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Metabolomic response of *Brassica rapa* submitted to pre-harvest bacterial contamination

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Abstract

Plants are continually challenged by natural pathogens. Among a number of environmental factors, pre-harvest contamination of plants with pathogens responsible for enteric diseases in humans is of major international concern. Despite the knowledge of how bacterial attack can affect the biological system of plants, little is known about the effect of the interaction of these bacteria on plant's metabolome. In order to investigate the metabolic change of *Brassica* induced by its response to different typically food borne bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri*, ^{1}H NMR and two-dimensional NMR spectra, coupled with principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were applied to *Brassica rapa* which had been subjected to these pathogens during plant growth. The metabolic changes were found to vary according to bacterial species; for example, Gram-positive and Gram-negative bacteria had a different effect on the *Brassica* metabolome. While threonine and GABA were found to be the discriminating metabolites in Gram-positive bacteria treated plants, those treated with Gram-negative bacteria exhibited a significant increase in sinapoyl-malate, caffeoyl-malate and histidine. The detailed study of the effect of type of bacteria showed that amino acids, alcohols, carbohydrates and phenols were discriminating metabolites. These results prove the potential of NMR-based metabolomics as a tool to study the interaction of these food-borne bacteria with vegetables.

Keywords: Metabolomic analysis; NMR; Multivariate data analysis; *Brassica rapa*; Food-borne bacteria; Pre-harvest contamination; Gram-positive and negative

1. Introduction

Brassicaceae species have been traditionally included among the important food crops in all Asian countries (Sasaki & Takahashi, 2002). Over the past decades *Brassica* production has increased, becoming an important source of oil and proteins for animal and human nutrition (Thiyam, Kuhlmann, Stockmann, & Schwarz, 2004). In addition to the nutritional benefits, they constitute a very rich source of health-promoting phytochemicals such as phenols, flavonoids, hydroxycinnamic acids, vitamins, glucosinolates, fiber, soluble sugars, fats and carotenoids (Vallejo, Gil-Izquierdo, Pearez-Vicente, & Garciaa-Viguera, 2004). Besides this, there is growing evidence that a higher intake of *Brassica* vegetables (e.g., broccoli, cabbage, kale, mustard greens, brussel-sprouts, cauliflower) could help to reduce the risk of cancer (Wang et al., 2004).

In the wild, plants are constantly interacting with external environmental factors. In particular, plants are exposed to the challenge posed by natural pathogens. Among a number of environmental factors, pre-harvest contamination of plants with bacteria that are responsible for enteric diseases in humans is of major international concern (Beuchat, 2006). There are many sources of pre-harvest contaminations. In recent years, biological products such as manure which might contain pathogenic microbes have been widely used as fertilizers in vegetable production, (Ingham et al., 2004). Digested urban sludge and livestock

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waste were also found applicable as basal dressing for the growth of leafy vegetables (Chui, Lam-Leung, Cheung, & Wu, 1992). This, added to the rapid change of environmental conditions which lead to different ecological interactions, may contribute to higher risks of contamination by food-borne pathogens (Anderson, Kenney, Millner, Beuchat, & Williams, 2006).

Microbial growth in food is of serious concern as it causes decay, loss of nutritional effect and organoleptic properties (Duarte, Delgadillo, & Gil, 2006). Bacteria are known to produce chemicals which can either promote or inhibit the growth of other organisms when interacting with plants (Kennedy, 1999). Some of these bacteria are pathogenic for the plants, destroying or diminishing their photosynthetic output for example. As a result of the bacterial attack, plants generate their own defense mechanism, triggering many complex biological processes. There are a number of reports on the changes at a genetic or protein level brought about by phytopathogenic microbes which are reflected in a profound alteration of the metabolic pool of the affected plants (Dangle & Jones, 2001). However, little is known about the interaction of the plants with bacteria that are pathogenic to humans, in particular, those that are responsible for enteric infections usually acquired by ingestion of fresh fruit and vegetables, such as *Escherichia coli* and *Salmonella* species, that have the potential for reproduction prior to consumption (Buck, Walcott, & Beuchat, 2003).

While there is evidence of the effects of plant pathogen infection on their biosynthetic system, but the interaction of plants with food-borne human pathogens is not clear (Hirano & Upper, 2000; Wachtel, Whitehand, & Mandrell, 2002). If the metabolome of host plants were quantitatively or qualitatively affected by these exogenous bacteria following a clearly distinguishable and constant pattern, this could constitute a good tool for the quality control of plants to be used as food. Thus, studies on the metabolic interaction of plants and micro-organisms including the total heterotrophs, *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus*, *E. coli*, *Salmonella* species, and *Shigella* species are necessary (Banerjee & Sarkar, 2003).

