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Exploitation of grape marc as functional substrate for lactic acid bacteria and bifidobacteria growth and enhanced antioxidant activity

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ABSTRACT

This study aimed at using grape marc for the growth of lactic acid bacteria and bifidobacteria with the perspective of producing a functional ingredient having antioxidant activity. *Lactobacillus plantarum* 12A and PU1, *Lactobacillus paracasei* 14A, and *Bifidobacterium breve* 15A showed the ability to grow on grape marc (GM) based media. The highest bacterial cell density (>9.0 CFU/g) was found in GM added of 1% of glucose (GMG). Compared to un-inoculated and incubated control fermented GMG showed a decrease of carbohydrates and citric acid together with an increase of lactic acid. The content of several free amino acids and phenol compounds differed between samples. Based on the survival under simulated gastro-intestinal conditions, GMG was a suitable carrier of lactic acid bacteria and bifidobacteria strains. Compared to the control, cell-free supernatant (CFS) of fermented GMG exhibited a marked antioxidant activity *in vitro*. The increased antioxidant activity was confirmed using Caco-2 cell line after inducing oxidative stress, and determining cell viability and radical scavenging activity through MTT and DCFH-DA assays, respectively. Supporting these founding, the *SOD-2* gene expression of Caco-2 cells also showed a lowest pro-oxidant effect induced by the four CFS of GMG fermented by lactic acid bacteria and bifidobacteria.

1. Introduction

Grape marc showed a significant impact on the environment due to the high phenols content considerably increasing both the chemical (COD) and biochemical (BOD) oxygen demands (Spigno and De Faveri, 2007), thus making its disposal a serious environmental problem with increasing cost for its treatment. In Europe, grape marc produced in the wine-making process – referred to as "fresh grape marc" – was mandatory utilized by the distilling industry. The Council Regulation (EC) No. 479/2008 and Commission Regulation (EC) No. 555/2008 allowed the producers to look for new opportunities for the exploitation of the fresh and also exhausted grape marc (Fiori and Florio, 2010). Italy has always had a great agricultural vocation either in quantitative or qualitative terms, and the Apulian region is leader at national and international levels in wine productive branches with significant economic and occupational outcomes (ISTAT 2015; OIV, 2015). Several studies suggested different grape marc processing to balance out waste treatment costs. The most of analyses were focused on the extraction of natural antioxidants (polyphenols compounds) with application in pharmacological, cosmetic, and food industries (Palenzuela et al., 2004; Pasqualone et al., 2014; Rockenbach et al., 2011; Sessa et al., 2013; Spigno and De Faveri, 2007), cellulose and hemicelluloses (Spigno et al., 2008), composting processes (Bustamante et al., 2009) and energetic exploitation (Fiori and Florio, 2010). Grape marc containing large amounts of hemicellulosic sugars and fatty acids was fermented by *Lactobacillus pentosus* for the production of lactic acid or biosurfactants (Portilla et al., 2007, 2008a, 2008b). In other food-chains, e.g. dairy industry, several applications were proposed as efficient and cost-effective methods to eliminate cheese whey by-product without negative

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environmental issues. Previously, whey was used to produce lactic acid (Arasaratnam et al., 1996; Mostafa, 1996) and ethanol by lactose converting lactic acid bacteria and genetically-engineered yeast (Domingues et al., 2001; Guimaraes et al., 2010), respectively. Production of microbial biomass (e.g., yeasts) using by-products (e.g., whey) were also proposed (Vamvakaki et al., 2010). In the last decades, the increasing interest for novel processes and products, providing ingredients with new functionalities and cost-effective manufacturing, emphasizes the potential of food-grade fermentations and microbial bioconversion for the production of functional foods/metabolites (De Vos, 2005; Gobbetti et al., 2010). Fermented functional foods have healthy effects on humans by interactions of ingested live microorganisms with the host (probiotic effect) or indirectly as the result of the ingestion of microbial metabolites synthesized during fermentation (biogenic effect) (Stanton et al., 2005; Arena et al., 2014). The use of food by-products to produce functional foods represent a new interesting opportunity. This is already done for whey which is currently used to produce functional beverages (Almeida et al., 2008; Madureira et al., 2005; Magalhães et al., 2010). The use of grape marc as substrate for production of biomass of lactic acid bacteria and bifidobacteria strains has very limited economic costs and due to its chemical composition may deserve interesting nutritional perspective for industrial applications (Iriti and Faoro, 2006). Previously, 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, 2008). Due to the antioxidant activity of grape marc, new opportunities of its use for the production of functional foods and/or compounds can be hypothesized.

This study aimed at using grape marc as substrate for the growth of lactic acid bacteria and bifidobacteria with the perspective of producing a functional ingredient, dietary supplement or pharmaceutical preparation.

