The enhancement of the growth ability and the viability of some probiotic bacteria in media with wild *Origanum vulgare* L. extract

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Abstract

The growth ability and the viability of the *Lactobacillus acidophilus* and *Lactobacillus casei* probiotic strains in media *Origanum vulgare* L., extract rich in phenolic constituents had a good antioxidant capacity in biological active compounds were evaluated. An extract of *Origanum vulgare* L. from wild Romanian mountains flora, obtained by pressurized liquid extraction (PLE), was used in order to show the benefic role of bioactive compounds on the two probiotic strains in culture and in the aggressive conditions of the simulated gastric juice. The results showed that the growth of *Lactobacillus acidophilus* and *Lactobacillus casei* was positively influenced by the addition of 20 mg/mL extract of *Origanum vulgare* L. in MRS broth. After 4 hours of cultivation at 37 °C, in anaerobe conditions the viability of probiotic bacteria in simulated gastric conditions (pH 2.0, time of 0, 30, 60 and 90 min and 50 rpm) in the presence of the same concentration of *Origanum vulgare* L., extract was evaluated. The resistance of probiotic bacteria in simulated gastric conditions is enhanced of 1.25 fold, after 30 minutes of incubation, comparing with control without added vegetal extract.

Keywords: probiotics, *Lactobacillus acidophilus*, *Lactobacillus casei*, wild *Origanum vulgare* L., phenolic compounds, pressurized liquid extraction (PLE)

Introduction

Probiotics are micro-organisms which when administered in adequate amounts confer a health benefit on the host [1]. In order to provide health benefits for probiotic bacteria it has been recommended that they must be present at a minimum level of 6 log CFU/g of food product or 7 log CFU/g at point of delivery or be eaten in sufficient amounts to yield a daily intake of 8 log CFU/g [2-4]. In recent years it has been proved that probiotic bacteria brings some protection against pathogen microorganisms, stimulates the immune system and it even contributes to the lessening of colon cancer [5]. *Lactobacillus* and *Bifidobacterium* probiotic species provide multiple functional benefits in the human large intestine. To accomplish the probiotic effect, lactic bacteria must survive viable and at a large number to the colon [6].

The species, *Lactobacillus acidophilus* and *Lactobacillus casei* are lactic bacteria mostly used in the dairy production and they have an important probiotic activity that brings benefits to the human body. However, the gastrointestinal tract provides aggressive conditions to probiotics, such as the acid pH in the stomach and the bile in the duodenum, that reduce the viability of these bacteria. Several system have been developed to simulate in vitro the physicochemical conditions like the ones in the gastrointestinal human tract and to allow the study of lactic bacteria viability [7].

Dietary phenolic compounds with biological effects are susceptible to be metabolized by intestinal bacteria during the gastrointestinal passage, prior being absorbed. The metabolic
activity of the gut microbiota on bioactive food components can modify the host exposure to these components and their potential health effects. However, research on the possible stimulatory role of phenolic compounds on beneficial intestinal bacteria growth is less study, more attention being paid to antimicrobial activity [8]. Therefore, the aim of the study was to evaluate the growth ability and the survival capacity of *Lactobacillus acidophilus* and *Lactobacillus casei* probiotic strains in presence of *Origanum vulgare* L. extract, during cultivation and in simulated gastric juice conditions.

**Materials and Methods**

**Samples, probiotic cultures and chemicals**

*Origanum vulgare* L. sample were obtained from Plafar market from Galati, Romania (dried using a traditional method, as follows: once collected, the plant was ventilated to remove humidity, covered with a blanket to avoid sunlight and let dry in a ventilated place for 20-30 days, at 20 °C, depending on the season) [9].

Probiotic lactic acid cultures, *Lactobacillus acidophilus* and *Lactobacillus casei* were provided from Chr. Hansen, Denmark, as freeze-dried commercial starters. The storage and maintenance of the cultures were carried out according to the recommendation of the manufacturers.

