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# Antilisterial activity of broccoli stems (*Brassica oleracea*) by flow cytometry

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**Abstract**

Studies have demonstrated the presence of antimicrobial compounds in vegetal species. However, natural components can be found not only in raw materials, but also in agro-industrial wastes. Their reuse as source of natural preservatives in food and beverage industry can promote sustainable practices since environmental risks caused by its discard are reduced. The antimicrobial activity of broccoli stems (*Brassica oleracea*) aqueous extracts (1:20 w/v) against *Listeria monocytogenes* was evaluated by detection of Minimal Inhibition Concentration (MIC) and flow cytometry analysis. The chemical composition of major compounds was detected by Gas Chromatography with Mass Spectrometry (CG-MS). Broccoli stems extract showed antimicrobial activity against *L. monocytogenes*, which MIC was 102.4 mg/mL. Analysis by CG-MS allowed the identification of organic acids, as ascorbic and malic acids, and phenolic compounds, as sinapinic, ferulic and caffeic acids. The use of flow cytometry to evaluate the antimicrobial activity of the extracts was very suitable, enabling to infer their action mechanisms. In face of the difficulty to avoid the food contamination by this pathogen, the study of vegetal residues as source of natural preservatives in food and beverage industry is very promising.



## Introduction

Natural products as source of health and welfare is a concept which has become frequent for consumers. In this sense, the demand for foods free of synthetic preservatives has caused changes in the beverage and food industry, which is interested in the potential of natural components for developing various new products (Moreira *et al*., 2006). The global commerce promotes the production and transportation of food worldwide, so the use of preservatives in foods becomes essential to assure quality foods supply (Davidson and Branen, 2005).

Studies have demonstrated the existence of antimicrobial compounds in vegetal materials, as leaves, flowers, seeds, peels (Baydar *et al*., 2004). These natural components can be found not only in raw materials, but also in agro-industrial wastes (Martin *et al*., 2012). Their reuse can promote sustainable practices since environmental risks caused by discard are reduced. Bioactive molecules produced by vegetal species are known as secondary metabolites, which play important roles in protection and defense (Harborne and Williams, 2000). In addition, phenolic compounds have been studied due to their antioxidant and antimicrobial properties (Luciano *et al*., 2008; Shin *et al.*, 2004).

*Listeria monocytogenes* is a food pathogen that has became the focus of studies in recent years, after appearance of cases and outbreaks in human beings (Lundén *et al*., 2004; Barbuddhe *et al*., 2012). This microorganism is largely spread in the nature and can contaminate production, processing and distribution of food, representing risks for food consumers and industry (Chae *et al*., 2006). Any raw animal food or fresh vegetal can present *L. monocytogenes* contamination, as raw and frozen meat, chicken, seafood, fruits and vegetables (Jay, 2005). In processed food, it occurs in raw and poorly pasteurized milk, besides dairy products, as cheeses and ice-cream. As a psychrotrophic bacteria, its growth is improved in chilled food (Nuttawee, 2009). In this study, broccoli stems extract was evaluated for the presence of antimicrobial compounds with activity against *L. monocytogenes* using flow cytometry and its chemical composition was determined by CG-MS.

## Materials and Methods

### *Vegetable sample*

Broccoli stems (*Brassica oleracea*) were collected from distribution centers of fruits and

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vegetable in the region of Piracicaba, State of São Paulo, Brazil. The samples were freeze-dried for 5 days at 60 - 100 μHg and at -50°C (Liotop® L101) and stored at -20°C until the use.

*Extraction procedure*

The freeze-dried broccoli stems were ground in mechanical mill (IKA® A11). For preparation of extracts, samples were immersed in destilled water (1:20 w/v), centrifuged at 5,000 rpm for 15 minutes and filtered in qualitative filter paper 12.5 μm (Qualy®). The aqueous extracts were freeze-dried (Liotop® L101) and stored under refrigeration until the time of use. For the antimicrobial analysis, the extracts were dissolved in Tryptic Soy Broth with Yeast Extract (0.6% w/w) (TSB+YE) (Difco®).

*Antimicrobial activity*

Antimicrobial activity was evaluated in *Listeria monocytogenes* ATCC 7644, from the collection of strains of the Laboratory of Hygiene and Dairy – "Luiz de Queiroz" School of Agriculture (ESALQ/USP). For Minimum Inhibitory Concentration (MIC/MBC), the microbroth dilution method in 96-well microplate was used (Clinical and Laboratory Standards Institute, 2007). The concentrations of extracts were obtained by 2-fold serial dilution in the microplate, resulting in initial concentrations of 102.4 mg/mL, after the addition of inoculated TSB (1-2 x $10^5$ CFU/mL). The final volume for each well was 200 L. The controls were composed as follows: positive control (200 μL of TSB + YE added of 0.12% chlorhexidine v/v) and negative control (200 μL of inoculated TSB+YE). Two hundred microliters of sterile TSB were used for broth sterility control. After incubation at 35°C for 24 hours, all wells received 30 μL of resazurin (0.01% w/v) in order to verify, through visual reading, in which wells bacterial growth was detected. Any evidence of color change was considered as indicative of bacterial growth (Cabral *et al*., 2009). The experiments were carried out in triplicate for each extract.

