Commensal symbiosis between a *Lactococcus lactis* strain and an *Enterococcus mundtii* strain increases cell yield in constituted broth

H. Kimoto-Nira,¹ H. Ohmori, and C. Suzuki

NARO Institute of Livestock and Grassland Science, Ikenodai 2, Tsukuba, Ibaraki 305-0901, Japan

ABSTRACT

To exert their beneficial effects, probiotics need to survive in the stringent conditions of the gastrointestinal tract. Symbiosis between different bacteria is a potential way of enhancing this survival. In developing new probiotic cultures, we investigated the synergic effect between *Enterococcus mundtii* IFO 13712 and 7 strains of *Lactococcus lactis*, many of which are widely used as starter bacteria for making dairy products and have probiotic properties. The growth yield of a mixed culture of *L. lactis* strain Y and IFO 13712 in de Man, Rogosa, and Sharpe broth was greater than that of a single culture. Supernatant from culture of strain IFO 13712 enhanced the growth of strain Y, but that of strain Y did not enhance the growth of strain IFO 13712. This commensalism phenomenon was confirmed by using a simpler tryptone-yeast extract-glucose (TYG) broth. Increased cell yield in mixed culture of the 2 strains compared with single cultures was observed in TYG broth in the presence of both Tween 80 and citrate but not in TYG broth alone or TYG broth containing either Tween 80 or citrate. Thus, the Tween 80 and citrate in the broth contributed to the commensalism. Metabolite analysis revealed that ethanol production in the co-metabolism of glucose and citrate by strain Y was suppressed by mixed culture in TYG broth containing Tween 80 and citrate, compared with that in TYG broth containing citrate alone. The mechanism supporting the observed commensal symbiosis between strains Y and IFO 13712 was the increase in availability of glucose for lactate production by strain Y because, in glycolysis, the pathway from glucose to lactate is energetic, whereas the pathway from glucose to ethanol is not. Whether growth stimulation of strain Y by mixing it with IFO 13712 in milk products will enhance the survival of strain Y in the intestine remains to be elucidated.

Key words: enterococci, lactococci, starter culture, symbiosis

INTRODUCTION

Probiotics are defined by the Food and Agriculture Organization and World Health Organization as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host.” It has been suggested that bacteria ingested as probiotics cannot affect the intestinal environment unless their populations reach a minimal threshold of 10^6 to 10^8 cfu/g in intestinal content (Marteau and Rambaud, 1993). For the bacteria to accumulate in the intestinal tract, bacterial growth needs to be enhanced.

Prebiotics have been defined as “nondigestible food ingredients that beneficially affect host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). Lactobacilli and bifidobacteria are among the intestinal bacteria stimulated by prebiotics (Haarman and Knol, 2006; Iwata et al., 2009). Prebiotics combined with probiotic bacteria in the form of synbiotics are capable of improving the gastrointestinal health of humans.

Enterococci are bacteria that occur naturally in the intestinal tract, and most strains therefore tolerate the conditions found in the intestinal tract. Some strains of enterococci have been isolated from fermented milk products (Ao et al., 2012; Faye et al., 2012) and have probiotic activities, such as the ability to reduce numbers of *Salmonella enteritidis* and coliforms (Bhardwaj et al., 2010) and to exert a bactericidal effect on diarrheagenic *Escherichia coli* (Miyazaki et al., 2010). In contrast, lactococci are not intestinal bacteria but can tolerate the conditions found in the intestinal tract (Klijn et al., 1995). Lactococci are widely used as starter bacteria in the manufacture of dairy products such as fermented milk and cheese. Furthermore, they have probiotic activities such as the improvement of skin condition in mice (Kimoto-Nira et al., 2007) and improvement of lipid metabolism in rats (Lee et al., 2005). Despite the ability of prebiotics to enhance the

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¹Corresponding author: anne@affrc.go.jp

