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GROWTH MODULATING PROPERTIES OF POLYPHENOLIC APPLE POMACE EXTRACT ON FOOD ASSOCIATED MICROORGANISMS

Christopher Beermann, Nadine Gruschwitz, Katharina Walkowski, Annekathrin Göpel*

Address(es): Prof. Dr. sc. nat. Christopher Beermann
[1]University of Applied Science Fulda, Faculty of Food Technology, Marquardstrasse 35, D-36039 Fulda, Germany, phone number:+49 661 96 40 506.

*Corresponding author: barryomogbai@yahoo.com

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ABSTRACT

Bacteriostatic effects of plant derived polyphenols are generally proposed for food protection against microbial spoiling. This study aimed at characterizing distinct growth modification and cell-lytic properties of an apple pomace extract (APE) containing short-chain and long-chain PP on food spoiling and fermenting starter bacteria.
APE contained 6.76 wt % PP, 0.46 wt % glucose, 1.69 wt % fructose, 1.26 wt % starch, 3.8 wt % sorbitol, and 0.64 wt % nitrogen with a pH-value of 4.1. APE caused growth modification of prominent bacterial food spoilers, yeasts, moulds and food fermenting starter bacteria was analyzed turbidometry (180° light absorption measurement at 600 nm wavelength). Cell-lytic activity of APE was measured by a SYTOX® Green fluorescence cell viability assay.
APE 1.5 w/w % reduced the growth of gram-positive and gram-negative food spoiling bacteria in dose-dependent manner up to 35.00%. *Bacillus subtilis* growth was reduced up to 10.53% comparable to 1.01 µg/ mL ampicillin or 0.144 mg/ mL sulfamethoxazol. In contrast, the growth of several fermenting starter bacteria increased at 1.5 w/w % APE up to 167.65% whereas expansion of yeasts and moulds were unaffected.
Neither specific cell-lytic activities of APE could be examined on gram-positive and gram-negative food spoiler nor food fermenting starter bacteria.
This study indicates that APE is a bacteriostatic but not a cell-lytic agent against food spoiling bacteria. Instead, the growth of specific lactic acid bacteria was supported by APE. Therefore, APE might stabilize explicit food fermentation processes.

Keywords: Anti-microbial; bacterial growth kinetic; bacteriostatic; cell membrane permeabilization; polyphenol; turbidometry

INTRODUCTION

Polyphenols (PP) are a heterogeneous group of versatile compounds, which occur in most plants for pigmentation as well as growth and reproduction regulation along with plants protecting properties from pathogens and predators. PP are important secondary metabolites often utilized in food application as antioxidants (Martin *et al.*, 2010). To date, bacteriostatic plant-derived PP are proposed for food protection from microbial spoiling. This also includes apple PP. This study aimed at characterizing specific growth inhibiting and cell-lytic properties of an apple pomace extract (APE) containing short-chain and long-chain PP on food spoiling and fermenting starter bacteria with focus on the cell-lytic activity, which might lead to new application opportunities in food. Additionally, anti-fungal properties against food associated moulds and yeasts were found.
PP are commonly found in fruits, vegetables, and plant-derived beverages such as fruit juices, coffee, tea, red wine, but also cereals and chocolate (Cushnie *et al.*, 2005; Scalbert *et al.*, 2005). As apples are available in large quantities all over the world with a high consumer acceptance apple extracts are an interesting source of PP. On average, one apple contains 0.01 – 1% phenolic compounds, based on fresh weight. The polyphenol content and the polyphenol composition vary depending on the cultivar and stage of maturation (Vieira *et al.*, 2009).
In general, PP are aromatic compounds substituted with several hydroxyl groups. PP can be classified according to their number of phenolic rings and the structural elements connecting these ring structure. Predominant classes in plants are phenolic acids, flavonoids, stilbenes and lignans. Apples contain several polyphenolic derivates and flavonoids (Bengoechea *et al.*, 1997). Flavonoids consist of two aromatic rings which are connected by three carbon atoms that form an oxygenated heterocycle and are divided into the following subgroups: flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (catechins and proanthocyanidins) (Manach *et al.*, 2004). The main phenolic compounds of apples are esters of caffeic and *p*-coumaric acids with quinic acid, flavanol monomers, di- and oligomers, quercetin glycosides and dihydrochalcones (Schieber *et al.*, 2001). The tested APE is dark brown and sweet flavored viscous syrup which is used in various dietary applications. It is prepared from freshly harvested, juiced and gently dried apple components. In addition to various fruit sugars such as fructose, glucose, sorbitol, fruit acids, and minerals, APE contains a high percentage of coloring secondary plant substances. Over the last ten years, several studies have investigated health beneficial effects of foods and herbs containing PP, like free radical scavenging abilities and the capacity to mitigate oxidative stress-induced tissue damage associated with chronic diseases (Martin *et al.*, 2010; Scalbert *et al.*, 2005). Besides this, PP seem to possess also anti-bacterial, anti-fungal and anti-viral effects (Cowan, 1999; Moreno *et al.*, 2006). Recent studies examined the anti-microbial effects of PP containing plant extracts against gram-positive and gram-negative bacteria. Cowan documented that the distinct PP structural groups specifically inhibit bacterial growth (Cowan, 1999). Due to the protective outer membrane gram-negative bacteria are usually more sensitive to PP compared to gram-negative ones (Rodriguez-Carpena *et al.*, 2011; Shan *et al.*, 2007). Aside from cell wall and cell membrane destabilizing aspects, different studies suggested that the polyphenolic effect related to microorganisms can be associated with the inactivation of microbial adhesins, enzymes or cell envelope transport proteins (Cowan, 1999; Scalbert, 1991). Therefore, polyphenolic bacteriostatic effects do not necessarily include cell damage but disturbing aspects within the metabolic activity. This approach aimed at characterizing the potential bacteriostatic and bacteriolytic properties of the APE regarding food associated bacteria, moulds, and yeasts. Further, the influence of APE on the growth kinetics of food fermented starter bacteria was in focus. For this, turbidometric measurements for growth-inhibitory properties and for cell-lysis a specific fluorescence assay with the SYTOX® Green dye were performed.
Anti-microbial effects of PP offer new applications in food manufacturing, medicine, and pharmacy (Konaté *et al.*, 2012; Taguri *et al.*, 2004). To avoid synthetic food conserving agents, distinct anti-microbial effects of polyphenolic APE on food associated microorganisms might offer new application opportunities on food protection and processing.

