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Quality perception and willingness to pay: The case of red wine with health-beneficial effects

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PAPER

Abstract

This paper attempts to identify consumers' preferences toward red wine quality cues and willingness to pay (WTP) for wine with health-beneficial effects, thus clarifying the complex process of purchasing habits and patterns. The data were analyzed using structural equation modeling. The findings from a case study conducted in Serbia show that consumer health-effect consciousness and household income are significant predictors of their WTP. On the other hand, health-conscious consumers are more inclined to intrinsic quality cues, while those who are willing to pay a higher price for a bottle of red wine are prone to extrinsic wine quality cues.

Keywords: consumer quality perception, red wine, structural equation modeling, willingness to pay, wine preferences

Introduction

The choice-making of wine is a difficult and complicated process since there is a diverse and vast range of wines available in the market (Agnoli *et al.*, 2016). Accordingly, numerous papers aimed to segment and cluster the wine market (Caracciolo and Furno, 2020; Hu and Ruimei, 2019), and thus indicate the importance of diverse wine quality attributes in the overall consumer preference. Quality perception is seen as a mediator between product characteristics and consumer preferences (Steenkamp, 1989; Tomic *et al.*, 2017). Consumer judgment about an entity's overall superiority (product, service, and process) is based on the cues of excellence (Lee and Hwang, 2016; Snoj *et al*. 2004). One of the biggest challenges for businesses and wine industry is to meet consumer requirements and the need to understand their decision-making when purchasing wine.

A extensive body of literature sheds light on the quality cues of wine (Rodrigues and Parr, 2018; Sáenz-Navajas *et al.*, 2013; Verdú Jover *et al.* 2004). For example, Verdú Jover *et al.* (2004) observed consumer preferences through 15 red wine quality cues and divided them into extrinsic and intrinsic elements. Intrinsic cues are defined as inherent characteristics of wine, including taste, color, acidity, level of alcohol, etc. (Hu and Baldin, 2018; Valentin *et al.*, 2016). On the other hand, extrinsic attributes are often associated with noninherent characteristics such as brand, price, and year of production, country of origin, grape variety, label, tradition, awards, and recommendation (Boncinelli *et al.*, 2019; Lu *et al.*, 2017; Sáenz-Navajas *et al.*, 2013, 2016; Williamson *et al.* 2016). Verdú Jover *et al.* (2004) determined seven quality dimensions that contain the majority of aspects used in the 15 initial cues: origin, image, presentation, age, harvest, sensitivity, and acuteness of bouquet. Next, Jaeger *et al.* (2009) recognized 13 intrinsic and extrinsic purchasing decision-making wine cues such as award, brand, origin, grape variety, taste, recommendation, information presented on the label, alcohol level, etc. For instance, Heatherly *et al.* (2019) and Valentin *et al.* (2016) emphasized color as a strong intrinsic predictor of consumer preference. However, different research studies have indicated that extrinsic cues are becoming the main determinants of wine quality (Balestrini and Gamble, 2006; Reynolds *et al.*, 2018).

Besides quality cues related to hedonistic and social beliefs, consumer beliefs about the health benefits of wine are gradually becoming an important factor of purchase (Samoggia, 2016). Nowadays, health-conscious customers are looking for food and beverages that are not only nutritional but also have extraordinarily health benefits (Rathi, 2018). The health effects of wine were pointed at as far back as in ancient Greece and Rome (Fiore *et al.*, 2019). Recent research has highlighted that red wine is considered a healthy drink (Chang *et al.*, 2016; Vecchio *et al.*, 2017), because it contains ingredients that support cardiovascular, neurodegenerative, and aging health, and reduce the risk of cancer, diabetes, Parkinson's, and Alzheimer's disease (Fiore *et al.*, 2019; Krstonošić *et al.*, 2019; Kuršvietienė *et al.*, 2016; Liberale *et al.*, 2019; Soares *et al.*, 2015). Nevertheless, customer opinions differ. Chinese consumers tend to drink more red wine for its potential health benefits, whereas Australian consumers are less likely to rate wine as a healthy product (Yoo *et al.*, 2013). The same belief about the positive health effects of wine was noticed among young adults in Portugal (Patrícia Silva *et al.*, 2014). However, Thach and Olsen (2006) revealed that a minority of youth in the United States highlighted the health-beneficial effects as the main reason for wine-consuming. Conversely, Agnoli *et al.* (2016) conclude that novice consumers fail to completely recognize the beneficial effects of wine on health (Mueller and Szolnoki, 2012). Regarding wine purchasing reasons, Bazzani *et al.* (2019) acknowledge that health-oriented customers prefer extrinsic cues referring to quality assurance information (marked on the label) such as hand-picked grapes, sustainable product certifications, and unfiltered wine. In the research conducted by Cavaliere *et al.* (2016), however, customers are keener on reading nutrition information on the bottle label. Furthermore, Bazzani *et al.* (2019) and Martin-Moreno *et al.* (2013) also found out that health-conscious consumers have an aversion toward a higher percentage of alcohol content. Most of the previously mentioned studies highlight red wine benefits, show that most people are aware of the curative effects of this wine, and indicate that health-oriented consumers pay more attention to information provided on label.

The positive consumer perception of wine quality and willingness to pay (WTP) can be associated with health-enhancing wines (Samoggia, 2016), organic wines (Jorge *et al.*, 2020), sustainable wines (Sellers-Rubio and Nicolau-Gonzalbez, 2016), or Old World producer wines (Giacomarra *et al.*, 2020). Researchers often point to consumers' attitudes toward tasty, healthy, and eco-friendly food that influence their shopping behavior (Jorge *et al.*, 2020). For example, consumers in Spain are willing to pay for resveratrol-enriched wine (Barreiro-Hurlé *et al.*, 2008), while it was also found that health-oriented consumers are willing to pay more for health-enhancing wine (Bisson *et al.*, 2002; Higgins and Llanos, 2015; Samoggia, 2016). Therefore, high values of health-related substances in wine tend to lead to increased consumption of the particular wine type (Fiore *et al.*, 2019). Finally, Samoggia (2016) observed that consumers, who doubted the favorable effect of wine on one's health, failed to perceive the positive relation between higher price and the health properties of wine.

Scientific literature has frequently examined the above-mentioned topics. To the best of our knowledge, however, few authors have jointly observed relationship between consumer consciousness of health-beneficial effects of wine, quality cues preferences, and WTP. Hence, the paper attempts to create and verify a conceptual model which explores consumers' perception regarding the following constructs: Intrinsic Wine Quality Perception (IWQP), Extrinsic Wine Quality Perception (EWQP), Health Effect Consciousness (HEC), and WTP, while also considering Monthly Household Income (MHI). The model tested on wine consumers in Serbia aimed to improve the conceptual models on the topic devised so far and fill in the literature gaps related to the habits and patterns of consumption of red wine.

Serbia is one of the major producers and consumers of grapes and wines in Southeast Europe (Radovanović *et al.*, 2019). According to Statistical Report on World Vitiviniculture (OIV, 2019), Serbia ranks 30th in the world in terms of wine consumption with 1.1 million hectolitres. Also, in 2018, Serbia imported $31.6 million worth of wine (Workman, 2019), that is, 0.1% of the world's total worth. The growth of wine market in Serbia thus presents a challenge for marketing and quality management professionals. Proper understanding of the consumer quality perception would therefore help retailers, producers, and supplying organizations to successfully design their marketing strategies for evolving markets in order to have a significant competitive advantage.

The next section of this paper provides a detailed overview of the proposed conceptual model and the related hypotheses. The results and discussion are given in the third section, while the concluding remarks are outlined in section four.

Methodology

The proposed conceptual model

In our research, we attempted to broaden some of the current conceptual models for exploring the factors that impact the consumer behavior of red wine. The proposed conceptual model consists of the following four

constructs: HEC, EWQP, IWQP, and WTP, and also takes into account MHI.

HEC construct points to the degree of consumer awareness expressed through the attitudinal questions on health issues and the impact of red wine on human health (Appendix 1). Another construct, EWQP, indicates the level of importance of extrinsic quality in consumer's wine purchasing decision, including tradition, price, awards, recommendation, grape variety, brand, label, year of production, and country of origin. By investigating purchase intention, Huang *et al.* (2018) stated that wine lovers prefer to use extrinsic characteristics. So, the first hypothesis we test in our study is as follows:

> **H1**. *Consumer consciousness of red wine health effect influences extrinsic wine quality perception*.

The third construct, IWQP, points to the degree of importance of intrinsic quality in consumer wine purchasing decision such as flavor, health ingredients, wine color, additives, percentage of alcohol, and acidity. According to Bazzani *et al.* (2019), Cavaliere *et al.* (2016), and Martin-Moreno *et al.* (2013), consumers who are conscious of the positive effects of wine on their health are more inclined to pay attention to intrinsic cues. Therefore, the next hypothesis we propose is as follows:

> **H2**. *Consumer consciousness of red wine health effect influences intrinsic wine quality perception*.

The fourth construct, WTP, is determined by questions linked to the amount of money buyers are willing to spend on a bottle of red wine. Some of the researchers observed the effects of extrinsic cues on the willingness to pay (Lee *et al.*, 2018; Nowak *et al.*, 2006; Veale and Quester, 2009). For instance, Nowak *et al.* (2006) indicate that consumers are willing to pay more for a famous wine brand. Hence, following is our third hypothesis:

> **H3**. *Consumer willingness to pay influences extrinsic wine quality perception*.

Generally, some studies have found that consumers are more willing to pay for intrinsic quality cues such as nutritional contents or freshness (Balineau, 2018). The same conclusion is observed in a study conducted by Barreiro-Hurlé *et al.* (2008) and Gabrielyan *et al.* (2014). Our next hypothesis is as follows:

> **H4**. *Consumer willingness to pay influences intrinsic wine quality perception*.

Even though some studies failed to find a significant relationship between health attitudes and willingness to buy, most authors discovered a positive relationship between WTP and consumer consciousness (Bisson *et al.*, 2002; Higgins and Llanos, 2015; Samoggia, 2016). Accordingly, following is our next hypothesis:

> **H5**. *Consumer consciousness of red wine health effect influences consumer willingness to pay*.

High willingness to pay for a bottle of wine may also relate to a higher household income (Hofmann *et al.*, 2018; Sogari *et al.*, 2016). Research indicates that the respondents with a higher household income are ready to pay more for a bottle of wine and turn to premium wine (Camillo, 2012; Onofri *et al.*, 2015). Consequently, following is the final hypothesis:

> **H6**. *Monthly household income influences consumer willingness to pay*.

The proposed conceptual model is graphically presented in Figure 1. For more details on the questions used to measure each construct, see Appendix 1.

Data analysis

To test the validity of the proposed conceptual model, we opted for the structural equation modeling (SEM). The SEM analysis is a multivariate statistical analysis based on the principles of factor analysis and regression or path analysis (Hox and Bechger, 1998; Kline, 2005). On one side, SEM reduces the dimensionality of the observed phenomenon, while on the other it provides insights on the relationship between the newly formed latent variables or constructs. Taking into account the benefits of the SEM analysis and the clear theoretical concept it is based on, this analysis has become a vastly applied approach for representing dependency in multivariate data (Kline, 2005).

So far, the SEM analysis has been conducted in the research field of wine consumption. For example, Vilela *et al.* (2018) used SEM to explore how the sensory profile of the respondent impacted his/her observation of the wine aroma, mouth feel, and flavor for three different types of Portuguese wine. Pestar Bizjak *et al.* (2018) observed how respondents from two Slovenian wine regions perceived the value of wine through emotional-social dimension, quality-price, and terroir and the impact of regiocentrism. Further, Bianchi (2015) proposed a conceptual model of consumer brand loyalty based on wine knowledge, wine experience, wine brand satisfaction, wine brand trust, and wine brand loyalty, and tested this in Chile.

The survey

Inspiration for the items of a questionnaire came from Bruwer *et al.* (2002); Cholette and Castaldi (2005);

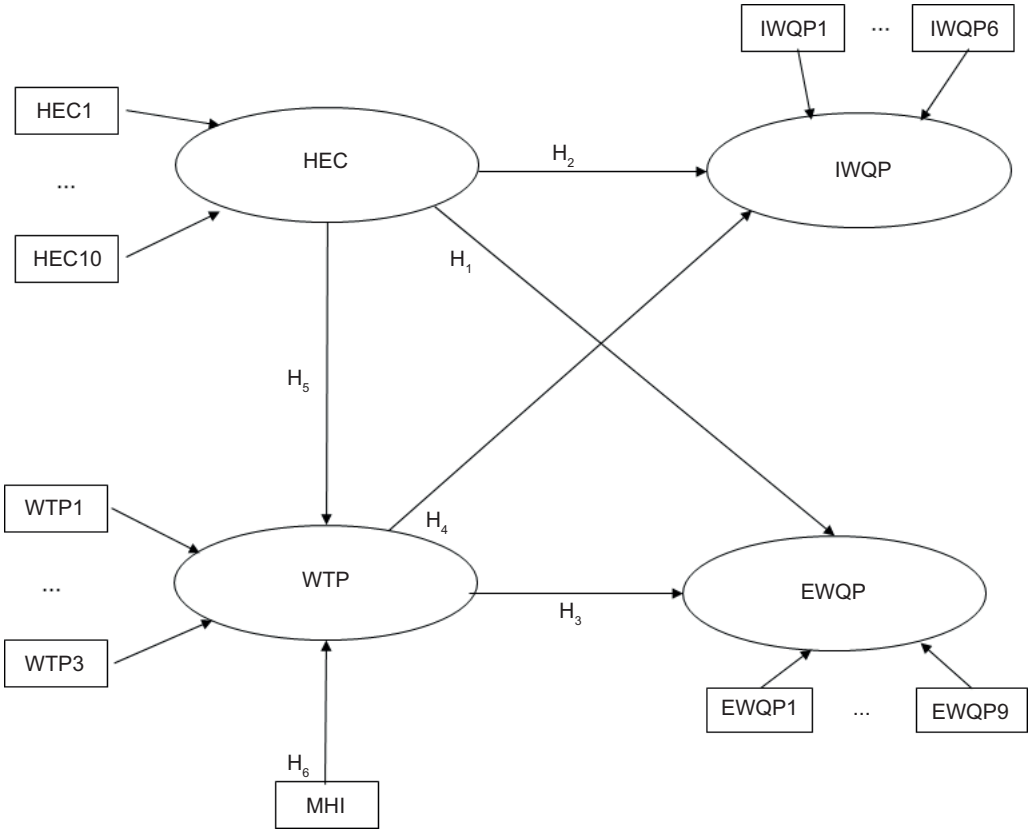

Figure 1. Conceptual model.

Johnson *et al*. (2017); Keller (2009); Lim *et al*. (2013); Sjostrom *et al*. (2016); Werdelmann (2014); and Yoo *et al*. (2013). The questionnaire was distributed online between September 2019 and January 2020. Some of the questions were adopted for the purpose of the study, while some scales were used in the original form. All the questions regarding respondents' opinions on red wine cues were measured on a 5-point Likert scale ranging from 1 (strongly disagree) to 5 (strongly agree). For more details on the questions used to measure each construct, and the obtained mean values and standard deviations (SD), see Appendix 1. The questionnaire was distributed on Facebook groups related to wine consumption and on LinkedIn. The questionnaire comprised the following five groups of questions: *Demographic information, Habits of red wine consumption, Health characteristics, Price,* and *Quality perception*. In the first section of the questionnaire, the respondents were asked some basic demographic information regarding gender, age, residence, highest completed degree, and household income. The rest of the questions were related to the frequency, quantity, occasions and place of consumption, and purchase of red wine, its characteristics, price, health effects, and perceptions of respondents about red wine consumption. Afterwards, statistical analysis using SPSS 25 and AMOS 22 was performed.

Results and discussion

Sample characteristics

A total of 605 responses were collected after closing the questionnaire. In order to obtain a sample of true wine consumers, we asked respondents whether they declare themselves as red wine consumers in the first place. In reply, 496 respondents (81.9%) declared themselves as consumers of red wine, while the remaining 109 (18.1%) responded in negative. The non-consumers of red wine were not eligible for our research, so their answers were removed from further analysis.

The sample consisted of 312 female respondents, who formed 62.9% of the sample, and 184 were male respondents (37.1%). A slight disproportion in the respondents' gender was observed, but the same was also found in the work of Bruwer (2004). Most of the respondents (64.7%) are in the age group 18–32 years, followed by the age group 33–45 years (24.8%). The remaining respondents were aged more than 46 years. This indicates that our sample comprised younger population, namely GenXers and Millennials. It is expected that GenXers would become the largest consumers of wine in 2021, while Millennials would take over in 2026 (Mcmillan, 2018). Accordingly, it

is reasonable to focus on all these segments. Similar age distribution of respondents within a sample was found in wine consumption research conducted by Bruwer (2004) and Bruwer *et al*. (2012). Respondents mainly came from Serbia (73.2%), followed by consumers from other countries of the region (Montenegro, Bosnia and Herzegovina, and Croatia). Taking a look at the highest educational degree, 51.0% of the respondents had a bachelor's degree, 22.4% had a master's degree, 15.7% completed high school, while 10.9% had PhD. We observed that the sample consisted of highly educated individuals, with 60.0% of surveyed millennials having at least a bachelor of science (BSc). This indicated that in this regard the sample was unbalanced. We believe that this occurred due to the sampling method used. Although this might be a limitation of the study, we believe that it would not distort the conclusions as we were observing health-enhanced wine which was more expensive and regarded as premium wine, which respondents with lower educational attainment and income did not often consume. Most respondents earned a monthly income between €1000 and €2000 (36.1%), 35.1% earned income below €1000, while the rest had a monthly income of above €2000. It was concluded that we covered a segment of the population which was young, educated, and had more than Serbia's average monthly income. Sample characteristics are provided in Table 1.

Table 1. Characteristics of the respondents who participated in the survey.

Variable	Frequency	Proportion
Gender		
Female	312	62.9%
Male	184	37.1%
Age group		
18–32	321	64.7%
33–45	123	24.8%
46–64	48	9.7%
65–75	4	0.8%
Educational attainment		
High School	92	15.7%
BSc	253	51.0%
MSc	111	24.2%
PhD	54	10.9%
Monthly household income		
Less than €1000	174	35.1%
€1000–2000	179	36.1%
€2000–3000	51	10.2%
€3000–4000	34	6.9%
€4000–5000	17	3.4%
Over €5000	41	8.3%

To provide additional insights on the consumers' attitudes toward the four explored constructs of the proposed model, we provide mean value and SD of each item of the constructs in Appendix 1.

Furthermore, we aimed to obtain insight on the respondents' attitudes and habits regarding consumption of red wine. Most of the respondents (32.7%) were consuming red wine for 1–5 years, followed by those who had been consuming for 6–10 years (28.8%). As for frequency of consumption of red wine, most of the respondents consumed it once every 2 months (38.3%), followed by those who consumed it on a monthly basis (19.4%) or once in a fortnight (16.3%). As for purchasing habits, most respondents (32.3%) consumed one to three bottles per year, followed by those who consumed over 10 bottles (27.4%). On the other hand, 39.3% of the respondents buy one to three bottles a year as a gift. Half of the respondents (50.8%) believed that the reasonable price of a red wine bottle is between €5 and €10, while 27.6% were ready to pay between €10 and €40. This suggests that a large percentage of our respondents had multiple years of moderate consumption and purchasing habits and were ready to pay for a good bottle of wine.

SEM model

The primary step in SEM analysis is to observe the internal consistency of the proposed latent variables. The most commonly used metric for internal consistency and scale reliability is Cronbach's alpha (Cronbach, 1951). The Cronbach's alpha provides a metric level up to which all the measured variables in a latent construct measure the same concept, and it takes values from 0 to 1. The closer the Cronbach's alpha is to 1, the higher the internal consistency (Peterson, 1994). As reported by relevant literature, the acceptable levels of Cronbach's alpha are in the range of 0.70–0.95 (Tavakol and Dennick, 2011). Besides Cronbach's alpha, average variance extracted (AVE) and construct reliability are used (Fornell and Larcker, 1981). The closer these indices are to 1, the better the internal consistency; thus, this shows that the scale is more reliable. The threshold for the acceptable level for AVE is above 0.5, while for composite reliability, it is above 0.7 (Fornell and Larcker, 1981; Wong, 2013).

The calculated Cronbach's alpha per latent variable and the number of items per scale are given in Table 2. As presented, the internal consistency ranges from 0.751 (WTP) to 0.984 (HEC). The composite reliability for all constructs is at the threshold of 0.7 or above. However, when it comes to AVE, EWQP's AVE is less than the threshold (0.447). Having in mind the obtained metrics of reliability and validity, we can conclude that the data are suitable for SEM analysis.

Additionally, we explored the normality of variables in the model, having in mind that the

Table 2. Obtained Cronbach's alpha, average variance extracted (AVE), and composite reliability per construct and the number of items per construct.

	HEC	IWQP	EWQP	WTP
No. of items	10	6	9	3
Cronbach's alpha	0.984	0.862	0.886	0.751
AVE	0.501	0.470	0.447	0.484
Composite reliability	0.904	0.841	0.876	0.697

Note: HEC - Health Effect Consciousness; IWQP - Intrinsic Wine Quality Perception; EWQP - Extrinsic Wine Quality Perception; WTP - Willingness to Pay

non-normality might significantly impact the result of analysis. According to Muthén and Kaplan (1985), 'if most variables have univariate skewness and kurtosis in the range −1.0 to +1.0, not much distortion is to be expected'. In our sample, out of 28 variables in the model, six proved to have issues with skewness and kurtosis in the range from −2 to 3. Taking into account that just several variables expressed skewness and kurtosis out of the suggested range, we continued with the analysis (Hallow, 1985).

The initial model had relatively poor fit to the data (Chi-square = 2643.028, df = 372, $P < 0.000$, Root Mean Square Error of Approximation (RMSEA) = 0.111, Comparative Fit Index (CFI) = 0.862, Tucker-Lewis Index (TLI) = 0.849). To evaluate the significance of the paths and indicators, we used critical ratios (C.R.). The value of C.R. above 1.96 or below −1.96 points out a two-sided significance at the 5% level (Hox and Bechger, 1998). Paths between HEC and EWQP and between IWQP and WTP had a C.R. below the defined threshold, so they were removed from the model. Additionally, we used modification indices to fine-tune our model. The final model had relatively good fit to the data (Chi-square = 1721.177, df = 328, $P < 0.000$, RMSEA = 0.092, CFI = 0.915, TLI = 0.895). The detailed model assessment is given in Table 3.

Concerning the construct IWQP, it initially had two predictors, HEC and WTP. However, WTP proved to be statistically insignificant, which would mean that hypothesis H4 was not confirmed. The obtained standardized coefficient of the impact of HEC on IWQP was 0.776, with C.R. = 12.888, indicating that HEC significantly impacted IWQP and the impact was positive and medium in strength, taking into account that the standardized coefficient could take a value of 0–1. This finding confirmed the assumption H2. The R^2 of this construct was 0.601, meaning that one predictor explained 60.1% of the variability of IWQP, thus creating a solid model. These results showed that more health-conscious consumers valued more the intrinsic characteristic of wine quality (focusing on flavor, healthy ingredients, color, percentage of alcohol, additives, and acidity). The results were in accordance with the research done by Bazzani *et al.* (2019) and Martin-Moreno *et al.* (2013). The obtained result might be due to the fact that health-oriented consumers were more likely to undertake actions and behaviors that could contribute to their health improvement and thus pay more attention to the intrinsic quality of wine cues (Cavaliere *et al.*, 2014).

Similar to IWQP, EWQP also initially had two predictors, HEC and WTP. In this case, HEC proved to be statistically insignificant, thus hypothesis H1 was not proved.

Table 3. Assessment of the model: construct, predictors, obtained unstandardized and standardized coefficients, C.R., R^2, and the decision on the related hypothesis.

Construct	Predictors	UnStd. Coeff.	Std. Coeff.	C.R.	R^2	Hypothesis
IWQP	HEC	0.203	0.776	12.880	0.601	H2—Approved
	WTP		Not significant			H4—Rejected
EWQP	HEC		Not significant		0.613	H1—Rejected
	WTP	0.542	0.783	16.449		H3—Approved
WTP	HEC	0.710	0.975	33.908	0.998	H5—Approved
	MHI	0.124	0.221	13.202		H6—Approved

Note: C.R. – Critical ratio, R^2 – R square, HEC - Health Effect Consciousness; IWQP - Intrinsic Wine Quality Perception; EWQP - Extrinsic Wine Quality Perception; WTP - Willingness to Pay; MHI - Monthly Household Income

The obtained standardized coefficient of the impact of WTP on EWQP was 0.783 with C.R. = 16.449, indicating that WTP significantly impacted EWQP statistically and that the impact was positive and medium in strength. The R^2 of this construct was 0.613, meaning that one predictor explained 61.3% of the variability of EWQP, thus creating a solid model. The results suggested that consumers who were more willing to pay a higher price for a bottle of red wine and its health effect were more inclined to extrinsic wine cues such as price, year of production, country of origin, brand image, grape variety, label, tradition, recommendation, and award. Correspondingly, hypothesis H3 hypothesis, about a positive impact of WTP on EWQP, was also confirmed. A similar relation was observed by Lee *et al.* (2018) and Veale and Quester (2009).

The construct WTP had two predictors, HEC and MHI, and both proved to be statistically significant. In the final model, HEC and MHI had a statistically significant positive impact with respective standardized coefficients of 0.975 and 0.221. MHI was left in the model, although its impact was weak as it was statistically significant and the goal was to obtain a good measurement model (Allen *et al.*, 2019; Milenković *et al.*, 2019). The R^2 of this construct was 99.8%, indicating that the two predictors explained a high percentage of variability of WTP. As in other research on wine consumption (Bisson *et al.*, 2002; Higgins and Llanos, 2015; Samoggia, 2016), the findings indicated that health-conscious consumers were willing to pay more for a bottle of red wine as well as for health-enhancing wine. In corroboration of the previous claims made by Hofmann *et al.* (2018), Hu and Ruimei (2019), Onofri *et al.* (2015), and Sogari *et al.* (2016), results included the observation that respondents with higher MHI seemed to have higher WTP. In other words, people who earned more were willing to pay more for a bottle of red wine. Hence, we can say that hypotheses H5 and H6 were proved.

The graphical interpretation of the results and the final model are given in Figure 2.

Conclusion

The way a user views the quality of a product is closely related to his/her needs and requirements. These needs include not only the physical benefit of the product but also the one that relates to the image a user creates of himself/herself for the use of a specific brand and all other aspects of the benefits of the delivered product that are considered valuable. Furthermore, a user's perception

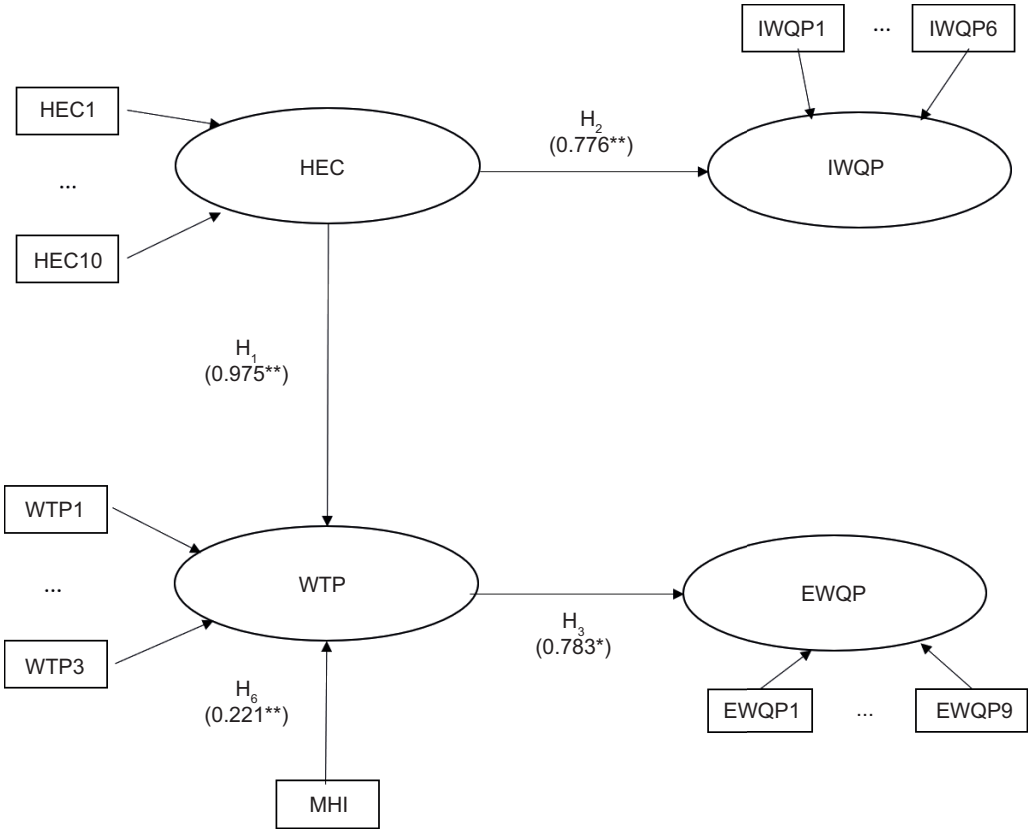

Figure 2. Final conceptual model. $^{**}P < 0.01$.

of a product quality and its characteristics can influence the creation of a different marketing strategy for the same product.

The purpose of this paper was to create and verify a conceptual model which explores consumers' perception of red wine. The design of this study imparted significant insights into the issue of consumer preference in the case of red wine. The research covered a segment of population that was young, educated, with an above-the-average Serbian monthly income, and having multiple years of moderate consumption and purchasing habits and was ready to pay for a good bottle of wine. By employing SEM, we developed a conceptual model that pointed to different influences between five constructs: IWQP, EWQP, HEC, WTP, and MHI. The findings established that the initial model was changed, and four out of six set hypotheses were confirmed. After the analysis, it was found that consumer quality perception has two predictors—HEC and WTP. Health-conscious consumers were keen on evaluating intrinsic quality cues, while respondents who would pay a higher price for red wine inclined to extrinsic quality cues. Moreover, health-oriented respondents and those with higher MHI depicted that they were willing to pay a higher price for red wine as well as for health-enhanced red wine.

Our study provided useful insights, but it was not without limitations. First, the sample size was a limitation of the study. Although we had almost 700 respondents after closing the survey, a larger sample could have been expected as we were exploring wine consumption, a product of mass consumption. As a remedy, we suggest a larger sample based solely on Serbian consumers or even a regional study in several Balkan countries in the future studies. Another possible limitation, as elaborated above, might be the sample structure, which was biased toward highly educated individuals. Also, convenience sampling based solely on the individuals who had Facebook and LinkedIn accounts limited our ability to generalise the findings of the study. This suggests that different approach to sampling could be taken in the future studies.

The obtained knowledge had a number of theoretical and managerial implications. Given that Serbia represents an evolving wine market, understanding consumers' willingness, consciousness, and perception could significantly contribute to the understanding of purchasing behavior. This would further help companies develop an appropriate marketing strategy and prepare for the Balkan market.

The future research should be directed toward revealing premium wines preferences, including tasting, and/or must focus on older population. A larger number of respondents from the Balkan region would allow for a comparative analysis, thus significantly improving the quality of the result. Also, another possible direction of the study could be the extension of the conceptual model by including new constructs such as wine knowledge, wine experience, or purchase intention (Bianchi, 2015).

The paper provided important information for policy makers to identify barriers, develop rules, policies, and initiatives, as well as labeling schemes to engage consumers in sustainable consumption of wines with health benefits. Our findings could benefit companies, wineries, and managers who plan to enter the growing wine market in Serbia and the Balkans. We believe that the proposed conceptual model for exploring the relationship between quality perceptions and willingness to pay would initiate further research on both factors that affected consumer decision-making process when purchasing red wine and the future improvements of the conceptual models regarding this topic.

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Conflict of interest

There are no conflicts of interest. The authors of this research strictly followed all ethical guidelines provided by the University of Belgrade.

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Appendix 1

Willingness to pay

Item	Mean	SD	Source
1. An acceptable price for a bottle of red wine at retail	2.20	0.869	Cholette and Castaldi (2005)
2. Reasonable price of red wine with curative effects	1.82	2.644	
3. I would pay more for health-enhanced red wine	3.17	2.253	Yoo *et al*. (2010)

Health effects consciousness

Item	Mean	SD	Source
4. Red wine can slow down the aging process and extend human life	3.33	2.024	Yoo *et al*. (2013)
5. Red wine can reduce the risk of certain diseases (cardiovascular and metabolic diseases and cancer)	3.52	2.047	
6. Red wine can cure certain diseases	2.61	2.130	
7. I think, red wine is a healthy alcoholic beverage	3.45	2.221	
8. Red wine has better health properties than other alcoholic beverage	3.67	2.227	
9. It is important to limit the amount of alcohol you consume	4.35	2.258	
10. I understand how much alcohol is considered healthy	3.66	2.335	
11. I know what moderate drinking is	4.22	2.430	
12. Red wine has more health-enhancing properties	3.56	2.453	
13. I would drink more red wine if I thought it was healthy for me	3.24	2.574	

Intrinsic wine quality perception

Item	Mean	SD	Source
14. Flavor	4.43	1.104	Bruwer *et al.* (2002); Werdelmann (2014); Yoo *et al*. (2013)
15. Health ingredients	3.51	1.239	
16. Color of wine	3.47	1.391	
17. Alcohol (%)	3.15	1.461	
18. Additives	3.08	1.569	
19. Acidity	3.43	1.540	

Extrinsic wine quality perception

Item	Mean	SD	Source
20. Price	3.80	1.116	Bruwer *et al.* (2002); Werdelmann (2014); Yoo *et al*. (2013)
21. Year of production	3.05	1.268	
22. Country of origin	3.17	1.342	
23. Brand image	2.93	1.328	
24. Grape variety	3.28	1.414	
25. Label	2.91	1.159	
26. Tradition	3.21	1.568	
27. Recommendation	3.95	1.500	
28. Awards	3.08	1.627	

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Riboflavin removal by commercial bentonites and charcoals in white and red wines

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PAPER

Abstract

Riboflavin (RF) represents one of the primary molecules undergoing photodegradation in wine, and its excited form acts as an intermediate in light-induced oxidation reactions responsible for the light-struck fault. A recent study has revealed bentonites (BENs) and charcoals (CHAs) as the most promising fining agents for removal of RF in model wine. This work explored their potential on both white and red wines, where polyphenols could interfere in the fining agent–RF interaction. A total of 11 BENs and 11 CHAs were compared. BENs exhibited a limited capacity, while decoloring carbons confirmed a great attitude for removal of RF in white wine, even at low dosages. Nevertheless, efficiency of CHAs shows a sensible reduction in red wine.

Keywords: bentonite, charcoal, red wine, riboflavin removal, white wine

Introduction

Shelf life of wine assumes a complicated meaning because of high diversity in both wine styles and typical characters searched by consumers. The quality of final product depends on both winemaking practices (fermentation and post-fermentation fining treatments) and storage in bottles. Maturation of wine should be strictly controlled to avoid undesirable chemical reactions, with particular attention to temperature and light conditions during transportation to the final seller and its often long storage. The post-fermentation is particularly critical for red wine because it commonly requires more time for maturation. Exposure to light could have two independent effects on wine: first, it could activate oxidative reactions, and second, it could cause heat-induced damages on direct exposure to sunlight (Jackson, 2011). In order to limit light-induced oxidation, the best protection is represented by storage in dark conditions and use of ultraviolet (UV)-masking bottles. It is known that green glass bottles can filter a larger spectrum of UV-Visible (VIS) wavelengths than uncolored bottles, thus reducing the rate of photodegradation reactions (Grant-Preece *et al.*, 2017). However, this technological requirement is in contrast with the consumer preference for uncolored bottles, which is rapidly growing along with market for rosè wines. Riboflavin (RF) assumes a particular relevance in wine's shelf life because of its implication in intermolecular photoreduction. In fact, RF is one of the principal responsible for the development of light-struck taste, a wine fault characterized by 'cooked-cabbage' aroma. RF is thermostable at the temperature of winemaking process, but it is highly photosensitive and can easily undergo photochemical degradation (Sheraz *et al.*, 2014). In aqueous solutions, RF is implicated in photosensitization reaction that acts following two mechanisms. The type 1 pathway results in the formation of two charged free radicals, RF and a

target molecule, through hydrogen- or electron-transfer reactions between the excited triplet state of RF ($^3RF_1$) and the substrate. When this substrate is methionine (Met), it leads to the formation of methional. While RF radical is involved in a recycling reaction, methional is unstable, and thus readily decomposes to methanethiol (MeSH) and acrolein. In addition, two molecules of methional can combine into dimethyl disulfide (DMDS). The type 2 process uses the energy transferred from $^3RF_1$ to oxygen to form singlet oxygen, which can then react with multiple biological substrates. In light-struck reactions, it oxidizes methionine sulfur, generating methionine sulfoxide (Silva *et al.*, 2019). MeSH and DMDS are the main compounds responsible for the 'cooked-cabbage' aroma of light-struck reaction; they have a perception threshold of 2–10 μg/L and 20–45 μg/L, respectively (Fracassetti *et al.*, 2019). In a recent study, Fracassetti and colleagues (2019) explored the relationship between Met and RF and verified that Met degradation could be avoided if RF concentration remains below 50 μg/L. In a different study, the same authors compared different fining agents with the aim to determine the clarification practice that could be the most efficient one for removal or lowering of RF (Fracassetti *et al.*, 2017). This study compared several fining agents, namely polyvinylpolypyrrolidone (PVPP), bentonite (BEN), zeolite, silica, kaolin, albumin, and charcoal (CHA), using different concentrations in model wine, and identified BEN and CHA as the most promising agents. However, the efficacy of fining agents seems strictly dependent on the media composition, as demonstrated by comparison of BEN, CHA, and zeolite performance in both model wine and a real Chardonnay wine (Fracassetti *et al.*, 2017). In this case, the CHA removal efficacy was lower in real wine at all the tested dosages. From the applicative point of view, it would be interesting to understand whether different categories of BENs and CHAs could be used successfully in real wines. Besides white wines, which are commonly preferred for RF studies because of being more subjected to light-struck fault, red wines represent an interesting case of study because, as reported by Lagunes *et al.* (2017), their extracts could act as photosensitizer if exposed to light, generating $^1O_2$, which in turn is able to oxidize other compounds. Moreover, polyphenols make more complex the removal of RF because of the high affinity of phenols to CHAs (Lisanti *et al.*, 2017). A fine balance between the quenching and the photosensitizing nature of red wine polyphenols is of particular interest for monitoring the removal of RF in this kind of wine. In the present study, different commercial BENs and CHAs, provided by different suppliers, were compared in two wines to explore deeply their ability to remove RF. In particular, it was verified whether this ability is correlated to other fining properties, such as protein removal and decolorizing (DEC) capacity.

Materials and methods

Chemicals and reagents

Methanol, *trifluoroacetic acid (TFA)*, tartaric acid, acetic acid, and sodium acetate were purchased from Sigma-Aldrich (Milano, Italy). Enocyanin powder (Enocianina GSE12 UC) was provided by EVER S.R.L. (Pramaggiore, Italy). Water of HPLC grade was obtained from Milli-Q system (Millipore Filter, Bedford, MA, USA).

Bentonites and charcoals

Eleven BENs, two calcic (CAL), one sodic–calcic (SOD-CAL), and eight sodic (SOD), and 11 CHAs, four deodorizing (DEO) and seven DEC, were provided by different commercial suppliers.

Wine selection

The wines were selected from different wine samples for their medium–high RF content. One white wine (Glera base wine, harvest 2018, produced by School of Oenology Cerletti, Conegliano (TV), Italy), with a content of 123.9 μg/L of RF, was chosen for BEN trials. This wine showed 9.6% alcohol and 6.5 g/L of titratable acidity. One white wine (Glera base wine) and another red wine (Wildbacher), both produced by Collalto winery (Susegana (TV), Italy), with 104.0 μg/L and 138.6 μg/L of RF, respectively, were chosen for CHA treatments. Glera wine (harvest 2018) showed 11.6% alcohol and 6.2 g/L of titratable acidity, while Wildbacher (harvest 2018) showed 12.5% alcohol and 5.1 g/L of titratable acidity.

Bentonite's protein adsorption trial

Deproteinization capability of BENs was evaluated according to modified Oeno 441-2011 Resolution (The International Organization of Vine and Wine [OIV], 2011). Trial solution was prepared using bovine serum albumin protein (BSA) 500 mg/L instead of ovalbumin 5 g/L, and the protein was dissolved into model wine (5 g/L tartaric acid, 12% v/v ethanol, pH = 3), whereas in the OIV method, the ethanol was absent. Eight BEN dosages (namely 10, 20, 30, 40, 50, 60, 70, 80 g/hL) were tested in order to determinate BEN adsorption curves. Samples were shaken and maintained in dark at 25°C for 30 min before performing protein quantification using a Pierce BCA Protein Assay Kit (Fischer Scientific Italia, Rodano (MI), Italy). After an incubation of 30 min at 37°C, samples were read using Microplate Reader (Euroclone) and data were elaborated by Software Manta and quantified over a calibration curve of BSA between 12.5 mg/L and 1000 mg/L.

Charcoal's decolorization power in enocyanin solution

Decolorizing power (DP) of a commercial CHA was evaluated applying the method reported in OIV 7/2007 (OIV, 2007) with little modifications. The enocyanin solution was prepared adding 4.5 g/L of enocyanin, 7 g/L of tartaric acid, 4 g/L of acetic acid, and 7 g/L of sodium acetate. The solution was stirred to allow complete dissolution and centrifuged for 10 min at 14,000 g. Supernatant was recovered and its absorbance was read in quartz cuvettes (2-mm path length) at three different wavelengths, namely 420, 520, 620 nm, using spectrophotometer ULTROSPEC 2100pro (Amersham Bioscience Europe GmbH, Cologno Monzese (MI), Italy). The color intensity (CI) was calculated as the sum of the three absorbance values standardized to a length path of 10 mm. Each CHA was added to 100 mL of enocyanin solution at a final concentration of 1 g/L. Samples were stirred for 30 min; after 10 min, they were collected into Eppendorf tubes and centrifuged for 10 min at 14,000 g to remove CHAs. Supernatant CI was measured as described for enocyanin solution. Finally, DP was expressed as percentage using the following equation:

$$DP = 100 \times \frac{CI1 - CI2}{CI1}, \quad (1)$$

where CI1 is the enocyanin solution color intensity and CI2 is its color intensity after CHA treatment. The specific DP of each CHA was calculated by three independent replications.

Bentonites and charcoals riboflavin removal in wine

Four BEN dosages were chosen for removal of RF in white wine (Glera wine with 123.9 µg/L of RF), namely 20, 40, 60, and 80 g/hL; the experiment was performed twice in 50-mL Falcon tubes. Tubes were vigorously shaken to allow dispersion of BEN and maintained at 25°C for 10 min before centrifugation at 14,000 g for 10 min. Removal assay was repeated in two independent tests. For CHAs, five dosages were selected for both red and white wines (Wildbacher and Glera, with 138.6 µg/L and 104 µg/L of RF, respectively), namely 10, 5, 2, 1, and 0.5 g/hL. The test was repeated for three times. Samples were shaken and kept in dark for 24 h. At the end of time contact, samples were mixed and centrifuged for 10 min at 14,000 g in order to assure removal of CHA. The supernatant obtained was used for both RF quantification and color intensity determination. For the latter analysis, control CI (CC) and samples CI (CS) were used in Eq. (1) in place of CI1 and CI2, respectively. Color intensity was calculated as 420-nm absorbance value for the white wine and sum of the 420, 520, and 620 nm absorbance values for the red wine.

Quantification of riboflavin in high-performance liquid chromatography (HPLC)

A Nexera HPLC system (Shimadzu) equipped with RF 20-A XS fluorescence detector was used. Filtered samples (20 µL) were separated in a Kinetex C18 (5 µm, 100 Å, 150 × 4.6 mm Phenomenex). Eluting solvents were as follows: (A) Milli-Q water and 0.1% of TFA v/v and (B) gradient-grade methanol and 0.1% of TFA v/v. The gradient program was 0–2 min, 30% B; 2–10 min, 30–60% B; 10–11 min, 60–100% B; 11–14 min, 100% B; 14–15 min, 100–30% B; and 15–18 min, 30% B. The flow rate was set to 0.6 mL/min and the column temperature was kept at 37°C. The RF was detected by fluorescence using 452 and 516 nm as excitation and emission wavelengths, respectively. RF was quantified using the external standard method. Data were acquired and processed with LabSolutions version 5.93.

Statistical analyses

R software (R version 3.0.1) was used for statistical analysis. Differences were evaluated by one-way ANOVA, Welch's ANOVA, and Kruskal–Wallis H test depending on data distribution. *Post hoc* analyses Tukey HSD test and Games–Howell test were used for ANOVA and Welch's ANOVA, respectively, while Dunn test with Holm correction was chosen as Kruskal–Wallis *post hoc* test. Correlations were tested using Pearson's correlation test. Statistical significance was attributed with $P < 0.05$ or a Confidence Interval of 0.95.

Results and discussion

Bentonite's deproteinization capacity

Bentonites are mainly used to remove proteins and avoid unpleasant haze in white wines. Selected BENs have been characterized for determining their performance in protein removal using modified Oeno 11/2003 (OIV, 2011) protocol. For this purpose BSA was considered more suitable than egg albumin because of its isoelectric point and absence of glycosylation, which make BSA more similar to wine proteins (Sarmento *et al.*, 2000). In addition, 12% ethanol was added to the test solution, as BEN is generally used in wine and it has been demonstrated that ethanol can influence the swelling of BEN, modifying its protein removal ability (Achaerandio *et al.*, 2001). Moreover, the quantity of protein used was 500 mg/L, more close to the real protein content in unstable white

Table 1. Summary of selected bentonites. For each bentonite, de-proteinization curve slope and related R^2 values are reported.

Sample ID	Category	Slope	R^2
BENT1	SOD	7.97	0.99
BENT2	SOD	7.17	0.99
BENT3	CAL	4.11	1.00
BENT4	CAL	2.44	0.94
BENT5	SOD-CAL	2.61	0.94
BENT6	SOD	6.75	0.96
BENT7	SOD	5.34	0.97
BENT8	SOD	5.30	0.97
BENT9	SOD	7.00	0.99
BENT10	SOD	4.99	1.00
BENT11	SOD	7.14	0.99

wines (Marangon *et al.*, 2011) than the 5-g/L of ovalbumin used in the original OIV method.

Removal curve slopes of 11 BENs belonging to CAL, SOD, and SOD-CAL categories were used to compare efficiency of BENs, the highest slope value corresponded to the best performing BEN. Data revealed a positive linear relationship between BEN dosage and quantity of protein removed in this range of concentration. Only in two cases, namely BENT4 and BENT5, R^2 was lower than 0.95 (Table 1) because of the lower absorbing capacity of these BENs. Statistical analyses revealed a significant difference between categories ($F_{(2,8)}$ = 10.5, P < 0.05) and *post hoc* test-grouped SOD-CAL with CAL being the reason of their low deproteinization capacity (Figure 1).

This trend is in accordance with Jönsson *et al.*'s (2009) findings, which demonstrated that SOD bentonites are characterized by about 10 times major swelling capability than CAL ones. The nature of cations arranged between

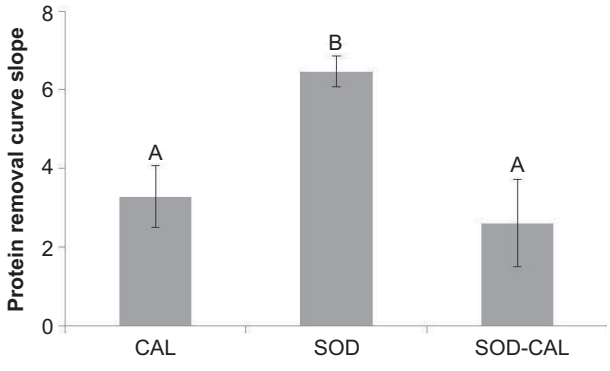

Figure 1. Slope index of protein removal curves. Least-square mean values and standard errors are presented. Different capital letters identify significantly different groups (P < 0.05) according to Tukey test.

montmorillonite lamellae strongly affects physical properties of BEN, resulting in a great difference in protein-binding ability. Sodium guarantees a major distance between BEN layers enhancing protein entrance and a higher availability of binding surfaces.

Bentonite's effect on removal of riboflavin

Attitude of BEN to removal of RF was recently studied in comparison to other fining agents (Fracassetti *et al.*, 2017). BEN was identified as one of the most useful fining agents. In fact, in the study conducted by Fracassetti *et al.* (2017), all the tested BENs (six, all from the same supplier) were able to remove about 60% of original RF in an RF-enriched model wine (350 µg/L of RF). For the screening presented in this work, BENs were chosen with the aim to explore a major variability of commercial products, and therefore 11 BENs furnished from seven different suppliers were selected. In Glera wine, BENs showed a limited removal of RF even at the highest dosage (on average, 28% of reduction at 80 g/hL) in accordance with the data reported by Fracassetti and colleagues (2017) when testing a calcic BEN at 100 g/hL in Chardonnay wine. Interestingly, the treatment with SOD-CAL BEN (BENT5) at 80 g/hL led to a sensible reduction, corresponding to about 60%, of RF (Fig. 2). Considering that white wines present a mean RF content of 115 µg/L (Cataldi *et al.*, 2002), this is the only BEN treatment that assured to decrease RF content below 50 µg/L, which is considered the threshold for light-struck taste risk (Fracassetti *et al.*, 2019). Nevertheless, several cases of white wine in which concentration of RF overcame 151 µg/L have been reported (Ournac, 1968; Pilcher, 1996), and it should be taken into account that high dosages of BEN could lead to severe side effects, that is, wine aroma depletion (Lambri *et al.*, 2012; Lira *et al.*, 2015).

