Cometabolism of Citrate and Glucose by *Enterococcus faecium* FAIR-E 198 in the Absence of Cellular Growth

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Citrate metabolism by *Enterococcus faecium* FAIR-E 198, an isolate from Greek Feta cheese, was studied in modified MRS (mMRS) medium under different pH conditions and glucose and citrate concentrations. In the absence of glucose, this strain was able to metabolize citrate in a pH range from constant pH 5.0 to 7.0. At a constant pH 8.0, no citrate was metabolized, although growth took place. The main end products of citrate metabolism were acetate, formate, acetoin, and carbon dioxide, whereas ethanol and diacetyl were present in smaller amounts. In the presence of glucose, citrate was cometabolized, but it did not contribute to growth. Also, more acetate and less acetoin were formed compared to growth in mMRS medium and in the absence of glucose. Most of the citrate was consumed during the stationary phase, indicating that energy generated by citrate metabolism was used for maintenance. Experiments with cell-free fermented mMRS medium indicated that *E. faecium* FAIR-E 198 was able to metabolize another energy source present in the medium.

Citrate metabolism is widespread among lactic acid bacteria (LAB), e.g., *Lactococcus lactis*, *Leuconostoc* spp., *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Oenococcus oeni* (8, 11, 17, 26, 28, 31, 32, 34, 35, 36, 37). The breakdown of citrate results in the production of acetate, formate, ethanol, acetaldehyde, acetoin, 2,3-butanediol, diacetyl, and carbon dioxide. Some of these compounds contribute to the flavor development in fermented foods. For instance, diacetyl production displays a distinct effect on the quality of dairy products, such as butter, buttermilk, and certain cheeses (8, 24). Also, the formation of eyes due to the production of carbon dioxide contributes to the texture development in some cheeses (25).

The ability to metabolize citrate is dependent on the presence of the enzyme citrate permease (CitP), which has been characterized in strains belonging to the genera *Leuconostoc*, *Oenococcus*, and *Lactococcus* (3, 13, 30, 47). Citrate can be used as the sole energy source or is cometabolized (17). When citrate is present as the sole energy source, uptake occurs via symport of divalent citrate and one proton or via uniport of monovalent citrate (21, 30). Also, it has been suggested that divalent citrate can be taken up via an antiport mechanism, which releases intermediates (e.g., pyruvate) or end products (e.g., acetate) of citrate metabolism in the medium (18). During cometabolism of citrate and a sugar (citrolactic fermentation), CitP catalyzes the exchange (antiport) of divalent anionic citrate and monovalent lactate. Citrate uptake is an electrogenic process, which together with the formation of a pH gradient across the cell membrane, results in the formation

of a proton motive force and hence generation of metabolic energy (3, 21).

Among different LAB genera, optimal citrate uptake occurs at low pH values, ranging from pH 4.0 to pH 5.5 (23, 28, 31). In the presence of sugar, citrate consumption has been shown to enhance growth of *Leuconostoc* spp. and, to a lesser extent, of *Lactococcus lactis* subsp. *lactis* (16, 41). In contrast, citrate does not affect the growth of *Lactobacillus casei* and *Lactobacillus plantarum* (28). Metabolism of citrate in the presence of a sugar is of importance for food quality, as citrate and sugar are both present in fermented food products, such as during the fermentation of milk that naturally contains 8 to 9 mM of citrate. In heterofermentative LAB, citrate is converted into pyruvate, which is further reduced to lactate. All reducing equivalents (NADH), which would normally be used for ethanol formation, are used for this reduction of pyruvate. This results in a metabolic switch of sugar degradation towards acetate production via the energy-generating acetate kinase pathway (6, 41). Consequently, in the presence of citrate, more energy is generated during sugar degradation, which can explain the observed growth activation of *Leuconostoc* spp. (38, 39). In homofermentative LAB, pyruvate is the common intermediate formed during sugar and citrate metabolism. In addition to lactate, acetate, via the acetate kinase pathway, can also be the end product of pyruvate degradation in these bacteria. In contrast to heterofermentative LAB, homofermentative LAB are able to generate energy when citrate is present as the sole energy source (17).

