Gutierrez Albanchez Enrique (Orcid ID: 0000-0002-2099-7464)

Biotic elicitation as a tool to improve berry (Strawberry and Raspberry) extract potential on metabolic syndrome related enzymes in vitro

Enrique Gutierrez-Albanchez[1,2], Ara Kirakosyan[2], Steven F. Bolling [2], Ana García-Villaraco[1], Javier Gutierrez-Mañero[1], Beatriz Ramos-Solano[1].

1. Pharmaceutical and Health Sciences Department, Faculty of Pharmacy, Universidad San Pablo-CEU Universities, Boadilla del Monte, Madrid, Spain.
2. Cardiovascular Research Center, University of Michigan, Ann Arbor, MI, USA.

ABSTRACT

BACKGROUND

Raspberry and strawberry are among the high added value food products relevant for human health due to abundance of polyphenols. Polyphenols are secondary metabolites and therefore, devoted to improve plant adaptation so polyphenol profile can ba triggered by different stimulus like beneficial bacteria. The aim of this study was two-fold, i) to evaluate the ability of two bacterial strains to modulate secondary metabolism in strawberry and raspberry, and ii) to explore the ability of plant extracts to modify enzymes' activities related to metabolic syndrome.

RESULTS

Total phenolics and anthocyanins contents were higher in strawberries than in raspberries, despite similar antioxidant capacity. Strawberry extracts performed better on the tested enzymes, except on ±-glucosidase inhibition capacity. *Bacillus*

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amyloliquefaciens stabilized effects of extracts in different time points, while the *Pseudomonas fluorescens* modified plant metabolism after more inoculations (spring) in both species, improving effects of raspberry extracts on ±-glucosidase, COX1 and COX2, and of strawberry on ±-amylase and COX1.

CONCLUSION

These two fruits are a good choice to be included in the diet since they improve the activity of metabolic syndrome related enzymes. Delivering either strain along plant growth modifies bioactives' profiles, improving effects of fruit extracts over human health.

Keywords: Metabolic syndrome, elicitation, berries, secondary metabolism, COX2.

INTRODUCTION

Metabolic syndrome is a health condition with high prevalence due to the lifestyle of modern society; according to the International Diabetes Federation, 20-25 % of the world adult populations have the risk factors that constitute the metabolic syndrome [1]. Metabolic syndrome can precede the development of type II diabetes and cardiovascular disease and includes phenotypes such as obesity, systemic inflammation, hyperlipidemia, and high and/or fluctuating blood glucose due to insulin resistance [2].

Hyperglycemia is related to ±-glucosidase and ±-amylase, which are responsible of starch and disaccharides cleavage, releasing glucose [3]. Hypertension is related to the angiotensin-converting enzyme (ACE), a part of the renin angiotensin system, which is the responsible of the fluid pressure regulation in the body [4]. Cycloxigenase-1&2 (COX-1&2) are enzymes involved in the production of prostaglandins, among other functions. They play a key role in inflammatory processes and cardiovascular dysfunction, including hypertension [5,6]; COX-1 is the constitutive enzyme with a basal expression in most cells [7, 8], while COX-2 is the inducible isoform, non-detectable in basal conditions; it is expressed in swollen tissues or somehow altered, and is responsible of the prostanoids synthesis [9].

Raspberry and strawberry are important crops because recent studies relate berry consumption with the prevention of certain chronic diseases [10, 11, 12]. In both cases, health benefits are associated to the high amounts of secondary metabolites like

polyphenols (flavonoids, anthocyanins, flavonols and phenolic acids) [13, 14]; within the wide range of health benefits from antioxidant properties to anti-inflammatory and anti-hypertensive effects, cardioprotective effect and blood glucose regulation [15, 16]. Several studies have reported that diet is a very promising path to prevent metabolic syndrome, even revert progression of type II diabetes, and also very important to reduce pharmacological treatments in type II diabetes patients [17].

On the other hand, due to their sessile nature, plants have developed a specific secondary metabolism aiming to improve adaptation capacity to the environment [18]. As environmental conditions change along the year, secondary metabolites also change, and so do flavonoids in order to improve plant adaptation capacity in response to environmental stress (biotic or abiotic) [19]. Beneficial bacteria may be used to trigger plant metabolism without compromising plant fitness, in a process called elicitation. This method has been successfully used in agriculture because it lowers chemical inputs as plants metabolism is activated for effective defense and improves growth ability under adverse conditions [20, 21, 22].

Previous studies in field conditions have shown that two bacterial strains, *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*, are able to trigger secondary metabolism in different plants [23, 20]. Therefore, the aim of this study was two-fold, i) to evaluate the ability of these two strains to modulate the bioactive levels in strawberry and raspberry produced in counter season (adverse conditions), and ii) to demonstrate

the superior ability of these extracts in exerting health benefit properties related to blood glucose regulation, hypertension, and inflammatory processes, comparing winter and spring samples, indicative of adverse and favorable conditions, respectively. To achieve this purpose, we will i) determine antioxidant properties, and anthocyanins and other polyphenols content; ii) determine the ability of berry fruit extracts to inhibit different enzymes related to these processes such as the two major carbohydrate digestive enzymes, ±-glucosidase and ±-amylase (glucose regulation), the angiotensin converting enzyme ACE (hypertension), and COX-1&2 (inflammation).