Table 1. *Enterococcus* and *Lactococcus* strains examined

Species	Strain	Origin	Source ¹
<i>Enterococcus mundtii</i>	13712	Silkworm	IFO
<i>Lactococcus lactis</i> ssp. <i>lactis</i>	19435	Unknown	ATCC
<i>L. lactis</i> ssp. <i>lactis</i>	527	Raw milk	Laboratory collection
<i>L. lactis</i> ssp. <i>lactis</i>	G50	Raw grass	Laboratory collection
<i>L. lactis</i> ssp. <i>lactis</i>	H46	Kimchi	Laboratory collection
<i>L. lactis</i> ssp. <i>lactis</i> bv. <i>diacetylactis</i>	N7	Commercial cheese starter	Laboratory collection
<i>L. lactis</i> ssp. <i>lactis</i> bv. <i>diacetylactis</i>	H59	Kimchi	Laboratory collection
<i>L. lactis</i> ssp. <i>lactis</i> bv. <i>diacetylactis</i>	Y	Commercial cheese starter	Laboratory collection

¹IFO = Institute for Fermentation Osaka (Osaka, Japan); ATCC = American Type Culture Collection (Manassas, VA).

value of enterococci or lactococci as probiotic bacteria, few reports on this topic have been published. In the present study, we attempted to increase growth of enterococci or lactococci by mixed cultivation of these species.

In the dairy industry, mixed cultures, rather than pure cultures, of lactic acid bacteria are typically used for making fermented milk products because pure strains often grow slowly in milk and mixed cultures can improve the flavor (Kothari et al., 1971; Frengova et al., 2000). Mixed starter cultures of lactococci with other lactic acid bacteria such as *Leuconostoc* spp. and lactobacilli are used, rather than single cultures. In addition, Picon and Nunez (2007) reported growth stimulation of a proteinase-positive *Lactococcus lactis* strain by a proteinase-negative *L. lactis* strain. If certain substances, such as prebiotics, released from enterococci or lactococci can be used to promote the growth of these probiotic species, then mixed fermentation of these 2 genera (*Lactococcus* and *Enterococcus*) in milk products might enhance their cell counts and thus their intestinal survival. Here, we investigated the growth stimulation of lactococci by enterococci as a model of this interaction.

MATERIALS AND METHODS

Strains and Culture Conditions

The 8 strains of lactic acid bacteria used included *Enterococcus mundtii* IFO 13712 (Institute for Fermentation Osaka, Osaka, Japan), *L. lactis* ssp. *lactis* ATCC 19435 (American Type Culture Collection, Manassas, VA), and 6 strains from our laboratory collection (Table 1). With the exception of strain ATCC 19435, the strains of lactococci were potential candidate probiotic bacteria based on previous reports from our laboratory and others. *Lactococcus* strain G50 induces the production of cytokines from a macrophage-like cell line, and oral administration of strain G50 to mice suppresses the production of serum immunoglobulin E

(Kimoto et al., 2004a). Oral administration of *L. lactis* ssp. *lactis* 527 and *L. lactis* ssp. *lactis* bv. *diacetylactis* N7 improves lipid metabolism in rats (Lee et al., 2005). *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* Y induces cytokine production by a macrophage-like cell line (Kimoto et al., 2004a). *Lactococcus lactis* ssp. *lactis* H46 can remove cholesterol from broth (Kimoto et al., 2004b), whereas *L. lactis* ssp. *lactis* bv. *diacetylactis* H59 produces bacteriocin (T. Okamoto, M. Nomura, H. Kimoto, M. Kobayashi, K. Mitani, and T. Narita, NARO Institute of Livestock and Grassland Science, Ibaraki, Japan, unpublished data). The strains were maintained by subculture of 1% inocula in deMan, Rogosa, and Sharpe (MRS) broth (Becton Dickinson, Sparks, MD). Cultures were incubated for 18 h at 30°C (for lactococci) or 37°C (for *E. mundtii* IFO 13712). The cultures were stored at 4°C between transfers and subcultured once before use.