Statistical analysis evidenced a significant difference among BENs only at 60 g/hL, and BENT5 confirmed to be the most efficient BEN. In two cases, BENT5 and BENT6, a statistically significant effect of dosage was registered (Figure 2). As also reported by Fracassetti and colleagues (2017), no evidence of different responses was observed between CAL and SOD. The statistical analysis revealed no significant correlation (r = -0.33, P = 0.328) between the percentage of removed RF at the highest BEN dosage and the protein removal curve slope, reinforcing the idea that protein and RF are linked to BEN surfaces through different mechanisms. BENs are known to link molecules by means of three different mechanisms: the first involves dipole bindings, the second is based on hydrogen bonding through the water bridge mechanism, and the third is based on Van der Waals forces (Luckham and Rossi, 1999). Probably, the chemical structure of RF, characterized by low polarity, induces the

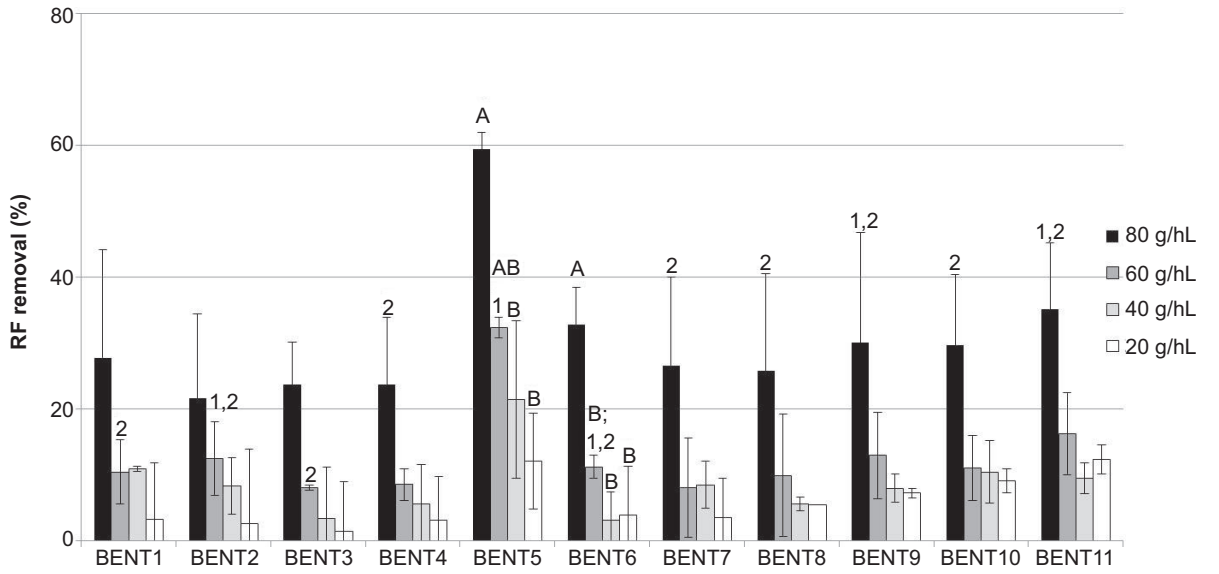

Figure 2. Riboflavin removal by bentonites. Mean values and standard deviation of two replicates are presented. Statistical differences between doses of bentonite are expressed by capital letters, while differences between bentonites at the same dosage are expressed by numbers (Tukey test, $P < 0.05$).

establishment of low polar bonds belonging to two latter types (Kasimova *et al.*, 2019).

Charcoal's decolorizing capacity

Active CHAs are commonly used in oenology to reduce organoleptic fault because of phenolic off-odors (Lisanti *et al.*, 2017) as well as fining agents to correct color intensity of white wines obtained by the vinification of red grapes. Their application depends on physical properties of CHA, in particular on the pore size, which strongly affects CHA permeability through molecular size exclusion. In fact, decolorizing CHAs are characterized by 20–500 Å macropores, while deodorizing CHAs show a dominance of small pores (Yahya *et al.*, 2015). Resolution Oeno 7/2007 (OIV, 2007) categorizes CHAs into the two groups based on the percentage of enocyanin removed from a model wine (decolorizing power). In particular, CHAs are assigned to DEC group if they are able to remove more than 40% of initial enocyanin, while they are recognized as DEO if the removed enocyanin is less than 40%. The decolorizing power of 11 selected CHAs was analyzed.

For all CHAs, decolorizing power test confirmed the category assignment declared in the technical datasheets. Four CHAs belonged to DEO, namely CHA3, CHA4, CHA8, and CHA11, while six CHAs were clearly assigned to DEC (CHA1, CHA5, CHA6, CHA7, CHA9, and CHA10; Figure 3). The ambiguous case of CHA2

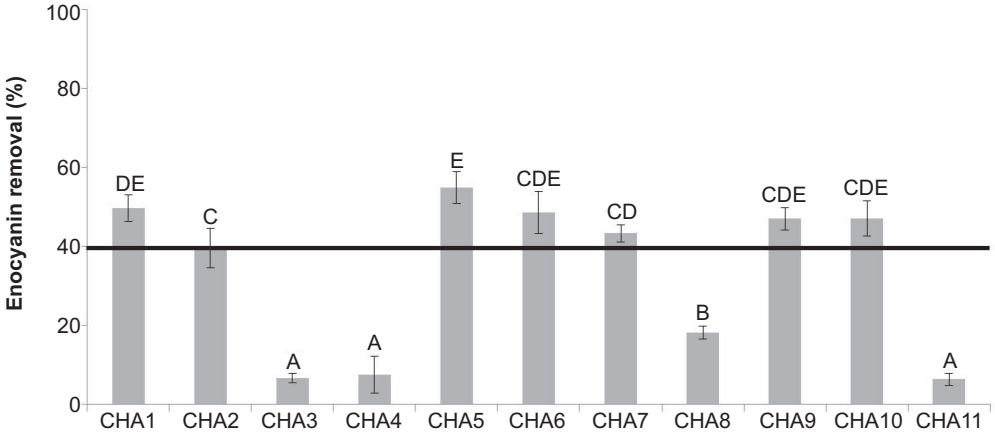

Figure 3. Percentage values of enocyanin removal. Mean values and standard deviations are presented. Each test was repeated for three times. Different capital letters identify significantly different groups ($P < 0.05$) according to *post hoc* Tukey test. Line corresponds to OIV threshold.

that reduced 40% of enocyanin content was ascribed to DEC in accordance with the statistical analysis, which grouped CHA2 with CHA6, CHA7, CHA9, and CHA10. Statistical analysis evidenced significant differences between samples ($F_{(10, 22)}$ = 108.8, P < 0.01) and allowed to differentiate decolorizing power ability even inside the two categories. For example, different from the other DEO, CHA8 revealed a singularly higher decolorizing power, which confirmed the specific supplier declaration of presence of high mesopores. Among DEC, CHA5 achieved the highest percentage of subtraction (almost 55%; Figure 3).

Charcoal's riboflavin removal in white wine

In a recent study, CHA was recognized as the best fining agent for removal of RF (Fracassetti *et al*., 2017). Nevertheless, CHA should be carefully used in order to avoid undesirable effects on wine, such as depletion of color and flavor. Therefore, in this work five dosages of low-range CHA (between 0.5 g/hL and 10 g/hL) have been used for comparison. In a preliminary test performed with different contact periods, CHAs showed the best vitamin removal property after 24 h of contact (Figure SI-1); therefore, the CHA samples are compared after 24 h. In white wine, the results evidenced that DEC have a better performance than DEO at all tested doses, achieving about 90% of RF removal (Figure SI-2). As reported for other absorption kinetics, RF showed a positive but nonlinear trend of reduction with respect to CHA concentration increment (Ribéreau-Gayon *et al*., 2006). Differences between decolorizing and deodorizing CHAs were statistically significant at all doses, and drop in doses corresponded to a progressive decrease in differences between average of categories (Figure SI-2). As indicated by Fracassetti *et al*. (2017), in real wine even the highest CHA concentration didn't allow the complete removal of RF. In fact, Fracassetti *et al*. (2017) reported that after 24 h of contact a large-pore CHA was able to remove 100% RF at 5 and 10 g/hL in a model wine, while it reached only 58% and 71% of RF removal in Chardonnay wine. When compared with Fracassetti *et al*. (2017), the decolorizing CHAs studied in the present work showed higher RF removal capacity in real wine. This phenomenon probably depends on differences in wine compositions because of grape varieties and winemaking processes.

Recently, it has been demonstrated that decreasing the final RF concentration below 50 µg/L drastically reduced the risk of light-struck development (Fracassetti *et al*., 2019), and therefore this concentration was chosen as a threshold for the evaluation of efficacy of CHAs. DEC assured sufficient removal of RF at 10 g/hL and 5 g/hL (residual RF concentration of 13.58 ± 5.24 µg/L and 31.25 ± 9.01 µg/L, respectively). On the other hand, DEO permitted to achieve the threshold only after treatments with the highest doses and only with two CHAs (CHA4 and CHA8 with a residual RF of 39.61 ± 2.80 µg/L and 34.95 ± 2.49 µg/L, respectively; Figs 4a and 4b). The percentage of RF reduction in samples treated with DEC varied between 85% and 94%, with the only exception of CHA2, which is the worst color removal DEC. Among DECs, CHA5 evidenced the best performance, reducing the RF value from 104 µg/L to 5.71 µg/L. It could be observed that CHAs demonstrated interesting variation in their efficiency depending on the dosage. CHA5 showed a reduction of only 10%, passing from 10 g/hL to 5 g/hL, other two CHAs, namely CHA1 and CHA10, showed a reduction of about 15%, while CHA6, CHA7, and CHA9 showed a marked reduction (higher than 20%). CHA2 lost 18% of its removal ability passing from 10 g/hL to 5 g/hL. This resulted in a greater difference registered among DEC at 5 g/hL, which showed an RF removal varying between 63.2% and 88.6%, corresponding to 41–15 µg/L of residual RF (Figure 4a).

DEO could be divided into two subcategories. CHA4 and CHA8 achieved a maximum RF reduction of about 65%. Differently, CHA3 and CHA11 evidenced a very low RF removal, without exceeding 25% even at 10 g/hL, leaving a residual vitamin concentration of 82.78 ± 5.63 µg/L and 79.15 ± 0.21 µg/L, respectively (Figure 4b) in the investigated white wine. Differences in RF adsorption could be attributed to specific surface availability between the two categories as well as within DEO. It is well known that the main difference between DEC and DEO lies in pore size (Yahya *et al*., 2015). Even if mesopores and micropores seem to be the main contributors in CHA's removal power, by extending the surface area, macropores could represent an indispensable way for large molecules to achieve internal surfaces. Recently, it has been demonstrated that reduction in large-size pores affects CHA affinity toward methylene green, a cationic dye showing high similarity to RF molecule structure (Tran *et al*., 2017). Most likely, pore configuration could also affect RF permeability and consequently its removal, confirming the previous finding of having close relationship between pore size and RF permeability (Kisler *et al*., 2001). Color intensity of white wine was evaluated after treating with 10 g/hL of CHA in order to quantify color depletion (CD) at 420 nm. Color depletion significantly differed between CHA categories ($F_{(1,31)}$ = 40.42, P < 0.05; data not shown); on average, DEO removed 5%, while DEC removed 8% of color. Pearson's correlation analysis between percentage of RF removal and color depletion at 10 g/hL revealed a positively significant correlation (r = 0.77, P < 0.05), which indicated that RF and flavan-3-ols/flavonoids have similar interaction with CHA. Concerning this, Gogoi and colleagues (2010) suggested that the catechin sequestration by activated carbon depended on external

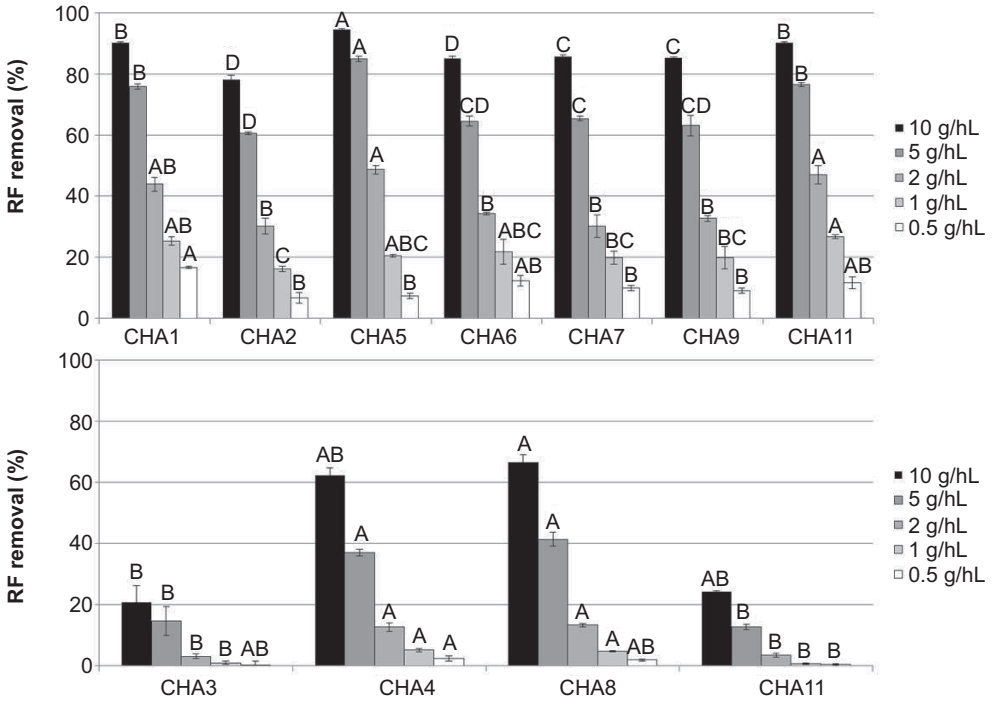

Figure 4. Removal of riboflavin in white wine. (a) Wine treated with decolorizing CHAs. Different capital letters identify statistically significant differences within carbon according to *post hoc* Games–Howell test (CI = 0.95, dosage 10 g/hL) and Tukey test (*P* > 0.05, other doses). (b) Wine treated with deodorizing CHAs. Different capital letters identify statistically significant differences within carbon according to *post hoc* Dunn test (CI = 0.95, dose: 10 g/hL and 0.5 g/hL) and Tukey test (*P* > 0.05, other doses). Mean values and standard deviations of three replications are expressed.

physicochemical parameters, for example, pH and competing compounds present in the solution that could interfere in the solute–sorbent association. This linkage was supposed to occur as an equilibrium between the hydrogen bonding (less important in the water environment because of high number of water hydrogen bonds) and the π-electron interaction between phenol ring and carbon backbone, which directly determines the bond strength.

Charcoal's RF removal in red wine

Quantity of RF in wine directly depends on grape variety and winemaking process (Cataldi *et al.*, 2002). On average, red wines revealed higher vitamin content with respect to both white and rosè wines. Although the light-struck phenomenon is not relevant in red wines, it has been demonstrated that RF-mediated oxidation could play a role in degradation of anthocyanins (Kim *et al.*, 2010). Moreover, CHAs are more often used in fining of red wine (Lisanti *et al.*, 2008). Therefore, CHA's ability to remove RF in red wine is of particular interest. As before, RF quantification was performed after 24 h of treatment, samples were kept in dark condition, and five dosages were tested. As before, DEC resulted in a higher RF removal capacity than DEO at all selected concentrations (Figure SI-3). The best performance was shown by DEC at 10 g/hL with a significant difference from DEO ($F_{(1,32)}$ = 53.82, $P < 0.05$). In general, CHAs evidenced a reduced ability of RF removal in red wine in comparison to white wine; in fact, DEC registered less than 25% of RF removal at the highest concentration. This effect could depend on other wine components which interfere in the RF–CHA interaction. These compounds likely belong to phenolics, which have been studied for their high affinity to CHA (Dąbrowski *et al.*, 2005). Deodorizing CHAs revealed no RF removal capacity in red wine; on the contrary, they seem to display a slight RF protection, as the final RF content in the treated samples was slightly higher than in the control (Figure SI-3). It is widely reported that spontaneous degradation of RF occurs during the first few hours of opening of bottle (Mattivi *et al.*, 2000) and that this phenomenon is accelerated in organic solvents (Sheraz *et al.*, 2014). Even by keeping the samples in the dark, a slight RF degradation occurred during the treatment; however, the presence of DEO seemed to prevent this degradation, as the RF content was greater at higher CHA concentrations. It can be assumed that the same RF degradation occurred in all the samples; although in DEC-treated samples the RF removal masked this effect, in DEO-treated samples the removal power was too low.

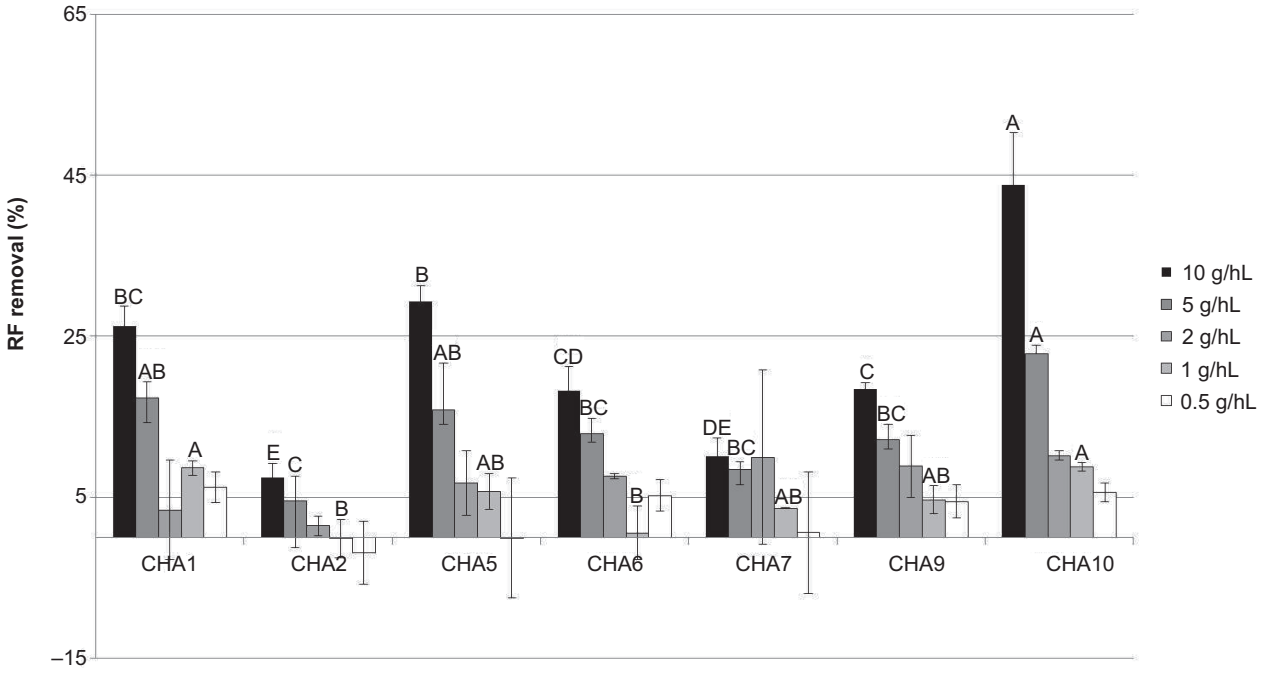

Figure 5. Riboflavin removal of decolorizing CHAs in red wine. Different capital letters identify statistically significant differences within DEC according to *post hoc* Tukey test ($P > 0.05$) at the dosages of 10 g/hL, 5 g/hL, and 1 g/hL. Mean values and standard deviations of three replications are expressed.

Even in DEO-treated samples, differences between categories were statistically significant at all dosages.

The analyses of individual CHAs belonging to DEC group revealed strong differences in their behaviors. In particular, CHA10 was identified as the best one for RF removal. In fact, it is the only one that reduced the RF value by 43% at 10 g/hL (corresponding to a final concentration of 77.96 ± 9.11 µg/L in treated wine; Figure 5). Considering the initial RF concentration of 138 µg/L, it means that about 60 µg/L was removed, similar to the depletion recorded in white wine at 5 g/hL. Two CHAs, namely CHA1 and CHA5, exhibited limited ability, reaching a little more than 25% of removal (Figure 5). In all other cases, the RF sequestering was very low. Color depletion at 10 g/hL significantly differed between CHA categories ($F_{(1,31)} = 85.19$, $P < 0.05$); DEO removed on average 2%, while DEC removed 9% of color intensity. The correlation between percentage of removed RF and color depletion at 10 g/hL highlighted a positively significant correlation ($r = 0.88$, $P < 0.05$). This suggests that the interaction between RF and polyphenols with CHA is characterized by a similar binding mechanism. In a dynamic equilibrium, this mechanism depends on the ionic strength (and pH) of the solvent that probably involved electron donor–acceptor interactions between the aromatic phenolic ring and the surface oxygens, dispersion effect between the aromatic phenolic ring and the π electrons of the graphitic structure, and, if ions are present, then electrostatic attraction and repulsion (Dąbrowski *et al.*, 2005).

Conclusion

This work explored the potential of commercial BENs and active CHAs in lowering of RF in real wines. BENs showed a reduced ability to sequester RF which appeared independent from their shied cation nature, and thus from their swelling properties. Among 11 different commercial BENs, only a sodic-calcic BEN (BENT5) revealed an interesting higher ability in RF removal. This work represents the first study in which a sodic-calcic BEN has been tested for this purpose. The future studies would define whether SOD-CAL matrices could play a role as RF removal agents. On the other side, activated CHAs confirmed their high attitude to remove RF, which for the first time was tested in both white and red wines. A great difference between deodorizing and decolorizing CHA was registered in both wines, which probably depends on CHA porosity. Additionally, in comparison to white wine, CHA RF removal was dramatically reduced in red wine. On average, decolorizing CHAs revealed 87% of RF removal in Glera wine to 22% in Wildbacher wine. This phenomenon has been attributed to complex interactions between CHA and wine phenols. Phenolic acids,

flavan-3-ols, and flavonoids could establish ʌ-electron interactions between phenolic ring and the active binding sites of CHA, and therefore could represent direct RF competitors. This phenomenon could also explain different RF removal efficiency of CHA fining in real wines (present work) compared to a model wine (Fracassetti *et al.*, 2017). To date, it is not clear whether the phenolic compounds belonging to different classes have the same effects on CHA–RF interaction.

Acknowledgment

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Supplementary information

Removal of Riboflavin by Commercial Bentonites and Charcoals in White and Red Wines

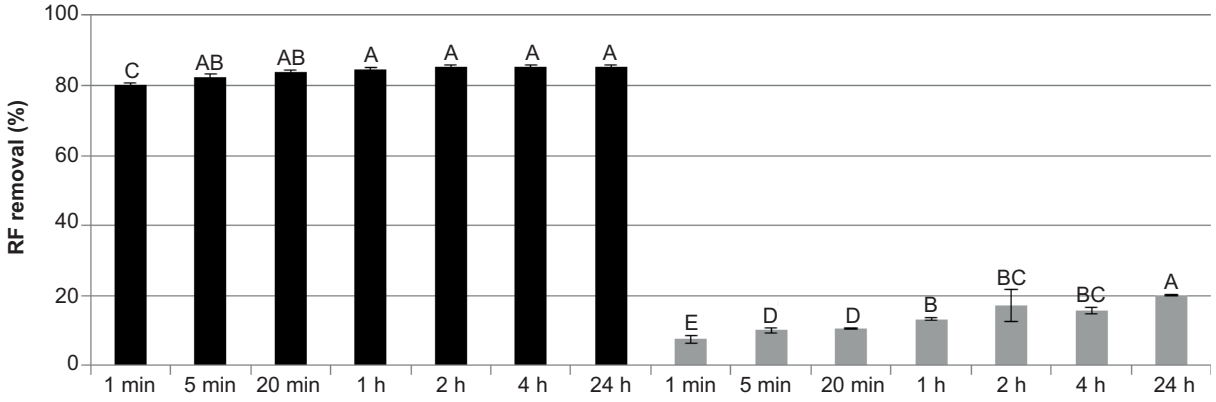

Figure SI-1. Trend of removal of riboflavin with CHA5 and CHA3 in white wine. Mean values and standard deviations are expressed. Each test was repeated for three times. Black: decolorizing CHA (CHA5), and gray: deodorizing CHA (CHA3). Different capital letters identify statistically significant differences within category according to Tukey test (*P* < 0.05).

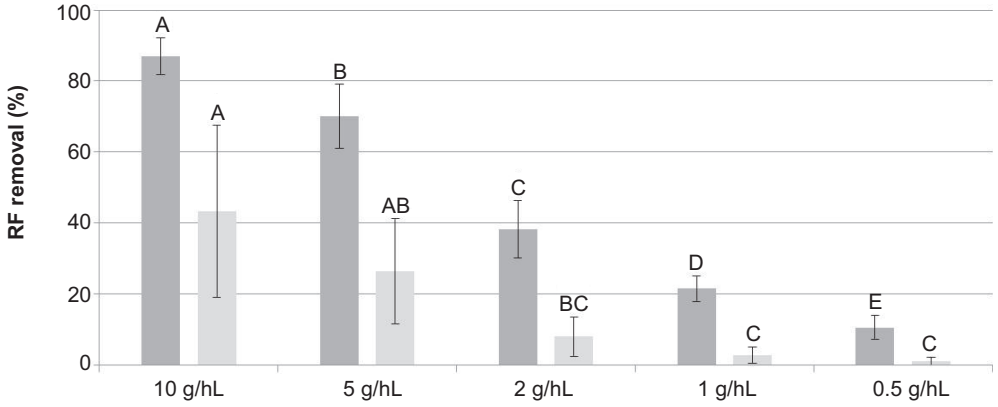

Figure SI-2. Comparison of removal of riboflavin with CHAs in white wine. Mean values and standard deviations are expressed. Each test was repeated for three times. Gray: decolorizing CHA, and light gray: deodorizing CHA. Different capital letters identify statistical significant differences within categories according to *post hoc* Dunn's test (CI = 0.95). CI: Color intensity.

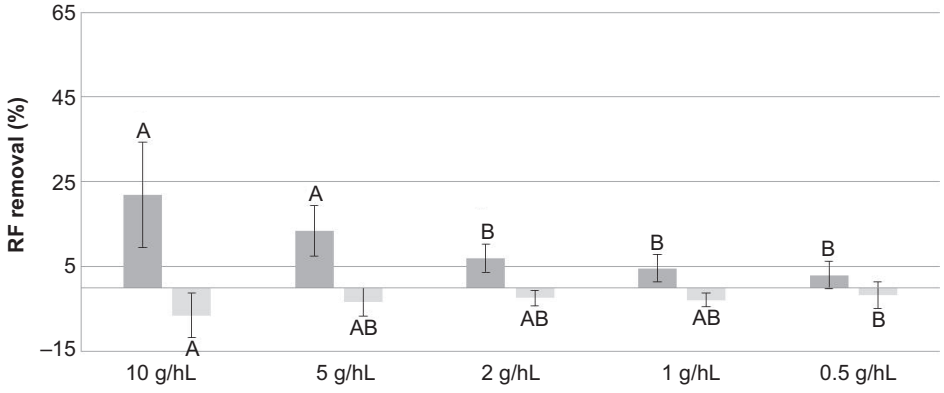

Figure SI-3. Comparison of removal of riboflavin with CHA in red wine. Mean values and standard deviations are expressed. Each test was repeated for three times. Gray: decolorizing CHA, and light gray: deodorizing CHA. Different capital letters identify statistically significant differences within category according to Dunn's test (CI = 0.95) and Tukey test (P < 0.05) respectively. CI: Color intensity.

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The influence of starter cultures on the lactic acid bacteria microbiota of Petrovac sausage

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PAPER

Abstract

Petrovac sausage (*Petrovská klobása*) is a high-quality fermented dry sausage produced traditionally in the municipality of Bački Petrovac (Vojvodina, Serbia). The product is characterised by specific and recognised texture, aroma and colour, produced without additives or preservatives. Lactic acid bacteria (LAB) microbiota plays an important role in production of the sausage. The aim of the paper is to monitor the changes in LAB during the production of Petrovac sausage. Samples of sausages were prepared without and with the addition of starter culture *Staphylococcus xylosus* as well as combined starter culture *Lactiplantibacillus plantarum* and *S. xylosus*, and produced at two different temperature ranges. A total number of 495 strains were isolated from 33 samples of Petrovac sausage during 120 days of production process. Characterisation of the isolates was performed by phenotypic tests, while molecular identification of the representative strains was done by 16S ribosomal DNA sequencing. The total number of LAB was about 8 log (Colony Forming Unit (CFU))/g in all samples, while the number of staphylococci was about 4 log CFU/g. Molecular identification confirmed that all isolates belonged to the following species: *Levilactobacillus brevis*, *Leuconostoc mesenteroides*, *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus. Lactobacilli* and *Leuconostoc* spp. dominate the total LAB strains, while *P. pentosaceus* was isolated at the lowest frequency.

Keywords: fermented sausages, lactic acid bacteria, Petrovac sausage, 16S rDNA sequencing

Introduction

The production of fermented sausages correlate with the diversity of microbiota present in meat batter as well as those added in the form of starter culture (Cocconcelli and Fontana, 2008; Toldra, 2002). Starter cultures contribute to the functional properties of fermented products and play a major role in the improvement of organoleptic, technological, nutritional and health characteristics of fermented sausages (Laranjo *et al.,* 2017). Different types of microorganisms (Lactic Acid Bacteria [LAB], staphylococci and micrococci, mould, and yeasts) can be used for autochthonous and commercial starter cultures in the production of fermented sausages (Casaburi *et al*., 2008; Kovacevic *et al.,* 2010). Combination of *Lactobacillus* spp. and *Staphylococcus* spp. used as starter cultures in the sausages production can contribute to the pleasant aroma of sausages and they possess antimicrobial properties against unwanted microorganisms and pathogen microbiota (Hosseini and Pilevar, 2017). Owing to acidification, lipolysis, and proteolysis, and production and development of volatile aroma compounds, LAB microbiota (*Lactobacillus sakei*, *Lactiplantibacillus pentosus*, *Lactobacillus curvatus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Levilactobacillus brevis* etc.) play an essential role of a starter culture in meat fermentation (Ammor and Mayo, 2007; Kumar *et al.,* 2017). Production of lactic and acetic acid reduces pH of the meat batter resulting in the formation of characteristic sausage aroma and consistency. The acidification process plays an important role in the inhibition and inactivation of pathogenic microorganisms contributing to the prolonged shelf life and safety of fermented sausages (Leistner, 1995; Martinovic and Veskovic-Moracanin.,

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2006). LAB species can produce bacteriocins as antimicrobial products of fermentation. *L. sakei*, *L. curvatus, L. plantarum* and *L. paracasei,* which are often used as starter cultures, contribute to the safety and stability of fermented sausages because of strong antibacterial activity against *Escherichia coli* and *Listeria monocytogenes* (Pidcock *et al.,* 2002; Veskovic-Moracanin, 2010). In addition to the safety of product, some strains of lactobacilli used as starter cultures (*L. sakei*, *L. curvatus* and *L. plantarum*) promote the degradation of peroxide (Martinovic and Veskovic-Moracanin., 2006).

Gram-positive cocci used as starter cultures (*Staphylococcus carnosus*, *Staphylococcus xylosus* and *Micrococcus varians*) play an important role in the reduction of nitrates and nitrites, decomposition of peroxides, lipolysis stabilisation and development of texture (Skocińska *et al.,* 2016). *S. carnosus* and *S. xylosus* as starter cultures contribute to the development of desirable colour and aroma in fermented sausages. Owing to antioxidant properties, growth on optimal salt concentrations and growth on optimal pH, *S. carnosus* and *S. simulans* are often used as starters in fermented sausages (Casaburi *et al*., 2005).

Dry-fermented sausages represent the result of physical, chemical, biochemical, microbiological and sensory changes that occur during the ripening of meat batter (Hammes *et al.,* 2008). Petrovac sausage (*Petrovská klobása*) is a traditional dry-fermented product made in Bački Petrovac (Vojvodina, Serbia). As a high-quality fermented product with appropriate texture, aroma and colour, it is produced without additives or preservatives, and protected by Protected Denomination of Origin (PDO) at the national level (Ikonic *et al*., 2015; Petrovic *et al.,* 2007). Petrovac sausage can be produced without adding starter cultures (Danilovic *et al*., 2018). The traditional production excludes the addition of starter cultures (Ikonic *et al*., 2016; Jokanovic *et al*., 2017, 2010).

The aim of this work was to monitor the changes in LAB microbiota in the samples of Petrovac sausage (*Petrovská klobása*) prepared without and with the addition of starter culture *S. xylosus* and combined starter cultures *L. plantarum* and *S. xylosus* and produced under controlled conditions in two different temperature ranges. For this purpose, isolation, characterisation and identification of LAB microbiota were performed.

Materials and methods

Fermented sausage technology and sampling procedure

Fermented sausages were produced according to the traditional recipe in the Agro-Industrial Complex (AIC) 'Bačka Topola' (Vojvodina, Serbia). Meat batter was made of minced pork (85%) and solid back fat tissue (15%) with addition of the following ingredients (w/w): red hot pepper (2.5%), salt (1.8%), garlic (0.2%), caraway seeds (0.2%) and sucrose (0.1%). The meat batter was divided into three equal parts: control sausages (H) (without addition of starter), sausages (I) (with the addition of combined starter cultures of *S. xylosus* and *L. plantarum*) and sausages (J) (with the addition of starter culture of *S. xylosus*). The initial number of starter cultures in meat batter was the same for LAB and coagulase-negative cocci (CNC) (4.5–5.0 log (CFU)/g). Autochthonous starter cultures were previously isolated from traditionally produced Petrovac sausage (Danilovic, 2012). The mixture was stuffed into artificial collagen casings. Smoking, drying and ripening of the sausages were carried out under controlled conditions in the ripening chamber at the temperature range of 14–16°C (tag 1) and ~10°C (tag 2). All experiments were performed in triplicate. Samples were collected after 0 (meat batter), 6, 15, 60, 90 and 120 days of production.

Isolation and enumeration of bacteria

For microbiological analysis, 10 g of each sausage sample was aseptically homogenised in 90 mL of sterile saline peptone water (8 g/L NaCl + 1 g/L peptone) (Urso *et al*., 2006). The enumeration of microorganisms was performed in triplicate by the successive serial dilution method and represented as the mean value. Dilutions were prepared and plated on nutrition agar (NA, Torlak, Belgrade, Serbia), de Man, Rogosa and Sharpe (MRS) agar (Torlak, Belgrade, Serbia) and Mannitol Salt Agar (MSA) plates for determining the total number of mesophilic bacteria, LAB and staphylococci, respectively. After the incubation of plates (48 h, 30°C) and enumeration, randomly selected colonies from MRS agar plates were streaked to new MRS agar plates for purification.

Phenotypic identification and characterisation of LAB isolates

Basic characterisation of the isolates was performed through Gram reaction, cell morphology and catalase test with H_2O_2 (30% v/v). Gram-positive and catalase-negative isolates were subjected to the following physiological tests: CO_2 production, arginine and esculin hydrolysis, bacterial growth on MRS agar plates at different temperatures (15°C and 45°C) for 72 h, bacterial growth on MRS agar plates supplemented with NaCl (4%, 6.5% and 8%) for 72 h, bacterial growth on bile esculin agar, synthesis of exopolysaccharides and the synthesis of bacteriocines.

Arginine hydrolysis was performed in arginine broth (g/L: tryptophan 5, L-arginine 3, glucose 0.5 and K_2HPO_4 2), while esculin hydrolysis was performed in esculin broth (Torlak, Belgrade, Serbia). After incubation, a few drops of phenyl-red were added to the arginine broth (red colour indicates a positive reaction, and yellow colour a negative one), and a few drops of 2% $FeCl_3$ solution to the esculin broth (a positive reaction is the appearance of a black precipitate). For preliminary identification of enterococci, isolates were grown on bile esculin agar (Rocheux's Medium, Himedia, Mubai, India). The appearance of black colonies indicate the presence of enterococci. Exopolysaccharide production was detected visually (appearance of mucous colonies) after incubation of isolates on a modified MRS medium supplemented with maltose, sucrose, galactose, fructose, lactose and glucose (Merck GmbH, Darmstadt, Germany) at a temperature of 30°C for 48 h.

The bacteriocinogenic activity was performed using the agar well diffusion assay. Soft nutrition agar (0.7% w/v), containing indicator strain, was poured into plates with thin layer of MRS agar. After hardening of the medium, small diameter wells (10 mm) were made into plates. Into each well, aliquot (50 µL) of the supernatant of overnight culture (16 h) was poured. Also, a crystal of pronase E was added close to the edge of the bacteriocin-containing well. The plates were incubated at 30°C for 24 h. Appearance of a clear inhibition zone around the well was recorded as a positive signal for production of bacteriocin. For detecting bactericiongenic activity, *Bacillus subtilis, Listeria monocytogenes* and *E. coli* were used as pathogenic microorganisms. Production of bacteriocin against any of the analysed strains was stated as positive.

Molecular identification of LAB isolates

Isolation of the total genomic DNA as well as (GTG)5-PCR fingerprinting was performed as described previously (Nikolic *et al.*, 2008). For 16S ribosomal DNA (rDNA) sequencing method, PCR amplifications with primers UNI 16SF (5'-GAG AGT TTG ATC CTG GC-3) and UNI 16SR (5'-AGG AGG TGA TCC AGC CG-3') were performed with a Taq DNA polymerase kit (Fermentas UAB, Vilnius, Lithuania). The amplification of the samples was performed through GeneAmp® PCR system 2700 (Applied Biosystems) operated with the following parameters: the initial duration of DNA for 7 min at 95°C, 32 cycles of denaturation of 1 min at 94°C, polymerisation with a duration of 8 min at 65°C, and the final extension of incomplete product with a duration of 16 min at 65°C. Plasmide profiles were monitored on 1.5% (w/v) agarose gel with ethidiumbromide at a constant voltage of 60 V (at 4°C for 20 h) (Versalovic *et al.*, 1994). Visualisation of PCR products was performed by applying CCD camera Biometra BDR2/5/6 (Bio Doc Analyze). Specific PCR products were analysed by electrophoresis on 1% agarose gel and purified using QIAquick PCR Purification KIT/250 (Qiagen, Hilden, Germany). Purified PCR amplicons were sequenced using Macrogen sequencing service in Seoul, South Korea. The results were compared with the data stored in the National Centre for Biotechnology Information (NCBI) gene databank using BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST).

Results and discussion

Petrovac sausage is an indigenous fermented sausage produced of minced meat and spices without preservatives with specific and recognisable characteristics. The sausage fermentation process is greatly affected by the changes in the development and composition of LAB and staphylococci microbiota. In order to determine the changes in LAB microbiota during the production of Petrovac sausage with the addition of starter cultures, sausage samples were prepared without starter culture (sausages H), with combined starter culture *L. plantarum* and *S. xylosus* (sausages I) and with starter culture *S. xylosus* (sausages J). Production of the sausages was performed under controlled conditions at a temperature range of 14–16°C (tag 1) and ~10°C (tag 2).

During the production of Petrovac sausage, the change in the number of mesophilic bacteria was almost identical to the change in LAB regardless of using starters. In the sausages prepared without starter cultures (sausages H), the number of initial LAB and aerobic mesophilic bacteria was about 5 log CFU/g, while the number of staphylococci ranged about 4 log CFU/g. The maximum value of LAB and aerobic mesophilic bacteria (8–9 log CFU/g) was reached after 15 days and it remained stable till the end of the production process. The number of staphylococci at the end of the process was lower and was about 3 log CFU/g (Figure 1H). Similarly, in the sausages prepared with combined starter culture of *S. xylosus* and *L. plantarum* (sausages I), the initial number of LAB was almost identical to the initial number of aerobic mesophilic bacteria (about 5 log CFU/g). During the production of sausages I, similar changes in the number of both LAB and aerobic mesophilic bacteria were observed as in sausages H. The number of staphylococci in sausages I was in the same range as in sausages prepared without starter cultures (3–4 log CFU/g) (Figure 1I). In sausages prepared with the addition of starter culture *S. xylosus* (sausages J), the number of staphylococci at the end of production was higher in all samples produced at 14–16°C (about 3 log CFU/g) than in the samples produced at ~10°C (about 2 log CFU/g). Changes in the number of LAB and aerobic mesophilic bacteria were almost identical as in sausages I (Figure 1J).

The number of LAB in sausages produced without starter culture (H) during the first days of fermentation was in accordance with the results obtained for Tunisian dry-fermented sausage produced without starter culture (4.3 log CFU/g). However, the initial number of LAB in sausages I and J (about 5 log CFU/g) was lower than the number of LAB obtained for Tunisian sausages produced with combined starter culture of *S. xylosus* and *L. plantarum* (7.3 log CFU/g) (Essid and Hassouna, 2013). The rapid increase in LAB during the first days of fermentation is also in accordance with the rapid increase in dry-fermented poultry sausages prepared without starter cultures (8.3 CFU/g), with starter culture of *S. xylosus* or *L. plantarum* (8.9 CFU/g) and mixed starter culture of *S. xylosus* and *L. plantarum* (8.8 CFU/g) (El Adabi *et al.,* 2014). Also, the maximum level of number of LAB during the first 15 days (8–9 log CFU/g) was in accordance with the results obtained for Tunisian sausages produced with combined starter culture of *S. xylosus* and *L. plantarum* (8.1 log CFU/g) (Essid and Hassouna, 2013). Rapid increase in the total number of aerobic mesophilic bacteria in all samples during the first days of production process was in accordance with the results obtained for the samples of Petrovac sausages produced under traditional conditions (5–8.5 log CFU/g) and for the samples produced under controlled conditions (5–7 log CFU/g) (Danilovic *et al.,* 2018). The total number of aerobic mesophilic bacteria was in

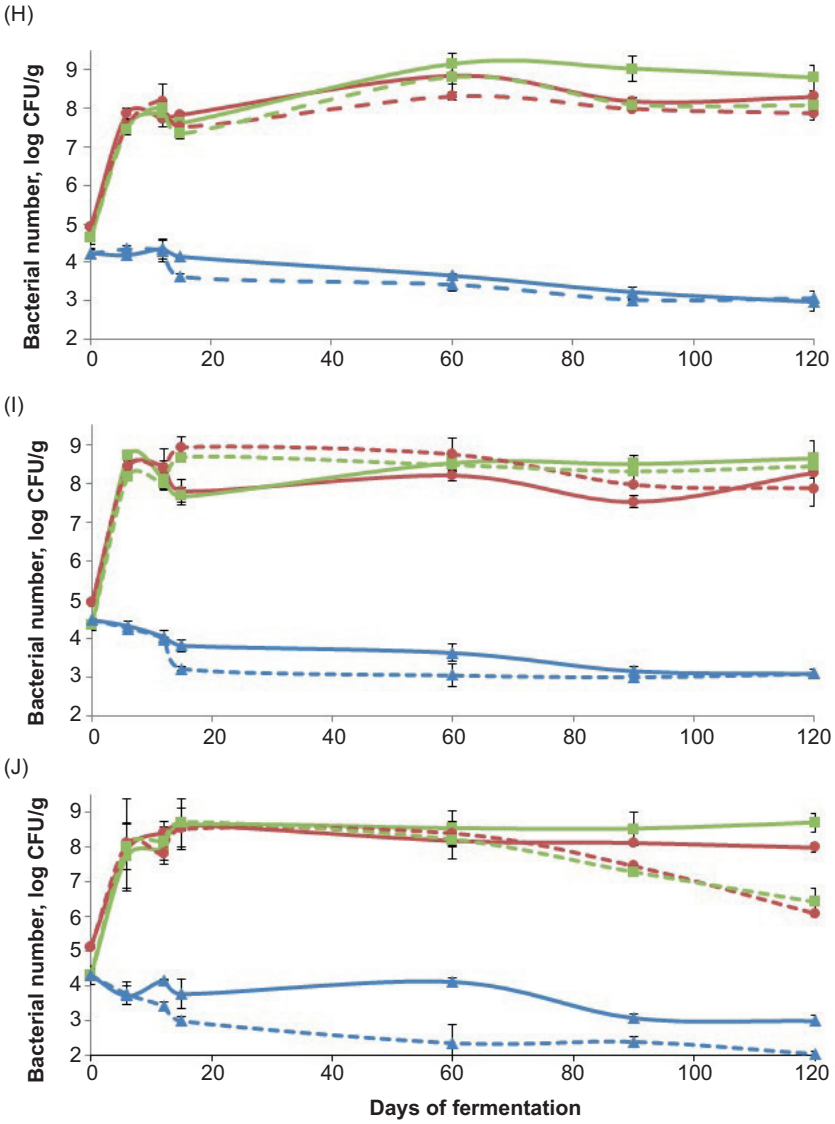

Figure 1. The number of aerobic mesophilic bacteria (●, red line), LAB (■, green line) and staphylococci (▲, blue line) during the production of Petrovac sausages prepared without starter cultures (H), with combined starter culture *S. xylosus* and *L. plantarum* (I), and with *S. xylosus* (J) and produced at 14–16°C (full line) and ~10°C (dashed line). Vertical error bars represent standard deviation.

accordance with the results obtained for Petrovac sausages produced under traditional and controlled conditions (7–8 log CFU/g; Danilovic *et al.*, 2018) and for the sausages produced from hot deboned meat (Danilovic *et al.*, 2011). Results obtained for Petrovac sausages indicated that LAB microbiota, being the dominant microbiota during the production process, were in accordance with the results of Casaburi *et al.* (2008), Casquete *et al.* (2012) and Zdolec *et al.* (2008). Domination of LAB microbiota in Petrovac sausages was in accordance with the results obtained for Alheira-fermented sausage produced in Portugal (Albano *et al.*, 2009), traditional Greek dry-fermented sausages (Ambrosiadis *et al.*, 2004; Papamanoli *et al.*, 2003), dry-fermented sausages produced with *L. sakei* (Bolumar *et al.*, 2006) and Tunisian dry-fermented beef sausage produced with combined starter culture of *S. xylosus* and *L. plantarum* (Essid and Hassouna, 2013). The initial number of staphylococci in sausages H, I and J (about 4 log CFU/g) was lower than the number of the same microbiota in Tunisian beef sausage produced without starter culture (5 log CFU/g) and with combined starter culture of *S. xylosus* and *L. plantarum* (7 log CFU/g) (Essid and Hassouna, 2013). The lower number of staphylococci at the end of production process was probably due to reduction in pH caused by lactobacilli (Johansson *et al.*, 1994; Lizaso *et al.*, 1999). Addition of starter culture had no effect on the total number of staphylococci. The higher number of staphylococci in the samples produced at higher temperature range (14–16°C) than the number presented in samples produced at lower temperature (~10°C) was in accordance with the results obtained for Italian fermented sausages, where the growth of *S. xylosus* was better at higher temperatures (Fiorentini *et al.*, 2010). Other results confirmed that increasing temperature from 10°C to 26°C increased growth of *S. xylosus*, *S. carnosus* and *S. equorum*, with strong synergy between temperature and pH (Søndergaard and Stahnke, 2002). The number of both aerobic mesophilic bacteria and LAB was identical regardless of the addition of starter cultures *S. xylosus* and *L. plantarum*. These results were in accordance with the results obtained for Tunisian dry-fermented sausages produced with the addition of starter cultures *S. xylosus* and *L sakei* (Najjari *et al.*, 2020).

A total of 495 Gram-positive and catalase-negative strains were isolated from 33 samples during the production of Petrovac sausage. Phenotypic grouping of strains by cell morphological characteristics divided all isolates into five groups (Table 1). The identity of the isolate was confirmed by (GTG)5-PCR and 16S rDNA sequencing. The 16S ribosomal RNA (rRNA) gene sequence analysis confirmed that all isolates belonged to *L. brevis*, *L. mesenteroides*, *L. plantarum* and *P. pentosaceus* species. DNA analyses of the PCR-amplified 16S rRNA gene fragments obtained from purified isolates during sausage production provided the fingerprints shown in Figure 2. The (GTG)5 fingerprints didn't show intraspecific biodiversity.

Gram-positive, catalase-negative and rod-shaped cells were classified as lactobacilli. Arginine-negative group of lactobacilli had the ability to grow well at 15°C and in the presence of 6%, 5% and 8% of NaCl. This group didn't

Table 1. Characterisation of LAB isolated during the production of Petrovac sausage.

Group	I	II	III	IV
No. of isolates	188	172	131	4
Cell morphology	rods	Rods	coccoid	cocci
CO_2 formation	–	–	+	–
Growth at				
45°C	–	–	–	–
15°C	+	+	+	+
Growth on NaCl				
4%	–	–		–
6.5%	+	+	+	+
8%	+	+	+	+
Hydrolysis of arginine	–	–	–	–
Hydrolysis of esculin	–	–	+	+
Black colonies on bile esculin agar	–	–	–	–
production of EPS from sucrose	–	+	+	–
Production of bacteriocines	v	V	–	v
Identified by 16S rDNA gene sequencing	*L. brevis*	*L. plantarum*	*L. mesenteroides*	*P. pentosaceus*

'+': positive; '–': negative; 'v': variable, 'EPS': exopolysaccharides.

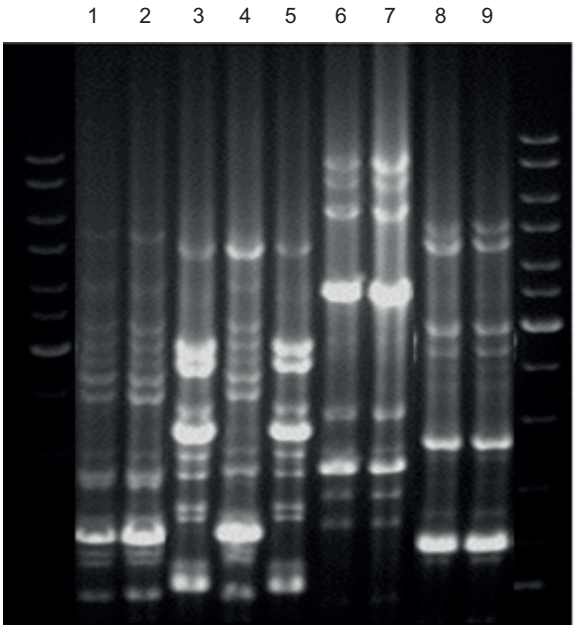

Figure 2. Reference PCR profiles of the amplified 16S rRNA gene of the isolates: *L. brevis* (1, 2, 4), *L. plantarum* (3, 5), *P. pentosaceus* (6, 7) and *L. mesenteroides* (8, 9).

produce CO_2 and was not able to grow at 45°C. On the basis of morphological characteristics, two groups of lactobacilli were observed. (GTG)5-PCR fingerprinting (Figure 2) confirmed that two groups belonged to *L. brevis* and *L. plantarum.* Some *L. brevis* and *L. plantarum* strains synthesised bacteriocines, which was in accordance to the data found in literature that these species could be active against *L. monocytogenes* (Tosukhowong *et al.*, 2011). Also, nitrite-reduction capability is one of the most important characteristics of *L. brevis* (Paik and Lee, 2014). Additionally, *L. plantarum* leads to rapid decrease of pH in fermented sausages and contributes to the organoleptic properties of the fermented product (Heinz and Hautzinger, 2007).

The arginine-negative and esculin-positive isolates that produced CO_2 from glucose and had the ability of forming slimy colonies on MRS agar plates with sucrose were identified by 16S rDNA sequencing as *L. mesenteroides. Leuconostoc* spp. produce lactic acid, acetic acid, dextran, acetaldehyde, diacetyl, ethanol and other metabolites that contributes to the development of aroma and flavour in production of fermented sausages (Lee *et al.,* 2006). As heterofermentative strains, *Leuconostoc* spp. produce CO_2, which is considered as one of the main causes in forming holes in meat products; this property classifies them as undesirable microbiota (Ammor and Mayo, 2007). *Leuconostoc* spp. may synthesise spectra of bacteriocines (mesentericin Y105, produced by *L. mesenteroides* spp. *mesenteroides*; leucocin A-UAL 187, produced by *L. gelidum*; carnosin 44A, produced by *L. carnosum*; and leuconocin S, produced by *L. paramesenteroides*) that exhibit strong microbial activity against *Listeria* spp (Stiles, 1994). The prevalence of *Leuconostoc* spp. in sausages is in correlation with the results obtained for Petrovac sausages produced from hot deboned meat (Danilovic *et al,* 2011) as well as for sausages ripened under the traditional and controlled conditions (Danilovic *et al.*, 2018).

