Research Article

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Biotic elicitation as a tool to improve strawberry and raspberry extract potential on metabolic syndrome-related enzymes *in vitro*

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Abstract

BACKGROUND: Raspberry and strawberry are high value-added food products that can contribute to human health due to the abundance of polyphenols that they contain. Polyphenols are secondary metabolites and therefore devoted to improve plant adaptation, these polyphenol profile can be induced applying different stimuli, such as certain bacteria. The aim of this study was twofold: (i) to evaluate the ability of two bacterial strains to modulate secondary metabolisms in strawberry and raspberry, and (ii) to explore the ability of plant extracts to modify enzyme activities related to metabolic syndrome.

RESULTS: Total phenolic and anthocyanin content was higher in strawberries than in raspberries, despite similar antioxidant capacities. Strawberry extracts performed better on the tested enzymes, except on α-glucosidase inhibition capacity. *Bacillus amyloliquefaciens* stabilized the effects of extracts at different points in time, and *Pseudomonas fluorescens* modified plant metabolism after more inoculations (spring) in both species, improving the effects of raspberry extracts on α-glucosidase, COX1, and COX2, and of strawberry on α-amylase and COX1.

CONCLUSION: It is good to include these two fruits in the diet because they improve the activity of metabolic syndrome-related enzymes. Applying either strain during plant growth modifies the bioactive profile of the plants, improving the effects of the fruit extracts on human health.

Supporting information may be found in the online version of this article.

Keywords: metabolic syndrome; elicitation; berries; secondary metabolism; COX2

INTRODUCTION

Metabolic syndrome is a health condition that is highly prevalent due to modern lifestyles. According to the International Diabetes Federation, 20–25% of the world's adult population has 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 α-glucosidase and α-amylase, which are responsible for starch and disaccharide cleavage, releasing glucose.[3] Hypertension is related to the angiotensin-converting enzyme (ACE), a part of the renin angiotensin system, which is responsible for 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] whereas COX-2 is the inducible isoform, non-detectable in basal conditions; it is expressed in swollen tissues, and is responsible for prostanoid synthesis.[9]

Raspberries and strawberries are important crops because recent studies relate berry consumption to the prevention of certain chronic diseases.[10–12] In both cases, health benefits are associated with high amounts of secondary metabolites like polyphenols (flavonoids, anthocyanins, flavonols, and phenolic acids).[13,14] There is a wide range of health benefits, from antioxidant properties to anti-inflammatory and anti-hypertensive effects, cardioprotective effects, and blood glucose regulation.[15,16] Several studies have reported that diet is a very promising path to prevent metabolic syndrome. It can even reverse the progression of type II diabetes, and is also very important for reducing pharmacological treatments in type-II diabetes patients.[17]

On the other hand, due to their sessile nature, plants have developed a specific secondary metabolism that improves their capacity to adapt to the environment.[18] As environmental conditions

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change throughout the year, secondary metabolites also change, and so do flavonoids, 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 the plant metabolism is activated for effective defense. It improves growth ability under adverse conditions.[20–22]

Previous studies in field conditions have shown that two bacterial strains, *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*, are able to trigger secondary metabolisms in different plants.[23,20] The aim of this study was therefore twofold: (i) to evaluate the ability of these two strains to modulate the bioactive levels in strawberry and raspberry produced in counter season, and (ii) to demonstrate the positive effects of these extracts on blood glucose regulation, hypertension, and inflammatory processes, comparing winter and spring samples, indicative of adverse and favorable conditions, respectively. To achieve this purpose we (i) determined antioxidant properties, and anthocyanin and other polyphenol content; (ii) determined the ability of berry fruit extracts to inhibit different enzymes related to these processes, such as the two major carbohydrate digestive enzymes, α-glucosidase and α-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 were used that were known to be effective in triggering secondary metabolisms in other species. One bacterial strain was *Bacillus amyloliquefaciens* (CECT 9371), which is a gram-positive sporulated bacillus isolated from the rhizosphere of *Pinus pinea*. It releases siderophores[24] triggering a defensive metabolism in *A. thaliana* against pathogen and salt stress.[25] The other bacterial strain was *Pseudomonas fluorescens* (Spanish Type Culture Collection accession number CECT 7620), a gram-negative bacillus isolated from the rhizosphere of *Nicotiana glauca*. It releases siderophores and chitinases, triggering defensive metabolisms in *Solanum lycopersicum* [26], *Arabidopsis thaliana*,[25] and *Glycine max*.[27] It also increases isoflavone content in *G. max* [28] and fruit yield in blackberries.[23]