MATERIAL AND METHODS

Polyphenolic apple pomace extract

The polyphenolic APE contained 6.76 wt % short- and long-chain PP, 0.46 wt % glucose, 1.69 wt % fructose, 1.26 wt % starch, 3.8 wt % sorbitol, and 0.64 wt % nitrogen with a pH value of 4.1 (Herbstreith & Fox, Neuenbürg, Germany).
The PP were quantified using the Folin-Ciocalteu method (Folin and Ciocalteu, 1927). For all functional measurements APE was diluted to 0.5, 1.0, 1.5, 3.0 wt % in sterile ringer solution (Merck, Darmstadt, Germany).

Microorganisms and culture media

Escherichia coli K12 was used as a representative of gram-negative, *Bacillus subtilis* and *Streptocaoccus uberis* represent gram-positive food spoiling bacteria, while *Lactobacillus acidophilus*, *Lb. brevis, Lb. delbrueckii* subsp. *bulgaricus*, *Lb. reuteri, Lb. sanfranciscensis,* and *Lactococcus lactis* were used as representatives for food fermenting starter bacteria. *Aspergillus flavus*, *A. niger*, *Mucor circinelloides*, *Penicillium expansum*, *P. italicum,* and *Rhizopus oligosporus* were utilized as representatives of moulds and *Pichia anomala* and *Saccharomyces cerevisiae* represents the group of yeasts. All organisms were obtained from the German Collection of Microorganisms and Cell Cultures (Leibnitz-Institute DSMZ).
All microbial inoculations with the food spoiling bacteria were prepared from overnight stock cultures in standard I broth (Carl Roth, Karlsruhe, Germany) at 30 °C. Lactic acid bacteria (LAB) were anaerobically cultivated in MRS broth (Carl Roth) and incubated at 30 °C for 48 h. The microbial inoculations for the bacteriostatic tests were adjusted photometrically with an absorbance between 0.15 and 0.3. The microbial inoculations for the bacteriolytic tests were adjusted at approximately 10^7 bacteria/mL validated by hemocytometric cell counting. Yeasts were incubated in malt extract broth (Merck) at 25 °C for 48 h. Moulds cultivated in malt extract broth were spread on malt extract agar (Merck) and incubated at 25 °C for 72 h.

Analysis of the bacterial growth modulating effect of APE

Agar diffusion test: Anti-microbial properties of APE were performed by agar diffusion tests. Casein soy pepton agar (Carl Roth) was used for standard culture plates, while LAB were cultured on MRS agar plates (Merck). After spreading 0.1 mL of a 24 h old bacteria suspension, a sterile filter plate containing APE was set onto the plate centre and cultivated at 30 °C for 72 h. The size of the bacteria free area around the filter plate is correlated to the anti-microbial effectiveness of the tested agent.

Turbidometric measurement of growth

The growth of bacteria was analyzed turbidometrically (180° white light absorption measurement) in transparent 100 well microtiter plates with flat bottom using the BioScreen C (Oy Growth Curves AB Ltd., Helsinki, Finland). The period of measurement was 20 h, measured in intervals of 15 min. The process temperature was 30 °C (wherein the applicable temperature range is generally at 15 - 42 °C). The wells were filled with 300 µL sample solution. The tests were performed at a wavelength of 600 nm. Before each measuring, the plate was automatically shaken for 3 s. The increase of absorbance intensity along time represents the growth of the tested organism. The growth rate is defined as the gradient of growth within the exponential phase of bacterial growth kinetic. Due to the high color intensity of APE, only solutions with a concentration up to 1.5% were measureable. Due to its lower optical density Reinforced Clostridial Medium (pH 6) (Carl Roth) broth instead of MRS broth was utilized for LAB. Samples containing only the test organism in broth represent maximal growth as positive control. Pure APE in culture medium represents the base line growth as negative control. Tests with the antibiotic ampicillin (Geno Technology, MO, USA) and the biostatic sulfamethoxazol (Sigma-Aldrich, Steinheim, Germany) were used as reference for the growth inhibiting effectiveness of APE. For these tests the growth kinetics of *Lb. brevis*, *B. subtilis*, *E. coli,* and *Sc. uberis* were measured. The turbidometric measurements were validated by microbiological agar diffusion tests.