Recently, citrate metabolism by *Enterococcus* spp. received increasing attention (12, 31, 32, 35, 36, 37). It has been assumed that citrate metabolism and lipolysis by enterococci are responsible for the aroma and flavor development in Mediterranean cheeses (5, 14, 15, 42). Many of these cheeses naturally contain high numbers of enterococci (4, 5, 7, 22, 44). Some enterococcal strains have been cultivated for their use as

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starter cultures in the manufacture of mozzarella and Cebreiro (5, 7, 29). *E. faecium* K77D has been accepted for use as a starter culture in fermented dairy products (2).

E. faecium FAIR-E 198, a strain isolated from Greek Feta cheese, is able to consume citrate both in the absence and presence of glucose (37). However, as these fermentations were not performed under pH-controlled conditions and as the pH of the medium influences citrate consumption, no clear conclusions can be drawn about the (simultaneous) consumption of citrate and glucose by *Enterococcus*. Also, the influence of citrate itself on growth and citrate metabolism is difficult to explain under non-pH-controlled conditions, as an increase in citrate concentration, and hence citrate metabolism, results in a higher final pH of the medium (37). As the strain *E. faecium* FAIR-E 198 has been shown to be γ -hemolytic, vancomycin sensitive, and cytotoxin negative, its use in food fermentation processes can be considered (9, 46).

The aim of this study was to perform citrate fermentations with *E. faecium* FAIR-E 198 at constant pH. First, citrate metabolism and growth of *E. faecium* FAIR-E 198 at different constant pH values were studied in a medium without glucose. Second, glucose was added, and its effects on growth and citrate metabolism were investigated. Finally, to know to what extent citrate influences cellular growth and citrate metabolism in the presence of glucose, fermentations were performed using different initial citrate concentrations.

MATERIALS AND METHODS

Strain and strain propagation. *E. faecium* FAIR-E 198 was used throughout this study. This strain is available in the catalogue of enterococci of the FAIR-E collection (45). The strain was stored at -80°C in de Man-Rogosa-Sharp (MRS) medium (Oxoid, Basingstoke, United Kingdom) containing 25% (vol/vol) glycerol as a cryoprotectant. To prepare the inoculum, the strain was propagated twice in MRS medium at 37°C for 12 h, followed by cultivation in the medium used for the fermentations later on. The transfer volume was always 1% (vol/vol).

Media. Fermentations were performed in modified MRS medium (mMRS), composed of the following (in grams liter $^{-1}$): peptone (Oxoid), 10; Lab Lemco (Oxoid), 8; yeast extract (VWR International, Darmstadt, Germany), 4; K_2HPO_4 , 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.038. Depending on the fermentations, the final citrate concentration ranged from 8.5 to 150 mM. Glucose was used at a final concentration of 0, 111, and 222 mM.

Cell-free fermented (CFF) mMRS medium was obtained from mMRS medium, in which *E. faecium* FAIR-E 198 was grown overnight at 37°C and without pH control. This fermented mMRS medium was then centrifuged ($25,000 \times g$, 30 min) to remove the cells. After adjustment to pH 6.5 with 10 N NaOH, the supernatant was filter sterilized. Finally, depending on the fermentation, citrate (25 and 50 mM) was added to this CFF mMRS medium.

Solid MRS medium, used to determine cell counts, was prepared by the addition of 15 g liter $^{-1}$ agar to MRS broth.

Fermentation conditions, online analysis, and sampling. Fermentations were performed in a 15-liter computer-controlled, in situ sterilizable, laboratory fermentor (Biostat C; B. Braun Biotech International, Melsungen, Germany). Sterilization was performed at 121°C for 20 min. Citrate and glucose were autoclaved separately (20 min at 121°C) and aseptically added to the fermentor. The total working volume of the fermentor was 10 liters. To keep the medium in the fermentor homogeneous, agitation was performed at 100 rpm. The fermentor was inoculated with 1.0% (vol/vol) of the inoculum culture (the initial cell count was 1.5×10^4 CFU ml $^{-1}$) that was prepared as described above. The temperature, pH, and agitation were computer controlled and monitored online (Micro MFCs for Windows NT software; B. Braun Biotech International). All fermentations were performed under microaerophilic conditions only by flushing the headspace of the fermentor with sterile air (4 liters min $^{-1}$). The pH was kept constant during all fermentations through automatic addition of 10 N NaOH and 2 N HCl. Bacterial growth was also followed online by registration of the CO_2 (as a percentage [vol/vol]) in the headspace of the fermentor, using an automatic gas analyzer (EGAS-8 exhaust gas analyzer system; B. Braun Biotech International),

equipped with an infrared detector (Hartman & Braun, Frankfurt am Main, Germany).