MATERIALS AND METHODS

Biological material: plants and bacterial strains

Two bacterial strains with a good background to trigger secondary metabolism in other species were used. One bacterial strain was *Bacillus amyloliquefaciens (B.amyloliquefaciens)* (CECT 9371) is a Gram positive sporulated bacilli isolated from the rhizosphere of *Pinus pinea*. It releases siderophores [24] triggering defensive metabolism in *A. thaliana* against pathogen and salt stress [25]. The other bacterial strain was *Pseudomonas fluorescens (P.fluorescens)* (Spanish Type Culture Collection accession number CECT 7620), a Gram negative bacilli isolated from the rizhosphere of *Nicotiana glauca*. It releases siderophores and chitinases, triggering defensive metabolism in *Solanum lycopersicum* [26], *Arabidopsis thaliana (A.thaliana)* [25] and *Glycine max (G. max)* [27]. It also increases isoflavones contents in *G. max* [28] and fruit yield in blackberry [23].

Inoculum was prepared in luria broth for *B.amyloliquefaciens*, and in nutrient broth for *P.fluorescens*, under shaking at 28ºC for 24h to achieve a 10^9 cfu/mL bacterial concentration, and diluted to deliver 0.5 L to plants at 10^7 ufc/mL, every two weeks throughout the plant cycle, in the irrigation system.

Fruits from two different species of berries, Raspberry (*Rubus idaeus* Var. Adelita) and Strawberry (*Fragaria x ananassa* Var. Fortuna) grown in the winter cycle were collected from production greenhouses in Huelva (Spain) in winter and spring 2016. Shortly, plants undergo an artificial cold period before transplant to greenhouses, in order to start their regular cycle and produce over the winter. Raspberry is transplanted in September and shows two production peaks in winter (December through February) and spring (April through June). Strawberries are transplanted in October and start with a slight production in January that increases markedly along spring until May. A total of 195 raspberry and 350 strawberry plants per treatment were in the trial; plants were arranged in 65 plants per replicate in raspberry, and 175 plants per replicate for strawberry. In both cases 125 g for each replicate of fruits from both plant species were harvested by hand. Raspberry was sampled in January and May, and strawberry in March and May. Fruits from each replicate were pooled and grounded to a fine powder with liquid nitrogen using a sterilized mortar and pestle. After that, 1 g from each sample was mixed with 9 mL of methanol 80%, then it was sonicated for 10 min and centrifuged for 5 min. Supernatants were lyophilized and resuspended in methanol 80%

to obtain a 10 mg/mL concentration for all analysis except for total phenolics, anthocyanins and angiotensin converting enzyme, for which the concentration was 25 mg/mL.

HPLC Analysis Conditions: Total Phenolics, Total Anthocyanins, and ACE.

A reverse phase HPLC system was used and consisted of a Shimadzu HPLC equipped with a photodiode array detector (The Shimadzu LC-10A Series, Japan) equipped with a C18 column (Phenomenex LunaTM column 150X4.6 mm; 5 μm). Total phenolics, total anthocyanins, and ACE analysis methods are described in supplementary material.

Total phenolics

Elute was analyzed at a wavelength of 280 nm, and column temperature was maintained at 40 ^{o}C. Samples were injected in duplicate; total phenolics were calculated by integrating area on a calibration curve ($y = 2*10^{7}x + 84535$; $R^2 = 0.99952$) of gallic acid. Results are expressed as g of gallic acid equivalents per kg.

Total anthocyanins

Elute was analyzed at 520 nm, and column temperature was maintained at 40 ^{o}C. Samples were injected in duplicate; total anthocyanins were calculated by integrating

area on a calibration curve ($y = 1*10^7x - 3975.5$; $R^2 = 0.99975$) of cyanidin-3-glucoside. Results are expressed as g of cyanidin 3-glucoside equivalents per kg.

Angiotensin Converting Enzyme (ACE)

Elute was analyzed at a wavelength of 226 nm, which was at the maximum absorbance of hippuric acid (HA), and column temperature was maintained at 30ºC. N-Hippuryl-His-Leu hydrate (HHL) and ACE were dissolved in 100 mM borate buffer (pH = 8.3) supplemented with 300 mM NaCl, and their concentrations were 5 mM and 1 U/mL, respectively. Reaction system: 20 µL HHL, 20 µL ACE, 40 µL ACE inhibitors (samples) and 40 µL 100 mM borate buffer (pH = 8.3). System was incubated at 37ºC for 30 min, and then 250 µL HCl 0.5 M were added. Samples were injected in duplicate; the inhibition rate was calculated as follows:

% Inhibition = ((HA blank area– HA sample area)/ HA blank area)*100.

±-glucosidase inhibition assay

Alpha-glucosidase inhibition was assessed using the method described by [29]. In brief, 90 µL of 0.1 M phosphate buffer (pH 7.5, 0.02% NaN_3), 10µ L test sample dissolved in Dimethyl Sulfoxide (DMSO), and 80 µL of enzyme solution (well concentration 0.08 U/mL) were added to each well. The mixture was incubated at 28° C for 10 min before adding 4-Nitrophenyl ±-D-glucopyranoside (PNPG) to a final volume of 200 µL (final well concentration 1.0 mM). The blank was carried out in a similar manner, with the test sample replaced by solvent. The hydrolysis rate of PNPG to release p-

nitrophenolate was monitored at 405 nm every 30 s for 35 min. Incubation and absorbance measurements were performed with a Eon Biotek Bioreader, controlled by Gen5 software. Samples were analyzed in duplicate; the ±-glucosidase inhibitory activity was expressed as percentage inhibition and was calculated using as follows:

% Inhibition = ((Slope blank– Slope sample)/ Slope blank)*100.

IC50 (Concentration at which inhibition is 50%) was calculated using three different % inhibitions at three different concentrations.

