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Synthesis of γ-aminobutyric acid (GABA) by *Lactobacillus plantarum* DSM19463: functional grape must beverage and dermatological applications

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Abstract Agriculture surplus were used as substrates to synthesize γ-aminobutyric acid (GABA) by *Lactobacillus plantarum* DSM19463 for the manufacture of a functional beverage or as a novel application for dermatological purposes. Dilution of the grape must to 1 or 4% (*w/v*) of total carbohydrates favored higher cell yield and synthesis of GABA with respect to whey milk. Optimal conditions for synthesizing GABA in grape must were: initial pH 6.0, initial cell density of Log 7.0 cfu/mL, and addition of 18.4 mM L-glutamate. *L. plantarum* DSM19463 synthesized 4.83 mM of GABA during fermentation at 30°C for 72 h. The fermented grape must also contain various levels of niacin, free minerals, and polyphenols, and Log 10.0 cfu/g of viable cells of *L. plantarum* DSM19463. Freeze dried preparation of grape must was applied to the SkinEthic® Reconstructed Human Epidermis or multi-layer human skin model (FT-skin tissue). The effect on transcriptional regulation of human beta-defensin-2 (*HBD-2*), hyaluronan synthase (*HAS1*), filaggrin (*FGR*), and involucrin genes was assayed through RT-PCR. Compared to GABA used as pure chemical compound, the up-regulation *HBD-2* was similar while the effect on the expression of *HAS1* and *FGR* genes was higher.

Keywords γ-Aminobutyric acid · Functional grape must · Human skin protection · *Lactobacillus plantarum*

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Introduction

During the last decade, fundamental studies opened a new field of research dealing with bioactive or biogenic substances derived from foods. γ-Aminobutyric acid (GABA), a non-protein amino acid, showed well-known physiological functions: neurotransmission, induction of hypotension, and diuretic and tranquilizer effects (Wong et al. 2003; Jacobs et al. 1993). GABA also exerted positive effects for treatment of sleeplessness, depression, and autonomic disorders (Okada et al. 2000), chronic alcohol-related symptoms (Oh et al. 2003) and stimulation of immune cells (Oh and Oh 2003). In addition, GABA showed anti-inflammation and fibroblast cell proliferation activities that promoted the healing process of cutaneous wounds (Han et al. 2007). It positively interfered with (1) the primary normal human keratinocytes through the osmolyte strategy for maintaining cell volume homeostasis under UV radiation (Warskulat et al. 2004); (2) the synthesis of hyaluronic acid; and (3) enhanced the survival rate of the dermal fibroblasts when exposed to H_2O_2 as the oxidative stress agent (Ito et al. 2007).

GABA is synthesized by glutamate decarboxylase (GAD; EC 4.1.1.15), a pyridoxal 5′-phosphate-dependent enzyme, that catalyzes the irreversible α-decarboxylation of L-glutamate to GABA. GAD was largely distributed in higher

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plants, animals, and bacteria (Ueno 2000; Komatsuzaki et al. 2005a). Some reports showed the presence of GAD activity in lactic acid bacteria also (Komatsuzaki et al. 2005a, b; Nomura et al. 1998; Cho et al. 2007). The sequence of the GAD gene was reported for *Lactobacillus brevis* (Park and Oh 2007), *Lactobacillus plantarum*, *Lactobacillus delbrueckii* subsp. *bulgaricus* (Makarova et al. 2006; Siragusa et al. 2007), *Lactobacillus paracasei* (Komatsuzaki et al. 2008), and *Lactococcus lactis* subsp. *lactis* (Nomura et al. 1999). Overall, GAD activity protected bacteria against low pH stress. After uptake of L-glutamate by the specific transporter, cytoplasmic decarboxylation resulted in the consumption of an intracellular proton. The net result was an increase in the pH of the cytoplasm due to the removal of hydrogen ions and a slight increase in the extracellular pH due to the exchange of extracellular glutamate for the more alkaline GABA. Furthermore, it was shown that ATP could be generated by L-glutamate metabolism in lactobacilli. GAD system seemed to provide metabolic energy by coupling electrogenic antiport and amino acid decarboxylation (Cotter and Hill 2003).

Owing the above physiological functions and the distribution of GAD in food grade microorganisms, several functional foods containing GABA were manufactured. These included several food matrices such as green tea, rice, and tempeh-like and dairy products, mainly fermented by lactic acid bacteria (Ohmori et al. 1987; Saikusa et al. 1994; Hayakawa et al. 2004; Inoue et al. 2003). Previously, the concentration of GABA of several Italian cheese varieties was determined (Siragusa et al. 2007). Lactic acid bacteria were isolated from the various cheeses and strains showing the highest GAD activity were mainly isolated from cheeses having the highest concentration of GABA. The sequence of the core fragment of GAD DNA was identified in several lactic acid bacteria, including *L. plantarum* DSM19463 (formerly *L. plantarum* C48). Some food substrates (such as whey milk and grape must) could be enriched in GABA by fermentation with GABA-producing starters (Nomura et al. 1998; Siragusa et al. 2007). Currently, the world surplus of wine is estimated to be 3.32×10^6 tons (http://www.fao.org/). Therefore, the alternative use of grape must as the substrate for biotechnological conversions has very limited economic costs and due to its chemical composition may deserve interesting nutritional perspective for industrial applications (Iriti and Faoro 2009). The consumption of grape must or juice in the human diet increased the serum antioxidant capacity (Zern et al. 2005), decreased peroxide formation and platelet aggregation, and enhanced flow-mediated vasodilation (Castilla et al. 2006; Castilla et al. 2008). Oral supplementation by concentrated red grape juice decreased the synthesis of NADPH oxidase-dependent superoxide in patients with end-stage renal disease (Garrow et al. 2000).

This study aimed at describing: (1) the synthesis of GABA by *L. plantarum* DSM19463 during agriculture surplus fermentation; (2) the manufacture of a functional grape must beverage; and (3) the dermatological effects of the fermented grape must enriched in GABA.

Materials and methods

Microorganism and substrates

L. plantarum DSM19463 (DSMZ-BP/7 0906; formerly *L. plantarum* C48) was isolated from cheese, identified, and characterized previously (Siragusa et al. 2007). Strain DSM19463 was routinely propagated and cultivated in MRS broth (Oxoid LTD, Basingstoke, Hampshire, England) at 30°C for 24 h. Twenty-four-hour-old cells were harvested by centrifugation (9,000×*g* for 15 min at 4°C), washed twice with sterile 0.05 M potassium phosphate buffer, pH 7.0, and re-suspended in an aliquot of diluted grape must or whey milk at the cell density of Log 9.0 cfu/mL.