At regular time intervals, samples were aseptically withdrawn from the fermentor to determine the optical density at 600 nm (OD_{600}), the cell dry mass (CDM), and cell counts (CFU). Concentrations of glucose, citrate, and different metabolites were determined by high-pressure liquid chromatography and gas chromatography coupled to mass spectrometry (see below). On the basis of these analyses, different biokinetic parameters were calculated (see below).

Fermentations. First, citrate metabolism by *E. faecium* FAIR-E 198 was studied in mMRS medium at 37°C and at constant pH 5.0, 6.0, 6.5, 7.0, and 8.0. During these fermentations citrate (50 mM) was added as the sole energy source. To determine the minimum and maximum pH values for growth and to obtain additional information on the growth of *E. faecium* FAIR-E 198 on citrate, additional static 100-ml fermentations in glass bottles using mMRS medium supplemented with 50 mM citrate were performed with different initial pH values (pH 4.5, 7.0, 8.0, 8.3, and 8.5), all carried out at 37°C without pH control. Second, the influence of different citrate concentrations (8.5, 50, 100, and 150 mM) in combination with glucose (111 and 222 mM) was investigated. All fermentations were performed in duplicate.

Analyses of citrate, glucose, and metabolites. Citrate, glucose, lactate, acetate, and formate concentrations were determined by high-pressure liquid chromatography, using a Waters chromatograph (Waters Corp., Milford, Massachusetts) equipped with a Waters 410 differential refractometer, a Waters column oven, a Waters 717plus autosampler, and Millenium software (version 2.10), as described previously (10). Briefly, cells and solid particles were removed from 1.5-ml samples (appropriately diluted with ultrapure water) by microcentrifugation ($13,000 \times g$, 20 min). Proteins were removed by the addition of an isovolume of 20% trichloroacetic acid, centrifuged ($13,000 \times g$, 20 min), and filtered through a nylon syringe filter (Euro-Scientific, Lint, Belgium). A 30- μl portion was injected into a Polyspher OA KC column (Merck) held at 35°C . A 0.005 N H_2SO_4 solution was used as the mobile phase at a fixed flow rate of 0.4 ml min $^{-1}$. Ethanol, acetoin, and diacetyl were determined by a dynamic headspace analyzer (HS-40; Perkin-Elmer, Ueberlingen, Germany) coupled to a QP 5050 gas chromatograph-mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia, Maryland), as described previously (36). Briefly, 5-ml samples of cell-free culture supernatant were incubated at 80°C for 20 min, purged, and pressurized with 35 ml of ultrapure helium per min. The isolated volatile compounds were driven through the transfer line (thermostat temperature, 100°C) and injected into an HP INNOWax capillary column (60 m by 0.25 mm) that was coated with cross-linked polyethylene glycol (film thickness, 0.25 μm) and connected without splitting to the ion source of the quadrupole mass spectrometer (interface line temperature, 250°C) operating in the scan mode within a mass range of m/z 35 to 300 at a rate of 1 scan s $^{-1}$. The carrier gas was helium (flow rate of 0.6 ml min $^{-1}$), and the injector temperature was set at 200°C . The temperature program was as follows: 35°C for 3 min, increase to 80°C at a rate of 5°C min $^{-1}$; 80°C for 5 min; increase to 180°C at a rate of 8°C min $^{-1}$; and 180°C for 5 min. Compounds were identified by computer matching of mass spectral data with data in the Shimadzu NIST62 mass spectral database and by comparing the retention times and mass spectra with those of standard compounds (Sigma-Aldrich, Steinheim, Germany). Quantification was performed by integrating the peak areas of total ion chromatograms using the Shimadzu Class 500 software and appropriate standard curves. The concentrations of both water-soluble and volatile metabolites were expressed as millimolar concentrations, and the yields of the different metabolites were determined after 24 h of fermentation.