±-amylase inhibition assay

The ±-amylase inhibition assay was conducted according to the method described by [30]. In brief, 80 µL of 0.1M-phosphate buffer (pH 6.0), 20 µL test sample dissolved in DMSO, and 80 µL of enzyme solution (well concentration 0.05 U/mL) were added to each well. After incubation at 37° C for 10 min, the reaction was started by adding 2-Chloro-4-nitrophenyl-±-D-maltotrioside (CNP-G3) to a final volume of 200 µL (final well concentration 1.0 mM). The blank was carried out in a similar manner, with the test sample replaced by solvent. Absorbance was measured at 405 nm every three minutes for 30 min on the same instrument as described above. Samples were analyzed in duplicate; the ±-amylase inhibitory activity was expressed as percentage inhibition and was calculated as follows:

% Inhibition = ((Slope blank– Slope sample)/ Slope blank)*100.

IC50 was calculated using three different % inhibitions at three different concentrations.

Antioxidant capacity

Antioxidant capacity was measured using commercially available Antioxidant Assay Kit (Cayman, Item No 709001) following the manufacturer´s instructions. This Kit relies on the ability of the antioxidants in the sample to inhibit the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) • (ABTS•) to ABTS by metmyoglobin; samples were measured in triplicate at 720 nm and 405 nm. Data is expressed as mM Trolox equivalents.

Cyclooxygenase (COX) inhibition assay

COX inhibitor activity was measured using commercially available COX Activity Assay (Cayman, Item No 760151) following the manufacturer´s instructions. This Kit measures the peroxidase activity of COX, assayed colorimetrically by monitoring the appearance of oxidized N, N, N, N-tetramethyl-p-phenylenediamine (TMPD); samples were measured in triplicate at 590 nm. Data is expressed as % of inhibition.

Statistical analysis

Two different types of multivariate analyses were performed with CANOCOTM v4.5 software to evaluate the most relevant factors in our data: Correspondence Analysis (CA) and distance based redundancy analysis (db-RDA). Firstly, ordinations provided by CA were carried out with average values of the replicates. Secondly, db- RDA [31] were carried out with data from the following parameters: antioxidant capacity, total phenols and anthocyanins, and inhibition of the following enzymes: ACE, COX1,

COX2, ±-amylase and ±-glucosidase, all of them calculated with values obtained from raspberries and strawberries extracts from spring and winter, and inoculated with two bacterial strains and the non-inoculated controls. This analysis (db-RDA) begins with the calculation of distances among replicates (Bray Curtis index), followed by principal coordinate analysis (PCoA), and finally, by redundancy analysis (RDA). db-RDA is a multivariate test of hypothesis which tests for the effect of treatments on a multivariate data table and indicates statistical significance of the ordination.

Two-way ANOVA was performed to evaluate differences among the treatments and sampling moment for the following parameters: i) antioxidant capacity; ii) total phenolics, iii) total anthocyanins, inhibition capacity on iv) ±-amylase v) ±-glucosidase vi) COX1, vii) COX2, viii) ACE; all of them include both sampling moments in which the effect of the two factors (sampling moment and bacterial treatment) and their interaction were analyzed using Statgraphics 5.1. When differences were significant ($p < 0.05$), the LSD Fisher post-hoc test was also performed [32].

RESULTS

Since a lot of variables have been considered, the first approach to organize data was to carry on a CA, a type of multivariate analysis that considers all data simultaneously organizing samples according to the degree of similarity (Figure 1). Ordination of samples on the plane defined by the first two axes that absorb 91.7% of the variance, separates raspberry samples towards the negative values of axis I, from strawberry

samples that remain in the positive values of the same. Within raspberry samples, controls separate from inoculated samples towards the negative values of axis II. In strawberry, separation between samples is not so clear. The separation of raspberry and strawberry indicated in the CA analysis is statistically significant according to db-RDB (supplementary material) ($p<0.05$).

Antioxidant capacity and bioactive content of extracts.

Antioxidant capacity, total phenolic contents and total anthocyanin contents of raspberries and strawberries (Table 1) grown in winter and spring, inoculated with the *Bacillus* and the *Pseudomonas* strain, as well as non-inoculated controls were determined.

The average antioxidant capacity ranged between 0.2 and 0.25 mM Trolox equivalents, being significantly higher in spring samples than in winter samples, both in raspberry and strawberry, consistent with the reported increase on antioxidant compounds along the plant cycle [33]. In raspberry, both strains increased the antioxidant capacity in winter samples and decreased it in spring; *P.fluorescens* showed a more intense effect than *B.amyloliquefaciens* that maintained the same values in both moments. In strawberry, *B.amyloliquefaciens* had a negative effect on antioxidant capacity in winter while *P.fluorescens* enhanced its potential; no effects were detected in spring samples.

Since the antioxidant capacity is related to total phenolics and anthocyanins, and these compounds are the main bioactives responsible from biological effects, HPLC quantification was carried out (Table 1).

Raspberries total phenolic contents in winter averaged 2.5 g kg^{-1}, and anthocyanins accounted for the greatest part of this content, representing 2 g kg^{-1} on average in winter controls (Table 1); in spring, total phenolic contents were more than two fold, and anthocyanins accounted for most of total phenolics in non-inoculated controls. Despite the fact that extracts from inoculated plants showed no significant changes on the amount of total phenolics or anthocyanins in winter samples, their antioxidant capacity (Table 1) increased under the influence of both strains. Only *P.fluorescens* spring samples caused a significant decrease on total phenolics and anthocyanins concentrations, in parallel to a decrease in antioxidant capacity.