Measurement of bacteriolytic effects of APE

Cell-lytic activity was observed by a SYTOX® Green fluorescence cell viability assay. SYTOX® Green (Molecular Probes, Carlsbad, CA, USA) is an unsymmetrical cyanine dye that intercalates with the DNA helix from eukaryotic and prokaryotic cells. Due to its hydrophilic character the dye is completely excluded from living cells (Roth *et al.*, 1997).
The detection of cell death was performed with the microplate reader Genios (Tecan, Crailsheim, Germany). Before each measurement, optimal gain value was automatically calculated by the instrument. The integration time was 20 µs, with 0 µs lag time and 4 flashes per measurement. The total measurement time was 24 h, being measured at intervals of 1 h. The samples were measured at room temperature (25 ± 5 °C). The plate was shaken circular for 3 s, and measured instantly.
The prepared bacterial suspensions of *E. coli*, *B. subtilis*, *Sc. uberis,* and *Lb. brevis* were centrifuged for 5 min at 3000 rpm and then washed twice in 0.85% NaCl solution to remove the nutrient medium. The resulting cell pellet was re-suspended in sterile ringer solution. The measurements were performed in black 96 well plates with flat bottom. The wells were filled with 240 µL sample solution and 10 µl of a 1:200 diluted SYTOX® Green solution (final concentration 1.0 µM). DNA-intercalated dye emitted light at 485 ± 20 nm wavelength (Roth *et al.*, 1997). The fluorescence of the samples was measured through a 535 ± 25 nm band pass filter. Cells treated with ethanol (70%), heat or nisin (Sigma-Aldrich) were used as control agents for maximal cell membrane dissolution (negative control).

Analysis of the antifungal effect of APE

The growth inhibition of yeasts was analyzed turbidometrically with the microplate reader Genios. The test was carried out at 25 °C for 40 h.
The growth inhibition of moulds was determined by a growth test on malt extract agar plates and on bread. Defined agar blocks (Ø 7 mm) with moulds were centrally placed on plates and bread slices containing 0, 4, or 8 wt % APE, respectively. The slices were incubated at 25°C and predefined humidity for 72 h.

Statistic analysis

Data were evaluated by GraphPad 5 Prism software. P-values were determined by one sided Wilcoxon-Mann-Whitney test. P-values ≤ 0.05 were defined as significant.

RESULTS

Bacterial growth modulating effect of APE

The purpose of this study was to examine the effect of a polyphenolic extract of freshly harvested, juiced, and gently dried apples on the growth of 9 different gram-positive and gram-negative bacteria food spoiling and food fermenting starter bacteria as well as food associated moulds and yeasts. The growth modulating effects of APE to bacteria were monitored by turbidometry within a concentration range of 0.5 up to 1.5% APE or 0.03 up to 0.10% phenol content, respectively. In order to quantitatively confirm the APE related bacterial growth inhibition, the effect was correlated to the standard anti-microbial components ampicillin and sulfamethoxazol (Tab 1).

Table 1 Bacteriostatic effect of APE on food associated bacteria correlated to reference substances

Bacterium	APE/ PP[1] [w/w %]	Growth rate[2]	Alteration [%]	Ampicillin [µg/ mL]	Sulfamethoxazol [mg/ mL]
B. subtilis	0.0/ 0.00	1.59 +/0.25		0.0	0.0
	0.5/ 0.03	1.50 +/0.23	-6.15*	0.96	0.137
	1.0/ 0.07	1.46 +/0.21	-8.47*	0.99	0.140
	1.5/ 0.10	1.43+/0.19	-10.53*	1.01	0.144
E. coli	0.0/ 0.00	3.98 +/0.25			0.0
	0.5/ 0.03	3.79 +/0.25	-4.83*		0.283
	1.0/ 0.07	3.742 +/0.16	-5.94*		0.287
	1.5/ 0.10	3.365 +/0.12	-15.51*		0.319
Sc. uberis	0.0/ 0.00	4.71 +/0.30			0.0
	0.5/ 0.03	3.94 +/0.23	-16.51*		0.244
	1.0/ 0.07	3.75 +/0.09	-20.41*		0.256
	1.5/ 0.10	3.05 +/0.14	-35.28*		0.315

Legend: [1]APE – apple pomace extract; PP – polyphenol; [2] gradient of growth within the exponential phase of bacterial growth kinetic; *difference between test and control: $p \leq 0.05$

The applied amount of these active compounds is inversely proportional to the bacterial growth within the exponential phase. It could be observed that APE significantly suppressed bacterial growth within the exponential phase of all tested food spoiling bacteria in concentration-dependent manner. The growth reduction rates were up to -35.00% compared to control which correlates to the effectiveness of 0.315 mg/mL sulfamethoxazol. The growth suppression of *B. subtilis* at 1.5% APE corresponded to the effectiveness of 1.01 µg/mL ampicillin and 0.144 mg/mL sulfamethoxazol. The inhibitory effect of 1.5% APE on the growth of *E. coli* and *Sc. uberis* was similar to the effect of approximately 0.3 mg/mL sulfamethoxazol on these strains.

