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Use of *Lactococcus lactis* subsp. *cremoris* NCDO 763 and α-ketoglutarate to improve the sensory quality of dry fermented sausages

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Abstract

The aim of the present work was to enhance the degradation of free amino acids in dry fermented sausages as precursors of volatile compounds responsible for the ripened flavour. For this purpose, *Lactococcus lactis* subsp. *cremoris* NCDO 763, its intracellular cell free extract (ICFE) and α-ketoglutarate were added to sausages. Papain was also used to increase the amount of free amino acids. When *L. lactis* was inoculated in sausages, an increase in the proteolytic phenomena was observed. The addition of α-ketoglutarate increased transamination phenomena in batches where it was added. The enhancement of these phenomena determined a noticeable rise in the content of glutamic acid (the main final product in transamination reactions) and a decrease, among other amino acids, of valine and leucine, with the formation of high amounts of their derivatives 2-methylpropanal and 3-methylbutanal. These aldehydes are responsible for the ripened flavour of dry fermented sausages. Sensory analysis showed an improvement of odour and flavour when *L. lactis* and α-ketoglutarate were combined. On the other hand, the intracellular cell free extract of *L. lactis* did not show any important activity in relation to amino acid breakdown even when used together with α-ketoglutarate and/or papain.

Keywords: Fermented sausages; *Lactococcus lactis* subsp. *cremoris*; α-Ketoglutarate; Papain; Amino acid breakdown; Volatile compounds

1. Introduction

Free amino acids, released by starter or endogenous proteases, directly participate in the basic taste of ripened foods (i.e. cheese, dry fermented sausages) and indirectly contribute to the development of their typical aroma since they are precursors of many volatile compounds such as acids, alcohols, aldehydes, ammonia, sulfur compounds, esters, etc. (Engels & Visser, 1994, 1996; Meynier, Novelli, Chizzolini, Zanardi & Gandemer, 1999; Ordóñez, Hierro, Bruna & de la Hoz, 1999; Stahnke, 1999). For this reason, many authors have attempted the acceleration of the ripening processes or the enhancement of the flavour of this kind of products through the addition of exogenous proteases (Díaz, Fernández, García de Fernando, de la Hoz, & Ordóñez, 1997; El-Soda & Pandian, 1991; Fox, Wallace, Morgan, Lynch, Niland, & Tobin, 1996; Hagen, Berdagué, Holck, Naes, & Blom, 1996; Naes, Holck, Axelsson, Andersen, & Blom, 1995; Zapelena, Zalacain, Paz de Peña, Astiasarán, & Bello, 1997; Zapelena, Ansorena, Zalacain, Astiasarán, & Bello, 1998). Nevertheless, these studies have shown that the sole increase in the amount of free amino acids is not enough to produce a significant increase in aroma compounds and it seems that, together with the addition of proteases, the mechanisms of amino acid degradation must also be favoured in order to yield higher amounts of volatile compounds (Díaz et al., 1997; Fernández, Ordóñez, Bruna, Herranz, & de la Hoz, 2000).

The degradation of amino acids during ripening of cheese and fermented meats is mainly due to microbial enzymes (Engels & Visser, 1996; Hinrichsen & Pedersen,

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1995; Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997) although chemical degradation (Strecker degradation) can also occur (Yvon et al., 1997). Main microbial degradation pathways include deamination, transamination and decarboxylation. These routes have been studied in numerous microorganisms, mainly from cheese origin (Alting, Engels, van Schalkwijk, & Exterkate, 1995; Christensen, Dudley, Pederson, & Steele, 1999; Hemme, Bouillanne, Métro, & Desmazeaud, 1982; Lee & Richard, 1984; Smit, Verheul, van Kranenburg, Ayad, Siezen, & Engels, 2000; Yvon, Berthelot, & Gripon, 1998; Yvon et al., 1997).

Lactococci, and especially *Lactococcus lactis*, are among the most important starters in the dairy industry. Strains used by the dairy industry are auxotrophic for certain amino acids and, although requirements are strain specific, they generally include isoleucine, valine, leucine, histidine and methionine (Dudley & Steele, 2001). Thus, lactococci possess proteolytic and peptidolytic enzymes to release amino acids from caseins and also a number of enzymes responsible for amino acid catabolism. Products of this catabolism are used by bacteria to generate energy and carbon, synthesise cell structures and regulate different metabolic processes (Lapujade, Cocaign-Bousquet, & Loubiere, 1998; Roudot-Algaron, & Yvon, 1998). The first step in amino acid catabolism is a transamination, which requires the presence of α-keto acid acceptor for the amino group, commonly α-keto acid acceptor for the amino group, commonly α-ketoglutarate (Yvon et al., 1998). This reaction is catalysed by aminotransferases and these enzymes have been demonstrated to initiate the catabolism of aromatic, branched-chain and sulfur amino acids either in chesse or under cheese-like conditions (Dias & Weimer, 1998; Engels et al., 2000; Gao, Oh, Broadbent, Johnson, Weimer, & Steele, 1997; Rijnen, Bonneau, & Yvon, 1999a; Roudot-Alagaron & Yvon, 1998; Yvon et al., 1997). Different authors have identified and characterised *L. lactis* aminotransferases (Atiles, Dudley, & Steele, 2000; Dudley & Steele, 2001; Engels et al., 2000; Gao & Steele, 1998; Rijnen et al., 1999b; Rijnen, Courtin, Gripon, & Yvon, 2000; Yvon et al., 1997; Yvon, Chambellon, Bolotin, & Roudot-Algaron, 2000). The resulting products of the transamination can suffer further transformations to yield, among other products, branched aldehydes (Olessen & Stahnke, 2000). The volatile fraction of many cured meat products contains those branched aldehydes, such as 2-methylpropanal and 2- and 3-methylbutanal which (by themselves or through further degradations) are responsible for the ripened aroma of these kind of products (Careri et al., 1993; Hinrichsen et al., 1995; Stahnke, Holck, Jensen, Nilsen, & Zanardi, 2000). These compounds derive from the catabolism of branched amino acids, valine, isoleucine and leucine, respectively (Montel, Masson, & Talon, 1998).