Growth in Single and Mixed Cultures

For each experiment, a tube containing 20 mL of MRS or tryptone-yeast extract-glucose (TYG) broth was inoculated with *E. mundtii* IFO 13712 at 10⁷ cfu/mL or the desired strain of *L. lactis* at 10⁷ cfu/mL, or both. The TYG broth consisted of 0.5% tryptone (Becton Dickinson), 0.5% yeast extract (Wako Chemical, Osaka, Japan), and 1.0% glucose supplemented with 1% sodium succinate and 1% sodium chloride. The pH of the TYG broth was adjusted to pH 6.86. Single and mixed cultures were incubated for 24 h at 37°C. To simulate the conditions in the intestine, these experiments were performed at 37°C, not at the optimal growth temperature (30°C) for lactococci. At the end of incubation, bacterial growth was determined by measuring the optical density of the cultures at a wavelength of 620 nm (OD₆₂₀) with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, NY) against an uninoculated broth blank. Bacterial growth was also determined from the dry weight of the cells. For measurement of dry weight, cells in the cultures

were collected by centrifugation ($2,800 \times g$, 20 min) at 4°C, washed twice with distilled water, and dried at 105°C for 4 h.

Contact-Free Coculture on Bacterial Growth

For this assay, a cell-culture insert (0.4- μ m pore size, Falcon type 3095, Becton Dickinson Labware, Oxnard, CA) was placed in 24-well tissue culture plates (Falcon type 2046). Growth medium was added to the culture well and the culture insert to a volume of 0.5 mL. Bacterial cells were seeded at a concentration of 10^7 cfu/mL to the culture well and the insert. The plate was incubated at 37°C. After 24 h of incubation, bacterial growth in the culture plate and culture insert was determined with a spectrophotometer (DU-640, Beckman Coulter Inc., Brea, CA), as described above. In this system, the 2 strains of bacteria do not contact each other, but bacterial metabolites from one strain are able to reach the cells of the other strain.

In an another experiment using culture plates, *Lactococcus* strain Y was cultured with the supernatant from *E. mundtii* IFO 13712-inoculated MRS broth or with the supernatant from strain-Y-inoculated MRS broth for 24 h at 37°C. These supernatants were adjusted to pH 6.5 with 1 N NaOH and sterilized by filtration (0.2 μ m). Bacterial growth was quantified as described above.

Substrate and Metabolite Analysis

Culture supernatants were stored at -20°C until the time of analysis for substrate and product. Residual glucose in the broth was measured enzymatically with a Glucose CII test kit (Wako). Lactate, citrate, acetate, and formate in the broth after culture were quantified by using a postcolumn HPLC system (LC-UV, Shimadzu, Tokyo, Japan) equipped with a Shim-pack SCR-102H column (8 mm $\times$ 300 mm; Shimadzu) fitted with a SCR-102H guard column and a CDD-10Avp conductivity detector (Shimadzu). The mobile phase contained 5 mM *p*-toluenesulfonic acid. The postcolumn buffer solution was composed of 5 mM *p*-toluenesulfonic acid, 100 μ M EDTA, and 20 mM Bis-Tris. Ethanol was quantified with an F-kit (Boehringer, Mannheim, Germany). Acetoin was measured by the method of Westerfeld (1945), and CO₂ was not quantified.

Cellular sugar content was determined by the phenol-sulfate method (Dubois et al., 1956). Cells were harvested and washed 3 times with distilled water and then resuspended in water. The cell suspensions were used to analyze the sugar content and measure the dry weight. Milligrams of glucose per milligram of dry cell

weight were calculated, and the results were expressed as percentage glucose.

Statistical Analysis

Data are expressed as means or means $\pm$ standard deviation from 3 samples and a representative from 2 independent experiments. Statistical analysis was performed on the differences between single and mixed cultures by using the *t*-test, and *P* < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Interaction of *E. mundtii* IFO 13712 with *Lactococci* to Increase Cell Yield

Seven strains of lactococci were cocultured with *E. mundtii* IFO 13712 in MRS broth. The OD₆₂₀ of a mixed culture of *Lactococcus* strain Y or N7 with strain IFO 13712 in MRS broth was markedly greater than that for each single *Lactococcus* and *Enterococcus* culture (Table 2). Because the increase in growth of strain Y in the presence of strain IFO 13712 was higher than that of strain N7 with strain IFO 13712, we selected strain Y for subsequent experiments. The dry weight of strains IFO 13712 and Y in mixed culture (900.0 ± 1.50 mg/L) was significantly higher than that of strain IFO 13712 (780.0 ± 17.6 mg/L; *P* = 0.0012) or strain Y (795.6 ± 20.4 mg/L; *P* = 0.0037) individually.