An investigation of the plant metabolome, that is, of all its metabolites, is an extremely complex task due to their large number and variety. It is unlikely that a single analytical method could provide information about all the metabolites in plants, since the diversity of their structures results in a wide range of physical properties and chemical characteristics, such as volatility, polarity, solubility, and chromatographic behaviour and detectability requires the use of multiple methods. Particularly in the case of the interaction between plants and bacteria, an unbiased method that allows the simultaneous detection of as wide an array of metabolites as possible is essential. In this context the use of metabolomics, a comprehensive profiling of metabolites achieved through the combination of analytical methods and the multivariate data analysis of results thus generated could be applied.

Among the analytical methods appropriate for the implementation of this technique, nuclear magnetic spectroscopy (NMR) is an optimum choice for the first step, that is, the acquisition of data of general metabolite composition of the sample. It is a quick, non-destructive method which simultaneously detects all proton-bearing compounds such as carbohydrates, amino acids, fatty acids, amines, esters, lipids etc. (Choi et al., 2004). Although ^{1}H NMR has a relatively low sensitivity compared to other methods such as mass spectrometry (MS), it has the advantage of allowing the detection of diverse groups of plant metabolites in a single run, as a result of which, many researchers have chosen to use it as a first macroscopic approach to metabolomic studies.

The aim of this research was therefore to study the effect of certain food-borne human pathogenic bacteria on *Brassica rapa* metabolome. To follow and detect these changes, ^{1}H NMR and two-dimensional NMR spectra, coupled with principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were applied.

2. Materials and methods

2.1. Preparation of MS 0.5 media

Seeds were grown on Murashige and Skoog (MS 0.5) solid medium (Murashige & Skoog, 1962) and the seedlings were transferred in conical flasks containing MS (0.5) liquid medium including vitamin B_5 and 0.3% (w/v) sucrose. Previous experiments were performed with different concentrations of auxin (2,4-dichlorophenoxyacetic acid) and cytokinin (6-benzylaminopurine) in order to select the best combination of these hormones for growth of *B. rapa* (var. Raapstelen) seedlings in liquid media. Optimum growth was observed in control samples, so no hormones were added to the seedlings in experimental conditions.

2.2. Plant and microbial material

B. rapa seeds of a registered cultivar (var. Raapstelen, Groene Gewone) were germinated in MS (0.5) media. After surface sterilization, the seeds were sown in MS (0.5) solid media in conical flasks under sterilized conditions and kept in cold storage (4 °C) in the dark overnight, after which they were transferred to the greenhouse and kept in 24-h daylight conditions. Five days later, the seedlings were transferred to a MS (0.5) liquid media in sterile conditions and kept in a shaker at 75 rpm in continuous light conditions. After nine days of plant growth, the liquid media were inoculated individually with 500 μl of different bacteria cultures (*Bacillus subtilis*, *S. aureus*, *E. coli*, *Salmonella typhimurium* and *Shigella flexneri*), with a bacterial concentration of 10^{8} ml^{-1}. The control sample was treated with 500 μl of the sterilized liquid broth used for bacteria culture, while an untreated sample of seedlings was kept as

a blank sample. After three days of the bacterial inoculation at room temperature, the plants were removed from flasks, washed thoroughly with running tap water and then with sterilized and deionized water, roots were removed and leaves were immediately frozen in liquid nitrogen. Prior to extraction all material was pulverized in liquid nitrogen using a mortar and pestle and freeze dried.

2.3. Extraction of plant material and NMR measurements

Fifty mg of freeze dried material were transferred to a microtube (2 ml) to which 1.5 ml of 50% methanol-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.05% TMSP (trimethyl silyl propionic acid sodium salt, w/v) was added. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min, and centrifuged at 13,000 rpm at room temperature for 5 min. Eight hundred μl of the supernatant was transferred to a 5 mm NMR tube.

^{1}H NMR and 2D J-resolved spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH-d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 s acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 μs), and relaxation delay (RD) = 1.5 s. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0.0 ppm, using XWIN NMR (version 3.5, Bruker). 2D J-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8 k for F2 using spectral widths of 5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin–spin coupling constant axis). A 1.5 s relaxation delay was employed, giving a total acquisition time of 56 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. J-Resolved spectra tilted by 45°, was symmetrized about F1, and then calibrated, using XWIN NMR (version 3.5, Bruker). ^{1}H–^{1}H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). The COSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions. Window function for COSY spectra was sine-bell (SSB = 0). The HSQC spectra were obtained with 1.0 s relaxation delay, 6361 Hz spectral width in F2 and 27,164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HSQC. The HMBC spectra were recorded with the same parameters as the HSQC spectrum except for 30,183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 Hz and 8 Hz, respectively.

2.4. Data analysis

The ^{1}H NMR spectra were automatically reduced to ASCII file. Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.3–10.0. The region of δ 4.75–4.9 and δ 3.28–3.34 was excluded from the analysis because of the residual signal of HDO and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. Principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umea, Sweden) with scaling based on Pareto and unit variance method, respectively.