Given the potential role of microbial enzymes for the generation of flavour compounds, different experiences have been carried out by adding either pure enzymes or microbial intracellular extracts to enhance the reactions occurring during ripening. These studies were primarily oriented to increase proteolysis and lipolysis but more recently they are centred on increasing amino acid and free fatty acid breakdown. Although some of them have been performed in sausages (Bruna, Fernández, Hierro, de la Hoz, & Ordóñez, 1999; Bruna, Fernández, Hierro, Ordóñez, & de la Hoz, 2000; Bruna, Fernández, Ordóñez, & de la Hoz, 2002; Bruna, Hierro, de la Hoz, Mottram, Fernández, & Ordóñez 2001a; Gossling, 1990), most of the studies have been carried out with microorganisms isolated from milk or cheese either in vitro or in cheese (Darwish, El Deeb, & Mashaly, 1989; Dias, & Weimer, 1998; Engels, & Visser, 1996; Ezzat, 1990; Roudot-Algaron & Yvon, 1998; Smit et al., 2000). However, the only activity of aminotransferases appears not to be sufficient to cause an intense degradation of amino acids (Yvon et al., 1997). Yvon et al. (1998), Rijnen et al. (1999b) and Tammam, Williams, Noble, and Lloyd (2000) studied the effect of the addition of exogenous α-ketoglutarate to cheese and Yvon et al. (2000) carried out experiences by using a lactic acid bacterial strain capable of producing α-ketoglutarate itself from their precursors in cheese. In these experiences, the conversion of amino acids to aroma compounds was highly improved.

Considering these premises, the present work was carried out. It is an attempt to use both procedures, i.e., the inoculation of a selected strain of *L. lactis* subsp. *cremoris* or the addition of its intracellular cell free extract, with the addition of α-ketoglutarate, in order to increase the conversion of amino acids into aroma compounds in dry fermented sausages and, as a consequence, to enhance the ripened flavour of these products. To increase the amount of free amino acids, a protease-papain- was also added to sausages.

2. Materials and methods

2.1. Preparation of sausages

2.1.1. Experience with L. lactis subsp. cremoris and α-ketoglutarate

To assess the role of *L. lactis* subsp. *cremoris* NCDO 763 as starter and the effect of α-ketoglutarate in the development of aroma compounds in sausages, the following experience was carried out. A salchichón-type mixture was prepared using the formula: (% w/w): pork (55), beef (13.49), pork fat (25), NaCl (2.5), dextrin (1.8), lactose (1.0), glucose (0.8), monosodium glutamate (0.25), sodium ascorbate (0.046), NaNO3 (0.0095), NaNO2 (0.0065), and equal amounts of whole

grain and ground black pepper (0.14). The ingredients were processed in a mincer equipped with an adjustable plate set at a hole diameter of 5 mm. The total mixture (20 kg) was divided into four parts, which were used to prepare four separate batches of fermented sausages: (1) batch C (control) consisted of the initial mixture alone; (2) batch S, to which *L. lactis* subsp. *cremoris* NCDO 763 (Unité de Recherches Laitières et Genetique Apliquée, INRA, France) was added as starter (1% v/w); (3) batch K, which was added with α-ketoglutarate (2.25 g/kg); and 4) batch SK which was added with the corresponding amounts of the starter and α-ketoglutarate. The amount of α-ketoglutarate (2.25 g/kg); and (4) batch SK which was added with the corresponding amounts of the starter and α-ketoglutarate. The amount of α-ketoglutarate added to sausages was calculated according to the experiences of Yvon et al. (1998) in cheese and it was previously assayed in vitro experiences on a free amino acid mixture in the usual conditions for sausage fermentation. For its addition to sausages, α-ketoglutarate was dissolved in 200 ml of phosphate buffer 0.2 M pH 5.5. To adjust the moisture content, the corresponding amount of phosphate buffer was added to the different batches. After sufficient mixing, the mixtures were introduced into synthetic sausage casings (45 mm in diameter) and left to ripen in an Ibercex ripening cabinet, model G-28 (A.S.L., San Fernando de Henares, Spain). The sausages were fermented at 22 °C and 90% relative humidity (R.H.) for 48 h. After this, the temperature and R.H. were slowly reduced to 18 °C and 80%, respectively, in 60 h. Finally, the sausages were dried at 12 °C and 80% R.H. until the end of the ripening process (a total of 22 days). The results recorded here are the mean data obtained with samples from three different manufacturing processes carried out with the same formulation and technology.

2.1.2. Experience with an intracellular cell free extract (ICFE) of L. lactis subsp cremoris

For the preparation of the ICFE, *L. lactis* subsp. *cremoris* NCDO 763 was grown at 32 °C for 48 h in Erlenmeyer flasks containing 250 ml MRS broth (Hispanlab, Madrid, Spain) to give a final volume of 8.5 l. From centrifugation at 3000×*g* for 20 min a pellet was obtained. Four-gram aliquots were taken from the pellet and, after mixing with 10 mL of ground glass and 20 ml of phosphate buffer 0.2 M pH 5.5, they were processed in a cellular disrupter (Braun MSK, Melsugen, Germany) for 1.5 min. The final mixture was filtered through Whatman n°45 filter paper and centrifuged at 28 000×*g* for 20 min. Finally, the filtrates obtained were combined and the resulting mixture was passed through a 0.45 μm filter (Millipore, Bedford, MA, USA) connected to a vacuum pump A final volume of 1L of filtered extract was obtained. The protein content, estimated by Lowry's method (Lowry, Rosenbrough, Farr, & Randall, 1951) was 2.81 mg/ml.

Salchichón-type dry fermented sausages were manufactured as described in 1, but six batches of sausages were prepared: (1) batch C (control) consisted of the initial mixture alone; (2) batch E which was like batch C but added with 250 ml of the ICFE; (3) batch P which added with 300 units/kg of papain (E.C. 3.4.22.2) (Sigma); (4) batch EK which was added with 250 ml of the ICFE and 2.25 g/kg of α-ketoglutarate; (5) batch EP which was added with 250 ml of the ICFE and 300 units/kg of papain; and (6) batch EKP, which was added with 250 ml of the ICFE, 2.25 g/kg of α-ketoglutarate and 300 units/kg of papain. The amount of papain was established according to previous experiences with different amounts of the enzyme, in which the most adequate texture and free amino acid profile were studied. One proteolytic unit was defined as the amount of enzyme that produced an increase of 1 unit in the absorbance at 440 nm/h using azocasein (Sigma) as substrate (8 g/l in Tris–HCl buffer 0.2 M, pH 6.5). For its addition to sausages, papain was dissolved in 200 ml of phosphate buffer 0.2 M, pH 6.6.