Inoculum was prepared in luria broth for *B. amyloliquefaciens*, and in nutrient broth for *P. fluorescens*, by shaking at 28 °C for 24 h to achieve a 10^9 cfu mL^{-1} bacterial concentration, and it was diluted to deliver 0.5 L to plants at 10^7 ufc mL^{-1}, every 2 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. The plants underwent an artificial cold period before transplant to greenhouses, 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; production is at a low level in January but increases markedly through spring until May. A total of 195 raspberry and 350 strawberry plants per treatment were in the trial; plants were arranged with 65 plants per replicate for raspberry, and 175 plants per replicate for strawberry. In both cases, 125 g for each replicate of fruits from both plant species was harvested by hand. Raspberries were sampled in January and May, and strawberries in March and May. Fruits from each replicate were pooled and ground 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 80% methanol, then it was sonicated for 10 min and centrifuged for 5 min. Supernatants were lyophilized and resuspended in 80% methanol to obtain a 10-mg mL^{-1} concentration for all analysis except for total phenolics, anthocyanins, and angiotensin-converting enzyme, for which the concentration was 25 mg mL^{-1}.

High-performance liquid chromatography (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 (Shimadzu LC-10A Series, Kyoto, Japan) and equipped with a C18 column (Phenomenex Luna™ column 150 × 4.6 mm; 5 µm California, United States). Total phenolics, total anthocyanins, and ACE analysis methods are described in the supplementary material.

Total phenolics

Eluent was analyzed at a wavelength of 280 nm, and column temperature was maintained at 40 °C. Samples were injected in duplicate; total phenolics were calculated by integrating the area on a calibration curve ($y = 2 * 10^7 x + 84\,535$; $R^2 = 0.99952$) of gallic acid. Results are expressed as g of gallic acid equivalents per kg.

Total anthocyanins

Eluent was analyzed at 520 nm, and column temperature was maintained at 40 °C. Samples were injected in duplicate; total anthocyanins were calculated by integrating the area on a calibration curve ($y = 1 * 10^7 x - 3975.5$; $R^2 = 0.99975$) of cyanidin-3-glucoside. Results are expressed as g of cyanidin 3-glucoside equivalents per kg.

Angiotensin converting enzyme (ACE)

Eluent was analyzed at a wavelength of 226 nm, which was the maximum absorbance of hippuric acid (HA), and column temperature was maintained at 30 °C. *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^{-1}, respectively. Reaction system is composed by 20 µL HHL, 20-µL ACE, 40-µL ACE inhibitors (samples) and 40-µL 100-mM borate buffer (pH = 8.3). The system was incubated at 37 °C for 30 min, and then 250 µL HCl 0.5 mol L^{-1} were added. Samples were injected in duplicate; the inhibition rate was calculated as follows:

$$\%\text{inhibition} = \left[(\text{HA blank area} - \text{HA sample area}) / \text{HA blank area}\right] * 100$$

α-Glucosidase inhibition assay

α-Glucosidase inhibition was assessed using the method described in Schmidt *et al.*[29] In brief, 90 µL of 0.1-mol L^{-1} phosphate buffer (pH 7.5, 0.02% NaN_3), 10 µL test sample dissolved in dimethyl sulfoxide (DMSO), and 80 µL of enzyme solution (well concentration 0.08 U mL^{-1}) were added to each well. The mixture was incubated at 28 °C for 10 min before adding 4-nitrophenyl α-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 an Eon Biotek bioreader (Bad Friedrichshall, Germany), controlled by Gen5 software. Samples were analyzed in duplicate; α-glucosidase inhibitory activity was expressed as percentage inhibition and was calculated as follows:

$$\%\text{inhibition} = \left[(\text{slope blank} - \text{slope sample})\,/\text{slope blank}\right) * \ 100$$