3. Results and discussion

A number of metabolites including amino acids, organic acids, carbohydrates and phenylpropanoids were identified from bacteria infected *B. rapa* using ^{1}H NMR and 2D J-resolved spectra together with other 2D spectra including ^{1}H–^{1}H COSY, HSQC and HMBC spectra (Table 1). The amino acids and organic acid region (δ 0.8–4.0) showed ^{1}H NMR signals of alanine, threonine, valine, malate, glutamate, glutamine, acetate and GABA (γ-amino-butyric acid). The strong signals of the amino acids and organic acids together with sugars made it easier to elucidate the signals by comparison with reference compounds. Most of the metabolites in the aromatic region (δ 6.0–8.5) were found to be secondary metabolites but low signal intensity and lack of reference compounds made it difficult to elucidate their structures. Based on previous reports (Liang, Kim et al., 2006), diverse malate conjugated phenylpropanoids were identified in *Brassica* leaves. The doublets in the range of δ 6.30–6.50 (J = 16.0) having COSY correlation with doublets at δ 7.30–7.85 (J = 16.0) and coupling with carbonyl carbons at δ 171 in HMBC spectrum are typical signal of H-8 of *trans*-phenylpropanoids. Four *trans*- and three *cis*-phenylpropanoids (Table 1) were identified using COSY, HMQC and HMBC spectra. However, the *cis*-forms of phenylpropanoids are considered to be artifacts of their *trans*- forms possibly produced during extraction or sample storage (Liang, Kim et al., 2006).

Clear differences were detected in the ^{1}H NMR spectra of treated plants when compared to both the blank and control samples. In the first place, fermentation products such as short chain alcohols or acids exhibited a high variability: high levels of 2,3-butanediol at δ 1.14 (d, J = 6.4) were detected in *B. subtilis* and *S. typhimurium* treated plants, while increased levels of acetate at δ 1.91 (s) were found in *B. subtilis*, *E. coli* and *S. flexneri* treated plants (Fig. 1). The fermentation product 2,3-butanediol has been reported to cause induced systemic resistance (ISR) of plants which might play a role in the triggering of the production of other metabolites (Ryu et al., 2004). These increased short chain alcohols and acids might not be products of plant biosynthesis but from the infecting bacteria.

Table 1
Characteristic ^{1}H chemical shifts (δ) and coupling constant (Hz) of *B. rapa* (grown in hydroponics) metabolites obtained from 1D and 2D NMR spectra in 50% methanol-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0)

Metabolite	Chemical (δ) shift and coupling constant (Hz)
Acetate	1.91 (s)
Alanine	1.48 (d, $J = 7.3$), 3.73 (q, $J = 7.2$)
γ-Amino-butyrate (GABA)	1.90 (m), 2.30 (t, $J = 7.2$), 3.01 (dd, $J = 8.4$, 6.3)
trans-Caffeoyl-malate	2.62 (dd, $J = 15.3$, 11.3), 5.21 (dd, $J = 11.5$, 2.7), 6.43 (d, $J = 16.1$), 6.84 (d, $J = 8.8$), 7.14 (dd, $J = 6.9$, 1.6), 7.64 (d, $J = 16$)
cis-Coumaroyl-malate	2.58 (dd, $J = 15.3$, 11.3), 5.18 (dd, $J = 11.5$, 2.7), 5.95 (d, $J = 13.0$), 6.80 (d, $J = 8.8$), 6.90 (d, $J = 9.2$), 6.93 (d, $J = 13.6$), 7.07 (d, $J = 8.5$), 7.60 (d, $J = 9.2$)
trans-Coumaroyl-malate	2.62 (dd, $J = 5.3$, 11.3), 5.21 (dd, $J = 11.5$, 2.7), 6.49 (d, $J = 15.9$), 6.84 (d, $J = 8.8$), 7.58 (d, $J = 9.1$), 7.67 (d, $J = 16.2$)
cis-Feruloyl-malate	2.58 (dd, $J = 15.3$, 11.3), 5.18 (dd, $J = 11.5$, 2.7), 5.99 (d, $J = 13.0$), 6.84 (d, $J = 8.8$), 6.94 (d, $J = 13.6$), 7.13 (dd, $J = 9.4$, 2.0), 7.83 (d, $J = 3.0$)
trans-Feruloyl-malate	2.62 (dd, $J = 15.3$, 11.3), 5.21 (dd, $J = 11.5$, 2.7), 6.49 (d, $J = 16.0$), 6.89 (d, $J = 8.2$), 7.13 (dd, $J = 8.8$, 2.1), 7.27 (d, $J = 2.3$), 7.66 (d, $J = 16.3$)
Fumarate	6.53 (s)
α-Glucose	3.4 (m), 3.47 (dd, $J = 9.8$, 3.6), 5.19 (d, $J = 3.8$)
β-Glucose	4.59 (d, $J = 8.0$)
Glutamate	2.13 (m), 2.46 (m), 3.72 (t, $J = 6.0$)
Glutamine	2.14 (m), 2.47 (m)
Histidine	3.16 (dd, $J = 6.5$, 1.5), 3.25 (d, $J = 8.2$, 1.5), 7.12 (d, $J = 1.6$), 7.93 (d, $J = 1.6$)
Malate	2.68 (dd, $J = 15.3$, 3.2), 2.38 (dd, $J = 16.3$, 6.8), 4.28 (dd, $J = 9.8$, 3.3)
cis-Sinapoyl-malate	2.58 (dd, $J = 15.3$, 11.3), 5.18 (dd, $J = 11.5$, 2.7), 5.93 (d, $J = 13.0$), 7.15 (s), 6.93 (d, $J = 13.6$)
trans-Sinapoyl-malate	2.62 (dd, $J = 15.3$, 11.3), 5.21 (dd, $J = 11.5$, 2.7), 6.93 (s), 6.50 (d, $J = 16.0$), 7.67 (d, $J = 16.3$)
Sucrose	3.44 (dd, $J = 9.9$, 8.5), 3.51 (m), 3.73 (d, $J = 6.8$), 4.05 (d, $J = 6.7$), 4.17 (d, $J = 8.5$), 5.4 (d, $J = 4.0$)
Threonine	1.33 (d, $J = 7.0$)
Valine	1.00 (d, $J = 7.1$), 1.05 (d, $J = 7.1$), 2.29 (m)