2.2. Microbial analysis

Total viable microorganisms were counted in Plate Count Agar (PCA) (Hispanlab) and the Micrococcaceae in Manitol Sal Agar (MSA) (Hispanlab), both incubated at 32 °C for 2 days. Lactic acid bacteria were grown in MRS agar (Hispanlab), pH 7.0 and pH 5.5 at 32 °C for 2 days. The growth of *L. lactis* was indirectly monitored by comparing counts in MRS agar at pH 7.0 with counts in MRS agar at pH 5.5, which inhibits *L. lactis* (Reuter, 1985).

2.3. Chemical analysis

Dry matter (D.M.) was determined by drying the sample at 110 °C to constant weight. Water activity (a_w) was determined using a Decagon CX1 hygrometer (Decagon Devices, Pullman, WA) at 25 °C. The pH was measured in a homogenate of the sample with distilled water (1:10, w/v) using a Crison Digit-501 pH meter (Crison Instruments, Barcelona, Spain).

Free amino acids were extracted as described by Yang and Sepúlveda (1985) and analysed by HPLC as described by Bruna, Ordóñez, Fernández, Herranz, and de la Hoz (2001a). After extraction, amino acids were derivatized with phenylisothiocyanate (PITC). Amines were extracted according to Spinelli, Lakritz, & Wasserman (1974) and analysed after derivatization with dansyl chloride (Ordóñez, de Pablo, Pérez de Castro, Asensio, & Sanz, 1991). The amino acids and amines derivatives were analysed in a Beckman System Gold Nouveau chromatograph (Fullerton, CA) equipped with a Waters

column (Milford, MA) Spherisorb S5 ODS2 (25 cm×4.6 mm, 5 μm particle size) maintained at 35 °C in a column oven (Jones Chromatography, Hengoed, UK). Detection was performed at 254 nm in both cases.

Ammonia levels were determined using the Boehringer kit for enzyme analysis (Boehringer Mannheim, Mannheim, Germany) following the manufacturers instructions for meat products.

A Purge and Trap concentrator Tekmar 3000 connected to a HP 5890 gas chromatograph coupled to a HP 5972 mass spectrometer were used for the volatile compounds analysis. Seven grams of sample were minced and thoroughly mixed with 10 g of Na_2SO_4. Eight grams of the mixture were transferred into a 25 ml fritless sparger and were purged under the following conditions: 30 ml/min flow of ultra pure helium was used as a purge gas, purge was 15 min at 30 °C controlled by a thermal sleeve. The compounds concentrated in the Tenax trap were thermally desorbed at 220 °C for 3 min. The transfer line and the valves were maintained at 180 °C. A CP-Sil 8 CB low bleed/MS fused silica capillary column (60 m×0.25 mm i.d., 0.25 μm film thickness; Chrompack, Middelburg, The Netherlands) was used with helium as the carrier gas at a flow rate of 1 ml/min. Immediately before the desorption of the trap, 1 μl of an internal standard (130.6 ng/μl 1,2-dichlorobenzene in methanol) were injected into the gas chromatograph. During the desorption period of 3 min, volatile compounds were cryofocused by immersing 15 cm of column adjacent to the heater in a solid CO_2 bath while the oven was held at 40 °C. The bath was then removed and chromatography achieved by holding at 40 °C for 2 min followed by a programmed rise to 280 °C at 4 °C/min and held for 5 min. A series of n-alkanes (C_6–C_{22}) was analyzed, under the same conditions, to obtain linear retention index (LRI) values for the aroma components.

The mass spectrometer was operated in electron impact mode with an electron energy of 70 eV and an emission current of 50 μA. Compounds were identified by first comparing their mass spectra with those contained in the HP Wiley 138 Mass Spectral Database and then comparing the LRI values with either those of authentic standards or with published values. Approximate quantities of the volatiles were estimated by comparing their peak areas with those of the 1,2-dichlorobenzene internal standard, obtained from the total ion chromatograms, using a response factor of 1.

2.4. Texture analysis

Texture analysis was done following the method described by Bourne (1978) in a TA-XT2i texture analyzer (Stable Micro Systems, Surrey, UK) equipped with a cylindrical probe P/25 to determine hardness, cohesiveness, adhesiveness, gumminess, chewiness and springiness and a reversible probe to determine the maximum cutting force and the cutting work. This procedure involved cutting samples approximately 1.5 cm long and 2.5 cm wide which were compressed twice to 50% of their thickness. The following parameters were defined: hardness (H) = maximum strength required to achieve compression; area of the first compression (A1) = total energy required for the first compression; area of the second compression (A2) = total energy required for the second compression, adhesiveness = area under the abscissa after the first compression; springiness (S) = height the sample recovers between the first and second compression; cohesiveness (C) = A2/A1; gumminess (G) = H×C; chewiness (Ch) = S×G; maximum cutting strength = maximum height on the cutting graph and total cutting work = area under the cutting curve.

2.5. Sensory analysis

Triangle and acceptance tests were carried out by a panel of 11 tasters selected among the members of the Departamento de Nutrición y Bromatología III (Higiene y Tecnología de los Alimentos) and previously trained in the sensorial assessment of meat products. Sensory analysis was performed in a tasting room designed according to ISO/DP 66.58 (ISO, 1981a) and consisting of an area for the preparation of the samples and six independent cabinets for the panellists. Triangle test was performed according to ISO (1981b) by the forced-choice option, where the panellist must choose an odd sample. All batches in each experience were compared among them by presenting to the panellists three samples advising them that one might be different and asking them to identify which was the different sample. Red light was used for this analysis. In relation to the acceptance test, this was performed by the hedonic rating option, presenting one sample at a time and asking to the panellists to rate the colour, texture, odour and flavour by using a non-structured hedonic scale in which samples were given scores of 1 (very poor) to 10 (excellent). The global quality was calculated from the expression: Overall quality = (Colour×0.1) + (Texture×0.25) + (Odour×0.15) + (Flavour×0.5). This expression was calculated taking into account the opinion of the tasters who, in a study on commercial fermented sausages, had been asked to assess the relative importance of the different sensory characteristics (Bruna et al., 2000).

2.6. Statistical analysis

ANOVA was used to search for significant differences between mean values of the different results. Comparison between batches was performed by the Student Newman-Keul's test ($P < 0.05$) using SigmaStat 1.1.