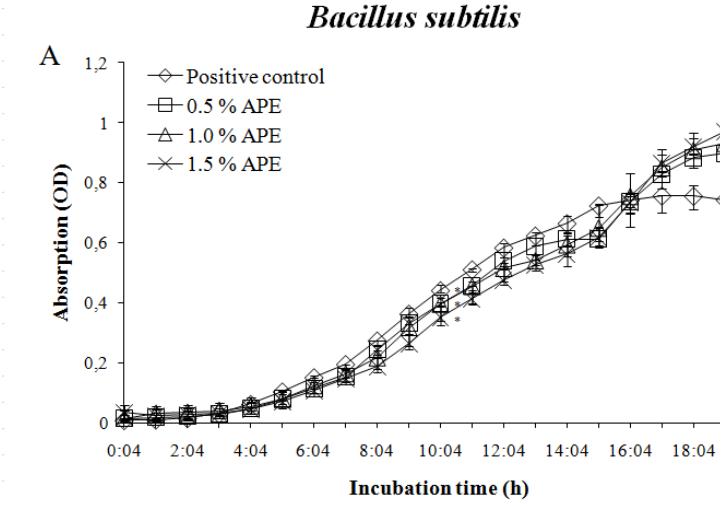
Interestingly, the influence of APE on the growth of *B. subtilis* is different (Fig 1A). Hereby, APE revealed the lowest effect of all tests. Besides this initial growth inhibiting effect, APE seems to induce a diauxic-like growth kinetic. Also, *B. subtilis* was less sensitive to sulfamethoxazol. Therefore, ampicillin was additionally measured as anti-microbial standard agent. The maximal growth reduction of -10.53 by APE correlates to 1.01 µg/ mL ampicillin. While *E. coli* (Fig 1B) was slightly more sensitive to the APE treatment with a growth inhibition of -15.51%, APE strongly inhibited the growth of *Sc. uberis* (Fig 1C). In contrast, the growth of several fermenting starter LAB was enhanced by APE in concentration-dependent manner (Tab 2).

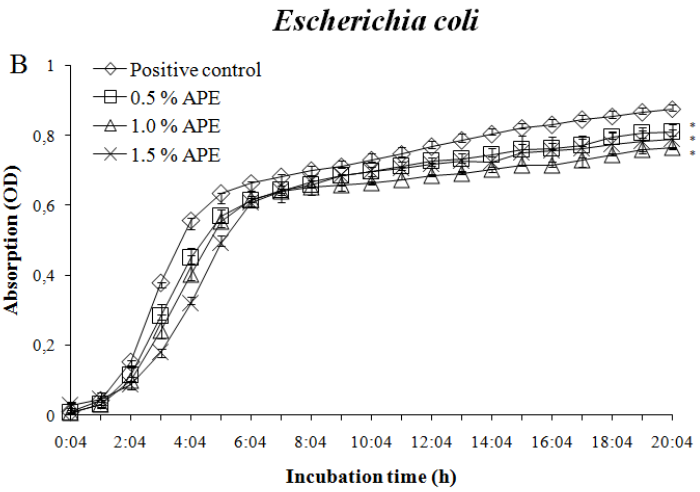
Table 2 Bacteriostatic effect of APE on lactic acid bacteria