Concentrated grape must (60%, *w/v*, of total carbohydrates), without SO_2 added, was diluted to the concentration of total carbohydrates of 0.3−4.5% (*w/v*) by distilled water or by the mixture of distilled water and fresh yeast extract (ratio of 1:1), added of 1 N NaOH to set the pH at 4.5 or 6.0, and sterilized in autoclave at 120°C for 15 min.

Fresh yeast extract was prepared according to the following protocol. Sixty grams of commercial baker's yeast were suspended in 300 mL of distilled water, sterilized in autoclave at 120°C for 30 min, stored at 4°C for 12 h, and centrifuged at 6,000×*g* for 10 min at 4°C to recover the supernatant, mainly containing the cytoplasm extract of baker's yeast.

Whey milk was supplied by local cheese making industry (Bari, Italy). The main composition of the whey milk was the following: lactose 4.8% (*w/v*), protein (0.8%, *w/v*), fat (0.4%, *w/v*), and pH 6.0. Whey milk was heat treated in autoclave at 100°C for 5 min and filtered through a 0.22 μm pore size filter.

Fermentation and determination of γ-aminobutyric acid

Grape must or whey milk were inoculated with 4% (*v/v*) of the cell suspension of *L. plantarum* DSM19463. The initial cell density was Log 7.0 cfu/mL. Substrates were supplemented with 18.4 mM L-glutamate (Sigma Chemical Co. Milan, Italy) and, in some cases, with 0.1 mM pyridoxal phosphate (Sigma Chemical Co.). Fermentation was allowed at 30°C for 72−96 h. Grape must was also fermented in the presence of high (Log 10.0 cfu/mL) and low (Log 6.0 cfu/mL) initial cell densities of *L. plantarum* DSM19463; two initial values of pH (4.5 and 6.0); two

temperatures (30 and 37°C) of incubation; and a total concentration of carbohydrates which ranged from 0.3 to 4.5% (*w/v*). Un-inoculated samples of grape must or whey milk containing glutamate (18.4 mM) and GABA (0.97 mM) were also made and incubated at two temperatures (30 and 37°C) for 72−96 h. Each batch of fermentation was carried out in triplicate.

At the end of fermentation, all samples of grape must or whey milk (10 mL) were diluted in 90 mL of sodium citrate (2%, *w/v*) solution. Serial dilution were made in quarter strength Ringer's solution and plated on MRS (Oxoid LTD) at 30°C for 48 h. The concentrations of GABA and L-glutamate were determined by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, England) as described by Siragusa et al. (2007). Thirty grams of grape must or whey milk were diluted in 90 mL of 50 mM phosphate buffer pH 7.0. The suspension was kept at 40°C for 1 h under gentle stirring (150 rpm) and centrifuged at 3,000×*g* for 30 min at 4°C. The supernatant was filtered through Whatman No. 2 paper, and the pH of the extract was adjusted to 4.6 using 1 N HCl. The suspension was centrifuged at 10,000×*g* for 10 min. Finally, the supernatant was filtered through a Millex-HA 0.22-μm pore size filter (Millipore Co., Bedford, MA, USA). A mixture of amino acids at known concentration (Sigma) was added with cysteic acid, methionine sulphoxide, methionine sulphone, tryptophan, ornithine, glutamic acid, and GABA, and used as external standard. Internal standard was produced by adding glutamate (18.4 mM) and GABA (0.97 mM) before incubation of the un-inoculated samples. Proteins and peptides from samples were precipitated by addition of 5% (*v/v*) cold solid sulfosalicylic acid, holding at 4°C for 1 h, and centrifuging at 15,000×*g* for 15 min. The supernatant was filtered through a 0.22 μm pore size filter and diluted, when necessary, with sodium citrate (0.2 M, pH 2.2) loading buffer. Amino acids were post-column derivatized with ninhydrin reagent and detected by absorbance at 440 (proline and hydroxyproline) or 570 nm (all the other amino acids).

Characterization of the fermented grape must

The concentration of total carbohydrates (glucose and fructose) was determined by enzymatic methods (DHIFF-CHAMB Italia Srl, Italia).

Fermented samples were also characterized for some nutritional/functional compounds (Kleijnen and Knipschild 1991; McDowell 2003; Bravo 1998; Katina et al. 2005). The concentrations of free Cu^{++}, Zn^{++}, and Mg^{++} were determined at the laboratory Redox SNC, Monza, Italy according to method of the inductively coupled plasma by using atomic absorption spectrophotometric (IRIS Intrepid, Thermo Elementhal, Thermo Fisher Scientific, Waltham, MA, USA) analysis and air/acetylene flame.

Niacin was determined by HPLC analysis as described by Ward and Trenerry (Ward and Trenerry 1997). The analysis was carried out with a 600E HPLC pump, model 700 WISP and a 996 photodiode array detector using a 4 mm C8 NOVAPAK Radial-PAK cartridge (8–100 mm). To avoid contamination, a C_{18} pre-column (Waters Corporation, Milford, MA, USA) was used. The mobile phase consisted of 15% methanol, 85% deionised water mixture containing 0.005 M PIC A Reagent. The eluent flow rate was 1.5 mL/min. Nicotinic acid was detected at 254 nm. Peak areas obtained from a Waters Millennium data system were used in the calculations.

Phenolic compounds were determined with the Folin-Ciocalteau method, using gallic acid as the standard (Spanos and Wrolstad 1990).

Skin model

SkinEthic® Reconstructed Human Epidermis (RHE) consisted of normal, human epidermal keratinocytes cultured to form a multi-layer, well-differentiated model of the human epidermis in vitro. The epidermal model used was represented by reconstituted human epidermal cultures which were fully differentiated by growth under air liquid interface for 17 days (surface 0.63 cm^2; biological origins: foreskin, age of donors: usually 1−4 years or abdomen, age of donors: 30−37 years). The epidermal model was inserted in a polycarbonate filter immerged in a serum-free, chemically defined medium (Rosdy and Clauss 1990; Rosdy et al. 1993).

A multi-layer human skin model (FT-skin) tissue (Phenion GmbH & Co. KG Frankfurt am Main, Germany) consisting of keratinocytes and fibroblasts belonging to the same donor was also used (O'Byrne et al. 2002). After arrival, the FT-skin model was immediately conditioned onto atmosphere containing 5% CO_2, 37°C, and humidity saturated. According to the manual's instruction, the FT-skin tissue was fully developed after 5 weeks and the model consisted of epidermis, basal membrane, and derma. The FT-skin tissue was characterized with respect to the expression of markers of differentiation at (1) epidermis (cytokeratin 10; filaggrin, FLG; transglutaminase; and involucrin, IVL); (2) derma-epidermis junctions (laminin; and collagen IV); and (3) dermal levels.