The constitutive plant metabolite content showed a very significant change. In particular, the level of GABA was clearly increased in *B. subtilis*, *S. flexneri*, and *S. aureus* infected plants as compared to other treatments and control/blank. An increase in sucrose, α-glucose, β-glucose, alanine, threonine, and some phenylpropanoids was also observed in bacteria treated samples. Grouping the observed metabolic alterations, it was found that there was a difference in the plants infected by Gram-positive and Gram-negative bacteria. While phenolic metabolites increased in all treated samples, high levels of histidine, feruloyl-malate and caffeoyl-malate were detected in Gram-negative infected plants only (*E. coli*, *S. flexner*, and *S. typhimurium* in this study) and Gram-positive bacteria (*B. subtilis* and *S. aureus*) infected plants displayed increased levels of coumaroyl-malate and fumarate. The increase of phenolic compounds might be explained as a generic response of all the plants to the infection by microorganisms (Liang, Choi, Kim, Linthorst, & Verpoorte, 2006).

Among several multivariate data analysis, principal component analysis (PCA) was first used to identify metabolic changes in an unbiased manner, after bacterial treatment of *Brassica* plants in this study. In the PCA score plot, control and blank samples were grouped together, showing that the broth by itself had no effect on the metabolome, while different bacterial treated plants were clearly discriminated from the control and blank samples. Additionally, each treated plant displayed metabolic changes which differed for each type of infecting bacteria as can be observed in Fig. 2. In the score plot there were four well defined groups corresponding to blank and control; *B. subtilis* and *E. coli*; *S. flexneri* and *S. aureus*; and *S. typhimurium* treated plants, all separated by PC1 and PC2 (Fig. 2). For the investigation of differentiating metabolites, a loading plot was used in which the correlation between grouping and correlated metabolites was shown. The primary metabolites contributing to the discrimination were found to be glutamic acid, glutamine, glucose, sucrose, alanine, threonine, GABA and acetate (Fig. 2). The highest glutamine and glutamate content was observed in control and blank plants but the treatment with *S. flexneri* and *S. aureus* was observed to increase the level of sucrose in *Brassica* leaves. In the case of *S. typhimurium,* glucose proved to be differentiating metabolite from control and other treated plants. Acetate, threonine, and GABA were responsible for the discrimination of *B. subtilis* and *E. coli* treated plants from other treatments both in PC1 and PC2 (Fig. 2).

A clear separation in PCA was observed between Gram-positive (*S. aureus* and *B. subtilis*) bacteria treated plants. The separation of *S. aureus* was due to cumaroyl malate, sucrose, α-glucose, β-glucose and glutamic acid. Separation of *B. subtilis* in PCA was determined by the presence of feruloyl-malate, sinapoyl-malate, threonine, alanine, GABA and histidine (Fig. 3). Similarly, a clear discrimination of Gram-negative (*E. coli, S. flexneri* and *S. typhimurium*) bacteria-treated plants was observed. Separation of *S. typhimurium* was due to sinapoyl malate and *S. flexneri* was separated due to signals of caffeoyl-malate,