*Flow cytometry*

Flow cytometry methodology (Paparella *et al.*, 2008) was used to analyze the conditions of bacterial cells exposed to broccoli stems extract. *L. monocytogenes* was reactivated in TSB+YE from stock culture at 35ºC for 24 hours. The bacterial culture was washed with Phosphate Buffered Saline (PBS), centrifuged at 8,000 rpm at 4ºC for 10 minutes, re-suspended in PBS and adjusted to 1-2 x $10^8$ CFU/mL. The concentration analyzed was the CIM (102.4 mg/mL), previously detected. For this

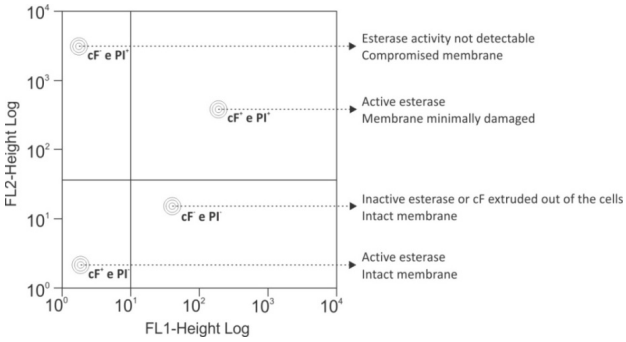


Figure 1. Region designations of cells stained with cFDA and PI. Adapted from Ananta *et al.* (2004).

analysis, broccoli stems were prepared using PBS. The controls were composed as follows: negative control (PBS) and positive control (heating at 70ºC for 30 minutes). The extracts were filtered in membrane 0.22 μm (Millipore®) to remove impurities. Three different parameters were used to evaluate the cellular viability during the extract action: 1, 3 and 5 hours of incubation. The cells were marked with esterase substrate Carboxyfluorescein Diacetate (cFDA) (Molecular Probes®, Eugene, Oregon) and collected in the FL1 channel (525 nm ± 20 nm), and marked with Propidium Iodide (PI) (Sigma-Aldrich®, Italy) and collected in the FL2 channel (620 nm ± 15 nm). The tests were carried out by FACScalibur™ (Becton Dickinson, San Jose, CA). For data collection, Cellquest™ Software (collection rate of 10,000 events/sample) was used, as well as Summit Software version 4.3 for data analysis. Forward Scatter (FSC) and Side Scatter (SSC) were measured on a logarithmic scale. The fluorochromes detection was evidenced in different intensities and four regions were assigned to each area of labeled cells ($cF^-PI^+$, $cF^+PI^+$, $cF^-PI^-$ $ecF^+PI^-$), according Ananta *et al.*, 2004 (Figure 1).

*Chemical composition*

The broccoli stems extracts were submitted to Gas Chromatography with Mass Spectrometry (GC-MS) in order to determine their chemical composition (Proestos *et al*., 2006; Markham *et al*., 1996). Chromatographic analysis: the extracts were analyzed by Shimadzu® gas chromatograph (Model GC-2010) coupled to a Shimadzu® mass spectrometer (QP 2010 Plus). The separation occurred in capillary column RTX5MS (30 m x 0.25 mm x 0.25 μm). The injector temperature was 280°C and the injection volume was 0.5 μl in "splitless" mode. The interface was maintained at 280°C and the detector operated in the "scanning" mode (m/z 40 - 800). Chromatographic conditions were: initial temperature of 80ºC (1 min) heating to 250°C, at a rate of 20°C/min (1 min), heating to 300°C (5 minutes) at a rate of 6°C/min,

heating to 310°C (10 minutes) at a rate of 15°C/min, and heating to 320ºC (10 minutes) at a rate of 20°C/min, totaling 40 minutes of analysis. The integration was done using the LabSolutions-CGMS software. Flavonoids, phenolic acids and derivatives were identified by comparison with data obtained from GC-MS, such as retention time and ionic fragmentation of authentic standards, silanized and eluted under the same conditions, and with the Wiley 8 Library.

## Results and Discussion

The antimicrobial analysis of extracts showed a high MIC (102.4 mg/mL). Several studies have demonstrated the biological properties of related species from Brassicaceae family, as antioxidant and antimicrobial activities (Llorach *et al.*, 2003; Ayaz *et al.*, 2008). Despite the high MIC value, we reinforce the potential for extraction of antimicrobial compounds in this material; the extraction optimization could increase the levels of this bioactive components.