Biokinetic analysis and modeling. The maximum specific growth rate (μ_{max}) and the specific death rate (k_d) were determined by linear regression (indicated by the correlation coefficient r^2) from the plots of $\ln \text{OD}_{600}$ versus time. The influence of acidity on the μ_{max} was then modeled using the equation of Rosso et al. (33). Modeling was performed by minimizing the sum of the least-square differences between modeled and experimental values using the solver function in Excel. The citrate consumption rate (r_{CA}) was determined by linear regression (indicated by the correlation coefficient r^2) from the plots of $\ln [\text{citrate}]$ versus time. The yield coefficients of acetate (Y_{AA}), formate (Y_{FA}), ethanol (Y_{ET}), acetoin (Y_{AC}), and diacetyl (Y_{DI}), based on citrate consumption, were calculated on a molar basis [mol product (mol citrate) $^{-1}$] after 24 h of fermentation. The yield of lactate (Y_{LA}) was based on glucose consumption (mole of lactate [mole of glucose] $^{-1}$). The carbon recovery ($100 \times \text{C mole of products formed} [\text{C mole of citrate consumed}]^{-1}$) was calculated with adjustment for CO_2 production that was derived from the theoretical stoichiometric balance: $[\text{CO}_2] = [\text{acetate}] + [\text{ethanol}] - [\text{formate}] + 2 \times [\text{acetoin}] + 2 \times [\text{diacetyl}]$.

FIG. 1. Influence of pH at a controlled temperature of 37°C on the maximum specific growth rate ($\mu_{\max}$) of *Enterococcus faecium* FAIR-E 198 in mMRS medium. Symbols indicate experimental values; the closed symbols (■) indicate the results of fermentations on a 10-liter scale, and the open symbols indicate the results of fermentations in 100-ml bottles (Δ). The solid line is drawn according to the model of Rosso et al. (33). The results shown are representative of the results of two experiments.

RESULTS

Influence of pH on growth and citrate metabolism. The highest $\mu_{\max}$ was detected in a pH range from constant pH 6.0 to pH 8.0, with an optimum at constant pH 6.7 (modeled value of $\mu_{\max}$, 1.11 h⁻¹) (Fig. 1). No growth was detected below pH 4.5 (the minimum pH). According to the model, no growth was expected above pH 9.8 (the maximum pH). The optimum maximum growth was observed between pH 6.0 and pH 7.0 (maximum OD₆₀₀ [OD_{600max}], 1.2). At constant pH 8.0 and pH 5.0, a lower OD_{600max} was noticed, namely, 0.6 and 0.4, respectively. For all fermentations, a short exponential phase was followed by a fast decrease in optical density and viable cells of the culture (Fig. 2A). The k_d was also dependent on the pH of the medium, with constant pH 6.0 resulting in the highest k_d (0.26 h⁻¹; Table 1). Except for the fermentation at constant pH 8.0, the strain was able to consume citrate at the different pH values tested (Table 1). At the end of the exponential growth phase of the fermentation at constant pH 5.0, the largest amount of citrate was consumed per unit of OD₆₀₀ (Table 1). At higher pH values, citrate consumption per unit of OD₆₀₀ was lower. Only 30% of citrate was consumed during the exponential growth phase of fermentations at constant pH 6.0 and pH 7.0, indicating that a compound other than citrate in the mMRS medium was limiting growth (Table 1). Furthermore, the metabolites produced by citrate degradation (acetate, formate, acetoin, ethanol, diacetyl, and carbon dioxide) mainly increased during the stationary phase (Fig. 2A and B). During citrate metabolism, mostly acetate, formate, and acetoin were formed, whereas ethanol and diacetyl were produced in much smaller amounts (Table 1). At constant pH 5.0, no formate was produced. The yields of acetate and formate were highest at constant pH 7.0. Although no citrate was metabolized at constant pH 8.0, the strain grew in mMRS medium and

produced only lactate (4.5 mM), indicating that a compound other than citrate in the mMRS medium was used as the energy source (Table 1). At the other pH values, lactate was also produced, ranging from 3.3 mM at constant pH 5.0 to 9.5 mM at constant pH 7.0. In contrast to the other metabolites, lactate was produced only during the exponential growth phase (Fig. 2A and B). Consequently, an unidentified energy source present in the mMRS medium seemed to be responsible for lactate formation, as most of the citrate was consumed during the stationary phase.