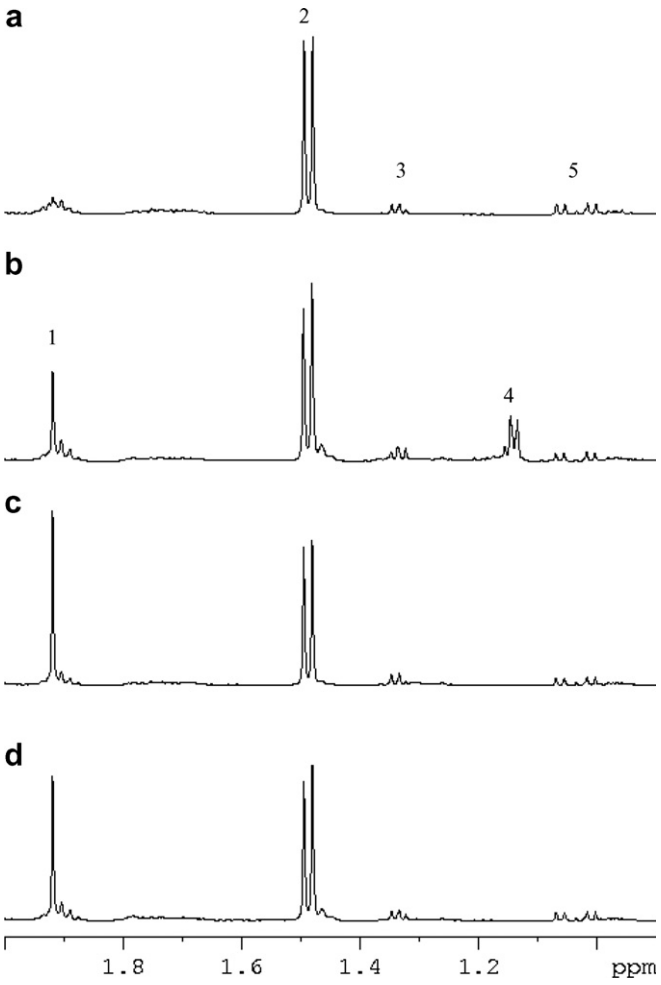

Fig. 1. ^{1}H NMR spectra (400 MHz), of control *Brassica rapa* leaves (a), infected with *Bacillus subtilis* (b), *Escherichia coli* (c), *Shigella flexneri* and (d) in the range of δ 0.9–2.0. 1: acetate; 2: alanine; 3: threonine; 4: 2,3-butanediol; 5: valine. The spectra were measured in the mixture of KH_2PO_4 in D_2O (pH 6.0)–methanol-d_4 (1:1).

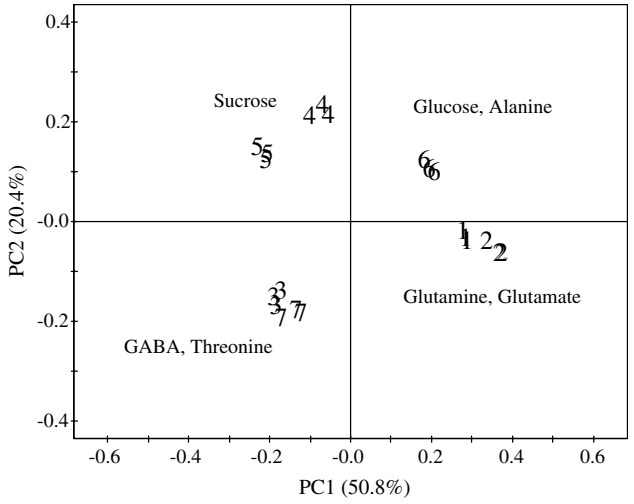

Fig. 2. Score plot of PCA based on whole range of ^{1}H NMR signals (δ 0.3–10.0). 1: Control (with 500 µl of sterilized broth); 2: blank (without bacteria and broth); plants treated by 3: *Bacillus subtilis*; 4: *Staphylococcus aureus*; 5: *Shigella flexneri*; 6: *Salmonella typhimurium*; 7: *Escherichia coli*.

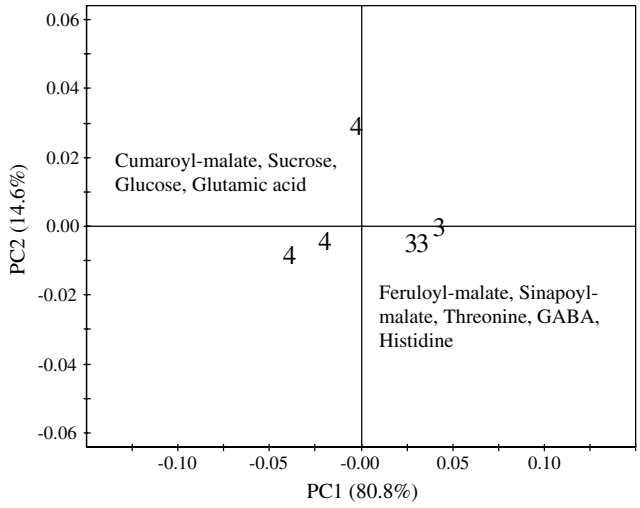

Fig. 3. Score plot of PCA based on whole range of ^{1}H NMR signals (δ 0.3–10.0). Plants treated by Gram-positive bacteria, 3: *Bacillus subtilis*; 4: *Staphylococcus aureus*.

cumaroyl-malate and histidine, while *E. coli* was separated due to feruloyl-malate and fumarate signals in PCA (Fig. 4).