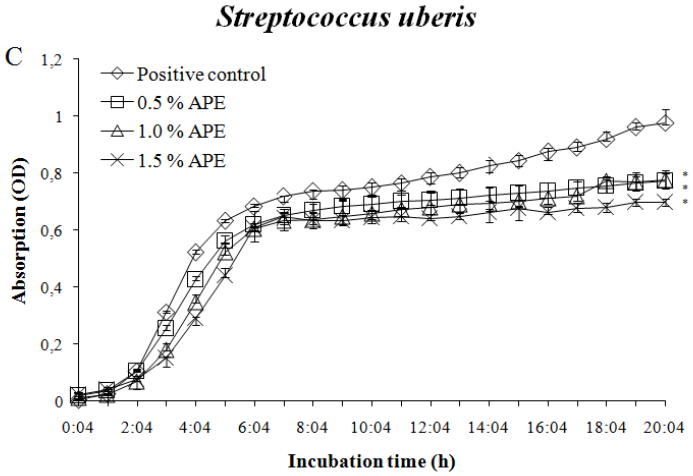
Bacteria	APE/ PP[1] [w/w %]	Growth rate[2]	Growth alteration [%]
Lb. brevis	0.0/ 0.00	0.60 +/0.08	
	0.5/ 0.03	1.07 +/0.17	+78.06*
	1.0/ 0.07	1.39 +/0.16	+132.25*
	1.5/ 0.10	1.66 +/0.12	+177.18*
Lb. reuteri	0.0/ 0.00	1.25 +/0.28	
	0.5/ 0.03	1.27 +/0.31	+1.25*
	1.0/ 0.07	1.41 +/0.27	+12.71*
	1.5/ 0.10	1.59 +/0.20	+26.73*
Lb. sanfranciscensis	0.0/ 0.00	0.29 +/0.26	
	0.5/ 0.03	0.65 +/0.22	+126.37*
	1.0/ 0.07	0.74 +/0.22	+160.48*
	1.5/ 0.10	0.77 +/0.23	+167.65*
Lb. delbrueckii subsp. *bulgaricus*	0.0/ 0.00	0.75 +/0.39	
	0.5/ 0.03	0.70 +/0.27	-7.28*
	1.0/ 0.07	0.60 +/0.29	-21.04*
	1.5/ 0.10	0.55 +/0.30	-27.61*
Lc. lactis	0.0/ 0.00	2.47 +/0.07	
	0.5/ 0.03	1.82 +/0.11	-26.25*
	1.0/ 0.07	1.26 +/0.13	-48.97*
	1.5/ 0.10	0.95 +/0.18	-61.70*

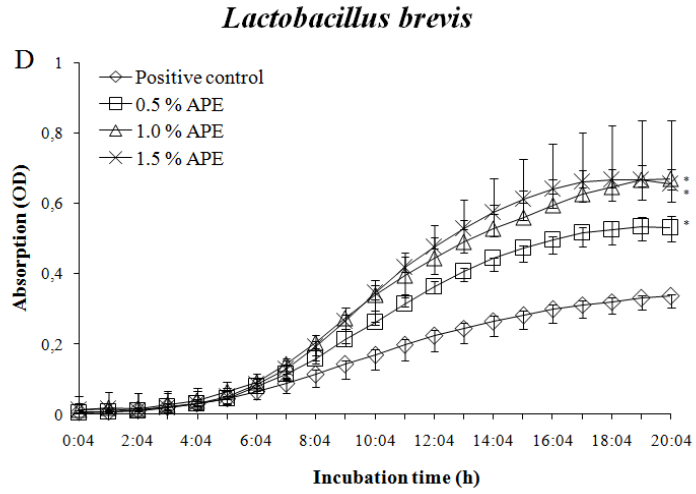
Legend: [1]APE – apple pomace extract; PP – polyphenol; [2] gradient of growth within the exponential phase of bacterial growth kinetic; *difference between test and control: $p \leq 0.05$

Especially, the growth of *Lb. brevis* (Fig 1D) significantly increased 177.18% from 0.32 (pure medium) to 0.71 (1.5% APE). Also, the growth of *Lb. reuteri* and *Lb. sanfranciscensis* was supported by APE. In contrast, *Lb. delbrueckii* subsp. *bulgaricus* showed a concentration-dependent growth inhibition. *Sc. uberis* and *Lc. lactis* were almost twice as strongly inhibited as *L. delbrueckii* subsp. *bulgaricus* (Fig 1E).









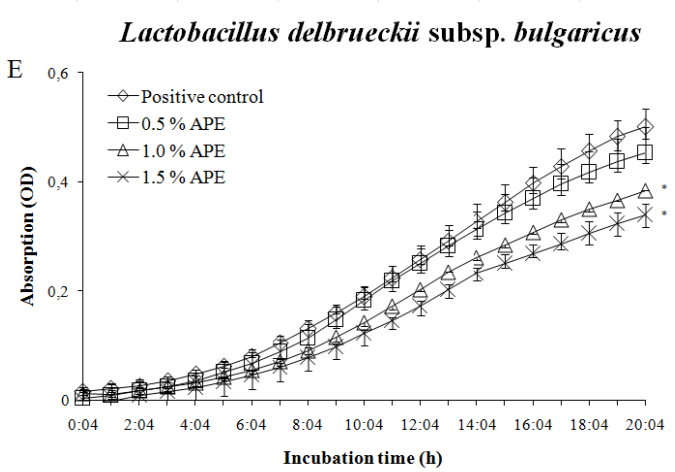
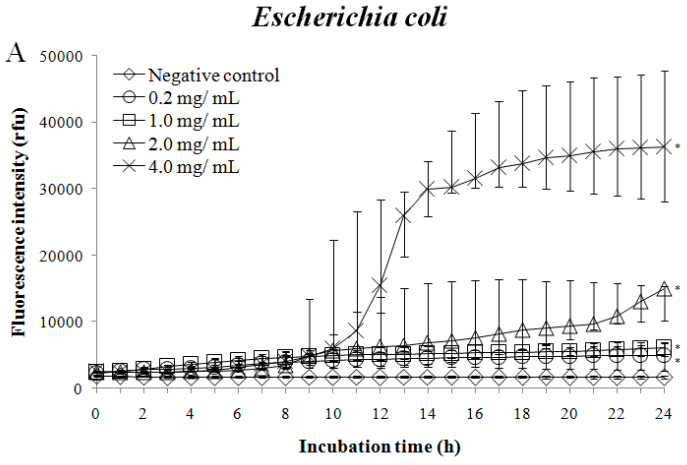