3. Results and discussion

3.1. Experience with L. lactis subsp. cremoris and α-ketoglutarate

As expected, lactic acid bacteria were the dominant microbiota over the ripening period (Fig. 1). At day 0, MRS pH 7.0, counts were higher in sausages inoculated with *L. lactis* subsp. *cremoris* NCDO 763 (batches S and SK), but similar counts were found in the final product in all batches. Starting at levels of 10^5 cfu/g (batches C and K at pH 7.0) or 10^6 cfu/g (batches S and SK at pH 7.0.), counts rose sharply during the fermentation stage and then stabilised around 10^8 cfu/g until the end of the ripening. These observations are in agreement with those described by other authors (Bruna et al., 1999, 2000, 2001b, 2002; Lücke, 1998; Roig-Sagués, Hernández-Herrero, López-Sabater, Rodríguez-Jerez, & Mora-Ventura, 1999). Fig. 1 also shows the results in batches S and SK incubated at pH 5.5. These counts were between 0.4 and 1 unit lower than those of the same batches at pH 7.0, which indicates a noticeable growth of *L. lactis*.

Inoculation of *L. lactis* and/or addition of α-ketoglutarate did not significantly affect general parameters such as pH, dry matter or water activity of dry fermented sausages (data not shown). Only batch SK showed a slightly higher decrease of pH during the fermentation stage (4.7 at day 5) compared to the remainder batches (about 4.9 at day 5), although final values were similar in all batches (4.9). In any case, the values and trends of the physicochemical parameters were similar to those reported in sausages from different countries (Díaz et al., 1997; Bruna et al., 1999, 2000, 2001b, 2002; Fernández et al., 1995; Samelis, Aggelis, & Metaxopoulos, 1993; Hierro, de la Hoz, & Ordóñez, 1997; Beriain, Lizaso, & Chasco, 2000).

Fig. 2 shows the changes in the total free amino acid content of the experimental sausages along ripening. As it can be observed, all batches recorded an increase in the amino acid content, which doubled initial levels at day 15 of ripening, to reach values about 2000–3000 mg/100 g D.M. at the end of the process. Batches inoculated with *L. lactis* (S and SK) showed higher amounts of free amino acids, which evidenced the proteolytic activity exerted by the starter. The final free amino acid content (3200 and 3300 mg/100 g D.M.) in the non inoculated sausages (batches C and K) was similar to the observations reported by Beriain et al. (2000) and Bruna et al. (2000, 2001b, 2002) but in general, all batches (even the non-inoculated ones) showed a much higher content than that observed by many authors in both conventional fermented sausages and sausages manufactured with different proteases (Díaz et al., 1997; Hierro, de la Hoz, & Ordóñez, 1999; Naes et al., 1995; Zapelena et al., 1997; Zapelena, Astiasarán, & Bello, 1999). These differences can be attributed to the different composition of sausages, the ripening conditions and the duration of the ripening process. Finally, the lower amino acid content found in batch SK (2750 mg/100 g D.M.) when compared to batch S (2900 mg/100 g D.M.) could be explained by the possible enhancement of the amino acid transformations due to the presence of α-ketoglutarate, which ultimately leads to the degradation of aromatic and branched chain amino acids to give aroma compounds (Yvon et al., 1998). This hypothesis could be supported by the volatile profile found in batch SK (Table 2) and the concentration of amines found in these sausages, which was the lowest of the four batches (Fig. 3). This fact suggests that no important amino acid decarboxylation took place in these sausages.

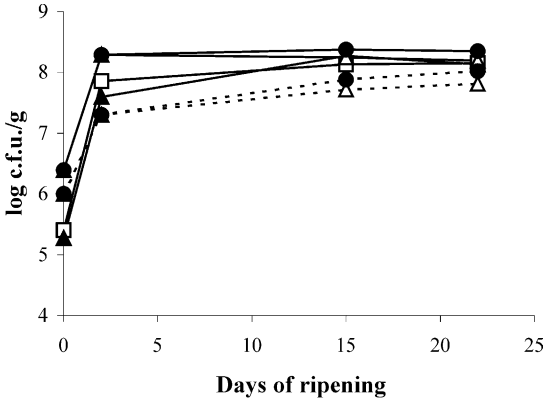

Fig. 1. Changes observed in lactic acid bacteria numbers during the ripening of dry fermented sausages. (□) control batch; (△) batch S (control batch inoculated with *Lactococcus lactis* subsp. *cremoris* NCDO 763); (▲) batch K (control batch added with α-ketoglutarate); (●) batch SK (control batch inoculated with with *L. lactis* subsp. *cremoris* NCDO 763 and added with α-ketoglutarate). Plain lines: counts at pH 7.0. Dotted lines: counts at pH 5.5.

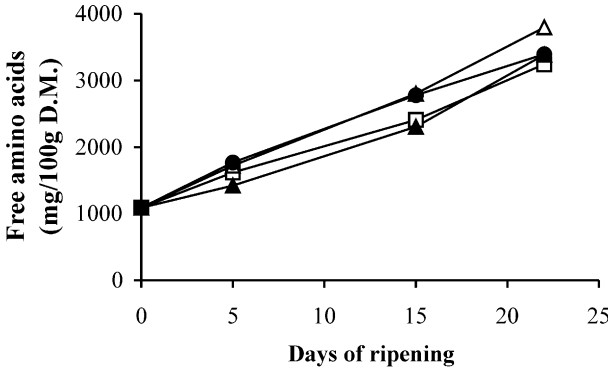

Fig. 2. Changes in the total free amino acid content (mg/100 g D.M.) during the ripening. (□) control batch; (△) batch S (control batch inoculated with *Lactococcus lactis* subsp. *cremoris* NCDO 763); (▲) batch K (control batch added with α-ketoglutarate); (●) batch SK (control batch inoculated with with *L. lactis* subsp. *cremoris* NCDO 763 and added with α-ketoglutarate).