Dimethylsulfoxide (DMSO, 99.9 % purity) was obtained from Fluke (Switzerland). 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95 % purity) was obtained from Sigma–Aldrich (Steinheim, Germany). Folin-Ciocalteau phenol reagent and sodium carbonate (Na$_2$CO$_3$) were acquired from Merck (Darmstadt, Germany) whereas gallic acid was supplied by Sigma–Aldrich (Steinheim, Germany).

**Pressurized liquid extraction (PLE)**

Extraction of bioactive compounds was performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller. Water was used as solvent and the extraction was performed at 150 °C, while the extraction time was 20 min. The extraction procedure has been described elsewhere [10]. The extraction cell was filled with sand between the sample to prevent the clogging of the system. The extract obtained were protected from light and stored under refrigeration until dried. For water evaporation a freeze-dryer (Labconco Corporation, Missouri, USA) was employed.

**Total phenols in extract determination**

Total phenols were estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/g dry matter, according to the Folin-Ciocalteau assay [11]. The total volume of reaction mixture was miniaturized to 10 mL. Six milliliters water and 100 μL of sample were mixed, to which 500 μL undiluted Folin-Ciocalteau reagent was subsequently added. After 1 min, 1.5 mL of 2 % (m/V) Na2CO3 were added and the volume was made up to 10 mL with water. After 2 h of incubation at 25 °C, the absorbance was measured at 760 nm in a UV-VIS Jenway 6300 spectrophotometer and compared to the gallic acid calibration curve (0.025 – 2 mg/mL) elaborated in the same manner. Data were presented as the average of triplicate analyses.

**DPPH radical scavenging assay**

The antioxidant activity of all the obtained extracts was measured using the DPPH radical scavenging assay based on the protocol by Brand-Williams et al. [12] and formerly described [13]. Briefly, a solution was prepared dissolving 23.5 mg of DPPH in 100 mL of methanol. This solution was further diluted 1:10 with methanol; different concentrations of wild *Origanum vulgare* L. extracts were tested and 0.1 mL of these solutions along with 3.9 mL of DPPH solution was placed in test tubes to complete the final reaction media (4
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reaction was completed after 4 h at room temperature and absorbance was measured at 516 nm in a UV-VIS Jenway 6300 spectrophotometer. Methanol was used to adjust zero and DPPH–methanol solution as a reference sample. The DPPH concentration remaining in the reaction medium was calculated from a calibration curve. The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50 % or EC50. Therefore, the lower the EC50 the higher the activity of the antioxidant. Measurements were done, at least, in triplicate.

**Probiotic bacteria cultivation**

Freeze-dried probiotic starter cultures were rehydrated in MRS broth and incubated at 37 °C for 1 h under aerobic condition. The cell number at the beginning of cultivation was 8 log CFU/mL for *Lactobacillus acidophilus* and 6 log CFU/mL for *Lactobacillus casei*. The *Origanum vulgare* L. extract was first dissolved in 20% DMSO.

To highlight the influence of the *Origanum vulgare* L. extract upon growth of *Lactobacillus acidophilus* and *Lactobacillus casei*, the following samples were designed: control 1 - culture in MRS broth; control 2 - culture in MRS broth with added 20 % DMSO solution; oregano extract sample – culture in MRS broth with 20 mg/mL *Origanum vulgare* L. extract dissolved in 20% DMSO solution. All the samples were incubated at 30 °C for 4 h under aerobic conditions. The resulting suspensions were serially diluted and plated in triplicate on MRS agar for counting purposes, initial and after 4 hours of incubation. Plates were incubated under anaerobic condition at 37 °C, for 3 days. Data, expressed as means from three independent experiments with two replicates.

**Cells viability testing**

Simulated gastric juice (SGJ) consisted of 9 g/L of sodium chloride containing 3.0 g/L of pepsin with pH adjusted to 2.0 with hydrochloric acid.

