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SHORT-CHAIN ORGANIC ACIDS PRODUCED ON GLUCOSE, LACTOSE, AND CITRATE MEDIA BY *ENTEROCOCCUS FAECALIS, LACTOBACILLUS CASEI,* AND *ENTEROBACTER AEROGENES* STRAINS

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Abstract

Three strains of Enterococcus faecalis, *three of* Lactobacillus casei *and two of* Enterobacter aerogenes, *isolated from commercial Palmita-type cheese were cultured in peptone–yeast extract broth with glucose (PYG), lactose (PYL), or citrate (PYC) added as the main carbon sources. The short-chain volatile and non-volatile organic acids were extracted and their concentration determined by GC with a FID detector. The identity of the acids was determined by their retention times and confirmed by GC mass spectral (MS) analysis. Only acetic acid and propionic acid among the volatile acids, and lactic and succinic acids among the non-volatile acids, were found. Results showed that all the strains produced high concentrations of lactic acid from lactose (130–730 mg%); differences were mainly found among enterococci strains. Lactobacilli produced considerably higher quantities of lactic acid from glucose (1200–1300 mg%) compared to enterococci and* Enterobacter *strains. Acetic acid was produced in PYL only by enterococci strains and at very low concentrations (6–9 mg%). Both enterococci and* Enterobacter *strains produced relatively high concentrations of acetic acid in PYC (65–120 mg%). Succinic acid was only produced by the Ea_2 strain of* E. aerogenes *in both PYL (45 mg%) and PYC (40 mg%). Propionic acid was only produced in PYC (16 mg%) by the Ea_2 strain.*

Key words: Organic acids, enterococci, lactobacilli, *Enterobacter.*

INTRODUCTION

Palmita-type cheese is made in Venezuela by using raw cow's milk. It is sold as a fresh cheese. Studies on this cheese (Ferrer *et al.*, 1987; Ferrer *et al.*, 1991) have shown the need for standardizing its manufacture. This lack of standardization partly explains the great variation in its physical–chemical quality along with some other factors, such as the quality of raw milk used, and particularly its endogenous microflora. Studies also showed the need for improving quality control during manufacture, since high counts of opportunist and potentially toxigenic bacteria, such as *E. coli* (2×10^6–2×10^8 MPN/g of cheese) and *S. aureus* (6×10^3–3×10^8 MPN/g of cheese) were found in the cheese. Recent studies (Colina, 1991; Sánchez, 1991) revealed that some of these strains produced enterotoxins *'in vitro'*. Although there are no public reports about intoxications produced by this type of cheese, this finding indicates that there is a potential risk for consumers.

Several cheeses in the world, initially manufactured from raw milk, are currently made from pasteurized milk, while maintaining their organoleptic properties and significantly improving their sanitary quality (Langsrud & Reinbold, 1973). Cabrera and Ferrer (1994) recently studied the possibility of producing Palmita-type cheese from pasteurized milk and starter cultures and the results were satisfactory. The microorganisms chosen as starter cultures belong to the genera *Enterococcus, Lactobacillus* and *Enterobacter.* Cheeses made with these starters exhibited good organoleptic properties and *S. aureus* and fecal coliforms counts were within the international limits for pasteurized cheese. However, the typical acidic flavor of the cheese was not attained (Raffe, 1994). Ferrer and Granados (1992) found that lactic, propionic, acetic and succinic acids were the most abundant short-chain organic acids in the commercial Palmita-type cheese. Ferrer *et al.* (1993) showed that those acids could be produced in skim milk from *Enterococcus, Lactobacillus* and *Enterobacter* strains isolated from the commercial cheese. These genera, along with *Staphylococcus* and

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Escherichia, are the most abundant bacteria in the cheese (Ferrer *et al.*, 1991). Eyes of the cheese are produced by coliform fermentation, mostly from *E. coli, Enterobacter* spp., and *Klebsiella pneumoniae.* Experiments for producing the eyes of the cheese with heterofermentative lactic acid bacteria and propionibacteria were unsuccessful, since neither the typical eyes nor the typical flavor were attained; however, the eyes could be produced by some strains of innocuous *Enterobacter* spp. which do not produce flavor defects.