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

α-Amylase inhibition assay

The α-amylase inhibition assay was conducted according to the method described by Trinh *et al.*[30] In brief, 80 µL of 0.1 mol L^{-1}-phosphate buffer (pH 6.0), 20-µL test sample dissolved in DMSO, and 80 µL of enzyme solution (well concentration 0.05 U mL^{-1}) were added to each well. After incubation at 37 °C for 10 min, the reaction was started by adding 2-chloro-4-nitrophenyl-α-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 3 min for 30 min on the same instrument as described above. Samples were analyzed in duplicate; the α-amylase inhibitory activity was expressed as percentage inhibition and was calculated as follows:

$$\%\text{inhibition} = \left[(\text{slope blank} - \text{slope sample})\,/\text{slope blank}\right] * \ 100$$

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

Antioxidant capacity

The antioxidant capacity was measured using a commercially available antioxidant assay kit (Cayman, Item No 709001, Cayman Chemical, Michigan, United States) 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-sulfonic acid) • (ABTS•) to ABTS by metmyoglobin; samples were measured in triplicate at 720 and 405 nm. Data are expressed as mM Trolox equivalents.

Cyclooxygenase (COX) inhibition assay

Cyclooxygenase inhibitor activity was measured using a commercially available COX activity assay (Cayman, Item No 760151, Cayman Chemical, Michigan, United States) 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 are expressed as percentage 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). First, ordinations provided by CA were carried out with average values of the replicates. Second, db-RDA[31] were carried out with data from the following parameters:

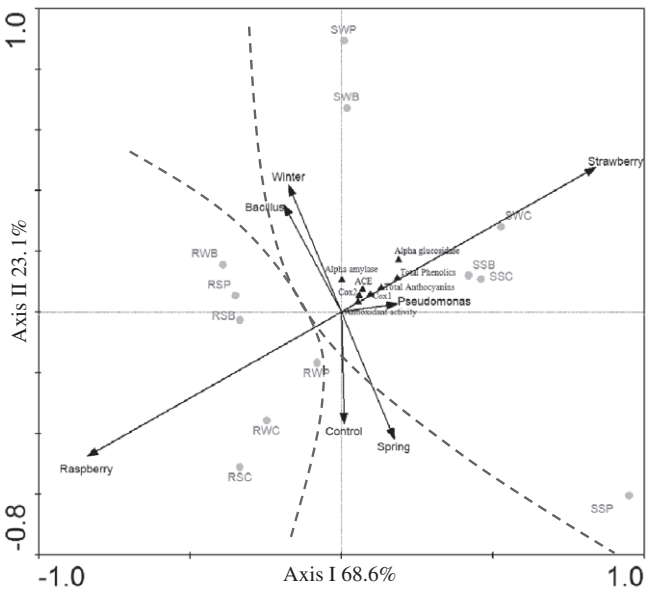

Figure 1. Correspondence analysis (CA) performed with data from quantitative determination of phenols and anthocyanins, antioxidant capacity, COX1&2, ACE, α-amylase and α-glucosidase, in different seasons (W, S). RWC, raspberry winter control; RWB, raspberry winter *B. amyloliquefaciens*; RWP, raspberry 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*.

antioxidant capacity, total phenols and anthocyanins, and inhibition of the following enzymes: ACE, COX1, COX2, α-amylase, and α-glucosidase, all of them calculated with values obtained from raspberry and strawberry 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). Distance-based redundancy analysis is a multivariate test of hypotheses, which tests for the effect of treatments on a multivariate data table and indicates the statistical significance of the ordination.