Table 1
Ammonia and selected free amino acids[a] (mg/100 g D.M.) of the experimental sausages corresponding to experience 1 (experience with *Lactococcus lactis* subsp. *cremoris* and α-ketoglutarate)

Amino acid	Day 0	Day 22			
	C[b]	C	S	K	SK
Aspartic acid	27.9	32.2b	37.4b	72.0a	41.8b
Glutamic acid	79.0	69.7b	43.7c	95.9a	119.4a
Hypoxanthine	31.8	60.4a	24.6c	44.8b	28.2c
Serine	64.2	122.8c	132.6c	220.2a	172.1b
Asparagine	28.0	97.5a	84.4a	98.4a	64.2b
Histidine	101.9	291.3a	314.4a	227.7b	227.9b
Taurine + GABA	54.3	250.5a	273.5a	252.5a	205.4b
Proline	65.3	24.0b	21.3b	32.9b	61.8a
Tyrosine	82.0	113.3c	426.8a	144.2c	362.3b
Valine	89.3	308.6b	357.7a	282.3b,c	262.6c
Cysteine	41.3	122.8b	186.7a	199.9a	199.0a
Isoleucine	64.8	127.4b	199.9a	109.8b	185.5a
Leucine	83.6	372.0a	336.1b	362.5a	302.4c
Phenylalanine	87.0	181.7b	229.8a	225.5a	156.4c
NH_3	13.8	37.2a	41.4a	39.6a	43.2a

Values in a row with different letters are significantly different ($P < 0.05$).

[a] Free amino acids which showed significant differences ($P < 0.05$) at the end of the ripening.

[b] C: control batch; S: control batch inoculated with *L. lactis* subsp. *cremoris* NCDO 763 (1% v/w); K: control batch added with 2.25 g/kg of α-ketoglutarate; SK: control batch inoculated with *L. lactis* and added with 2.25 g/kg of α-ketoglutarate.

Table 1 shows the ammonia content and the amino acid profiles of the experimental sausages referred to those amino acids which showed significant differences among batches. In order to establish the effect of the starter on the amino acid composition, batches S (sausages only inoculated with *L. lactis*) and batch C (control sausages) were compared. By the addition of the starter, a significant increase in the content of tyrosine (×3.7), isoleucine (×1.6) cysteine (×1.5), phenylalanine (×1.3) and valine (×1.2) was noticed in batch S, while in these sausages a sharp decrease in the content of glutamic acid (×0.55) was observed. The increases of valine and isoleucine are easy to explain due to the proteolytic and peptidolytic activity of *L. lactis*, since the strains commonly used as starters are auxotrophic for valine, leucine, isoleucine (Dudley & Steele, 2001). The important decrease in the amount of free glutamic acid seems to indicate that the aminotransferases of *L. lactis* did not operate in sausages; otherwise, a significant increase of this amino acid would have been observed. Perhaps, there was not enough α-ketoglutarate present in the

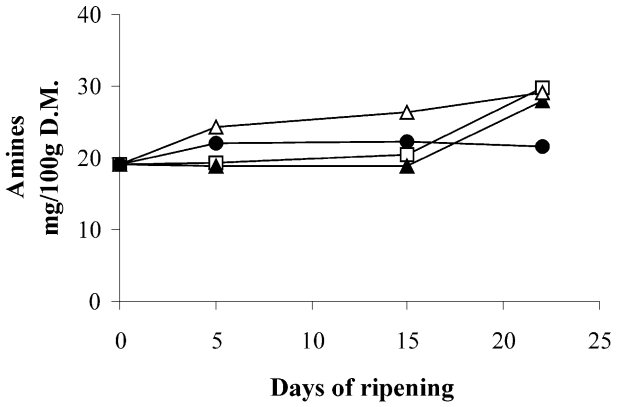

Fig. 3. Changes in the total amine content (mg/100 g D.M.) during the ripening. (□) control batch; (△) batch S (control batch inoculated with *Lactococcus lactis* subsp. *cremoris* NCDO 763); (▲) batch K (control batch added with α-ketoglutarate); (●) batch SK (control batch inoculated with *L. lactis* subsp. *cremoris* NCDO 763 and added with α-ketoglutarate).

Table 2
Volatile compounds (ng/100 g sausage) that showed significant differences ($P < 0.05$) among dry fermented sausage batches after 22 days of ripening

	C[a]	K	S	SK
Alchohols				
Ethanol	226c	1873b	1358c	2600a
2-Propanol	149a	107b	159a	161
1-Propanol	451a	402b	498a	475a
1-Penten-3-ol	1154a	955a	794b	826a,b
3-Methyl-1-butanol	69b	255a	245a	231a
1-Pentanol	203b	242b	341a	299a
Aldehydes				
2-Methylpropanal	60c	591a	347b	608
3-Methylbutanal	288d	1249c	2174b	3284a
2-Methylbutanal	n.d.	464a	n.d.	454a
Hexanal	2968a	2320b	2286b	2540a,b
Heptanal	206b	933a	399b	776a
Esters				
Ethyl acetate	436d	908c	1256b	1647a
Eythyl butanoate	n.d.	n.d.	1883a	621b
Ethyl 3-methylbutanoate	n.d.	n.d.	176a	100a

[a] C: Control batch; S: control batch inoculated with *Lactoccus lactis* subsp. *cremoris* NCDO 763; K: control batch added with α-ketoglutarate; SK: control batch inoculated with *L. lactis* and added with α-ketoglutarate. Values in a row with different letters are significantly different ($P > 0.05$).
n.d.: not detected.

medium to give rise to the transamination reactions (Yvon et al., 1998, 2000).

On the other hand, the addition of α-ketoglutarate alone (batch K) caused, in comparison with the control sausages, a decrease of the branched-chain amino acids valine (×0.9), isoleucine (×0.86) and leucine (×0.97). It seems, therefore, that α-ketoglutarate mobilised the branched chain aminotransferases (Bca T) (Yvon et al., 2000) of the lactic acid bacteria that spontaneously colonised these sausages (Larroutoure, Ardaillon, Pépin, & Montel, 2000). These observations are in agreement with the increase in glutamic acid (×1.3) reported in sausages of batch K, as it is the main acceptor for microbial aminotransferases (Yvon et al., 1998).