Strawberry extracts (Table 1) were richer than raspberries on total phenolics (average 10 g kg^{-1} of dry weight) and anthocyanins, which accounted for 50% of total phenolic content. Differences between winter and spring extracts were not as marked as in raspberries. In winter samples, bacterial treatments significantly decreased total phenolics, while anthocyanins significantly increased on *B.amyloliquefaciens* treated extracts and significantly decreased on those from *P.fluorescens*, revealing different plant targets for each strain; in spring samples, *B.amyloliquefaciens* did not affect total phenolics as compared to controls and again significantly increased total anthocyanins.

Effects on blood glucose regulating enzymes, ±-glucosidase and ±-amylase

Non-inoculated winter raspberry extracts (Figure 2a) were significantly more effective inhibiting ±-glucosidase than springs'; except for *P.fluorescens* winter extracts, bacterial treatments significantly improved extract's inhibition capacity. Raspberry winter samples inoculated with *B.amyloliquefaciens* (figure 2a) were the most effective, associated to an increased antioxidant capacity due to compounds other than total phenolics or anthocyanins since both show non-significant differences (Table 1); interestingly, raspberry winter and spring samples inoculated with *B.amyloliquefaciens* caused the same effect, supporting the metabolic stabilization in the plant induced by this strain.

As regards to strawberry (Figure 2b), spring extracts were more effective inhibiting ±-glucosidase. *P.fluorescens* did not alter effects on this enzyme neither in winter or spring samples. Interestingly, and although only strawberry winter samples inoculated with *B.amyloliquefaciens* modified the effects on ±-glucosidase, improving in winter and worsening in spring, *B.amyloliquefaciens* extracts obtained reproducible results both in winter and spring. Nevertheless, strawberry extracts were less effective than raspberry, averaging IC50 values of 10 mg/mL and 4 mg/mL, respectively.

The most effective extracts to inhibit ±-amylase activity were raspberries' with lower IC50 values, although average values in strawberries' (1.6 mg/mL) were similar to

raspberries' (1.3 mg/mL). Bacterial treatments significantly increased IC50 values in raspberry and strawberry winter samples, therefore decreasing inhibiting activity (Figure 2c, d); however, the only significant improvement was strawberry spring samples inoculated with *P.fluorescens* extracts, associated to lower phenolics and anthocyanins (Table 1). Hence, a deeper metabolic characterization will be carried out on this extract to find specific inhibitors of ±-amylase.

Effects on antihypertensive enzyme, ACE

As regards to the ACE inhibition capacity, strawberry extracts were more effective than raspberries (Figure 3). In strawberry (Figure 3b), spring extracts performed better than winter extracts inhibiting ACE, and all bacterial treatments decreased the capacity of these extracts to inhibit the enzyme; however, consistent effects were achieved under the influence of the two bacteria in both sampling moments. In raspberries (Figure 3a), winter extracts were more effective and bacterial treatments lowered this potential; a different effect was found in spring extracts, being significantly enhanced by *B.amyloliquefaciens*.

Effects on anti-inflammatory related enzymes: COX-1 and COX-2

Raspberry extracts inhibited COX-1 activity by around 35%, irrespective of the sampling moment; bacterial treatments improved this activity similarly at both moments, and *P.fluorescens* always achieved best results (Figure 4a). Strawberry extracts were more effective than raspberries inhibiting this enzyme (average 47%),

irrespective of the sampling moment; in winter, bacterial treatments decreased the capacity to inhibit COX-1, although it was increased on spring samples, when *P.fluorescens* strawberry extracts significantly increased inhibition capacity to 50% (Figure 4b).

As regards to COX-2 activity, raspberry extracts were able to inhibit COX-2 by 30% in winter and spring, and bacterial treatments significantly improved the inhibition capacity in both sampling moments, irrespective of the bacterial strain (Figure 4c). Strawberry extracts were more effective inhibiting COX-2 (Figure 4d) by 35% although non-significant differences were found among any treatment, as anticipated by the bidirectional ANOVA (Table 2, supplementary material).

DISCUSION AND CONCLUSION

Despite the fact that extracts from inoculated plants showed no significant changes on the amount of total phenolics or anthocyanins in winter samples, their antioxidant capacity increased under the influence of both strains, so it is evident that biotic stimuli change plant metabolism, affecting other bioactive profiles different to phenols and anthocyanins, and therefore, effects on enzyme activities are expected to be different to controls. Only *P.fluorescens* spring samples caused a significant decrease on total phenolics and anthocyanins concentrations, in parallel to a decrease in antioxidant capacity, supporting the relationship between these compounds and antioxidant capacity

[34]. The decrease in phenols and anthocyanins may be due to bacterial activation of the biosynthetic pathway beyond anthocyanins, since these compounds have been shown to play a role in plant defense either directly, behaving as phytoalexins, or as precursors of the real phytoalexins [35,36]. Although anthocyanins have a strong antioxidant capacity, the newly formed products may not show this ability, consistent with the loss of antioxidant capacity detected; nevertheless, a beneficial effect on other targets should not be ruled out yet, because health benefits may be achieved by a more specific mechanism than the basic mechanism of ROS removal from the system [37].

B.amyloliquefaciens increases anthocyanin contents in strawberry, contributing to the good quality of the fruit since it will show a better color due to anthocyanins, which increases market quality, with higher bioactive contents along the production period [20].