A 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) α-amylase (v) α-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

As a lot of variables have been considered, the first approach to organize data was to carry out a CA, a type of multivariate analysis that considers all data simultaneously, organizing samples according to the degree of similarity (Fig. 1). The 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, which remain

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 the LSD Fisher test ($P < 0.05$)

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	$0.20 \pm 0.001(a)$	$2.57 \pm 0.039(a)$	$1.96 \pm 0.009(a)$
RWB	$0.22 \pm 0.002(b)$	$2.65 \pm 0.087(a)$	$2.27 \pm 0.005(a)$
RWP	$0.23 \pm 0.0004(c)$	$2.69 \pm 0.024(a)$	$1.94 \pm 0.003(a)$
RSC	$0.24 \pm 0.002(d)$	$6.35 \pm 0.028(c)$	$4.93 \pm 0.18(c)$
RSB	$0.22 \pm 0.001(b)$	$6.18 \pm 0.089(c)$	$4.91 \pm 0.017(c)$
RSP	$0.20 \pm 0.003(a)$	$5.41 \pm 0.088(b)$	$4.27 \pm 0.185(b)$
SWC	$0.19 \pm 0.004(wx)$	$10.01 \pm 0.002(y)$	$5.13 \pm 0.322(x)$
SWB	$0.18 \pm 0.006(w)$	$9.28 \pm 0.044(wx)$	$5.87 \pm 0.160(y)$
SWP	$0.20 \pm 0.004(x)$	$9.10 \pm 0.009(w)$	$4.15 \pm 0.091(w)$
SSC	$0.22 \pm 0.003(y)$	$11.03 \pm 0.020(z)$	$5.74 \pm 0.125(y)$
SSB	$0.23 \pm 0.002(y)$	$10.89 \pm 0.206(z)$	$6.58 \pm 0.115(z)$
SSP	$0.23 \pm 0.002(y)$	$9.56 \pm 0.060(z)$	$5.31 \pm 0.115(xy)$

in the positive values of the same. Within raspberry samples, controls separate from inoculated samples towards the negative values of axis II. In strawberries, 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 content and total anthocyanin content of raspberries and strawberries (Table 1) grown in winter and spring, inoculated with the *Bacillus* and the *Pseudomonas* strains, 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, which is consistent with the reported increase in antioxidant compounds along the plant cycle.[33] In raspberries, both strains increased the antioxidant capacity in winter samples and decreased it in spring; *P. fluorescens* showed a more intense effect than *B. amyloliquefaciens,* which maintained the same values in both moments. In strawberry, *B. amyloliquefaciens* had a negative effect on antioxidant capacity in winter whereas *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).

The total phenolic content of raspberries 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 content was more than twice this and anthocyanins accounted for most of total phenolics in non-inoculated controls. Despite the fact that extracts from inoculated plants showed no significant changes in 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 phenolic and anthocyanin concentration, in parallel to a decrease in antioxidant capacity.

Strawberry extracts (Table 1) were richer than raspberries in 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 phenolic content, while anthocyanins significantly increased in *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, α-glucosidase and α-amylase

Non-inoculated winter raspberry extracts (Fig. 2(a)) were significantly more effective in inhibiting α-glucosidase than spring extracts; except for *P. fluorescens* winter extracts, bacterial treatments significantly improved extracts' inhibition capacity. Raspberry winter samples inoculated with *B. amyloliquefaciens* (Fig. 2(a)) were the most effective, associated with increased antioxidant capacity due to compounds other than total phenolics or anthocyanins because 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.