As next step for metabolomic analysis, the partial least square-discriminant analysis (PLS-DA) was performed based on pre-input information, i.e. unlike the unbiased system used for PCA, information is classified prior to its input. The most important information obtained from PLS-DA is the correlation between two data sets, in this case, the investigation of ^{1}H NMR signals and their classification such as control, blank, Gram-positive and Gram-negative treated plants. For the classification required by PLS-DA three groups were applied. Group 1 was for control and blank samples, group 2 for Gram-positive bacteria treated *Brassica*, and group 3 for Gram-negative treated

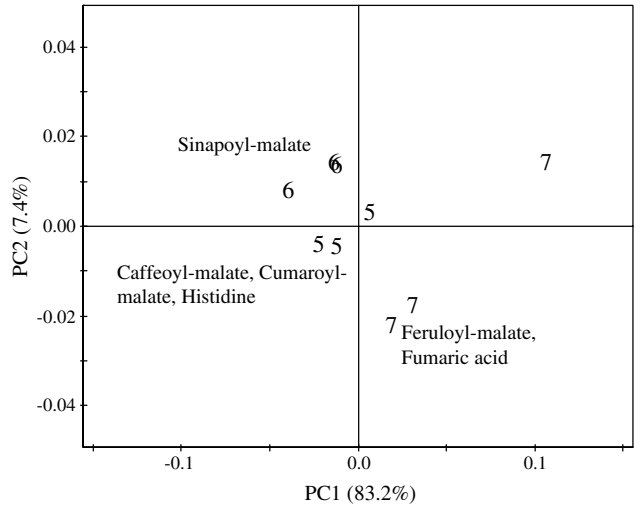

Fig. 4. Score plot of PCA based on whole range of ^{1}H NMR signals (δ 0.3–10.0). Plants treated by Gram-negative bacteria 5: *Shigella flexneri*; 6: *Salmonella typhimurium*; 7: *Escherichia coli*.

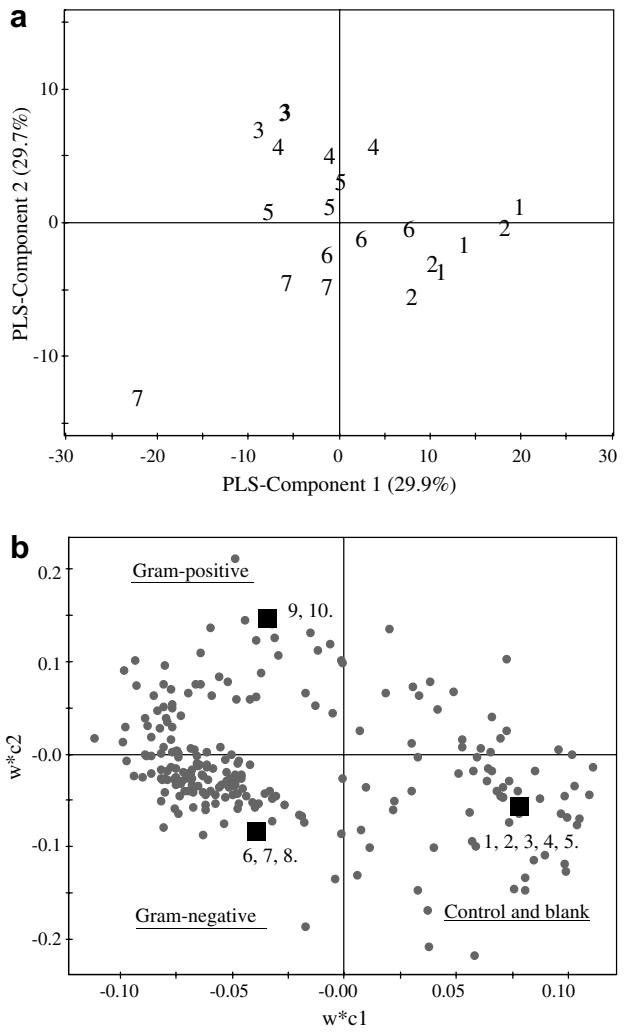

Fig. 5. Score (a) and loading plot (b) of PLS-DA based on whole range of ^{1}H NMR signals (δ 0.3–10.0). 1: Control (with 500 μl of sterilized broth); 2: blank (without bacteria and broth); plants treated by 3: *B. subtilis*; 4: *Staphylococcus aureus*; 5: *Shigella flexneri*; 6: *Salmonella typhimurium*; 7: *Escherichia coli*. Control and blank region in loading plot contains glucose (1), sucrose (2), valine (3), glutamine (4), and glutamate (5). Region of Gram-negative bacterial treatment in PLS-DA loading plot contains sinapoyl-malate (6), caffeoyl-malate (7), and histidine (8).Region of Gram-positive bacterial treatment in PLS-DA loading contains threonine (9) and γ-amino-butyric acid (GABA) (10).

ones. In the PLS-DA score plot those three groups were well separated (Fig. 5a). The identification of characteristic metabolites in each group loading plot of PLS-DA is displayed in Fig. 5b. Threonine and GABA were found to be the discriminating metabolites in Gram-positive bacterial treated plants. However, in the case of Gram-negative bacterial treated ones, sinapoyl-malate, caffeoyl-malate and histidine were clearly increased, while sugar, glucose, glutamine and glutamate levels were decreased.