Finally, when both treatments were combined (batch SK), a decrease in the amount of some of the amino acids for which aminotransferases are more specific, was observed, i.e. leucine (×0.9), tyrosine (×0.8), phenylalanine (×0.7) and valine (×0.7), when comparing batch SK and S. Accordingly, a significant increase in the amount of glutamic acid (×2.7) was reported. It is well known that leucine, isoleucine and valine are intensely degraded by the branched-chain aminotransferase of *L. lactis* subsp. *cremoris* NCDO 763 (Yvon et al., 2000), and leucine also by the aromatic aminotransferase (Ara T) (Yvon et al., 1997). These activities could explain the decrease of valine and leucine observed in batch SK, also enhanced by the addition of α-ketoglutarate. It must be noted that the content of isoleucine was also lower in batch SK in comparison with batch S, but probably, the release of this amino acid by the proteases and peptidases during the ripening was so high that no significant decrease was observed in comparison with the control sausages. The decrease of valine and leucine and the increase of glutamic acid are in agreement with the observations of Yvon et al. (1998) and Rijnen et al. (1999b, 2000) in semi-hard cheeses. Valine and leucine have been reported to be precursors of volatile compounds such as short-chain fatty acids (isobutyrate and isovalerate, respectively) (Rijnen et al., 1999b), branched aldehydes (2-methylpropanal and 3-methylbutanal respectively) and their respective alcohols via either Strecker degradation (Barbieri et al., 1992; Hofmann, Münch, & Schieberle, 2000; Ventanas et al., 1992) or microbial metabolism (Hinrichsen & Pedersen, 1995). Branched aldehydes have been associated with the ripened flavour of sausages (Careri et al., 1993). Yvon et al. (1998) and Rijnen et al. (1999b) found in their studies on cheese added with α-ketoglutarate significant amounts of these compounds. In recent work, Larrouture et al. (2000) in different meat starter cultures (*Lactobacillus* spp., *Carnobacterium* spp., *Staphylococcus* spp. and *Pediococcus* spp.) and Tammam et al. (2000) in non-starter Lactobacillus spp. from cheese, demonstrated that these organisms can also produce these compounds via transamination from branched-chain amino acids, especially in the presence of α-ketoglutarate. Thus, degradation of amino acids such as valine and leucine can strongly influence flavour development. The results obtained in the volatile analysis performed in the present work seem to support these observations.

The changes in ammonia content during ripening are also shown in Table 1. Although the ammonia content was slightly higher in the inoculated sausages, no significant differences were found between batches. Therefore, no important deaminative activity was exhibited by the starter. The ammonia content markedly increased in all batches during the fermentation stage (data not shown), reaching final values of about 40 mg/100 g D.M. These concentrations are within the range considered as normal for dry fermented sausages (Flores, Marcus, Nieto, & Navarro, 1997; Huang & Lin, 1992).

Fig. 3 shows the changes in the amine content during the ripening of the experimental sausages. Two patterns were observed. No significant differences were found between batches C, K and S, where from initial levels of 19 mg/100 g D.M. a final concentration around 30 mg/100 g D.M. was reached, which indicates that decarboxilase activity was mainly due to the spontaneous microbiota present in sausages. The increase in the amine content was more noticeable in the last week of the ripening in the non-inoculated sausages (batch C and S) than in batch S. Decarboxylative phenomena and, consequently, the increase in amine content are normal during the ripening of sausages, although the increases observed in the present work (about 50%) and the final content of batches C, K and S were lower than those observed by many authors (Ayhan, Kolsarici, & Ozkan, 1999; Bover-Cid, Schoppen, Izquierdo-Pulido, & Vidal-Carou, 1999; Eerola, Maijala, Roig-Sagués, Salminen, & Hirvi, 1996). On the other hand, batch SK showed a final amine content similar to the initial values, which suggests that the presence of α-ketoglutarate directed the microbial transformation of amino acids to other pathways different to decarboxylation. The most abundant amines at the end of the ripening were tryptamine, putrescine and spermine (data not shown). Tryptamine proceeds from deamination of tryptophan and putrescine can derive from arginine. Putrescine is normally present in vacuum packaged meats (Edwards, Dainty, & Hibbard, 1983) and it is formed in the metabolic pathways of pseudomonas and, especially, enterobacteria (Edwards, Dainty, Hibbard, & Ramantanis, 1987), although this origin is not very probable in sausages since pH and a_w are not appropriate for the growth of these organisms. Putrescine is a precursor of spermine. Although there is a great variation in the results obtained by different authors, our observations partially coincide with those previously reported by Bruna et al. (2000, 2001b, 2002).

In the present work, 37 volatile compounds were identified in the headspace of the experimental sausages

after 22 days of ripening: 11 aldehydes, 11 terpenes, seven alcohols, four esters, three sulfur compounds, three hydrocarbons and one furan. The number of volatile compounds observed in the present work is markedly lower compared to the observations of other authors (Ansorena, Gimeno, Astiasarán, & Bello, 2001; Bruna et al., 2000, 2002, 2001a; Edwards, Ordóñez, Dainty, Hierro, & de la Hoz, 1999; Meynier et al., 1999; Montel, Reitz, Talon, Berdagué, & Rousset-Akrim, 1996). Table 2 shows the volatile compounds (14) that showed significant differences ($P<0.05$) among batches. A significant increase of some branched aldehydes in the experimental sausages was observed compared to the control ones. The inoculation of *L. lactis* (batch S) determined the generation of high amounts of 2-methylpropanal and 3-methylbutanal, which was highly enhanced by the addition of α-ketoglutarate (batch SK), together with the formation of 2-methylbutanal (batch K). As previously mentioned, these aldehydes are responsible for the ripened aroma of sausages and derive from the microbial breakdown of their amino acid precursors valine and leucine respectively (Barbieri et al., 1992; Hinrichsen & Pedersen, 1995; Hofmann, Münch, & Schieberle, 2000; Ventanas, Córdoba, Antequera, García, López-Bote, & Asensio, 1992). The increase of the reported branched aldehydes in the volatile profile of the experimental sausages coincides with the decrease in the concentration of valine and leucine in batches S, K and SK, and also isoleucine in batch K. As an example, one of the main volatile compounds detected in the experimental sausages, 3-methylbutanal, increased about 4-fold by the addition of α-ketoglutarate, 7-fold by the inoculation of *L. lactis* and 11-fold when both treatments were combined. In the same way, the generation of 3-methyl-1-butanol, formed by reduction of 3-methylbutanal, increased about 4-fold by the addition of α-ketoglutarate, 7-fold by the inoculation of *L. lactis* and 11-fold when both treatments were combined. In the same way, the generation of 3-methyl-1-butanol, formed by reduction of 3-methylbutanal, was enhanced by the addition of the starter and/or α-ketoglutarate. Rijnen et al. (1999b) and Banks et al. (2001) also observed an increase in the volatile compounds derived from leucine, valine and isoleucine as a result of the inoculation of *L. lactis* and/or the addition of α-ketoglutarate to cheese.