With regard to strawberry (Fig. 2(b)), spring extracts were more effective inhibiting α-glucosidase. *Pseudomonas fluorescens* did not alter effects on this enzyme, either in winter or spring samples. Interestingly, and although only strawberry winter samples inoculated with *B. amyloliquefaciens* modified the effects on α-glucosidase, improving in winter and worsening in spring, *B. amyloliquefaciens* extracts obtained reproducible results both in winter and spring. Nevertheless, strawberry extracts were

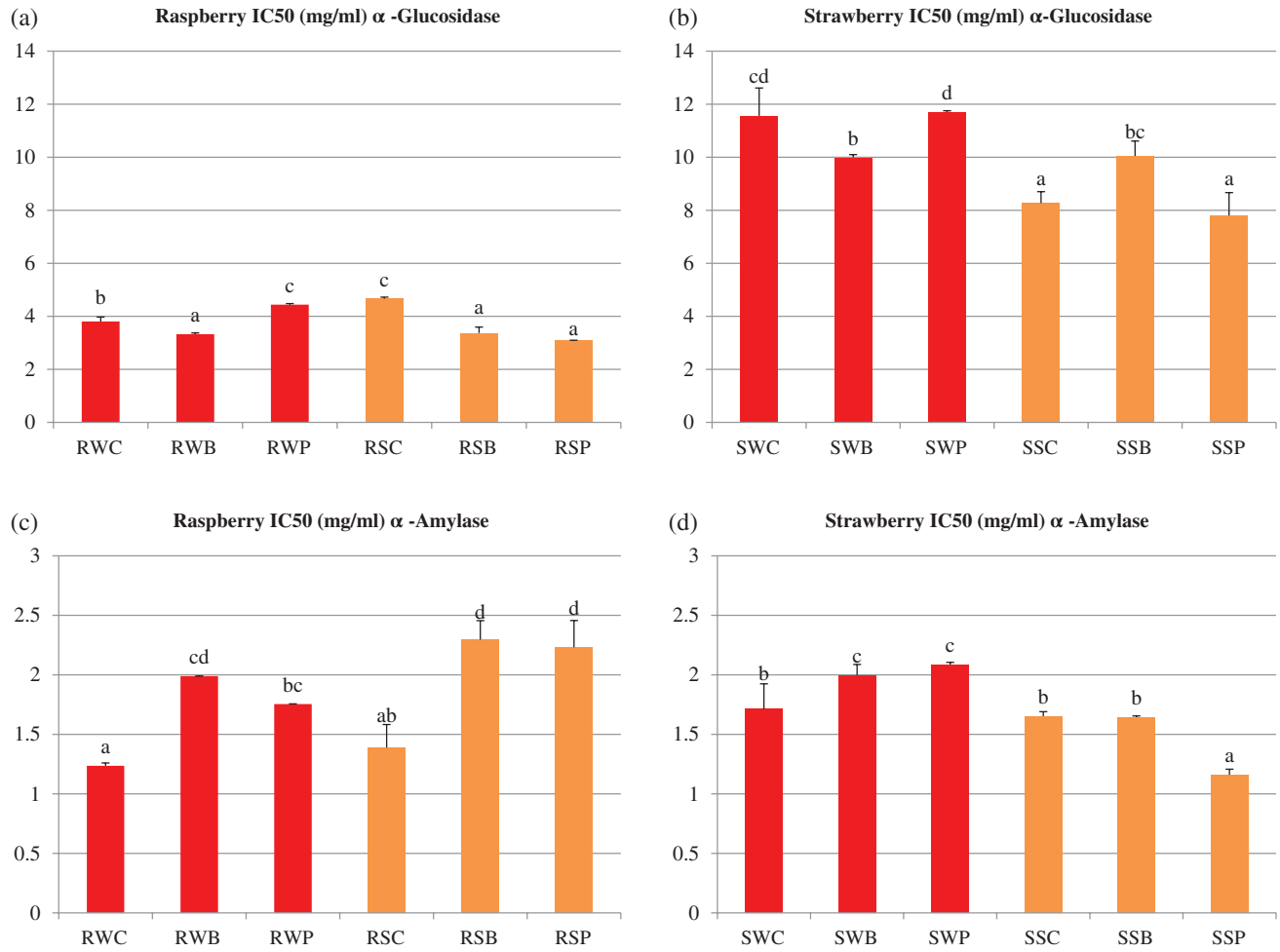

Figure 2. IC50 α-glucosidase (a and b), and α-amylase (c and d) obtained with raspberry and strawberry extracts. RWC, raspberry winter control; RWB, raspberry winter *B. amyloliquefaciens*; RWP, raspberry winter *P. fluorescens*; RSC, raspberry spring control; RSB, raspberry spring *B. amyloliquefaciens*; 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 the LSD Fisher test ($P < 0.05$).

less effective than raspberry, averaging IC50 values of 10 and 4 mg mL^{-1}, respectively.