Plant disease resistance to pathogens such as fungi, bacteria, and viruses often depends on whether the plant is able to recognize the pathogen. Recognition of pathogens triggers a large range of inducible defense mechanisms that are believed to contribute to overall resistance in the plant (Mehdy, 1994). Plants, for example, can synthesize secondary metabolites as a defensive response. It has been observed that the level of production of phenolic compounds is particularly sensitive to the type of attacking bacteria (Vaquero, Alberto, & Nadra, 2007). This is of course, a relevant factor for a plant that will be included in human diet, since these compounds are particularly bioactive and have pronounced effects (Williamson, Day, Plumb, & Couteau, 2000). By comparing different spectra the increase in the production of GABA, phenylpropanoids, glutamine, glutamate, sugars and amino acids was confirmed. A clear separation in PCA of plants submitted to the different treatments shows that bacterial strains differ in their ability to induce resistance in *B. rapa* leaves. These pathogens have developed sophisticated mechanisms to interact with their hosts through a specialized protein secretion system, which has been identified in several Gram-negative pathogenic bacteria including the plant pathogens *Pseudomonas* spp., *Erwinia* spp. and *Xanthomonas* spp. and the animal pathogens *Salmonella* spp., *Pseudomonas aeruginosa*, *Shigella* spp., *Yersinia* spp., and *E. coli* spp. (Boquet & Lemichez, 2003).

The decrease in the quantity of sugars produced in all bacteria-treated plants can be attributed to the impairment of photosynthesis by the bacteria, while sugars can also be utilized for primary and secondary metabolism. The decrease in amino acids in different treatments could be explained by the carbohydrate starvation (Hodges & Robinson, 1977), especially in the case of *B. subtilis* and *E. coli* treated plants. Production of GABA, a non protein producing amino acid derived from glutamate catabolism after abiotic and biotic stresses was also observed in all treatments. It is thought that it acts as a signal molecule (Beuve et al., 2004).

Increase of primary and secondary metabolites in infected plants suggested that biotic stress by these tested human pathogenic bacteria could cause induced systemic resistance (ISR) in *Brassica* leaves. In general, these compounds are either absent or present in very low concentrations in healthy plants. However, upon infection their concentration increases considerably depending on the invading bacteria.

4. Conclusion

Plant response to bacterial stress depends on the type of invading bacteria. The set of metabolites affected by different microorganisms differed, probably reflecting the chemical environment of the invaded tissue and the mechanism of action of the infecting bacteria (Vereecke et al., 1997). The present results show the potential of NMR to study the interaction of food-borne bacteria and vegetables and that of NMR-based metabolomics as a promising tool for the pre-harvest studies of vegetables. Further work should be carried out on a specific group of metabolites. For example, glucosinolate content, as these bioactive compounds are present in small concentrations in *Brassica*

and the response of the plant in terms of glucosinolate production should be evaluated. An increased insight into plant-microbe interaction may allow the detection of contaminants, in this case, pathogenic bacteria in vegetables and fruits, thus helping ensure its safety for human consumption.

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References

Anderson, G. L., Kenney, S. J., Millner, P. D., Beuchat, L. R., & Williams, P. L. (2006). Shedding of foodborne pathogens by *Caenorhabditis elegans* in compost-amended and un-amended soil. *Food Microbiology, 23*, 146–153.

Banerjee, M., & Sarkar, P. K. (2003). Microbiological quality of some retail spices in India. *Food Research International, 36*, 469–474.

Beuchat, L. R. (2006). Vectors and conditions for preharvest contamination of fruits and vegetables with pathogens capable of causing enteric diseases. *British Food Journal, 108*, 38–53.

Beuve, N., Rispail, N., Laine, P., Cliquet, J. B., Ourry, A., & Deunff, E. L. (2004). Putative role of γ-aminobutyric acid (GABA) as a long-distance signal in up-regulation of nitrate uptake in *Brassica napus* L. *Plant, Cell & Environment, 27*, 1035–1046.

Boquet, P., & Lemichez, E. (2003). Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis? *Trends in Cell Biology, 13*, 238–246.

Buck, J. W., Walcott, R. R., & Beuchat, L. R. (2003). Recent trends in microbiological safety of fruits and vegetables. *Plant Health Progress*. doi:10.1094/PHP-2003-0121-01-RV.