Glucose regulation by ±-glucosidase and ±-amylase is a good strategy to prevent high glucose blood levels. Taking into account that IC50 values for acarbose (drug use for diabetes mellitus type II treatment) are 0.5811mg/mL for ±-glucosidase, and 0.11µg/mL ±-amylase [30], our results indicate the potential of these extracts to manage hyperglycemia, especially when delivered through the diet, being raspberries more effective. Among bacterial treatments, *B.amyloliquefaciens* improved the inhibition potential for ±-glucosidase in raspberry and strawberry winter extracts, while no improvement was shown for ±-amylase, except for *P.fluorescens* in strawberry spring extracts. Total anthocyanins and total phenolics don´t seem to be related to the

inhibitory effects, supporting the notion of changes in secondary metabolism in other groups of compounds [35].

Results obtained for ACE inhibition showed a potential relationship between ACE inhibition and total phenolics and anthocyanins, since lower bioactives in raspberry spring samples inoculated with *P.fluorescens* result in a decreased inhibition capacity.

The anti-inflammatory effect of these extracts showed good results in the inhibition of COX1 and COX2 (around 30-40% inhibition). The two strains improved the effect of the control extract on COX2 inhibition, both in raspberry and strawberry. Interestingly COX2 is the inducible form of inflammatory process; hence a diet rich in berries could prevent inflammation. These results don´t seem to be related to the total phenolics or anthocyanins reinforcing the modification of other metabolic pathways.

In view of the above data, anthocyanins and total phenolics do not seem to be the underlying compounds responsible of the extracts effect. Hence, a deeper metabolic characterization should be carried out on this extract to find specific phenolic compounds or other secondary metabolites responsible of these effects.

In summary, total phenolics and anthocyanins contents were higher in strawberries than raspberries, despite similar antioxidant capacity. Strawberry extracts perform better on the tested enzymes, except for ±-glucosidase inhibition capacity. Bacterial inoculations

along plant growth modify strawberry and raspberry metabolism, modifying extract composition that results in modification of marker enzymes' activity; total phenolics and anthocyanins are not fully responsible for the improvement. *B.amyloliquefaciens* induces changes in strawberry and raspberry metabolism that improves inhibition potential of ±-glucosidase and COX2; it stabilizes effects of extracts at different time points. *P.fluorescens* modifies plant metabolism after more inoculations (spring) in both plants, improving effects of raspberry extracts on ±-glucosidase, COX1 and COX2, and of strawberry on ±-amylase and COX1.

Based on effects on metabolic syndrome related enzymes, these two fruits are a good choice to be included in the diet, and they are even better when either bacterial strain are used during production. Nevertheless, much remains to be elucidated before understanding of the effects of particular compounds and whether the elicitation process results in improved extract potential to modify metabolic syndrome related enzymes.

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Figure captions.

Figure 1. Correspondence analysis (CA) performed with data from quantitative determination of phenols and anthocyanins, antioxidant capacity, COX1&2, ACE, ±-amylase and ±-glucosidase, in different seasons (W, S). RWC, Raspberry Winter Control; RWB, Raspberry Winter *B.amyloliquefaciens*; RWP, Raspberry Winter *P.fluorescens*; RSC, Raspberry Spring Control; RSB, Raspberry Spring *B.amyloliquefaciens*; RSP, Raspberry Spring *P.fluorescens*; SWC, Strawberry Winter Control; SWB, Strawberry Winter *B.amyloliquefaciens*; SWP, Strawberry Winter *P.fluorescens*; SSC, Strawberry Spring Control; SSB, Strawberry Spring *B.amyloliquefaciens*; SSP, Strawberry Spring *P.fluorescens*.

Figure 2. IC50 ±-glucosidase (a and b), and ±-amylase (c and d) obtained with raspberry and strawberry extracts. RWC, Raspberry winter Control; RWB, Raspberry winter *B.amyloliquefaciens*; RWP, Raspberry winter *P.fluorescens*; RSC, Raspberry spring Control; RSB, Raspberry spring *B.amyloliquefaciens*; RSP, Raspberry spring *P.fluorescens*; SWC, Strawberry winter Control; SWB, Strawberry winter *B.amyloliquefaciens*; SWP, Strawberry winter *P.fluorescens*; SSC, Strawberry spring Control; SSB, Strawberry spring *B.amyloliquefaciens*; SSP, Strawberry spring *P.fluorescens*. Different letters denote statistically significant differences according to LSD Fisher test ($P < 0.05$).

Figure 3. ACE %inhibition. RWC, RWC, Raspberry winter Control; RWB, Raspberry winter *B.amyloliquefaciens*; RWP, Raspberry winter *P.fluorescens*; RSC, Raspberry spring Control; RSB, Raspberry spring *B.amyloliquefaciens*; RSP, Raspberry spring *P.fluorescens*; SWC, Strawberry winter Control; SWB, Strawberry winter *B.amyloliquefaciens*; SWP, Strawberry winter *P.fluorescens*; SSC, Strawberry spring Control; SSB, Strawberry spring *B.amyloliquefaciens*; SSP, Strawberry spring *P.fluorescens*. Different letters denote statistically significant differences according to LSD Fisher test ($P< 0.05$).

Figure 4. COX-1 & 2 % inhibition. RWC, Raspberry winter Control; RWB, Raspberry winter *B.amyloliquefaciens*; RWP, Raspberry winter *P.fluorescens*; RSC, Raspberry spring Control; RSB, Raspberry spring *B.amyloliquefaciens*; RSP, Raspberry spring *P.fluorescens*; SWC, Strawberry winter Control; SWB, Strawberry winter *B.amyloliquefaciens*; SWP, Strawberry winter *P.fluorescens*; SSC, Strawberry spring Control; SSB, Strawberry spring *B.amyloliquefaciens*; SSP, Strawberry spring *P.fluorescens*. Different letters denote statistically significant differences according to LSD Fisher test ($P< 0.05$).