Figure 1 Growth modulation induced by APE solutions in *B. subtilis* (A), *E. coli* (B), *Sc. uberis* (C) in standard-I-broth and *Lb. brevis* (D), *Lb. delbrueckii* subsp. *bulgaricus* (E) in Reinforced Clostridial Medium measured turbidometrically. Samples containing only the test organism in broth represent maximal growth as positive control. Values are medians with range of quintuplicate measurements of three independent representative experiments (*difference between test and control: $p \leq 0.05$)

Bacteriolytic effect of APE

As bacteriostatic activity does not necessarily include the dissolution of the cellular membrane the cell-lytic activity of APE was measured by a SYTOX® Green fluorescence cell viability assay. Figure 2 illustrates the median of all independent three measurements. Possible cell membrane permeabilization was assessed after a 24 h treatment of *E. coli*, *Sc. uberis,* and *Lb. brevis* with 0.5 to 3.0% APE with 0.03 and to 0.20% phenol content respectively. The fluorescence emission of APE-treated cells was related to ethanol- or heat-treated cells as positive control. The basic fluorescence intensity varies depending on the microorganism strains measured, whereas living cells had a slightly higher fluorescence signal, like *E. coli*, 1845.3 rfu, *Sc. uberis*, 2019.7 rfu and *Lb. brevis*, 2147.2 rfu compared to 1563.4 rfu of dye in ringer solution only.
APE related dissolution of cell membranes was correlated to the effectiveness of nisin, a cell membrane active commonly food conserving agent, within a concentration range of 0.2 mg/mL up to 4.0 mg/mL. Figure 2 illustrates the kinetics of bacteriolysis over time. Nisin binds to the lipid A portion of the bacterial outer surface which leads to a destruction of the cell membrane semi-permeability (Papadopoulou *et al.*, 2005). The nisin related cell-lysis of *E. coli* (Fig 2A) and *Sc. uberis* (Fig 2B) increased in dose-dependent manner. Maxima were reached in *E. coli* with 37386.3 rfu and in *Sc. uberis* with 30015.3 rfu.



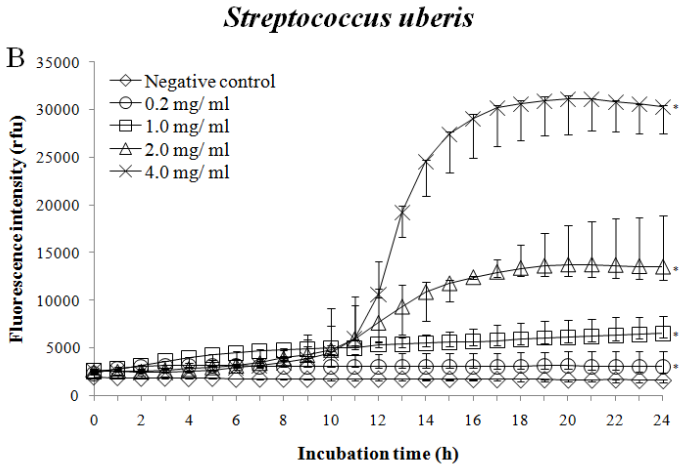


Figure 2 Membrane permeabilization induced by nisin in E. coli (A) and Sc. uberis (B) in ringer solution measured by SYTOX® Green fluorescence assay. Values are medians with range of quadruplicate measurements of three independent representative experiments. Samples containing only the test organism in broth represent minimal cell membrane dissolution as negative control (*difference between test and control: $p \leq 0.05$).

No cell membrane disturbing activities of any concentration of APE could be found by all food spoiling and food fermenting starter bacteria tested. No significant difference in fluorescence emission was observed even in comparison to the negative control (data not shown).

Anti-fungal effect of APE

The growth of all moulds and yeasts tested was never influenced by APE neither on plates nor on bread (data not shown).

DISCUSSION

Anti-microbial effects of plant extracts, like rosemary (Moreno *et al.*, 2006), avocado (Rodríguez-Carpena *et al.*, 2011) and brown algae (Nagayama *et al.*, 2002) are widly discussed in several studies. These extracts contain bioactive substances, like terpenoids, lectins, polypeptides, alkaloids, phenolics and PP which protect the plant against microorganisms, insects and herbivores (Cowan, 1999). In many studies especially PP were examined for bacteriostatic (Moreno *et al.*, 2006; Taguri *et al.*, 2004) or bacteriolytic (Konaté *et al.*, 2012; Nagayama *et al.*, 2002) effects against microorganisms. Several studies demonstrated that the anti-microbial effect of PP is concentration dependent. Polyphenole-rich methanol extract was more effective against yeast, gram-positive and gram-negative bacteria than the watery rosemary extract with less PP (Moreno *et al.*, 2006). Also, Papadopoulou *et al.* (2005) examined that the anti-microbial effect is related to the total phenolic content of red and white wine phenolic extracts. In accordance to this, the bacteriostatic effect of APE was directly dependent on the PP concentration. This approach investigated growth modulating and cell lytic activities of APE with 6.76% short- and long-chain PP on selected food spoilers, food fermenting starter bacteria, moulds and yeasts.