Only four isolates (0.8%) were esculine-positive cocci. They all had the ability to grow at 15°C as well as on MRS agar plates with addition of NaCl (6%, 5% and 8%). Some of cocci produced bacteriocines (Table 1). Esculine-positive cocci, which formed tetrads, were identified by 16S rDNA sequencing as *P. pentosaceus* (Figure 2). As a result of low catabolism of amino acids, pediococci don't play a major role in the formation of organoleptic properties in fermented sausages (Leroy *et al*., 2006). Among pediococci, *P. acidilactici* and *P. pentosaceus* were often isolated from European sausages (Albano *et al.* 2007; Kozachinski *et al.* 2008). *P. acidilactici* produced pediocin that inhibits the growth of food-borne pathogens *L. monocytogenes* and *Clostridium perfringens* in Spanish dry-fermented sausages (Nieto-Lozano *et al*., 2010). In addition, *P. pentosaceus* showed strong inhibitory effect against *S. aureus* (Erdogrul *et al*., 2002). *P. pentosaceus* and *P*. acidilactici are commonly used as starters in the United States in producing dry sausages (Rantsiou and Cocolin, 2006). Besides bacteriocines, some strains of pediococci produce EPS (Semjonovs and Zikmanis, 2008). The low frequency of isolation of pediococci is correlated with the results obtained for Bosnian Sudzuk, Alheira sausage and Croatian sausage (Albano *et al.,* 2009; Kozachinski *et al.,* 2008). In Petrovac sausages produced from hot deboned meat, pediococci were isolated at the highest percentage after ninth day of production process (Danilovic *et al.,* 2011).

Total isolated LAB microbiota constituted *L. brevis* (37.9%), *L. plantarum* (34.7%), *L. mesenteroides* (26.4%) and *P. pentosaceus* (0.8%). Sausages prepared without starter cultures (H1 and H2) were characterised by the prevalence of *leuconostoc* spp. during the first 15 days of fermentation regardless of temperature. Complete replacement of *leuconostoc* spp. was observed after 15 days and lactobacilli were the dominant microbiota. On the 60th day of production process, *L. plantarum* rapidly increased up to 80% in sausages H1, while in sausages H2, almost equal distribution of *L. brevis* and *L. plantarum* was detected. Later stages of production process were characterised by the prevalence of *L. plantarum. P. pentosaceus* was isolated only from sausages H2 in a 90-day-old sample with a representation of 1.4% (Figure 3). On the other hand, in sausages prepared with the addition of combined starter cultures of *S. xylosus* and *L. plantarum* (sausages I1 and I2), the highest percentage of

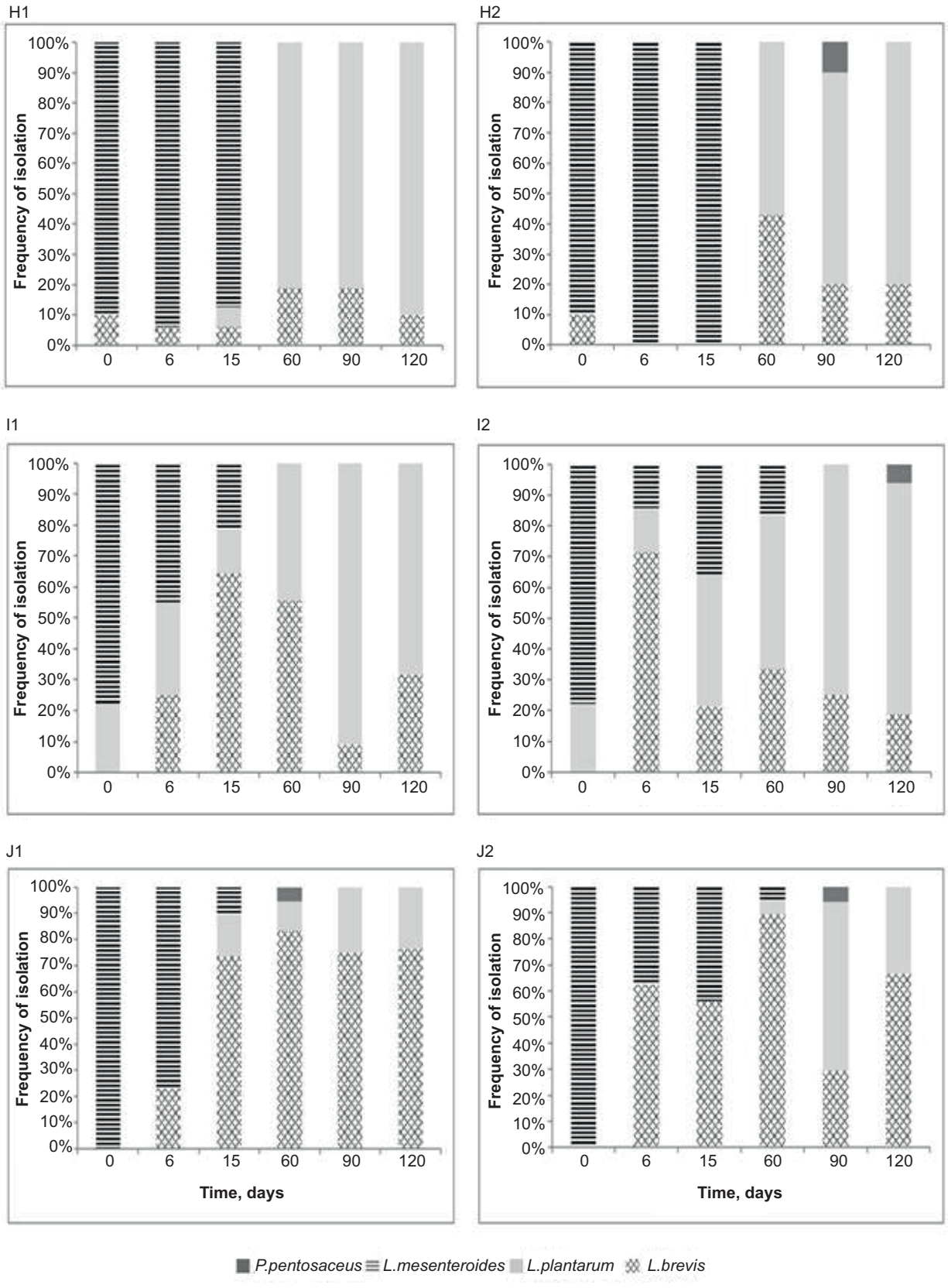

Figure 3. Changes in microbial population during the production of Petrovac sausages prepared without starter cultures (sausages H), with combined starter cultures of *S. xylosus* and *L. plantarum* (sausages I) and with starter culture of *S. xylosus* (sausages J) produced at 14–16°C (samples with tag 1) and ~10°C (samples with tag 2).

leuconostoc strains was detected only in the meat batter. *Leuconostoc* spp. decreased immediately after preparation of sausage mixture but remained still up to the 15th day of production in sausages I1 and up to the 60th day of production in sausages I2. After this period, depletion of leuconostoc strain was observed and lactobacilli were the dominant microbiota (Figure 3). Pediococci were isolated only at the end of production process in sausages I2 with a share of 1%. In sausages prepared with the addition of starter culture of *S. xylosus* (sausages J1 and J2), the domination of *L. brevis* was detected at all stages of production process except in the meat batter, where the full presence of *L. mesenteroides* (100%) was detected. *P. pentosaceus* was detected on the 60th and 90th day of production in sausages J1 and J2, respectively. This increase in the content of lactobacilli was observed during production, with the presence of 100% lactobacilli in the sample after 120 days of production.

During the production of Petrovac sausage, the prevalence of *L. mesenteroides* was observed in the meat batter prepared with and without adding starter cultures. Also, *L. mesenteroides* strains were present during the early stages of fermentation process regardless of temperature. The high frequency of *L. mesenteroides* at the beginning of production process was in accordance with the results obtained for Serbian traditional fermented sausages Sremski kulen, Lemeski kulen (Vasilev *et al*., 2015) and Užička sausage (Borovic *et al*., 2017). On the contrary, these results were not in accordance with the results obtained for Italian fermented sausage (Comi *et al*., 2005; Urso *et al*., 2006), where low frequency of *leuconostoc* spp. was detected at the beginning of the production process. Regardless of the production conditions, in all sausages, lactobacilli were the dominant microbiota from 15 days till the end of production process. This is in accordance with the results obtained for Užička sausage (Borovic *et al*., 2017). Also, the high frequency of lactobacilli was presented in Sremski and Lemeški kulen (77.1 and 54.3%, respectively). *L. brevis* was the most dominant lactobacilli species in these sausages (61.5% and 57.9%, respectively) (Vasilev *et al*., 2015). High frequency of *L. brevis* was in accordance with the results obtained for traditional fermented Užička sausage (Borovic *et al*., 2017); *P. pentosaceus* was isolated in the smallest percentage in the final stages of production, while in sausages ripened under traditional and controlled conditions, pediococci were present only in the meat batter (1.7% of the total microbiota) (Danilovic *et al.,* 2018). Pediococci were isolated in small percentage from Iberian dry-fermented sausages—Salcichon and Chorizo (Benito *et al.,* 2008) and Italian fermented sausages—Salami (Bonomo *et al.,* 2008). On the contrary, Pediococci were isolated at high frequency from the fermented sausages produced in the United States, where *P. acidilactici* and *P. pentosaceus* are commonly added as starter cultures (Anba-Mondoloni *et al*., 2015).

Conclusion

Petrovac sausage is an artisanal Serbian sausage appreciated for its sensory characteristics. In order to preserve the quality of the industrial production process, there is a need to understand the effect of starter cultures on the level of microbiota and composition. The results indicate that application of starter culture *S. xylosus* and combined starter culture *S. xylosus* and *L. plantarum* didn't influence the total number of LAB during process. On the other hand, temperature range of 14–16°C increased the number of staphylococci, compared with the application of ~10°C temperature. Comparison of the effect of different starter cultures with the composition of microbiota resulted in the achievement of similar microbiota composition as for traditional sausages when combined starter culture was used. According to the results, combined starter culture of *S. xylosus* and *L. plantarum* could be the most promising solution for the production of Petrovac sausage, although further sensory analysis is required to be conducted.

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Quality and safety evaluation of new tomato cultivars

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PAPER

Abstract

This paper is aimed to provide a quality and safety assessment of new cherry tomato cultivars (*Solanum Lycopersicum var. cerasiforme*): *Bamano, Dulcemiel*, and *Sugarland.* Eight biogenic amines, total phenolics, total carotenoids, lycopene, and antioxidant activity (2,2-diphenyl-1-picrylhydrazyl [DPPH] and 2,2-azino-bis (3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt [ABTS] assays) were determined. Comparison with control cultivars demonstrated lower pH values, and total contents of biogenic amines and antioxidant compounds while having higher soluble solid concentration. Moreover, multivariate statistical analyses (principal component analysis and cluster analysis) were applied to the results. Different results allowed for a successful differentiation of new cultivars. Therefore, the chosen compounds resulted in suitable markers for quality and safety assessment of tomatoes.

Keywords: antioxidants, biogenic amines, carotenoids, food safety and quality, phenolics, tomato

Introduction

Tomato (*Solanum Lycopersicum*) is an annual plant whose berries are used widely, either processed or raw, for food and beverage. Tomato plants are native to South America, and their cultivation in the Mediterranean countries dates back to the 17th century (Peralta and Spooner, 2014). Italy is the first tomato-producing country in Europe and one of the top 10 producers in the world (Food and Agriculture Organization Corporate Statistical Database [FAOSTAT], 2019). Italy also tops the list for global export of processed tomatoes ahead of China (Istituto Servizi Mercato Agricolo Alimentare, 2017). In Italy, tomatoes are cultivated especially in the central and southern regions, where small-size tomato varieties are much appreciated (Masetti *et al.*, 2014; Carillo *et al.*, 2019). Tomato is of great value in the global vegetable consumption, and recently, there has been an increase in the spread of new small-size tomato cultivars. Small-size tomatoes are preferred for fresh consumption than regular-size tomatoes, and consumers choose the same for their organoleptic proprieties (Liu *et al.*, 2019). Tomato is one of the most studied crops, and their genetic improvement is constant. The breeding programs led to the offspring of new varieties that can meet the industry and/or consumer preferences.

Moreover, through new cultivars, disease resistance is achieved. Recently, new hybrid cultivars of tomatoes named *Bamano*, *Dulcemiel*, and *Sugarland* have been introduced in Central Italy. These cultivars are trying to expand the fresh agronomic market with products characterized by unique organoleptic and nutritional properties. Usually, seed companies evaluate prime properties (e.g., size, color, sugars, etc.) under different stress and environmental conditions, followed by researchers' early characterization (Ingallina *et al.*, 2020c). However, in order to valorize the final product, quality and safety assessment is highly recommended. Quality assessment is necessary to determine molecular markers, typical of

a sample, that can establish the sample's origin or the good state of storage (Giuggioli *et al.*, 2016). Antioxidant compounds are usually used to evaluate food quality (Armenta and de la Guardia, 2016). Phenolic compounds, secondary metabolites of many plants, are ubiquitous in the vegetable domain and they are one of the most extensively studied groups of natural compounds. Their dietary intake is highly recommended, and they have anti-microbial and anti-carcinogenic effects (Coyago-Cruz *et al.*, 2018). These compounds have already been detected in good quantity in commercial tomatoes, especially in small-size varieties (Selli *et al.*, 2014). Tomatoes have a significant antioxidant activity afforded by phenolic compounds and antioxidants such as carotenoids, lycopene, and vitamins (Szabo *et al.*, 2018). Total phenolic content, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assays are established as quick and robust tools for characterizing antioxidants in the fractions of hydrophilic tomatoes. They are used widely in an explorative and preliminary assessment of vegetables and fruits (Fanasca *et al.*, 2006; Preti *et al.*, 2017). Besides, to characterize the antioxidants present in the lipophilic fraction, such as carotenoids (including lycopene), Ultraviolet–Visible spectroscopy (UV-Vis) methods are used generally (Ingallina et *al.*, 2020a).

Other metabolites present in small amounts in food are often used as markers of food safety. Among these compounds, biogenic amines (BAs) are widely used as food safety markers because of their presence in food and their effect on the human body. BAs are the result of the decarboxylation of amino acids, but their presence in food can also be related to spoilage. In addition, BAs can induce several negative physiological reactions, and the investigation of their levels in food is important for consumers' health and the formulation of diets (Kalač, 2014). Some BAs, such as histamine (HIS) and tyramine (TYR), pose potential risks to human health, that is, 'scombroid food poisoning' and 'cheese crisis' (Al Bulushi *et al.*, 2009). However, not all BAs are dangerous for human health. For example, serotonin (SER) plays an essential role to regulate mood, sleep, body temperature, sexuality, and appetite in the central nervous system (Hano *et al.*, 2017). Therefore, its presence in food could be an exciting feature. Notwithstanding that presence of BAs is regulated in some foods and drinks, some authors have suggested BAs to be food quality markers (Silla Santos, 1996).

In this work, a quality and safety assessment of three new tomato cultivars is proposed. At first, soluble solid concentration (SSC) and pH were determined to evaluate physicochemical characteristics of the tomato cultivars. Thereafter, an evaluation of antioxidants present in hydrophilic and lipophilic fractions was carried out. The hydrophilic antioxidant fraction was tested by *in vitro* antioxidant activity through scavenging of DPPH- and ABTS-free radicals and total phenolic content by the Folin–Ciocâlteu method. Total contents of carotenoids and lycopene were analyzed in lipophilic fraction by UV-Vis methods.

Moreover, the profile of eight BAs was evaluated in tomato samples by high-performance liquid chromatography with fluorescence detection (HPLC-FD) after dansyl chloride derivatization. The BAs studied were spermine (SPM), spermidine (SPD), putrescine (PUT), and cadaverine (CAD) for polyamines, whereas β-phenylethylamine (β-PEA), HIS, SER, and TYR were studied for monoamines. The above-mentioned analyses were also conducted on samples from two traditional cultivars of tomatoes used for fresh market and canning industry.

Finally, a multivariate statistical analysis (principal component analysis [PCA] and cluster analysis [CA]) was conducted on the bioactive compound profiles of tomatoes to highlight natural differentiation of samples coming from the new cultivars.

Materials and methods

Materials

Methanol (CH_3OH), n-Hexane (C_6H_{14}), water (HPLC grade), acetonitrile (HPLC grade), Folin–Ciocâlteu reagent ($H_3[P(W_3O_{10})_4]/H_3[P(Mo_3O_{10})_4]$), ABTS, DPPH, potassium persulfate, sodium bicarbonate ($NaHCO_3$), gallic acid ($C_7H_6O_5$), perchloric acid ($HClO_4$), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), and ammonium hydroxide (NH_4OH) were purchased from Sigma Aldrich Chemical Co. The eight BAs—HIS, SER, SPM, SPD, PUT, β-PEA, CAD, and TYR—were supplied by Supelco (Bellefonte, PA, USA) as well as the derivatizing agent, dansyl chloride, and the internal standard, 1,7-diaminoheptane (IS).

Sampling

Tomato samples were supplied by eight different farmers with similar pedo-climatic conditions, located in the south of Lazio region (Italy), which were harvested in 2016. Tomato seeds (Bamano and Dulcemiel) were supplied by Syngenta, Basel, Switzerland and Rijk Zwaan, De Lier, The Netherlands (Sugarland). Two samplings per cultivar were prepared for each farm. A total of 48 samples were collected (Bamano, n =16; Dulcemiel, n = 16; and Sugarland, n = 16). Moreover, other 11 samples were collected from selected cultivars for fresh market and canning industry. After acquisition, samples were

Table 1. Physical characteristics of new tomato cultivars.

Cultivar	Color	Size	Fruit weight	Picture
Bamano	Bright orange	Elongated shape (2.5 ± 0.5 × 4.5 ± 0.5 cm)	11 ± 1 g	
Dulcemiel	Green with honey shades	Round shape (2.5 ± 0.5 × 3.5 ± 0.5 cm)	15 ± 1 g	
Sugarland	Deep shiny red	Round shape (1.5 ± 0.5 × 2.5 ± 0.5 cm)	12 ± 1 g	

homogenized by an Ultra-Turrax system and stored at –18°C. The physical characteristics of each cultivar are reported in Table 1.

Physicochemical parameters

The SSC was determined with a portable refractometer (RS PRO; Milan, Italy) at 20°C and expressed as °Brix. The pH was measured with a pH-meter (Hach Company; Loveland, CO, USA).

Determination of Biogenic Amines

Extraction

Biogenic amines were extracted according to a previously optimized tomato products method (Chiacchierini *et al.*, 2006). About 8 g of sample, previously added with 0.1 mL of IS (100 mg/L), was extracted with 10 mL of 0.6 M $HClO_4$, homogenized for 3 min, and centrifuged at 2,700 *g* for 10 min. The supernatant was filtered through a 0.20-µm membrane Millipore filter and collected in a flask. The residue was added with 10 mL of 0.6 M $HClO_4$, mixed, and again centrifuged for 10 min. Then the second extract was filtered and added to the first one. The final volume was adjusted to 25 mL with 0.6 M $HClO_4$.

Derivatization

An aliquot of 1 mL of the final extract was derivatized according to procedures reported by Ingallina *et al.* (2020b). About 200 µL of 2M NaOH, 300 µL of saturated $NaHCO_3$ solution, and 2 mL of dansyl chloride solution (10 mg/mL in acetone) were added in a tube. After shaking, the samples were left in dark for 60 min at 45°C. About 100 µL of 25% NH_4OH was added to stop the derivatizing reaction. The final volume was adjusted to 5 mL by adding acetonitrile. The dansylated extract was filtered using 0.22-µm filter (Polypro Acrodisc, Pall Gelman Laboratory, USA) and injected into the HPLC system (Ingallina *et al.*, 2020b).

Chromatographic setup

Chromatographic separation was achieved by a system consisting of a LC-10 ATVP binary HPLC pump, a Supelcosil LC-18 column (Supelco, 5-µm particle size, 150 × 2.1-mm I.D.) equipped with a Supelguard LC-18 guard column (Supelco Inc., Bellefonte, PA, USA), and an RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan). The injector was fitted with a 20-µL loop. The chromatographic data were collected and processed using Class-VP software (Shimadzu). The analysis was conducted as described in previous work. Fluorescence detection was set at 320 nm for excitation and 523 nm for emission. Identification of the BAs was based on their retention time and adding of standards. The quantification was performed using the internal standard calibration method by linear regression analysis ($R^2 > 0.995$).

Extraction of hydrophilic antioxidant compounds

Sample extractions for antioxidant activity and total phenolic content were prepared from 2 g of tomatoes in 20 mL of methanol. Samples were homogenized in an Ultra-Turrax for 3 min and centrifuged at 2,400 *g* for 5 min (Fratoddi *et al.*, 2018).

Determination of total phenolic content (Folin–Ciocâlteu)

Total Phenolic Content (TPC) was determined using the Folin–Ciocâlteu method (Fratoddi *et al.*, 2018), modified for tomatoes as follows: 1 mL of methanolic extract was

added to 0.25 mL of Folin–Ciocâlteu reagent and 0.5 mL of Na_2CO_3 water solution (7.5% w/v) in a 10-mL volumetric flask. The final volume was reached with purified water. Spectrophotometric analysis was performed at λ = 750 nm after 45 min of incubation in dark at room temperature. TPC was expressed as milligrams of gallic acid equivalent (GAE) per kg. The final results were obtained through a calibration curve ranging from 15 to 500 mg/L (R^2 = 0.9925).

Determination of antioxidant activity

The DPPH and ABTS assays were based on the same mode of action, and they are common *in vitro* antioxidant tests (Tonolo *et al.*, 2019). The disappearance of radical was determined by measuring absorbance at 515 nm (DPPH) and 734 nm (ABTS) as described previously (Preti *et al.* 2017); the absorbance was measured in 1-cm path length cuvettes, using a UV-Vis spectrophotometer (Jenway, Stone, UK).

Results were expressed as inhibition rate and were calculated based on Equation 1:

$$I\% = \frac{A_0 - A_f}{A_0} \times 100, \quad (1)$$

where A_0 is the radical cation's initial absorbance, and A_f is the absorbance after the addition of sample extract.

Lipophilic antioxidant extraction

Briefly, 7 mL of ethanol:hexane mixture (4:3 v:v) was added to 0.1-g homogenized sample in a glass tube (protected from light). The lycopene extraction was conducted by agitating the mixture for 1 h (darkness) at 200 rpm. Thereafter, 1 mL of distilled water was added to the mixture and stirred by inversion. The hexane fraction was then collected in an amber vial.

Total carotenoids

The total carotenoid content was determined at 449 nm (Ingallina *et al.*, 2020a). The results were compared with a standard solution of β-carotene in n-hexane, and the quantification of total carotenoids was achieved by the linear regression (r^2 = 0.9962) and expressed as milligram of β-carotene (mg BCE).

Lycopene determination

The lycopene determination was performed by measuring the hexane phase absorbance at 472 nm in a spectrophotometer. The lycopene content was calculated with the Lambert–Beer Law as described in Equation 2:

$$\text{Lycopene (mg / kg)} = \frac{Abs \times MW \times 2.7}{w \times E}, \quad (2)$$

where *Abs* is the absorbance reading, *MW* is the molecular weight, 2.7 refers to the volume (in mL) of the hexane phase, *w* is the sample weight, and *E* is the molar extinction coefficient of lycopene in hexane (185.3 mM/cm). Results were expressed as mg/kg of lycopene (fresh weight, FW) (Antolinos *et al.*, 2020).

Statistical analysis

All the experiments were conducted in triplicate and expressed as mean ± standard deviation. T-test, correlations, and chemometric data analyses (PCA and CA) were performed with JMP software (ver. 15.2, SAS Institute, Cary, NC, USA).

Results and discussion

Physicochemical properties

The presence of phytochemicals in tomatoes, such as carotenoids and phenolic compounds, mineral salts, and organic and fatty acids content is closely related to their health-promoting properties. Therefore, these are used in quality and safety assessment. These compounds are biosynthesized and accumulated in fruits, and their content is influenced by environmental factors, cultural practices, and genetic aspects, such as different cultivars (Antolinos *et al.*, 2020). In this respect, pH and SSC were evaluated in the examined samples, and the results are reported in Figure 1. The new cultivar samples had significantly lower pH values ($P < 0.01$) compared to control cultivars, even if the difference was 8–10%. These results suggest a possible use of specific consumers satisfaction related to their organoleptic and sensorial features. Moreover, the highest SSC was found for Sugarland cultivar, followed by Bamano and Dulcemiel. The resulting SSC values of new cultivars were statistically different from that of the control, indicating greater soluble solid compounds.

Biogenic amines

The evaluation of BAs in fresh vegetables has been recently explored in literature, tomatoes included (Sánchez-Pérez *et al.*, 2018). According to Sánchez-Pérez *et al.* (2018), the BAs found in tomatoes were HIS (n.d.–22 mg/kg FW); TYR (n.d.–6.38 mg/kg FW); PUT (5.3–35.5 mg/kg FW), and CAD (n.d.–2.33 mg/kg FW).

In this study, contents of eight BAs were determined in three new cultivars of cherry tomatoes; their profiles are shown in Figure 2.

The chromatograms exhibited different trends for the three cultivars. An appreciable peak resolution was achieved (Palomino-Vasco *et al.*, 2019; Ramos *et al.*,

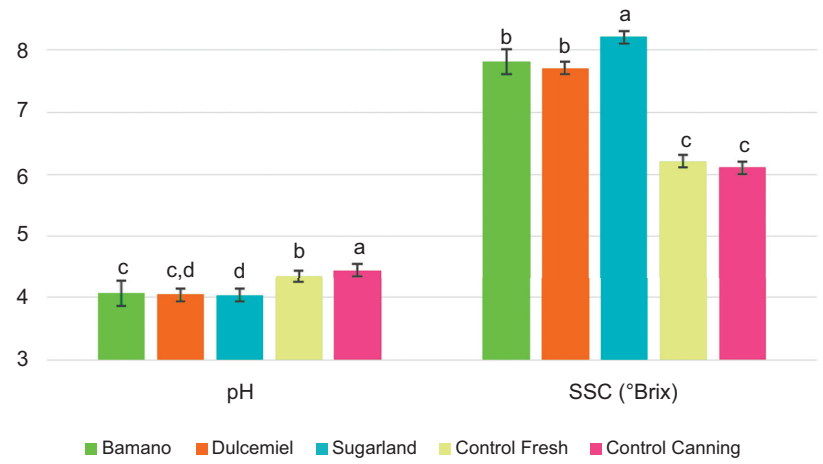

Figure 1. Determination of physicochemical properties of different tomato cultivars: pH and soluble solid concentration (SSC) (°Brix). Samples not connected by the same letter are significantly different.

2020). The quantification of BAs in new cultivars and control tomatoes is summarized in Table 2. Table 2 also describes the T-test results (α = 0.95) of each variable for the five categories of the sample analyzed.

Among new cultivars, the highest total BA contents in tomatoes was determined in Sugarland (275.2 ± 11.10 mg/kg), followed by Dulcemiel (201.01 ± 1.71 mg/kg) and Bamano (137.36 ± 1.98 mg/kg). These contents were comparable with the control canning cultivar, in spite of the fact that the fresh control had a higher total BA values.

The amount of HIS, PUT, and CAD of the new cultivars (<LOQ: 0.57 mg/kg, 0.16–5.75 mg/kg, and 1.15–2.41 mg/kg, respectively) was in agreement with results from literature, while TYR was below the limit of quantification for all the samples. Compared with the control cultivars, the HIS values were comparable with that of the canning cultivar and were lower than that of the fresh cultivar

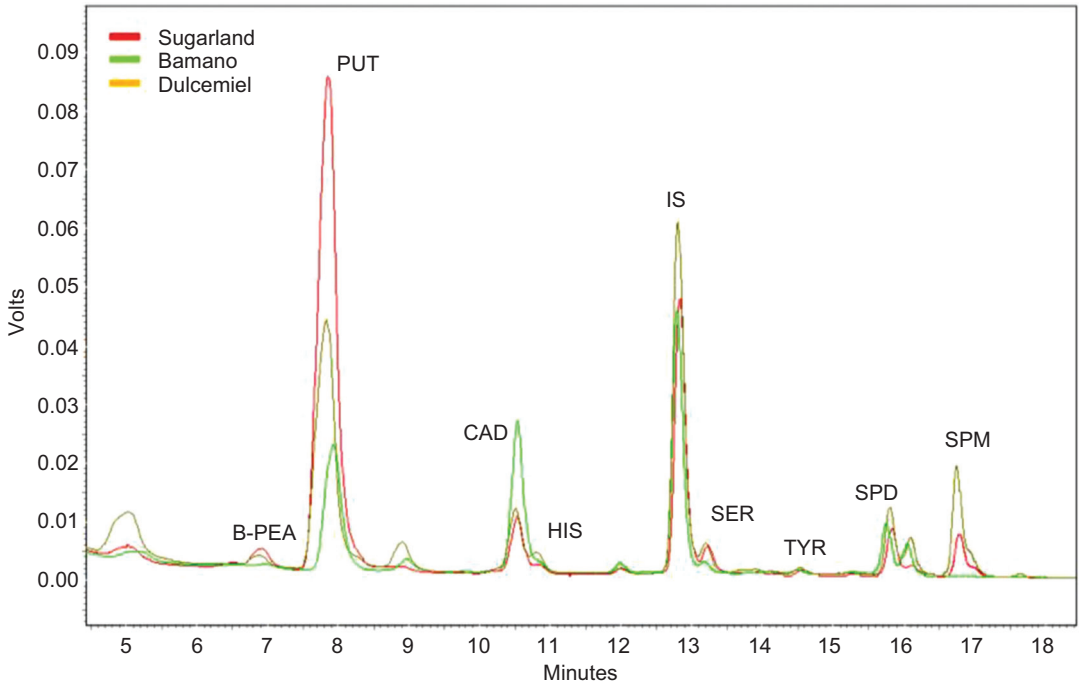

Figure 2. Chromatographic profiles of biogenic amines determined in tomato samples: Sugarland (red trace), Bamano (green trace), and Dulcemiel (dark yellow trace). β-PEA: β-phenylethylamine; PUT: putrescine; CAD: cadaverine; HIS: histamine; IS: internal standard; SER: serotonin; TYR: tyramine; SPD: spermidine; and SPM: spermine.

Table 2. Quantitative results of biogenic amines in tomato samples (mg/kg). Samples not connected by the same letter are significantly different.

	Bamano	Dulcemiel	Sugarland	Control fresh	Control canning
β-PEA	$1.16^{b} \pm 0.05$	$0.17^{c} \pm 0.001$	$0.17^{c} \pm 0.01$	$1.47^{a} \pm 0.12$	$1.13^{b} \pm 0.10$
PUT	$0.16^{c} \pm 0.01$	$0.56^{c} \pm 0.03$	$5.75^{b} \pm 0.14$	$11.17^{a} \pm 4.17$	$4.98^{b} \pm 0.21$
CAD	$2.41^{a} \pm 0.09$	$1.15^{b} \pm 0.01$	$1.22^{b} \pm 0.02$	$0.94^{c} \pm 0.15$	$0.70^{d} \pm 0.03$
HIS	$<LOQ^{c}$	$0.57^{a,b} \pm 0.02$	$0.33^{b,c} \pm 0.01$	$1.01^{a} \pm 0.41$	$0.10^{b,c} \pm 0.06$
SER	$132.47^{d} \pm 2.05$	$197.27^{c} \pm 1.71$	$266.87^{b} \pm 11.16$	$379.51^{a} \pm 4.06$	$146.81^{c,d} \pm 8.67$
TYR	$<LOQ^{c}$	$<LOQ^{c}$	$<LOQ^{c}$	$1.29^{a} \pm 0.09$	$0.67^{b} \pm 0.08$
SPD	$0.29^{b} \pm 0.01$	$0.37^{b} \pm 0.02$	$0.33^{b} \pm 0.01$	$8.32^{a} \pm 0.40$	$8.32^{a} \pm 0.85$
SPM	$0.87^{b} \pm 0.04$	$0.91^{b} \pm 0.06$	$0.53^{c} \pm 0.02$	$0.80^{b,c} \pm 0.03$	$1.16^{a} \pm 0.47$
Total BAs	$137.36^{d} \pm 1.98$	$201.01^{c} \pm 1.71$	$275.2^{b} \pm 11.10$	$404.53^{a} \pm 9.45$	$164.67^{c,d} \pm 10.53$

β-PEA: β-phenylethylamine; PUT: putrescine; CAD: cadaverine; HIS: histamine; SER: serotonin; TYR: tyramine; SPD: spermidine; SPM: spermine; total BAs: total biogenic amines; LOQ: limit of quantification.

(1.01 ± 0.41 mg/kg). Besides, in the control cultivar, TYR was also observed (0.67 – 1.29 mg/kg). It is essential to underline BAs' shallow levels such as HIS and TYR, frequently reported as dangerous in the human diet (Linares *et al.*, 2016). Although, the HIS and TYR contents were not dangerous, a lower concentration in new cultivars allowed products with lesser contamination at the processing stage.

Polyamines, such as SPD and SPM, play a role in increasing shelf life of tomatoes. The gene expression related to SPD and SPM would reduce the post-harvest senescence and decay (Handa and Mattoo, 2010; Nambeesan *et al.*, 2010). Thereafter, the low amount of SPD (0.29–0.37 mg/kg) and SPM (0.53–0.91 mg/kg) found in all new cultivars could be a desirable feature. They could be related to a natural over expression of some metabolic pathways in these cherry tomato varieties, contributing to the elongation of shelf life. Also, the lowest concentrations in new cultivars, compared to the control, suggest these tomato cultivars' eligibility in the supply chain.

Finally, an interesting remark should be made about the SER content. The SER content was the major contributor to the total contents of BAs established in the samples, starting from 132.47 ± 2.05 mg/kg (96%) for Bamano to 197.27 ± 1.71 mg/kg (98%) for Dulcemiel and 266.87 ± 11.16 mg/kg (97%) for Sugarland. These results were in agreement with already published results (Riga *et al.*, 2016). A similar trend in SER content was also found in control cultivars (90–93%), although in slightly lower proportions. However, the excellent SER content in tomato fruits is related to its several physiological functions in plants (e.g., growth regulator, protection against pathogens, etc.). In plants, SER is produced from tryptophan, and demonstrates some positive effects on the human body. Daily assumption of SER-rich vegetable varieties has demonstrated, *inter alia*, useful anti-obesity and anxiety control effects (Islam *et al.*, 2016). Moreover, it has been proved that the SER content tends to decrease in processed tomato products. Therefore, tomato cultivars relatively rich in SER could be of interest for the tomato industry (Hano *et al.*, 2017).

Antioxidants evaluation

Nowadays, several features, such as being rich in nutrients or having physiological benefits, are searched in foods. Among these, antioxidant compounds are the most interesting nutrients for human health, and are considerably present in fruits and vegetables (Dudonné *et al.*, 2009). Moreover, these compounds are widely used to evaluate food quality. The hydrophilic and lipophilic fractions were examined to evaluate antioxidants in new tomato cultivars.

TPC assay was chosen to quantify phenolics' content in hydrophilic fraction, essential components of antioxidant compounds in tomatoes (Fanasca *et al.*, 2006). The antioxidant activity was also tested by two different *in vitro* anti-radical assays—ABTS and DPPH (Campestrini *et al.*, 2019). Moreover, these two radicals are sensitive to different types of antioxidants. Consequently, their combined use consented to an effective evaluation of antioxidant activity.

The results and significant differences are shown in Table 3. For TPC, Sugarland had the highest results with 303.15 ± 21.62 mg GAE/kg, followed by Dulcemiel and Bamano (256.39 ± 6.63 and 242.18 ± 6.6 mg GAE/kg, respectively). TPC results were in accordance with previously reported tomato results, especially for cherry tomatoes (Raffo *et al.*, 2002; Riga *et al.*, 2016).

Table 3. Quantitative results of evaluation of antioxidants in tomato samples. Samples not connected by the same letter are significantly different.

	Bamano	Dulcemiel	Sugarland	Control fresh	Control canning
TPC (mg GAE/kg)	242.18^{d}± 6.60	256.39^{d}± 6.63	303.15^{c} ± 21.62	369.98^{b} ± 12.37	458.97^{a} ± 3.11
DPPH (I%)	88.61^{b}± 3.42	93.38^{a}± 3.47	91.25^{a} ± 1.36	93.46^{a} ± 1.34	92.04^{a} ± 0.74
ABTS (I%)	60.34^{b}± 1.92	26.63^{d} ± 1.54	32.50^{c}± 2.49	96.82^{a} ± 0.29	98.64^{a} ± 0.38
TCC (mg BCE/kg)	40.12^{c} ± 2.69	33.12^{d} ± 0.99	54.12^{b} ± 1.36	142.00^{a} ± 5.05	143.17^{a} ± 4.99
Lycopene (mg/kg)	29.25^{c} ± 7.48	12.12^{d} ± 1.25	48.88^{b} ± 2.95	127.80^{a} ± 1.79	128.50 ^{a} ± 1.38

TPC: total phenolic content; TCC: total carotenoids content; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: diammonium salt; GAE: gallic acid equivalent.

The trend of TPC results agreed with DPPH radical scavenging assay for antioxidant activity, proving a high radical inhibition by the three cultivars. The lowest result was achieved by Bamano cultivar (88.61, I%). A similar result was reported by Lu *et al.* (2020), who established that TPC values could be positively correlated with the DPPH values (Lu *et al.*, 2020). However, Bamano variety had demonstrated the highest ABTS scavenging activity results (60.34, I%), followed by Sugarland (32.50, I%) and Dulcemiel variety (26.63, I%). In Bamano samples, these results could be explained by a more significant presence of other chemical compounds with antioxidant activity not included in the phenolic compounds, such as vitamin C or anthocyanins (Pataro *et al.*, 2015; Marengo *et al.*, 2017).

Total carotenoids and lycopene contents were evaluated in the lipophilic fraction of antioxidants by UV-Vis methods. Among new cultivars, Sugarland had the highest content of carotenoids (54.12 ± 1.36 mg BCE/kg) and lycopene (48.88 ± 2.95 mg/kg), followed by Bamano (40.12 ± 2.69 mg BCE/kg, 29.25 ± 7.48 mg/kg) and Dulcemiel (33.12 ± 0.99 mg BCE/kg, 12.12 ± 1.25 mg/kg). These values of compounds in new tomato cultivars were compared with the literature data (D'Evoli *et al.*, 2013). It is also appropriate to highlight that lycopene is the major carotenoid in cherry tomatoes, representing 40–90% of the total carotenoid contents in new cultivars.

The values obtained were significantly lower than that of control, except for DPPH assay results for Sugarland and Dulcemiel cultivars.

Multivariate analysis

Different profiles of bioactive compounds found in tomatoes had suggested the hypothesis that some of the compounds detected for quality and safety assessment could also be typical of a cultivar (Uarrota *et al.*, 2014). Therefore, their presence as a potential authenticity marker of the tomato variety was investigated (Bajoub *et al.*, 2016). For this purpose, PCA and CA were used to explore data matrices in order to highlight a natural grouping among samples (Marengo *et al.*, 2017). Autoscaling pretreatment was conducted in the data matrix composed of experimental results (Nur Azira *et al.*, 2014). The PCA results are reported in Figure 3: Sugarland is represented by circles, Bamano by squares, Dulcemiel by crosses, control fresh by stars, and control canning by triangles. In PCA, the first two principal components (PC1 and PC2) accounted for 82.9% of the total variability (Liu *et al.*, 2013). In the scores plot, all cultivars were clearly separated (Šamec *et al.*, 2016). New cultivars are located in the left part of the diagram, Sugarland and Dulcemiel in the upper part, while Bamano in the lower one. Control cultivars were located on the right, fresh cultivar samples on the top, and the canning ones on the lower part. As highlighted by the scores plot, PC1 differentiated new cultivars (Sugarland, Bamano, and Dulcemiel) from the control. This PC was highly influenced by physicochemical properties (pH and SSC), carotenoids (including lycopene), phenolic compounds, and SPD and TYR for BAs. Separation among each cultivar was enabled by PC2, whereby BAs (SER, BAI, HIS, and β-PEA) and DPPH anti-radical assays were the major contributors.

To characterize new cultivars, PCA was recalculated by excluding control cultivars. The scores and loadings' plot of this analysis are given in Figure 4.

The loadings' plot pointed out for Dulcemiel samples was positively correlated with DPPH, SPD, and HIS variables. It is clear in Tables 2 and 3 that Dulcemiel cultivar had the highest content in these compounds. Sugarland demonstrated a positive correlation with SER, BAI, PUT, TPC, SSC, total carotenoids, and lycopene. Moreover, Sugarland had a high negative correlation with SPM. Samples of the Bamano cultivar were positively correlated with CAD, β-PEA, pH, and ABTS content. Therefore,

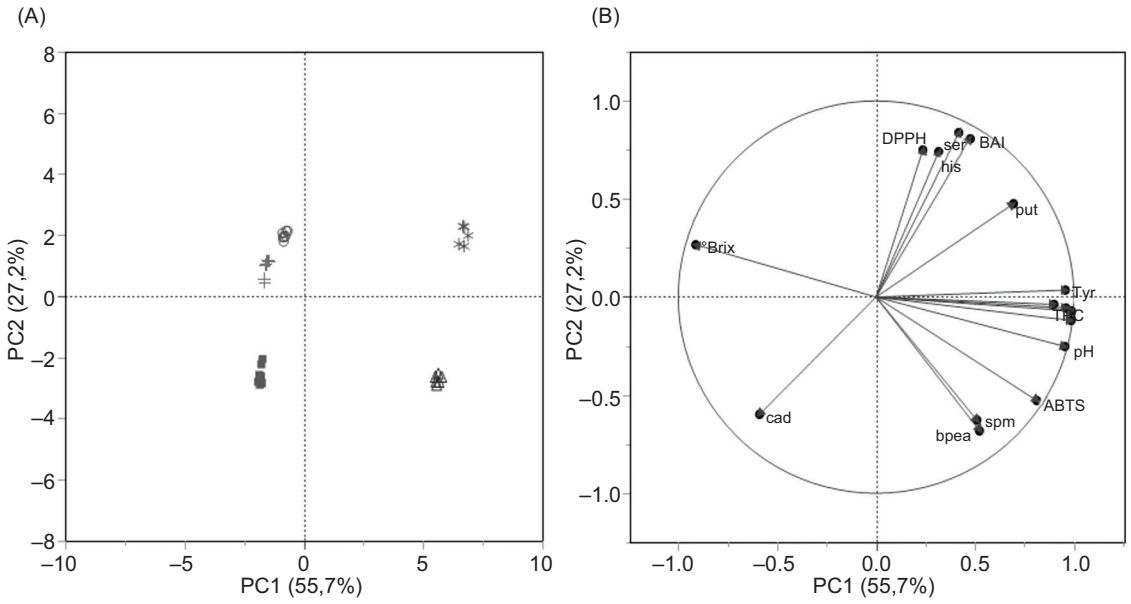

Figure 3. (A) Principal components analysis (PCA) scores and (B) loading plots of tomato samples: Sugarland (circles), Bamano (squares), Dulcemiel (crosses), control fresh (stars), control canning (triangles).

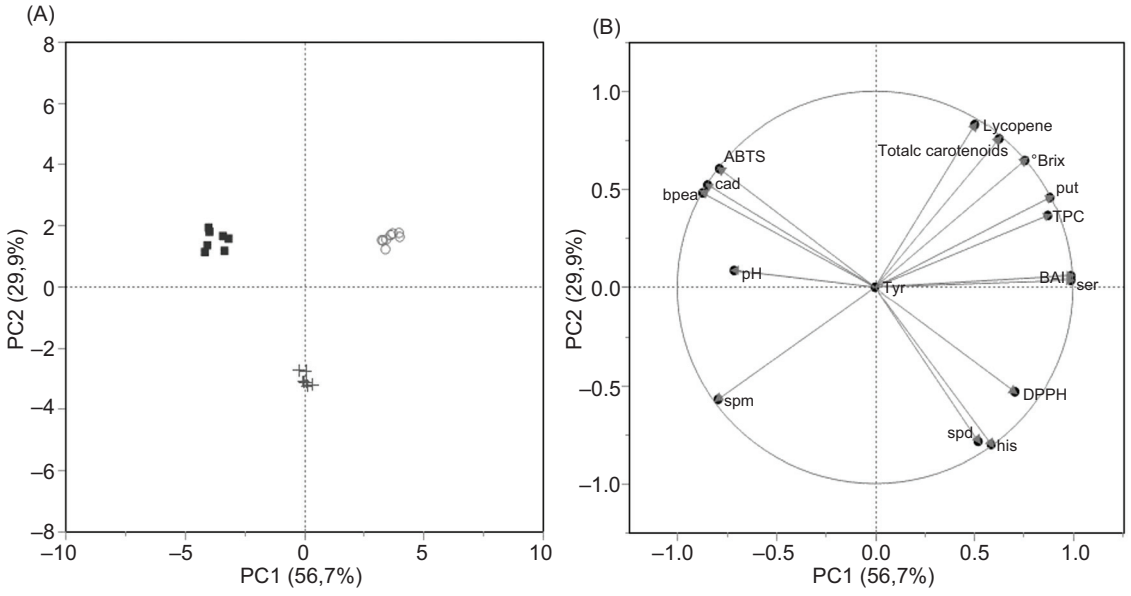

Figure 4. (A) Principal components analysis (PCA) scores and (B) loading plots of tomato samples: Sugarland (circles), Bamano (squares), and Dulcemiel (crosses).

results of the PC explorative analysis were in accordance with the experimental ones (Guerreiro *et al.*, 2013).

The good results of PCA analysis were also confirmed by CA, reported in Figure 5. This analysis pointed out general similarities or differences in the profile of bioactive compounds of the investigated cultivars. The first level of dendrogram demonstrates two clusters: the first one comprises control cultivars, and the second one by three new cultivars (Bamano, Dulcemiel, and Sugarland). At the lower level of dendrogram, the first cluster is divided in two parts by separating control cultivars as the samples used for fresh market and that for canning industry. The second cluster (three new cultivars) was also divided into two parts: the first part consisting of Bamano samples, and the other one comprising Dulcemiel and Sugarland samples. Therefore, these two cultivars exhibited similar contents to the compounds examined herein. CA and PCA results demonstrated differentiation among new cultivars and the control.

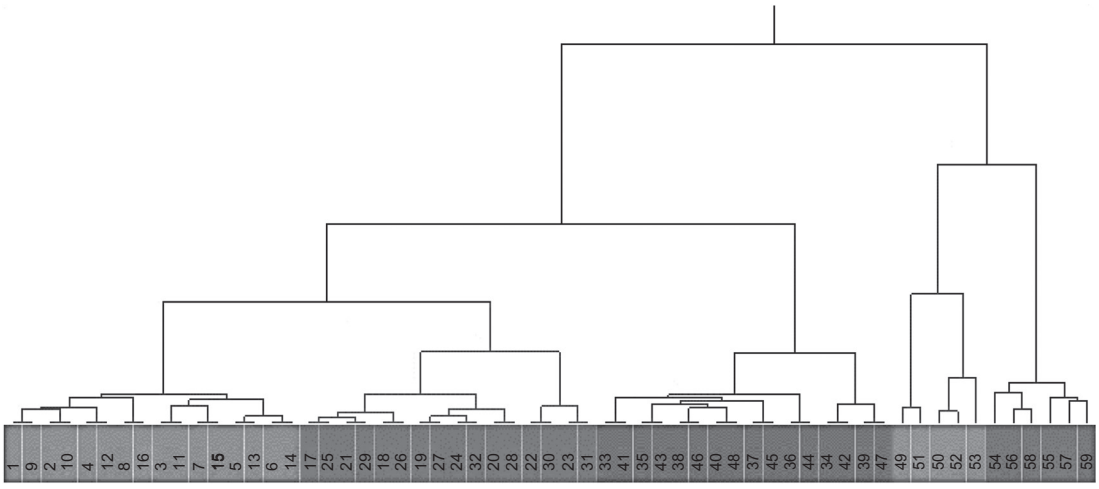

Figure 5. Cluster analysis: Sugarland (samples 1–16), Dulcemiel (samples 17–32), Bamano (samples 33–48), control fresh (samples 49–53), and control canning (samples 54–59).

Conclusion

This study assessed the quality and safety of three new cherry tomato cultivars through bioactive compounds evaluation. All the samples came from the same Italian region to minimize differences because of production factors (e.g., climate, soil, etc.). BAs and antioxidant fractions were investigated in Bamano, Dulcemiel, and Sugarland varieties, besides evaluation of physicochemical characteristics. The results were also compared with two control cultivars usually involved in the fresh market and canning industry. The new cultivars had meager amount of HIS (<LOQ 0.57 ± 0.02 mg/kg) and TYR (<LOQ) as well as an interesting amount of SER (132.47 ± 2.05 mg/kg–266.87 ± 11.16 mg/kg). Therefore, quality features were assessed in addition to the absence of spoilage indicators. The antioxidant evaluation was conducted by TPC, anti-radical assays, and total carotenoids and lycopene contents. Comparison with control cultivars demonstrated different physicochemical properties, lower content in total BAs, and lower antioxidant compounds. Finally, a chemometric evaluation of bioactive compounds was conducted by PCA and CA. Different profiles of analyzed compounds enabled a successful differentiation of new cultivars and the control. Therefore, the chosen bioactive compounds resulted in suitable markers for quality and safety assessment of analyzed samples. However, this research could be a good start for new possible investigations. Each variety needs additional experimentation for full characterization of antioxidant fraction and a shelf-life study to evaluate the post-harvest decay. An interesting aspect could be the application and assessment of these tomatoes in processed products.

Conflict of interest

The authors declare that they have no conflict of interest in the subject matter or materials discussed in this manuscript.

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Composition and nutritional evaluation of amino acids in *Mimai qu* rice wines

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Abstract

An amino acid analyzer was used to detect free amino acids (FAA) in *Mimai Qu* rice wines (SMW and DMW) and control wine samples (Chinese rice wine [CRW] and Japanese sake [JNS]). It was found that CRW had the highest total amino acid (TAA) content (~2814 mg/L), followed by SMW (~2509 mg/L) and DMW (~1474 mg/L), while JNS had the least (~917 mg/L). Amino acid ratio coefficient method (SRCAA), linear regression method, cluster analysis (CA) and principal component analysis (PCA) were used for evaluating the nutritional value of amino acids in wine samples, giving similar results. SMW had the highest nutritional value, followed by CRW and DMW and JNS.