We also investigated the interaction between strain Y and *Enterococcus faecalis* ATCC strains 27274 (obtained from ATCC) and IFO 12964 and IFO 12968 (ob-

Table 2. Bacterial growth in single and mixed cultures (in de Man, Rogosa, and Sharpe broth) of *Enterococcus mundtii* IFO 13712 and *Lactococcus* strains¹

Bacterial strain	OD ₆₂₀	
	Single	Mixed
IFO 13712	1.54	ND ²
ATCC 19435	1.01	1.35
527	1.40	1.40
G50	1.60	1.56
H46	1.74	1.74
N7	1.56	1.72
H59	1.83	1.87
Y	1.49	1.73

¹*Enterococcus mundtii* IFO 13712 and *Lactococcus* strains (see Table 1 for species designations) were cultured in single and mixed fermentations (mixed cultures included the listed *Lactococcus* strain plus IFO 13712). Values were determined by measuring the optical density (OD) at a wavelength of 620 nm after a 24-h incubation at 37°C. Each value is the mean of 3 samples.

²Not done.

tained from IFO). Mixed cultures of each of these *Enterococcus* strains and strain Y did not increase growth compared with that of each single culture (data not shown), suggesting that the symbiosis is not specific to *Enterococcus* species.

The sugar contents of strain IFO 13712 or strain Y cells grown in MRS broth were compared with that of mixed-culture cells. The sugar content of strain IFO 13712 cells (dry weight) was $21.2 \pm 2.40\%$, that of strain Y cells was $22.6 \pm 0.80\%$, and that of the mixed-culture cells was $20.8 \pm 1.55\%$; these values did not differ significantly. This result suggests that polysaccharides, such as those in the capsules of bacterial cells, were not responsible for the increase in dry weight after mixed culture of these strains.

Contact-Free Coculture of Strain IFO13712 or Strain Y

Table 3 shows the growth of strain IFO 13712 or strain Y mixed with either strain in a cell-free culture system. Growth of strain Y in the culture insert and culture plate was enhanced by coculture with a cell-free filtrate of strain IFO 13712, whereas the growth of strain IFO 13712 in the culture insert and culture plate was not enhanced by coculture with a cell-free filtrate of strain Y.

Culture filtrate from MRS culture of strain IFO 13712 or Y was prepared, the pH was adjusted, and the filtrate was added to MRS broth inoculated with strain Y. Growth was determined by time interval, and a growth curve was obtained. The maximal growth rate of strain Y mixed with cell-free filtrate of strain IFO 13712 ($0.173 \pm 0.010/\text{h}$) was significantly ($P < 0.01$) higher than that of strain Y mixed with cell-free filtrate

of strain Y ($0.112 \pm 0.004/\text{h}$). This interaction between strains IFO 13712 and Y was indicative of commensalism—one strain producing or releasing compounds that promote the growth of the other (Meers 1973).

de Man, Rogosa, and Sharpe broth has many components. We therefore used TYG broth (a simpler medium) in our subsequent experiments to attempt to reveal the mechanism by which coculture of strains IFO 13712 and Y showed enhanced growth yield compared with each single culture. In TYG broth, a mixed culture of strains Y and IFO 13712 had a greater growth yield compared with a single culture of strain Y but not IFO 13712 (Table 4)—an additive, but not synergistic, effect. The fact that the MRS broth contained 0.1% Tween 80, and TYG broth did not, led to us hypothesize that Tween 80 was involved in the commensal symbiosis. To test the hypothesis, we prepared TYG broth with 0.1% Tween 80. A mixed culture of strains Y and IFO 13712 in TYG broth containing 0.1% Tween 80 did show an increase in growth yield over a single culture of strain Y but not strain IFO 13712 (Table 4); again, an additive, but not synergistic, effect. Therefore, we needed to study other components of the medium.