Citrate consumption in the presence of glucose. In the presence of glucose (111 mM), the growth of *E. faecium* FAIR-E 198 was enhanced in mMRS medium containing 50 mM of citrate (Fig. 2A and C). Doubling of the glucose concentration (222 mM) of mMRS medium resulted in a longer growth phase and an increased maximum biomass, i.e., from 2.4 g CDM liter⁻¹ after 10 h of fermentation for 111 mM glucose to 3.9 g CDM liter⁻¹ after 12 h of fermentation for 222 mM of glucose. This was also reflected by the higher OD_{600max}, indicating that growth was limited by glucose during fermentations with 111 mM of this energy source (Table 2). In the presence of glucose (111 mM), more acetate and less acetoin were formed compared to growth in mMRS medium without added glucose (Fig. 2B and D). Increasing the initial citrate concentration from 8.5 mM, i.e., the concentration present in common MRS medium, to 50 mM did not result in an increased biomass formation, although more citrate was consumed (Fig. 3). Moreover, increasing the citrate concentration in the fermentation medium from 50 to 150 mM resulted in lower OD_{600max} and $\mu_{\max}$ values (Table 2). In mMRS medium with 50 mM of citrate, the maximum citrate consumption rate in the presence of glucose ($r_{CA} = 0.28$ h⁻¹, $r^2 = 0.935$) was higher than that in the fermentation without glucose ($r_{CA} = 0.11$ h⁻¹, $r^2 = 0.988$). Doubling the glucose concentration did not change the citrate consumption rate ($r_{CA} = 0.26$ h⁻¹, $r^2 = 0.974$). However, increasing the citrate concentration slowed down r_{CA} from 0.43 h⁻¹ ($r^2 = 0.927$) when 8.5 mM citrate was present to 0.04 h⁻¹ ($r^2 = 0.985$) when 150 mM citrate was present. When all the glucose was consumed, 88, 34, 28, and 25% of the initial citrate concentrations were consumed in the case of the presence of 8.5, 50, 100, and 150 mM of citrate (final concentration), respectively. This indicated a simultaneous consumption of glucose and citrate. The onset of citrate consumption was later than the onset of glucose consumption (Fig. 3B). Production of lactate as end product of glucose metabolism stopped when all the glucose was consumed (Fig. 2D). After 24 h of fermentation, most of the citrate was consumed, except for the fermentation with 150 mM of citrate (Fig. 3B).

Growth and citrate metabolism in cell-free fermented mMRS medium. To verify whether an unknown compound in mMRS medium (with no added glucose or citrate) was responsible for growth and lactate production of *E. faecium* FAIR-E 198, fermentations were carried out in mMRS medium and CFF mMRS medium. In both media, without the addition of glucose or citrate, growth still occurred, indicating that an unknown nutrient present in the media was responsible for energy generation (Table 3). The growth rates and OD_{600max} values in CFF mMRS medium were lower than in mMRS medium, as more nutrients were available in mMRS medium. In the case of the addition of 25 mM citrate, an increase in

FIG. 2. Growth and citrate metabolism of *Enterococcus faecium* FAIR-E 198 in mMRS medium at 37°C and a constant pH 6.5, with 50 mM citrate alone (A and B) and with 50 mM citrate (C and D) plus 111 mM glucose as added energy sources. Bacterial growth (A and C) is represented by OD₆₀₀ (▲) and CFU (10⁸; ●). Citrate and glucose metabolism (B and D) is presented by citrate (mM; ●), glucose (mM; ◆), acetate (mM; ○), lactic acid (mM; ◇), formate (mM; ▲), acetoin (mM; ■), and ethanol (mM; □). The CO₂ production (A and C) was measured in the headspace (as a percentage [vol/vol]; □). The results shown are representative of the results of two experiments.

$\mu_{\max}$ and OD_{600max} were observed in both media. Increasing the concentration of citrate to 50 mM did not result in more growth. At OD_{600max}, the consumption of citrate in mMRS medium was doubled compared with that in CFF mMRS me-

dium. The biomass was also doubled in mMRS medium. When the concentration of citrate was doubled in mMRS medium, more citrate was consumed after 24 h of incubation, although no increase in biomass was observed (Table 3).