The most effective extracts to inhibit α-amylase activity were raspberries with lower IC50 values, although average values in strawberries (1.6 mg mL^{-1}) were similar to raspberries (1.3 mg mL^{-1}). Bacterial treatments significantly increased IC50 values in raspberry and strawberry winter samples, thereby decreasing inhibiting activity (Fig. 2(c), (d)); however, the only significant improvement was strawberry spring samples inoculated with *P. fluorescens* extracts, associated with lower phenolics and anthocyanins (Table 1). Hence, a deeper metabolic characterization will be carried out on this extract to find specific inhibitors of α-amylase.

Effects on antihypertensive enzyme, ACE

With regard to the ACE inhibition capacity, strawberry extracts were more effective than raspberries (Fig. 3). In strawberry (Fig. 3(b)), spring extracts performed better than winter extracts in 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 at both sampling times. In raspberries (Fig. 3(a)), winter extracts were more effective and bacterial treatments lowered this potential; a different effect was found in spring extracts, which were enhanced significantly 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 time; bacterial treatments improved this activity similarly at both times, and *P. fluorescens* always achieved best results (Fig. 4(a)). Strawberry extracts were more effective than raspberries in inhibiting this enzyme (average 47%), irrespective of the sampling time; in winter, bacterial treatments decreased the capacity to inhibit COX-1, although it was increased in spring samples, when *P. fluorescens* strawberry extracts significantly increased inhibition capacity to 50% (Fig. 4(b)).

With regard to COX-2 activity, raspberry extracts were able to inhibit COX-2 by 30% in winter and spring, and bacterial treatments significantly improved inhibition capacity at both sampling times, irrespective of the bacterial strain (Fig. 4(c)). Strawberry extracts were more effective in inhibiting COX-2 (Fig. 4(d)) by 35% although non-significant differences were found among the treatments, as anticipated by the bidirectional ANOVA (supporting information Table S2).