The FSC and SSC graphic of *L. monocytogenes* demonstrated the status of this population under different conditions (Figure 2). The incubated samples with broccoli stems extract indicated an altered standard to cell morphology in comparison with the negative control (PBS). This changes in the cells morphology provided information about possible mechanisms of action for the vegetal extract. R1 region represents viable population, without any membrane damage (negative control) (Figure 2A). R2 and R3 regions represent conditions of granulosity increase and size and granulosity increase, respectively. In the positive control, we observed a short appearance of the R3 region (Figure 2B). For samples submitted to broccoli stems extracts, the appearance of R2 e R3 regions was considerable (Figure 2C).

This substantial increasing can be explained by two reasons: size and granulosity rising or aggregates formation from cells at different physiological status (viable, dead and damaged cells). Thus, to evaluate the real condition of *L. monocytogenes* population on R3 region, we optimized data from granulosity graphic, excluding cells aggregates and obtaining the percentage of individual cells for the three incubation periods. The percentage of individuals cells of *L. monocytogenes* population under broccoli stems extract action decreased considerably from period of 1 hour of incubation (12.0%) to 3 hours (9,5%) and 5 hours (1,9%). Thus, the extract was capable to form cell aggregates and inhibit the bacterial growth.

The delimitated regions from granulosity and size graphics (R1, R2 and R3) were analyzed

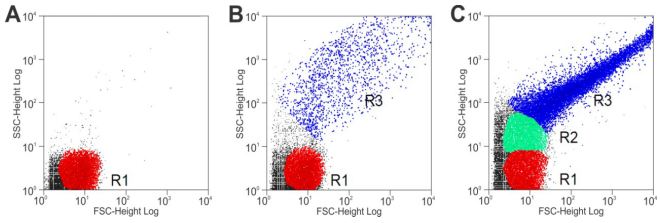


Figure 2. Size (FSC) and granulosity (SSC) of *L. monocytogenes* populations by flow cytometry. (A) Negative control (PBS), (B) Positive control (chlorhexidine 0.12% v/v) and (C) Broccoli stems extract (102.4 mg/mL). Red, green and blue colors express R1, R2 (increase of size) and R3 (increase of size and granulosity) regions, respectively. Black regions represent debris and artifacts.

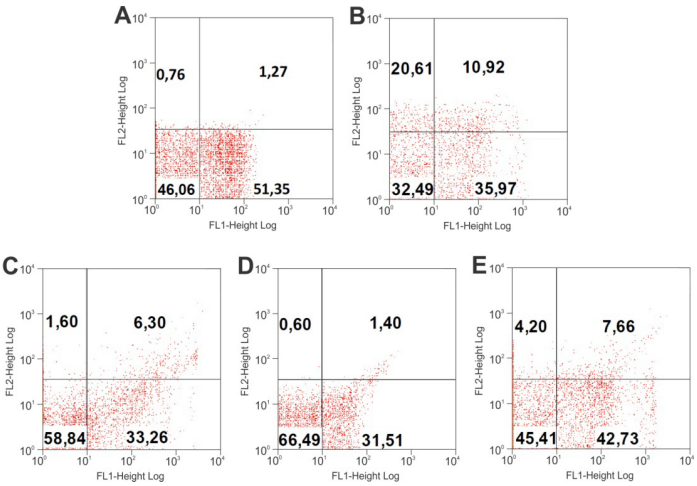


Figure 3. Fluorescence of *L. monocytogenes* populations on R1+R2 region submitted to broccoli stems extracts by flow cytometry (FL1 vs. FL2). (A) Negative control (PBS), (B) Positive control (heating at 70ºC for 30 minutes), (C) 1 hour, (D) 3 hours and (E) 5 hours of incubation.

for fluorescence intensity to the CFDa and PI fluorochromes (Figure 3). As the R1 and R2 regions from this graphic showed the same fluorescence percentage for both markers, populations related to these regions were analyzed together. Therefore, the following results express cell conditions relative to subpopulations present only in these areas, which comprise size and granulosity similar to those of the negative control and increase of size and granulosity. The combined use of the markers exposed 4 different areas of labeled cells, which represent the status of the bacterial population evaluated. In the negative control (PBS), the subpopulation of dead cells consisted of 0.76% of the cell total, viable cells 51.35% and damage cells, 1.27% (Figure 3A). The positive control (heating at 70ºC for 30 minutes) showed subpopulations values of 20.61%, 35.97% and 10.92%, respectively (Figure 3B). These results demonstrated considerable functionality for esterase, as opposed to findings in previous study (Paparella *et*