Keywords: brewed wine, cluster analysis, linear regression analysis, principal component analysis, ratio coefficient method

Introduction

Mimai Qu rice wines, prepared from crushed wheat malt (25%) and steamed rice (75%) using the Japanese koji process with *Aspergillus oryzae* Su-16 and *Aspergillus oryzae* AOK139 as inoculum (China application patent No.: CN201810216324), is a novel fermentation starter (http://pss-system.cnipa.gov.cn/sipopublicsearch/portal/uilogin-forwardLogin.shtml). As for Chinese rice wine (CRW), the brewing properties of *Mimai Qu* are similar to those of traditional wheat *Qu*. However, *Mimai Qu* rice wine is made from purified microbes, and the hygiene of the production environment and food safety are much higher than that of wheat *Qu*. Mature *Mimai Qu* rice wine is white in color (the wheat *Qu* of CRW shows yellow color) with a delicate fragrance that is distinct from the complex aroma of wheat *Qu*. *Mimai Qu* rice wine is transparent, light yellow in color and has a fresh and elegant mild fruity flavor, but without the so-called "soy sauce flavor" of CRW (Wang and Xu, 2005). Its taste and flavor are between those of CRW and Japanese sake (JNS). A small-scale tasting survey conducted in the Sichuan Province of China revealed its popularity than CRW among young people.

Japanese Sake, a Japanese alcoholic beverage, is generally prepared from the polished Japonica rice and koji with an alcohol content of 13–17% v/v (Sato and Kohsaka, 2017). It has a refreshing taste and fruity aroma, and its market is widely distributed in Japan, the United States, Southeast Asia and some European countries (Mimura *et al.*, 2014). The production of JNS requires partial grinding of the outer layer of rice to produce "polished rice." The rate of polished rice refers to the ratio of the mass of polished rice to that of original mass. The lower the rate of polished rice, the lower the content of protein and lipid in rice, thereby making it more suitable for brewing high-quality sake such as Daiginjo (Okuda, 2019; Yamashita, 1997). CRW has originated in China and is one of the world's oldest alcoholic beverages with a history of thousands of years (Mcgovern *et al.*, 2004). It is brewed using glutinous rice, wheat *Qu* (fermentation starter) and water. CRW has a mellow and rich taste, and contains various nutrients such as amino acids,

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oligosaccharides, short peptides etc., and therefore is also called "liquid cake" (Wang, 1998). Currently, 90% of CRW consumption is concentrated in eastern China, including Zhejiang Province, Jiangsu Province and Shanghai, which pose regional restrictions for aging consumer groups (Jiao *et al*., 2017; Xu *et al*., 2013). After the 2010s, because of lack of innovation in the production process of CRW, its taste and flavor are not considered attractive by the current young and middle-aged consumers, thus gradually losing these huge main consumer groups (Jiao *et al*., 2017). In 2018, two *Mimai Qu* rice wines (DMW and SMW) were creatively developed using *Mimai Qu* and polished Japonica rice varieties as raw materials by combining the processing techniques of sake and CRW in our laboratory to attract young people and broaden the consumer groups and consumption areas of CRW.

Amino acids are basic components of protein, providing significant nutrition, and are divided into essential amino acids (EAA) and non-essential amino acids (NEAA) (Cui *et al*., 2014; Omar *et al*., 2017). Generally, men prefer to drink moderate-alcohol beverages, such as CRW, with an average intake of 1000–1500 mL/d (Yang, 2021), containing 3–5 g/d of free amino acids (FAA). Moreover, FAA is a non-negligible nutrition source, as it can be easily assimilated and utilized by the human body. Contents and composition of amino acids are important factors in the nutritional value of foods (González-Castro *et al*., 1997; Okada *et al.*, 2017). Therefore, this study intended to detect the content and composition of FAA in *Mimai Qu* rice wine, and assess its amino acid nutritional value using different analytical methods. This study would provide a theoretical basis for further analyzing the nutritional profiles of this novel rice wine.

Materials and Methods

Materials

Chinese Rice Wine (CRW): Traditional CRWs produced in the Shaoxing city of China were purchased from Wal-Mart Supermarket, Zigong City, Sichuan Province. The raw materials included glutinous rice, wheat *Qu* and water. The alcohol content of this wine was 13–16% v/v, and the total sugar content was 20–30 g/L. The age of the wine was 3–5 years. In all, three CRW wine samples, such as Guyuelongshan, Kuaijishan and Tapai, were analyzed in this study.

Japanese Sake: Sakes were purchased from Japan. The raw materials included water, Yamadanishiki rice and rice koji, with a polishing ratio of 50–70%. The alcohol content was 14–16% v/v and the total sugar content was 20–35 g/L. The age of sake was 1–5 years. Three sake wine samples were analyzed in this study: company A, B, and C produced in the cities of Yamaguchiken, Kobe, and Kyoto of Japan, respectively.

Mimai Qu rice wine (dry type), abbreviated as DMW, was brewed in 2018 with *Mimai Qu* rice and water as raw materials at a polishing ratio of 70%. *Brewing method*: The rice was soaked for 45 min after polishing and then steamed, followed by spreading and cooling to 35–40°C. *Mimai Qu*, equal to 12% of rice, and commercial mineral water at a volume equal to that of rice were added as raw materials. The initial temperature of the mash was controlled at 20–25°C, and after 6 h, lactic acid equivalent to 0.7% of the mass of mash was added to perform acidification. *Saccharomyces cerevisiae* (pre-activated into yeast mash) equivalent to 0.1% of raw material was then added and mixed evenly. Seeding mash was achieved after a day of culture. The feeding was carried out as per the three-step feeding method described in Table 1. After the third feeding, fermentation of DMW was commenced officially and completed in 20 days (Figure 1). DMW was light yellow and transparent in color, with a fresh and elegant flavor without the so-called "soy sauce flavor" of CRW. Additionally, it had a light fruity aroma. Its alcohol content was 16–18% v/v and the total sugar content was 6–9 g/L. The wine storage time was 1 year. Four DMW wine samples were analyzed in this study.

Mimai Qu rice wine (semi-sweet; SMW): The raw materials and brewing process were essentially the same as that for DMW, except that in the course of each feeding, 30% of the brewed water was replaced by aged CRW (15% v/v; total sugar content: 6 g/L). Therefore, SMW

Table 1. Three-step feeding method for DMW brewing.

Ingredients	Seeding mash	First feeding	Second feeding	Third feeding	Total
Rice (kg)	50	100	200	400	750
Mimai Qu (kg)	6	20	40	80	146
Water (L)	50	160	320	640	1170
Lactic acid (L)	7.5	0	0	0	7.5
Yeast culture solution (L)	5.5	0	0	0	5.5

DMW: *Mimai Qu* rice wine (dry type).

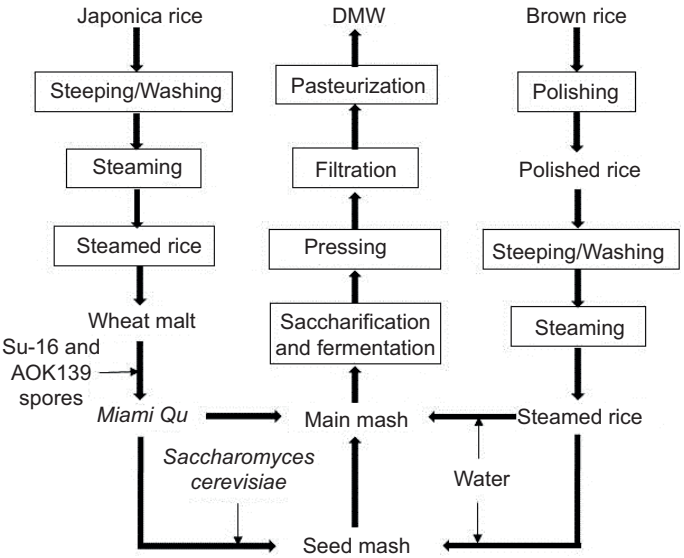

Figure 1. The brewing process of DMW (*Mimai Qu* rice wine, dry type).

was with a brewing characteristic of "wine made from wine" and contained more oligosaccharides with a semi-sweet taste. The alcohol content in SMW was 14–16% v/v and the total sugar content was 40–60 g/L with the storage age of 1 year. Four SMW wine samples were analyzed in this study.

Methods

Amino acids determination method

Free amino acids in the test sample were analyzed by L-8900 amino acid automatic analyzer (HITACHI, Japan), with a 53-min short program (including the time for washing and rinsing columns).

Wine sample pretreatment: After filtration, 40 mL of 10% trichloroacetic acid (Sigma-Aldrich Trading Co. Ltd, Shanghai, China) was added to every 10 mL of wine sample to remove proteins, followed by centrifugation in a high-speed centrifuge at 5000 rpm for 10 min. The supernatant solution was dried thrice in an oven at 50°C to remove alcohol, after which 0.02 mol/L of HCL (China National Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) solution was added to a final volume of 10 mL. After centrifugation, 1 mL of supernatant was taken and tested directly on the machine.

Evaluation Methods for Amino Acid Nutrition

Amino acid ratio coefficient method

The WHO/FAO meeting in 1973 pointed out that the amino acid composition of proteins in eggs and human milk is most suitable for absorption by human body and has high nutritional value. Therefore, the composition and content of EAA in eggs and human milk were used as templates to evaluate the amino acid ratio (RAA; Equation 1) and the ratio coefficient (RC; Equation 2) in foods to derive the score of ratio coefficient (SRC; Equation 3) of amino acids. This is called the score of ratio coefficient of amino acid (SRCAA) method, which is employed to evaluate the amino acid nutritional value of the food (FAO/WHO, 1973).

$$\text{RAA} = \frac{\text{EAA content of the food to be evaluated}}{\text{The corresponding EAA content recommended by WHO/FAO}}, \quad (1)$$

$$\text{RC} = \frac{\text{RAA}}{\text{Mean value of RAA}}, \quad (2)$$

$$\text{SRC} = 100 - (\text{CV} \times 100), \quad (3)$$

where CV is the standard deviation coefficient of RC. CV = RC standard deviation/RC mean.

Linear regression analysis

Linear regression is a statistical analysis method that analyzes the strength of correlation between two or more sets of data. The correlation coefficient "R" reflects the degree of correlation in the data. Linear regression analysis is able to investigate the degree of correlation between the content of seven types of EAA in wine samples and the recommended pattern of WHO/FAO through the R-value. The larger the R-value, the greater the degree of correlation and higher nutritional value of the wine sample (Zhang *et al*., 2017).

Cluster analysis and principal component analysis

The score of ratio coefficient of amino acid and linear regression analysis methods were adopted to examine similarities between seven types of EAA in the wine samples and the WHO/FAO-recommended model, which provided a good evaluation parameter. However, the manual calculation workload of the data was large and no investigation was carried out on the proportion of EAA, namely EAA/TAA and EAA/NEAA. Therefore, for a more comprehensive evaluation of the nutritional value of amino acids in brewed wines, EAA/TAA and EAA/NEAA were also included within the scope of investigation (the two indicators were 0.4 and 0.6 according to the WHO/FAO-recommended model). Cluster analysis (CA) and principal component analysis (PCA) were employed for analysis and evaluation of EAA/TAA and EAA/NEAA. Since software is used in evaluation, the amount of manual calculation is greatly reduced. CA (Euclidian distance) is a method for data classification analyzing the similarity of observed data and clusters data to optimal groups. Observations in each category are different, with some level of similarity. The system clustering method can cluster wine samples and the WHO/FAO-recommended model separately. The wine sample that was grouped with the recommended model first had higher similarity with the recommended model as well as higher nutritional value (Biglari *et al*., 2009). PCA is a factor analysis method that reduces the number of factors in a dataset into several major components and ensures that most of the information is retained. Using the load map of PCA enables one to identify the wine sample that is closer to the WHO/FAO-recommended model. In the coordinate load map, the wine sample that is closer to the WHO/FAO-recommended model point has a greater nutritional value (Shin *et al*., 2010).

Data Analysis

All analyses were performed in triplicate. All statistical analyses were accomplished using DPS Statistical Software for Windows, version 15.10 (DPS 15.10, Hangzhou Rui Feng Information Technology Co. Ltd., Zhejiang, China) (Tang and Zhang, 2013). Significant differences were analyzed by Duncon's analysis of variance with $P < 0.05$.

Results and Discussion

Amino acids and their contents in the tested wine samples

Table 2 lists 17 FAAs found in wine samples (Tryptophan was degraded at low pH and was not detected). The result indicated that the value for each type of wine was the mean of three to four samples, which could reduce the analysis and table width.

Apart for methionine (Met) that was not detected in JNS and DMW, other 16 amino acids were present in all wine samples. As shown in Table 2, among the tested samples, the highest TAA (2814.4 mg/L) was found in CRW. TAA in SMW was the second highest at 2509.8 mg/L, while DMW had a TAA content of 1474.1 mg/L. The lowest TAA was found in JNS at 916.5 mg/L ($P < 0.05$). The most abundant amino acid was proline (Pro), followed by alanine (Ala) and asparagine (Asp). The most abundant amino acid found in JNS was arginine (Arg). The amino acids that ranked second and third highest were Ala and Asp, respectively. Ala had the highest content in SMW, followed by Asp and leucine (Leu) (Pro). The highest amino acid content in DMW was that of Pro, with Ala and Asp ranking second and third highest, respectively ($P < 0.05$). CRW uses traditional wheat *Qu* as a starter with a protein content of about 8–11% (Ye *et al*., 2018; Zhang *et al*., 2019a), which is higher than that of rice. Moreover, since rice is not polished, its protein content is higher than that of polished rice. Therefore, more protein was decomposed or hydrolyzed into amino acids by microorganisms under acidic conditions during the fermentation process, resulting in a higher TAA in CRW. Rice koji is used as a starter to produce JNS, which has a lower protein content than wheat koji. When the polishing ratio reached 50%, the amount of external protein was greatly reduced, resulting in a lower TAA content in brewed sake to only one-third of CRW. Since some wheat was included in both SMW and DMW koji, the TAA in these wines was higher than that in JNS but lower than that in CRW. In the brewing process, rice wine partially replaced brewing water, which also added some amino acids, resulting in higher TAA in SMW when compared with DMW.

Zhang *et al.* (2017) measured the amino acid content of hulless barley *Zajiu* using the automatic amino acid analyzer, and found that the TAA content in traditional *Zajiu* was 2875 mg/L, which was equivalent to SMW. The EAA/TAA of SMW was 33.84%, about 10% lower than traditional *Zajiu*. This may be due to the raw material for *Zajiu* being hulless barley, while that for the wine samples in this study were mainly rice and a small amount of wheat. Difference in brewing process may also contribute to this variation (Zhang *et al*., 2019b).

Luo *et al.* (2017) determined the amino acid content of beer sold in Lanzhou, China, by capillary electrophoresis. It was found that the total amount of amino acids in beer was about 260–370 mg/L. Kabelová *et al.* (2008) compared the amino acid content found in Czech Republic beer and foreign brand beer by the HPLC method and found that the TAA content of Czech Republic beer was

Table 2. Individual amino acids in the tested wine samples (mg/L).

FAA	SMW (*n* = 4)	DMW (*n* = 4)	JNS (*n* = 3)	CRW (*n* = 3)
Thr	90.2 ± 7.8^{a}	55.4 ± 6.1^{b}	28.8 ± 6.6^{c}	94.2 ± 11.2^{a}
Val	158.6 ± 13.3^{a}	68.5 ± 7.4^{c}	38.4 ± 9.7^{d}	116.2 ± 16.6^{b}
Met	20.4 ± 3.6^{b}	nd	nd	28.4 ± 5.5^{a}
Ile	105.4 ± 9.2^{a}	45.2 ± 6.9^{b}	24.1 ± 5.2^{c}	91.1 ± 12.6^{a}
Leu	257.1 ± 19.3^{a}	110.7 ± 9.4^{b}	71.9 ± 11.2^{c}	240.1 ± 28.7^{a}
Phe	89.4 ± 7.7^{b}	60.7 ± 7.3^{c}	37.6 ± 6.3^{d}	116.3 ± 15.7^{a}
Lys	128.4 ± 16.1^{a}	72.9 ± 8.2^{b}	31.2 ± 6.6^{c}	124.0 ± 18.2^{a}
Asp	317.3 ± 19.6^{a}	178.9 ± 20.2^{b}	108.3 ± 14.7^{c}	282.5 ± 32.9^{a}
Ser	143.7 ± 9.7^{b}	88.1 ± 6.6^{c}	48.6 ± 8.5^{d}	176.4 ± 22.6^{a}
Glu	102.2 ± 11.5^{b}	53.9 ± 4.1^{c}	29.2 ± 6.3^{d}	167.7 ± 21.4^{a}
Gly	225.2 ± 16.1^{a}	139.8 ± 17.2^{b}	73.8 ± 11.1^{c}	208.9 ± 27.2^{a}
Ala	355.0 ± 23.8^{a}	187.6 ± 24.4^{b}	112.2 ± 24.0^{c}	325.6 ± 41.3^{a}
Cys	19.1 ± 2.4^{a}	9.1 ± 1.1^{b}	3.8 ± 1.1^{c}	4.7 ± 1.6^{c}
Tyr	155.0 ± 16.2^{a}	124.0 ± 11.5^{b}	83.7 ± 12.5^{c}	150.3 ± 19.6^{a}
His	70.9 ± 8.8^{a}	36.4 ± 3.7^{b}	21.7 ± 4.3^{c}	62.5 ± 11.3^{a}
Arg	14.8 ± 2.2^{d}	58.5 ± 7.4^{c}	124.7 ± 18.0^{b}	231.7 ± 17.2^{a}
Pro	257.1 ± 22.8^{b}	184.3 ± 16.6^{c}	78.6 ± 11.6^{d}	393.8 ± 27.8^{a}
TAA	2509.8 ± 19.4^{b}	1474.1 ± 17.7^{c}	916.5 ± 13.7^{d}	2814.4 ± 17.3^{a}
EAA	849.5 ± 7.5^{a}	413.5 ± 3.2^{b}	231.9 ± 4.4^{c}	810.3 ± 9.6^{a}
NEAA	1660.3 ± 12.4^{b}	1060.6 ± 9.7^{c}	684.6 ± 4.5^{d}	2004.1 ± 11.2^{a}
EAA/TAA (%)	33.8 ± 2.2^{a}	28.0 ± 1.8^{b}	25.3 ± 1.4^{bc}	28.8 ± 2.2^{b}
EAA/NEAA (%)	51.2 ± 3.3^{a}	39.0 ± 3.4^{b}	33.9 ± 2.4^{bc}	40.4 ± 3.9^{b}

FAA: free amino acid; SMW: *Mimai Qu* rice wine (semi-sweet); DMW: *Mimai Qu* rice wine (dry type); JNS: Japanese sake; CRW: Chinese rice wine; TAA: total amino acids; EAA: essential amino acids; NEAA: non-essential amino acids; nd: not detected; Thr: threonine; Val: valine; Met: methionine; Ile: isoleucine; Leu: leucine; Phe: phenylalanine; Lys: lysine; Asp: asparagine; Ser: serine; Glu: glutamine; Gly: glycine; Ala: alanine; Cys: cysteine; Tyr: tyrosine; His: histidine; Arg: arginine; Pro: proline.

higher than that of foreign beer (~450 mg/L vs ~257 mg/L). This result was consistent with the results of Redruello *et al*. (2017). The amino acid content in beer was much lower than that in the wine samples used in this study. This may be due to the brewing process of beer, as the amount of water added was four to seven times of the mass of raw materials, while for the *Mimai Qu* wine or JNS production in this study, the amount of water did not exceed twice the mass of raw materials.

Results of SRCAA method

The nutritional value of amino acids in brewed wine is reflected by the proportion of TAA and EAA. A better estimate is the degree of compatibility of EAA and the standard pattern of amino acids most easily absorbed by the human body. WHO/FAO pointed out that when cysteine and tyrosine (Tyr) are abundant in diet, they can partially replace methionine and phenylalanine (Phe). Therefore, phenylalanine and tyrosine, and methionine and cysteine (Cys) were consolidated in the SRCAA method. Based on this, the optimal ratio of EAA in food was proposed as follows: threonine:cystine + methionine:valine:isoleucine:leucine:phenylalanine + tyrosine:lysine:tryptophan = 8:7:10:8:14:12:11:2 (FAO/WHO, 1973). When the ratio of EAA in food is close to this ratio, it is more easily absorbed by the body, with higher nutritional value. As shown in Table 3, among the wine samples, SMW presented the smallest RC variation coefficient with the highest SRC (~66) and higher nutritional value, followed by CRW (~60) and DMW (~46). The SRC value of JNS (~33) was only half of that for SMW, showing the lowest nutritional value ($P < 0.05$). The first limited amino acids in the wine samples were methionine + cystine.

Evaluation results based on linear regression method

The R-value was used to establish the correlation between contents of seven EAA in the wine samples and the recommended pattern of WHO/FAO. The R-value of SMW was the largest (0.938), indicating that its EAA is closest to the WHO/FAO-recommended model, with higher nutritional value. The R-value of CRW ranked

Table 3. RAA, RC and SRC of the tested wine samples from SRCAA methods.

Wine sample Indicator	SMW		DMW		JNS		CRW	
	RAA	RC	RAA	RC	RAA	RC	RAA	RC
Thr	0.564	0.819	0.347	0.958	0.180	0.866	0.589	0.914
Val	0.793	1.152	0.343	0.947	0.192	0.924	0.581	0.902
Lle	0.657	0.957	0.283	0.781	0.151	0.726	0.570	0.884
Leu	0.918	1.334	0.395	1.092	0.257	1.236	0.858	1.332
Lys	0.584	0.848	0.331	0.916	0.142	0.683	0.564	0.876
Met + Cys	0.282	0.410*	0.065	0.180*	0.027	0.131*	0.237	0.367*
Phe + Tyr	1.019	1.480	0.770	2.127	0.505	2.434	1.111	1.725
SRC	66.818[a]		46.409[c]		33.807[d]		60.692[b]	

RAA: amino acid ratio; RC: ratio coefficient of amino acids; SRC: the most limiting amino acids of corresponding wine sample; SMW: *Mimai Qu* rice wine (semi-sweet); DMW: *Mimai Qu* rice wine (dry type); JNS: Japanese sake; CRW: Chinese rice wine.
*Mean values within rows without a common superscript lowercase letter indicate significant differences ($P < 0.05$).

second (0.900), while no significant difference was observed between R-values of CRW and SMW ($P < 0.05$). The ranked order of the other two wine samples with respect to the R-value was DMW (0.775) and JNS (0.752) ($P < 0.05$). These results were consistent with the SRCAA results except for the significant difference between CRW and SMW in the SRCAA method ($P < 0.05$).

Cluster analysis and principal component analysis evaluation results

Cluster analysis and PCA are the analytical tools in current statistical analysis to study the hidden connections between samples with unclear data and grouping relationships (Granato *et al.*, 2018). It can solve many problems of data analysis involving food science by in-depth mining and data analysis (Brown, 2017). The CA is an exploratory data analysis tool that investigates the similarity of data's potential structure and then divides them into groups (Govender and Sivakumar, 2019). The PCA reduces multiple variables in the sample into several main components through dimensionality reduction, with most of the information being retained for data analysis. Based on the results of CA analysis of amino acid data, the wine samples and the WHO/FAO-recommended model were divided into two categories (Figure 2). The first category consisted of SMW, CRW and WHO/FAO-recommended model, while the second category included JNS and DMW. The first category could be divided into two sub-categories: SMW and CRW in one group, and WHO/FAO-recommended model in another group. It was concluded that the amino acid nutritional values of SMW and CRW were similar and were initially grouped into one class with the WHO/FAO-recommended model, indicating that the amino acid composition of the

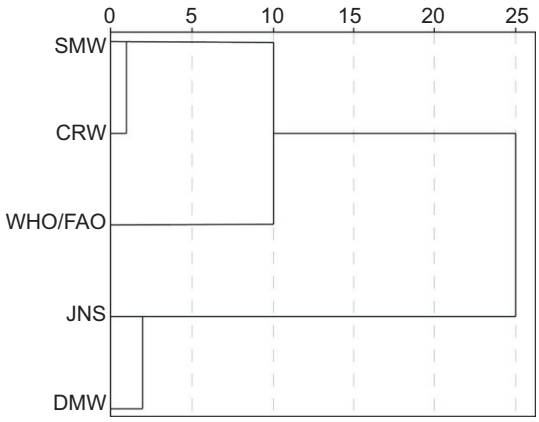

Figure 2. Cluster analysis results of the tested wine samples based on their amino acid data. WHO/FAO: the WHO/FAO-recommended amino acid model; JNS: Japanese sake; CRW: Chinese rice wine; SMW: *Mimai Qu* rice wine (semi-sweet); DMW: *Mimai Qu* rice wine (dry type).

two wine samples was closer to that of the WHO/FAO-recommended model. Since the amino acid nutritional values of DMW and JNS were similar, they were placed in the same group, and were quite different from the WHO/FAO-recommended model.

Based on the amino acid test data and via PCA, it was found that PC1 and PC2 explained more than 90% of the total variance. Therefore, PC1 and PC2 were selected for further investigation. Among the wine samples, SMW was the most related sample to the WHO/FAO-recommended model (Figure 3), with a significant coefficient of 0.001, followed by CRW with a significant coefficient of 0.002. DMW ranked third (with a significant coefficient of 0.006), and JNS ranked last (with a

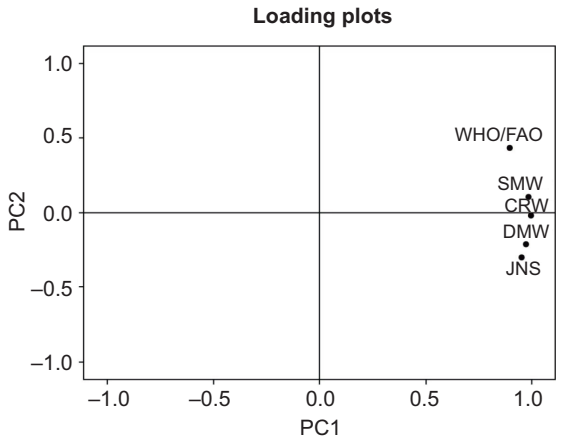

Figure 3. PC1 vs PC2 plots of principle component analysis based on the amino acid data of the tested wine samples. SMW: *Mimai Qu* rice wine (semi-sweet); DMW: *Mimai Qu* rice wine (dry type); JNS: Japanese sake; CRW: Chinese rice wine.

significant coefficient of 0.013). In other words, SMW had the highest nutritional value of amino acids, followed by CRW, DMW and JNS. It can also be seen from Figure 3 that in PC1, all samples were positively correlated with WHO/FAO-recommended model. In PC2, DMW and JNS were negatively associated with WHO/FAO-recommended model, and CRW had a slight negative correlation with WHO/FAO-recommended model (correlation coefficient was –0.017). SMW was positively correlated with WHO/FAO-recommended model. The point on the load map corresponds to the vector projection of that sample on PC1 and PC2.

The above results showed that when two indicators EAA/TAA and EAA/NEAA were considered, the results of CA and PCA were consistent with those of SRCAA and linear regression. Since the indicators were more comprehensive, the conclusions from CA and PCA analysis were more persuasive.

Conclusion

An amino acid analyzer was employed to detect FAAs in brewed wines. Simultaneously, the nutritional value of amino acids was evaluated by multiple methods. The results showed that TAA, EAA and NEAA contents were highest in CRW, while the proportion of EAA was lower than that found in SMW. The amino acid content and EAA ratio in DMW ranked third, while JNS ranked last. Considering nutritional evaluation by different methods, the results obtained by the SRCAA method, linear regression method, and CA and PCA analysis were consistent with one another. The EAA composition of SMW was closest to the WHO/FAO-recommended model, indicating the highest nutritional value of amino acids in SMW, followed by CRW, DMW and JNS.

Disclosure Statement

Authors have no potential conflicts of interest.

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Comparison of social media platforms in terms of marketing performances of food companies

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RESEARCH ARTICLE

Abstract

The objective of this study was to evaluate to what extent social media platforms are effective on the marketing performances of food companies. Facebook was the most effective platform in terms of some performance criteria such as time-saving, easy access to customers, customer feedback, brand awareness, marketing costs, order taking frequency, and sales amount. The most effective platforms after Facebook in terms of marketing performance are Instagram and Twitter, respectively. Marketing costs and product sales are factors that affect the attitude of food companies towards social media platforms.

Keywords: food, marketing, social media, social media marketing

Introduction

The research of new marketing methods and the rapid development of technology have improved the marketing techniques. More access to people with the Web has started to move marketing to the digital environment. The convenience of Web marketing for product promotion, services, and to reach potential customers has made companies adopt this method. Companies that promote their marketing activities digitally started to offer product and service promotions at a low cost. Internet marketing is not limited to space and time, makes it more attractive.

Technological changes and constantly changing consumer demands have created new avenues in marketing. Companies use those sites for marketing which has increased usage. For example use of social media (SM) and the awareness of its ease of use attracted companies to these platforms. According to the report published by the SM analysis company 'We Are Social' for 2020, 4.5/ 7.7 billion world population use the Internet, out of which 3.8 billion are active SM users. The active SM users in the world increased by 9.2% compared with the previous year (We Are Social, 2020). Because of the increase in SM users, companies have started to show their presence on SM platforms (SMPs) to reach users.

Companies that can promote their products and services with minimal marketing expense have started to continue their marketing activities on SM to increase their brand awareness. The use of SM increases the rate of interaction with existing and potential consumers. SM marketing (SMM) is the new media marketing channel that uses SMPs to interact with customers (Yao *et al.*, 2019). SMM makes a significant contribution to companies in terms of customer relationship management by allowing rapid consumer feedback. Companies prefer SMM for fast target audience reach for unique products and for contacting potential customers. Today, SM is also a product research tool for conscious and interested consumers in product research.

SMM has become a preferred marketing channel in many sectors and , has increased marketing food sector products on SM. The global epidemic i.e., the coronavirus (COVID-19); has also increased the tendency of companies to use digital marketing channels for marketing food

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products. However, advertising many nonfood products in the digital environment is easier versus food products owing to seasonal variations, fluctuations in production amount, durability of products, and cultural differences. It is not difficult to predict that the impact of COVID-19 and similar shocks have increased the high consumers' demand in the digital environment. However, without considering the short-term effects of these shocks, it is pivotal to study the effects of SM, one of the most important digital marketing channels, on the marketing performance of companies in the food sector.

This study studied the effect of SMPs on the marketing performance of food companies. This evaluation differs from previous studies in some ways. Previous studies investigating different aspects of this topic have been analyzed within the scope of one or more SMPs where Facebook is predominant (Ainin *et al.*, 2015; Aspasia and Ourania, 2015; Say, 2015; Nyarkoa and Altıntaş, 2015; Saad and Badran, 2016; Francisco, 2016; Yurttadur and Sari, 2017; De Vries *et al.,* 2018; Pantano *et al.*, 2019; Bernal Jurado *et al.*, 2019). In this study, the marketing performance of food companies was analyzed concerning seven SMPs, including Facebook (FB), Twitter (TW), Instagram (IG), YouTube (YT), Google Plus (G+), LinkedIn (LI), and Blogs. In previous studies, the effect of SMM on companies was generally examined within the scope of sales increase (Ainin *et al.*, 2015; Nyarkoa and Altıntaş, 2015; Say, 2015; Canovi and Pucciarelli, 2019) and marketing costs (Ainin *et al.*, 2015; Yurttadur and Sari, 2017; Barišić and Vujnović, 2018; Yao *et al.*, 2019). This study more comprehensively analyzed the effect of SMM on companies with performance criteria such as time-saving, easy access to customers, customer feedback, brand awareness, marketing costs, order taking frequency, and sales amount. Another different aspect of this study from previous studies is that research was conducted on companies operating in different subsectors of the food industry. In previous studies, it is seen that the food sector has been examined in general terms or together with nonfood sectors. This study examined a total of five different subfood sectors.

Materials and Methods

Data acquisition

The primary data of this study were obtained from survey interviews with different food subsector companies that actively use SMPs. Five subsectors of food that use SM intensely in the food industry in Turkey were selected that included confectionery (CONF), milk and dairy products (MDP), olive and olive products (OOP), dry food and pulses (DFP), and coffee and tea (CT). Information obtained from SMPs, statistics published by SM analysis companies, and previous research on the subject helped generate the secondary data of this study. A survey was conducted by selecting food brands that actively use SMPs in each subsector. The authors planned to interview 100 companies (20 per sub-sector). Additional surveys were also conducted to eliminate the negativity that may arise from incomplete and erroneous surveys. A total of 101 questionnaires were taken into consideration for data analysis after obtaining the feedback and accuracy levels of the survey. Valid questionnaires received from each subsectors included 19 for CONF, 21 each for MDP and OOP and, 20 each for DFP and CT.

During the selection of food companies in each sector, their FB followers were also considered. Companies with at least 1000 followers were included in the context of the research. For general evaluation of each subsector, companies with different number of followers were selected (between 1000 and 100,000). An online survey was conducted to obtain data from companies using Google Form and was shared with the respective company officials for survey completion.

Statistical analysis

The data obtained from the surveyed food companies were presented using the five-point Likert scale. The Kruskal-Wallis (KW) test was used to test whether the data means differ in terms of the food subsectors examined. The reason for using this test is that the data do not show normal distribution. Kalaycı (2006) defines the KW test as a nonparametric alternative to a one-way analysis of variance between groups.

Results and Discussion

General information about the examined food companies

Table 1 gives the general information about the examined food companies. The legal structure of food companies showed that most of the companies (56.40%) operated as limited companies. The rate of food companies operating as sole proprietorships and joint-stock companies was 38.60% and 5%, respectively.

The activity period of food companies showed that the majority (88.0%) have been operating for 10 years or more, 9.90% of the companies for 4–9 years, and 2% for 1–3 years. Grouping based on the number of employees revealed that more than half of the companies (51.50%) have a workforce of 10–49 people, 41.60% of the companies, however, had 50–249 people.

The distribution of domestic and foreign sales of food companies showed that the share of companies with

Table 1. General characteristics of food companies surveyed.

Variables	Frequency	Percentage (%)
Legal structure		
Joint stock company	5	5.00
Limited liability company	57	56.40
Sole proprietorship	39	38.60
Operating period (years)		
1–3	2	2.00
4–9	10	9.90
≥10	89	88.10
Number of employees		
1–9	6	5.90
10–49	52	51.50
50–249	42	41.60
≥250	1	1.00
Share of domestic sales as a percentage of total sales (%)		
50–75	8	7.92
76–99	40	39.60
100	53	52.48
Average domestic sales rate (%)	92.76	
Share of food products in total sales (%)		
<50	3	2.97
50–75	6	5.94
76–99	67	66.34
100	25	24.75
Average share of food sales (%)	87.38	

domestic sales was high. The portion is 92.76% on average, and 52.48% of the companies make all their sales domestically. In general, the share of food products in the total turnover of companies was high. The average share of food products in the total turnover of the companies interviewed is 87.38%. The share of food products in the turnover of 66.34% of the companies varies between 76–99%, and 24.75% of the examined companies got their entire turnover from the food products sale.

Evaluation of SMPs in terms of marketing performance

Following the opinions of the examined companies, a comparison of SMPs for marketing performance was conducted. Some performance criteria such as time-saving, easy access to customers, customer feedback, brand awareness, marketing costs, order taking frequency, and sales amount were used to compare SMPs.

Companies need to carry out, follow, and interpret marketing activities in a shorter time. It was observed that companies had started preferring SM applications to perform these activities faster as they wanted to reach more customers in less time.

Effectiveness of SMPs in terms of timesaving

When the data were analyzed to get an idea about the time-saving platform for companies marketing activities, the FB platform led the list, followed by IG and TW, respectively. In general, companies in different subsector groups find FB, IG, and TW effective for saving time in marketing, and no statistically significant difference was found between the groups. YT and G+ were evaluated as moderately effective, but LI and Blogs are less effective. However, a statistically significant difference of opinion among subsector groups in terms of time savings was noted (Table 2). Companies in the CT and CONF sectors found these four platforms less effective in terms of saving time.

Effectiveness of SMPs for easy customer access

When the opinions of companies regarding the effectiveness of SMPs for easy access to customers were examined,

Table 2. Evaluation of social media platforms in terms of time-saving.

SMPs	CONF		MDP		OOP		DFP		CT		Total		KW test P value
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
FB	4.26	1.05	4.57	0.60	4.43	0.60	4.25	0.55	4.45	0.69	4.40	0.71	0.483
TW	3.37	1.46	4.24	0.62	3.76	1.22	4.05	0.39	3.70	1.42	3.83	1.12	0.465
IG	3.37	1.57	4.19	0.68	4.00	0.84	3.70	0.86	4.05	1.05	3.87	1.06	0.298
YT	2.16	1.07	3.71	0.90	3.24	1.04	3.60	0.88	3.00	1.12	3.16	1.13	0.000*
G+	2.68	1.29	3.62	0.92	3.43	0.98	3.45	1.00	2.80	1.15	3.21	1.12	0.023**
LI	2.21	1.08	3.24	1.09	3.14	1.01	3.35	1.09	2.45	1.32	2.89	1.19	0.005*
Blogs	2.11	1.10	3.33	1.06	3.24	1.00	3.35	1.09	2.70	1.26	2.96	1.18	0.003*

$\bar{x}$: mean score of the Likert scale by the level of effectiveness of each SMP (1: not at all effective to 5: highly effective); SD: standard deviation.
*Statistical significance at 1%.
**Statistical significance at 5%.

FB with a mean score of 4.52 was determined to be quite effective . Platforms found to be moderately effective in terms of easy access to customers were IG, TW, and YT, respectively (Table 3). In general, it was understood that the FB platform was more convenient for food companies to reach their customers. The number of FB users in Turkey is high, causing many customers to focus on this platform. LI (1.87) stands out as the least effective platform concerning easy access to customers as it is primarily business-oriented.

Effectiveness of SMPs concerning customer feedback

When the opinions of customer feedback on SM were examined, the FB platform was effective in customer feedback with an average score of 4.61 followed by the IG (3.62), and TW was moderately effective (3.29). YT, G+, LI, and Blogs platforms were found to be minimally effective concerning customer feedback (Table 4). The FB, was quite impressive for customer feedback, as it allows users to comment and share, directly message the companies, and provides more notifications from the customers. The IG also offers users the opportunity to comment and send messages to the relevant company. Unlike FB and IG, the sharing of users via text messages is limited on the TW, and the option to send direct messages to the company is often not available. A statistically significant difference was observed between the group evaluations for TW, YT, and Blogs platforms. Compared with the other groups, companies operating in the CONFEC industry did not find TW, YT, and Blogs platforms very effective for customer feedback.

Effectiveness of SMPs for brand awareness

When the impact of SMPs for increasing brand awareness was analyzed, the FB platform led with an average score of 4.58. Large number of users attribute to its popularity. The other two platforms that were effective in increasing brand awareness were IG (4.22) and TW (4.07), respectively. Other SMPs scored 3.64 (YT), 3.43 (G+), 3.23 (Blogs), and 3.21 (LI) concerning their effect on increasing brand awareness (Table 5).

The evaluations made according to the 5-point Likert scale used in the survey study indicated that the

Table 3. The effectiveness of social media platforms in terms of easy access to customers.

SMPs	CONF		MDP		OOP		DFP		CT		Total		KW test P value
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
FB	4.47	1.12	4.62	0.59	4.57	0.60	4.75	0.55	4.20	0.83	4.52	0.77	0.146
TW	3.32	1.16	3.38	1.16	3.29	1.31	3.65	1.04	3.10	1.17	3.35	1.16	0.657
IG	3.63	1.12	3.81	0.98	3.90	1.00	3.90	1.17	4.00	0.97	3.85	1.03	0.829
YT	2.32	1.11	2.52	1.03	2.62	1.02	2.40	1.10	2.75	1.21	2.52	1.08	0.586
G+	2.32	1.00	2.57	0.98	2.48	0.98	2.20	0.77	2.45	1.23	2.41	0.99	0.841
LI	1.84	1.07	2.05	0.92	2.14	0.91	1.70	0.57	1.60	0.88	1.87	0.89	0.170
Blogs	1.89	0.94	2.62	1.02	2.52	0.87	2.00	0.65	2.45	1.05	2.31	0.95	0.022*

$\bar{x}$: mean score of the Likert scale by the level of effectiveness of each SMP (1: not at all effective to 5: highly effective); SD: standard deviation.
*Statistical significance at 5%.

Table 4. The effectiveness of social media platforms in terms of customer feedback.

SMPs	CONF		MDP		OOP		DFP		CT		Total		KW test P value
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
FB	4.63	0.76	4.67	0.48	4.71	0.46	4.70	0.47	4.35	0.75	4.61	0.60	0.385
TW	2.47	1.39	3.57	1.08	3.52	1.33	3.75	0.97	3.05	1.43	3.29	1.31	0.027**
IG	2.95	1.58	3.90	1.00	3.71	1.27	3.75	1.07	3.75	1.12	3.62	1.24	0.301
YT	1.63	1.01	2.76	1.09	2.33	1.15	2.50	1.00	2.80	1.24	2.42	1.16	0.003*
G+	2.05	1.13	2.48	1.03	2.57	1.21	2.15	0.93	2.45	1.23	2.35	1.11	0.459
LI	1.58	1.07	2.00	0.95	2.05	1.02	1.90	0.85	2.30	1.22	1.97	1.03	0.130
Blogs	1.53	1.02	2.48	0.98	2.43	1.16	2.30	0.92	2.45	1.23	2.25	1.11	0.004*

$\bar{x}$: mean score of the Likert scale by the level of effectiveness of each SMP (1: not at all effective to 5: highly effective); SD: standard deviation.
*Statistical significance at 1%.
**Statistical significance at 5%.

Table 5. The effect of social media platforms on brand awareness.

SMPs	CONF		MDP		OOP		DFP		CT		Total		KW test P value
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
FB	4.53	0.96	4.62	0.59	4.57	0.60	4.55	0.60	4.65	0.59	4.58	0.67	0.969
TW	3.68	1.00	4.33	0.66	3.81	1.03	4.40	0.60	4.10	1.12	4.07	0.93	0.070
IG	4.00	1.11	4.48	0.68	4.14	0.79	4.00	1.17	4.45	0.69	4.22	0.91	0.353
YT	2.89	1.24	4.05	0.67	3.57	1.08	3.55	1.05	4.10	1.02	3.64	1.09	0.004*
G+	2.68	1.25	3.86	0.79	3.48	1.03	3.50	1.05	3.55	1.19	3.43	1.12	0.020**
LI	2.42	1.30	3.81	0.81	3.48	0.87	3.25	1.21	3.00	1.56	3.21	1.24	0.014**
Blogs	2.26	1.28	3.86	0.79	3.29	0.96	3.25	1.21	3.40	1.47	3.23	1.25	0.003*

$\bar{x}$: mean score of the Likert scale by the level of effectiveness of each SMP (1: not at all effective to 5: highly effective); SD: standard deviation.
*Statistical significance at 1%.
**Statistical significance at 5%.

Table 6. The effect of social media platforms on reducing marketing costs.

SMPs	CONF		MDP		OOP		DFP		CT		Total		KW test P value
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
FB	4.21	1.23	4.43	0.60	4.29	0.72	4.35	0.59	4.40	0.82	4.34	0.80	0.926
TW	4.00	1.25	4.24	0.62	4.00	1.00	4.30	0.57	4.05	1.19	4.12	0.95	0.951
IG	3.89	1.33	4.24	0.62	4.24	0.70	4.05	0.76	4.25	0.97	4.14	0.89	0.823
YT	3.37	1.34	3.95	0.67	3.90	0.94	3.85	0.81	4.00	0.92	3.82	0.96	0.478
G+	3.32	1.34	3.81	0.75	3.90	0.94	3.75	0.79	3.95	0.94	3.75	0.97	0.385
LI	2.68	1.38	3.71	0.96	3.67	0.91	3.60	0.88	3.50	1.43	3.45	1.17	0.091
Blogs	2.63	1.38	3.71	0.96	3.76	0.89	3.65	0.93	3.75	1.16	3.51	1.14	0.031*

$\bar{x}$: mean score of the Likert scale by the level of effectiveness of each SMP (1: not at all effective to 5: highly effective); SD: standard deviation.
*Statistical significance at 5%.

companies had a positive opinion about the effect of SMPs on brand awareness. A statistically significant difference was found between groups for other SMPs other than FB, IG, and TW. The companies in the CONFEC and MDP sectors were effective in this difference. Companies in the CONFEC industry found SMFs other than FB, IG, and TW had minimal effect of increasing the awareness of their brands. On the other hand, the opinions of the companies in the MDP sector on this issue are more positive versus other subsector groups.

Effectiveness of SMPs in terms of marketing cost reduction

When the effect of SMPs on reducing marketing costs was analyzed, FB once again led the race with an average of 4.34, followed by IG (4.14) and TW (4.12). Table 6 shows the effectiveness of SMPs on reducing marketing costs. The effect of these platforms in reducing marketing costs was noted as 3, which is above the neutral value according to the 5-point Likert scale average. The KW test was conducted to determine whether there is a difference between the evaluations of the company groups regarding the effect of SMPs in reducing marketing costs. A statistically significant difference was found for the Blogs platform. Compared with other food subsectors, companies in the CONFEC industry found the Blogs platform's decreased effect on reducing marketing costs.

According to the results mentioned above, the examined food companies think that SMPs are generally effective in reducing marketing costs. This result is consistent with the findings obtained in previous studies (Ainin *et al.*, 2015; Yurttadur and Sari, 2017; Barišić and Vujnović, 2018; Yao *et al.*, 2019). In particular, the FB platform was evaluated as highly effective in reducing marketing costs, which can be attributed to its free content sharing and easy tracking of user comments.

Effectiveness of SMPs concerning increasing product sales

When the effect of increasing product sales was examined, FB again led with an average of 4.11, followed by moderate product sales by IG (3.26) and TW (3.15).

Table 7. The effect of social media platforms in increasing product sales.

SMPs	CONF		MDP		OOP		DFP		CT		Total		KW test P value
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
FB	4.37	0.83	4.43	0.87	3.90	1.09	4.20	0.95	3.65	1.35	4.11	1.06	0.206
TW	3.00	1.20	3.57	0.87	3.00	1.38	3.40	1.10	2.75	1.59	3.15	1.26	0.336
IG	3.21	1.36	3.71	0.96	3.05	1.47	3.30	1.34	3.00	1.62	3.26	1.36	0.615
YT	1.63	0.68	2.33	1.11	2.14	1.35	1.90	0.91	1.90	1.21	1.99	1.09	0.309
G+	2.11	1.05	2.14	0.91	2.05	1.28	1.85	0.81	1.75	0.97	1.98	1.01	0.561
LI	1.68	0.67	1.95	0.86	1.76	1.00	1.70	0.73	1.45	0.69	1.71	0.80	0.348
Blogs	1.63	0.68	2.10	0.94	1.95	1.12	1.75	0.72	1.50	0.69	1.79	0.86	0.249

$\bar{x}$: mean score of the Likert scale by the level of effect of each SMP (1: not at all effective to 5: highly effective); SD: standard deviation.

Table 7 reports the impact of YT, G+, Blogs, and LI platforms in enhancing sales.

FB was the most effective platform in the sales increase because of marketing their products via SM, which attributes to the consumers' emphasis on visuality in marketing food products through SM. Ainin *et al.* (2015) and Say (2015) also revealed that FB effectively increased product sales. Ainin *et al.* (2015) showed that the use of FB had a positive effect on the sales volume of SMEs in Malaysia. Say (2015) determined that the companies in the convenience food sector in Turkey increase their online sales with campaigns supported by FB. IG was placed second concerning the effect of increasing product sales because of its visual density like FB.

Attitude of food companies towards SMM

Fifteen statements were presented to companies during the survey study to measure the attitude of companies. Likert scale responses of companies for these statements were tested with reliability analysis. In the analysis, Cronbach's Alpha value, the general reliability coefficient, was determined as 0.873. Since this value was between $0.80 \leq \alpha < 1.00$, the scale was found to be reliable.

The responses of food companies to some statements through SMM are shown in Table 8. The statements to which the companies mostly agree were: providing brand awareness, the convenience of offering products and services to target regions, presenting campaigns and activities at the appropriate time, increasing the competitive power, strengthening the status of the company, reducing marketing expenses, increasing loyal customers, providing tips about the market, and increasing sales.

The statement that the interviewed companies least agreed was about the price. Companies hardly agreed with the view that SMM provides a higher price than traditional marketing. Besides, companies believe that marketing food products on SM are more difficult than other product categories.

In general, there is no statistically significant difference between companies in different food subsectors in terms of their level of agreement with some statements related to SMM. There is a statistical difference of opinion among companies for statements that "social media enables customers to make better decisions" and "social media is preferred over other marketing channels." However, the degree to which companies agree with both statements is high.

Table 9 shows the correlation analysis results of the relationship between the general attitude of companies towards SMM. These results revealed that the attitude of companies towards SM is not in a statistically significant relationship with the size of the companies, operating period of the companies, and the SMM experience of the companies.

The correlation analysis was used to determine the attitude change toward SM according to the company size. However, there was no significant relationship found between company size and attitude toward SM. This aspect was not examined in previous studies. However, some studies investigated the relationship between company size and SM use. Aspasia and Ourania's (2014) study on the Greek food sector found a positive relationship between company size and the adoption of SM tools. According to the authors, this is because large firms allocate more staff and budget to SM. Braojos-Gomez *et al.* (2015) and Pantano *et al.* (2019) state that small companies with low financial resources must improve their SM skills to gain a competitive advantage in SM. Tarsakoo and Charoensukmongkol (2019) argue that both small and large companies use SM to add value to their business activities. But many difficulties that limit the capabilities of small companies in terms of effective SMM.

On the other hand, the increasing effect of SM on reducing marketing costs positively increases the attitude of

Table 8. Level of agreement of food companies with some statements on social media marketing.

