud, & Haguenauer-Tsapis, 1994): we therefore hypothesized that stress conditions, as in tomato pulp, could limit uracil degradation.

In order to test this hypothesis, we tried to create a situation of stress in the MRS broth with added uridine, first by reducing the pH of the medium from 6.2 to 4.34 and then by lowering its sugar concentration. The reduction of pH did not permit any explanation because it inhibited microbial growth. The results of the second assay are shown in Fig. 4, where uridine and uracil evolution, during the growth of *L. plantarum* and *L. fermentum* in MRS

broth enriched with uridine and a lower glucose concentration (MRS 0.2 and 0.02% glucose + uridine), is depicted. The formation of uracil does effectively correspond to the complete disappearance of the uridine; furthermore, uracil concentration remains constant over time, as already observed for tomato products.

4. Conclusions

The validity and reliability of uracil as an index of LAB contamination of tomato products was confirmed. In fact, the quantity of uracil formed in tomato pulp during the growth of some strains of lactic acid bacteria did not decrease even after very long incubation periods. Differently, the uracil formed in a culture medium is degraded or used by the very microorganisms that produce it, but a lack of glucose in the broth creates a stressful condition which prevents uracil decrease.

Since an anaerobic spore-forming microorganism, such as *Cl. pasteurianum*, produces uracil, even if only in low quantities, finding it in a tomato product (when the absence of LAB is proven) may indicate that the thermal treatment has been ineffective in destroying its spores.

The results obtained by repeating selected tests in tomato pulp, performed using different microbial strains from the same species, showed that microbial diversity has a determinant effect on the quantity of uracil produced.

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