After fermentation, grape must diluted to 1% (*w/v*) of total carbohydrates was freeze dried or subjected to centrifugation (9,000×*g* for 15 min at 4°C), filtered through 0.22 μm pore size filter to remove microbial cells and freeze dried. The freeze dried preparations were dissolved in distilled water to get concentrations of GABA of 0.86 or 2.59 mM and 50 μl of these solutions were added to the SkinEthic® RHE or FT-skin tissue. The two above concentrations were selected since representative of low to medium-high concentrations of GABA. GABA, as pure chemical compound, at the lowest

concentration (0.86 mM) was also tested to discriminate the effect of GABA from the other compounds contained in the fermented grape must. Incubation at 37°C was allowed under different conditions: (1) 24 h; (2) 24 h followed by washing with saline solution (0.9%, *w/v*) and further incubation for 24 h for stressing the tissue response; (3) 48 h; and (4) 72 h. SkinEthic® RHE and FT-skin tissue treated using only the saline solution were used as the negative controls. After treatment, SkinEthic® RHE and FT-skin tissue were washed with saline solution and the tissues stored at −80°C for further RNA extraction.

Transcriptional regulation of human beta-defensin-2, hyaluronan synthase, filaggrin, and involucrin genes

RNA was extracted with the RNAqueous kit according to the manufacturer's protocol (Applied Biosystems, Monza, Italy). The cDNA was synthesized from 2 μg RNA template in a 20 μL reaction volume using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Ten microliters of total RNA were added to the Master Mix and subjected to reverse transcription in a thermal cycler (Applied Biosystems ABI PRISM 7500 Real Time PCR System) under the following conditions: 25°C for 10 min, 37°C for 60 min, and 85°C for 5 s. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using TaqMan® assay. The cDNA was amplified using the TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan gene expression assay. The following Taqman gene expression assays were used: DEFB4 Hs00175474-m1 (human beta-defensin-2 (*HBD-2*)); HAS1 Hs00155410-m1 (hyaluronan synthase, *HAS1*); FLG Hs00863478-g1 (filaggrin, *FLG*); IVL Hs00846307-s1 (involucrin, *IVL*); and Hs999999-m1 (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*). Human *GAPDH* was used as the housekeeping gene. PCR amplifications were carried out using 25 ng of cDNA in a 25 μL of total volume. In particular, the mixture reaction contained 12.5 μL of 2× TaqMan Universal PCR Master Mix, 1.25 μL of 20× TaqMan gene expression assay, 6.25 μL of water and 5 μL of cDNA. PCR conditions were 95°C for 10 min followed by 40 amplification cycles (95°C for 15 s; 60°C for 1 min). Analyses were carried out in triplicate. Standard curve was generated by plotting the threshold cycle values against the Log of the amount of cDNA. The average value of target gene was normalized using *GAPDH* gene and the values were expressed as the relative quantification data (RQ).

Immunohistochemical analysis of HBD-2

SkinEthic® RHE was treated as described above, removed from the insert using a sharp scalpel and fixed with 4% (*w/v*) formaldehyde solution (Sigma Chemical Co.). Paraffin was removed, and sections of SkinEthic® RHE were re-hydrated and boiled onto 10 mM citrate buffer pH 6.0 for antigen retrieval. Then, sections were stained with the primary antibody specific for HBD-2 (rabbit polyclonal, dilution 1:50, FL-64—Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibody enhancer and horseradish peroxidase (HRP) polymer (Thermo Fusher Scientific USA) were used for signal amplification. Diaminobenzidine (Thermo Fisher Scientific) was used as the chromogen substrate for HRP. Following development, sections were counterstained with hematoxyline and mounted under cover slips for examination. After drying, sections were used for imaging under light microscopy. SkinEthic® RHE treated with phenoxyethanol (1%, *w/w*) was used as positive control.

Detection of HAS1 and FLG proteins by ELISA

FT-skin tissue was treated as described above, removed from the insert, snap frozen, and stored at −70°C overnight. Pre-warmed phosphate-buffered saline (0.9 mL) was added twice to remove residual sample. The lower matrix of each culture was removed using forceps and the triplicate FT-skin were placed into 1 mL protein lysis solution (8 M urea, 1 M thiourea, 4% *w/v* CHAPS, 40 mM tris base, 50 mM dithiothreitol, 100 μL protease inhibitors, Sigma, Poole, UK) and homogenized using hand-held homogenizer. The samples were snap frozen in liquid nitrogen and stored at −70°C. HAS1 and FLG proteins were quantified by ELISA in 96-wells, round-bottom plates, according to the manufacturers' recommendations. Anti-HAS1 Antibody ELISA Kit was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Human Anti-FLG Antibody ELISA Kit was purchased from Cusabio Biotech Co., Ltd. (Barksdale Professional Center Newark, DE, USA). The absorbance was read at 450 nm after 2 h.

Statistical analysis

All data were obtained at least in triplicates. Percentages were arcsine-transformed for data analysis (Winer et al. 1991). Analysis of variance was carried out on transformed data followed by the separation of means with Tukey's HSD using a statistical software Statistica for Windows (Statistica 6.0 per Windows 1998, StatSoft, Vigonza, Italia). Letters indicate significant different groups ($p<0.05$) by Tukey's test.

Results

Substrate selection

Preliminarily, grape must diluted to 1% (*w/v*) of total carbohydrates with distilled water, pH 6.0, and whey