Probiotic bacteria were cultivated in MRS broth, for 4 hours, at 37 °C, and then 0.2 mL of cells suspension of *Lactobacillus acidophilus* or *Lactobacillus casei* were mixed in 10 mL of SGJ and incubated for 30, 60 and 90 minutes at 37 °C with constant agitation at 50 rpm. Triplicate culture samples was mixed with SGJ with added 20 mg/mL *Origanum vulgare* L. extract, and then incubated at 37 °C and sampled 30, 60 and 90 minutes. Surviving bacteria were counted by pour plate techniques in MRS agar by anaerobically incubation at 37 °C, for 3 days according to methods described by Chavari et al. [2]. Data, expressed as means from three independent experiments with two replicates.

**Results and discussions**

The applicability of PLE as an advanced environmentally friendly extraction technique for the extraction and characterization of native Romanian plants such as wild oregano has been demonstrated [14]. In an attempt to identify the compounds responsible of the antioxidant activity and also for stimulatory role on probiotic cells growth found in *Origanum vulgare* L., a characterization by LC–MS of the PLE extract have been reported previously by Miron et. al [15]. The total phenolic content and antioxidant capacity of different aromatic plants from Romania were measured. Some compounds that are partially responsible for the antioxidant activity observed were identified. The main antioxidant compound identified in oregano was rosmarinic acid; this compound is well-known by its potent antioxidant activity [16]. Although other important compounds were also identified, such as phenolic acids (protocatechuic, caffeic, chlorogenic, homovanilic, hydroxybenzoic, caffeic ethyl ester and syringic acids) and flavonoids (luteolin-7-O-glucuronide, luteolin, naringenin). Typical phenolics that possess antioxidant activity are known to be mainly
phenolic acids and flavonoids. These type of phenolic compounds are widely distributed on nature and are well known by their functional properties, among others, a potent antioxidant activity.

The results provide useful information like the potential use of plants as a natural source of antioxidants and as a value-added product in the preparation of functional food ingredients and/or for enrichment of certain products.

By use PLE a Origanum vulgare L. extract have been obtained with a high antioxidant activity (EC50 10.06 ± 0.16 μg/mL) and high total phenols content (173.65 ± 6.87 mg gallic acid/g extract). This extract was used as functional ingredient to evaluate the growth ability and viability in simulated gastric juice of Lactobacillus acidophilus and Lactobacillus casei, Ch. Hansen commercial starters.

The influence of the Origanum vulgare L. extract on the growth ability of Lactobacillus acidophilus and Lactobacillus casei

Starting from an inoculum of 8.57 log CFU/mL for Lactobacillus acidophilus and 6.32 log CFU/mL for Lactobacillus casei, after 4 hours of cultivation, at 37 °C, in MRS broth and MRS broth supplemented with 20 mg/mL Origanum vulgare L. extract the count clearly showed that vegetal extract rich in phenolic compounds stimulate the growth of the probiotic bacteria.

The figure 1 shows that DMSO solution (control 2) inhibits the cell growth in probiotics compared to the culture in MRS broth (control 1) (from 8.71 to 8.69 log CFU/mL) but the cultivation of Lactobacillus acidophilus strain on MRS broth with Origanum vulgare L. extract dissolved in DMSO led to a growth compared to control from 8.71 to 8.94 log CFU/mL.

Figure 1. The influence of the Origanum vulgare L. extract on the growth ability of Lactobacillus acidophilus and Lactobacillus casei.

As can be observed control 2 containing 20 % DMSO solution, the solvent used to dissolve oregano extract, inhibits the probiotic cells growth by 15 % compared to control 1. Nevertheless, the cultivation of Lactobacillus acidophilus bacteria on MRS broth with Oregano vulgare L. extract seemed to be more active leading to a growth by 163 % compared to control 1. These data could indicate that the polyphenols compounds are responsible for the stimulatory role on probiotic cells growth as previously described by Hervert-Hernández et al. [8].

Figure 2 shows the results after 4 hours cultivation at 37 °C of Lactobacillus casei. As in the experiment above presented, it can be seen that control 2 slightly inhibits the growth of the probiotic Lactobacillus casei, but on the other hand it can be observed that sample with Origanum vulgare L. extract stimulated the growth of the probiotic compared to control 1.
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from 6.45 to 6.69 log CFU/mL. Compared to control 1, the oregano extract sample has a 200% growth of the probiotic cell number.