Statements	CONF		MDP		OOP		DFP		CT		Total		KW test P value
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
SM increases brand awareness	4.16	0.90	4.52	0.51	4.43	0.68	4.30	0.66	4.40	0.60	4.37	0.67	0.645
It is easier to reach the target audience with SM	3.89	0.99	4.43	0.60	4.43	0.60	4.20	0.62	4.30	0.86	4.26	0.76	0.236
SM is effective for campaigns	3.87	1.13	4.14	0.48	4.38	0.59	4.55	0.60	4.30	0.66	4.25	0.74	0.100
SM gives a competitive edge	3.81	0.93	4.27	0.76	4.36	0.72	4.31	0.62	4.38	0.57	4.23	0.74	0.161
SM strengthens the status of the company	3.84	0.83	4.43	0.51	4.43	0.60	4.15	0.49	4.10	0.79	4.20	0.68	0.059
SM reduces marketing expenses	3.74	0.99	4.38	0.50	4.19	0.75	4.15	0.67	4.40	0.60	4.18	0.74	0.120
SM increases loyal customers	3.69	1.06	4.19	0.60	4.38	0.67	4.05	0.51	4.20	0.77	4.11	0.76	0.163
SM provides market-related tips	4.06	0.91	4.14	0.57	4.19	0.60	3.90	0.55	4.20	0.62	4.10	0.66	0.400
SM increases sales	3.95	0.78	4.00	0.77	3.81	1.08	3.75	0.85	4.05	1.05	3.91	0.91	0.704
SM enables customers to make better decisions	3.57	0.76	3.95	0.59	4.05	0.67	3.64	0.74	4.20	0.77	3.89	0.73	0.023**
Selling on SM is easy	3.84	0.90	3.73	0.62	3.98	0.71	3.68	0.56	4.19	0.70	3.88	0.71	0.078
I prefer SM to other marketing channels	3.28	1.14	3.71	0.64	3.86	0.57	3.38	0.49	4.05	0.69	3.66	0.77	0.008*
SM offers special products to customers	3.03	1.00	3.11	0.86	3.05	0.90	3.12	0.21	3.18	1.31	3.10	0.91	0.673
SM is a good option for marketing food products.	2.90	1.07	2.57	1.00	2.52	0.95	2.52	0.73	2.58	1.40	2.62	1.04	0.365
Sellers on SM get higher prices	2.01	1.23	1.84	1.13	1.51	0.71	1.73	0.82	2.20	1.34	1.85	1.08	0.526

$\bar{x}$: mean score of likert scale by the level of agreement with statements (1: not at all effective to5: highly effective); SD: standard deviation.
*Statistical significance at 1%.
**Statistical significance at 5%.

Table 9. Correlation analysis results between the attitude of companies towards social media.

		Attitude of companies towards SM	Size of the companies	Operating period of the companies	SMM experience of the companies	Effect of SM on reducing marketing costs	Effect of SM on increasing product sales
Attitude of companies towards SM	Pearson Correlation	1.000	0.114	−0.182	−0.030	0.216**	0.317*
	Sig. (2-tailed)	–	0.255	0.069	0.763	0.030	0.001

*Correlation is significant at the 1% level (2-tailed).
**Correlation is significant at the 5% level (2-tailed).

companies toward SMM (r = 0.216; P = 0.030). Analysis findings also revealed a statistically significant and positive relationship (r = 0.317, p = 0.001) between the increasing effect of SM on product sales and attitude toward SM. Although the relationship between them is not strong, according to the size of the correlation coefficients, the effect of SM, both to reduce marketing costs and increase product sales positively affects the attitudes of companies toward SM. However, the highest degree of relation with the attitude of companies towards SM is the increasing effect of SM on product sales.

Conclusion

This study examined the effectiveness of SMPs on the marketing performance of food companies. According to the outcomes, FB is the most effective platform for performance criteria such as time-saving, easy access to customers, customer feedback, brand awareness, marketing costs, order taking frequency, and sales amount. The most effective platforms after FB in terms of marketing performance are IG and TW, respectively. LI, Blogs, and G+ are the platforms with the least performance.

The marketing performance of food companies varies according to SMPs. The use of all SM platforms for marketing purposes will waste the time of companies. Hence, a company should first determine which SMP their current and target customers use more. In the next stage, these companies should conduct their marketing activities over the SMP chosen. A food company that is engaged in marketing activities on a platform where there are no current and target customers will not reach the SM usage purpose. Since the content offered by the food company cannot reach current and target customers, SMM will not have an impact on the product sales of the company. Besides, companies need to follow the SM activities of their competitor companies while continuing their marketing activities on SM. Food companies to examine the content on SM provided by competitors that produce similar products and their feedback.

Enhancing the company's knowledge on the use of SM and SMM will aid in increasing the marketing effectiveness of food companies on SM. In general, the food companies make intensive marketing initiatives on SM. However, they do show their competence in using SMPs. It has been observed that some companies have incorrect/ no or nonsuitable information entered in their SM accounts. Food companies should start operating on SM after doing a good research on using the functional features of SMPs as every SMP has options specific to the platforms it offers. Since marketing strategies will change according to platforms, preliminary research is required on this subject. In addition, visually intensive shares for food products should be presented to the consumer. Hence, food companies need to pay attention to the quality and remarkable features of the content offered on SM. Rapid and positive feedback of food companies on SM will be a supportive effort to achieve the SMM goal.

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Conflict of Interest

No potential conflict of interest was reported by the authors.

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Citrus species: Modern functional food and nutraceutical-based product ingredient

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REVIEW

Abstract

Citrus is the most cultivated fruit crop in the world and occupies a place of considerable importance in the country's economy. Almost 33% of the *citrus* fruits are processed for juice production; however, a great amount of wastes, including peels, segment membranes, and seeds are also produced. Indeed, *citrus* fruits consist of 45% juice, 26% pulp, 27% peels, and 2% seeds. Pruning, a cultural practice involving the removal of tree branches and limbs, was applied to improve fruit's quality. A large amount of leaves are produced through pruning. These agri-food matrices contain a wide range of bioactive phytochemicals compared to fruits. The present review covers the past 5 years of research carried out in chemistry, health properties, and applications in food and nutraceutical industries of all portions of *citrus* fruit and its major bioactive compounds. Additionally, patents are also included.

Keywords: bioactive compounds, by-products, *citrus,* health properties, juice, peels, pulp, seeds

Introduction

Citrus is the most cultivated fruit tree in the world and occupies a place of considerable importance in country's economy. The *citrus* fruits are processed for juice production (45%), and a great amount of waste, including peels (27%), pulp (26%) and seeds (2%), is produced (Mahato *et al*., 2018).

Food waste is defined as the by-product obtained from various industrial, agricultural and other activities of food sector. Especially, food-processing industries produce large quantities of by-products, which are difficult to dispose of as they have a high demand for biological oxygen. Indeed, waste disposal has high costs and, also a potential negative impact on the environment (Kumar *et al*., 2017).

These agri-food matrices contain a wide range of bioactive phytochemicals with different structures and functionality that could be used as ingredients for food, food supplements or active bioactive compounds in pharmaceutical products (Rombaut *et al*., 2014).

In food industry, *citrus* by-products and their value-added compounds, including polyphenols, vitamins, microelements and fiber are utilized as natural additives with the following properties: antimicrobials, antioxidants, colorants and flavoring agents (Mahato *et al*., 2019). These wastes have gained increasing interest for further exploitation on the production of food additives, supplements with high nutritional value and pharmaceutical products.

The recent data were collected from several scientific databases (PubMed, Science Direct, SciFinder, Scopus, Elsevier, SpringerLink, ReserchGate and Google Scholar) from 2015 to 2020 using the following keywords: *citrus*, *citrus* by-products, juice, seed, peels, leaves, and

essential oils. This work is structured by dividing the text in *Citrus* portion and discussions on the phytochemical profile and biological activities of each. Additionally, patents are also included. The information collected would be useful for research on *citrus* species and for food and nutraceutical industries interested in using ingredients with health potential.

Juice

Antioxidant activity

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in the pathogenesis of many human diseases, and antioxidants play a crucial role in restoring the physiological oxidative balance and modulating biological pathways and membrane functions (Smeriglio *et al*., 2018). The *citrus* genus is recognized for its protective effects against free radical-induced damage. Barberis *et al*. (2020) analyzed the antioxidant potential of fresh squeezed *pompia, lemon* (cv. Lisbon) and orange juices (cv. Hamlin, Sanguinello, and Moro). Among these, Pompia juice had a marked effect against ROS, and a moderate capacity to reduce ROS damages on cell membrane. On the contrary, orange juices resulted much less effective. The *in vitro* antioxidant potential (Table 1) of fresh squeezed *citrus* × *clementina* juices, collected from different areas of Calabria, was recently evaluated (Leporini *et al*., 2020a). Results revealed that juice obtained from fruits collected in Corigliano Calabro exhibited the highest radical activity, with the concentration giving 50% inhibition (IC_{50}) values of 81.13 and 27.82 mg/mL for 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) tests, respectively. The same juice exhibited the highest protection of lipid peroxidation. These results are similar to the previous study conducted by Loizzo *et al*. (2018) that reported the antioxidant activity of fresh squeezed *citrus* × *clementina* juices from fruits collected in flood plains, hills, and coastal plains of Sibari (Calabria, Italy). The following trend of radical potency was found: flood plain > coastal plain > hill.

Haraoui *et al*. (2020) compared the antioxidant activity of fresh squeezed juices derived from different *citrus* fruit varieties. All investigated samples possessed radical scavenging activity with IC_{50} values comparable to positive control ascorbic acid and butylated hydroxytoluene (BHT). Among them, *Citrus maxima* and *Citrus aurantium* juices showed the highest DPPH radical scavenging activity (IC_{50} = 0.42 and 0.44 mg/mL, respectively). The same trend was observed in β-carotene bleaching test with percentage exceeding to 80.55%, followed by *Citrus sinensis* cv. Sanguinelli and *Citrus limon*.

The antioxidant ability of fresh squeezed *Citrus limon* L. Burm. cv. Femminello comune juice was analyzed by Loizzo *et al*. (2019), founding the IC_{50} values of 40.3 and 46.5 g/mL in DPPH and ABTS test, respectively, and 49.7 mg Fe (II)/g in the ferric-reducing ability power (FRAP) test. More recently, the antioxidant ability of two *Citrus sinensis* cultivars (Sanguinelli and Salustiana) was demonstrated by applying ABTS, DPPH, and ORAC assays (Ordóñez-Díaz *et al*., 2020). *Citrus sinensis* cv. Sanguinelli extracts were 40% more active than *Citrus sinensis* Salustiana samples.

Recently, Ali *et al.* (2020) reported that animals treated with 0.75% hydrogen peroxide in drinking water with daily drenching with 1 mL lemon juice, exhibited enhancement in hemoglobin concentration, red blood cells count, white blood cells count, and total proteins, and reduction in the level of aspartate aminotransferase and alanine aminotransferase. These findings clearly confirmed the protective and antioxidant features of lemon juice on hematological and biochemical parameters of the oxidatively stressed female mice.

Metabolic syndrome

Metabolic syndrome (MS) is a clustering characterized by abdominal obesity, high blood pressure, high blood sugar, high serum triglycerides (TG), low serum, and high-density lipoprotein (HDL) that directly increase the risk of cardiovascular disease, type 2 diabetes mellitus (T2DM), and all-cause mortality (Kaur *et al*., 2014).

Recently, the beneficial effects of *Citrus bergamia* juice were evaluated using an experimental animal model of MS and cardiovascular risk (De Leo *et al.,* 2020). Results demonstrated that daily oral treatment reduced TG levels, cardiovascular risk, and showed protective effects on hepatic steatosis, probably due to the reduction of oxidative stress and inflammation. Previously, Impellizzeri *et al.* (2015) tested the *in vivo* anti-inflammatory activity of bergamot juice extract. Mice treated with this extract were more resistant to induction of colitis and reduction in the expression of important inflammatory mediators, tumor necrosis factor-alpha (TNF-α) and interleukin-1 β (IL-1β), was observed.

The effects of a bergamot phytocomplex (Patent No. EP3116520A1) was investigated by Di Folco *et al*. (2018). Each tablet provided 200-mg bergamot juice dry extract, 120-mg phytosterols, 80-mg artichoke leaf extract, and 20-mg vitamin *citrus*. After 6 months of administration, patients in the intervention group showed a significant reduction in fasting blood glucose compared to the simple dietary intervention alone.

Table 1. Biological effects of *Citrus* juices.

Juice	Biological activity	Mechanism	TPC, TFC, TCC, and/or main abundant identified compounds	References
In vitro				
Citrus aurantifolia	Antioxidant	Radical scavenging Ferric-reducing power	TPC = 52 mg GAE/L; TFC = 29.5 mg QE/L	Oboh *et al.*, 2015b
	Antibacterial	*S. aureus, S. epidermis*, *M. luteus, E. faecalis, B. subtillis, P. aeruginosa, K. pneumonia S. thypii,* and *C. diphtheriae* inhibition	NR NR NR	Abdallah, 2020 Fadillah *et al.*, 2020 Azhara *et al.*, 2020
Citrus aurantium	Antioxidant	Radical scavenging	TPC = 295.37 mg GAE/g; FC = 26.08 mg QE/g TPC = 0.58 mg GAE /mL; TFC = 0.43 mg RE/mL; Neohesperidin = 144.85 mg/mL; Naringin = 79.19 mg/mL; Hesperidin = 4.68 mg/mL.	Haraoui *et al.*, 2020 Chen *et al.*, 2020
	Antibacterial	*S. aureus* inhibition		Haraoui *et al.* 2020
Citrus grandis	Antioxidant	Radical scavenging	TPC = 0.49–1.27 mg GAE /mL; TFC = 0.35–1.17 mg RE/mL; Naringin = 40.82–419.28 mg/mL; Neohesperidin = 37.52–42.54 mg/mL; Hesperidin = 3.14–12.17 mg/mL; Diosmin = 14.79–21.38 mg/mL; Tangeretin = 3.09–3.64mg/mL.	Chen *et al.*, 2020
Citrus hystrix	Antibacterial	*S. aureus* inhibition	NR	Kusumawardhani *et al.*, 2020
Citrus limon	Antioxidant	Radical scavenging	TPC = 151.7 mg GAE/L; TFC = 30.8 mg QE/L; Eriocitrin = 16.7 mg/100 mL; Hesperidin = 14.1 mg/100 mL.	Loizzo *et al.*, 2019
	Metabolic syndrome	Inhibition of α-amylase and β-glucosidase enzymes		Loizzo *et al.*, 2019
	Antibacterial	*S. aureus, S. epidermis*, *M. luteus, E. faecalis* and *B. subtillis* inhibition.	TPC = 231.16 mg GAE/g; TFC = 25.04 mg QE/g.	Haraoui *et al.* 2020
	Neuroprotective	AChE and BChE inhibition	Diosmetin 6,8-di-C-glucoside = 5.35 mg/100 mL; Hesperetin 7-*O*-rutinoside = 3.11 mg/100 mL.	Gironés-Vilaplana *et al.*, 2015
Citrus maxima	Antioxidant	Radical scavenging	TPC = 350.05 mg GAE/g; TFC = 55.38 mg QE/g.	Haraoui *et al.*, 2020
	Antibacterial	*S. aureus, S. epidermis*, *M. luteus, E. faecalis* and *B. subtillis* inhibition.		Haraoui *et al.* 2020
Citrus medica	Antioxidant	Radical scavenging	TPC = 0.30–1.37 mg GAE /mL; TFC = 0.19–0.68 mg RE/mL; Hesperidin = 42.13 mg/mL; Eriocitrin = 28.46 mg/mL; Narirutin = 28.46 mg/mL; Didymin = 12.50 mg/mL; Tangeretin = 3.76 mgmL.	Chen *et al.*, 2020
Citrus paradisi	Antioxidant	Radical scavenging	TPC = 153.08 mg/mL; TFC = 390.21 mg/mL; Naringin = 287.15 mg/mL; Narirutin = 37.07 mg/mL; Naringenin = 31.25 mg/mL; Poncirin = 17.32 mg/mL; Neohesperidin = 13.48 mg/mL.	Sicari *et al.*, 2018

(continues)

Table 1. Continued

Juice	Biological activity	Mechanism	TPC, TFC, TCC, and/or main abundant identified compounds	References
Citrus reticulata	Antioxidant	Radical scavenging	TPC = 0.30–1.37 mg GAE /mL; TFC = 0.19–0.68 mg RE/mL; Hesperidin = 50.53–141.85 mg/mL; Naringin = 26.57–78.39 mg/mL; Neohesperidin = 69.19 mg/mL; Didymin = 1.73–10.33 mgmL; Eriocitrin = 27.17–78.39 mg/mL; Tangeretin = 3.43–4.32 mg/mL.	Chen *et al.*, 2020
Citrus sinensis	Antioxidant	Radical scavenging	TPC = 0.47–0.67 mg GAE /mL; TFC = 0.23–0.92 mg RE/mL; Hesperidin = 94.98–173.11 mg/mL; Eriocitrin = 28.38–46.95 mg/mL; Narirutin = 28.38–46.95 mg/mL; Didymin = 1.73–10.33 mg/mL; Tangeretin = 3.52–3.67 µg/mL.	Chen *et al.*, 2020
Citrus × *clementina*	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	TPC = 17.58–54.65 mg CAE/100 mL; TFC = 18.16–51.48 mg QE/100 mL; TCC = 18.23–53.54 mg β-carotene E/100 mL; Neohesperidin = 80.26–110 mg/100 mL; Hesperidin = 40–81.08 mg/100 mL; Naritutin = 6.25–8.50 mg/100 mL. TPC = 29.74–44.20 mg GAE/100 mL; TCC = 42.89–75.45 mg β-carotene E/100 mL; Neohesperidin = 72.96–116.50 mg/100 mL; Hesperidin = 55.24–69.52 mg/100 mL; Didymin = 3.65–5.65 mg/100 mL	Leporini *et al.*, 2020a Loizzo *et al.*, 2018
	Metabolic syndrome	Inhibition of α-amylase, β-glucosidase and lipase enzymes		Leporini *et al.*, 2020a Loizzo *et al.*, 2018
	Antibacterial	*M. luteus* and *B. subtillis* inhibition	TPC = 75.60 mg GAE/g; TFC = 20.51 mg QE/g	Haraoui *et al.* 2020
In vivo				
Citrus aurantifolia	Metabolic syndrome	Reduction in plasma TC, TG, and LDL-c levels and increase in plasma HDL-cholesterol levels.		Oboh *et al.*, 2015b
Citrus bergamia	Metabolic syndrome	Reduction TG levels, cardiovascular risk, oxidative stress and inflammation, protective effects on hepatic steatosis.	Neohesperidin 182.3 mg/mL; Neoeriocitrin = 165.0 mg/mL; Naringin =160.1 mg/mL Not reported	De Leo *et al.*, 2020 Impellizzeri *et al.*, 2015
Citrus lemon	Antioxidant	Reduction in ROS levels	Hesperidin = 77.1 mg/L; Isorhamnetin 3-*O*-rutinoside = 44.9 mg/L; Rhoifolin = 31.4 mg/L; Eriocitrin = 29.9 mg/L; Diosmin = 25.7 mg/L.	Barberis *et al.*, 2020
Citrus sinensis	Antioxidant	ROS scavenger	Hesperidin = 422.8 mg/L; Naringin = 132.6 mg/L; Narirutin = 100.1 mg/L	Barberis *et al.*, 2020
	Metabolic syndrome	Reduction in body mass index	1 tablet/die [day] containing 400 mg of Morosil®	Cardile *et al.*, 2015

NR: not reported; TPC: total phenolics content; TFC: total flavonoids content; TCC: total carotenoids content; ROS: Reactive Oxygen Species.

The edible portion of hybrid Tacle®, a crossbreeding of *Citrus* × *clementina* and Tarocco tetraploids, was able to influence anthropometric values and lipid and glucose metabolism in a rat model having obesity and MS. For this reason, it could be included in dietary supplements for the management of metabolic disorders (Casacchia *et al*., 2019). A promising anti-obesity potential of fresh squeezed *Citrus* × *clementina* juices against lipase enzymes with IC_{50} values in the range of 179.32–197.69 mg/mL was recently confirmed (Leporini *et al*., 2020a). Moro juice (*Citrus sinensis*) extract (Morosil®, 400 mg/die [day]) was able to induce a significant reduction in body mass index (BMI) after 4 weeks of treatment (Cardile *et al*., 2015).

One important therapeutic approach for suppressing postprandial hyperglycemia is to reduce or bring down dietary carbohydrate digestion and absorption. The inhibition of carbohydrate-hydrolyzing enzymes, α-glucosidase and α-amylase, in the digestive tract also determined reduction in the rate of glucose absorption and consequently blunting the post-prandial plasma glucose rise (Tundis *et al*., 2010). The fresh squeezed *Citrus lemon* exhibited a promising hypoglycemic inhibitory potential with the IC_{50} values of 31.1 and 35.3 mg/mL against α-amylase and α-glucosidase enzymes, respectively (Loizzo *et al*., 2019) whereas values in the range of 67.19–103.43 μg/mL against α-glucosidase were found for fresh squeezed *Citrus* × *clementina* juices from different areas of collection (Leporini *et al*., 2020a). The hypoglycemic ability of *Poncirus trifoliata* juice, related to the genus *citrus,* was investigated against α-amylase and α-glucosidase enzymes (Tundis *et al*., 2016), with the IC_{50} values of 138.14 and 81.27 μg/mL, respectively.

2.3. Antibacterial activity

The development of antibiotic resistance by pathogenic microorganisms necessitated the quest for alternative drug therapy. Medicinal plants are traditionally recognized as conventional medicines, and numerous studies have confirmed their antibacterial activity (Gavarić *et al*., 2015). Haraoui *et al.* (2020) investigated the bacteriostatic action of *citrus* variety juices. Fresh squeezed *citrus limon* juice exhibited inhibition zone of 27.66 mm on *Micrococcus luteus*, followed by *Citrus aurantium* with an area of 24.66 mm against *Staphylococcus aureus.* Interesting results were observed also for *Citrus maxima* and *citrus* × *clementina* with inhibition zones of 23.00 and 17.66 mm, respectively, against *M. luteus.* The lime fresh squeezed juice as antibacterial agent was confirmed against *Salmonella thypii* (Fadillah *et al*., 2020) and *Corynebacterium diphtheriae* (Azhara *et al*., 2020).

Growth of *S. aureus* was inhibited by fresh squeezed *Citrus hystrix* juice (Kusumawardhani *et al*., 2020). Abdallah (2020) suggested *Citrus aurantifolia* as natural antibacterial agent against *S. aureus*, *S. epidermis*, *Pseudomonas aeruginosa,* and *Klebsiella pneumonia*. Low antibacterial activity was found for *Citrus sinensis*.

Prebiotic effects

The prebiotic effect of orange juice could be due to its positive effect on the intestinal microbiota and metabolic biomarkers of young women (aged 28.5 years) (Lima *et al.*, 2019). Indeed, daily intake of orange juice (300 mL/day for 2 months) did not change women's body composition, but improved blood biochemical parameters, such as low-density lipoprotein (LDL)-cholesterol, glucose, and insulin sensitivity. Additionally, orange juice positively modulated the composition and metabolic activity of microbiota, increasing the population of *Bifidobacterium* spp. and *Lactobacillus* spp and reduction of Enterobacteria. Reduction in ammonium (NH_4^+) and increase in the production of short-chain fatty acids were also demonstrated. More recently, this prebiotic effect in healthy female volunteers after intervention with 300-mL/day orange juice for 60 days was confirmed (Fidélix *et al*., 2020). Orange juice stimulated the growth of *Lactobacillus* spp. in the intestinal microbiota and improved glucose metabolism due to the probiotic effect of these bacteria.

Daily supplementation of juices of two oranges (cv. Cara Cara and cv. Bahia) with different flavanone content for 7 days in healthy volunteers resulted in increase in the abundance of Lachnospiraceae and Ruminococcaceae that represented the two most abundant phylum Firmicutes' families present in the gut environment (Brasili *et al*., 2019). Interestingly, after intake of Cara Cara juice positive correlations were found between *Lachnospiraceae* and butyrate, as well as between the most abundant short-chain fatty acids present in the colon, including acetate, butyrate, and propionate (Brasili *et al*., 2019).

Pulp

Antioxidant activity

The radical scavenging ability of eight *citrus* pulp methanol extracts, namely *Citrus sinensis* cv. Hamlin, cv. red blood, cv. succuri, *Citrus limetta* mosambi, *Citrus reticulata* tangerine, *Citrus paradise* macfed, *Citrus aurantium* L., and *Citrus jambhiri* lush (Table 2), were investigated (Rehman *et al*., 2020a). Among these, *Citrus sinensis* cv. succuri had the highest values (65.3%). Costanzo *et al*. (2020) compared the antioxidant potential of powdered *Citrus reticulata*, *Citrus japonica*, and

Table 2.. ***Citrus*** **pulp and biological potential.**

Pulp	Extract	Biological activity	Mechanism	TPC, TFC, TCC, and/or main abundant identified compounds	References
Citrus aurantifolia	50% Ethanol Methanol 10% (KOH, saponification)	Antioxidant	Lipophilic antioxidant capacity	Hesperidin = 23–338 mg/100 g FW; Naringin = 0–271 mg/100 g FW; Neohesperidin = 51–158 mg/100 g FW. Luteolin = 0.02–0.21 mg/100 g FW; (*all-E*)-Zeaxanthin = 0.02–0.03 FW.	Ernawita *et al.*, 2017 Ernawita *et al.*, 2016
		Metabolic syndrome	Inhibition of α-amylase and β-glucosidase enzymes		Ernawita *et al.*, 2017
		Antibacterial	*K. pneumoniae, S. aureus* inhibition		Ernawita *et al.*, 2017
	Ethanol, methanol, and acetone		*E. coli, Klebsiella, Pseudomonas, Salmonella* inhibition	NR	Bhuiyan *et al.*, 2019
Citrus aurantium	Ethanol	Antioxidant	Radical scavenging	TPC = 10.45 mg GAE/g DW; TFC = 7.14 mg RE/g DW; Neohesperidin = 517.10 mg/100 g DW; Naringin = 326.44 mg/100 g DW; Diosmin = 189.16 mg/100 g DW; Hesperidin = 53.05 mg/100 g DW.	Chen *et al.*, 2020
Citrus bergamia	Ethanol	Antioxidant	Radical scavenging	TPC = 208.02 mg GAE/g FW.	Fratianni *et al.*, 2019
		Antibacterial	*E. coli, L. monocytogenes, P. aeruginosa, S. aureus,* and *P. carotovorum.* inhibition		Fratianni *et al.*, 2019
Citrus grandis	Ethanol	Antioxidant	Radical scavenging	TPC = 4.52–7.65 mg GAE /g DW; TFC = 3.67–8.25 mg RE/g DW; Naringin = 199.95–777.65 mg/100 g DW; Neohesperidin = 34.29–52.20 mg/100 g DW; Hesperidin = 2.73–13.00 mg/100 g DW; Diosmin = 25.52–62.6 mg/100 g DW; Eriocitrin = 15.95–19.77 mg/100 g DW.	Chen *et al.*, 2020
Citrus hystrix	50% Ethanol Methanol 10% (KOH, saponification)	Antioxidant	Lipophilic antioxidant capacity	Hesperidin = 74 mg/100 g FW; Neohesperidin = 75 mg/100 g FW. Luteolin = 0.21 mg/100 g FW; (*all-E*)-α-Carotene = 0.04 mg/100 g FW; (*all-E*)-Zeaxanthin = 0.02 mg/100 g FW.	Ernawita *et al.*, 2017 Ernawita *et al.*, 2016
		Metabolic syndrome	Inhibition of α-amylase and β-glucosidase enzymes		Ernawita *et al.*, 2017
		Antibacterial	*K. pneumoniae,* and *S. aureus* inhibition		Ernawita *et al.*, 2017
Citrus japonica	80% Methanol	Antioxidant	Radical scavenging	NR	Costanzo *et al.*, 2020

Citrus limon	Ethanol, methanol, and acetone	Antibacterial	*S. aureus, E. coli, Klebsiella, Pseudomonas, Salmonell* inhibition	NR	Bhuiyan *et al*., 2019
Citrus macroptera	Ethanol, methanol, and acetone	Antibacterial	*K. pneumoniae,* and *Salmonella* inhibition	NR	Bhuiyan *et al*., 2019
Citrus medica	Ethanol	Antioxidant	Radical scavenging	TPC = 148.02 mg GAE/g FW.	Fratianni *et al.*, 2019
		Antibacterial	*E. coli, L. monocytogenes, P. aeruginosa, S. aureus,* and *P. carotovorum* inhibition	TPC = 3.89–8.07 mg GAE /g FW; TFC = 1.89–5.08 mg RE/g FW; Naringin = 10.63 mg/100 g FW; Neohesperidin = 22.96 mg/100 g FW; Hesperidin = 18.55–136.39 mg/100 g FW; Diosmin = 17.36–21.64 mg/100 g FW; Eriocitrin = 23.76–24.11 mg/100 g FW; Narirutin = 26.85 mg/100 g FW.	Chen *et al.*, 2020 Fratianni *et al.*, 2019
Citrus nobilis	Methanol 10% (KOH, saponification)	Antioxidant	Lipophilic antioxidant capacity	(9Z)-Violaxanthin = 2.76 mg/100 g FW; (*all-E*)-Violaxanthin = 1.93 mg/100 g FW; (*all-E*)-Antheraxanthin = 0.64 mg/100 g FW.	Ernawita *et al*., 2016
	50% Ethanol	Metabolic syndrome	Inhibition of α-amylase and β-glucosidase enzymes		Ernawita *et al*., 2017
		Antibacterial	*K. pneumoniae,* and *S. aureus* inhibition		Ernawita *et al*., 2017
	Benzene, ethanol, and methanol		*B. cerus, S. aureus, S. epidermidis, P. vulgaris, S. typhimurium, P. aeruginosa, C. albicans* and *T. viride* inhibition	NR	Sharma and Tyagi, 2019
Citrus reticulata	Ethanol 80% Ethanol	Antioxidant	Radical scavenging Ferric-reducing power	TPC = 4.25–8.55 mg GAE/g DW; TFC = 5.21–10.90 mg RE/g DW; Naringin = 7.68–160.03 mg/100 g; DW Neohesperidin = 19.86–287.4 mg/100 g DW; Hesperidin = 13.83–415.59 mg/100 g DW; Naringin = 7.68–160.03 mg/100 g DW; Eriocitrin = 23.92–45.50 mg/100 g DW; Narirutin = 23.91–120.03 mg/100 g DW; Tangeretin = 1.68–3.84 mg/100 g DW; TPC = 127.33 mg GAE /g extract; TFC = 0.87 mg QE /g extract.	Chen *et al.*, 2020 Bentahar *et al.*, 2020

(continues)

Table 2.. Continued

Pulp	Extract	Biological activity	Mechanism	TPC, TFC, TCC, and/or main abundant identified compounds	References
Citrus sinensis	Ethanol 80% Ethanol 50% Ethanol	Antioxidant Metabolic syndrome	Radical scavenging Ferric-reducing power Inhibition of α-amylase	TPC = 4.98–9.49 mg GAE/g DW; TFC = 5.32–9.81 mg RE/g DW; Hesperidin = 103.14–385.37 mg/100 g DW; Eriocitrin = 13.85–24.55 mg/100 g DW; Narirutin = 41.62–153.48 mg/100 g DW; Didymin = 4.19–13.15 mg/100 g DW; TPC = 159.66 mg GAE/g extract; TFC = 0.85 mg QE/g extract. TPC = 207.69 mg CAE/g FW; TFC = 85.9 mg QE/g FW	Chen *et al.*, 2020 Bentahar *et al.*, 2020 Casacchia *et al.*, 2019
	Benzene, ethanol, and methanol	Antibacterial	*B. cerus, S. aureus, S. epidermidis, P. vulgaris, S. typhimurium, P. aeruginosa, C. albicans* and *T. viride* inhibition		Sharma and Tyagi, 2019
Citrus × *clementina*	80% Methanol 50% Ethanol	Antioxidant	Radical scavenging Lipid peroxidation inhibition	NR TPC = 90.01 mg CAE/g; FW TFC = 41.30 mg QE/g FW. β-cryptoxanthin = 232.4 μM Phytoene = 62.7 μM 9-Z-Violaxanthin = 60.5 μM Antheraxanthin = 39.0 μM Hesperidin = 3.3 μM Naringenin-7-*O*-glucoside = 1.4 μM Narirutin = 1.0 μM	Costanzo *et al.*, 2020 Casacchia *et al.*, 2019 Cilla *et al.*, 2018
	50% Ethanol	Metabolic syndrome	Inhibition of α-amylase and lipase enzyme		Casacchia *et al.*, 2019

NR: not reported; TPC: total phenolics content; TFC: total flavonoids content; TCC: total carotenoids content.

citrus × *clementina* tissue. In the *citrus* × *clementina* pulp extracts, the total antioxidant capacity (TAC) was found to be three-fold higher (7.1 mmol TE/mg fresh weight [FW]) compared to *Citrus reticulata* (2.6 mmol TE/mg FW) and six-fold higher compared to *Citrus japonica* (1.2 mmol TE/mg FW). Similarly, *Citrus sinensis* and *Citrus reticulata* fruits possessed good antioxidant activity studied by the hydroxyl radical scavenging activity and reducing power capacity methods (Bentahar *et al*., 2020).

Fratianni *et al*. (2019) indicated that *Citrus bergamia* and *Citrus medica* cv. *Salò* homogenized pulp extracts exhibited the highest antioxidant potential compared to *Citrus medica.* Previously, the *in vitro* lipophilic antioxidant capacity of seven *citrus* pulp extracts was reported (Ernawita *et al*., 2017). Among them, jeruk makin (*Citrus aurantium*) showed the highest antioxidant capacity (19.5 µmol TE/100 g), followed by jeruk calung pulp extracts (*Citrus aurantium*) and jeruk nipis (*Citrus aurantiifolia*) (10.7 and 10.6 µmol α-TE/100 g, respectively). Previously, *in vivo* studies have reported that *Citrus macroptera* ethanol pulp extract possessed a significant lipid-lowering activity and a significant diminution of lipid peroxidation in liver and kidney tissues was observed (Paul *et al*., 2015). The protective effect against oxidative stress of pulp bio-accessible fractions of oranges from Navel and Cara oranges cultivars as well as clementine was also demonstrated (Cilla *et al*., 2018). These fractions act by pre-serving cell viability, correct cell cycle progression, mitochondrial membrane potential, and diminishing ROS level and lipid peroxidation.

Recently, the effect of dried orange pulp on antioxidants level in the plasma was evaluated (Allam Sabbah *et al*., 2020). Results demonstrated that the value of total antioxidant capacity, as a biomarker of oxidative stress, was gradually increased (ranged from 0.420 to 0.433 mm/L) by increasing the level of dried orange pulp supplementation (25, 50, and 75%). Additionally, a reduction of total lipids values was observed.

Metabolic syndrome

The α-amylase inhibition activity of seven *citrus* pulp extracts was analyzed by Ernawita *et al*. (2017). Makin and jeruk nipis pulp extracts exhibited the lowest IC_{50} values (18.8 and 19.4 mg/mL, respectively), while calung extract possessed less activity (IC_{50} = 56.2 mg/mL).

Oral administration of *Citrus hystrix* and *Citrus maxima* pulp extracts (5, 50, 300, and 2,000 mg/kg body weight (BW) in streptozotocin (STZ)-induced diabetic rats for 14 days was able to reduce blood glucose, TG level, and serum cholesterol. Additionally, the HDL-cholesterol level was found to improve (Arumugam *et al*., 2019).

The lipase inhibitor activity of *citrus* pulp extracts was reported by Casacchia *et al.* (2019), founding the IC_{50} values of 86.30, 105.90, and 67.20 mg/mL, respectively, for *Citrus clementina*, *Citrus sinensis*, and their hybrid called Tacle®.

Antibacterial activity

Citrus bergamia, *Citrus medica,* and *Citrus medica* cv. *Salò* pulp extracts were described as antibacterial agents against *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa, S. aureus*, and *Pectobacterium carotovorum* (Fratianni *et al*., 2019). The antibacterial activity of various *citrus* pulp extracts was also reported by Ernawita *et al*. (2017). Among them, jeruk makin and jeruk nipis showed the highest inhibitor capacity against *Klebsiella pneumoniae* (IC_{50} = 3.3 and 4.1 mg/mL) and *S. aureus* (IC_{50} = 2.6 and 3.1 mg/mL). In addition, *Citrus limon*, *Citrus aurantifolia*, and *Citrus macroptera* pulp extracts were screened for antimicrobial activity against *S. aureus*, *E. coli*, *Klebsiella* sp., *Pseudomonas* sp., and *Salmonella* sp. (Bhuiyan *et al*., 2019). *Citrus macroptera,* a taxonomic synonym of *Citrus hystrix* (kaffir lime) known for its antioxidant, nutritious, and therapeutic uses (Paul *et al.,* 2017) ethanol extracts exhibited the highest zone of inhibition (14 mm) against *Klebsiella* sp. while *Citrus aurantifolia* methanol extract showed the highest zone of inhibition (8 mm) against *Salmonella* sp.

Citrus seed

Antioxidant potential

Bitter orange, blonde orange, sweet orange, lemon, and mandarin seed ultrasound methanol extracts (Table 3) were investigated for their antioxidant ability but no differences were found in the radical scavenging activity (Falcinelli *et al*., 2020). Conversely, Costanzo *et al.* (2020) demonstrated that powered *Citrus reticulata* seed extract tissue showed the highest antioxidant capacity (55.6 mmol TE/mg FW) compared to *Citrus japonica* seed extract tissue (3.2 mmol TE/mg FW). The radical scavenging ability of eight *citrus* seed extracts were investigated (Rehman *et al*., 2020a). Among them, *Citrus jambhiri* lush, *Citrus sinensis* cv. red blood, and *Citrus reticulata* tangerine possessed the highest activity (54.3, 53.6, and 53.3%, respectively). Previously, the following ranking of radical scavenging effect was demonstrated: lemon seeds extract > orange seeds extract > mandarin seeds extract (İnan *et al*., 2018).

Table 3. Bioactivity of *Citrus* seed.

Seed	Extract	Biological activity	Mechanism	TPC, TFC, TCC, and/or main abundant identified compounds	References
Citrus aurantium	Methanol	Antioxidant	Radical scavenging	TPC = 2.5 GAE/g DW	Falcinelli *et al.*, 2020
		Anti-inflammatory	Anti-edematogenic effects		
		Antibacterial	*Proteus* and *Pseudomonas* inhibition	NR	Aladekoyi *et al.*, 2016
Citrus aurantium	Methanol	Antioxidant	Radical scavenging	TPC = 107 mg/100 g; TFC = 20.8 mg/100 g.	Rehman *et al.*, 2020c
Citrus jambhiri	Methanol	Antioxidant	Radical scavenging	TCC = –25 mg/g FW	Costanzo *et al.*, 2020
Citrus jambhiri	Methanol	Antioxidant	Radical scavenging	TPC = 129 mg/100 g; TFC = 22.8 mg/100 g.	Rehman *et al.*, 2020c
Citrus junos		Anti-inflammatory	Inhibition of NO production	Not reported	Ko *et al.*, 2020
Citrus limon	Methanol	Antioxidant	Radical scavenging Restoration of antioxidant defense system	TPC = 1.2 GAE/g DW TPC = 152.70–212.30 mg GAE/kg	Falcinelli *et al.*, 2020 İnan *et al.*, 2017
		Metabolic syndrome	Reduction of glucose and lipid levels	NR	Demir and Celik, 2019
		Antibacterial	*Klebsiella*, *Proteus* and *Pseudomonas* inhibition	NR	Aladekoyi *et al.*, 2016
Citrus limetta	Methanol	Antioxidant	Radical scavenging	TPC = 99.1 mg/100 g; TFC = 19.37 mg/100 g.	Rehman *et al.*, 2020c
Citrus maxima	Ethanol	Antibacterial	*S. aureus*, *E. coli*, and *B. subtilis* inhibition	TFC = 1602.740 mg/kg.	Sahlan *et al.*, 2018
Citrus paradise	Ethanol	Antibacterial	*S. aureus*, *E. coli*, *S. typhimurium, S. enteritidis, P. aeruginosa*, *K. pneumoniae, Citrus utilis, and B. cereus* inhibition	TFC = 483.562 mg/kg.	Sahlan *et al.*, 2018
Citrus reticulata	Methanol	Antioxidant	Radical scavenging	TPC = 112 mg/100 g; TFC = 21.5 mg/100 g.	Rehman *et al.*, 2020c
	Methanol			TPC = 2.4 GAE/g DW. TCC = –10 mg/g FW. TPC = 152.70–212.30 mg GAE/kg.	Falcinelli *et al.*, 2020 Costanzo *et al.*, 2020 İnan *et al.*, 2017
Citrus sinensis	Methanol	Antioxidant	Radical scavenging	TPC = 101–118 mg/100 g; TFC = 20.1–22.60 mg/100 g.	Rehman *et al.*, 2020c
Citrus sinensis	Methanol			TPC = 1.3 GAE/g DW	Falcinelli *et al.*, 2020
	n-Hexane	Metabolic syndrome	Reduction of fasting blood glucose, serum TG, serum cholesterol, HDL	NR	Chilaka *et al.*, 2015
	Ethanol	Antibacterial	*S. aureus*, *Enterococcus faecalis, P. aeruginosa, E. coli* and *Citrus albicans* inhibition	NR	Oikeh *et al.*, 2020

NR: not reported; TPC: total phenolics content; TFC: total flavonoids content; TCC: total carotenoids content.

Metabolic Syndrome

It has been reported recently that lemon seed extract could prevent diabetic complications due to reduction in glucose and lipid profile levels and restoration of antioxidant defense system (Demir and Celik, 2019). Previously, a reduction of blood glucose in alloxan-induced diabetic rats was observed after treatment with emulsified sweet orange seed oil (1000 mg/kg BW). In addition, this seed oil improved sugar and lipid profile with reduction of serum TG, cholesterol, and increased HDL-cholesterol in diabetic rats (Chilaka *et al.*, 2015).

Anti-inflammatory effects

Nitric oxide (NO) is recognized as a mediator and regulator in pathological reactions, especially in acute inflammatory responses (Terao, 2009). The development of substances to prevent the overproduction of NO has become a new research target to treat chronic inflammatory diseases. Ko *et al.* (2020) reported the anti-inflammatory effect of *Citrus junos* seed oil. NO production was suppressed by 53% at a concentration of 0.05% that does not show cytotoxicity. The possible anti-inflammatory and antinociceptive activity of *Citrus aurantium* seed oil, obtained by using Soxhlet apparatus with *n*-hexane, was evaluated by using formalin-induced paw licking, edema, and myeloperoxidase activity assessment (Azadeh *et al*., 2019). The results showed that seed oil exhibited anti-inflammatory properties in the first and second phases of formalin test, anti-edematogenic effects but exerted no effects on myeloperoxidase activity.

Antibacterial potential

Recently, the antibacterial activities of *Citrus sinensis* seed oil, obtained by Soxhlet apparatus using *n*-hexane as solvent and ethanol extract, was studied (Oikeh *et al*., 2020). The results showed that the non-oil extract had better antibacterial activity against *S. aureus*, *Enterococcus faecalis*, and *E. coli*. On the contrary, the seed oil had better activity against *Salmonella* spp. Similar susceptibility was found for *P. aeruginosa*. Previously, it was demonstrated that *Citrus sinensis* seed oil obtained by Soxhlet apparatus using *n*-hexane as solvent possessed antibacterial activity against *S. aureus* and *Candida albicans (*Olabanji *et al*., 2016). Buket *et al*. (2018) also reported that the lemon, orange, and grapefruit cold-pressed seed oil had inhibition zones ranging from 6.62 to 11.00 mm against pathogenic bacteria such as *S. aureus*, *E. coli*, *S. typhimurium, Salmonella enteritidis, P. aeruginosa*, *K. pneumoniae, Candida utilis*, and *Bacillus cereus Holl*. The antimicrobial and antifungal activities of aqueous and ethanolic grapefruit seed extracts were confirmed against *S. aureus*, *E. faecalis*, *Bacillus subtilis*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *C. albicans* (Eryilmaz *et al*., 2018). The ethanolic extract of pomelo seeds also gives positive results with growth-inhibition effect on *Bacillus subtilis*, *S. aureus*, and *E. coli* (Sahlan *et al*., 2018). Oil extracted from lemon, lime, and bitter orange seeds possessed different antimicrobial potential. Similar activity was found for *Staphylococcus*, but only lemon seed oil has activity against *Klebsiella* and the highest zone of inhibition against *Proteus*, while bitter orange has a maximum zone of inhibition (0.25 mm) against *Pseudomonas* (Aladekoyi *et al*., 2016).

Citrus peels

Antioxidant effects

Recently, the antioxidant potential of *citrus* × *clementina* peel extracts, collected from different areas of Calabria and obtained by using different methodologies, was studied (Leporini *et al*., 2020a). Results demonstrated that sample from Cetraro obtained by ultrasound extraction in ethanol possessed the highest antioxidant activity (Table 4). Interestingly, the *citrus* × *clementina* juice enriched with this extract (20% v/w) increased its antioxidant potential. Similarly, Pereira *et al*. (2020) reported the increase of beer antioxidant activity after addition of orange peels extract.

Huang *et al*. (2020) compared the antioxidant ability of eight *citrus* peel extracts: grapefruit, pomelo, kumquat, mandarin, ponkan, tangerine, lemon, and sweet orange. The most active samples were ponkan extract in DPPH and FRAP assays (386.25 and 466.14 μmol TE/g of extract, respectively), tangerine extract in ABTS assay (689.43 μmol TE/g), and pomelo in ORAC assay (1964.0 μmol TE/g). An inhibition of 92.87% was reported for *Citrus hystrix* peels extract against DPPH radical (Ramli *et al*., 2020).

Citrus sinensis and *Citrus aurantium* peels' extracts were investigated as potent antioxidant agents against lipid peroxidation (Rafiq *et al*., 2018). Furthermore, the bergamot extract showed a higher ABTS radical inhibition with a value of 136.3 mmol TE/g dry weight (DW). The capacity of *Citrus medica* Diamante hydroalcoholic peels extract to inhibit both DPPH and ABTS radicals (IC_{50} = 0.81 and 3.48 mg/mL, respectively) was also demonstrated by Menichini *et al*. (2016). In β-carotene, this extract exhibited an IC_{50} value of 0.23 mg/mL. Da Silva *et al*. (2018) studied the antioxidant potential of pomelo peels cv. Toranja Buraram in *n*-hexane, ethyl acetate, acetone, ethanol, methanol, and methanol:water (80:20). The ethyl acetate and methanolic extracts presented the highest antioxidant activity *in vitro* by DPPH (IC_{50} = 298.3 and 303.8 μg/mL, respectively), ABTS assay (IC_{50} = 298.2 and 296.4 μg/mL, respectively), and FRAP (IC_{50} = 234.6 and 398.1 μg/mL, respectively).

Long *et al*. (2021) evaluated the antioxidant effects of ethanol extract and its three subfractions—petroleum ether, ethyl acetate, and water extracts—of *Citrus sinensis* cv. Gannanzao peels. The ethyl acetate extract exhibited the best antioxidant potential compared to four extracts in all antioxidant assays with the IC_{50} values of 38.33 mg/mL and 8.47 mg/mL in DPPH and ABTS tests, respectively, and value of 21.54 mM Trolox equivalents (TE)/mg DW in FRAP assay. The results correspond to those reported by Guo *et al*. (2020) for *Citrus sinensis* cv. Newhall peels extract.

Table 4. Biological activity of *Citrus* peels.