TABLE 1. Influence of pH on growth, citrate consumption, and product formation of *Enterococcus faecium* FAIR E-198 in mMRS medium at 37°C with 50 mM of citrate as the sole energy source

pH	Growth parameter			Citrate consumption (mM)		Product yield ^a (mol product [mol citrate] ⁻¹)					C recovery ^c (%)
	$\mu_{\max}^b$ (h ⁻¹)	k_d^b (h ⁻¹)	OD _{600max}	At OD _{600max}	After 24 h	Y_{AA}	Y_{FA}	Y_{ET}	Y_{AC}	Y_{DI}	
5.0	0.38 (0.990)	0.01 (0.937)	0.4	11.9	14.6	1.35	0.00	0.04	0.25	0.02	84
6.0	1.07 (0.987)	0.26 (0.993)	1.2	14.6	49.4	1.32	0.27	0.10	0.34	0.02	104
6.5	1.15 (0.990)	0.18 (0.974)	0.9	13.1	48.2	1.35	0.23	0.06	0.24	0.01	97
7.0	1.04 (0.995)	0.10 (0.997)	1.0	14.8	42.8	1.72	0.40	0.15	0.19	<0.01	97
8.0*	0.89 (0.973)	0.03 (0.909)	0.6	0.0	0.0	—	—	—	—	—	—

^a The yield coefficients for acetate (Y_{AA}), formate (Y_{FA}), ethanol (Y_{ET}), acetoin (Y_{AC}), and diacetyl (Y_{DI}) were calculated after 24 h of fermentation. —, not relevant, as no citrate was consumed.

^b The maximum specific growth rate ($\mu_{\max}$) and the specific death rate (k_d) were determined by linear regression from the plots of ln OD₆₀₀ versus time. The correlation coefficient r^2 is shown in parentheses.

^c The carbon recovery ($100 \times \text{C mole of products formed} [\text{C mole of citrate consumed}]^{-1}$) was calculated with adjustment for CO₂ production that was derived from the theoretical stoichiometric balances. —, not relevant as no citrate was consumed.

TABLE 2. Influence of citrate and glucose concentration on growth and citrate consumption of *Enterococcus faecium* FAIR-E 198 in mMRS medium at 37°C and constant pH 6.5

Glucose/citrate concn (mM)	Growth parameter		Citrate consumption (mM) after 24 h
	$\mu_{\max}^a$ (h ⁻¹)	OD _{600max} /maximum cell count (CFU ml ⁻¹)	
111/8.5	1.39 (0.983)	11.3/2.3 × 10 ⁹	8.5
111/50	1.49 (0.983)	11.0/1.8 × 10 ⁹	50.7
111/100	1.01 (0.996)	7.9/4.0 × 10 ⁸	100.3
111/150	1.00 (0.991)	7.2/2.0 × 10 ⁸	119.6
222/50	1.36 (0.996)	13.7/2.9 × 10 ⁹	53.0

^a The maximum specific growth rate ($\mu_{\max}$) was determined by linear regression from the plots of ln OD₆₀₀ versus time. The correlation coefficient r^2 is shown in parentheses.

DISCUSSION

Citrate metabolism by LAB is important, as the end products, such as acetate, diacetyl, acetaldehyde, and acetoin determine the flavor of many fermented foods (17). The ability to consume citrate has been well documented for *Leuconostoc mesenteroides* subsp. *mesenteroides* (3, 6, 41) and *Lactococcus lactis* subsp. *lactis* biovar diacetylactis (18, 20, 41). More recently, studies on citrate metabolism of *Enterococcus* strains have been performed (31, 32, 36, 37), as this can be of importance for the development of the aroma and flavor in many cheeses (5, 27, 42, 43).