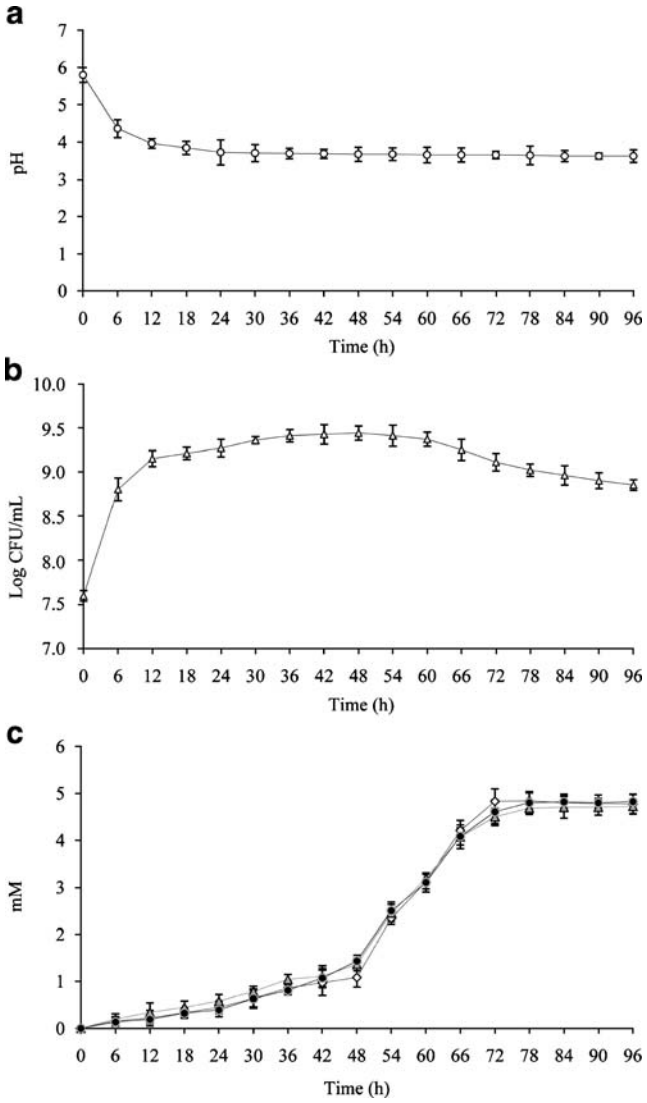

Fig. 1 Kinetics of acidification (pH units; **a**), growth (Log cfu/mL; **b**), and γ-aminobutyric acid synthesis (mM; **c**) during fermentation of grape must, diluted to 1% (*w/v*) of total carbohydrates with the mixture of distilled water and fresh yeast extract (ratio 1:1), by *L. plantarum* DSM19463. *Unfilled square* fermentation at 30°C for 96 h; *filled circle* fermentation at 37°C for 96 h; *filled diamond* fermentation at 30°C for 96 h with 0.1 mM pyridoxal phosphate. Data are the means of three independent experiments±standard deviations (n=3) analyzed in duplicate

milk, pH 6.0, were used as substrates for growing *L. plantarum* DSM19463. After 48 h at 30°C, the density was 8.1±0.2 and 8.5±0.3 Log cfu/mL for cells grown in grape must and whey milk, respectively. When grape must was diluted with the mixture of distilled water and fresh yeast extract (ratio of 1:1), the cell density of *L. plantarum* DSM19463 increased to 9.4±0.2 Log cfu/mL. The addition of yeast extract (0.5−1.0 g/L, *w/v*) to whey milk did not increase the cell yield with respect to whey milk alone. During growth in grape must diluted with distilled water and fresh yeast extract, and whey milk, the synthesis of GABA was ca. 0.89 mM and ca. 0.11 mM, respectively. Based on the above results, grape must was chosen as the substrate for both the manufacture of the functional beverage and the application for dermatological purposes.

Optimization of the synthesis of GABA

Grape must, diluted to 1% (*w/v*) of total carbohydrates with the mixture of distilled water and fresh yeast extract, was supplemented with 18.4 mM L-glutamate and fermentation was allowed at 30°C for 96 h. The acidification of the medium was completed during 24 h of fermentation reaching the constant value of pH 3.72 (Fig. 1a). The stationary phase of growth was reached after 24–30 h of fermentation leading to the final cell density of 9.4 Log cfu/mL (Fig. 1b). After 48 h of fermentation, the viability of cells of *L. plantarum* DSM19463 slightly decreased and reached 8.83 ±0.2 Log cfu/mL at 96 h. The synthesis of GABA by *L. plantarum* DSM19463 progressively increased up to 4.83 mM found at 72 h of fermentation (Fig. 1c). No interferences by other compounds on the determination of glutamate and GABA were found.

Probably due to the endogenous amount of pyridoxal phosphate in grape must (Castor 1953), the addition of 0.1 mM pyridoxal phosphate to the diluted grape must did not modify the synthesis of GABA (Fig. 1c). When the initial value of pH was set to 4.5, the synthesis of GABA at 72 h decreased to 0.39 mM. This value of pH also had a negative effect on cell yield that decreased to Log 8.03± 0.1 cfu/mL (data not shown). The increase of the temperature

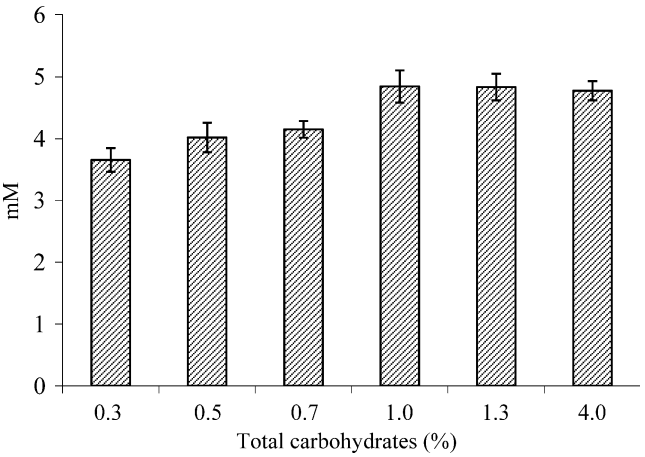

Fig. 2 Effect of the concentration of carbohydrates (0.3–4%, *w/v*) on the synthesis of γ-aminobutyric acid (mM) after fermentation (30°C for 72 h) of grape must, diluted with the mixture of distilled water and fresh yeast extract (ratio 1:1), by *L. plantarum* DSM19463. Data are the means of three independent experiments±standard deviations (n=3) analyzed in duplicate

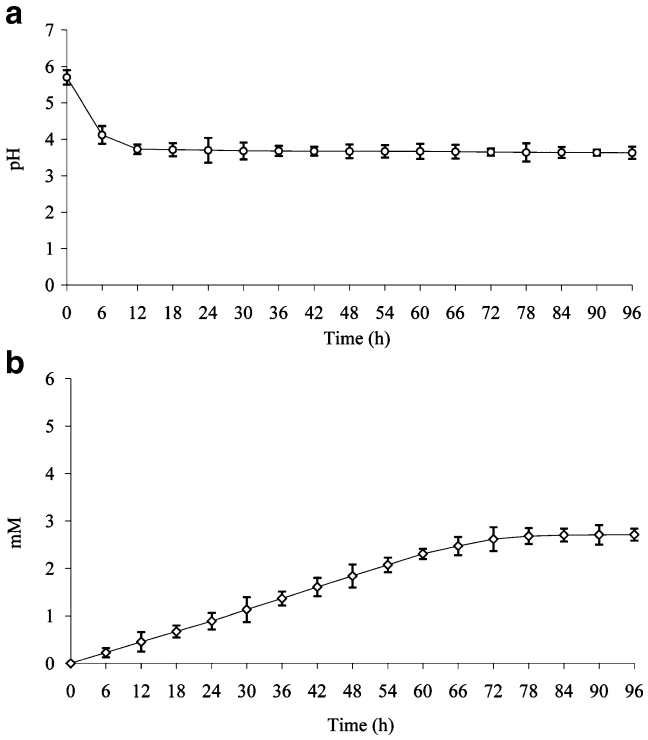

Fig. 3 Kinetics of acidification (pH units; **a**) and γ-aminobutyric acid synthesis (mM; **b**) during fermentation (30°C for 96 h) of grape must, diluted to 1% (*w/v*) of total carbohydrates with the mixture of distilled water and fresh yeast extract (ratio 1:1), by *L. plantarum* DSM19463. The initial cell density of *L. plantarum* DSM19463 was Log 10.0 cfu/mL. Data are the means of three independent experiments±standard deviations (n=3) analyzed in duplicate