Peels	Extract/Essential oil	Biological activity	Mechanism	TPC, TFC, TCC, and/or main abundant identified compounds	References
Citrus aurantifolia	96% Ethanol	Antibacterial Anti-inflammatory	*S. typhi* inhibition Reduction of IL-6 levels	NR	Kasim *et al.*, 2020 Kasim *et al.*, 2020
	70% Ethanol		Inhibition of paw edema.	NR	Pallavi *et al.*, 2018
Citrus aurantium	Ethanol	Antioxidant Metabolic syndrome	Radical scavenging Inhibition of lipid peroxidation	TPC = 18.15 mg GAE/g FW; TFC = 17.09 mg RE/g FW; Neohesperidin = 1620.77 mg/100 g DW; Naringin = 879.33 mg/100 g DW; Hesperidin = 35.88 mg/100 g DW; Naruritun = 18.72 mg/100 g DW. NR	Chen *et al.*, 2020 Rafiq *et al.*, 2018
	80% Ethanol		Reduction of BW, lipid droplets regulating adipogenesis and thermogenesis.	Naringin = 0.916 mg/mL; Neohesperidin = 0.657 mg/mL	Park *et al.*, 2019
	70% Ethanol	Anti-inflammatory	Inhibition of paw edema.		Pallavi *et al.*, 2018
Citrus grandis	Ethanol	Antioxidant	Radical scavenging	TPC = 8.79–14.93 mg GAE/g FW; TFC = 8.25–14.03 mg RE/g FW; Naringin = 694.15–1676.31 mg/100 g DW; Neohesperidin = 28.42–73.04 mg/100 g DW; Hesperidin = 7.39–33.39 mg/100 g DW; Naruritun = 2.72–6.37 mg/100 g DW; Eriocitrin = 20.42–34.49 mg/100 g DW; Diosmin = 6.02–1.29 mg/100 g DW.	Chen *et al.*, 2020
	70% Ethanol	Anti-inflammatory	Inhibition of paw edema.		Pallavi *et al.*, 2018
Citrus limon	70% Ethanol	Antioxidant	Radical scavenging	TPC = 198.52 mg GA/g extract; TFC = 183.13 mg/g extract; Hesperidin = 84.24 mg/g extract; Eriocitrin = 84.80 mg/g extract; Narrutin = 13.56 mg/g extract.	Huang *et al.*, 2020
Citrus maxima	70% Ethanol	Antioxidant	Radical scavenging	TPC = 138.93 mg GA/g extract; TFC = 416.54 mg/g extract; Naringenin = 386.37 mg/g extract; Rhoifolin = 28.54 mg/g extract.	Huang *et al.*, 2020
	95% Ethanol	Metabolic syndrome	Reduction the blood glucose level, total cholesterol, TG, and LDL-C. Inhibition of lipase enzyme	NR	Ani and Ochu, 2020 Huang *et al.*, 2020
Citrus medica Diamante	70% Ethanol	Antioxidant	Radical scavenging Inhibition of lipid peroxidation	Apigenin = 62.8 mg/Kg FW; Hesperitin = 30.4 mg/Kg FW; Neringenin = 18.6 mg/Kg FW; Quercetin = 18.2 mg/Kg FW.	Menichini *et al.*, 2016

		Metabolic syndrome	Reduction of serum glucose, cholesterol and TG.		Menichini *et al.*, 2016
Citrus paradisi	70% Ethanol	Antioxidant	Radical scavenging	TPC = 179.13 mg GA/g extract; TFC = 474.55 mg/g extract; Naringenin = 252.13 mg/g extract; Neohesperidin = 182.32 mg/g extract; Narirutin = 14.80 mg/g extract; Hesperidin = 6.55 mg/g extract.	Huang *et al.*, 2020
	Water, 80% ethanol, and *n*-hexane	Metabolic syndrome	Reduction in the cholesterol and TG levels	Nobiletin = 18.13 µg/mL.	Fayek *et al.*, 2017
Citrus reticulata	70% Ethanol	Antioxidant	Radical scavenging	TPC = 215.11 mg GA/g extract; TFC = 192.22 mg/g extract; Hesperidin = 150.96 mg/g extract; Narirutin = 16.79 mg/g extract; Eriocitrin = 11.33 mg/g extract.	Huang *et al.*, 2020
	Ethanol		Ferric-reducing power	TPC = 10.58–23.46 mg GAE/g FW; TFC = 7.57–21.37 mg RE/g FW; Naringin = 6.31–77.99 mg/100 g DW; Neohesperidin = 0–745 mg/100 g DW; Hesperidin = 39.98–1893.73 mg/100 g DW; Naruritun = 16.21–145.56 mg/100 g DW; Eriocitrin = 10.32–268.69 mg/100 g DW; Diosmin = 3.29–38.73 mg/100 g DW.	Chen *et al.*, 2020
	Water *n*-butane	Metabolic syndrome	Reduction in body mass index, body fat percentage and in waist circumference, TC and TG levels. Reduction of blood glucose level and plasma insulin level. Inhibition of lipase enzyme	Hesperidin = 40 mg Naringenin = 28 mg Quercetin = 26 mg Rutin = 25 mg Nobiletin = 32.28% Tangeritin = 22.82%	Kamel *et al.*, 2019 Guo *et al.*, 2016 Huang *et al.*, 2020
	Alkaline hot water Dichloromethane and ethyl acetate	Anti-inflammatory	Inhibition of NO Inhibition of COX1 and COX2	Narirutin = 0.26–4.52 mg/g extract; Hesperidin = 7.02–26.81 mg/g extract; Nobiletin = 0.39–7.79 mg/g extract; Tangeretin = 0.19–3.37 mg/g extract. NR	Chen *et al.*, 2017 Hamdan *et al.*, 2020
Citrus sinensis	70% ethanol 95% Ethanol, petroleum ether, ethyl acetate, and water	Antioxidant	Radical scavenging	TPC = 149.42 mg GA/g extract; TFC = 186.81 mg/g extract; Hesperidin = 148.63 mg/g extract; Narirutin = 21.49 mg/g extract;	Huang *et al.*, 2020
	95% Ethanol, petroleum ether, ethyl acetate, and water		Ferric-reducing power	Didymin = 7.18 mg/g extract. TPC = 0.12–0.49 mM GAE/ mg DW; TFC = 1.29–4.20 mM HE/ mg DW; Sinensetin = 0–121.3 mg/mg; Hesperidin = 1.56–21.23 µg/mg; Eriocitrin = 0–4.20 mg/mg;	Long *et al.*, 2021

(*continues*)

Table 4. Continued

Peels	Extract/Essential oil	Biological activity	Mechanism	TPC, TFC, TCC, and/or main abundant identified compounds	References
				Narirutin = 0.94–6.27 mg/mg; Tangeretin = 0.18–2.72 mg/mg. TPC = 18.71–91.55 mg GAE/g DW; TFC = 3.62–86.91 mg QE/g DW; Sinensetin = 0–36.92 mg/mg DW; Nobiletin = 0–35.54 mg/mg DW; Narirutin = 1.27–20.21 mg/mg DW; Hesperidin = 1.65–42.56 mg/mg DW.	Guo *et al.*, 2020
	0.5 g and 1 g of CitrusiM® Methanol Water, 80% ethanol, and *n*-hexane 50% Ethanol	Metabolic syndrome	Reduction of fat, and increase lean mass reducing waist circumference. Reduced blood glucose and plasma insulin. Reduction in the cholesterol and TG levels. Inhibition of α-amylase and lipase enzymes	NR Rutin = 1248.3 mg/g DW;*p*-Coumaric acid = 957.4 mg/g DW; Protocatechuic acid = 326.3 mg/g DW; Ferulic acid = 316.0 mg/g DW; Naringenin = 220.7 mg/g DW; Vanillic acid = 112.2 mg/g DW. Nobiletin = 73.15 mg/mL. TPC = 177.16 mg CAE/g FW; TFC = 65.9 mg QE/g FW;	Kegele *et al.*, 2019 Sathiyabama et al, 2018 Fayek *et al.*, 2017 Casacchia *et al*., 2019
	Methanol and ethanol	Anti-inflammatory	Inhibition of edema.	Not reported	Osarumwense *et al.*, 2017
	Benzene, ethanol, and methanol	Antibacterial	*B. cerus, S. aureus, S. epidermidis, P. vulgaris, S. typhimurium, P. aeruginosa, C. albicans* and *T. viride* inhibition *E. coli* and *B. subtillis* inhibition	NR	Sharma et Tyagi, 2019 Guo *et al.*, 2020
Citrus tumida	HDF+ 5% peel powder	Metabolic syndrome	Suppression BW gain	NR	Sato *et al.*, 2019
Citrus unshiu	Methanol	Antioxidant Metabolic syndrome	Radical scavenging Ferric-reducing power Inhibition of α-glucosidase and lipase enzymes	Hesperidin = 50027 mg/g DW; Narirutin = 9284 mg/g DW; Nobiletin = 103.8 mg/g DW; Tangeretin = 55.5 mg/g DW.	Kim *et al.*, 2020 Kim *et al.*, 2020
	Fermented dried	Anti-inflammatory	Inhibition of LPS-induced NO, iNOS, COX-2 protein, TNF-γ and IL-6	NR	Kim *et al.*, 2019a
Citrus × *clementina*	Ethanol and 80% ethanol	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	TPC = 3.45–8.75 mg CAE/g FW; TFC = 2.47–6.05 mg QE/g FW; TCC = 9.66–39.84 mg b-carotene E/g FW; Hesperidin = 155.28–1093.36 mg/100 g FW; Sinensetin = 19.56-37.99 mg/100 g FW; Tangeretin = 5.43–9.60 mg/100 g FW; Luteolin = 3.02–8.58 mg/100 g FW.	Leporini *et al.*, 2020a

	50% Ethanol	Metabolic syndrome	Inhibition of α-amylase, β-glucosidase and lipase enzymes	TPC = 109.86 mg CAE/g FW; TFC = 61.3 mg QE/g FW;	Leporini *et al.*, 2020a Casacchia *et al.*, 2019
Citrus aurantifolia	Essential oil	Antioxidant	Radical scavenging	Limonene = 42.35% γ-terpinene = 15.44% β-pinene = 12.57%	Lin *et al.*, 2019
		Metabolic syndrome	Improve the serum TC, TG, LDL-c, alanine aminotransferase, and aspartate transaminase levels		Lin *et al.*, 2019
Citrus aurantium	Essential oil	Antioxidant	Radical scavenging	Limonene = 85.22%; β-myrcene = 4.30; α-pinene = 1.28%.	Taneva *et al.*, 2019
			Ferric-reducing power		Farahmandfar *et al.*, 2020
			Increase in mRNA gene expression of Cu-Zn SOD, CAT, and GPx	Limonene = 81.19%; Linalool = 4.06%; β-myrcene = 3.07.	Hsouna *et al.*, 2018
		Antibacterial	*E. coli, P. aeruginosa, L. monocytogenes, S. aureus, B. subtilis, C. albicans* and S. paratyphi B inhibition	Limonene = 48.7%; Linalool = 32.4%; β-myrcene = 1.2%. Limonene = 61.85%; γ-Terminene = 9.15%; Octanal = 5.28%; α-pinene = 3.02%.	Guo *et al.*, 2018
		Anti-inflammatory	Reduction of NO production		Hsouna *et al.*, 2018
			Reduction of cell migration, cytokine production and protein extravasation	Limonene = 31.1%; γ-terpinene = 10.8%; β-pinene = 8.5%; Neral = 7.1%.	Amorim *et al.*, 2016
Citrus bergamia	Essential oil	Antioxidant	Radical scavenging	NR	Lombardo *et al.*, 2020
			Ferric-reducing power		
			Chelate pro-oxidant metal		
		Anti-inflammatory	Reduction of IL-1α, IL-6, TNF-γ nitrite/nitrate and PGE_2		Lombardo *et al.*, 2020
Citrus limon	Essential oil	Antioxidant	Radical scavenging	Limonene = 61.72%; 3-Carene = 13.67%; α-Pinene = 13.97%.	Guo *et al.*, 2018
		Metabolic syndrome	Inhibition of α-amylase and β-glucosidase enzymes	Limonene = 53.07%; β-pinene = 9.53%; Borneol = 5.57%:	Oboh *et al.*, 2017
		Anti-inflammatory	Reduction of cell migration, cytokine production and protein extravasation	Limonene = 53.9%; β-Pinene = 13.1%; Sabinene = 3.4%.	Amorim *et al.*, 2016

(*continues*)

Table 4. Continued

Peels	Extract/Essential oil	Biological activity	Mechanism	TPC, TFC, TCC, and/or main abundant identified compounds	References
		Antibacterial	*E. coli*, *Fusobacterium necrophorum*, *Trueperella pyogenes, S. areus* inibition *P. aeruginosa, L. monocytogenes, B. subtilis, C. albicans* and *S. paratyphi* B inhibition	Limonene = 65.59% β-Pinene = 15.06%; γ-Terpinene = 7.93.	Braga *et al.*, 2020 Guo *et al.*, 2018
Citrus lumia	Essential oil	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	Limonene = 48.90%; Linalool = 18.24%. Linalyl anthranilate = 10.96%.	Smeriglio *et al.*, 2018
Citrus medica	Essential oil	Antioxidant	Radical scavenging	Limonene = 48.94%; α-pinene = 2.88%; Myrcene = 2.29%.	Guo *et al.*, 2018
		Antibacterial	*E. coli, P. aeruginosa, L. monocytogenes, S. areus, B. subtilis, C. albicans* and *S. paratyphi* B inhibition		Guo *et al.*, 2018
Citrus paradisi	Essential oil	Antioxidant Antibacterial	Radical scavenging *E. coli, S. aureus, P. aeruginosa and Citrus albicans* inhibition	Limonene = 91.78%; δ-3-carene = 2.07%.	Denkova-Kostova *et al.*, 2020
Citrus reticulata	Essential oil	Antioxidant	Radical scavenging	Limonene = 61.72%; 3-Carene = 13.67%; α-Pinene = 13.97%.	Guo *et al.*, 2018
		Metabolic syndrome	Improve the hypercholesterolemia, and hepatic steatosis. Reduction in serum total cholesterol, LDL-C, hepatic TC and TG levels.	Limonene = 84.89%; δ-3-carene = 3.14%. Limonene = 76.58%; γ-Terpinene = 12.88%. β-Myrcene = 2.45%.	Denkova-Kostova *et al.*, 2020 Konglong *et al.*, 2020

		Antibacterial	*E. coli, P. aeruginosa, L. monocytogenes, S. areus, B. subtilis, Citrus albicans* and S. paratyphi B inhibition		Guo *et al.*, 2018
Citrus sinensis	Essential oil	Antioxidant	Radical scavenging Inhibition of lipid peroxidation	Limonene = 95.11%; Myrcene = 1.07%.	Magalhães *et al.*, 2019
		Metabolic syndrome	Inhibition of α-amylase and β-glucosidase enzymes	Limonene = 92.14%; β-Myrcene = 2.70%.	Oboh *et al.*, 2017
		Antibacterial	*E. coli, P. aeruginosa, L. monocytogenes, S. areus, B. subtilis, C. albicans* and *S. paratyphi* B inhibition	Limonene = 79.28%; 3-carene = 7.76%; β-Pinene = 2.28%.	Guo *et al.*, 2018
		Anti-inflammatory	Reduction of edema	Limonene = 80.5%; *trans*-b-ocimene = 6.5%; Linalool = 2.7%.	Thandiswa *et al.*, 2020
Citrus unshiu	Essential oil	Antioxidant	Radical scavenging	Limonene = 64.21%; g-Terpinene = 9.44%; Myrcene = 8.37%; a-Pinene = 4.98%.	Guo *et al.*, 2018
		Antibacterial	*E. coli, P. aeruginosa, L. monocytogenes, S. areus, B. subtilis, C. albicans* and *S. paratyphi* B inhibition		Guo *et al.*, 2018
Citrus × *clementina*	Essential oil	Antioxidant Metabolic syndrome	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation Inhibition of α-amylase and β-glucosidase and lipase enzymes.	Limonene = 61.31%; Linalool = 3.29–6.64%; Myrcene = 3.56–9.10%.	Leporini *et al.*, 2020a

NR: not reported; TPC: total phenolics content; TFC: total flavonoids content; TCC: total carotenoids content.

Recently, the antioxidant activities of *Citrus reticulata*, *Citrus paradise*, and *Citrus lemon* peels' essential oils were reported (Denkova-Kostova *et al*., 2020). The radical scavenging potential on DPPH radical revealed that the highest percentage of inhibition was found in the grapefruit (87.5%), followed by lemon and tangeretine with values of 86.1% and 78.0%, respectively. The antioxidant potential of grapefruit, lemon, mandarin, and orange essential oils was also investigated by Raspo *et al*. (2020). Mandarin exhibited the highest activity in ABTS test, grapefruits exhibited the highest activity in FRAP test, and lemon exhibited the highest activity in DPPH test.

Citrus lumia essential oil showed a strong antioxidant activity in different assays, with the following order of potency (expressed as IC_{50}): β-carotene (22 μg/mL) > ORAC(46 μg/mL) > DPPH (104 μg/mL) > Folin-Ciocalteu (181 μg/mL) > FRAP (202 μg/mL) > Trolox equivalent antioxidant capacity (TEAC) (233 μg/mL) (Smeriglio *et al*., 2018). For *Citrus aurantium* peels' essential oil, an inhibition percentage of 88.1% against DPPH radical was observed (Tevena *et al*., 2019). A lower activity was reported for bitter orange with an inhibition percentage of 31.33% (Farahmandfar *et al*., 2020).

Metabolic syndrome

Recently, the effects of *Citrus reticulata* peels' water extract (800 mg) administered to obese adolescents were analyzed (Kamel *et al*., 2019). In this clinical trial, the extract showed a reduction in BMI, body fat percentage, and waist circumference after 4 and 8 weeks of supplementation. Additionally, a reduction of total cholesterol (TC) and TG levels was observed. Huang *et al*. (2020) compared the *in vitro* anti-obesity ability of grapefruit, pomelo, kumquat, mandarin, ponkan, tangerine, lemon, and sweet orange peels' extracts. Among them, the most active sample was sweet orange, followed by tangerine and ponkan with the IC_{50} values of 87.25, 109.44, and 126.62 mg/mL, respectively, against lipase enzymes. Also, for *Citrus unshiu* peels' water extract, an inhibitor effect was reported for lipase activity (IC_{50} = 507.01 μg/mL) (Kim *et al*., 2016). Better results were observed for *citrus* × *clementina* peels extract with values in the range of 112.06–191.91 mg/mL (Leporini *et al*., 2020a). In particular, peels extract from Cetraro, obtained by ultrasound extraction EtOH, exhibited the strongest hypolipidemic activity. Additionally, this extract increased the hypolipidemic activity of *citrus* × *clementina* juice when added at a concentration of 20% (w/v). Oboh *et al*. (2017) reported that the lemon peels' essential oil exhibited stronger inhibitory activity on α-amylase and α-glucosidase activities (IC_{50} values of 8.16 and 7.56 μg/mL, respectively) compared to orange peels' essential oil (IC_{50} values of 11.51 and 11.53 μg/mL, respectively).

The effectiveness of *Citrus maxima* peels ethanol extract was suggested recently in the management of diabetes (Ani and Ochu, 2020). Indeed, the administration of this extract (600 mg/kg BW/day) for 14 days decreased the blood glucose level (70.17%), TA (30.86%), TG (10.58%), and LDL-cholesterol (10.20%). Additionally, an increase of HDL-cholesterol (4.43%) was observed. Dietary ingestion of *Citrus tumida* Hort. ex Tanaka peels powder (5% w/w) suppressed BW gain by decreasing epidydimal, perirenal, and subcutaneous fat weights (Sato *et al*., 2019). A similar effect, that is a significant decrease of BW, was observed for *Citrus aurantium* extract (100 mg/kg/day) administered for 8 weeks. Additionally, the same treatment in 3T3-L1 adipocytes determined a reduction of lipid droplets regulating adipogenesis and thermogenesis *via* AMP-activated protein kinase alpha (AMPKα) pathway (Park *et al*., 2019). Kegele *et al*. (2019) investigated the effects of CitrusiM® (*Citrus sinensis* dried extract) on body composition: percentage of lean mass and percentage of fat mass. This extract determined a significant reduction of fat, and increase in lean mass reducing waist circumference after a dose of 0.5 or 1 g/day. Similarly, the obese mice treated with supplementation of 0.25% and 0.5% of *Citrus reticulata* extract in food for 12 weeks exhibited a reduction of 21% and 34% in BW, respectively (Guo *et al*., 2016). This effect was probably due to the action of *citrus* phytochemicals on metabolism of glucose and fatty acids.

Administration of *Citrus sinensis* methanol peels extract at doses of 50 and 100 mg/kg in diabetic rats reduced fasting blood glucose by 56.1% and 55.7%, respectively, and plasma insulin levels by 22.9% and 32.7%, respectively (Sathiyabama *et al*., 2018). *Citrus medica* Diamante hydroalcoholic peels extract was tested in db/db mouse model for leptin deficiency. This mutation confers susceptibility to obesity, insulin resistance, and T2DM. Administration of 600 mg/kg of Diamante peels extract significantly decreased the serum glucose level (Menichini *et al*., 2016). This extract was rich in phenolic compounds that are known to posses several actions to improve glucose tolerance as reported below. The *in vivo* reduction of blood glucose and plasma insulin levels was demonstrated for both *Citrus reticulata* and *Citrus sudachi* peels extract (Guo *et al*., 2016; Kobayashi *et al*., 2017). In particular, *Citrus sudachi* exerted its effect via reduction of TNF-α mRNA expression.

Literature showed that *citrus* genus was able to counteract the effect of high cholesterol level (Favela-Hernández *et al*., 2016). Recently, the hypocholesterolemic effects of mandarin peels' aqueous and *n*-hexane extracts was demonstrated (Fayek *et al*., 2017). The results showed that these extracts decrease the cholesterol level by 59.3% and 56.8%, respectively. A reduction in cholesterol and TG levels was also observed with *Citrus medica* cv.

Diamante peels' hydroalcoholic extract of (300 and 600 mg/kg/day) administered in Zucker diabetic rats for 4 weeks (Menichini *et al*., 2016). Successively, Konglong *et al*. (2020) demonstrated that *Citrus reticulata* peels' essential oil was able to ameliorate hypercholesterolemia and hepatic steatosis. In addition, a reduction in serum TC, LDL-cholesterol, and hepatic TC and TG levels was observed after supplementation (0.5% and 0.75%).

Anti-inflammatory activity

Fermented dried *Citrus unshiu* peel extracts were investigated for its anti-inflammatory activities in murine macrophages and moisturizing effects in human keratinocytes (Kim *et al*., 2019a). Results evidenced that *Citrus unshiu* peels extract, rich in polyphenolic compounds, was able to suppress lipopolysaccharide (LPS)-induced NO without exerting cytotoxic effects on RAW 264.7 cells. Moreover, extracts inhibited the expression of inducible Nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) protein, TNF-α, and IL-6. The inhibition of NO without compromising cell viability was also reported for *Citrus reticulata* peels alkaline hot water extract (IC_{50} 1.04–2.74 mg/mL) (Chen *et al*., 2017).

Recently, the anti-inflammatory effect of *Citrus sinensis* peels' hydroalcoholic and methanol extracts was confirmed by Osarumwense *et al*. (2017). Interestingly, methanol extract was more active than hydroalcoholic extract, and a positive control drug (Indomethacin) with an inhibition of 95% on carrageenan induced rat paw edema at a concentration of 40 mg/kg. Similarly, after 4 h of edema induction, the oral administration (300 and 500 mg/kg BW) of pomelo peels methanol extract determined an inhibition of paw edema by 34.47% and 38.68%, respectively (Ibrahim *et al*., 2019). The same model of paw was used by Pallavi *et al*. (2018), establishing that intraperitoneal (i.p.) doses (250 and 500 mg/kg) of pomelo peels extract inhibited paw edema (17% and 48%, respectively).

The mandarin dichloromethane and ethyl acetate peels' extracts against COX-1 and COX-2 were tested (Hamdan *et al*., 2020). The dichloromethane extract was more active against COX-1 (IC_{50} = 25.5 mg/mL) than ethyl acetate extract (IC_{50} = 28.79 mg/mL); conversely against COX-2, the ethyl acetate extract had the highest activity (IC_{50} = 3.55 mg/mL).

Citrus limon essential oil exhibited anti-inflammatory activity (30 or 10 mg/kg oral [p.o.]) by reducing cell migration, cytokine production, and protein extravasation induced by carrageenan (Amorim *et al*., 2016). Treatment (200 and 50 mg/kg) with sweet orange dried peels essential oil evidenced a significant reduction of edema in rats (Thandiswa *et al*., 2020). *Citrus bergamia* essential oil, without furanocoumarins fraction, reduced levels of IL-1β, IL-6, and TNF-α in the paw homogenates, nitrite/nitrate, and prostaglandin E2 (PGE2) contents in exudates, and possesses antioxidant properties (Lombardo *et al*., 2020).

Antiproliferative activity

Selim *et al*. (2019) investigated the cytotoxicity activity of *Citrus reticulata* peels 70% ethanolic extract against human breast carcinoma, hepatocellular liver carcinoma (HepG2), and colon carcinoma and determined the IC_{50} values of 34, 9.9, and 30 mg/mL, respectively. Previously, the anti-cancer effects of *Citrus medica (2 morphotypes), Citrus sinensis, Citrus maxima, Citrus limon,* and *Citrus reticolata* peels' water extracts were studied (Nair *et al*., 2018). Among these, *Citrus reticolata* had significant activity against Dalton's lymphoma ascites (DLA) cell-inducing cell cycle arrest of DLA in G0/G1 phase.

Antibacterial potential

The *in vivo* antibacterial activity of *Citrus hystrix* ethanol peels extract against *S. typhimurium* was demonstrated by Zulvikar *et al.* (2020). In particular, the bacterial loads of this pathogen in the ileum, liver, and spleen decreased after 24 h of administration of the extract (16 mg daily for 3 days in a mouse). Lime peels extract was used to inhibit the colonization and growth of bacteria *S. typhi* in Balb/c mice. Doses of 510 and 750 mg/kg BW decreased the number of *S. typhi* colonies; even maintenance for 20 days after the intervention showed no bacterial growth (Kasim *et al*., 2020). Sharma and Tyagi (2019) analyzed benzene, ethanol, and methanol peels' extracts of *Citrus nobilis* and *Citrus sinensis* against four Gram-positive and four Gram-negative bacteria and two fungal pathogens. The minimum inhibitory concentration (MIC) values in the range of 18–40 μg/mL were found against *Bacillus cerus, S. aureus, S. epidermidis, Proteus vulgaris, S. typhimurium, P. aeruginosa, C. albicans*, and *Trichoderma viride* for *Citrus nobilis* ethanolic extract, while less activity was reported for methanol and benzene extracts. The same observation was made for *Citrus sinensis* extracts, and, in particular, the MIC values in the range of 20–50 μg/mL were observed for ethanolic extract. The results were in accordance with Rehab *et al*. (2018) that reported antibacterial and antifungal effects of *Citrus sinensis* peels' hot, cold, and ethanol extracts against *S. aureus, E. coli, P. aerogenes, B. cereus*, and *C. albicans*. Interestingly, the green synthesis of zinc oxide nanoparticles using *Citrus sinensis* peel extract was proposed by Gao *et al*. (2020) in food packaging application as nanocoatings on fresh strawberries with similar antibacterial characteristic of commercial zinc oxide nanoparticles.

The antimicrobial potential of *Citrus sinensis* L. and *Citrus limonia* Osbeck methanol, ethyl acetate, ethanol, and distilled water peels extracts was also evaluated by Saleem and Saeed (2020) against six Gram-positive *(S. aureus, Aeromonas hydrophila, Enterococcus faecalis, Streptococcus pyogenes, Listeria monocytogenes*, and *Lactobacillus casei),* six Gram-negative (*P. aeruginosa, K. pneumoniae, Serratia marcescens, E. coli, P. vulgaris*, *and S. typhi*), two microscopic filamentous fungi (*Aspergillus niger* and *Penicillium citrinum*), and two yeasts (*C. albicans* and *Saccharomyces cerevisiae*). Interestingly, the zone of inhibition is well comparable with amoxicillin, used as a positive control. In addition, the yellow lemon extract exhibited the highest antimicrobial activity compared to orange peels, and resulted more effectively on Gram-negative bacteria as compared to Gram-positive bacteria. Strawberries treated with *Citrus limon*, *Citrus sinensis*, and *Citrus reticulata* essential oils showed the highest TAC and physicochemical parameters compared to untreated fruits. This effect extends the shelf-life and delays the fruit senescence (Shehata *et al*. 2020).

The antimicrobial effects of tangerine, grapefruit, and lemon peels' essential oils on the growth of saprophytic and pathogenic microorganisms were compared by Denkova-Kostova *et al*. (2020). The highest inhibitory activity was observed for grapefruit, followed by tangerine and lemon essential oil, with MIC values in the range of 60–60 ppm against *E. coli*, *S. aureus*, *P. aeruginosa*, and *Citrus albicans*. Similarly, grapefruit and lemon have respective MIC values of 0.35 mg/mL and 0.33 mg/mL against *E. coli* (Raspo *et al*., 2020). The antimicrobial effect of bitter orange essential oil against Gram-positive and Gram-negative selected bacterial strains was studied by Farahmandfar *et al*. (2020). MIC values of 20, 40, and 10 mg/mL were found, respectively, for *E. coli*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes*.

The addition of *Citrus medica* essential oil to the wines (0.010%) determined reduction in microbial counts compared to untreated wine, and is thus proposed as bio-preservative. In particular, it the antimicrobial activity of enriched wine against the common spoilage bacteria and yeasts/molds such as *Gluconobacter cerinus*, *Oenococcus oeni*, *Pediococcus pentosaceus*, *Dekkera bruxellensis*, *Candida zemplinina*, *Hanseniaspora uvarum*, *Pichia guilliermondii*, or *Zygosaccharomyces bailii* was studied and inoculated (Mitropoulou *et al*., 2020).

Leaves

Antioxidant effects

The antioxidant activity of leaf methanol–water extracts of 10 varieties of *citrus* fruits was reported by Haraoui *et al*. (2020). All investigated samples exhibited radical scavenging activity with IC_{50} values in the same order of positive controls such as ascorbic acid and BHT. Among them, *Citrus maxima* and *Citrus aurantium* leaves showed the highest DPPH radical scavenging activity with the IC_{50} values of 0.51 and 0.57 mg/mL, respectively (Table 5). More recently, the antioxidant activity of methanol leaves extract and ethyl acetate fraction of *Citrus pseudolimon* was examined (Kumar *et al.*, 2019). The ethyl acetate fraction displayed greater DPPH radical scavenging activity than the methanol leaves extract with the IC_{50} values of 278.60 and 313.20 μg/mL, respectively. The IC_{50} values of 476.39 and 498.26 μg/mL were also found in H_2O_2 scavenging assay. The methanol extract of *Citrus medica* leaves was also investigated for its capacity to inhibit DPPH radical (Shojaemehr *et al*., 2020). Similar values were observed in extracts (IC_{50} = 0.111 mg/mL) and ascorbic acid (IC_{50} = 0.109 mg/mL) used as control.

The *citrus* × *clementina* leaves subjected to different extractions were investigated for their antioxidant potential (Leporini *et al*., 2020b). The hydroalcoholic extract obtained by using ultrasound-assisted maceration had the highest antioxidant DPPH, ABTS, FRAP, and β-carotene bleaching values. Previously, methanol and aqueous leave extracts of *Citrus clementina*, *Citrus limon*, *Citrus hamlin*, *Citrus navel*, *Citrus aurantifolia*, *Citrus aurantium*, and *Citrus grandi* were investigated for their antioxidant activity (Khettal *et al*., 2017). Among aqueous extracts, *Citrus limon* had an important DPPH radical scavenging activity (IC_{50} = 35.35 μg/mL), while *Citrus clementina* exhibited the highest ABTS radical scavenging activity (IC_{50} = 1,174.43 μM TE/g) and ferric-reducing potential (IC_{50} = 30.60 mg butyl-hydroxyanisole equivalents (BHAE)/g). Regarding methanolic extracts, *Citrus clementina* showed the highest antioxidant activity in all assays with the IC_{50} values of 41.85 μg/mL, 378.63 μM TE/g DM, and 13.85 mg BHAE/g DM for DPPH, ABTS radicals scavenging activities, and ferric-reducing potential, respectively. The antioxidant potential of *Citrus macroptera* leaf methanol extract has been recently demonstrated by Lala *et al*. (2020) that reported the capacity of this extract to reduce ROS, which was generated on HepG2 cell line.

Previously, Bonesi *et al*. (2018) investigated six *citrus* petitgrain essential oils for their antioxidant properties. In this study, *Citrus aurantium* petitgrain oil demonstrated the strongest radical scavenging activity in DPPH assay with an IC_{50} value of 27.2 μg/mL, followed by *citrus* × *clementina* oil with an IC_{50} value of 39.0 μg/mL, while in β-carotene bleaching test, the highest antioxidant capacity was observed with *Citrus sinensis* oil with the IC_{50} values of 176.3 and 51.3 μg/mL after 30 and 60 min of incubation, respectively. Less activity was reported for clementine essential oils by Leporini *et al.* (2020b).

Table 5. Biological properties of *Citrus* leaves

Leaves	Extract/Essential oil	Biological activity	Mechanism	TPC, TFC, TCC, and/or main abundant identified compounds	References
Citrus aurantifolia	Water and methanol	Antioxidant	Radical scavenging Ferric-reducing power	TPC = 5.77–106.05 mg GAE/g DW; TFC = 2.72–38.36 mg QE/g DW.	Khettal *et al.*, 2017
	Ethanol	Metabolic syndrome	Reduction in the total serum cholesterol	NR	Cyndi *et al.*, 2016
Citrus aurantium	Water and methanol	Antioxidant	Radical scavenging Ferric-reducing power	TPC = 7.77–69.97 mg GAE/g DW; TFC = 5.08–11.99 mg QE/g DW.	Khettal *et al.*, 2017
Citrus grandis	Water and methanol	Antioxidant	Radical scavenging Ferric-reducing power	TPC = 2.48–68.23 mg GAE/g DW; TFC = 1.04–13.06 mg QE/g DW.	Khettal *et al.*, 2017
Citrus limon	Water and methanol 80% Methanol	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	TPC = 3.83–98.06 mg GAE/g DW; TFC = 2.83–38.73 mg QE/g DW. TPC = 30.51 mg GAE/g; TFC = 14.64 mg QE/g.	Khettal *et al.*, 2017 Haraoui *et al.*, 2020
	Water	Metabolic syndrome	Reduction of the BW and plasma insulin levels	NR	Thomas et Kamath, 2017
Citrus macroptera	Methanol	Antioxidant	Reduction of ROS	TPC = 24.55 mg GAE/g extract; TFC = 10.76 mg QE/ g extract.	Lala *et al.*, 2020
		Anti-inflammatory	Reduction of edema		
		Antibacterial	*Staphylococcus* sp. and *Klebsiella* sp. inhibition		
Citrus maxima	80% Methanol	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	TPC = 91.76 mg GAE/g; TFC = 16.98 mg QE/g.	Haraoui *et al.*, 2020
	Ethanol	Metabolic syndrome	Reduction of TG, TC, HDL, LDL, VLDL serum level and BW	NR	Dinesh and Hegde, 2016
		Antibacterial	*M. luteus, S. epidermis, B. subtilis,* and *E. fecalis* inhibition		Haraoui *et al.*, 2020
Citrus medica	Methanol	Antioxidant	Radical scavenging	TPC = 102.7 mg GAE/g extract; TFC = 3.95 mg GAE/g extract.	Shojaemehr *et al.*, 2020
		Antibacterial	*B. subtilis, B. cereus, S. aureus, M. luteus, E. faecalis, P. aeruginosa, K. pneumoniae, S. typhi and E. coli* inhibition		
Citrus pseudolimon	Methanol	Antioxidant	Radical scavenging	TPC = 10 mg GAE/g extract TFC = 7.9 mg GAE/g extract	Kumar *et al.*, 2019
		Metabolic syndrome	Inhibition of α-amylase and β-glucosidase enzymes. Reduction of blood glucose level		
Citrus sinensis	80% Methanol	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	TPC = 35-09-62.59 mg GAE/g; TFC = 3.67–6.39 mg QE/g.	Haraoui *et al.*, 2020

(*continues*)

Table 5. continued

Leaves	Extract/Essential oil	Biological activity	Mechanism	TPC, TFC, TCC, and/or main abundant identified compounds	References
		Metabolic syndrome	Inhibition of lipase enzyme	TPC = 209.27 mg GAE/g FW; TFC = 65.02 mg QE/ g FW.	
		Antibacterial	*M. luteus, S. epidermis, B. subtilis,* and *E. fecalis* inhibition		Haraoui *et al.*, 2020
Citrus unshiu	Methanol	Metabolic syndrome	Inhibition of lipase enzymes	NR	Itoh *et al.*, 2019
Citrus × *clementina*	80% Methanol Ethanol and 80% Ethanol	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	TPC = 31.43 mg GAE/g; TFC = 4.84 mg QE/g. TPC = 13313–45.54 mg GAE/g FW; TFC = 5.50–29.16 mg QE/g FW; Hesperidin = 174.91–656.66 mg/100 g FW; Rutin = 4.24–68.52 mg/100 g FW; Isoquercitrin = 7.73–52.01 mg/100 g FW; Sinensetin = 7.24–35.69 mg/100 g FW; Tangeretin=7.46–41.76 mg/100 g FW;	Haraoui *et al.*, 2020 Leporini *et al.*, 2020a
		Metabolic syndrome	Inhibition of α-amylase, β-glucosidase and lipase enzymes		Leporini *et al.*, 2020a
		Antibacterial	*M. luteus, B. subtilis,* and *E. fecalis* inhibition		Haraoui *et al.*, 2020
Citrus aurantifolia	Essential oil	Antioxidant	Radical scavenging	Limonene = 63.35%; Geraniol = 6.23%; Citral = 4.35%.	Al-Aamri *et al.*, 2018
		Metabolic syndrome	Reduction in fasting blood, hepatic glucose, TC, triacylglycerol and LDL-c	Limonene = 57.84%; Neral = 7.81%; Linalool = 4.74%.	Ibrahim *et al.*, 2018
		Antibacterial	*S. aureus, and P. aeruginosa* inhibition *E. coli, S. typhi*, and *B. cereus* inhibition	Limonene = 30.11%; b-pinene=19.27%; b-Ocimene = 3.48%:	Chi *et al.*, 2020 Al-Aamri *et al.*, 2018
Citrus aurantium	Essential oil	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	Sabinene=39.81%; Linalool = 13.75%; g-Terpinen = 7.43%; d-3-carene=6.55%	Bonesi *et al.*, 2018
		Antifungal	*C. albicans* inhibition		Nidhi *et al.*, 2020
Citrus bergamia	Essential oil	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	Linalyl acetate = 70.51%; Linalool=10.25%; Geranyl acetate = 3.04%.	Bonesi *et al.*, 2018
Citrus grandis	Essential oil	Antioxidant Antibacterial	Radical scavenging *S. areus*, *S. typhi*, and *B. cereus* inhibition	Limonene = 21.87%; α-caryophyllene = 6.75%; β-ocimene = 6.35%.	Chi *et al.*, 2020

Citrus limon	Essential oil	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	Limonene = 30.57%; Geranial = 14.44%; β-pinene = 14.98%:	Bonesi *et al.*, 2018
		Antibacterial	*Listeria* inhibition *S. aureus, E. coli,* and *B. subtilis* inhibition	Geraniol = 298.65 mg/mL; Limonene=256.87 mg/mL; Geranial = 98.39 mg/mL; Neral = 86.81 mg/mL. NR	Fancello *et al.*, 2020 Saeb *et al.*, 2016
Citrus reticulata	Essential oil	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	Sabinene = 39.81%; Linalool = 13.75%; γ-terpinene = 7.43%; δ-3-carene = 6.55%:	Bonesi *et al.*, 2018
		Antibacterial	*S. aureus, E. coli,* and *B. subtilis* inhibition	NR	Saeb *et al.*, 2016
Citrus sinensis	Essential oil	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	Limonene = 13.77%; β-pinene = 16.93%; β-ocimene = 7.48%. Sabinene = 49.67%; β-ocimene = 9.25%; Limonene = 5.48%	Chi *et al.*, 2020 Bonesi *et al.*, 2018
		Anti-inflammatory	Reduction of edema	Sabinene = 20.4%; Terpinen-4-olo = 13.2%; Limonene = 7.5%; δ-3-carene = 6.55%.	Thandiswa *et al.*, 2020
		Antibacterial	*S. areus*, *S. typhi*, and *B. cereus* inhibition		Chi *et al.*, 2020
Citrus × *clementina*	Essential oil	Antioxidant	Radical scavenging Ferric-reducing power Inhibition of lipid peroxidation	Sabinene = 27.84%; Linalool = 19.76%; Limonene = 6.44%:	Bonesi *et al.*, 2018
		Metabolic syndrome	Inhibition of α-amylase, α-glucosidase and lipase enzymes	Linalool = 15.80%; Limonene = 6.41%; β-Ocimene = 6.52%; δ-3-carene = 6.33%.	Leporini *et al.*, 2020b Leporini *et al.*, 2020b

NR: not reported; TPC: total phenolics content; TFC: total flavonoids content; TCC: total carotenoids content; ROS: reactive oxygen species.

Metabolic syndrome

In recent decades, numerous *in vitro* and *in vivo* studies have demonstrated the importance of genus *Citrus* in the prevention of T2DM. Recently, the hypoglycemic effects of *citrus* × *clementina* leaves extract has been reported by Leporini *et al*. (2020b), who found the IC_{50} values of 64.37–247.61 mg/mL in α-amylase enzyme and the IC_{50} values of 51.61–282.65 mg/mL against α-glucosidase. In particular, hydroalcoholic extract obtained by ultrasound-assisted maceration from Corigliano Calabro leaves was found to be the most active. The addition of this extract to the juice increased its hypoglycemic (+37% and +25% against α-glucosidase and α-amylase, respectively) and hypolipidemic (+17% against lipase) potential. The inhibitory activity of *Citrus unshiu* leaf methanol extract on pancreatic lipase enzyme was reported by Itoh *et al*. (2019) that showed an IC_{50} value of 44 μg/mL.

Citrus pseudolimon methanol leave extracts and ethyl acetate fraction possessed a hypoglycemic potential (Kumar *et al*., 2019). The ethyl acetate fraction displayed a greater inhibition against α-glucoside (84.18%) in comparison to the methanol extract (82.94%). The IC_{50} values of 83.66% and 78.52% for ethyl acetate and methanol extract, respectively, were found against α-amylase. In addition, the authors indicated that oral administration of methanol leaves extract (200 mg/kg) and ethyl acetate fraction (100 mg/kg) for 21 days decreased the fasting blood glucose level in diabetic rats. Aqueous extract of *Citrus limon* leaves was tested against STZ-induced diabetic rats. This extract, orally administered at doses of 50 mg/kg BW and 100 mg/kg BW for 28 days, decreased BW and plasma insulin levels and increased blood glucose levels (Thomas and Kamath, 2017). The hypocholesterolemic effects of *Citrus aurantifolia* was reported by Cyndi *et al.* (2016). Indeed, the ethanol extract of leaves determined reduction in TC serum in mice, with the most significant reduction at a dosage of 3.5 g/kg BW. Similarly, the oral administration of *Citrus maxima* leaves extract (200 and 400 mg/kg BW) in obese rats determined reduction in TG, TC, HDL, LDL, and very low-density lipoprotein (VLDL) serum levels and BW (Dinesh and Hegde, 2016).

Antibacterial effect

More recently, Haraoui *et al.* (2020) investigated the antibacterial activity of leaves methanol water extracts obtained from *Citrus aurantium*, *Citrus maxima*, *Citrus lemon*, *Citrus Clementine*, and *Citrus sinensis* cv. Sanguinelli, Thomson, Washington, Portuguese, Double Fine, and Jafa. *M. luteus* resulted in the most sensitive Gram-positive bacteria to the action of *Citrus aurantium* and *Citrus sinensis* cv. Jaffa leave extracts with an inhibition area of 20.00 mm and 16.00 mm, respectively. For Gram-negative bacteria, the best results were observed for the *Citrus lemon* extract with an inhibition area of 15.66 mm (*P. aeruginosa*) and 15.33 mm (*E. coli*).

The antibacterial effects of different extracts obtained from *Citrus medica* leaves were tested. Interestingly, the inhibitory activity of methanol extract on *B. cereus*, *E. coli*, and *E. aerogenes* was more potent than the gentamicin used as a positive control (Shojaemehr *et al*., 2020).

Citrus aurantium leaves essential oil demonstrated strong antifungal activity against two strains of *Citrus albicans* with MIC values of 0.15–0.31% (v/v) (Nidhi *et al*., 2020). Interestingly, *Citrus limon* var pompia leaves essential oil showed specific anti-listeria activity on ricotta salata cheese (Fancello *et al*., 2020). Recently, De Oliveira Filho *et al*., (2020) proposed a chitosan films enriched with *Citrus limonia* leaves essential oil as an active packaging material for food preservation for its capacity to: (a) reduce the moisture content and water vapor permeability; (b) decrease the visible light transmission rate values; (c) change the color of bioactive films significantly, remaining darker and yellowish; and (d) inhibit *S. aureus*. Similarly, the addition of lemon essential oil to chitosan coatings enhanced fermentative process during storage, with modification of strawberry fruit aroma composition notably appreciated (Perdones *et al*., 2015).

Bioactive compounds

Polyphenolic compounds are a wide group of metabolites that originate from the secondary metabolism of plants. These are considered as potent antioxidants for their capacity to increase catalase activity, trap reactive oxygen species, and to act as a metal chelator. Additionally, they determined the inhibition of chain lipid peroxidation by trapping peroxyl radical and quickly reacted with peroxy nitrite (Pisoschi and Pop, 2015). Flavonoids and phenolic acid (Figure 1) are dominant bioactive compounds found in *citrus*. In particular, peels are rich in flavone aglycons and polymethoxy flavones, rarely found in other plants. Polyphenols are present in both edible and nonedible parts of the fruits (Singh *et al*., 2020). In addition, *citrus* fruit is a good source of carotenoids (Figure 2) compounds recognized for their beneficial effects on human health (Ikoma *et al*., 2016).

Citrus by-products represented a rich source of essential oils that possessed a wide range of antioxidant, antimicrobial, and antidiabetic properties, and thus used in pharmaceutical and food industries (Bora *et al*., 2020).

Flavonoids

Flavonoids are secondary metabolites in plants, with a multitude of functions: They regulate the development of plants, their pigmentation, and protect them from UV-light. Furthermore, they act as defense and signaling between plants and microorganisms (Mathesius, 2018).

Hesperidin

Hesperidin is one of the main flavanone glycosides know in *citrus* fruits. Great attention has been focused on hesperidin and its aglycone form, hesperetin, which plays an important role in the prevention of diseases associated with oxidative stress such as obesity, diabetes, inflammation, and cancer (Barreca *et al*., 2017). Its antioxidant mechanism was correlated to direct ROS scavenging, transition metal ion chelation, and its ability to increase cellular glutathione content. De Souza *et al*. (2016) compared the antioxidant activity of hesperidin, hesperetin, and G-hesperidin *in vitro* and *in vivo*, administrating each of these for 30 days at 1 mmol/kg body mass to Wistar male rats. The aglycone form has the greatest inhibitory activity of xanthine oxidase by increasing superoxide dismutase (SOD) activity in the liver of animals. Recently, the antioxidant activity of hesperidin, and its ability to inhibit pancreatic lipase enzyme, was studied (Huang *et al*., 2020). Results demonstrated that hydrogen bonds and van der Waals forces played major roles in the interaction of hesperidin and lipase.

The metabolic effects of hesperidin were also demonstrated by Sahnoun *et al*. (2017) and Zeng *et al*. (2018), who reported its ability to inhibit α-amylase, α-glucosidase, and lipase enzyme with the IC_{50} values of 111 and 1 μM, and 688.25 μg/mL, respectively. In a randomized double-blind controlled clinical trial design, 23 subjects with T2DM consumed 500 mg/day hesperidin supplement for 8 weeks. Hesperidin supplementation led to significant decrease in fasting blood glucose and glycosylated hemoglobin (HbA1c). A significant increase in serum insulin and decrease in TG were also observed in the hesperidin-treated group (Eghtesadi *et al*., 2016). Similarly, the supplementation with hesperidin (500 mg/day for 8 weeks) in T2DM patients resulted in reduction of fasting blood glucose, TC, and HbA1c, and at the same time a significant increase in serum insulin (Mohammadi *et al*., 2016). A dose of 100 mg or 500 mg of hesperidin for 6 weeks in subjects with hypercholesterolemia decreased serum TG and LDL levels (Li and Schluesener, 2017). In addition, intra-gastric hesperidin attenuates the increased level of plasma cholesterol, LDL-cholesterol VLDL-cholesterol, TG, free fatty acids, and phospholipids, and decreased levels of high-density lipoprotein-cholesterol (HDL-c) (Homayouni *et al.*, 2017).

In an *in vivo* study, hesperidin and naringin increased the production and release of insulin from the islet cells and decreased intestinal glucose absorption (Mahmoud *et al*., 2015). In addition, hesperidin and hesperetin inhibited two gluconeogenesis enzymes, alanine aminotransferase and aspartate aminotransferase, indicating their effectiveness in treating diabetes mellitus (Zareei *et al*., 2017).

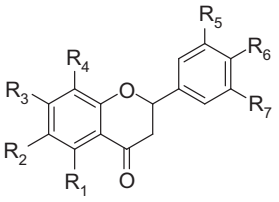
The therapeutic potential of hesperidin has been confirmed recently (Rehman et al., 2020b). This flavanone improved leptin and insulin resistance, IL-6 and TNF-α more significantly compared to the reference drug Orlistat used in high fat diet (HFD)-induced obese rats. In addition, the treatment with 500-mg hesperidin significantly reduced the plasma levels of C-reactive protein and serum amyloid A in individuals with MS (Homayouni *et al*., 2017). Moreover, hesperidin reduced symptoms of MS and improved cardiac function in HFD-induced MS in rats (Prasatthong *et al*., 2021). Indeed, treatment with hesperidin (15 or 30 mg/kg) ameliorated cardiac dysfunction and hypertrophy in rats, restored the insulin signaling pathway, and IRS/Akt/GLUT4 protein expression.

The consummation (500 mL) of orange juice enriched with hesperidin had positive effects on blood and pulse pressures in mildly hypertensive individuals (Valls *et al*., 2021). The results are in accordance with a recent study in which high blood pressure was attenuated by hesperidin (50 mg/kg BW). Regulation in the expressions of TNF-α, COX-2, and PGE2 with improvement of oxidative stress by increasing glutathione reductase and decreasing malondialdehyde (MAD) was also observed (Khidr *et al*., 2020).

Previously, the cardioprotective effect of hesperidin was investigated by Haidari *et al*. (2015). Administration of 600 mg/day of hesperidin decreases levels of adiponectin and HDL-cholesterol and increases E-selectin in patients with myocardial infarction.

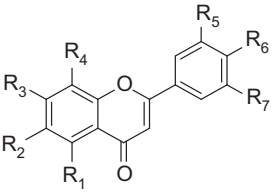
The neuroprotective activity of hesperidin was evaluated by Thenmozhi *et al*. (2015). In this study, administration of 100 mg/kg of hesperidin along with aluminum chloride ($AlCl_3$) injection for 60 days significantly reduced the concentration of ROS in hippocampus and cortex, the AchE activity, the protein expressions of amyloid precursor protein, the levels of both Ab_{1-42} and b and g secretases. Recently, Li and Schluesener (2017) demonstrated that administration of 100 mg/kg of hesperidin for 10 days significantly attenuated α-amyloid deposition and microglial activation in brain of transgenic mice.

The combination of diosmin and hesperidin exerted analgesic and/or anti-inflammatory effects (Patent No.



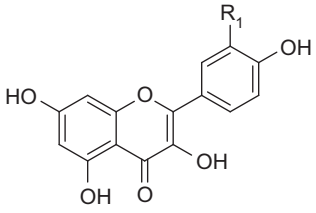
NAME	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Naringenin	OH	H	OH	H	H	OH	H
Hesperetin	OH	H	OH	H	OH	OCH_3	H
Narirutin	OH	H	O-Rut	H	H	OH	H
Naringenin	OH	H	O-Neo	H	H	OH	H
Poncirin	OH	H	O-Neo	H	H	OCH_3	H
Eriocitrin	OH	H	O-Rut	H	OH	OH	H
Neoeriocitrin	OH	H	O-Neo	H	OH	OH	H
Hesperidin	OH	H	O-Rut	H	OH	OCH_3	H
Neohesperidin	OH	H	O-Neo	H	OH	OCH_3	H
Didymin	OH	H	O-Rut	H	H	OCH_3	H

(a) Structure of flavones



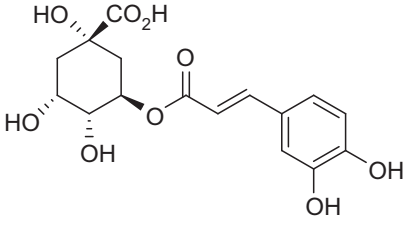
NAME	R_1	R_2	R_3	R_5	R_5	R_6	R_7
Apigenin	OH	H	OH	H	H	OH	H
Luteolin	OH	H	OH	H	OH	OH	H
Sinensetin	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3	H
Tangeretin	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	H
Nobiletin	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	H

(b) Structure of flavones

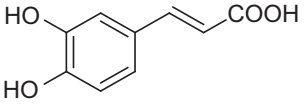


NAME	R_1
Quercetin	OH
Kaempferol	OH
Rutin	OCH_3

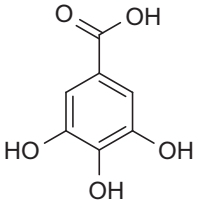
(c) Structure of flavonols



(A)



(B)



(C)

(d) Chlorogenic (A), gallic (B) and caffeic acid (C)

Figure 1. The main phenolic constituents of *Citrus* species.

WO2015019334) as reported by López Muñozmaría *et al*. (2015). This application was used for the treatment of different kinds of pain: moderate to severe pain, chronic pain, and/or neuropathic pain. No occurrence of adverse effects was observed.