Bacterial growth modulating effect of APE

The turbidometric data confirm that APE distinctively influenced the growth of food associated bacteria. The growth of food spoilers was inhibited in dose-dependent manner, wherein all gram-positive bacteria except *B. subtilis* were more inhibited than gram-negative *E. coli*. According to various studies, gram-positive bacteria are more sensitive to polyphenolic extracts than gram-negative ones (Ikigai *et al.*, 1993; Papadopoulou 2005; Rodríguez-Carpena *et al.*, 2011; Taguri *et al.*, 2004). In an investigation of 46 dietary spices and medicinal herb extracts, gram-positive bacteria are more sensitive concerning PP (Shan *et al.*, 2007). This difference might be due to the explicit cell wall structures of gram-positive and gram-negative bacteria. In gram-positive bacteria PP have to diffuse through the thick peptidoglycane cell wall to reach the proteoplast. Although gram-negative bacteria possess few murein layers, the cell wall sacculus is embedded in a periplasmatic gel where several enzymes are associated (Konaté *et al.*, 2012; Shan *et al.*, 2007). A protective lipopolysaccharide shield wrapped the outer bacterial surface which might act as an effective barrier for hydrophobic anti-microbials, such as PP. Additionally, enzymes of the periplasmatic gel might hydrolyze PP coming from outside (Konaté *et al.*, 2012; Shan *et al.*, 2007).
Interestingly, the growth kinetic of *B. subtilis* markedly differs from the other kinetics measured. Within the first 15 h incubation time, the effectiveness of 1.5% APE against *B. subtilis* corresponded to 1.01 µg/mL ampicillin and 0.144 mg/mL sulfamethoxazol. Afterwards, *B. subtilis* revealed a diauxic-like second

exponential phase. Environmental induced enzyme expression of *B. subtilis* might allow diauxic growth kinetic whereas PP of APE might be metabolized. Experiments to induce this diauxic growth kinetic with the different carbohydrates of APE without PP failed (data not shown). Several bacterial species can metabolize PP (Hervert-Hernández *et al.*, 2009).

Moreover, it is assumed that phenolic compounds increased absorption of carbohydrates and other nutrients (García-Ruiz *et al.*, 2011). Therefore, PP can also enhance microbial growth. In accordance to this, all tested food spoilers and food fermenting starter bacteria *Lb. delbrueckii* subsp. *bulgaricus* and *Lc. lactis* were concentration-dependently inhibited. On the contrary, *Lb. brevis*, *Lb. reuteri,* and *Lb. sanfranciscensis* were supported in growth by APE. Several studies with LAB demonstrated that PP examine both, growth inhibitory (Campos *et al.*, 2003; García-Ruiz *et al.*, 2011) and stimulatory effects (Alberto *et al.*, 2004; Hervert-Hernández *et al.*, 2009). Difference of growth inhibition levels by wine PP, especially flavonols and stilbenes, in *Lb. hilgardii*, *Pediococcus pentosaceus,* and *Oenococcus oeni* have been examined (García-Ruiz *et al.*, 2011). Hydroxycinnamic acid, hydroxybenzoic acids and their esters exerted a medium inhibitory effect, whereas phenolic alcohols and flavanol-3-ols were less effective. In contrast, gallic acids or catechins seem to promote growth of LAB (Hervert-Hernández *et al.*, 2009; Alberto *et al.*, 2004). Also, grape pomace extract, grape seed extractable PP and tannic acid had stimulating effects on the growth of *Lb. acidophilus* (Hervert-Hernández *et al.*, 2009). We could demonstrate that *B. substilis* contaminated leaven could be stabilized. Counts of Bacilli were reduced and the lactic acid starter bacteria were not influenced in growth. The dough became not soft and stringy (data not shown).

In several studies, the influences of the biochemical composition and the molecular structure on the effectiveness of PP have been discussed (Cowan, 1999; Hervert-Hernández *et al.*, 2009; Moreno *et al.*, 2006; Scalbert, 1991). He described that simple phenols and phenolic acids, like caffeic and cinnamic acids from tarragon and thyme, are effective against bacteria, fungi and viruses. Further, the toxicity of hydroxylated phenols, like catechol and pyrogallol against microorganisms is assumed to depend on formation and number of the hydroxyl groups linked to the core structure.