of fermentation to 37°C did not modify the synthesis of GABA (Fig. 1c). The effect of the concentration of total carbohydrates on the synthesis of GABA is shown in Fig. 2. The production of GABA by *L. plantarum* DSM19463 increased progressively from 3.65 mM (0.3%, *w/v* of total carbohydrates) to 4.83 mM (1%, *w/v* of total carbohydrates). No glucose and fructose were found in the medium after 72 h of fermentation. No further increase of GABA was found when the concentration of total carbohydrates was increased from 1 to 4% (*w/v*; Fig. 2). The synthesis of GABA slightly decreased when the initial concentration of total carbohydrates was higher than 4% (*w/v*; data not shown). When grape must diluted to 4% (*w/v*) was used, the residual concentration of glucose and fructose was ca. 2.5% (*w/v*). When the initial cell density of *L. plantarum* DSM19463 was increased to Log 10.0 cfu/mL the acidification of the medium was completed during 12 h of fermentation reaching the value of pH 3.73 (Fig. 3a) and the concentration of GABA decreased compared to those found using 7.0 Log cfu/mL as initial cell density (2.66 mM). However, the production of GABA constantly increased in the first 60 h. No further increased of GABA was found after 72 h (Fig. 3b). Cell density lower than Log 7.0 cfu/mL decreased the concentration of GABA and also caused delay of the fermentation process (data not shown).

The productivity of GABA was determined under optimal conditions: 72 h of fermentation at 30°C, initial pH 6.0, grape must diluted to 1 (*w/v*) of total carbohydrates and added of 20 mM L-glutamate, and initial cell density of Log 7.0 cfu/mL. The value of productivity of GABA was 59±1.28 μM/h. The highest productivity was found at 48–72 h of fermentation (0.17 mM/h) and the synthesis of GABA stopped at 72 h. After 72 h, no L-glutamate was found in the fermented grape must. Stoichiometric conversion of L-glutamate (1 mol) into GABA (1 mol) suggested that approximately 10 mM of the consumed L-glutamate were used for other biosynthetic purposes. When grape must diluted to 4% (*w/v*) of total carbohydrates was used, no significant differences (P>0.05) were found for productivity of GABA (data not shown).

Table 1 Concentration of γ-aminobutyric acid (g/kg of dry matter, d.m.), niacin, and minerals (mg/kg of d.m.), total polyphenols (g/kg of d.m.), and viable cells of *L. plantarum* DSM19463 (Log cfu/g of d.m.) in grape must diluted to 1% (*w/v*) of total carbohydrates and fermented grape must diluted to 1% or 4% (*w/v*) of total carbohydrates

Compound/lactic acid bacteria	Grape must (1% of total carbohydrates)	Fermented grape must (1% of carbohydrates)	Fermented grape must (4% of carbohydrates)
GABA	0.65±0.21a	8.9±0.18b	9.0±0.37b
Niacin	255±1.98a	258±2.87a	3835.0±7.54b
Zn^{++}	276±4.13a	281±3.11a	4205.0±8.35b
Cu^{++}	10.8±0.22a	11.1±0.16a	155.3±0.89b
Mg^{++}	1503±32.4a	1550±17.9a	23173.0±40.9b
Total polyphenols	18.7±0.87a	20.9±0.34a	303.4±1.58b
L. plantarum DSM19463	nd	10.0±0.3a	10.0±0.8a

Data are the means of three independent experiments±standard deviations (n=3) analyzed in duplicate. For each row, letters indicate Tukey's test significant different groups (P<0.05)

nd not determined

Both preparations (diluted to 1 or 4%, *w/v*, of total carbohydrates) of grape must mainly contained ca. 8.9 g/kg of dry matter (d.m.) of GABA and Log 10.0 cfu/g of d.m. of viable cells of *L. plantarum* DSM19463 (Table 1). Various levels of niacin, minerals, and polyphenols were also present in the preparations (Table 1). Lactic acid fermentation did not cause a decrease of the above compounds with respect to the grape must. Obviously, a significantly ($P<0.05$) higher concentration (ca. 15 times) of the above compounds was found in the preparation made with grape must diluted to 4% (*w/v*) of total carbohydrates.

Transcriptional regulation of human beta-defensin-2, hyaluronan synthase, filaggrin, and involucrin

Preliminarily, the fermented and freeze dried grape must was assayed for toxicity towards SkinEthic® RHE and FT-skin tissue at the GABA concentrations of 0.86 or 2.59 mM. The saline solution used as the negative control and nonfermented grape must did not cause variation of the level of expression of the *HBD-2* gene (Fig. 4a and b). SkinEthic® RHE treated with the fermented and freeze dried grape must corresponding to 0.86 mM of GABA showed the highest expression of *HBD-2* gene after 24 h of treatment followed by washing with saline solution and further incubation of 24 h (RQ=6) or after 48 h of treatment (Fig. 4a). Expression of *HBD-2* gene further increased by the addition of fermented and freeze dried grape must corresponding to 2.59 mM of GABA (Fig. 4b). No statistically differences ($P>0.05$) were found in the expression of *HBD-2* gene between fermented and freeze dried and GABA as pure chemical compound. The up-regulation of HBD-2 was further confirmed by immunohistochemical analysis using the primary antibody for HBD-2 (Fig. 5a–h). Compared to negative control (Fig. 5a), the expression of HBD-2 was found in all SkinEthic® RHE treated with fermented and freeze dried grape must containing 0.86 (Fig. 5b–d) or 2.59 mM (Fig. 5f–h) of GABA. According to RT-PCR data, the highest expression of HBD-2 was found using 2.59 mM GABA after 48 and, especially, 72 h of incubation. In particular, the chromogen coloration of the panel H approached that of the positive control phenoxyethanol (panel e). Also in this case, treatment of SkinEthic® RHE with 0.86 or 2.59 mM of GABA as pure chemical compound gave an immunohistochemical response almost similar to that shown in panel h. Concentrations of GABA, as pure chemical compound or contained in the fermented grape must, higher than 2.59 mM slightly increased the chromogen coloration (data not shown).