Ethyl esters are also very important from a sensory viewpoint, giving fruity and ripened notes to sausages (Barbieri et al., 1992; Meynier et al., 1999; Montel et al., 1996). Ethyl esters are formed from aldehydes that are oxidised to their corresponding acids, which are further esterified with alcohols by microorganisms (Stahnke, 1994). The detection of some ethyl esters such as ethylbutanoate and ethyl-3-methylbutanoate in batches S and SK could be explained by the esterase activity of *L. lactis*. On the other hand, some authors have found ethyl acetate to be the most abundant ester in dry fermented sausages (Bruna et al., 2000; Mateo & Zumalacárregui, 1996; Stahnke, 1994; Meynier et al., 1999). Ethyl acetate was detected in all batches, although its content was much higher in the inoculated batches (S and SK). Its presence in batches C and K could be attributed to the activity of the spontaneus lactic acid microbiota.

Table 3 shows the texture profile of experimental sausages. Significant, although not consistent differences were observed between batches, which could be related to the degree of proteolysis registered in sausages according to the activity of the dominant microbiota. Inoculated batches (S and SK) showed higher values for hardness than control sausages, but the degree of proteolysis in these batches was similar to that observed in the control sausages and no correspondence with the sensory analysis was found. On the other hand, batch K evidenced a slight decrease of hardness and related parameters (gumminess and chewiness), adhesiveness and cutting work. No explanation has been found for this behaviour since the degree of proteolysis, pH and a_w of these sausages were very similar to the other batches, but these results are in total agreement with the observations of the taste panel in the sensory analysis (Fig. 4).

Table 3
Texture analysis (mean ± standard deviation) of dry fermented sausages after 22 days of ripening

Parameter	C[a]	S	K	SK
Hardness (N)	89.8 ± 8.8b	103.2 ± 13.0a	79.5 ± 8.6b	102.1 ± 11.0a
Adhesiveness (N/s)	−0.93 ± 0.36b	−0.85 ± 0.41b	−0.34 ± 0.13a	−0.84 ± 0.52b
Cohesiveness	0.38 ± 0.033a	0.28 ± 0.058b	0.27 ± 0.082b	0.27 ± 0.031b
Springiness (m)	0.0054 ± 0.00030a	0.0038 ± 0.0147b	0.0040 ± 0.00091b	0.0036 ± 0.0048b
Gumminess (N)	37.4 ± 0.70a	29.9 ± 7.05b	22.3 ± 8.50b	27.2 ± 5.96b
Chewiness (J)	0.202 ± 0.0074a	0.118 ± 0.0652a	0.090 ± 0.0429b	0.086 ± 0.0444b
Cutting force (N)	75.3 ± 38.9b	116.2 ± 37.4b	107.6 ± 64b	143.6 ± 35.8a
Cutting work (J)	427.5 ± 84.9b	697.1 ± 127.2a,b	511.3 ± 72.4b	870.4 ± 68.2a

Values in a row with different letters are significantly different ($P<0.05$).

[a] C: Control batch; S: control batch inoculated with *Lactoccus lactis* subsp. *cremoris* NCDO 763; K: control batch added with α-ketoglutarate; SK: control batch inoculated with *L. lactis* and added with α-ketoglutarate.

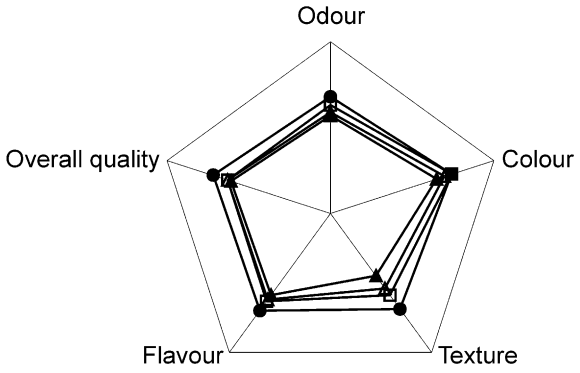

Fig. 4. Sensory analysis of experimental sausages at the end of the ripening (scale 1–10). (□) control batch; (△) batch S (control batch inoculated with *Lactococcus lactis* subsp. *cremoris* NCDO 763); (▲) batch K (added with α-ketoglutarate); (●) batch SK (inoculated with *L. lactis* subsp. *cremoris* NCDO 763 and added with α-ketoglutarate).

In the triangle taste, the tasters were able to significantly differentiate ($P<0.05$) batch SK (inoculated with *L. lactis* and supplemented with α-ketoglutarate) from control sausages and batch K (only supplemented with α-ketoglutarate) (data not shown). On the other hand, in the acceptance test (Fig. 4), significant differences ($P<0.05$) were also found for odour and flavour of sausages belonging to batch SK, apart from texture. As a consequence, the overall quality of these sausages was also significantly better. These observations can be related to a more intense amino acid breakdown beginning with a higher transaminative activity of the starter by the presence of α-ketoglutarate and the corresponding accumulation of volatile compounds. St. Paulin cheeses containing α-ketoglutarate have also been found more odorous than control samples (Yvon et al., 1998). Finally, in agreement with the results of the instrumental texture analysis, the texture of sausages only supplemented with α-ketoglutarate (batch K) was significantly worse than in the other batches.

3.2. Experience with an intracellular cell free extract (ICFE) of L. lactis subsp. cremoris

Changes in the microbial and physico-chemical parameters were similar to those described in the above reported experience and no differences were found among batches (data not shown).