de Man, Rogosa, and Sharpe broth contains 0.2% ammonium citrate, whereas TYG broth does not. To clarify the effect of citrate on commensal symbiosis, we first added 0.2 or 0.4% ammonium citrate to TYG broth and adjusted it to pH 6.86 with 1 N HCl. At 0.2% ammonium citrate, mixed culture of strains Y and IFO

Table 3. Bacterial growth in single and mixed cultures (in de Man, Rogosa, and Sharpe broth) of *Enterococcus mundtii* IFO 13712 and *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* strain Y in culture inserts (upper side) and culture plates (lower side)¹

Culture method	Strain	OD ₆₂₀
Culture insert	IFO 13712	1.53
Culture plate	IFO 13712	1.38
Culture insert	Y	1.98
Culture plate	IFO 13712	1.31
Culture insert	IFO 13712	1.39
Culture plate	Y	1.59
Culture insert	Y	1.78
Culture plate	Y	1.47

¹Strains IFO 13712 and Y were cultured in single and mixed fermentations. Values were determined by measuring the optical density (OD) at a wavelength of 620 nm after a 24-h incubation at 37°C. Each value is the mean of 3 samples.

Table 4. Bacterial growth in single and mixed cultures of *Enterococcus mundtii* IFO 13712 and *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* strain Y in different media¹

Medium and culture	Dry weight (mg/L)	P-value
TYG broth		
IFO 13712	691.1 $\pm$ 3.85	<0.01
Y	591.1 $\pm$ 7.70	<0.01
Mixed	644.4 $\pm$ 10.2	
TYG plus 0.1% Tween 80		
IFO 13712	575.6 $\pm$ 10.2	0.19
Y	355.6 $\pm$ 21.4	<0.05
Mixed	517.8 $\pm$ 51.7	
TYG plus 0.4% ammonium citrate		
IFO 13712	720.0 $\pm$ 3.67	<0.01
Y	755.6 $\pm$ 3.85	0.13
Mixed	746.7 $\pm$ 6.67	
TYG plus 0.1% Tween 80 and 0.4% ammonium citrate		
IFO 13712	693.3 $\pm$ 13.3	<0.01
Y	642.2 $\pm$ 13.9	<0.01
Mixed	851.1 $\pm$ 10.2	

¹Strains IFO 13712 and Y were cultured in single and mixed fermentations in different broths (tryptone-yeast extract-glucose, TYG, supplemented with Tween 80, ammonium citrate, or both) for 24 h at 37°C. Values were expressed as dry weight, as described in the Materials and Methods. Each value is the mean $\pm$ SD from 3 samples. A *t*-test was applied to single versus mixed fermentation in each broth.

13712 did not show an increase in growth yield over either single culture (data not shown). Similarly, at 0.4%, mixed culture of strains Y and IFO 13712 did not show an increase in growth yield over either single culture (Table 4). Finally, TYG broth with both 0.1% Tween 80 and 0.2 or 0.4% ammonium citrate was prepared. With 0.1% Tween 80 and 0.4% ammonium citrate, mixed culture of strains Y and IFO 13712 showed a significant increase in growth yield over each single culture (Table 4); this increase was not seen with the broth containing 0.1% Tween 80 and 0.2% ammonium citrate (data not shown). These results suggest that both 0.1% Tween 80 and 0.4% ammonium citrate had a positive influence on growth of these strains in mixed culture.

Analysis of Metabolites from Pure and Mixed Fermentation of Strains IFO 13712 and Y

Lactococcus and *Enterococcus* are homofermentative lactic acid bacteria. Metabolism in homolactic acid bacteria leads to >90% conversion of sugars to lactate. Under certain conditions, such as galactose utilization (Garrigues et al., 1997) and aerobic conditions (Smart and Thomas, 1987), other metabolites besides lactate, such as acetate, formate, ethanol, and CO₂, are produced; this process is generally called mixed-acid fermentation. *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis*, a citrate-utilizing lactic acid bacterium, is a component of mixed starter cultures used in the dairy industry to manufacture fermented products. The breakdown of citrate results in the production of CO₂,

acetate, and acetoin. Flavor compounds such as acetoin have been reported in dairy products (Callon et al., 2005; Chammass et al., 2006). For example, diacetyl and its reduced form, acetoin, are of interest for their organoleptic properties and are responsible for notes of a fermented buttery flavor.