Since 0.86 mM of GABA were enough for increasing the production of HBD-2, this concentration was used to assay the expression of hyaluronan synthase, filaggrin, and involucrin genes. After 72 h of exposure to the fermented and freeze dried grape must, a significant ($P<0.05\%$) over-expression of *HAS1* was found. This was higher than that found with GABA as pure chemical compound (Fig. 6a). The highest expression of *HAS1* was found after 24 h. Compared to the negative control, a significant ($P<0.05$) up-regulation of the *FLG* gene was also found. In this case, the level of expression increased during time and the highest value was found at 72 h (Fig. 6b). The expression of the *FLG* gene by fermented and freeze dried grape must was significantly ($P<0.05$) higher than that found with GABA as pure chemical compound. Compared to the negative control, no statistical ($P>0.05$) variations of the level of expression of the *IVL* gene were found with both

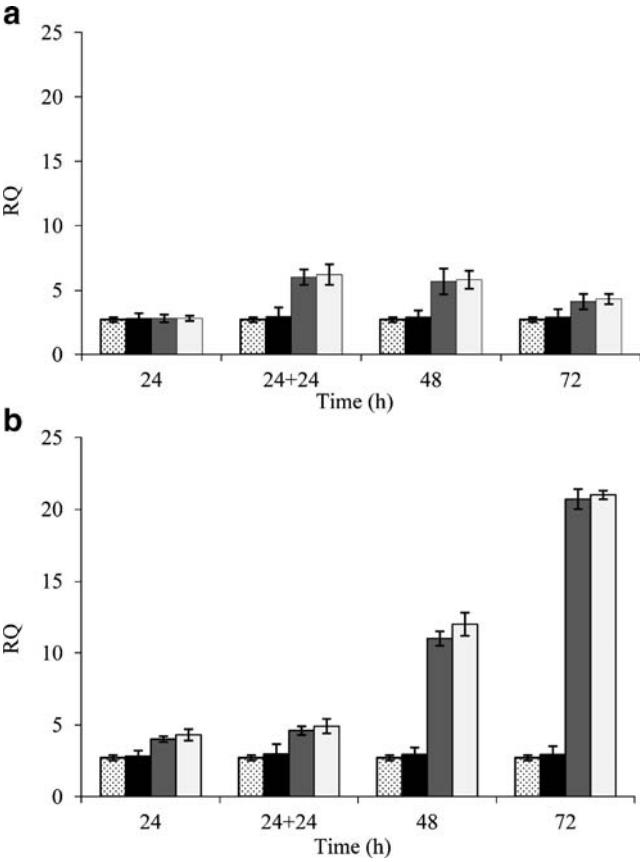

Fig. 4 Expression of the human beta-defensin-2 (*HBD-2*) gene in the SkinEthic® Reconstructed Human Epidermis as determined by RT-PCR. Concentrations of 0.86 mM (**a**) or 2.59 mM (**b**) of γ-aminobutyric acid were used. RHE treated using only saline solution (negative control; *light shaded*); grape must diluted to 1% (*w/v*) of total carbohydrates with the mixture of distilled water and fresh yeast extract (ratio 1:1; *filled block*);GABA 0.86 mM (*filled block*); grape must, diluted to 1% (*w/v*) of total carbohydrates with the mixture of distilled water and fresh yeast extract (ratio 1:1), fermented by *L. plantarum* DSM19463, and containing 0.86 mM of GABA, (*filled block*). Analyses were carried out after incubation at 37°C for 24 h; 24 h followed by washing with saline solution (0.9%, *w/v*) and further incubation for 24 h (24+24); 48 h; and 72 h. Expression rates were calculated as the relative quantification data. The data are the means of three independent experiments±standard deviations ($n=3$)

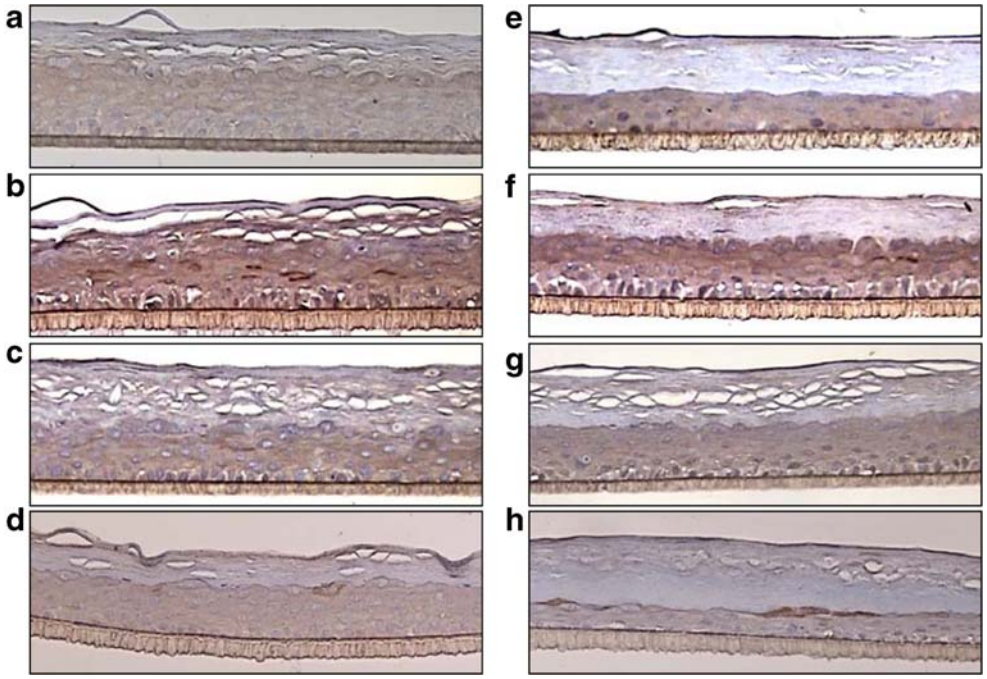

Fig. 5 Immunohistochemical analysis of the human beta-defensin-2 protein in the SkinEthic® Reconstructed Human Epidermis as determined by primary antibody (rabbit polyclonal, dilution 1:50, FL-64—Santa Cruz Biotechnology, Santa Cruz, CA, USA). Concentrations of 0.86 mM (**b**, **c**, and **d**) or 2.59 mM (**f**, **g**, and **h**) of γ-aminobutyric acid contained in the grape must, diluted to 1% (*w/v*) of total carbohydrates with the mixture of distilled water and fresh yeast extract (ratio 1:1), and fermented by *L. plantarum* DSM19463 were used. Analyses were carried out after incubation at 37°C for 24 (**b** and **f**); 48 (**c** and **g**); and 72 h (**d** and **h**). **a** RHE treated using only saline solution (negative control). **e** SkinEthic® RHE treated with phenoxyethanol (1%, *w/w*; positive control)

fermented and freeze dried grape must and GABA as pure chemical compound (Fig. 6c).