In this study, fermentations with *E. faecium* FAIR-E 198 were performed to investigate citrate metabolism under different physicochemical conditions. Growth and citrate metabolism of *E. faecium* FAIR-E 198 were dependent on the pH of the medium, with an optimum for growth around constant pH 6.5. Citrate metabolism was observed in a pH range from constant pH 5.0 to pH 7.0. Although growth still occurred at constant pH 8.0, no citrate was metabolized. Fermentations without any added energy source confirmed the presence of an unknown energy source in mMRS medium, which has also been found responsible for growth of *E. faecalis* (32). During fermentations containing only citrate, this unknown energy source seemed to limit the biomass formation, as most of the citrate was consumed during the stationary phase. Citrate consumption by both growing and nongrowing cells but without contribution to biomass formation has also been observed for *Lactobacillus casei* and *Lactobacillus plantarum* (28). Only when a small amount of citrate was added (25 mM) to mMRS and CFF mMRS medium was growth of *E. faecium* FAIR-E 198 stimulated, although most of the citrate was consumed during the stationary phase. In contrast, citrate contributes to growth of *E. faecalis* FAIR-E 239 in the absence of glucose (31, 32).

At constant pH 5.0, citrate consumption per unit of OD₆₀₀ was highest. This can be explained by the activity of citrate permease (CitP), which is optimal between pH 4.5 and pH 5.5, as has been shown in *Lactococcus lactis* subsp. *lactis* (13, 23). Similarly, optimal consumption of citrate by *Lactobacillus plantarum*, *L. casei*, and *E. faecalis* was observed at low pH values (19, 28, 31). In the latter species, citrate metabolism still occurred at pH 8.5 (31). However, it has been shown that CitP from *L. lactis* subsp. *lactis* does not function at pH 7.0 (13). In

TABLE 3. Growth and citrate consumption of *Enterococcus faecium* FAIR-E 198 in cell-free fermented mMRS medium and mMRS medium at 37°C and at an initial pH 6.5

Medium ^a	Growth parameter		Citrate consumption (mM)	
	$\mu_{\max}^b$ (h ⁻¹)	OD _{600max}	At OD _{600max}	After 24 h
CFF mMRS	0.75 (0.979)	0.29		
CFF mMRS + 25 mM citrate	1.04 (0.999)	0.44	3.6	17.1
CFF mMRS + 50 mM citrate	1.10 (0.999)	0.44	3.1	19.6
mMRS	1.06 (0.945)	0.36		
mMRS + 25 mM citrate	1.13 (0.999)	0.95	7.2	24.7
mMRS + 50 mM citrate	1.14 (0.999)	0.94	6.7	43.8

^a CFF mMRS medium is derived from filter-sterilized, cell-free fermented mMRS medium.

^b The maximum specific growth rate ($\mu_{\max}$) was determined by linear regression from the plots of ln OD₆₀₀ versus time. The correlation coefficient r^2 is shown in parentheses.

this study, *E. faecium* FAIR-E 198 was still able to consume citrate at constant pH 7.0 but not at pH 8.0. These data indicate that a variety of CitP enzymes among LAB exist, which are active in different pH ranges, or that the uptake of citrate can take place by distinct transport mechanisms.

On the basis of the study of Sarantinopoulos et al. (37), it is difficult to state that citrate in the presence of glucose contributes to an increased biomass formation by *E. faecium* FAIR-E 198, as higher levels of citrate increased the buffering capacity of the fermentation medium, and hence the growth and glucose consumption of this strain. However, during pH-controlled fermentations (this study), no increase in biomass was noticed when 50 mM of citrate was added to mMRS medium containing 111 mM of glucose. When growth of *E. faecium* FAIR-E 198 stopped due to glucose limitation, most of the citrate was not consumed at that time, indicating that citrate metabolism by this strain did not contribute to growth but only to maintenance of the cells. Furthermore, higher concentrations of citrate negatively affected growth. A possible explanation is that increased levels of intracellular citrate inhibit the activity of phosphofructokinase, a key enzyme of glycolysis. Alternatively, chelation of minerals (e.g., manganese) by citrate present in the medium can affect the uptake of minerals necessary for the metabolism of the cell (40). This detrimental effect of citrate on bacterial growth has not been observed in *Lactococcus lactis* (23). This difference may be explained by the higher citrate concentrations used in the present study (8.5 to 150 mM) compared with the 2 to 20 mM range used in most studies with *L. lactis* (13, 18, 23). Furthermore, in *L. lactis*, citrate in combination with glucose has been found to increase biomass formation at low pH (pH 5.0), due to the generation of a strong proton motive force under acidic conditions (23). This situation cannot be completely ruled out in the case of *E. faecium* FAIR-E 198, as cometabolism was investigated at optimal growth conditions (pH 6.5).