Choi, Y. H., Kim, H. K., Hazekamp, A., Erkelens, C., Lefeber, A. W. M., & Verpoorte, R. (2004). Metabolomic differentiation of *Cannabis sativa* cultivars using ^{1}H NMR spectroscopy and principal component analysis. *Journal of Natural Products, 67*, 953–957.

Chui, V. W. D., Lam-Leung, S. Y., Cheung, M., & Wu, V. K. C. (1992). The use of sewage sludge as basal dressing for vegetable cultivation. *Environment International, 18*, 201–209.

Dangle, J. L., & Jones, J. D. G. (2001). Plant pathogens and integrated defense responses to infection. *Nature, 411*, 826–833.

Duarte, I. F., Delgadillo, I., & Gil, A. M. (2006). Study of natural mango juice spoilage and microbial contamination with *Penicillium expansum* by high resolution ^{1}H NMR spectroscopy. *Food Chemistry, 96*, 313–324.

Hirano, S. S., & Upper, C. D. (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* – A pathogen, ice nucleus, and epiphyte. *Microbiology & Molecular Biology Reviews, 64*, 624–653.

Hodges, C. F., & Robinson, P. W. (1977). Sugar and amino acid content of *Poa pratensis* infected with *Ustilago striiformis* and *Urocystis agropyri*. *Physiologia Plantarum, 41*, 25–28.

Ingham, S. C., Losinski, J. A., Andrews, M. P., Breuer, J. E., Breuer, J. R., Wood, T. M., et al. (2004). *Escherichia coli* contamination of vegetables grown in soils fertilized with noncomposted bovine manure: Garden-scale studies. *Applied & Environmental Microbiology, 70*, 6420–6427.

Kennedy, A. C. (1999). Bacterial diversity in agroecosystems. *Agriculture, Ecosystems & Environment, 74*, 65–76.

Liang, Y.-S., Choi, Y. H., Kim, H. K., Linthorst, H. J. M., & Verpoorte, R. (2006). Metabolomic analysis of methyl jasmonate treated *Brassica rapa* leaves by two-dimensional NMR spectroscopy. *Phytochemistry, 67*, 2503–2511.

Liang, Y.-S., Kim, H. K., Lefeber, A. W. M., Erkelens, C., Choi, Y. H., & Verpoorte, R. (2006). Identification of phenylpropanoids in methyl jasmonate treated *Brassica rapa* leaves using two-dimensional nuclear magnetic resonance spectroscopy. *Journal of Chromatography A, 1112*, 148–155.

Mehdy, M. C. (1994). Active oxygen species in plant defense against pathogens. *Plant Physiology, 105*, 467–472.

Murashige, T., & Skoog, F. (1962). Arevised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15*, 473–497.

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Kloepper, J. W., & Pare, P. W. (2004). Bacterial Volatiles Induce Systemic Resistance in Arabidopsis[1]. *Plant Physiology, 134*, 1017–1026.

Sasaki, K., & Takahashi, T. (2002). A flavonoid from *Brassica rapa* flower as UV absorbing nectar guide. *Phytochemistry, 61*, 339–343.

Thiyam, U., Kuhlmann, A., Stockmann, H., & Schwarz, K. (2004). Prospects of rapeseed oil by-products with respect to antioxidative potential. *Les Comptes Rendus Chimie, 7*, 611–616.

Vallejo, F., Gil-Izquierdo, A., Pearez-Vicente, A., & Garciaa-Viguera, C. (2004). In vitro gastrointestinal digestion study of broccoli inflorescence phenolic compounds, glucosinolates, and vitamin C. *Jouranl of Agricultural & Food Chemistry, 52*, 135–138.

Vaquero, M. J. R., Alberto, M. R., & Nadra, M. C. M. (2007). Antibacterial effect of phenolic compounds from different wines. *Food Control, 18*, 93–101.

Vereecke, D., Messens, E., Klarskov, K., Bruyn, A. D., Van Montagu, M., & Goethals, K. (1997). Patterns of phenolic compounds in leafy galls of tobacco. *Planta, 201*, 342–348.

Wachtel, M. R., Whitehand, L. C., & Mandrell, R. E. (2002). Prevalence of *Escherichia coli* associated with a cabbage crop inadvertently irrigated with partially treated sewage wastewater. *Jouranl of Food Protection, 65*, 471–475.

Wang, L. I., Giovannucci, E. L., Hunter, D., Neuberg, D., Su, L., & Christiani, D. C. (2004). Dietary intake of cruciferous vegetables, glutathione S-transferase (GST) polymorphisms and lung cancer risk in a Caucasian population. *Cancer Causes & Control, 15*, 977–985.

Williamson, G., Day, A. J., Plumb, G. W., & Couteau, D. (2000). Human metabolic pathways of dietary flavonoids and cinnamates. *Biochemical Society Transactions, 28*, 16–22.