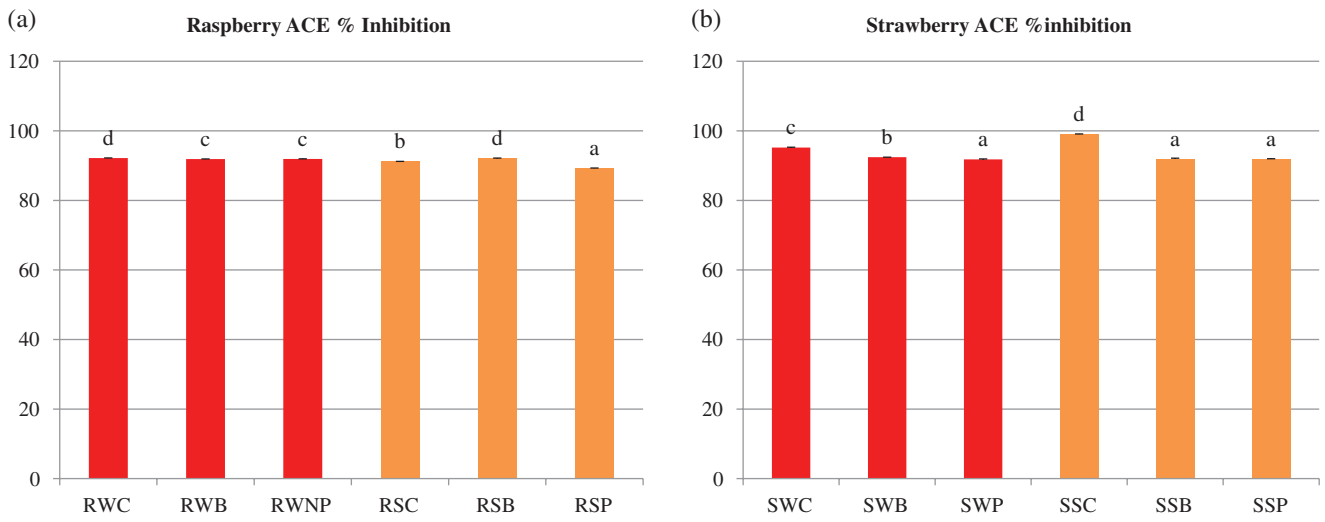

Figure 3. ACE %inhibition. RWC, RWC, raspberry winter control; RWB, raspberry winter *B. amyloliquefaciens*; RWP, raspberry winter *P. fluorescens*; RSC, raspberry spring control; RSB, raspberry spring *B. amyloliquefaciens*; RSP, raspberry spring *P. fluorescens*; SWC, strawberry winter control; SWB, 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 the LSD Fisher test ($P < 0.05$).