Tables

Table 1. Antioxidant capacity of extracts (mM Trolox equivalents) and bioactive contents in Raspberry and Strawberry (g Kg^{-1}). RWC, Raspberry winter Control; RWB, Raspberry winter *B.amyloliquefaciens*; RWP, Raspberry winter *P.fluorescens*; RSC, Raspberry spring Control; RSB, Raspberry spring *B.amyloliquefaciens*; RSP, Raspberry spring *P.fluorescens*; SWC, Strawberry winter Control; SWB, Strawberry winter *B.amyloliquefaciens*; SWP, Strawberry winter *P.fluorescens*; SSC, Strawberry spring Control; SSB, Strawberry spring *B.amyloliquefaciens*; SSP, Strawberry spring *P.fluorescens*. Different letters denote statistically significant differences between treatments for each parameter (a, b, c for Raspberry; x, y, z for Strawberry), according to LSD Fisher test ($P< 0.05$).

	0.20± 0.001 (a)	2.57± 0.039 (a)	1.96± 0.009 (a)
	0.22± 0.002 (b)	2.65± 0.087 (a)	2.27± 0.005 (a)
	0.23± 0.0004 (c)	2.69± 0.024 (a)	1.94± 0.003 (a)
	0.24± 0.002 (d)	6.35± 0.028 (c)	4.93± 0.18 (c)
	0.22± 0.001 (b)	6.18± 0.089 (c)	4.91± 0.017 (c)
	0.20± 0.003 (a)	5.41± 0.088 (b)	4.27± 0.185 (b)
	0.19± 0.004 (wx)	10.01 ± 0.002 (y)	5.13 ± 0.322 (x)
	0.18± 0.006 (w)	9.28 ± 0.044 (wx)	5.87 ± 0.160 (y)
	0.20± 0.004 (x)	9.10 ± 0.009 (w)	4.15 ± 0.091 (w)

	0.22± 0.003 (y)	11.03± 0.020 (z)	5.74 ± 0.125 (y)
	0.23± 0.002 (y)	10.89 ± 0.206 (z)	6.58 ± 0.115 (z)
	0.23± 0.002 (y)	9.56 ± 0.060 (z)	5.31 ± 0.115 (xy)