On one side, phenolic compounds were suspected to inactivate cellular enzymes and influence microbial metabolic activity (Alberto *et al.*, 2004; Moreno *et al.*, 2006). For example, oxidized PP, like quinones, inhibit enzymes due to specific reactions with enzymatic sulfhydryl groups and irreversibly bind to nucleophilic amino acids which again inactivate the protein functions (Cowan, 1999). It is suspected that the anti-microbial effect of quinones is due to binding to surface-exposed adhesions, cell wall polypeptides, or membrane-associated enzymes. Another group of anti-microbial PP are flavones with similar protein-complex-forming properties such as quinones (Cowan, 1999). Tannins, occurring in bark, wood, leaves, fruits and roots, bind to proteins but also polysaccharides. Anti-microbial effects of tannins are the ability to inactivate microbial adhesins, enzymes, and transporter proteins. It is also suggested that anti-microbial effects of polyphenolic compounds, like tannins, associate to substrate and metal ion deprivation (Alberto *et al.*, 2004; Cowan, 1999; Hervert-Hernández *et al.*, 2009; Scalbert, 1991).

On the other side, lipophilic flavonoids, like catechins from tea, disrupt membranes of microorganisms (Cushnie and Lamb, 2005). Apple polyphenols from byproducts, like peels and seeds, are suspected to penetrate the cell membrane and cause thereby a cell-lysis (Ayala-Zavala *et al.*, 2010). The APE used in this study contained undefined short- and long-chain PP with possibly metabolic disturbance and membrane disruptive properties.

Bacteriolytic effect of polyphenolic APE

Cell membrane perforating properties of apple derived PP gram-negative *E. coli* as well as gram-positive *Sc. uberis,* and *Lb. brevis* were determined by SYTOX® green fluorescent dye assay. This dye perfundates the injured cell membrane barrier and intercalates with the bacterial DNA. Hereby, the fluorescence intensity of the DNA-dye complex increases up to 500 times related to the basis signal which is detectable by microplate fluorometry (Roth *et al.*, 1997).

Several studies indicated that PP perforate bacterial cell membranes (Campos *et al.*, 2009; Ikigai *et al.*, 1993). The lipophilic character and the degree of ionization of PP determine the effectiveness (Campos *et al.*, 2009). Several prominent membrane-permeabilization models, especially for membrane-lytic peptides, have been discussed by Shai (1999). For example, the *barrel-stave model* describes the formation of transmembrane aqueous pores by bundles of amphipathic α-helices. The *carpet mechanism* suggests that lytic peptides unspecifically bind onto the surface and push through the membrane. Other penetration mechanisms are the *detergent model* and the *toroidal pore model* (Shai, 1999). Cell membrane mechanisms for PP are not clearly shown to date. García-Ruiz *et al.* (2009) suggested that the cell membrane damage leads to cell death, due to disturbed transport and energy-depending processes and metabolic pathways. For example, epigallocatechin gallate, prominent in green tea, possess bactericidal effect due to the direct membrane perturbation of polyphenole (Ikigai *et al.*, 1993). Another study observed that polyphenolic acids like hydroxycinnamic acids particularly p-coumaric acid increase the membrane permeability of *Lb. hilgardii* and *Oc. oeni* from wine (Campos *et al.*, 2009).

Although nisin, ethanol and heat treated cells revealed cell-lysis (Ruiz *et al.*, 2009), no cell membrane disturbing activities of any concentration of APE could be found by all food spoiling and food fermenting starter bacteria tested. Campos *et al.* (2009) demonstrated that phenolic acid induced cell membrane damage is reversible if cells are transferred in a growth medium afterwards. This might also be valid for polyphenols. As some nutrients are available for the bacteria during the tests this might be one explanation. However, inactivation of bacteria often involves more than one single mechanism. Here, the specific PP composition of APE principally seems to disturb essential bacterial metabolic activities, like inhibition of the oxidative phosphorylation (Scalbert *et al.*, 2005).

Anti-fungal effect of APE

Some studies determined inhibitory effects on the growth of yeasts by carnosic acid (Moreno *et al.*, 2006). PP, like caffeic acid, eugenol and tannins also showed anti-fungal effects (Cowan, 1999). However, Papadopoulou *et al.* (2005) observed that yeast strains were more resistant to wine phenolics than bacteria. They concluded that the difference in the resistance against polyphenolic is related to a different membrane structure and the protein synthesis. In accordance to this, the growth of yeasts and moulds was never influenced.

CONCLUSION

In conclusion, this study demonstrated that the polyphenolic APE has both bacteriostatic and no bacteriolytic effects on food spoiling bacteria and also growth stimulatory effects on selected food fermenting starter LAB. Additionally, its sweetening and flavoring functions APE might be beneficial to stabilize fermentation processes in food manufacturing, for example in the wine production. In order to influence the malolactic fermentation of oenological LAB, PP might be an alternative for sulfites (García-Ruiz *et al.*, 2011; García-Ruiz *et al.*, 2009).

New application opportunities might also arise in food preservation, functional food or pharmaceutical product applications. In order to avoid synthetic food conserving agents, phenolic plant extracts can be considered as a source of natural anti-microbial agents. And, with regard to the raising antibiotic-resistance problem of microorganisms, plant-derived anti-microbials might be a valuable source to open a new research field for apple derived PP.

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