Supplementation of a mixture of *Imperata cylindrical, Citrus unshiu markovich*-hesperidin, and *Evodia officinalis Dode*-Evodiamine for 12 weeks significantly reduced the BW, body fat mass, and waist circumference in overweight subjects (Cho *et al*., 2017). Recently, it was

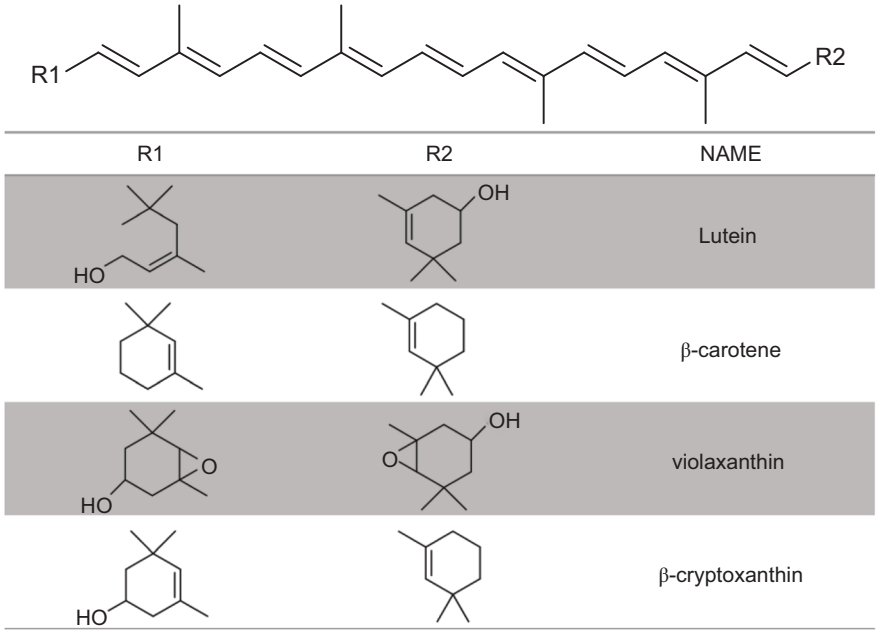


Figure 2. The most abundant *Citrus* carotenoids.

reported that hesperidin ameliorates hepatic dysfunction and dyslipidemia in male Wistar rats exposed to cadmium chloride (Aja *et al*., 2020).

Hesperetin

Recently, hesperetin showed cellular antioxidant activity with a value of 23.57 µmol of QE/100 µmol (Huang *et al*., 2020). Both hesperidin and hesperetin were able to reduce oxidative stress directly by scavenging intracellular ROS and increase natural antioxidant defense system with particular reference to glutathione (Dhanya and Jayamurthy, 2020). In addition, these flavonoids inhibited the non-enzymatic glycation of proteins involved in the formation of advanced glycation end-products which have an important role in developing diabetes. Previously, Jayaraman *et al*. (2018) investigated the anti-hyperglycemic, antioxidant, and anti-hyperlipidemic effects of hesperetin against STZ-induced experimental rats. Supplementation with 40 mg/kg of hesperetin for 45 days determined a significant decline in plasma glucose level and a marked improvement in insulin and glycogen secretions.

Hesperetin is also known to induce apoptosis in cancer cells primarily through activation of caspase-9 (Farooqi *et al*., 2015). This compound revealed significant cytotoxicity for HeLa cell line, and its anticancer ability was revalidated by *in silico* molecular docking study, which exhibited strong interaction with E6 protein of HPV16 cervical carcinoma with significant binding energy (Prakash *et al*., 2020). The capacity of hesperetin to attenuate testicular alteration in Wistar rats was also reported through inhibition of inflammation, oxidative stress, and apoptosis (Samie *et al*., 2018).

Interestingly, Li *et al*. (2018; Patent No. CN108815154A) investigated the ability of hesperetin to inhibit chloride channel and propose its use for the treatment of diarrhea, heart disease, pulmonary disease, stomach, brain, and mental diseases, rhinitis, ontological disease, and eye disease drug development.

Hesperetin administered orally (50 mg/kg/day for 46 days) reduced ROS, DNA fragmentation, serum glucose, MDA levels, and caspase 3 activity. In addition, this compound potentiated testicular antioxidant system with consequent increase in glutathione levels, ferric-reducing antioxidant power, catalase (CAT), SOD, and glutathione peroxidase (GPx) activity in diabetic rats (Samie *et al*., 2018). Shagirtha *et al*. (2017) has recently demonstrated the neuroprotective properties of hesperetin. The oral administration of this flavanone (40 mg/kg BW for 21 days) protected the brain of Wistar rats by increasing the levels of enzymatic antioxidants such as CAT, SOD, GPx, and glutathione-s-transferase (GSTs). In addition, hesperetin reduced oxidative stress, neuroinflammation, and motor dysfunction as well as amyloidogenesis and cognitive dysfunction in mice with positive effect against Parkinson's and Alzheimer's diseases (Khan *et al*., 2020).

Neohesperidin

The *in vivo* hypoglycemic and hypolipidemic effects of neohesperidin on KK-A(y) mice were studied (Jia *et al*., 2015). Treatment with neohesperidin significantly

decreased serum glucose, fasting glucose, glycosylated serum protein, and insulin resistance. Moreover, this bioactive compound significantly decreased TC, serum TG, leptin level, and inhibited lipid accumulation. Lv *et al*. (2015) also noted that naringin and neohesperidin mainly inhibited amylose digestion. In addition, the neohesperidin administration (50 mg/kg/day) attenuates weight gain, low-grade inflammation, and insulin resistance in mice, as well as restored gut barrier damage and metabolic endotoxemia (Lu *et al*., 2020).

A novel pharmaceutical use of neohesperidin in the preparation of drug for treating bronchial asthma or diseases caused by Th1/Th2 cell immune imbalance was disclosed (Shi and Yang, 2018; Patent No. CN108478586).

The efficacy of *citrus* flavonoids on MS resulted in the commercialization of Bergavit®, a standardized extract containing 150 mg of main active flavonoids of bergamot juice (16% of neoeriocitrin, 47% of neohesperidin, and 37% of naringin). This supplement was administrated at a fixed daily dose for 6 months in patients with moderate hypercholesterolemia. Results revealed reduction in TG, TC, and LDL-cholesterol (Toth *et al*., 2016).

It has been demonstrated recently that the neohesperidin inhibited Angiotensin II-induced myocardial contractile dysfunction, and reduced hypertension, myocardial hypertrophy, fibrosis, SOD production, and inflammation (Zhang *et al*., 2020).

Naringenin

Naringin, as reported by Sahnoun *et al*. (2017), showed an excellent inhibition for α-amylase and α-glucosidase enzyme, with IC_{50} values of 8.0 μM and 0.55 μM, respectively. Lim *et al*. (2018) studied the protective effects and molecular mechanisms of naringin in diabetic mice. The results showed that this flavanone ameliorated hyperglycemia and protected STZ-induced β-cell death by inhibiting both intrinsic and extrinsic apoptotic pathways. These protective effects have been related to the ability of naringin to reduce ROS and pro-inflammatory cytokines accumulation. It was suggested recently that antioxidant and anti-inflammatory properties of naringenin could confer hepatoprotective effects after oral treatment with 60 mg/kg BW (Kometsi *et al*., 2020).

In a clinical study, administration of naringin (400 mg/capsule/day) for 8 weeks in hypercholesterolemic individuals resulted in reduced concentration of plasma TC and LDL-cholesterol. Naringin exerted its effect by inhibiting gluconeogenesis and upregulation of AMPK, hence metformin-like effects. In addition, it increased glucose uptake in skeletal muscles, ameliorated pro-inflammatory reactions, and prevented metabolic dysregulation and atherosclerosis (Nyane *et al*., 2017). Moreover, naringenin decreased blood glucose, serum lipid, and ameliorated glucose tolerance through down-regulating oxidative stress and inflammation in STZ-induced rats (Jia *et al*., 2015).

Liang *et al.* (2015; Patent No. CN104940932A) reported the protective effects of naringenin and naringin during radiotherapy. Additionally, the use of naringenin and its derivative in preventing Alzheimer's disease and other cognitive disorders was reported (Liao, 2018; Patent No. CN108785301A).

The administration of naringenin (50 mg/kg/day) increased the serum level of insulin and consequently glucose uptake, improved lipid profile, TNF-α, IL-6, normalized level of NO, and increased SOD level (Rehman *et al*., 2020c). These effects were confirmed by Wu *et al*. (2016); they showed how this compound inhibited the expression of cytokine signaling, iNOS, COX-2, and release of NO and pro-inflammatory cytokines in microglial cells. A direct effect of this flavanone determined downregulation of genes involved in *de novo* lipogenesis, lipolysis, and triglyceride synthesis/storage. Moreover, narirutin and didymin are able to inhibit lipase enzyme with the IC_{50} values of 58.98 and 67.30 μg/mL, respectively (Zeng *et al*., 2018).

Didymin

Didymin acted as an anticancer agent by inhibiting phthalate-mediated invasion, migration, and proliferation of breast cancer cells (Hsu *et al.,* 2016), and as a scavenger of free radicals (Lin *et al.,* 2016). More recently, Ali *et al*. (2019) demonstrated that didymin was also able to inhibit α-glucosidase and α-amylase enzymes and increase glucose uptake. In addition, didymin reduced the expression of two key enzymes involved in the gluconeogenesis such as glucose 6-phosphatase and phosphoenolpyruvate carboxy-kinase with a consequent decrease of glucose production. Recently, it was found that didymin prevented hyperglycemia-induced ROS, production of lipid peroxidation product MAD, hyperglycemia-induced monocyte-endothelial cell adhesion, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation. In addition, this compound inhibited the release of various inflammatory cytokines and chemokines (Kirtikar *et al*., 2018).

Eriocitrin

Eriocitrin is known as a strong antioxidant agent (Smeriglio *et al*., 2019). It has been shown that a major role is played by its two hydroxy groups that are bound to the B ring in *ortho* position with respect to each other (Diab *et al*., 2015). This flavanone (200 mg/kg) showed protective effects against inflammation and oxidative stress in C57BL/6J mice, and may therefore prevent metabolic alterations associated with the development of cardiovascular diseases (Ferreira *et al*., 2016).

More recently, Kwon and Choi (2020) proposed a possible eriocitrin mechanism of action. In this study, dietary supplementation with eriocitrin (0.005%) in C57BL/6N mice for 16 weeks improved adiposity by increasing adipocyte fatty acid oxidation, energy expenditure, mRNA expression of thermogenesis-related genes in brown adipose tissue and skeletal muscle, and decreasing the expression of lipogenesis-related genes in white adipose tissue. The supplementation with eriocitrin also decreased hepatic lipogenesis and prevented hyperlipidemia whereas increased hepatic fatty acid (FA) oxidation and fecal lipid excretion. Moreover, eriocitrin supplementation improved insulin resistance, glucose tolerance, and decreased hepatic gluconeogenesis and pro-inflammatory responses. Previously, Liu *et al*. (2019a; Patent No. CN109806272A) proposed eriocitrin as potential α-glucosidase inhibitor.

Nobiletin

Nobiletin is one of the most abundant polymethoxylated flavones. This compound was investigated for its capacity to improve and prevent obesity and metabolic diseases. Recently, the application of nobiletin in preparation treatment of gastric accommodation disorder remedies was reported (Li, 2019; Patent No. CN108619130B). This compound selectively relaxes stomach smooth muscles, promotes the recovery of physiological gastrointestinal motility, and calms stomach upset. As a new therapeutic agent, it has provided and presented great market prospects and economic value.

Sahnoun *et al*. (2017) reported the carbohydrate hydrolyzing enzymes inhibitory activity of nobiletin with the IC_{50} values of 42.0 μM and 50.0 μM against α-amylase and α-glucosidase, respectively. This flavone was also able to inhibit lipase with an IC_{50} value of 26.28 mg/mL (Zeng *et al*., 2018), with IC_{50} value being better than that those reported for the positive control. In db/db diabetic mice, oral administration of nobiletin (200 mg/kg BW for 10 weeks) significantly attenuated BW gain, decreased fasting glucose levels, improved glucose tolerance and insulin sensitivity, and diminished serum TG levels (He *et al*., 2016). Moreover, nobiletin was able to reduce the protein peroxisomal acyl-coenzyme A oxidase 1, carnitine palmitoyltransferase-1, and ameliorated fatty acids β-oxidation *via* AMPK (Lone *et al*., 2018). In addition, treatment with this compound at 10–100 mg/kg BW for 8 weeks in obese mice accelerated lipid catabolism in adipose tissues.

Recently, it was found that nobiletin improved cognitive deficits and the pathological features of Alzheimer's disease, such as Aβ pathology, hyperphosphorylation of tau, and oxidative stress (Nakajima and Ohizumi, 2019). In addition, nobiletin ameliorated motor and cognitive deficits in Parkinson's disease models. Qi *et al.* (2019) also demonstrated that oral administration of nobiletin (100 mg/kg/day for 6 weeks) ameliorated LPS-triggered memory deficit regarding synaptic dysfunctions and neuronal loss, and inhibited the microglial activation and pro-inflammatory cytokine secretion (IL-1β, COX-2, TNF-α, and iNOS). In addition, in BV-2 microglia cells, the action of this flavone decreased pro-inflammatory cytokines secretion, and channeled modulation of mitogen-activated protein kinase (MAPKs), phosphatidylinositol 3-kinase/phosphorylated protein kinase B (PI3K/Akt), and NF-κB signaling pathways. Interestingly, nobiletin promotes antioxidant and anti-inflammatory responses and elicits protection against ischemic stroke *in vivo* with increase in the expression of SOD and glutathione (GSH) which are responsible of antioxidant endogenous defense systems. Moreover, a reduction in the levels of NF-κB and MDA was also observed (Zhang *et al*., 2016).

Wen-Zhe *et al*. (2015; Patent No. US9808477B2) detected a pharmaceutical composition for multidrug-resistant cancer treatment comprising *citrus* methoxyflavone (nobiletin) and chemotherapeutic drug. In addition, Chen and Wang (2015; Patent No. CN105030559A) proposed application of nobiletin in preparation of health products or medicines for prevention and/or treatment of oral cancer. The experiments showed that these compounds possessed an obvious effect on inhibiting proliferation of human oral epidermoid carcinoma cells through the anti-proliferation effects of hesperetin, naringenin, and nobiletin on human oral epidermoid carcinoma cells.

Tangeretin

Both nobiletin and tangeretin ameliorated ROS production and lipid peroxidation in mutant *Saccharomyces cerevisiae* deficient in glutathione synthase, SOD, or CAT (Wang *et al*., 2018). Similarly, a significant decrease in ROS content, with increase in the activities of SOD, CAT, and GPx through inhibition of NF-κB pathway in rats' insulinoma cell line (INS-1) pre-treated with tangeretin (0, 10, or 20 μM) for 12 h was also observed (Liu *et al*., 2019b).

Recent report has elucidated the anti-obesity capacity of tangeretin via inhibition of pancreatic lipase. This compound inhibited the enzyme with an IC_{50} value of 57.31 mg/mL (Zeng *et al*., 2018). Moreover, tangeretin ameliorated insulin resistance and increased glucose uptake by attenuating obesity-induced inflammation in adipose tissue through reduction of NO production, the expression of IL-6, IL-1β, TNF-α, iNOS, and COX-2 in 3T3-L1 adipocytes and macrophage cell line (Shin *et al*., 2017). Sahnoun *et al.* (2017) evaluated the inhibitory activities of tangeretin on carbohydrate metabolism key enzymes. This pentamethoxy flavone showed the IC_{50} values of 141.0 μM and 14.8 μM against α-amylase and α-glucosidase, respectively.

Recently, the neuroprotective effect of tangeretin against cerebral ischemia-reperfusion injury was demonstrated

(Yang *et al*., 2020). This compound downregulated the inflammatory and pro-inflammatory cytokines and oxidative stress parameters in the serum and brain tissues of rats with suppression of IL-1β, TNF-α, and IL-6. Lee *et al*. (2018; Patent No. KR102015221B1) proposed the application of tangeretin for the prevention and treatment of post-traumatic stress disorder. This compound showed an excellent anti-anxiety effect, and was consequently included in the pharmaceutical composition of foods as an active ingredient. Moreover, tangeretin was an active ingredient for alleviating, preventing, or treating renal fibrosis or cirrhosis of kidney glomerulus or albuminuria (Young-Hee and Min-Kyung, 2018; Patent No. KR101949471B1).

Sinensetin

The effects of sinensetin on lipid metabolism in mature 3T3-L1 adipocytes without causing cytotoxicity were reported by Kang *et al.* (2015). This compound showed anti-adipogenic property by downregulation of sterol regulatory element-binding protein 1c, and lipolytic property with increase of lipase enzyme. Moreover, sinensetin inhibited insulin-stimulated glucose uptake by decreasing the phosphorylation of insulin receptor substrate, and increased the phosphorylation of AMPK and acetyl-CoA carboxylase. It also upregulated mRNA expression of carnitine palmitoyltransferase-1a, suggesting that sinensetin enhances fatty acid β-oxidation through AMPK pathway. In addition, it was found that sinensetin quenched the fluorescence of α-glucosidase, and inhibited α-glucosidase and non-enzymatic glycation (Liu *et al*., 2020). Kim *et al*. (2019b) reported the anti-inflammatory activities of sinensetin on LPS-stimulated L6 skeletal muscle by regulating NF-κB.

Recently, the application of sinensetin as an active ingredient for preventing, ameliorating, or treating liver cancer or gastric cancer has been proposed (Kim and Lee, 2018; Patent No. KR20190050535A).

Luteolin

Sangeetha (2019) reported the antioxidant activity of luteolin and demonstrated how this polymethoxyflavone protects the pancreas and promotes insulin secretion. In addition, luteolin suppressed oxidative damage, lipid peroxidation, and increased antioxidant enzymes such as CAT and SOD (Xu *et al*., 2019). Antioxidant properties of luteolin are also proved in the central nervous system (CNS). The inhibition of gastric secretion and reduction of pepsin activity by luteolin was reported by Dai and Li (2018; Patent No. CN108309971B). In particular, the preparation includes 3–5 parts of luteolin and 1–2 parts of schisandrin B as active ingredients, and the dosage form of the compound preparation was preferably tablets, capsules, injections, and granules.

Quercetin

Quercetin has been used as a nutritional supplement and may have beneficial effects against a variety of diseases. Several *in vitro* and *in vivo* studies have evidenced its biological functions. Recently, Doustimotlagh *et al*. (2020) suggested the ability of quercetin (50 mg/kg/day for 10 days) to cause a significant decrease in protein carbonyl, hydroxyproline, and to regulate the GPx activity. Therefore, quercetin acted as an enzyme inducer by renewing the glutathione peroxidase activity and inhibiting the oxidation of proteins, and hence decreases ROS production. These results confirmed the positive role of quercetin in attenuating the liver damage and degeneration. Milanezi *et al.* (2019) analyzed the antioxidant activity of quercetin-capped gold nanoparticles. Quercetin-capped gold nanoparticles (IR_{50} 0.37 μg/mL) exhibited greater activity than free quercetin (IR_{50} 0.57 μg/mL) by NO free radical scavenging assay.

Similarly, quercetin vesicular formulations (Eudragit-coated liposomes) were capable of ensuring optimal protection against oxidative stress in human intestinal cells by reducing ROS production, as reported by Caddeo *et al*. (2019). Its antioxidant capacities were correlated to the presence of two antioxidant pharmacophores in the molecule that had optimal configuration for free radical scavenging. The high antioxidant potential of quercetin was also confirmed in superoxide test with the IC_{50} values of 0.025 mM versus 0.243 mM, for quercetin and kaempferol, respectively. Increasing *in vivo* studies have proved that quercetin acted as an antioxidant because of its ability to ameliorate antioxidant defenses, decrease free radical formation, and inhibit xanthine oxidase and lipid peroxidation (Shi *et al*., 2019).

Literatures data show that quercetin was able to reduce glucose levels when it was administered at a minimum dose of 30 mg/kg BW for 14 days (Yang and Kang, 2018). Additionally, this compound potentiated insulin secretion induced by glucose and glibenclamide and protected β-cells against oxidative damages (Shi *et al*., 2019). It was reported recently that the oral administration of quercetin (25 and 50 mg/kg) for 28 days remarkably reduced the level of blood glucose, HbA1c, hepatic glycogen, and restored the activity of glucose-6-phosphatase and hexokinase in diabetic rats (Oyedemi *et al*., 2019). Eid *et al*. (2015) proposed the use of quercetin as an anti-diabetic compound, since this flavonoid could act through the stimulation of GLUT4 translocation in the skeletal muscle and the inhibition of glucose-6-phosphatase in hepatocytes.

In a human study of 12-week, Lee *et al*. (2016) used 100 mg/day/subject of quercetin to treat obesity and showed that this compound diminished the total body fat, and decreased the BMI of overweight or obese subjects. In

addition, quercetin ameliorated mitochondrial functions in adipose tissue of HFD-induced obese mice by increasing the levels of oxidative stress-sensitive transcription factor and antioxidant enzymes (Kobori *et al*., 2016).

Kaempferol

The protective effect of kaempferol against oxidative stress in STZ-induced diabetic rats was evaluated by Al-Numair *et al*. (2015). Kaempferol administration (100 mg/kg BW) to diabetic rats reduced plasma glucose, insulin, and lipid peroxidation products enzymatic such as SOD, CAT, GPX, and GSTs.

Another study (Alkhalidy *et al*., 2018) demonstrated that oral administration of kaempferol (50 mg/kg/day than corresponding human equivalent dose of 240 mg/day for 60 kg) ameliorated blood glucose control in obese mice as well as reduced hepatic glucose production and improved insulin sensitivity. Additionally, these authors have found that kaempferol was a direct inhibitor of pyruvate carboxylase and suppressed gluconeogenesis in HepG2 cells. Torres-Villarreal *et al*. (2019) studied the kaempferol effects (60 μM for 21 days) in order to evaluate its lipolytic and anti-adipogenic potential. The results of anti-obesity effects showed that kaempferol modulated adipogenic differentiation in 3T3-L1 cells through promoting downregulation of *Cebpa* gene expression and decreased lipid accumulation in mature adipocytes for its positive effects on *Pnpla2* and *Lipe* mRNA levels.

Rutin

Rutin is considered a strong antioxidant agent; in fact, it acts as free radical scavenger, metal ions chelator, and reducing agent (Kaurinovic *et al*., 2019).

In STZ-induced diabetic rats, oral administration of 50 or 100 mg/kg BW of this compound decreased fasting blood glucose as well as HbA1c levels. Moreover, chronic administration of 200 mg/kg BW of rutin reduced (30–40%) the prevalence of diabetes in STZ-treated mice (Ghorbani, 2017). In addition, rutin treatment (50 mg/kg) for 24 weeks arrested the biochemical disturbances of diabetic retinopathy, lowering vascular endothelial growth factor (VEGF), TNF-α, and increasing TAC in the retina (Gupta *et al*., 2019). This compound also acted in reducing adiposity, increasing energy expenditure, and improving glucose homeostasis in obese mice (Yuan *et al*., 2017).

The positive effects of rutin on lipid profile was also proved (Wang *et al*., 2015). Glucose and lipid metabolism are strictly correlated. The most important clinical manifestation of this interaction is diabetic dyslipidemia characterized by high level of TG, LDL, and VLDL. Rutin, among its antidiabetic effects, decreased serum levels of TG and VLDL, and increased the level of HDL. Additionally, rutin decreased ROS formation, advanced glycation end-product precursors, and production of inflammatory cytokines. The anti-inflammatory activity of rutin was recently confirmed by Su *et al*. (2019). Authors evidenced the inhibition of NF-κB pathway and understatement of endoplasmic reticulum stress.

Phenolic acids

Phenolic acids are a diverse class of phenolic compounds made by plants. They act as agents of plant defense, and are, indeed, immensely important in plant–microbe interactions/symbiosis (Mandal *et al*., 2010).

Chlorogenic acid

Chlorogenic acid is an important bioactive dietary polyphenol. Several studies have reported the ability of chlorogenic acid to act in metabolic disease through different mechanisms of action. Recently, use of chlorogenic acid in the treatment of metabolic disorders was proposed (Kodimule, 2018; Patent No. US20190111015A1).

Chlorogenic acid supplementation in hypercholesterolemic rats at a dose of 20 or 90 mg/kg BW for 12 weeks suppressed serum lipid levels, while a dosage of 10 mg/kg significantly reduced total LDL-cholesterol and increased HDL-cholesterol by upregulating the expression of PPAR-γ gene (Huang *et al*., 2015). Additionally, administration of chlorogenic acid at a dose of 80 mg/kg BW for 12 weeks decreased percentage of body fat, fasting plasma glucose, and HbA1c level via modulation of adiponectin receptor signaling pathways (Jin *et al*., 2015). Recently, Di Wang *et al*. (2019) reported that chlorogenic acid (100 mg/kg/day BW) taken for 4 weeks ameliorated the survival rate after myocardial infarction and demonstrated that this compound showed a protective effect on myocardial infarction by reducing inflammatory response, exerting antioxidant activity, and minimizing weight gain. Similarly, chlorogenic acid (100 or 150 mg/day) reduced oxidative-induced damage and increased antioxidant protection in the inflamed paw skin, and reduced lipid peroxidation in serum (Mitrea *et al*., 2020). The effect of chlorogenic acid (100 mg/kg BW for 13 weeks) on energy balance in obese mice has been studied recently (He *et al*., 2020). This compound reduced food intake, increased body temperature, thermal dissipation, brown adipose tissue activity, and improved glucose tolerance. The anti-obesity effect of chlorogenic acid was also observed in male Sprague–Dawley rats at a dose of 20 or 90 mg/kg BW for 12 weeks (Huang *et al*., 2015). Oboh *et al*. (2015a) evaluated the inhibitory effects of chlorogenic acid on α-amylase and α-glucosidase enzymes. This compound showed the IC_{50} values of 9.10 μg/mL and 9.24 μg/mL for α-amylase and α-glucosidase,

respectively. Additionally, the same authors suggested its antioxidant properties with an IC_{50} value of 38.83 μg/mL.

Caffeic acid

The antioxidant protection of caffeic acid and chlorogenic acid against oxidative stress was studied *in vivo* using BY4741 strain and SOD and glutathione-deficient mutants of *S. cerevisiae* (Prudêncio *et al*., 2019). In the cell viability tests, caffeic acid showed higher stress tolerance, with a 106% increase in *S. cerevisiae* BY4741. However, in the SOD mutant, the effect of chlorogenic acid was stronger than caffeic acid, with a 3.3-fold increase. Conversely, in the glutathione-deficient mutant both treatments showed a similar level of protection. Arriagada *et al*. (2019) proposed the use of a hybrid nano-carrier consisting of core-shell silica nano-spheres linked to the surface with caffeic acid. These nano-spheres characterized by a potentiated antioxidant property accept the caffeic acid alone.

Gallic acid

Gallic acid was able to restore vitamin C and GSH levels in the pancreas of STZ-treated rats (Kahkeshani *et al.*, 2019). Yang (2018; Patent No. CN108464949A) disclosed a kind of antioxidant lightening compositions and its applications. The antioxidant lightening compositions include element of orange peels (tangeretin) and gallic acid.

Carotenoids

Carotenoids are a group of natural tetraterpenoid pigments distributed widely in plants. They play essential roles: (a) in photosynthesis and photoprotection; (b) as precursors for the biosynthesis of phytohormones; and (c) as signaling molecules to mediate plant development and responses to environmental cues (Sun *et al*., 2018).

In humans, carotenoids were recognized for their biological activities associated with the reduction of risk of developing chronic diseases such as cancer, cardiovascular and neurodegenerative diseases as well as metabolic disease. Additionally, these compounds acted as antioxidants and protected the cells against free radicals formed in the tissues. Some of these compounds are vitamin A precursors (Cardoso et al., 2017).

β-Carotene (Figure 2) is an intense orange-colored pigment used as a food coloring agent (Milne, 2005). In nature, β-carotene is a vitamin A precursor, which is synthesized from carotenoids via the action of enzyme β-carotene 15,150-monooxygenase. The beneficial effects of β-carotene-fortified synbiotic food intake on metabolic status were studied in T2DM patients (Asemi *et al.,* 2016). The β-carotene-fortified synbiotic food also contains *Lactobacillus sporogenes* (1×10^7 CFU), 0.1-g inulin, and 0.05-g β-carotene. Results showed that this synbiotic food had favorable effects on homeostatic model assessment of insulin resistance, insulin, TG, VLDL-cholesterol, and TC/HDL-cholesterol ratio, and NO and glutathione levels. Antioxidant immune response, and anti-inflammatory, anti-diabetic, and antitumor activities of β-carotene are also reported (Torregrosa-Crespo *et al.,* 2018). In addition, existence of a positive effect of β-carotene on insulin sensitivity in obese patients through a positive regulation of adiponectin, either directly or via its pro-vitamin, was also suggested (Ben Amara e*t al*., 2015).

Lutein (β, ε-carotene-3,30-diol) acted as a powerful antioxidant, prevented HFD-induced atherosclerosis in apoE-deficient mice by inhibiting NADPH oxidase and increasing PPAR-γ gene expression (Han *et al*., 2015). Additionally, it protects dopaminergic neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced apoptotic death and motor dysfunction by ameliorating mitochondrial disruption and oxidative stress (Nataraj *et al*., 2016).

β-Cryptoxanthin is used as a coloring agent for food products in certain countries. It is associated with the E number, E161C β-Cryptoxanthin, obtained from its common food sources. It exhibits high bioavailability, and β-cryptoxanthin-rich foods might be considered equivalent to β-carotene-rich foods as a source of retinol (Burri *et al*., 2016). Recently, Dhuique-Mayer *et al*. (2020) suggested that *citrus* × *clementina* juice enriched in β-cryptoxanthin (43 μg/g), hesperidin (2,850 μg/g), and pectin (376 mg/100 g) can be used for prevention of MS/T2DM. Moreover, the cancer preventive effects of β-cryptoxanthin have been described (Leoncini *et al*., 2016). The study included over 6,000 subjects with oral, laryngeal, and pharyngeal cancers. The treatment with β-cryptoxanthin determined a reduction of at least 18% in the rate of oral and pharyngeal cancers and a reduction of 17% in the rate of laryngeal cancer.

Lycopene, one of most potent oxygen-quenching reagents among carotenoids, possessed the ability to inhibit the reactions initiated by free radicals, such as peroxy radicals or hydroxyl radicals. Indeed, cellular enzymes glutathione S-transferase, superoxide dismutase, and quinone reductase were activated by lycopene with consequent protection cells against ROS (Supatra, 2019). Owing to its antioxidant potential, lycopene (a) facilitated cell-to-cell communication at sites called "gap junctions" and consequently prevent cancer from developing; (b) stimulated the immune system; (c) regulated the endocrine communication pathways; and (d) regulated the cell reproductive cycle, preventing development of cancer (Supatra, 2019). Caseiro *et al*. (2020) also reported the ability of lycopene

to protect lipids, proteins, and DNA from oxidative damage, and stimulate the modulation of cell growth and the expression of connexin 43, insulin-like growth factor-1 and/or blood levels of insulin-like growth factor-binding proteins, as well as intermediate levels in the immune system and inflammatory processes. In addition, lycopene improved insulin sensitivity through inhibition of signal transducer and activator of transcription 3/Srebp-1c-mediated lipid accumulation and inflammation in mice fed with HFD (Zeng *et al*., 2017).

Terpenes

Terpenes are the largest class of natural products applied in industrial sector as flavors, fragrances, and spices as well as used in perfumery and cosmetics. In plants, these act as defense against biotic and abiotic stresses, or they are treated as signal molecules to attract insects for pollination (Singh and Sharma, 2015).

Ameh and Obodozie-Ofoegbu (2016) reported the utilization of *citrus* essential oil as flavorings in carbonated cola and *citrus* soft drinks. In particular, lemon–lime sodas contain *Citrus limon, Citrus aurantifolia*, and *Citrus aurantium* essential oils as main flavorings, while orange sodas contain *Citrus aurantium* oil as the main flavoring constituent.

The chemical variation of each component in *citrus* essential oil is based on variety, season, and geographical position as well as the ripening phase of the fruit (Bora *et al*., 2020). The major components are monoterpenes (Figure 3), and D-limonene is the most abundant element. This monocyclic terpene is consumed by humans as an ingredient of traditional foods and is listed in the Code of Federal Regulations, as Generally Recognized as a Safe (GRAS) and used as a flavoring agent (Roberto *et al*., 2010).

The ameliorative effects of limonene on cadmium-induced genotoxicity in cultured human peripheral blood lymphocytes has been demonstrated recently (Verma *et al*., 2019). In this *in vitro* study, at concentrations of 20 and 100 Mm, it reduced the sister chromatid exchange frequency and peroxidation of lipids.

D-limonene reduced weight gain percentage, TC, LDL, and VLDL, and increased the level of HDL-cholesterol (Khan *et al*., 2019). In addition, the monoterpene (400 mg/kg) increased the levels of thiobarbituric acid (TBARS), SOD, CAT, and (GSH) in the liver tissue after treatment for 28 days. These results agreed with those reported by Yu *et al*. (2017); the authors observed how treatment with 50 or 100 mg/kg of D-limonene increased the levels of endogenous antioxidant enzymes. The treatment with limonene (50 mg/kg) displayed anti-inflammatory activity through decreasing TNF-α, IL-6, and IL-1β levels and increasing the level of IL-10 (De Souza *et al*., 2019). Additionally, this compound determined reduction in gastric ulcer area (93%) and myeloperoxidase activity. Increase in GPx activity was also observed. Limonene has also reported its ability to protect PC12 cells against corticosterone-induced neurotoxicity by activating the AMPK pathway (Tang *et al*., 2019). In fact, reductions were observed in MDA

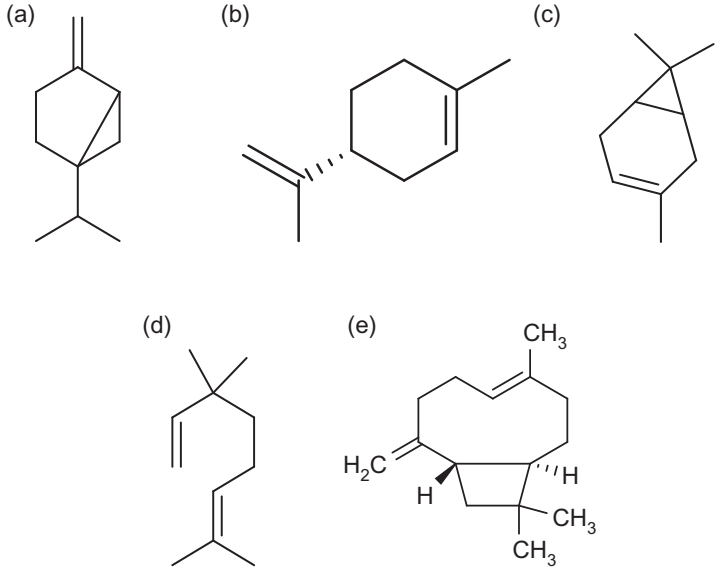


Figure 3. Chemical structure of main monoterpenes of *citrus* essential oils: (a) sabinene, (b) limonene, (c) δ-3-carene, (d) linalool, (e) β-caryophyllene.

and NO levels, NADPH oxidase activity, iNOS, COX-2, IL-6, IL-1β, TNF-α, and expressions of pro-apoptotic proteins.

Another monoterpene found particularly abundant in *citrus* essential oil is sabinene which acts as a potential modulator of bacterial resistance. It could act in synergism with antibiotics to reduce MIC values against bacterial strains of PA03 and SA358 (Matias *et al*., 2016).

Linalool is an acyclic monoterpene tertiary alcohol (Figure 3) and is one of the most investigated aroma compounds. Currently, linalool and citral are mainly used as flavoring and natural preservatives due to their antimicrobial and antifungal ability. Indeed, they were used to extend the short shelf-life of seafood products and cheese because of their capacity to reduce populations of microorganisms, especially Enterobacteriaceae (Bora *et al.*, 2020). At a concentrations of 0.1%, linalool exhibited antimicrobial activity against different strains such as *S. aureus*, *E. coli*, *B. subtilis*, and *Pasteurella multocida*, with major activity against Gram-positive bacteria than Gram-negative bacteria.

Baldissera *et al*. (2017) evaluated the effect of β-caryophyllene on hypercholesterolemia in rats and the possible effect on hepatic antioxidant enzymes. Administration of β-Caryophyllene at a dose of 1.0 mL/kg for 3 days reduced the levels of TC, LDL-cholesterol, and TG, inhibited the HMG-CoA reductase activity, and increased the antioxidant system of ROS and TBARS levels. These results agree with those reported by Basha and Sankaranarayanan (2016), who investigated the effect of β-caryophyllene on hyperglycemia. Oral administration of this compound (200 mg/kg BW) for 45 days reduced the level of glucose and increased the level of insulin, with restored antioxidant status enhancing the activity of CAT, SOD, and GPx as well as inhibition of pro-inflammatory cytokines, TNF-α and IL-6. It has been recently demonstrated that β-caryophyllene reduced PGE2 and iNOS production and COX-2 expression (Hu *et al*., 2017). Varga *et al*. (2018) have successively evidenced that at a dose of 10 mg/kg BW, this compound improved the chronic and binge alcohol-induced liver injury and inflammation by attenuating the pro-inflammatory phenotypic "M1" switch of Kupffer cells and diminishing the expression of E-Selectin, P-Selectin, and neutrophil infiltration. Additionally, it ameliorated the hepatic metabolic dysregulation, such as protein hyperacetylation, steatosis, and PPAR-γ – gene signaling. These protective effects were correlated to activation of type-2 cannabinoid receptor. Interaction with this receptor causes the expression of vascular cell adhesion molecule-1 mediated by the JAK2/STAT1/IRF-1 pathway (Zhang *et al*., 2017).

Conclusion

A critical review of recent studies on the health properties of different portions of *citrus* fruits and their major bioactive compounds was reported. It was interesting to observe that not only the edible portion but also its by-products are characterized by high biological value. A large number of *in vitro* and *in vivo* studies have suggested an inverse relationship between increased consumption of *citrus* fruits and lowered risk of chronic diseases correlated to their large contents in polyphenols responsible for a wide range of beneficial effects in humans. The extraction of this bioactive compound, its addition to food, and development of nutraceuticals have gained increasing interest. In addition, *citrus* essential oils are frequently used as natural alternatives to synthetic preservatives for food safety, packaging, and preservation.

Interest in this plant genus is evidenced by the numerous patents and nutraceutical/pharmaceutical products already on the market for the prevention and treatment of numerous pathological conditions, including those related to metabolic disorders.

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Anti-staphylococcal effect of cinnamaldehyde in milk

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PAPER

Abstract

The survival of *Staphylococcus aureus* in inoculated (10^5 colony forming units [CFU]/mL) 3.2% and 0.5% fat ultra-high temperature-pasteurized milk samples containing 0%, 0.05%, or 0.1% cinnamaldehyde stored at 4°C or 10°C was evaluated within 15 days. *S. aureus* populations reached 7.92 (0.5% fat) and 7.95 (3.2% fat) log CFU/mL in control milk samples stored at 10°C, while in milk sample stored at 4°C, *S. aureus* counts remained almost unchanged. At the end of the study, the number of this pathogen decreased by 1.52–4.04 log CFU/mL in milk treated with cinnamaldehyde. The greatest anti-staphylococcal effect was achieved in low-fat milk at 10°C and treated with 0.1% cinnamaldehyde.

Keywords: antibacterial activity, cinnamaldehyde, fat, milk safety, *Staphylococcus aureus*

Introduction

Milk and milk products, being highly nutritious foods, are excellent media for the growth of many spoilage and pathogenic microorganisms (Noël *et al.*, 2016), including *Staphylococcus aureus*. This pathogen commonly exists in dairy production plants (Xing *et al.*, 2016), and it is one of the most important causative infective agents of clinical and subclinical mastitis in dairy cattle (Nam *et al.*, 2011; Basanisi *et al.*, 2017). *S. aureus* presents an important public health burden since it is one of the major pathogens responsible for food intoxication (Jans *et al.*, 2017).

In spite of the fact that pasteurization kills *S. aureus*, it has little effect on thermostable enterotoxins, which generally preserve their biological activity after exposure to heat (Jablonski and Bohach, 1997; Jørgensen *et al.*, 2005). The presence of *S. aureus* in raw milk before processing is a concern because different physical and chemical production techniques are applied during processing and ripening of milk products to prevent growth of this pathogen and production of enterotoxins. Nevertheless, if one of these limiting factors fails, there is a risk of accumulation of staphylococcal enterotoxins (Jørgensen *et al.*, 2005). Thus, it is important to control growth of *S. aureus* in raw milk and raw milk products.

In order to ensure milk safety and prolong milk's shelf life, while also improving its sensorial characteristics, the dairy industry is developing minimum processing techniques (Cava *et al.*, 2007). It has been suggested

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that addition of plant extracts, including cinnamon, can enhance microbiological safety, and it positively affects the sensory attributes of processed dairy products and milk-based desserts such as rice pudding and vanilla cream pudding (Tayel *et al.*, 2015; Lianou *et al.*, 2018). When added to butter, cinnamon (3%) lowered microbial growth during storage and exhibited antioxidant activity, thus retarding the spoilage of butter by positively influencing its sensorial characteristics (Vidanagamage *et al.*, 2016). Thus, cinnamon could be successfully incorporated in butter as a natural preservative instead of synthetic preservatives.

Cinnamon contains 85.3–90.5% cinnamaldehyde (Doyle and Stephens, 2019). Together with eugenol, isoeugenol, vanillin, and safrole, cinnamaldehyde is one of the best studied phenylpropenes (Nazzaro *et al.*, 2013). Trans-cinnamaldehyde exhibits a wide range of beneficial effects, including antibacterial, antifungal, antioxidant, anti-inflammatory, anti-diabetic, neuroprotective, and antitumor (Masghati and Ghoreishi, 2018; Doyle and Stephens, 2019), while cis-cinnamaldehyde, the geometrical isomer of trans-cinnamaldehyde, exhibits antifungal properties (Doyle and Stephens, 2019). Essential oil (EO) of cinnamon has found application in food industry because of its various components, including cinnamaldehyde, a major ingredient of cinnamon bark oil (Masghati and Ghoreishi, 2018). Most essential oils and their components, including trans-cinnamaldehyde, are generally recognized as safe (GRAS) and accepted by consumers (Burt, 2004). Owing to their antibacterial and antioxidant properties, essential oils can be used as potential natural preservatives in different foods, including flavored drinks (Cava *et al.*, 2007). Flavored milk has increased in popularity in recent years; nevertheless, there are few data available in literature about the effect of adding essential oils directly to milk before cheese-making (Licon *et al.*, 2020).

The focus of the present study was to determine whether trans-cinnamaldehyde could be a potential natural antibacterial agent in milk, hence ultra-high temperature (UHT)-pasteurized milk was used as a matrix to eliminate any possible interactions with the microbiota normally present in raw milk. The aims of the study were to: (1) evaluate the anti-staphylococcal effect of different concentrations of cinnamaldehyde (0.05% and 0.1%) on *S. aureus* in milk; and (2) determine the influence of different fat contents (0.5% and 3.2% milk fat) and different storage temperatures on survival of *S. aureus* in milk.

Materials and Methods

Trans-cinnamaldehyde and *S. aureus* culture, UHT-pasteurized milk samples containing 0.5% and 3.2% fat were bought from a local supermarket. Cinnamaldehyde (CA) (98% purity) was purchased from Carl Roth, Germany and stored at 4°C prior to use. *S. aureus* was obtained from the American Type Culture Collection (ATCC 25923).

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of cinnamaldehyde was determined in a non-milk matrix using sterile U-bottom 96-well microplates. The bacterial inoculum density was set to 0.5 on the McFarland scale, then further diluted 10 times in sterile saline; 5 µL of this suspension was inoculated into 0.1 mL of Cation-Adjusted Mueller–Hinton Broth (CAMHB; Becton, Dickinson and Company, Sparks, USA) to reach a final *S. aureus* ATCC 25923 inoculum of 5×10^4 colony forming units (CFU)/well. Cinnamaldehyde was diluted in dimethyl sulfoxide (Serva, Heidelberg, Germany) and added to CAMHB in the levels of 2560–1.25 µg/mL by two-fold dilution in 96-well microtitre plates. After inoculation, plates were incubated for 24 h at 37°C. The MIC was the lowest concentration of cinnamaldehyde that did not show any visual growth of *S. aureus* after macroscopic evaluation, and it was expressed in µg/mL (Clinical and Laboratory Standards Institute [CLSI], 2006). The plates were prepared in triplicate.

Sample preparation and storage conditions

Milk containing 0.5% or 3.2% fat was analyzed for *S. aureus* to confirm the absence of this pathogen. Approximately 5 log CFU/mL of *S. aureus* was inoculated into *S. aureus*-free milk containing 0.5% or 3.2% milk fat. The concentration of the inoculum was verified by the standard plate count method and determined as 5.55–5.60 log CFU/mL. To study the survival of *S. aureus* in milk, different concentrations of cinnamaldehyde (0.05% and 0.1%) were added to milk samples with 0.5% (reduced fat) and 3.2% (whole milk) milk fat, whereas controls were without cinnamaldehyde but were inoculated with *S. aureus*. The selection of these concentrations of cinnamaldehyde was based on previous sensory evaluations (Babic *et al.*, 2019). After addition of cinnamaldehyde, all milk samples were divided into halves and stored in sterile glass bottles at 4°C and 10°C for 15 days. This temperature of 10°C was selected as an abuse temperature. The milk samples are described in Table 1.

Microbiological and pH analysis

All milk samples were examined on storage days 0, 3, 6, 9, 12, and 15. For bacterial enumeration, 25 mL of milk

Table 1. Experimental design.

Medium	Cinnamaldehyde	Temperature	Milk samples
Milk containing 0.5% fat with *S. aureus*	0%	4°C	1. Milk containing 0.5% fat with *S. aureus* and without cinnamaldehyde stored at 4°C.
	0.05%		2. Milk containing 0.5% fat with *S. aureus* and 0.05% cinnamaldehyde stored at 4°C.
	0.1%		3. Milk containing 0.5% fat with *S. aureus* and 0.1% cinnamaldehyde stored at 4°C.
	0%	10°C	4. Milk containing 0.5% fat with *S. aureus* and without cinnamaldehyde stored at 10°C.
	0.05%		5. Milk containing 0.5% fat with *S. aureus* and 0.05% cinnamaldehyde stored at 10°C.
	0.1%		6. Milk containing 0.5% fat with *S. aureus* and 0.1% cinnamaldehyde stored at 10°C.
Milk containing 3.2% fat with *S. aureus*	0%	4°C	7. Milk containing 3.2% fat with *S. aureus* and without cinnamaldehyde stored at 4°C.
	0.05%		8. Milk containing 3.2% fat with *S. aureus* and 0.05% cinnamaldehyde stored at 4°C.
	0.1%		9. Milk containing 3.2% fat with *S. aureus* and 0.1% cinnamaldehyde stored at 4°C.
	0%	10°C	10. Milk containing 3.2% fat with *S. aureus* and without cinnamaldehyde stored at 10°C.
	0.05%		11. Milk containing 3.2% fat with *S. aureus* and 0.05% cinnamaldehyde stored at 10°C.
	0.1%		12. Milk containing 3.2% fat with *S. aureus* and 0.1% cinnamaldehyde stored at 10°C.

was transferred into a sterile Stomacher bag and 225 mL of Buffered Peptone Water (BPW; Merck, Germany) was added. The contents of each bag were homogenized in a Stomacher blender (Stomacher 400 Circulator, Seward, UK) for 2 min. Serial decimal dilutions were prepared and 0.1 mL of appropriately diluted suspension was plated on Baird Parker agar (Oxoid CM 275, Basingstoke, Hampshire, UK) with egg yolk tellurite emulsion (Oxoid CM 275, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 h according to EN ISO 6888-1 (International Organization for Standardization [ISO], 1999). The number of colonies was counted, and results were recorded as colony forming units per milliliter.

The pH of milk samples was measured using a portable pH meter (Testo 205; Testo AG, Lenzkirch, Germany). The pH meter was calibrated with standard buffer solutions of pH 4.0 and 7.0 prior to use.

Statistical analysis

Six randomized milk samples from each group were analyzed on each examination day. Number of microorganisms were transformed into logarithms (log) before statistical analysis. Statistical analysis of the results was conducted using the SPSS 20.0 software (IBM, Chicago, IL, USA). The *S. aureus* counts were expressed as mean ± standard deviation. A three-way ANOVA analysis was used to investigate factor effects (concentrations of cinnamaldehyde, temperature, and fat%) and interactions among them on log-transformed *S. aureus* counts. Statistical differences between examined groups were determined by Tukey's *post hoc* multiple comparisons test. $P < 0.05$ was considered statistically significant.

Results and Discussion

Anti-staphylococcal effect of cinnamaldehyde in milk during storage

The MIC of cinnamaldehyde against *S. aureus* was 160 µg/mL, showing that cinnamaldehyde was able to inhibit growth of this pathogen at low concentrations in the non-milk matrix used. Alves *et al.* (2016) reported a cinnamaldehyde MIC of 100 µg/mL against *S. aureus*, in agreement with the result of the present study. Nevertheless, in spite of the good antibacterial effect *in vitro*, hydrophobic essential oil constituents are impaired by interactions with food matrix components, hence higher concentrations are needed to achieve the same antibacterial effect in food (Hyldgaard *et al.*, 2012). Thus, in the present study, approximately 4- and 9-fold higher concentrations (0.05% and 0.1%) of cinnamaldehyde than the obtained MIC were added to milk samples.

Significant ($P < 0.05$) antibacterial activity against *S. aureus* was found in milk samples at the cinnamaldehyde concentrations used (0.05% and 0.1%) when compared with the controls without cinnamaldehyde (Table 2).