We analyzed the metabolites from single and mixed cultures of the test strains in each broth (Table 5). In *E. mundtii* IFO 13712, lactate was the main end product from glucose in the presence or absence of citrate; the citrate was not consumed. In *Lactococcus* strain Y cultured in TYG broth or TYG plus Tween 80, lactate, acetate, formate, and ethanol were produced from glucose; acetoin was detected in the TYG broth but not in the TYG plus Tween 80 broth. Notably, although *Lactococcus* strain Y showed mixed-acid fermentation of glucose, it is unlikely that the type of fermentation in glycolysis was responsible for the symbiosis between strains IFO 13712 and Y. We ruled out this possibility because we also observed an increase in the OD value when strain IFO 13712 was cultured with N7 (Table 2), but strain N7 is a glucose homofermentative bacterium (Kimoto et al., 1999).

In co-metabolizing glucose and citrate, strain Y produced lactate, acetate, formate, ethanol, and acetoin (Table 5). In mixed culture, the main metabolic organic acids were also lactate, acetate, formate, and ethanol. We observed differences in lactate, formate, and ethanol production between mixed culture in TYG containing citrate and mixed culture in TYG containing both Tween 80 and citrate. In TYG containing

Table 5. Fermentation products from single and mixed cultures of *Enterococcus mundtii* IFO 13712 and *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* strain Y in different media¹

Medium and culture	Consumed (mM)		Metabolite (mM)				
	Glucose	Citrate	Lactate	Acetate	Formate	Ethanol	Acetoin
TYG broth							
IFO 13712	25.1	0.00	53.6	1.28	1.14	5.29	0.82
Y	14.4	0.00	21.4	7.85	13.0	33.7	0.34
Mixed	21.8	0.00	45.6	3.66	4.78	19.7	0.40
TYG plus 0.1% Tween 80							
IFO 13712	23.9	0.00	48.0	0.69	0.62	5.92	0.84
Y	10.8	0.00	15.3	2.68	4.23	19.2	ND ²
Mixed	22.3	0.00	45.3	1.23	1.44	10.8	0.26
TYG plus 0.4% ammonium citrate							
IFO 13712	29.8	0.00	60.1	0.86	1.76	6.50	0.75
Y	18.1	18.6	26.2	36.8	23.5	41.4	0.50
Mixed	24.6	18.6	38.4	35.0	20.6	40.4	0.64
TYG plus 0.1% Tween 80 and 0.4% ammonium citrate							
IFO 13712	30.7	0.00	57.1	0.94	1.35	7.38	0.96
Y	16.0	17.4	22.2	36.2	22.3	43.1	0.48
Mixed	35.0	17.4	61.9	29.0	11.9	11.7	0.92

¹Strains IFO 13712 and Y were cultured in single and mixed fermentations in different broths (tryptone-yeast extract-glucose, TYG, supplemented with Tween 80, ammonium citrate, or both) for 24 h at 37°C. Each value is the mean of 3 samples.

²Not detected.

citrate, lactate was produced partly from glucose, and the ratio of lactate produced to glucose consumed was 1.56; acetate, formate, and ethanol were produced from citrate and glucose. In TYG containing Tween 80 and citrate, the ratio of lactate to glucose was 1.77; the amounts of acetate, formate, and ethanol produced from citrate and glucose were less than that produced in mixed culture in TYG containing citrate. Considering that an increase in cell yield in mixed culture was observed only in the TYG containing both Tween 80 and citrate, increased availability of glucose for lactate production by strain Y must have been the mechanism supporting the observed commensal symbiosis between strains Y and IFO 13712. We arrived at this conclusion because in glycolysis, the pathway from glucose to lactate is energetic, whereas the pathway from glucose to ethanol is not. The mechanism by which ethanol production was suppressed by coculture of strains IFO 13712 and Y in TYG broth containing Tween 80 and citrate is not clear.