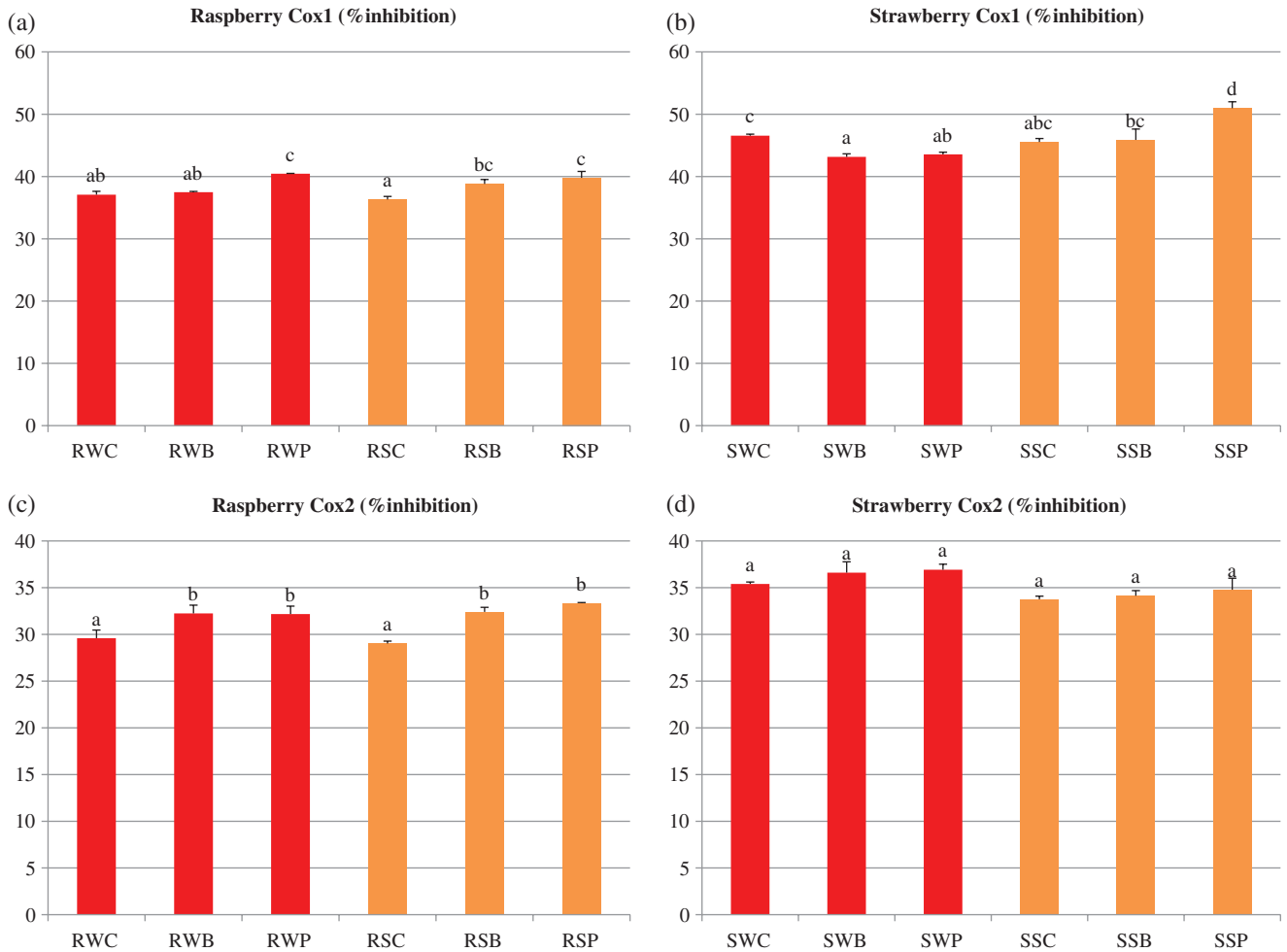

Figure 4. COX-1 & 2% inhibition. RWC, raspberry winter control; RWB, raspberry winter *B. amyloliquefaciens*; RWP, raspberry winter *P. fluorescens*; RSC, raspberry spring control; RSB, raspberry spring *B. amyloliquefaciens*; RSP, raspberry spring *P. fluorescens*; SWC, strawberry winter control; SWB, 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 ($P < 0.05$).

DISCUSSION AND CONCLUSION

Despite the fact that extracts from inoculated plants showed no significant changes in 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 that are different from phenols and anthocyanins, and therefore, effects on enzyme activities are expected to be different from controls. Only *P. fluorescens* spring samples caused a significant decrease in total phenolics and anthocyanin concentrations, in parallel with 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 because 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, which is consistent with the loss of antioxidant capacity that was 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 reactive oxigen species (ROS) removal from the system.[37]

Bacillus amyloliquefaciens increases the anthocyanin content in strawberry, contributing to the good quality of the fruit, which will show a better color due to anthocyanins. This increases market quality, with the bioactive content increasing during the production period.[20]

Glucose regulation by α-glucosidase and α-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 0.5811 mg mL^{-1} for α-glucosidase, and 0.11 µg mL^{-1} α-amylase,[30] our results indicate the potential of these extracts to manage hyperglycemia, especially when delivered through the diet, raspberries being more effective. Among bacterial treatments, *B. amyloliquefaciens* improved the inhibition potential for α-glucosidase in raspberry and strawberry winter extracts, while no improvement was shown for α-amylase, except for *P. fluorescens* in strawberry spring extracts. Total anthocyanins and total phenolics do not 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, because lower bioactives in raspberry spring samples inoculated with *P. fluorescens* resulted in 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 raspberries and strawberries; hence a diet rich in berries could prevent inflammation. These results do not seem to be related to the total phenolic or anthocyanin content, reinforcing the hypothesis that results are due to a modification of other metabolic pathways.

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

In summary, the total phenolic and anthocyanin content was higher in strawberries than raspberries, despite similar antioxidant capacity. Strawberry extracts performed better on the tested enzymes, except for α-glucosidase inhibition capacity. Bacterial inoculations during plant growth modified strawberry and raspberry metabolism, modifying extract composition, which resulted in modification of marker enzyme activity. Total phenolic and anthocyanin content was not fully responsible for the improvement. *Bacillus amyloliquefaciens* induced changes in strawberry and raspberry metabolism, which improved the inhibition potential of α-glucosidase and COX2. It stabilized the effects of extracts at different time points. *Pseudomonas fluorescens* modified plant metabolism after more inoculations (spring) in both plants, improving the effects of raspberry extracts on α-glucosidase, COX1, and COX2, and of strawberry on α-amylase and COX1.

Based on their 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 is used during production. Nevertheless, much remains to be understood about the effects of particular compounds and whether the elicitation process improves the potential of extracts to modify metabolic syndrome-related enzymes.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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