![Figure 2](image_url)

**Figure 2.** The effect of the vegetal extract addition on the growth of the *Lactobacillus casei*

The cultivation of the two probiotic bacteria species (*Lactobacillus acidophilus* and *Lactobacillus casei*) that was carried out in MRS broth, led to the conclusion that the stimulation of the probiotic bacteria growth by the *Origanum vulgare* L. extract is significant both in the case where *Lactobacillus acidophilus* was used as cultivation strain and in the case where *Lactobacillus casei* was used.

**The *Origanum vulgare* L. extract influence on the viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in simulated gastric juice**

The presence of biologic compounds of the vegetal extracts could be reasons for the successful survival of bacteria during the simulated gastric juice tests.

The viability of *Lactobacillus acidophilus* and *Lactobacillus casei* cells during simulated gastric digestion in the presence of *Origanum vulgare* L. extract is presented in figures 3 and 4.

The cell number of the *Lactobacillus acidophilus* in sample was higher than control 1, from 7.75 to 7.86 CFU/mL, after 30 minutes. After 60 minutes of incubation in the same experimental conditions, the cell number of the sample was also higher than the control 1, from 7.67 to 7.86 CFU/mL. From these results, after 30 and 60 minutes of incubation respectively, a reduction of the cell number of *Lactobacillus acidophilus* was observed for all the three variants (figure 3).

![Figure 3](image_url)

**Figure 3.** The influence of the *Origanum vulgare* L. extract of the viability *Lactobacillus acidophilus* in simulated gastric juice
Comparing the cell number of the sample with that of the control 1, at 30 minutes, it is observed that these are 130 % higher than control 1. After 60 minutes of incubation in the same experimental conditions, the cell number in the sample is 151 % higher than control 1.

Comparing the cell viability at 30 minutes, 60 minutes and 90 minutes a stimulation effect is observed on Lactobacillus casei in presence of the vegetal polyphenols (sample) comparing with the controls. Thus, the growth of the cell number compared to the control 1 is from 3.43 to 4.3 log CFU/mL after 30 minutes incubation in gastric juice, from 1.95 log to 1.97 log CFU/mL after 60 minutes and from 1.15 to 1.6 log CFU/mL after 90 minutes (figure 4).

**Figure 4.** The Origanum vulgare L. extract influence of the viability Lactobacillus casei in simulated gastric juice

Comparing the cell viability at 30 minutes, 90 minutes and 120 minutes a viability stimulation effect is observed on Lactobacillus casei in sample. Stability of probiotic bacteria Lactobacillus casei was positive affected by phenolic compounds compared to control 1.

In the presence of Origanum vulgare L. extract, both strains showed an enhanced survival in comparison with the negative controls samples.

The high recovery rate of the total probiotic bacterial population maintained in presence of the Origanum vulgare L. extract could be explained by the positive and protective action of biologic active compounds upon cells physiology and resistance.

**Conclusions**

These results are preliminary and demonstrate that Origanum vulgare L. extract is a suitable stimulative and protective mixture of phenolic compounds upon probiotic bacteria, Lactobacillus acidophilus and Lactobacillus casei (Chr. Hansen commercial starters).

The growth of both probiotic strains can be stimulated in media with added Origanum vulgare L. extract, obtained by pressurized liquid extraction.

The resistance of tested strains by incubation in simulated gastric juice is also positively influenced by adding 20 mg/mL Origanum vulgare L. extract after 30 minutes of incubation. After 60 and 90 minutes respectively the viability drastically decreases, but the protective effect of the extract is positive compared with the control.

To improve the positive effects, an optimization of the extract concentration is necessary for the next steps of the study. Origanum vulgare L. extract acts as a food vector by sustaining adequate populations of viable bacteria, and improves their survival in gastric digestion.

The development of new probiotic foods should consider not only the intrinsic characteristics of effective bacterial strains but also the ability of the food matrix to protect the bacterial cells all the way through the gastrointestinal tract.
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Complementary investigations are necessary to assess the effectiveness of these extracts in food systems by microencapsulation of *Origanum vulgare* L. extract and probiotics.

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**References**