As expected, the addition of papain to sausages gave rise to a significantly higher amount of free amino acids in batches P, EP and EPK in comparison to batches E and EK, with contents above 2000 mg/100 g D.M. in sausages containing the protease (Table 4). Amino acids

Table 4
Ammonia, total and selected free amino acids[a] (mg/100 g D.M.) of the experimental sausages corresponding to experience 2 (experience with an intracellular cell free extract of *Lactoccus lactis* subsp. *cremoris*)

Amino acid	Day 0	Day 22					
	C	C	E	P	EK	EP	EKP
Aspartic acid	46.4	123.1c	125.7c	200.3a	119.6c	143.2b	157.3b
Glutamic acid	56.1	85.2c	168.0b	144.5b	208.1a	161.9b	172.6b
Hypoxantine	5.9	5.7b	5.5b	10.3a	4.9b	5.7b	9.6a
Serine	12.7	20.8c	22.4c	39.1c	11.0d	64.1b	81.3a
Asparagine	5.5	12.4c	16.2c	21.2c	11.3c	45.8a	56.1a
Glycine	26.6	38.6b	48.1b	73.4a	25.4c	76.4a	81.9a
Glutamine	67.4	68.5c	63.0c	108.7b	46.5d	114.8b	135.7a
Taurine + GABA	15.7	15.3b	23.1b	20.6b	17.5b	64.7a	58.8a
Threonine	64.1	83.1b	93.7b	144.6a	44.6c	153.5a	145.1a
Alanine + Arginine	11.4	24.8b	25.0b	48.6a	15.0a	21.7b	26.4b
Proline	22.1	710.0b	700.0b	1336.4a	596.7c	534.8c	544.0c
Tyrosine	14.0	18.2c	21.3c	32.3b	13.0c	45.2a	57.6a
Valine	23.5	43.9c	47.6c	72.1b	29.0d	102.9a	97.9a
Methionine	23.0	25.5d	22.6d	31.5c	16.0d	72.4b	122.1a
Cysteine	45.6	33.2d	73.2b	133.4a	27.9d	30.4d	46.3c
Isoleucine	24.3	31.2c	25.6c	43.2b	55.5b	210.2a	211.9a
Leucine	19.6	53.2d	58.0d	83.3c	37.3e	137.3b	162.3a
Phenylanine	27.5	28.4b	31.4c	46.4b	23.6c	97.8a	93.4a
Tryptophan	24.7	30.1c	37.1c	63.9b	17.1d	115.4a	74.4b
Lysine	16.6	16.7d	32.1c	48.2b	20.5d	44.3b	76.7a
Total	666.3	1422.9c	1639.6c	2702.0a	1365.5b	2099.3a	2411.4a,b
NH_3	13.1	32.4b	36.9a,b	42.3a	38.6a,b	44.7a	49.1a

C: control batch; E: control batch added with an intracellular free extract (ICFE) of *L. lactis* subsp. *cremoris* NCDO 763; P: control batch added with papain; EK: control batch added with the ICFE and α-ketoglutarate; EP: control batch added with the ICFE and papain; EKP: control batch added with the ICFE, α-ketoglutarate and papain. Values in a row with different letters are significantly different ($P<0.05$).

[a] Free amino acids which showed significant differences ($P<0.05$) at the end of the ripening.

which showed significant differences ($P<0.05$) among batches and the ammonia content are also reported in Table 4. The addition of the bacterial extract combined with α-ketoglutarate (batch EK) determined a marked increase in the amount of glutamic acid, while a majority of amino acids significantly decreased; among them valine, leucine and tryptophan. As previously reported, branched chain and aromatic aminotransferases of *L. lactis* show a great affinity for these amino acids and the decreasing tendency might indicate that the extract exerted a slight aminotransferase activity in the presence of α-ketoglutarate. However, the only addition of the extract did not show the same effect, probably due to the lack of an acceptor for the transamination reactions. On the other hand, the addition of papain increased the content of all amino acids in all batches where it was added. In this case, no apparent enhancement of the transamination phenomena was observed when the protease was combined with the extract and α-ketoglutarate (batch EPK), probably because of the higher release of amino acids due to the proteolytic activity or maybe because papain hydrolysed the aminotrasferases of the extract. In any case, the amino acid profile of the experimental sausages analysed in this experience did not allow establishing the degradation mechanisms and, consequently, any clear conclusions.

Sausages added with papain and/or extract showed a significantly higher ammonia content (Table 4). In sausages supplemented with papain (batches P and EP), this increase can be attributed to a more intense metabolic activity of the microorganisms due to the larger amount of available free amino acids (Bruna et al., 1999, 2002; Hagen et al., 1996; Naes et al., 1995). On the other hand, the increase in the ammonia content in batches E and EK also suggests the presence of a slight deaminative activity in the extract. Obviously, batch EPK showed the highest ammonia content at the end of the ripening.

The sensory analysis performed at the end of the ripening showed that neither the addition of the extract, nor its use together with papain or α-ketoglutarate lead to an improvement of the sensory quality of the experimental sausages. As it can be seen in Fig. 5, the highest scores in the sensory analysis corresponded to the sausages added with the intracellular extract and α-ketoglutarate (batch EK), although no significant differences were found between these sausages and those from the control batch (C).

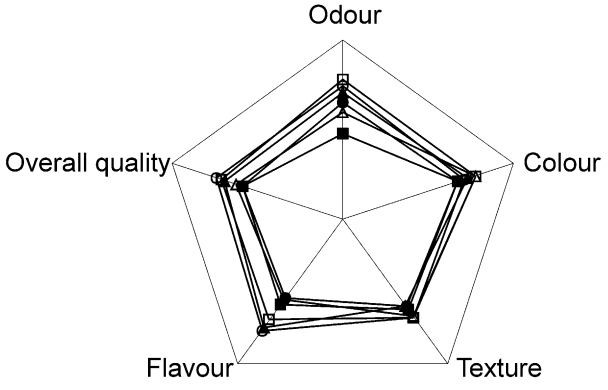

Fig. 5. Sensory analysis of experimental sausages at the end of the ripening (scale 1–10). (□) control batch; (△) batch E (control batch added with an ICFE of *Lactococcus lactis* subsp. *cremoris* NCDO 763); (▲) batch P (control batch added with papain); (○) batch EK (control batch added with an ICFE of *L. lactis* subsp. *cremoris* NCDO 763 and α-ketoglutarate); (●) batch EP (control batch added with an ICFE of *L. lactis* subsp. *cremoris* NCDO 763 and papain); (■) batch EKP (control batch added with an ICFE of *L. lactis* subsp. *cremoris* NCDO 763, α-ketoglutarate and papain).

4. Conclusions

The results obtained in the present work indicate that the use of *L. lactis* subsp. *cremoris* NCDO 763 together with α-ketoglutarate increased the content of the volatile compounds responsible for the ripened flavour and led to an improvement of the sensory quality of dry fermented sausages. The addition of an intracellular cell free extract of *L. lactis* in combination with α-ketoglutarate did not show the same effect.

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