In a wide range of citrate concentrations (8.5 to 150 mM), cometabolism of glucose and citrate by *E. faecium* FAIR-E 198

FIG. 3. Growth (OD₆₀₀) (A) and citrate and glucose metabolism (mM) (B) of *Enterococcus faecium* FAIR-E 198 in mMRS medium at 37°C and a constant pH 6.5 in the presence of 111 mM glucose and with the addition of various concentrations of citrate (8.5 mM [●, ○], 50 mM [◆, ◇], 100 mM [▲, △]), and 150 mM [■, □] citrate). The closed symbols in panel B represent the citrate concentration, while the open symbols represent the glucose concentration. The results shown are representative of the results of two experiments.

occurred, which confirms the previous results for fermentations at free pH (37). Cometabolism has also been found in other LAB, such as *Lactococcus lactis* (20, 23, 30), *Lactobacillus casei* (28), *Lactobacillus plantarum* (19, 28), and *Leuconostoc* spp. (6, 39). Furthermore, the citrate consumption rate in *E. faecium* FAIR-E 198 was enhanced when 50 mM glucose was added to the medium. Lactate from glucose breakdown may be responsible for an increased CitP activity (citrate-lactate exchange) (23). Adding more glucose did not change the citrate consumption rate, and citrate metabolism slowed down at higher levels of citrate. At these higher citrate levels, the maximum biomass concentration decreased as well, which explains the lower amount of energy needed for maintenance of the cells during the stationary phase. Recently, in several strains of *E. faecium* and *E. faecalis*, it has been found that glucose prevents citrate metabolism until glucose has been exhausted, indicating catabolite repression (31, 32). However, in the present study, *E. faecium* FAIR-E 198 was able to consume citrate simultaneously with glucose, even when more

glucose was added, although citrate consumption started later than glucose consumption.

Within the pH range of pH 5.0 to 7.0, citrate metabolism by *E. faecium* FAIR-E 198 resulted in the formation of acetate, formate, ethanol, acetoin, diacetyl, and carbon dioxide, metabolites that were also produced at free pH (37). However, the results of the present study showed that the yields of these metabolites were dependent on the pH of the medium. For instance, at constant pH 7.0 the highest yields of acetate and formate were found, while the yields of acetoin and diacetyl were the lowest. Similarly, acetoin production by *Lactococcus lactis* subsp. *lactis* occurs only at lower pH, whereas acetate is mainly produced at higher pH (18). No formate was produced at constant pH 5.0 by *E. faecium* FAIR-E 198, which can be explained by the lower activity of pyruvate formate lyase at low pH (1). Consequently, this low activity at pH 5.0 can be the reason for the lower yield of ethanol, as pyruvate dehydrogenase is the only enzyme left to convert pyruvate into acetyl coenzyme A (17).

In this study, it has been demonstrated that *E. faecium* FAIR-E 198 was able to consume citrate, even at high concentrations (>50 mM), conditions that have not been tested before in LAB. *E. faecium* FAIR-E 198 was able to cometabolize glucose and citrate, as is the case for *Lactococcus lactis* subsp. *lactis* and *Leuconostoc* spp. Despite the production of acetate as the main end product of citrate metabolism, this strain was not capable of growing on citrate as the sole energy source, in contrast with *L. lactis* subsp. *lactis* (18, 41), and citrate did not activate growth, in contrast with *Leuconostoc* spp. (38, 39). Under the conditions of pH and citrate used in the present study, it was shown that *E. faecium* FAIR-E 198 used citrate as the energy source for cell maintenance. Therefore, the production of typical aroma compounds, such as acetoin and diacetyl, which was dependent on the different physicochemical conditions tested, may contribute to the flavor properties of cheeses. This is of economic importance for the quality of Mediterranean-type cheeses in that no high cell counts of enterococci are to be expected during cheese ripening while flavor attributes are still produced. Indeed, enterococci are naturally present in several Mediterranean cheeses, and because of their poor growth in milk, they may cometabolize the citrate present in milk for cell maintenance, the end products of which influence the organoleptic properties of dairy products, without contribution to growth during ripening.

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