The objective of this research was to study the production of short-chain organic acids by selected strains of *E. faecalis, L. casei* and *E. aerogenes* when grown on carbon sources that included glucose, lactose and citrate. The selected strains are currently used as starters for the production of Palmita-type cheese on a pilot-plant scale. This information would be useful to help understand the biochemical behavior of these strains in milk. It would also help control the production of acids in the cheese manufactured with those cultures.

METHODS

Cultures

Three strains of *Enterococcus,* three of *Lactobacillus,* and two of *Enterobacter* that can produce short-chain organic acids characteristic of commercial Palmita-type cheese were selected (Raffe, 1994).

Cultures were isolated from commercial Palmita-type cheese in KF *Streptococcus* agar, *Lactobacillus* agar and Bilis Red Purple agar, for enterococci, lactobacilli and *Enterobacter*, respectively. Cultures were identified by morphology, Gram and biochemical tests as previously reported (Ferrer *et al.*, 1991).

All microorganisms were cultured in lactose broth (DIFCO), incubated at 37°C for 18 h. Glass vials with screw caps containing 10 ml of PYC (peptone–yeast–citrate), PYG (peptone–yeast–glucose), or PYL (peptone–yeast–lactose) were inoculated with 0·5 ml of the stock cultures and incubated at 37°C for 48 h. Average counts of log-phase cultures were approximately 1×10^9/ml, 2×10^9/ml and 1×10^8/ml, for enterococci, lactobacilli and *Enterobacter* strains, respectively. The basal peptone yeast medium was recommended by Lombard and Dowell (1982). The pH of the final media was adjusted to 7·2. The concentrations of the main carbon sources were 1% glucose (BBL), 2% lactose (BBL) and 0·65% diamonium citrate (Fisher).

Volatile and non-volatile acids

The Lombard and Dowell (1982) method was used to extract and separate the acids by GC. Formic acid was not determined because a FID detector was used. Volatile acids (acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acids) were extracted with diethyl ether and non-volatile acids (lactic, succinic, oxalic, fumaric and malonic acids) were methylated and then extracted with chloroform.

Determination of acid concentrations

The concentration of each acid was determined by injection of 1·5 μl of each extract (in triplicate) into a 3300 Varian GC with a FID detector connected to a 4400 Varian integrator.

For separation of volatile acids, a 2 m × 1/8 in. stainless-steel column (Supelco) packed with 15% FFAP in 80/100 Chromosorb W/WA was used. Non-volatile acids were separated using a 2 m × 1/8 in. stainless-steel column (Supelco) packed with 15% DGES in 80/100 Chromosorb W/WA. Nitrogen (30 ml/min) was the carrier gas and air (300 ml/min) and hydrogen (30 ml/min) were used as the flammable gas mixture. Injection port, column and detector temperatures were 200, 160 and 250°C, respectively, for volatile acids and 270, 180 and 190°C, respectively, for non-volatile acids. Standard solutions of acids (Aldrich) were prepared (1·5 and 10 mM for all acids except for lactic acid, which was prepared at 100, 150, and 200 mM) to plot the calibration curves. Chromatographic determinations were carried out on extracts of non-inoculated media and of PY medium inoculated with each strain. The first set was the blank and the last set was done to assure that extracted organic acids were not produced by metabolism of peptone or other compounds present in the media. The identity of the acids was determined by retention time and by GC-MS analysis. The GC-MS analysis was performed in a 3400 Varian gas chromatograph connected to a Finnigan Magnum mass spectrometer detector. The chromatograph was fitted with a Nukol (Supelco) 60 m × 0·25 mm capillary column for volatile acids and a Supelco 30 m × 0·25 mm DB-1701 capillary column for non-volatile acids. Helium was used as the carrier gas at 40 cm/s. The oven was held at 100°C for 5 min, then raised to 250°C at 10°C/min for volatile acids, and was run isothermally at 120°C for non-volatile acids. The injector was set at 250°C in both cases. One microliter of samples (prepared as described above) was injected into the chromatograph. The mass spectrometer was operated in the positive-ion electron-impact ionization mode with an electron energy of 70 eV.