Table 1. Chemical composition of broccoli stems extracts

| Compounds[a] | RT[b] | Percentage of relative area[c] |
|---|---|---|
| Malic acid | 7.24 | 0.92 |
| ρ-Phenyl-benzoic acid | 9.20 | 3.99 |
| Ascorbic acid | 9.93 | 24.79 |
| Ferulic acid | 10.72 | 2.02 |
| Caffeic acid | 10.98 | 1.16 |
| Sinapinic acid | 11.73 | 6.33 |

[a] All compounds identified showed similarity percentage > 80%
[b] RT: retention time in minutes
[c] peak area in relation to total percentage of peak areas

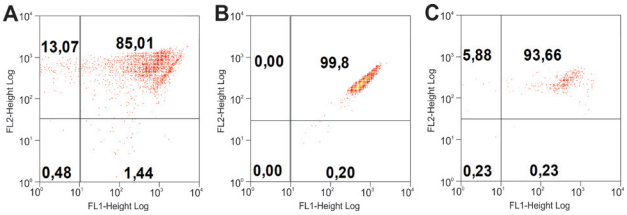


Figure 4. Fluorescence of *L. monocytogenes* populations on R3 region submitted to broccoli stems extracts by flow cytometry (FL1 vs. FL2). (A) 1 hour, (B) 3 hours and (C) 5 hours of incubation.

*al.*, 2008). For the bacterial populations submitted to broccoli stems extract on R1+R2 region, the levels of dead and damage cells decreased with the rise of incubation period (Figure 3C-D). The viable cells increased considerable, ranging from 33.26% (1 hour of incubation) to 31.51% (3 hours) and 42.73% (5 hours).

The R3 region showed a fluorescence percentage different of the R1 and R2 region; therefore, its analysis was realized separately. The most cells present in this region were damaged, ranging from 85,1% (1 hour of incubation) to 99,8% (3 hours) and 93,66% (5 hours) (Figure 4). All flow cytometry results proved the occurrence of morphologically changes in the cells, when exposed to extracts. By this technique was possible to discriminate the rate of intact and damage cells, demonstrating the physiological heterogeneity of the *L. monocytogenes* population. The main effect of the extracts on bacterial cells was the inhibition since the number of viable cells increased and the number of dead/damage cells decrease in the last period evaluated (5 hours of incubation). Damaged cells can recover its initial conditions or die (Yousef and Courtney, 2003). In generally, it adopts a latent status, but remains live and can to restore itself and return to normal status depending on environmental conditions (Paparella *et al.*, 2008). We observed that bioactive components from extracts interfered on reproductive growth of the bacterial population, without expressive bactericidal activity.

The chemical composition of broccoli stems extracts analyzed by CG-MS technique is presented in Table 1. Phenolic acids (ferulic, sinapinic and caffeic acid), malic and ascorbic acid were their major components. Sinapinic acid was the most abundant among the phenolic acids (6.33%); in others studies, this compound was found in cauliflower and kale's bioactive extracts (Llorach *et al.*, 2003; Ayaz *et al.*, 2008), species from the same broccoli family (*Brassicaceae*). Acid ferulic found in fermentation less was associated to antimicrobial activity against *L. monocytogenes* and *Staphylococcus aureus* (Martin *et al.*, 2012). The ascorbic acid, an organic acid widely present in vegetal materials, was found in high concentrations in broccoli stems extract (24,79%). This component is known like a important antioxidant; previous studies have indicated their potential to increase the antimicrobial activity from others compounds (Davidson and Branen, 2005; Golden *et al.*, 1995). Despite the low concentrations (1.16%), caffeic acid present in the extracts was observed in others vegetable materials with antilisterial activity, as grape marcs, peanut peel and guava bagasse (Martin *et al.*, 2012; Anastasiadi *et al.*, 2008).

The mechanism of action of phenolic compounds is not yet completely elucidated, but its effect can be related to cell membrane/wall disruption, action on genetic material, enzymatic inactivation and active transport's interruption (Sikkema *et al.*, 1995; Burt *et al.*, 2004). Lysis of membrane cell appears to be the primary mechanism of action of phenolic compounds (Beltrame *et al.*, 1988). Probably, the cells of different sizes detected by flow cytometry were due to cell disruption and leakage of internal contents when submitted to the extract action. Consequently, the cells became incapable to control the balance osmotic, resulting in input of external liquid and increasing the size.

## Conclusion

Broccoli stems extract presented antimicrobial potential against *L. monocytogenes*. Despite the high levels found for the CIM, this antibacterial activity could be improved by optimization of the extraction procedures. Flow cytometry was very useful to reveal possible mechanisms involved in the inhibitory action of the extract on *L. monocytogenes*, enabling the identification of viable, dead and damaged cells. In face of the difficulty to avoid the food contamination by this pathogen, the study of vegetal residues as source of natural preservatives in food and beverage industry is very promising.

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