A good agreement was found between proteins and level of mRNA. The highest production of HAS1 and FLG proteins was found in FT-skin tissues treated with fermented and freeze dried grape must (Fig. 7a and b).

Discussion

First, this study showed the synthesis of GABA by *L. plantarum* DSM19463 using grape must. Under optimal condition, the production of GABA (ca. 4.85 mM) was similar to that previously shown for other lactic acid bacteria (Nomura et al. 1999; Komatsuzaki et al. 2005a, b). Using synthetic media (MRS or M17), *L. paracasei* (Komatsuzaki et al. 2005a), *L. brevis* (Inoue et al. 2003), and *L. lactis* subsp. *lactis* (Nomura et al. 1999) synthesized GABA at concentrations of 5.97, 4.92, and 0.0006 mM, respectively. *L. paracasei* NFRI 7415, isolated from sushi, was used for the production of GABA in MRS broth and during the preparation of a fermented beverage made from rice and bovine milk (Komatsuzaki et al. 2005a, b). The concentration of GABA in the beverage was 1 g/kg of dry matter. During RSM fermentation at 30°C for 24 h, lactic acid bacteria isolated from cheeses synthesized concentrations of GABA (0.15–0.97 mM; Siragusa et al. 2007) higher than those found for other cheese starters in skim milk (Nomura et al. 1998) and *Bifidobacterium longum* (Ueno et al. 1997). The optimal conditions for synthesizing GABA varied depending on the strain of lactic acid bacteria and the environmental conditions. The pH is the environmental parameter of fermentation with the most pronounced effect (Komatsuzaki et al. 2005a). Based on the results of this study, the initial value of pH 6.0 allowed the highest synthesis of GABA in *L. plantarum*. The GAD activity in *L. paracasei* showed a shaped pH profile from 4.5 to 5.5, but it was relatively high at pH 4.0 (Nomura et al. 1999). Initial pH of 5.0 was also the optimum for *Lactobacillus buchneri* grown in MRS (Cho et al. 2007). Overall, it was found that high GABA-producing strains (e.g., *L. paracasei* and *L. brevis*) showed elevated GAD activity below pH 4.0 (Gut et al. 2006). On the contrary, low levels of GAD activity were found for low GABA-producing strains (e.g., *L. lactis* subsp. *lactis*) at pH below 4.0 (Nomura et al. 1998). These results suggested that high concentration of GABA might be important for lactic acid bacteria that showed marked acid tolerance (Sanders et al. 1998; Sayed et al. 2007; Kandárová et al. 2006). The highest amount of GABA was synthesized during the late-stationary phase of growth. This could be due

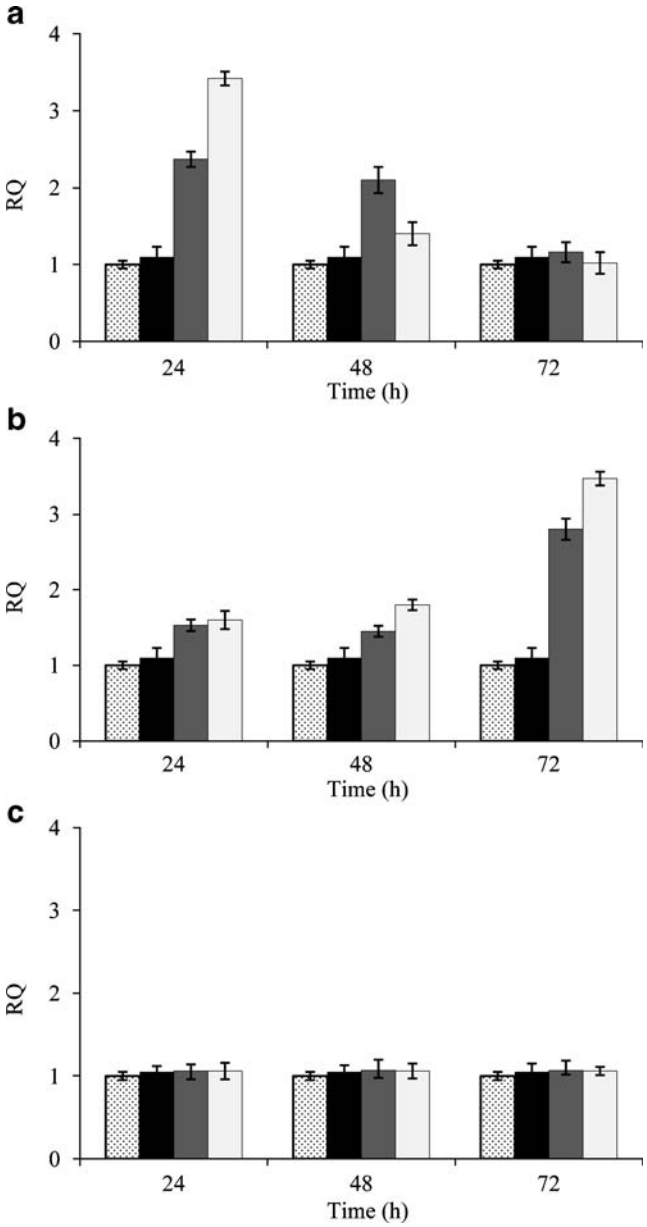

Fig. 6 Expression of the human hyaluronan synthase (*HAS1*; **a**), filaggrin (*FLG*; **b**) and involucrin (*IVL*; **c**) genes in the multi-layer human skin model (FT-skin) tissue as determined by RT-PCR. Concentrations of 0.86 mM of γ-aminobutyric acid were used. FT-skin tissue treated using only saline solution (negative control; *light shaded*); grape must diluted to 1% (*w/v*) of total carbohydrates with the mixture of distilled water and fresh yeast extract (ratio 1:1; *filled block*); GABA 0.86 mM (*filled block*); grape must, diluted to 1% (*w/v*) of total carbohydrates with the mixture of distilled water and fresh yeast extract (ratio 1:1), fermented by *L. plantarum* DSM19463, and containing 0.86 mM of GABA (*filled block*). Analyses were carried out after incubation at 37°C for 24, 48, and 72 h. Expression rates were calculated as the relative quantification data. The data are the means of three independent experiments±standard deviations (n=3)

to the induction of *gad* gene expression under stress conditions (acidity and starvation; Cotter and Hill 2003; Shao et al. 2008; Castanie-Cornet and Foster 2001). In *Escherichia coli*, it was hypothesized that GAD protein produced in exponential phase cells must undergo some form of stationary phase processing in order to become active (Castanie-Cornet and Foster 2001). Based on the results of this study, it seemed that the highest synthesis of GABA may be reached at the stationary phase of growth using growing cells (7 Log cfu/mL) instead of resting cells (10 log cfu/mL) (Castanie-Cornet and Foster 2001).