Intra- and inter-assay precisions were determined and expressed as coefficients of variation. Intra-assay precision was determined using six replicate GC analyses of a standard solution of acids and inter-assay precision by analyses of six separate samples obtained from a mother culture of one strain. The recovery of acids was also determined as previously described by Ferrer and Granados (1992).

Acid concentrations were expressed in mg/100 ml (mg%) of culture medium.

RESULTS AND DISCUSSION

The conditions of the chromatographic assays carried out in this work gave good resolutions for all the acids. The identity of the acids determined by retention time was confirmed by the GC-MS analysis. Intra-assay precision for all acids was between 0·5 and 1·85%. Intra-assay precisions were 3·6, 4·2, 0·5 and 11·2%, for acetic, propionic, lactic and succinic acids, respectively. Recovery values for each acid were (in %): 96, acetic; 97, propionic; 101, lactic; 96, oxalic; 92, malonic; 95, fumaric; and 96, succinic acid. Calibration curves showed correlation coefficients higher than 98%.

Table 1 contains the results of the organic acids produced by all the strains in the three media studied. When a peak appears showing a concentration lower than 5 mg%, it corresponds to 'traces'. The only acids found were the non-volatile lactic and succinic acids and the volatile acetic and propionic acids.

Enterococcus faecalis produced a high concentration of lactic acid in PYG and PYL. The only other acid produced by these strains in those media was acetic acid, which was produced at low concentrations by strains S_4 and S_7 in PYL. However, *E. faecalis* produced high concentrations of acetic acid in PYC. These bacteria are homofermentative and produce L-lactic acid as the principal final product from glucose fermentation, but Garg and Mital (1991) have reported that at pH > 6 enterococci produce formic acid, ethanol, acetic acid and lactic acid (the pH of PYL is 7·2). In addition, our results are consistent with studies that confirm acetic acid as a by-product of citrate metabolism in enterococci (Bassette *et al.*, 1967; Jensen *et al.*, 1975; El-Gendy *et al.*, 1983). Cultures of the same strains in skim milk (Raffe, 1994) produced an average of 505 mg% of lactic acid, 24 mg% of acetic acid, and 8 mg% of succinic acid. Results from this study indicate that lactose and citrate are simultaneously metabolized in skim milk; lactic acid would be produced from lactose and acetic acid from citrate. Co-metabolism of these substrates has been reported for several lactic acid bacteria, such as *Leuconostoc* spp. (Cogan, 1987) and *Lactobacillus* spp. (Fryer, 1970). It has been reported that some strains of *E. faecalis* (Raffe, 1994; Ferrer & Granados, 1992), including some studied in this research, produce small amounts of succinic acid in skim milk. However, succinic acid was not a by-product of glucose, lactose and/or citrate metabolisms in this study. It was not produced on PY in 48 h either (results not shown). Production of propionic acid by some strains of *E. faecalis* has also been reported (Ferrer & Granados, 1992). In this work, propionic acid was not produced. Nakae and Elliot (1965) have reported the production of propionic acid by some enterococci strains from amino acids, although longer incubation times were used. Lactic acid was produced in trace amounts from citrate in two strains of enterococci. Since pyruvate is the key intermediate in the metabolism of citrate and lactose, lactic acid may be produced from pyruvate obtained from citrate (Starrenburg & Jeroen, 1991).

Lactobacillus casei did not grow on PYC in 48 h, although the media contained peptone. It has been reported that some strains of *L. casei* grow on citrate as their sole carbon source and some do not (Thornhill & Cogan, 1984; Kaneuchi *et al.*, 1988). Some strains use citrate in the presence of glucose or lactose with the amount of acetic acid indicating whether citrate has been utilized. However, they have reported that, in most cases, glucose and lactose simply enhance citrate utilization due to faster growth of lactobacilli on carbohydrate. In this study, citrate was not mixed with glucose or lactose. *Lactobacillus casei* strains only produced lactic acid in PYG and in PYL, as expected, and the concentration of lactic acid produced in PYL was considerably less than in PYG. Since *L. casei* is also a galactose fermenter (Chassy & Thompson, 1983; Hickey *et al.*, 1986), it indicates that lactose hydrolysis is limiting. The concentration of lactic acid produced by *L. casei* was the highest found in this work (≥1200 mg%). The fact that the inoculum of lactobacilli was higher