An increase in cell yield in mixed cultures of the 2 strains was observed only in TYG containing both Tween 80 and citrate. This finding brings up 2 questions. First, what is the role of Tween 80 in the broth in this symbiosis? Tween 80 is a nonionic detergent. We found that the cell yield of strain IFO 13712 or Y was lower in TYG broth containing Tween 80 in plain TYG broth. It is likely that symbiosis occurred in the presence of some growth suppression. Second, what is the role of the citrate in the broth in this symbiosis? Growth stimulation by citrate has been observed in *L. lactis* ssp. *lactis* bv. *diacetylactis* (Cogan, 1981; Starrenburg and Hugenholtz, 1991; Kimoto et al., 1999). In consideration of the molecular energetics of biochemical pathways, the concept developed by Bauchop and Elsdén (1960) on yield of cells per mole of energy source or substrate has been widely used (Loubiere et al., 1992; Russell and Cook, 1995). We calculated the molar growth yield from glucose (Y_G) for each strain and for mixed culture in each broth (Table 6). In TYG broth containing both Tween 80 and citrate, the Y_G of the mixed culture did not differ from that of strain IFO 13712, but it was significantly ($P < 0.05$) lower than that of strain Y. The growth activation observed in *Leuconostoc* spp. by citrate is related to activation of the energetic acetate kinase pathway under sugar and citrate co-metabolism (Schmitt et al., 1990; Schmitt and Divies, 1991). In our study, the Y_G values from strain Y grown on TYG broth and on TYG with 0.4% citrate did not differ (Table 6). This result indicates that strain Y was not able to use citrate as an energy source in the presence of glucose: the energetic pathway in mixed culture was glycolysis alone, under glucose and citrate co-metabolism.

Table 6. Molar growth yields from glucose (Y_G) in single and mixed cultures of *Enterococcus mundtii* IFO 13712 and *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* strain Y in different media

Medium and culture ¹	Y_G ²	<i>P</i> -value
TYG broth		
IFO 13712	27.6 ± 1.38	0.22
Y	41.5 ± 6.22	0.07
Mixed	29.7 ± 1.99	
TYG plus 0.1% Tween 80		
IFO 13712	24.1 ± 0.52	0.65
Y	33.4 ± 5.57	0.07
Mixed	23.3 ± 2.67	
TYG plus 0.4% ammonium citrate		
IFO 13712	24.2 ± 0.77	<0.05
Y	42.0 ± 4.31	<0.05
Mixed	30.4 ± 2.21	
TYG plus 0.1% Tween 80 and 0.4% ammonium citrate		
IFO 13712	22.6 ± 1.21	0.14
Y	40.5 ± 4.16	<0.05
Mixed	24.3 ± 0.99	

¹Strains IFO 13712 and Y were cultured in single and mixed fermentations in different broths (tryptone-yeast extract-glucose, TYG, supplemented with Tween 80, ammonium citrate, or both) for 24 h at 37°C. Each value is the mean ± SD from 3 samples. A *t*-test was applied to single versus mixed fermentation in each broth.

²The Y_G values were calculated by dividing dry weight by glucose consumed.

CONCLUSIONS

Lactococci are frequently used in the manufacture of dairy products such as cheese and fermented milk. A trial was conducted on a certain strain of *E. mundtii* to evaluate it as a probiotic and its growth in milk (Botes et al., 2008). We propose the development of new, mixed starter cultures for dairy products by using lactococci and enterococci. Here, we observed enhancement of cell yield by coculture of *L. lactis* strain Y and *E. mundtii* IFO 13712. This is the first report on symbiosis between the 2 species. We need to determine whether the survival of strain Y in vivo is enhanced by mixing this strain with *E. mundtii* IFO 13712 in milk culture, for use as a probiotic.

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