2. Materials and methods

2.1. Microbial strains and culture conditions

Nine strains belonging to *Lactobacillus* genus (*Lactobacillus rossiae* DSM15814, *Lactobacillus reuteri* DSM20016, *Lactobacillus plantarum* 12A and PU1, *Lactobacillus rhamnosus* SP1, *Lactobacillus casei* FC1-13, *Lactobacillus paracasei* 14A) and *Bifidobacterium* genus (*Bifidobacterium breve* 15A and *Bifidobacterium animalis* 13A) were used. Strains belong to the Culture Collection of the Department of Soil, Plant and Food Sciences of the University of Bari Aldo Moro, Italy. The exceptions were for *L. rossiae* DSM15814 and *L. reuteri* DSM20016 (belonging to the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, Germany) and for *L. rhamnosus* SP1 (Sacco S.r.l., Cadorago, Italy). Strains were isolated from sourdoughs (*L. rossiae* DSM15814), cheeses (*L. plantarum* PU1, *L. casei* FC1-13) and human faeces (*L. reuteri* DSM20016, *L. plantarum* 12A, *L. rhamnosus* SP1, *L. paracasei* 14A, *B. breve* 15A and *B. animalis* 13A). As shown by in vivo test, all strains survived during the gastro-intestinal transit and adhered to human intestinal mucosa. *L. rossiae* DSM15814 and *L. reuteri* DSM20016 are known to produce the vitamin cobalamin (De Angelis et al., 2014; Sriramulu et al., 2008). *L. rhamnosus* SP1 was commercially available in the probiotics catalog of Sacco S.r.l. *L. casei* FC1-13 showed *in vitro* angiotensin-I converting enzyme (ACE)-inhibitory activity (Nejatia et al., 2013). Strains were propagated into de Man, Rogosa and Sharpe (MRS, Oxoid, Basingstoke, Hampshire, England). The only exception was for *L. rossiae* DSM 15814 that was propagated into modified MRS broth with the addition of fresh yeast extract (5%, v/v) and 28 mM maltose to a final pH of approximately 5.6 (De Angelis et al., 2002). Strains were cultured at 30 °C (lactobacilli) or 37 °C (bifidobacteria) for 24 h in anaerobic conditions.

2.2. Grape marc based media and growth of lactic acid bacteria and bifidobacteria strains

Industrial red grape marc, from the “Negroamaro” variety harvested in South Apulia in 2013 was collected. The grape marc derived from a winemaking process including a 7 days-maceration and a soft pressing with an hydraulic press. The average sugar content was of 5.0 g/kg of red grape marc. The red grape marc was homogenized with peptone water (0.4% yeast extract (w/v) and 0.1% peptone (w/v)) in Omni-mixer Homogenizer (Cole-Parmer, Genercontrol S.p.A., Milan, Italy) (rate 1:10) (GM). The homogenate GM was bring to pH ca. 6.0 with 5 N NaOH and sterilized (121 °C for 15 min). All strains were inoculated in GM and in GM added of 1% of glucose (GMG). Lactic acid bacteria and bifidobacteria cells used for the inoculum were cultured in conditions described above. After 24 h at 30 °C or 37 °C cells were centrifuged 9000×*g* for 10 min and washed in sterile 20 mM of potassium phosphate buffer at pH 7.0. Each strain was inoculated in the GM or GMG at the final cell density of ca. 7 log CFU/mL. Fermentation was carried out for 24 h at 30 °C (*Lactobacillus*) or 37 °C (*Bifidobacterium*) in anaerobic condition. GM and GMG without inoculum were also incubated for 24 h at 30 °C and 37 °C and used as control. Each trial was performed in triplicate.

The pH was determined with a pH meter (Model 507, Crison, Milan, Italy) after fermentation (T24). The growth of lactobacilli and bifidobacteria on GM and GMG after 24 h of incubation was determined by plating onto MRS agar. After 24 h at 30 °C and 37 °C only *L. plantarum* 12A and PU1, *L. paracasei* 14A and *B. breve* 15A showed acidification and growth on both media. In addition, GMG showing the highest growth of lactic acid bacteria and bifidobacteria cells was selected for further studies.

2.3. Chemical characterization of grape marc fermented by lactic acid bacteria and bifidobacteria

Samples of GMG fermented by selected lactic acid bacteria and bifidobacteria and un-inoculated and incubated GMG (control) were centrifuged at 9000×*g* for 10 min, and the supernatant was filtered through a Millex-HA 0.22-μm-pore-size filter (Millipore Co., Bedford, MA) (cell-free supernatant, CFS). Total titratable acidity (TTA) and volatile acidity were determined by OenoFossTM automatic analyzer (Foss, Hillerød, Denmark). Analyses of carbohydrates, organic acid and glycerol were carried out through HPLC (High Performance Liquid Chromatography), using an ÄKTA Purifier™ system (GE Healthcare Bio-Sciences, Uppsala, Sweden) equipped with a UV detector (Zeppa et al., 2001) and a Perkin Elmer 200a refractive index detector. Minerals, vitamins and fiber of GMG samples were determined by using MP/C/473 2006 official method for phosphorus determination; ITISAN 1996/34 for calcium, magnesium, sodium and iron; AOAC 975.03.1988 for potassium; AOAC 991.42 1994 for insoluble fiber; AOAC 993.19 1996 for soluble fiber; MP/C/640 2006 for vitamins B1 and PP. The total and individual Free Amino Acids (FAA) contained in the CFS samples were analyzed using a Biochrom 30 series amino acid analyzer (Biochrom Ltd., Cambridge Science Park, England) with a sodium cation-exchange column (20 by 0.46 cm [inner diameter]) (Siragusa et al., 2007).

2.4. Concentration of total phenols and low-molecular weight phenol composition

The total phenols content (TPC) of CFS samples was determined