Based on the chemical and microbiological characteristics, the fermented grape must enriched with GABA might have an interest as functional beverage. A daily intake of fermented milk (10 mg of GABA) for 12 weeks decreased the blood pressure by 17.4 mmHg in moderately

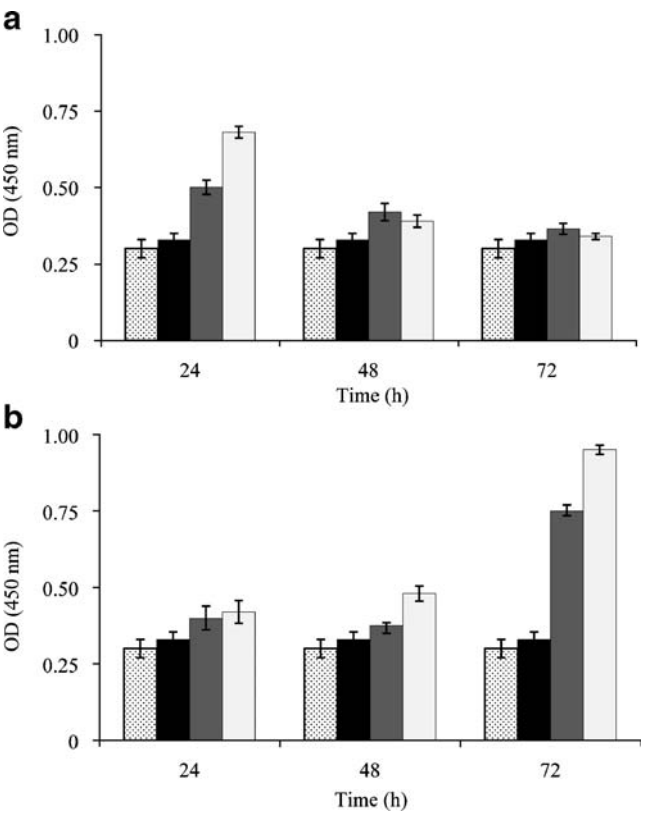

Fig. 7 Human hyaluronan synthase and filaggrin protein expression in the multi-layer human skin model (FT-skin) tissue as determined by ELISA analysis. Concentrations of 0.86 mM of γ-aminobutyric acid were used. FT-skin tissue treated using only saline solution (negative control, light shaded); grape must diluted to 1% (*w/v*) of total carbohydrates with the mixture of distilled water and fresh yeast extract (ratio 1:1; *filled block*); GABA 0.86 mM (*filled block*); grape must, diluted to 1% (*w/v*) of total carbohydrates with the mixture of distilled water and fresh yeast extract (ratio 1:1), fermented by *L. plantarum* DSM19463, and containing 0.86 mM of GABA (*filled block*). Analyses were carried out after incubation at 37°C for 24, 48, and 72 h. The data are the means of three independent experiments± standard deviations (n=3)

hypertensive patients (Inoue et al. 2003; Park and Oh 2005). The level of GABA (10 mg) contained in 20 mL of fermented grape must is the effective daily dose to get anti-hypertensive activity (Inoue et al. 2003).

First, this study showed that GABA has the capacity to induce the expression of some human genes involved in skin protection. SkinEthic® RHE is histologically similar to in vivo human epidermis and features a functional permeability barrier which is one of the main functions in viable skin. The European Centre for the Validation of Alternative Methods judged the SkinEthic® RHE model as reproducible, both within and between laboratories, and over time (Coquette and Poumay 2009). Following an approach similar to that of this study, SkinEthic® RHE was recently used to analyze the regulation of *HBD-2* gene expression in response to microbial lipopolysaccharides (LPS; Chadebech et al. 2003). As the barrier organ, human skin is always in contact with the environment and is covered with a characteristic microbiota (Noble 1992). Resident microorganisms are present in low numbers. Chemical compounds synthesized in the uppermost parts of the skin may control the growth of microorganisms (Schröder and Harder 2006). HBD-2 is a cystein-rich cationic low molecular weight antimicrobial peptide discovered in psoriatic lesional skin (Harder et al. 1997). Since inducible, it was intriguing to speculate that HBD-2 is a dynamic component of the local epithelial defense system of the skin. Healthy tissue epithelial cells express *HBD-2* gene at low levels. Nevertheless, it was strongly up-regulated by treatments of cultured epithelial cells with proinflammatory cytokines (e.g., TNF-α and IL-1β), bacterial LPS, bacteria, and yeasts or chemical mediators of skin inflammation (e.g., phorbol esters; Diamond et al. 1996; Tarver et al. 1998). This study showed that expression of HBD-2 was markedly induced by GABA. Induction was shown both by RT-PCR and immunohistochemical analyses. Since largely used for other applications (Pernet et al. 2003; Selleri et al. 2007), primary antibody specific for HBD-2 was used. Furthermore, it was clearly shown that up-regulation of HBD-2 was due only to the presence of GABA in the diluted and fermented grape must. As previously found by other authors (Ito et al. 2007), the GABA preparation also stimulated the synthesis of HAS1. HAS1 is involved in the synthesis of hyaluronan which is a glycosaminoglycan polymer responsible for the water content of skin (Stern and Maibach 2008). Contrary to the up-regulation of HBD-2, the stimulation of HAS1 was higher with the GABA preparation with respect to the GABA used as pure chemical compound. Based on our experimental results, other grape must components and especially microbial cells/metabolites might have an effect on the stimulation of the *HAS1* and *FLG* genes. Within the human keratinocyte differentiation markers, FLG plays an important role in the barrier function of the skin (Enomoto et al. 2008). The up-regulation of HBD-2, HAS1, and FLG by exogenous and noninflammatory related chemical compounds might open completely new strategies for antimicrobial therapy in cosmetics. In conclusion, grape must enriched of GABA by fermentation with *L. plantarum* DSM19463 would be of interest for food and cosmetic industries since it should be considered a health-oriented product with potential anti-hypertensive effect and dermatological protection.

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