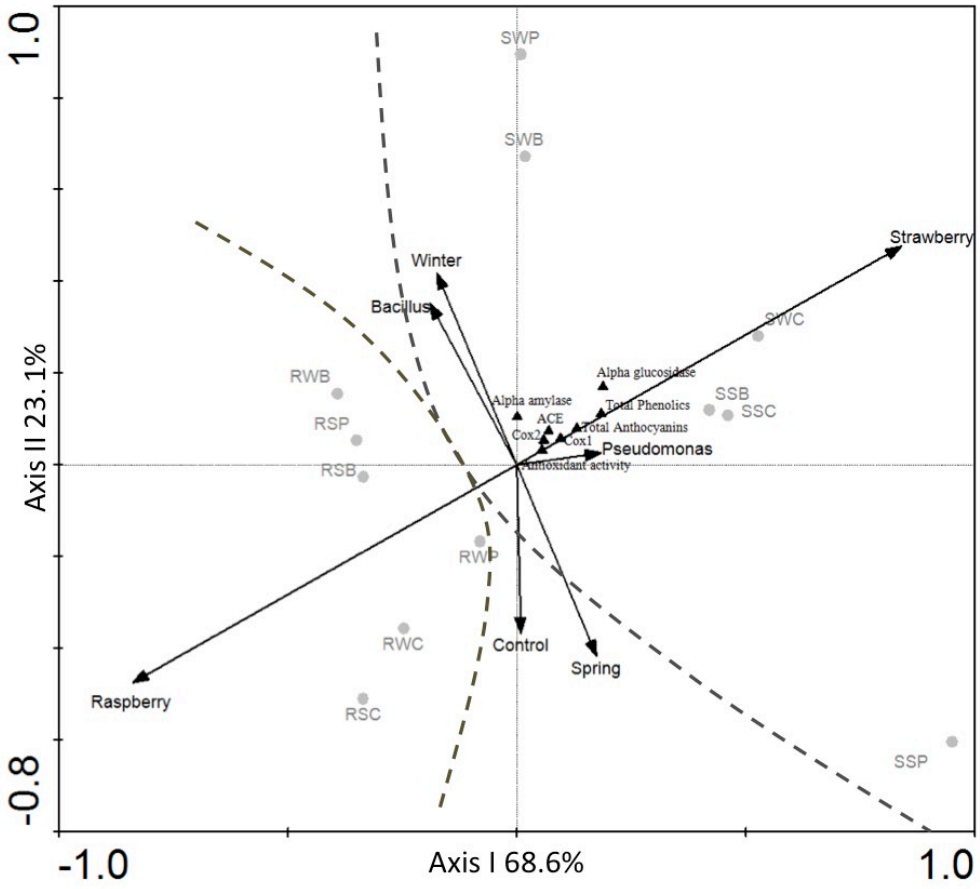
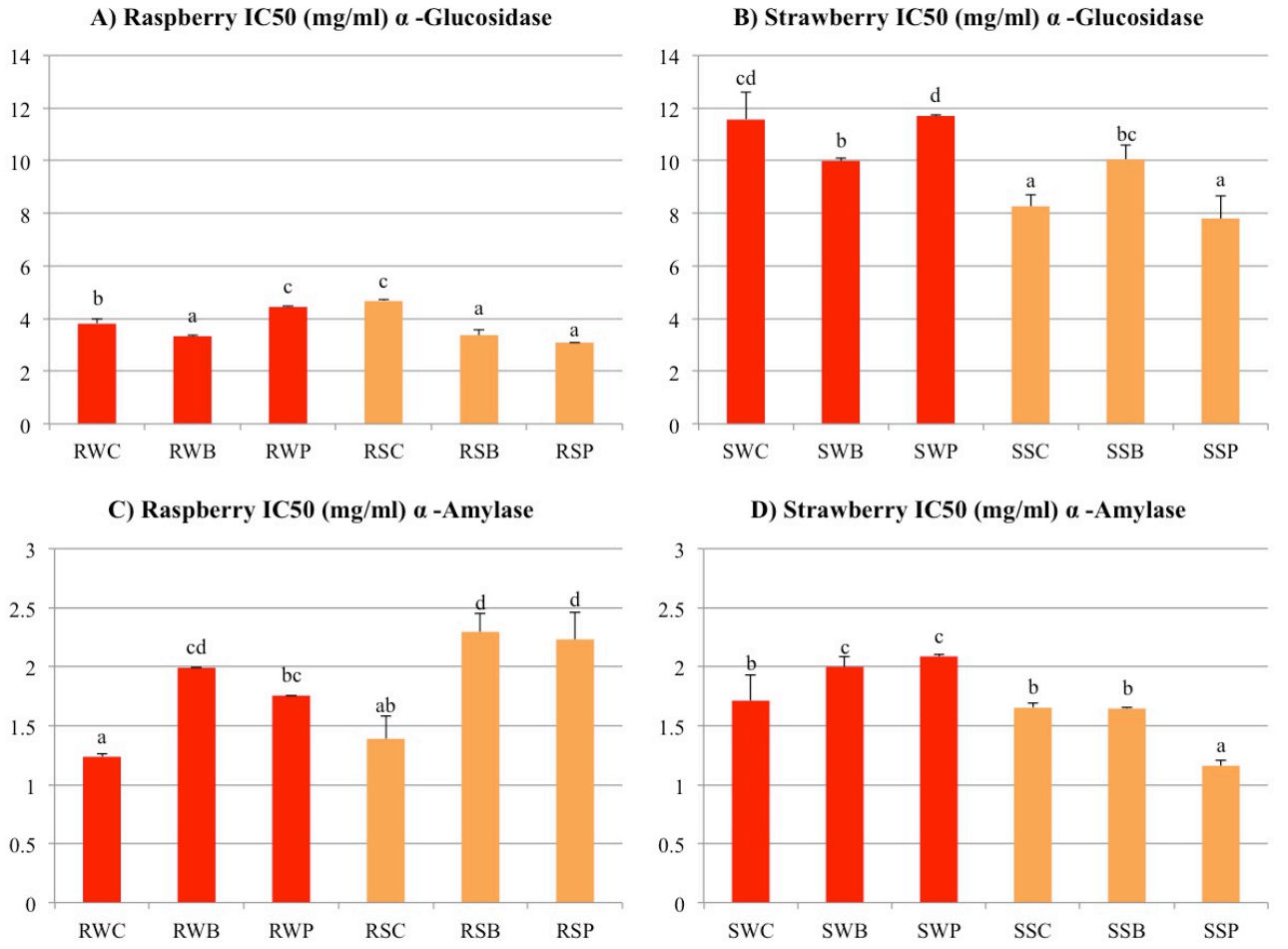


Figure 1..jpg



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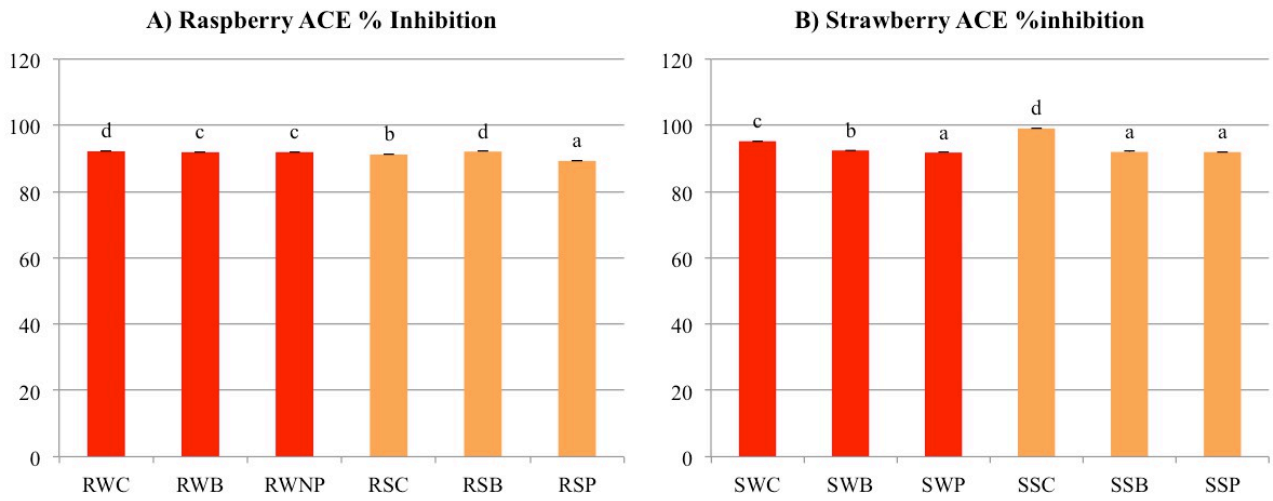