Table 1. Organic acids detected in PYG, PYL and PYC media (mg/100 ml)[a]

Strain	Lactic acid			Succinic acid			Acetic acid			Propionic acid		
	PYG	PGL	PYC	PYG	PYL	PYC	PYG	PYL	PYC	PYG	PYL	PYC
S_4	410	130	—	—	—	—	—	6	100	—	—	—
S_7	380	490	t	—	t	—	—	9	80	—	—	—
S_{11}	370	730	t	—	—	—	—	t	70	—	—	—
L_1	1200	310	*	—	—	*	—	t	*	—	—	*
L_2	1300	360	*	—	—	*	—	t	*	—	—	*
L_3	1200	410	*	—	t	*	—	t	*	—	—	*
Ea_2	440	300	—	9	45	40	—	t	120	—	—	16
Ea_3	460	450	—	—	—	t	—	t	65	—	—	t

[a] Butyric, isobutyric, valeric, isovaleric, caproic, oxalic, fumaric and malonic acids were not detected in any of the samples. PYG: peptone–yeast-extract–glucose broth. PYL: peptone–yeast-extract–lactose broth. PYC: peptone–yeast-extract–citrate broth. —: Non-detectable. t: Traces. *: No growth.

could explain this result. When these strains were cultured in skim milk (Raffe, 1994) they produced 520 mg% of lactic acid, 20 mg% of acetic acid and 6 mg% of succinic acid. In this study, lactic acid was produced in high concentrations, but acetic acid was produced in trace amounts by the three strains in PYL and succinic acid was produced in trace amounts by L_3 in PYL. Since milk contains both lactose and citrate, citrate could be used to produce acetic and succinic acids. Acetic acid could be certainly produced from citrate (Drinan *et al.*, 1976). With respect to succinic acid, Kaneuchi *et al.* (1988) found that many strains of lactobacilli may produce succinic acid from citrate, but the 10 citrate-utillizing strains of *L. casei* used in their study failed to do so. Therefore, the origin of succinic acid remains unclear.

Enterobacter aerogenes strains produced similar results in PYG and PYL. A mixed-acid fermentation occurred as shown by the production of both lactic and acetic acids at high concentrations. The lactic acid concentration was very similar to that observed for enterococci in PYG and lower than that of lactobacilli in PYG. Both *Enterobacter* strains produced acetic, succinic and propionic acids in PYC, although Ea_3 only produced trace amounts of the last two acids.

When *E. aerogenes* strains were cultured in skim milk for 24 h, they produced 52 mg% of lactic acid and trace amounts of acetic and succinic acids (Raffe, 1994). Since in this study the production of succinic and acetic acids was good in PYC, it suggests that *E. aerogenes* does not use citrate when lactose is present, as in milk during the manufacture of cheese. In addition, the strain that produced propionic acid in PYC did not produce it in skim milk (Raffe, 1994). The production of formic, acetic and succinic acids from citrate by *Enterobacter* has been reported in the literature (MacFaddin, 1984).

According to the results shown, lactic acid, typical of Palmita-type cheese, may be produced by lactobacilli, enterococci and enterobacteria found in the cheese. Acetic acid may be produced from citrate by enterococci and *Enterobacter* strains and from lactose by enterococci. Succinic and propionic acids can be produced by *Enterobacter* spp. Succinic acid might be produced by some strains of enterococci and lactobacilli, since one strain of each genus produced trace amounts of the acid.

The differences among the strains and among the species, in fermenting carbon sources to different organic acids, suggest that it would be advisable to continue screening cultures for the preparation of mixed cultures for the manufacture of Palmita-type cheese with desirable sensory properties. In addition, trials with media containing both lactose and citrate could help answer some of the questions arising from this study.

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