Figure 3..jpg

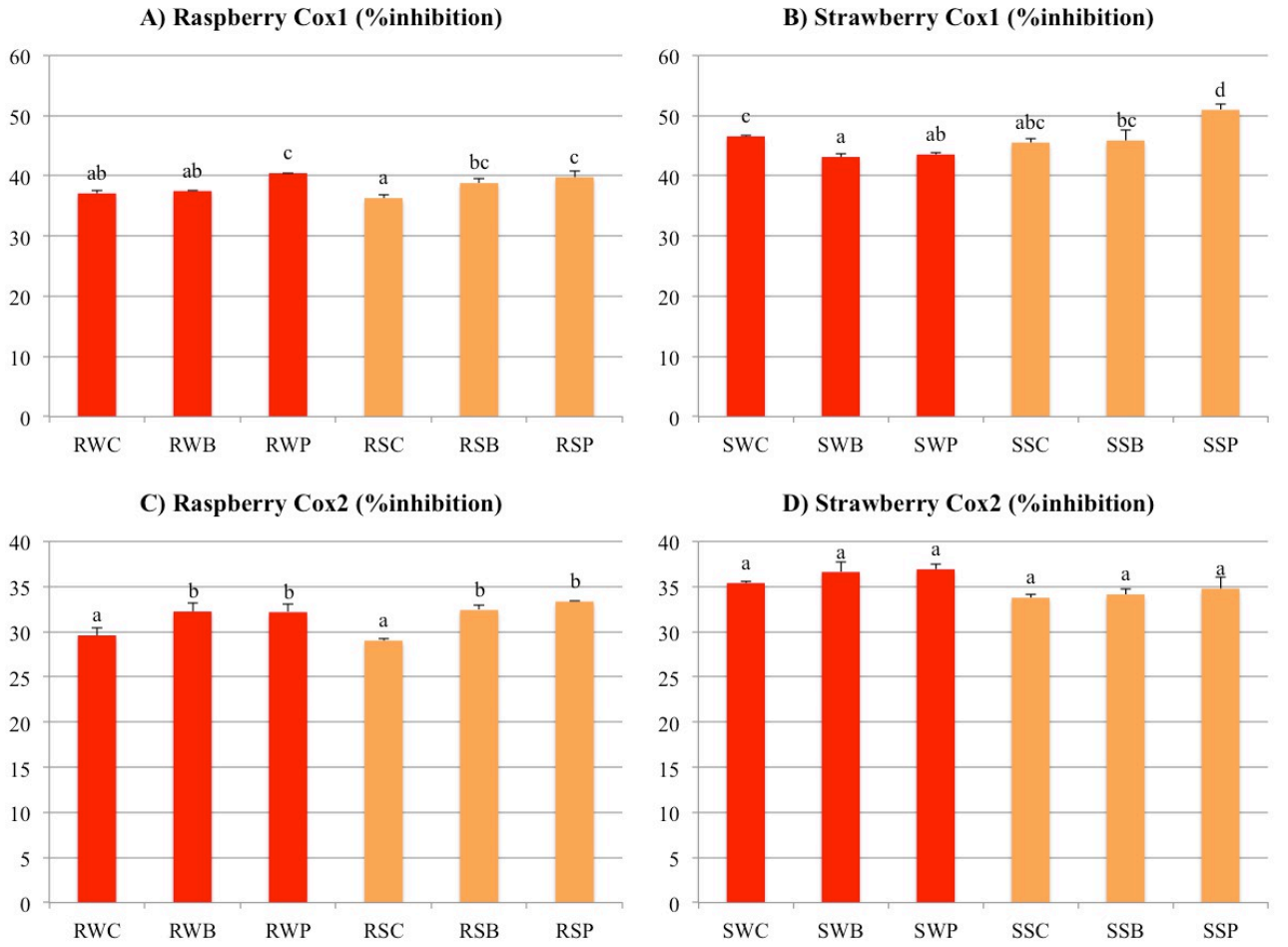

Figure 4..jpg

Sample	Antioxidant Capacity (mM Trolox equivalents)	Total phenolics (g kg^{-1} dry weight of Gallic acid equivalents)	Total anthocyanins (g kg^{-1} dry weight of Cyanidin 3-glucoside equivalents)
RWC	0.20± 0.001 (a)	2.57± 0.039 (a)	1.96± 0.009 (a)
RWB	0.22± 0.002 (b)	2.65± 0.087 (a)	2.27± 0.005 (a)
RWP	0.23± 0.0004 (c)	2.69± 0.024 (a)	1.94± 0.003 (a)
RSC	0.24± 0.002 (d)	6.35± 0.028 (c)	4.93± 0.18 (c)
RSB	0.22± 0.001 (b)	6.18± 0.089 (c)	4.91± 0.017 (c)
RSP	0.20± 0.003 (a)	5.41± 0.088 (b)	4.27± 0.185 (b)
SWC	0.19± 0.004 (wx)	10.01 ± 0.002 (y)	5.13 ± 0.322 (x)
SWB	0.18± 0.006 (w)	9.28 ± 0.044 (wx)	5.87 ± 0.160 (y)
SWP	0.20± 0.004 (x)	9.10 ± 0.009 (w)	4.15 ± 0.091 (w)
SSC	0.22± 0.003 (y)	11.03± 0.020 (z)	5.74 ± 0.125 (y)
SSB	0.23± 0.002 (y)	10.89 ± 0.206 (z)	6.58 ± 0.115 (z)
SSP	0.23± 0.002 (y)	9.56 ± 0.060 (z)	5.31 ± 0.115 (xy)

Table 1..jpg