Initial *S. aureus* counts ranged from 5.55 to 5.60 log CFU/mL. On day 0, *S. aureus* counts were significantly ($P < 0.05$) higher in controls than in milk samples with cinnamaldehyde at 4°C and at 10°C, indicating the immediate antibacterial effect of cinnamaldehyde. Regardless of fat content, in control milk samples without cinnamaldehyde stored at 4°C, with the exception of a slight decrease observed on day 3, the *S. aureus* populations remained almost unchanged for 15 days compared with the initial populations in milk samples. Nevertheless, at 10°C, *S. aureus* counts increased to approximately 7.92

Table 2. ***S. aureus*** **counts (log CFU/mL) in milk with and without added cinnamaldehyde (CA), stored at 4°C and 10°C (mean ± SD), and the significance of interactions between cinnamaldehyde, storage temperature, and milk fat.**

CA concentration	Temperature	Fat	Days					
			0	3	6	9	12	15
0%	4°C	0.5%	5.56 ± 0.06^{a}	5.43 ± 0.10^{ac}	5.46 ± 0.06^{a}	5.48 ± 0.04^{a}	5.57 ± 0.11^{a}	5.56 ± 0.05^{a}
		3.2%	5.60 ± 0.07^{a}	5.45 ± 0.09^{a}	5.49 ± 0.10^{a}	5.50 ± 0.06^{a}	5.64 ± 0.08^{a}	5.63 ± 0.06^{a}
	10°C	0.5%	5.55 ± 0.08^{a}	6.98 ± 0.10^{b}	7.21 ± 0.05^{b}	7.49 ± 0.07^{b}	7.47 ± 0.07^{b}	7.92 ± 0.07^{b}
		3.2%	5.57 ± 0.09^{a}	7.00 ± 0.16^{b}	7.23 ± 0.15^{b}	7.43 ± 0.07^{b}	7.45 ± 0.08^{b}	7.95 ± 0.10^{b}
0.05%	4°C	0.5%	5.40 ± 0.06^{b}	5.32 ± 0.07^{adce}	5.15 ± 0.07^{c}	4.62 ± 0.09^{c}	4.44 ± 0.05^{c}	3.95 ± 0.09^{c}
		3.2%	5.32 ± 0.07^{bc}	5.23 ± 0.04^{def}	5.11 ± 0.08^{c}	4.89 ± 0.07^{d}	4.28 ± 0.06^{d}	4.08 ± 0.07^{c}
	10°C	0.5%	5.21 ± 0.05^{cd}	5.26 ± 0.05^{cdef}	4.51 ± 0.05^{d}	3.81 ± 0.05^{e}	3.04 ± 0.07^{e}	2.45 ± 0.07^{d}
		3.2%	5.34 ± 0.07^{bc}	5.30 ± 0.10^{adef}	4.87 ± 0.08^{eg}	4.00 ± 0.13^{f}	3.90 ± 0.08^{f}	3.23 ± 0.07^{e}
0.1%	4°C	0.5%	5.28 ± 0.07^{bc}	5.20 ± 0.08^{ef}	4.94 ± 0.06^{g}	4.46 ± 0.08^{g}	4.10 ± 0.09^{g}	3.11 ± 0.07^{e}
		3.2%	5.26 ± 0.06^{bc}	5.18 ± 0.05^{ef}	5.04 ± 0.08^{cg}	4.56 ± 0.06^{cg}	4.26 ± 0.08^{d}	3.78 ± 0.07^{f}
	10°C	0.5%	5.11 ± 0.08^{d}	5.14 ± 0.06^{f}	4.08 ± 0.07^{f}	3.62 ± 0.08^{h}	2.51 ± 0.06^{h}	1.51 ± 0.04^{g}
		3.2%	5.27 ± 0.06^{bc}	5.25 ± 0.07^{ef}	4.48 ± 0.08^{d}	3.18 ± 0.09^{i}	3.11 ± 0.07^{e}	2.61 ± 0.10^{h}
Conc. CA × *Temp.			NS	**	**	**	**	**
Conc. CA × Fat%			NS	NS	**	**	**	**
Conc. CA × Temp. × Fat%			*	NS	**	**	**	**

$^{a–i}$Different superscript letters in the same column, $P < 0.05$.
NS: Not significant.
*$P < 0.05$; **$P < 0.001$.

log CFU/mL (0.5% milk fat) and 7.95 log CFU/mL (3.2% milk fat) by the end of storage (day 15) in milk samples without cinnamaldehyde. Growth of *S. aureus* is possible at temperatures above 8°C at optimum pH values ranging between 6.0 and 7.0 (Valero *et al.*, 2009). In all milk groups studied, the pH was within the optimal range (Figure 1) and enabled *S. aureus* to grow and survive at the utilized storage temperatures.

In contrast, *S. aureus* counts decreased during 15 days' storage in all milk samples with added cinnamaldehyde. The decrease was less pronounced during the first 3 days of storage, and during this time no significant differences ($P > 0.05$) in *S. aureus* numbers were recorded between milk samples stored at 4°C and those stored at 10°C. From day 6 until the end of storage period (day 15), significantly greater *S. aureus* decrease ($P < 0.05$) was recorded in milk samples with added cinnamaldehyde stored at 10°C than in comparable milk samples stored at 4°C. At the end of the study, in milk samples treated with cinnamaldehyde, *S. aureus* numbers had decreased by 1.61 log CFU/mL (0.5% milk fat with 0.05% cinnamaldehyde at 4°C), 2.45 log CFU/mL (0.5% milk fat with 0.1% cinnamaldehyde at 4°C), 1.52 log CFU/mL (3.2% milk fat with 0.05% cinnamaldehyde at 4°C), 1.82 log CFU/mL (3.2% milk fat with 0.1% cinnamaldehyde at 4°C), 3.1 log CFU/mL (0.5% milk fat with 0.05% cinnamaldehyde at 10°C), 4.04 log CFU/mL (0.5% milk fat with 0.1% cinnamaldehyde at 10°C), 2.34 log CFU/mL (3.2% milk fat with 0.05% cinnamaldehyde at 10°C), and 2.96 log CFU/mL (3.2% milk fat with 0.1% cinnamaldehyde at 10°C). The anti-staphylococcal effect of cinnamaldehyde found in the present study was in agreement with previous reports. Alves *et al.* (2016) reported that growth of *S. aureus* was inhibited by the combination of nisin and cinnamaldehyde in pasteurized 3% fat milk stored at 4°C for 6 days.

The mechanism of cinnamaldehyde's antibacterial action is known and well described. The antibacterial activity of cinnamaldehyde is attributed to a free hydroxyl group (Nazzaro *et al.*, 2013). Cui *et al.* (2016) reported that after treating *S. aureus* with cinnamon essential oil, cell membrane injury and leakage of intracellular material were observed. Loss of ATP and DNA were detected because of bacterial cell membrane damage. Some reports indicate that cinnamaldehyde inhibits the membrane-bound ATPase activity (Usta *et al.*, 2003; Gill and Holley, 2004). Di Pasqua *et al.* (2006) found that trans-cinnamaldehyde causes changes in the composition of fatty acid and large increase in the proportion of saturated fatty acids in membrane phospholipids. Shen *et al.* (2015) evaluated the effect of cinnamaldehyde on inner membrane permeability of *S. aureus* by measuring β-galactosidase activity. The authors found that β-galactosidase activity increased with increase in cinnamaldehyde concentration, leading to the conclusion that effects on membranes are dose-dependent. In our previous pilot study (Babic

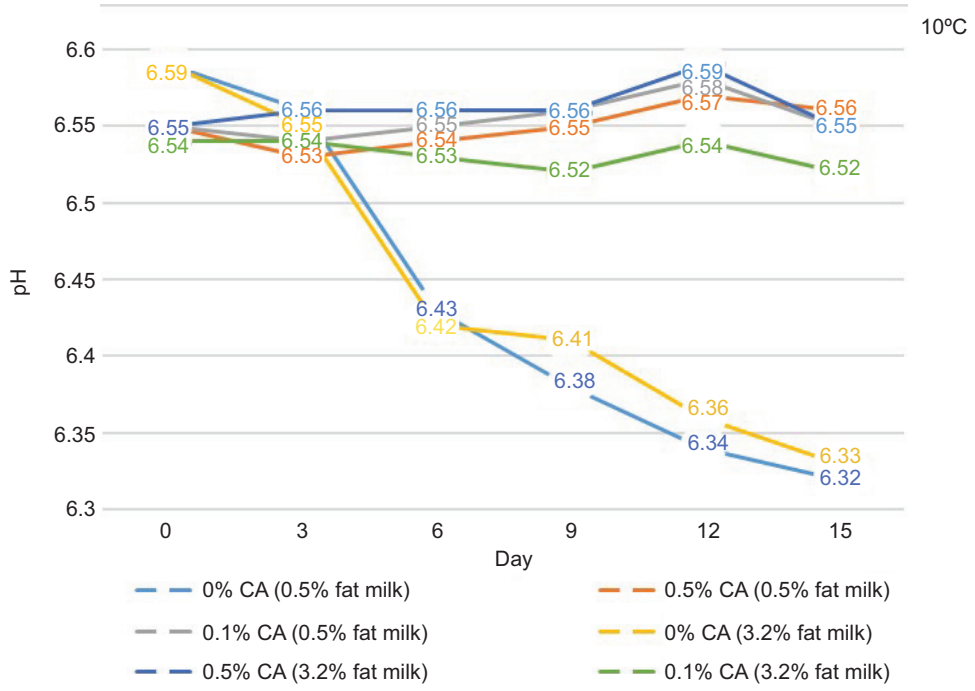

Figure 1. pH of milk stored at 10°C.

et al., 2019), we found that the antibacterial effect of cinnamaldehyde was dependent on its concentration in 1.5% fat milk inoculated with 10^3 CFU/mL *S. aureus* stored at 4°C for 12 days. The same observation was made in the present study, showing significantly ($P < 0.05$) higher inhibition with 0.1% than 0.05% cinnamaldehyde used, but only for milk samples stored at the same temperature. It is supposed that essential oils are more effective when added at higher concentrations because after interactions with food matrix components (e.g. proteins and fats), more of the essential oil remains to interact with the bacterial cells (Hyldgaard *et al.*, 2012; Boskovic *et al.*, 2017).

One of the most important findings of the present study was the greater bacteriostatic effect of cinnamaldehyde at higher temperature. Significantly greater *S. aureus* decrease ($P < 0.05$) was recorded in milk samples with cinnamaldehyde stored at 10°C than in milk samples with same concentration of cinnamaldehyde stored at 4°C. With the expected exception of day 0, the interactions of storage temperature and cinnamaldehyde concentration ($P = 0.001$; factorial ANOVA) on *S. aureus* counts ($P = 0.207$; factorial ANOVA) were statistically significant. At 4°C, 0.05% cinnamaldehyde decreased the number of *S. aureus* to 3.95 log CFU/mL in low-fat milk and to 4.08 log CFU/mL in whole milk, while at 10°C, this concentration of cinnamaldehyde decreased *S. aureus* counts to 2.45 log CFU/mL in low-fat milk and to 3.23 log CFU/mL in whole milk. When added at higher concentration (0.1%), cinnamaldehyde reduced the initial *S. aureus* population to 3.11 log CFU/mL in low-fat milk and to 3.78 log CFU/mL in whole milk in samples stored at 4°C, while a significantly lower number ($P < 0.05$) of *S. aureus* was recorded in milk samples treated with the same concentration of cinnamaldehyde and stored at 10°C (1.51 log CFU/mL in low-fat milk and 2.61 log CFU/mL in whole milk).

One possible explanation for this temperature-dependent antibacterial effect of cinnamaldehyde is that bacteria are metabolically more active at higher temperatures. Consequently, growth and death rates are higher at higher temperature (Smith-Palmer *et al.*, 1998; Yuste and Fung, 2003; Guler and Seker, 2009). In addition, the lower growth rate of bacteria at lower temperatures can make them less susceptible to antimicrobials (Martinsen *et al.*, 1992). Also, at lower temperatures, essential oils have lower diffusion rates, and this reduces the efficiency of their antibacterial activity (Wojtys and Jankowski, 2004; Leja *et al.*, 2019). Even a small change in temperature causes significant changes in the efficiency of their action, which is why the doses of essential oils must be significantly higher at lower temperatures (Leja *et al.*, 2019). These effects are in agreement with the results of present study. In milk samples with the same amount of fat, the anti-staphylococcal effect of 0.05% cinnamaldehyde was significantly ($P < 0.05$) more pronounced at 10°C than the effect of 0.1% cinnamaldehyde at 4°C (Table 2). In addition, Smith-Palmer *et al.* (1998) reported that the target site of cinnamon essential oil can change, and oil penetration to the interior of the cell can be reduced due to alterations in membranes at lower temperatures. Higher antibacterial

activities of essential oils at higher temperatures have been reported previously. Guler and Seker (2009) reported that the effect of cinnamon on *Bacilluscereus* reductions in UHT-pasteurized milk during 28 days was significantly lower when milk samples were stored at 4°C than at 25°C. Yuste and Fung (2003) found that addition of 0.3% cinnamon in apple juice was more effective against *S. aureus* during storage at higher temperatures. The initial contamination was lower (4.34–4.37 log CFU/mL) than in the present study, and counts decreased below detection limits in apple juice stored at 20°C after only 1 day, but it took 7 days of storage at 5°C to obtain the same results. In the present study, the greatest anti-staphylococcal effect was achieved in low-fat milk stored at 10°C and treated with 0.1% cinnamaldehyde, as *S. aureus* numbers were reduced by more than 4 log CFU/mL.

Moreover, the effect of interaction between cinnamaldehyde concentration and fat content in milk on *S. aureus* numbers was significant ($P < 0.0001$) from day 6 until the end of storage, while for the first 3 days no interaction was observed (day 0, $P = 0.423$; day 3, $P = 0.370$; factorial ANOVA). At the end of storage, significant differences ($P < 0.05$) between the *S. aureus* counts in whole milk (3.2%) and low-fat milk (0.5%) were found for the same concentrations of cinnamaldehyde. However, no significant differences ($P > 0.05$) in *S. aureus* counts were found between milk samples stored at the same temperatures without cinnamaldehyde, regardless of content of milk fat. Thus, cinnamaldehyde was more effective in inhibiting the pathogen in low-fat milk than in high-fat milk, which is also consistent with literature. Cava-Roda *et al.* (2012) found significant differences between whole milk (3.9% fat) and skimmed milk (0.3% fat) for inhibiting *Escherichia coli* O157:H7 and *Listeria monocytogenes* using the same concentration of vanillin (which also belongs to a group of phenylpropenes, as does cinnamaldehyde). The authors also reported that there was no effect of content of milk fat on pathogen numbers in control milk samples without vanillin. Therefore, the antibacterial effects of essential oils and other antimicrobial agents are likely to decrease or even limited by the amount of fat in the matrix (Liu and Yang, 2012; Boskovic *et al.*, 2017, 2019). It has been suggested that fats may form a protective layer around bacterial cells and absorb essential oils, leaving the water phase free of antimicrobial agents, and therefore lowering the antibacterial activity (Tassou *et al.*, 1995; Smith-Palmer *et al*., 2001; Perricone *et al.*, 2015).

pH of milk during storage

The initial pH of milk samples ranged from 6.54 to 6.59. Cinnamaldehyde initially caused the milk pH to drop very slightly (from 6.59 to around 6.55). Milk pH did not change significantly during storage at 4°C.

However, when milk without cinnamaldehyde was stored at 10°C, decline in pH was observed regardless of the fat content. In fact, on day 3, the pH of milk without cinnamaldehyde stored at 10°C (Figure 1) decreased slightly compared with the initial pH values, but from day 6, significant ($P < 0.05$) pH declines were measured. The pH of this milk kept on decreasing throughout the 15 days' storage, reaching pH values of 5.32 (0.5% milk fat) and 5.33 (3.2% milk fat). Growth of *S. aureus* in these milk samples (Table 2) matched decline in pH. Under aerobic conditions, *S. aureus* can ferment milk sugar and lactose, creating acids responsible for the storage-induced decline in milk pH (Medveďová and Valík, 2012). The pH of milk samples with cinnamaldehyde added and stored at 10 °C did not significantly differ between each other.

Conclusion

These results indicate that it could be possible to use cinnamaldehyde as a natural anti-staphylococcal agent in milk beverages. *S. aureus* numbers in milk were affected by cinnamaldehyde in a dose-dependent manner. Cinnamaldehyde showed a greater antibacterial effect against *S. aureus* in low-fat milk than in whole milk. Temperature had a strong effect on the anti-staphylococcal effect of cinnamaldehyde; hence, the lower concentration of cinnamaldehyde in milk stored at 10°C tended to have a better anti-staphylococcal effect than the higher concentration of cinnamaldehyde in milk stored at 4°C. Nevertheless, even if the results of our study are promising, and if flavored milk is becoming increasingly popular, further investigations are required to determine the antibacterial effectiveness of cinnamaldehyde in raw milk and dairy products and to conduct sensory analysis of final products.

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Conflicts of Interest

The authors have no conflicts of interest for this article.

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Effects of high linolenic acid diet supplemented with synthetic or natural antioxidant mix on live performance, carcass traits, meat quality and fatty acid composition of *Longissimus thoracis et lumborum* muscle of medium-heavy pigs

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PAPER

Abstract

We studied the effect of a high linolenic acid diet supplementation with synthetic (vitamin E + selenium) or vegetal mix rich in natural antioxidants (grape skin + oregano) on live performances, carcass and meat quality, fatty acid composition and oxidative stability of intramuscular lipids of *Longissimus thoracis et lumborum* muscle in medium-heavy pigs. Neither carcass traits nor chemical proximate composition of meat was affected by dietary treatments. Linseed dietary inclusion reduced the n-6:n-3 polyunsaturated fatty acids ratio and increased long-chain n-3 precursor, fundamental for human health. Our results offer new opportunities to use products more acceptable by consumers and are more eco-friendly.

Keywords: extruded linseed, fatty acids profile, grape skin extract, oregano extract, oxidative stability of pork, vitamin E

Introduction

Polyunsaturated fatty acids (PUFA), including n-6 and n-3 groups, are essential for physiological functioning and health of humans and domestic animals (Delgado-Lista *et al.*, 2012). Western diets are deficient in n-3 PUFA and contain excessive amounts of n-6 PUFA, resulting in a high n-6:n-3 ratio ranging from 10:1 to 20:1, while an optimal recommended ratio is 1:1–4:1. High values of ratio can be the cause of various diseases (Simopoulos, 2002, 2010; Leslie *et al.*, 2015). Many studies have shown that n-3, mainly eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) fatty acids (FA), have an anti-inflammatory effect and play a beneficial role in a number of human diseases, including autoimmune diseases, diabetes, tumours, Alzheimer's disease and stroke (Boston *et al.*, 2004; Fritsche, 2006; Wall *et al.*, 2010).

α-Linolenic acid (ALA, 18:3 n-3) is a precursor of n-3 PUFA group; EPA, docosapentaenoic (DPA, 22:5 n-3) and DHA fatty acids are synthesized from ALA through consecutive elongation and desaturation.

In general, animal fats are lacking in n-3 PUFA, while contain n-6 PUFA abundantly. Hence, pork products provide too much of n-6 while lacking in n-3 PUFA (Wood *et al.*, 2008; Kouba and Mourot, 2011). Owing to a high content of saturated fatty acids (SFA) and an

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unfavourable n-6:n-3 PUFA ratio (Liu and Kim, 2018), pork consumption has been associated with an increased risk of chronic diseases (Egeberg *et al.*, 2013; Klurfeld, 2015).

However, genetic factors (Cameron *et al.*, 2000; Piedrafita *et al.*, 2001; Wood *et al.*, 2008), sex, age, live weight at slaughter (Lebret and Mourot, 1998; Lo Fiego *et al.*, 2005b; Minelli *et al.*, 2019) and feeding strategies (Lo Fiego *et al.*, 2005a) can influence deposition of lipids in pig tissues and their fatty acid composition. It is widely accepted that nutrition is the main factor influencing deposition of lipid and fatty acid in monogastric animals. Dietary FA can significantly modify the fatty acid profile of adipose tissues in pigs. In fact, many studies have confirmed the enrichment of pig tissues with n-3 PUFA by incorporating linseed in pig diets (Kouba *et al.*, 2003; Corino *et al.*, 2008; Minelli *et al.*, 2020) or its derivatives rich in ALA (Guillevic *et al.*, 2009; Musella *et al.*, 2009; Corino *et al.*, 2014).

However, increasing PUFA and lowering SFA contents raises the susceptibility of meat to lipid oxidation, leading to undesirable effects such as deterioration of its sensorial quality and nutritional value (Rivas-Cañedo *et al.*, 2013; Chamorro *et al.*, 2015). To counteract this effect, dietary addition of antioxidants, such as vitamin E and selenium, is widely used in swine feeding. Supranutritional levels of vitamin E may improve oxidative and colour stability of meat during storage (De la Fuente *et al.*, 2009; Kasapidou *et al.*, 2012; Muíño *et al.*, 2014). Moreover, the combination of vitamin E and selenium can build a complex antioxidant system capable of protecting against free radicals and lipid oxidation products (Surai and Fisinin, 2015). However, now consumers feel more reasonable the use of natural antioxidant products in animal feeding (Gladine *et al.*, 2007; Brenes *et al.*, 2016). Among these, oregano extract has shown a considerable antioxidant effect (Botsoglou *et al.*, 2003) as well as antimicrobial and anti-inflammatory activity (Cheng *et al.*, 2017). Oregano antioxidant action is attributed to the presence of different phenolic active compounds such as thymol and carvacrol (Tuttolomondo *et al.*, 2013).

Another very rich natural source of antioxidants is represented by various by-products of winery, such as grape skin and seeds, that are rich in polyphenolic compounds characterized by a high antioxidant activity (Selani *et al.*, 2011).

Furthermore, the wine industry produces a large amount of residues in a short period of the year whose disposal is expensive and involves problems related to pollution (Bustamante *et al.*, 2008).

The inclusion of these by-products as an alternative to synthetic antioxidants could be an interesting nutritional and environment-friendly strategy.

The aim of this research was to study the effects of inclusion of linseed in the growing–finishing diet of medium-heavy pigs and of the supplementation with supranutritional levels of a synthetic antioxidant complex (vitamin E and selenium) or vegetal mix rich in natural antioxidants (grape skin and oregano) on live performance, carcass and meat quality, and on fatty acid composition and oxidative stability of intramuscular lipids of *Longissimus thoracis et lumborum* (LTL) muscle.

MATERIALS AND METHODS

Ethics approval

All the experimental procedures performed in this study were in accordance with the Italian legislation and did not require special animal care authorizations, that is the decision of the Animal Welfare Committee of Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA; 14 September 2016), according to the Italian Legislative Decree of 4 March 2014 n. 26 art. 2, point F.

Animals, diets and growth performances

Forty-eight Italian Large White pigs, balanced for gender and weight, housed in 16 pens (9 m^2 concrete floor pens) with three animals in each pen, were evenly assigned to four different dietary treatments (12 pigs per diet; four replicates). The diets were similar for energy and protein levels with the same lysine/digestible energy ratio. The composition of the diets is shown in Table 1. The control group (C) received barley/soybean diet. In the three treatment (linseed) groups (experimental groups), 5% of barley was substituted for 5% of extruded linseed, either unsupplemented (L) or supplemented with a synthetic antioxidant complex (LSA) containing 200 ppm of α-tocopheryl acetate and 0.21 ppm of selenium (supported on calcium carbonate), or added with 5 g/kg of vegetal mix rich in natural antioxidants (LNA), providing 3 g/kg of grape skin extract and 2 g/kg of oregano extract, adhering to the amounts suggested by the manufacturer for food supplementation. Water was always available. The trial lasted for 104 days starting from an average live body weight (LBW) of 79.9 ± 5.8 kg (6 months of age), till slaughter at 150.5 ± 9.9 kg LBW. From starting weight and up to 113 ± 10.6 kg LBW, the subjects were fed at 7.5% of metabolic weight, calculated as $LBW^{0.75}$; thereafter, till slaughtering, the pigs were fed at 8.5% of $LBW^{0.75}$.

The natural extracts from grape skin are normally used as supplement, nutraceutical, or for food colouring; the total amount of polyphenols in the antioxidant mix of LNA group was 14.3 g/L expressed as gallic acid

Table 1. Ingredients (%), proximate composition (% on dry matter basis) and fatty acid composition (% of total fatty acids) of dietary treatments.

		C		L		LSA		LNA	
		1st	2nd	1st	2nd	1st	2nd	1st	2nd
Ingredients									
Extruded linseed	%	0.00	0.00	5.00	5.00	5.00	5.00	5.00	5.00
Barley meal	%	85.50	91.00	80.50	86.60	80.30	86.40	80.50	86.60
Soybean meal	%	11.00	5.50	11.00	5.00	11.00	5.00	11.00	5.00
L-Lysine	%	0.31	0.29	0.30	0.29	0.30	0.29	0.30	0.29
DL-Methionine	%	0.06	0.04	0.06	0.03	0.06	0.03	0.06	0.03
L-Threonine	%	0.05	0.04	0.05	0.03	0.05	0.03	0.05	0.03
Calcium carbonate	%	1.18	1.13	1.19	1.15	0.89	0.85	1.19	1.15
Dicalcium phosphate	%	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Salt (NaCl)	%	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Vitamin/mineral pre-mix[1]	%	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vit E + Se pre-mix[2]	%	—	—	—	—	0.50	0.50	—	—
Vegetal ext. (grape skin + oregano)[3]	%	—	—	—	—	—	—	0.3+0.2	0.3+0.2
Proximate composition									
Dry Matter (DM)	%	88.30	89.50	88.60	89.80	88.70	89.90	88.80	90.00
(on DM basis)									
Digestible energy	MJ/kg	13.35	13.26	13.63	13.54	13.60	13.52	13.63	13.54
Crude protein	%	16.87	12.55	17.89	13.20	17.98	13.58	17.93	13.03
Crude fat	%	2.00	1.73	4.30	3.86	4.21	4.00	4.41	3.98
Crude fibre	%	4.76	4.50	4.91	5.08	5.01	4.65	5.26	4.97
Ashes	%	5.87	4.50	5.86	5.89	6.26	5.98	6.15	5.40
Fatty acid (FA) composition	**% (of total FAs)**								
C14:0	%	0.47	0.39	0.25	0.21	0.25	0.22	0.26	0.22
C16:0	%	29.01	24.25	18.13	15.20	17.78	15.59	18.80	15.31
C16:1	%	0.49	0.34	0.17	0.15	0.17	0.17	0.02	0.15
C18:0	%	2.03	1.51	4.00	3.18	3.88	3.34	4.16	3.23
C18:1n-9	%	14.92	13.50	20.60	18.12	20.24	18.45	21.29	18.26
C18:2n-6	%	47.55	53.67	33.50	34.69	33.91	34.09	32.52	34.47
C18:3n-3	%	4.77	5.70	22.83	28.02	23.25	27.73	22.38	27.95
C20:1	%	0.74	0.64	0.53	0.41	0.52	0.42	0.57	0.41

C: control group; L: experimental group with 5% of extruded linseed; LSA: experimental group with 5% of extruded linseed, 200 ppm vitamin E + 0.21 ppm selenium; LNA: experimental group with 5% of extruded linseed and vegetal extract 5 g/kg of feed (3.00 g of grape skin extract + 2.00 g of oregano extract).
1st = feed administered from an average weight of 79.9 to 113.4 kg (growing period); 2nd = feed administered from an average weight of 113.4 kg to the slaughter (finishing period).
[1]Vitamin/mineral: Providing the following nutrients (per kg diet as-fed): vitamin A, 15.000 IU; vitamin D3, 2.000 IU; vitamin E (α-tocopheryl acetate), 50 mg; vitamin K, 2.5 mg; vitamin B1, 2 mg; vitamin B2, 5 mg; vitamin B5, 15 mg; vitamin B6, 4 mg; vitamin B12, 0.036 mg; niacin, 25 mg; folic acid, 1 mg; biotin, 0.15 mg; choline, 346 mg; Cu, 15 mg; Fe, 150 mg; Mn, 25 mg; Co, 0.4 mg; I, 1.5 mg; Zn, 100 mg; and Se, 0.1 mg.
[2]Vitamin E + selenium: Providing the following nutrients (per kg diet as fed): vitamin E (α-tocopheryl acetate), 200 mg, and Se, 0.21 mg, supported on calcium carbonate.
[3]Vegetal extract was added directly to the water of the diet and not mixed with the complete feed.

equivalent (GAE). A complete characterisation of phenolic compounds of vegetal extracts used is reported in a previous paper (Martini *et al.*, 2020). Grape skin extract was supplied by Enocianina Fornaciari s.n.c. (Reggio Emilia, Italy) and oregano extract was supplied by Phenbiox s.r.l. (Bologna, Italy). All diets were distributed in wet form (water:feed ratio of 3:1) and the vegetal extract mix was diluted in the water of the diet. During the trial, the average daily feed intake (ADFI) per pen was monitored, and the feed conversion rate (FCR) per pen was calculated.

Slaughtering and sampling

The pigs were weighed individually after an overnight fasting, and slaughtered in a commercial abattoir by exsanguination after electrical stunning, in agreement with the Council Regulation (EC) No. 1099/2009 on the protection of animals at the time of killing. Each carcass, after slaughtering, was graded in agreement with EUROP grid carcass grading, using Fat-o-Meater device (MIPAAF, 2018). The hot carcasses were weighed, the pH_1 (45 min *post-mortem*) value at the last rib level was measured and the hot carcass yield was calculated as hot carcass weight (kg)/slaughter LBW (kg)×100. Subsequently, each carcass was dissected into commercial cuts, which were weighed and cold-stored at 0–4°C for about 24 h. At 24 h *post-mortem*, the refrigerated LTL muscle from each half left carcass was transported to laboratory and sliced in four subsamples: the first to be used for pH_2 (24 h *post-mortem*), colour and drip loss analysis; the second for evaluation of oxidative stability, moisture, intramuscular lipid and protein content of raw meat; the third one for cooking loss and shear force analysis and oxidative stability of cooked meat; and the last one was vacuum-packed (Elegen, Reggio Emilia, Italy) and stored at –20°C until lipid extraction for fatty acid analysis.

Instrumental analysis

At 24 h *post-mortem*, pH values and colour parameters were measured directly on the fresh muscle. The pH value was recorded using a portable Crison pH-meter equipped with a Xerolite electrode (Crison Instruments, Alella, Spain). Meat colour was measured by Minolta CM-600d spectrophotometer (Konica Minolta Holdings Inc., Osaka, Japan) using illuminant D65 and an 8-mm diameter aperture. After calibration with a white calibration plate, five different points on each sample were analysed and the measurements were averaged. The results were expressed as the CIE Lab three coordinates: L^* – 'lightness', a^* – 'redness', and b^* – 'yellowness'. Further, Chroma (C^*), the expression of saturation index and colour intensity, was calculated as $\sqrt[2]{a^{*2}+b^{*2}}$, and the Hue angle (H^*) was calculated as arctan (b^*/a^*).

Drip loss (%) was evaluated on LTL muscles (starting at 24 h *post-mortem*) according to Honikel (1998, slightly modified). To evaluate cooking loss, a sample (approx.100 g) of LTL muscle was vacuum-packed and cooked in a water bath till the core temperature reached 70°C. After cooling, the samples were weighed, and the cooking loss was calculated as the percentage of initial sample weight. The Warner–Bratzler shear force (WBSF) was determined on cooked samples according to Honikel (1998). Briefly, from each LTL-cooked muscle, six cylindrical cores (Ø, 1.50 cm) were cut parallel to the longitudinal orientation of muscle fibres. The measurements were averaged, and the peak force was expressed as kilogram. The working conditions of the Zwick Z50 kN testing machine (model BT1-FB050TN, Zwick Roell, Kennesaw, GA, USA) were as follows: 1-kN load cell equipped with a V-shaped blade with a triangular hole of 60°; and a constant speed of 250 mm/min.

Lipid oxidation analysis

The lipid oxidation of fresh and cooked LTL samples was evaluated in duplicate according to Siu and Draper (1978, slightly modified). Minced sample, 2.5 g, was blended and homogenised with 12.5 mL of distilled water for 2 min at 9,500 rpm using an Ultra-Turrax tissue homogenizer (IKA, Germany). Before centrifugation, at 2,000 rpm at 4°C for 20 min, 12.5 mL of 10% trichloroacetic acid (TCA) solution (Sigma-Aldrich, Milan, Italy) was added. The supernatant was collected after decantation through a paper filter (Whatman No. 541), and 4 mL of clear filtrate was transferred into 15-mL pyrex tubes; 1-mL 0.06 M 2-thiobarbituric acid (TBA, Sigma-Aldrich, Milan, Italy) was added and the samples were kept for 90 min in a water bath at 80°C; the samples were cooled before reading. At the same time, the blank was run (2-mL distilled water + 2-mL TCA solution + 1-mL TBA). Absorbance at 532 nm was measured against blank sample using a Jasco spectrophotometer (Model V550, UV/VIS, Tokyo, Japan). Using 1,1,3,3 tetraethoxypropane (TEP, Sigma-Aldrich, Milan, Italy) as a standard, thiobarbituric acid reactive substances (TBARS) was expressed as milligram of malondialdehyde (MDA) per kilogram of muscle.

Chemical composition of fresh meat and feed

The analyses of moisture, ether extract and crude protein were performed on LTL muscle according to the Association of Official Analytical Chemists methods (AOAC, 1995). The results were expressed as percentage of wet matter.

Analyses for the determination of proximate composition of feeds (dry matter, ash, crude protein, crude fat and crude fibre) were carried out according to the AOAC methods (AOAC, 1995) and the results were expressed on dry matter basis. Energy values of feeds were calculated according to Sauvant *et al.* (2004).

Fatty acid profile of fresh meat

Total lipids from LTL muscle were extracted according to the Folch *et al.* (1957) method. According to

Ficarra *et al.* (2010), 25 mg of lipid extract was methylated with methanolic potassium hydroxide (KOH) solution 2N (KOH supplied by Carlo Erba, Milan, Italy, and methanol supplied by ITW Reagents, Barcelona, Spain) and an aliquot of tridecanoic acid (C13:0) (Larodan Fine Chemicals AB, Malmö, Sweden) was added as internal standard. For determining the FA profile, TRACE™ Gas Chromatograph (GC) Ultra (Thermo Electron Corporation, Rodano, Milano, Italy) equipped with Flame Ionization Detector, a PVT injector and TR-FAME column (30-m long, 0.25-mm i.d., 0.2-µm film thickness), supplied by Thermo Scientific (Rodano, Milano, Italy), was used. Methylated sample, 1 µL, was injected into GC with a split flow rate of 10 mL/min, operating at a constant flow of 1 mL/min of helium as a carrier gas. Detector and injector had the same operating temperature of 240°C. After 2 min, the program temperature was increased at a rate of 4°C per minute from 140°C to 250°C and maintained for 5 min. The Chrom-card software (version 2.3.3, Thermo Electron Corporation Rodano, Milano, Italy) was used to record, identify and integrate the peaks of fatty acid methyl esters (FAMEs). To identify the retention period of FAMEs, a solution of standard FA mixed with the known quantity of standard was used (Supelco^r 37 Component FAME mix, PUFA standard n.2, Animal Source, Supelco, Bellafonte, PA, USA, and single FAMEs standard, Larodan, Fine Chemicals AB, Malmö, Sweden). The amount of each FAME was expressed as FAME relative percentage with respect to the total amount of FAMEs.

Moreover, atherogenic index, AI = [C12:0 + (4 × C14:0) + C16:0] / [n-6 PUFA + n-3 PUFA + monounsaturated FA (MUFA)] (Ulbricht and Southgate, 1991), and thrombogenic index, TI = [C14:0 + C16:0 + C18:0] / [(0.5 × MUFA) + (0.5 × n-6 PUFA) + (n-3 PUFA / n-6 PUFA)] (Ulbricht and Southgate, 1991), were calculated.

Statistical analysis

The statistical analysis was performed using the mixed model procedure of SAS (SAS Institute Inc., Cary, NC, USA). The statistical model included dietary treatments, gender and their interactions as fixed effects and pen as a random effect. The interactions were not statistically significant; therefore, they were removed from the statistical model. Live performance data as average daily gain (ADG), ADFI, FCR, and slaughter weight were covariate for starting LBW. Moreover, for ADFI and FCR, the pen was considered as an experimental unit. Carcass weight and dressing percentage were covariates for slaughter weight, while the fatty acid composition was a covariate for intramuscular fat content (IFC). When a significant ($P < 0.05$) treatment effect was observed, Tukey's multiple comparison test was performed to compare mean values.

Results and Discussion

Performance and carcass characteristics

The experimental diets did not influence ADG and slaughter LBW ($P > 0.05$) (Table 2).

These results confirm the results reported by Corino *et al.* (2008) in pigs fed with a diet containing 5% of extruded linseed and slaughtered at 100 or 160 kg, and Kouba *et al.* (2003) in pigs fed for 20, 60 or 100 days with a diet containing 6% of whole crushed linseed. Moreover, Juàrez *et al.* (2010), feeding pigs with 5%, 10% or 15% of extruded flaxseed, reported no statistical differences on ADG and final LBW but found a slight improvement of feed efficiency with an increased dietary level of flax. In our study, the control group showed the highest and the LNA group the lowest FCR values ($P < 0.05$). The

Table 2. Effect of dietary treatment and gender on live performance (data covariate for initial LBW).

	Dietary treatments					Gender		
	C	L	LSA	LNA	SEM	Gilts	Barrows	SEM
Initial LBW (kg)	77.4	82.3	80.1	79.7	2.59	79.8	79.9	1.64
ADG (kg)	0.67	0.68	0.68	0.70	0.035	0.70	0.68	0.025
ADFI (kg/day)	2.50^{a}	2.46^{ab}	2.46^{a,b}	2.42^{b}	0.019	—	—	—
FCR (kg/kg^{-1})	3.81^{a}	3.51^{b}	3.59^{b}	3.47^{b}	0.066	—	—	—
Slaughter LBW (kg)	148.9	150.9	150.6	151.7	2.52	151.5	149.6	1.86

C: control group; L: experimental group with 5% of extruded linseed; LSA: experimental group with 5% of extruded linseed, 200 ppm vitamin E + 0.21 ppm selenium; LNA: experimental group with 5% of extruded linseed and vegetal extract 5 g/kg of feed (3.00 g of grape skin extract + 2.00 g of oregano extract).
SEM: standard error of mean values; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion rate (ADFI/ADG); LBW: live body weight.
Feed conversion ratio: Pens were considered as experimental units.
^{a,b}Different letters in the same line indicate statistically different mean values ($P < 0.05$).

ADFI was higher in the control group with statistical differences ($P < 0.05$) only when compared with the LNA group (2.50 vs. 2.42 kg*day^{-1}). We could assume that the high content of polyphenols was the main reason for these results. Fiesel *et al.* (2014) stated that the presence of plant products in growing pigs' diet improves the gain:feed ratio, since polyphenols not only cause alteration in the microbial composition of the gut but also exert anti-inflammatory action.

In our study, dietary n-6:n-3 PUFA ratios were 9.8:1 in the control group and averaged as 1.35:1 in linseed groups (data not reported in the table). The groups with lower n-6:n-3 PUFA ratios showed slight improvement in feed efficiency ($P < 0.05$), and this result agrees with Duan *et al.* (2014) and Li *et al.* (2015). The authors stated that n-6:n-3 ratios of 1:1 and 1:5 compared with 10:1 improved feed efficiency of finishing pigs. These authors assumed that improvement in feed efficiency could be due to the anti-inflammatory effect of n-3, which spares energy and nutrients for tissue deposition.

Gender had no effect ($P > 0.05$) on any of the parameters evaluated. Since feed intake was recorded per pen, and both sexes were housed in each pen, the gender-wise statistical analysis of ADG and FCR was not possible.

Inclusion of dietary linseed (Table 3) did not affect ($P > 0.05$) carcass characteristics, as already observed in previous studies (Kouba *et al.*, 2003; Karolyi *et al.*, 2012).

Although differences were not statistically significant, the backfat thickness was slightly higher in the control group, and the lean meat percentage was lesser than in the linseed-fed groups (33.5 mm vs. avg. 30.9 mm, and 50.2% vs. avg. 51.4%). The same trend was recorded by Duan *et al.* (2014), who reported a significant decrease in adipose tissues and an increase in lean tissue masses with a decrease in dietary n-6:n-3 ratio from 10:1 to 1:1.

The effect of gender was significant only on the percentage of perirenal fat, higher in barrows (1.95% vs.1.59%; $P < 0.05$).

Table 3. Effect of dietary treatment and gender on carcass traits (carcass weight and dressing percentage were covariate for slaughter weight, while all carcass traits were covariate for hot carcass weight).

	Dietary treatments					Gender		
	C	L	LSA	LNA	SEM	Gilts	Barrows	SEM
Hot carcass weight (kg)	124.82	129.26	127.03	127.75	2.493	128.11	126.32	1.765
Hot carcass yield (%)	85.02	84.59	84.28	84.23	0.503	84.36	84.70	0.359
Backfat thickness (mm)	33.53	31.09	30.72	30.93	1.615	31.45	31.68	1.109
Lean meat content (%)	50.23	51.33	51.31	51.41	0.815	51.08	51.06	0.278
Lean cuts (%)[1]								
• Thigh	26.89	26.73	27.06	27.17	0.314	27.09	26.84	0.222
• Loin	18.61	17.62	18.02	17.62	0.369	18.00	17.94	0.259
• Neck	7.90	8.17	8.00	7.79	0.249	8.06	7.87	0.169
• Shoulder	14.48	14.52	14.15	14.34	0.182	14.35	14.40	0.128
• Total lean cuts	67.90	67.03	67.23	66.92	0.480	67.55	66.99	0.340
Adipose cuts (%)[1]								
• Backfat	4.50	5.06	5.22	5.53	0.285	5.17	4.98	0.200
• Belly	13.38	13.47	13.49	12.98	0.389	13.37	13.29	0.275
• Jowl	6.35	6.49	6.78	6.69	0.240	6.49	6.67	0.165
Perirenal fat	1.73	1.74	1.81	1.81	0.115	1.59[b]	1.95[a]	0.080
Total adipose cuts	25.94	26.77	27.31	26.98	0.513	26.54	26.96	0.363
Others (head, feet, tail) (%)[1]	6.13	6.20	5.46	6.10	0.580	5.91	6.05	0.410

C: control group; L: experimental group with 5% of extruded linseed; LSA: experimental group with 5% of extruded linseed, 200 ppm vitamin E + 0.21 ppm selenium; LNA: experimental group with 5% of extruded linseed and vegetal extract 5 g/kg of feed (3.00 g of grape skin extract + 2.00 g of oregano extract).
SEM: standard error of mean values.
[1]Percentage of hot carcass weight.
[a,b]Different letters in the same line indicate statistically different mean values ($P < 0.05$).

Raw and cooked meat characteristics

The effects of dietary treatment and gender on chemical and physical characteristics of LTL muscle are shown in Table 4.

In agreement with Corino *et al.* (2014) and Minelli *et al.* (2020), the pH values of LTL muscle were not influenced by dietary treatments. The colour parameters a^*, b^*, C^*, and H^* were the only parameters affected by dietary treatments. The control group showed higher a^* (3.16 vs. 1.88), b^* (12.40 vs. 11.59) and C^* (12.88 vs. 11.79) values but lower H^* (76.04 vs. 81.01) values with respect to the LSA group ($P < 0.05$). The control group yielded redder and yellower meat than the LSA group, whilst the other two diets with linseed showed intermediate and not statistically different values. Probably, vitamin E might have affected variation of colour parameters. Our data conflicted with the results of Hasty *et al.* (2002), who reported a tendentially ($P > 0.05$) linear increase of b^* value with increasing levels of dietary α-tocopheryl acetate, while Asghar *et al.* (1991) observed that a dietary supplementation of vitamin E at a level of 200 IU/kg led to an increase in the redness of pork without affecting yellowness. In general, we found that all linseed groups have tendentially lower a^*, b^* and C^* values and higher H^* value, and this indicated that extruded linseed could produce tendentially decoloured meat. The moisture, ether extract, drip loss, MDA and protein content of raw meat, and shear force and MDA content in cooked meat were not affected by dietary treatments.

Oxidative stability during meat storage and cooking is an important factor for both shelf-life and consumer safety. In this research, the MDA content increased by about four times with the cooking process, but no difference ascribable to diet or sex was found in raw or cooked meat, although unsupplemented extruded linseed showed the highest value ($P > 0.05$). These results confirmed the previous findings in pigs fed with similar diets (Minelli *et al.*, 2020).

Yet by imposing more challenging experimental conditions, other authors obtained quite different results. Among these authors are Botsoglou *et al.* (2012), who evaluated pork chops containing lipids with far higher proportions of n-6 and n-3 PUFA than our LTL samples. They observed that lipid oxidation in both raw and cooked chops was significantly alleviated in the samples obtained from the subjects that had received a dietary supplementation of α-tocopheryl acetate, 200 mg/kg feed.

Table 4. Effects of dietary treatment and gender on chemical and physical characteristics of raw and cooked *Longissimus thoracis et lumborum* muscle.

	Dietary treatments					Gender		
	C	L	LSA	LNA	SEM	Gilts	Barrows	SEM
pH_1	6.49	6.37	6.37	6.37	0.096	6.34	6.47	0.062
pH_2	5.60	5.55	5.58	5.54	0.020	5.55	5.58	0.014
L^*	52.78	52.69	53.34	53.20	0.755	53.66	52.34	0.533
a^*	3.16^{a}	2.71^{a,b}	1.88^{b}	2.07^{a,b}	0.379	2.02^{b}	2.89^{a}	0.268
b^*	12.40^{a}	11.94^{a,,b}	11.59^{b}	11.92^{a,b}	0.277	11.85	12.08	0.196
Chroma (C^*)	12.88^{a}	12.35^{a,b}	11.79^{b}	12.16^{a,b}	0.312	12.08	12.51	0.221
Hue angle (H^*)	76.04^{b}	77.80^{a,b}	81.01^{a}	80.51^{a,b}	1.672	80.73^{a}	76.95^{b}	1.18
Drip loss (%)	2.45	3.37	2.89	2.63	0.464	3.05	2.62	0.329
Cooking loss (%)	21.88	23.23	21.15	20.95	0.985	21.34	22.27	0.698
MDA, raw meat (mg/kg)	0.104	0.115	0.083	0.164	0.044	0.094	0.138	0.116
MDA, cooked meat (mg/kg)	0.401	0.501	0.401	0.380	0.0505	0.393	0.448	0.0358
Moisture (%)	68.98	68.24	67.98	68.75	0.655	68.65	68.33	0.461
Ether extract (%)	1.62	1.58	1.39	1.75	0.158	1.40^{b}	1.78^{a}	0.109
Protein (%)	23.40	23.62	23.81	22.88	0.344	23.42	23.44	0.243
WBSF (kg)	6.58	6.40	6.33	5.93	0.366	6.28	6.34	0.259

C: control group; L: experimental group with 5% of extruded linseed; LSA: experimental group with 5% of extruded linseed, 200 ppm vitamin E + 0.21 ppm selenium; LNA: experimental group with 5% of extruded linseed and vegetal extract 5 g/kg of feed (3.00 g of grape skin extract + 2.00 g of oregano extract).
SEM: standard error of mean values; MDA: Malondialdehyde; WBSF: Warner Bratzler Shear Force.
^{a,b}Different letters in the same line indicate statistically different mean values ($P < 0.05$).

Further, Martini *et al.* (2020), evaluating a subsample of this research, reported in meat grilled at 140°C for 5 min a dramatic increase of about eight times in MDA with respect to raw meat. The sharpest increase was found in the L group that showed statistically different results from all other groups, while no difference was found in C, LSA and LNA groups.

Thus, rearing and processing conditions play a pivotal role in the oxidative stability of meat enriched with n-3 PUFA. However, in our experimental conditions, the natural antioxidants were as effective as the synthetic ones in preventing formation of advanced lipid oxidation end products.

All groups showed an MDA content lower than 1.0 mg/kg of meat, which is considered the maximum acceptable threshold for rancidity (Rahman *et al.*, 2015).

Regarding the effect of gender on colour parameters, the redness value was higher in barrows than in gilts (2.89 vs. 2.02; $P < 0.05$) and H* was lower in barrows (76.95 vs. 80.73; $P < 0.05$). Our results are in agreement with previous studies (Alonso *et al.*, 2009; Daza *et al.*, 2018). These authors reported that a* value was directly related to myoglobin content that was higher in barrows than in gilts, and consequently their meat resulted redder globally.

LTL muscle in barrows had higher content of ether extract (1.78% vs. 1.40%; $P < 0.05$). This matched with other findings (Alonso *et al.*, 2009; Lo Fiego *et al.*, 2010; Daza *et al.*, 2018) and was largely expected, given that castration promoted the intramuscular fattening of meat (Barton-Gade, 1987).

Intramuscular fatty acid composition

Dietary treatments did not significantly affect the proportion of total SFA and MUFA ($P > 0.05$) (Table 5).

Among SFA, the percentage of 12:0 and 16:0 tended to be lower in the control group but statistically different ($P < 0.05$) from the LSA group (–0.01 and –0.95 percentage points, respectively). Among MUFA, 17:1 tended to be higher in the control group but statistically different from the L group (+0.06; $P < 0.05$). Irrespective of the type of antioxidant used, inclusion of extruded linseed in the diet increased ($P < 0.05$) the proportion of all n-3 PUFA except DHA, in agreement with Guillevic *et al.* (2009) and Minelli *et al.* (2020). Further, DHA was lower ($P < 0.05$) in the LSA group than in the L group (0.07% vs. 0.10%, respectively). Moreover, linseed dietary inclusion reduced ($P < 0.05$) the n-6:n-3 PUFA ratio and the proportion of all n-6 PUFA, except 18:2 n-6 and 20:2 n-6 ($P > 0.05$). The total content of PUFA was lowest in the LSA group and statistically different ($P < 0.05$) compared with the L group. The thrombogenic index (TI) decreased ($P < 0.05$) with linseed feeding but only in the L group compared with the control group.

In general, main variations in FA composition ascribable to linseed feeding are as follows: an increase of n-3 PUFA, mainly ALA and its derivates EPA and DPA, originated from elongase and desaturase reactions; decrease of n-6 PUFA, mainly 22:4 n-6, probably accounted for by the higher affinity of Δ6 desaturase for n-3 substrates (Lee *et al.*, 2016) and decrease of n-6:n-3 ratio below 4, the maximum threshold indicated by Simopoulos (2002) to avoid adverse health consequences. Our results agree with previous works (Riley *et al.*, 2000; Minelli *et al.*, 2020).

The FA composition of intramuscular fat did not differ significantly between LSA and LNA groups. Regarding the n-6:n-3 ratio, it did not vary in linseed-fed groups, although antioxidant supplementation led to a numerical increase of this ratio, with the highest value found in the LSA group.

This could be due to different effects of diets enriched with n-3 PUFA and natural or synthetic antioxidants in modulating the expression of pig skeletal muscle genes involved in *de novo* synthesis of FA and metabolism of lipids. Vitali *et al.* (2019) reported a more evident stimulation of the expression of genes involved in controlling muscle metabolism when n-3 dietary PUFA are administered in association with polyphenols-enriched diet.

Pigs' gender affected the total MUFA content, higher in barrows than in gilts (47.11 vs. 45.29; $P < 0.05$), specifically 18:1 n-7 and 18:1 n-9. Moreover, barrows had the lowest amount of PUFA (14.34 vs. 16.52, $P < 0.05$) and total n-6 content (11.80 vs. 13.57; $P < 0.05$), confirming previous findings (Alonso *et al.*, 2009; Lo Fiego *et al.*, 2010; Okrouhlá *et al.*, 2013). No difference was observed for total n-3, n-6:n-3 PUFA ratio, and atherogenic and thrombogenic indices.

Conclusion

Our study confirms that 5% of dietary-extruded linseed in pig diet is a suitable means to increase n-3 PUFA content and reduce n-6:n-3 PUFA ratio in LTL muscle, an important aim from the point of view of human nutrition, without affecting live performances and carcass traits. The technological characteristics of carcass and meat were not impaired by the ameliorated PUFA ratio. The main qualitative characteristics and chemical composition of the muscle were neither affected by linseed feeding nor by inclusion of synthetic or natural

Table 5. Effect of dietary treatments and gender on fatty acid profile (% of total fatty acid) of *Longissimus thoracis et lumborum* muscle.

	Dietary treatments					Gender		
	C	L	LSA	LNA	SEM	Gilts	Barrows	SEM
C10:0 (capric)	0.12	0.11	0.12	0.11	0.005	0.11^{b}	0.12^{a}	0.004
C12:0 (lauric)	0.07^{b}	0.08^{a,b}	0.08^{a}	0.08^{a,b}	0.003	0.07^{b}	0.08^{a}	0.002
C14:0 (myristic)	1.19	1.23	1.27	1.28	0.034	1.20^{b}	1.29^{a}	0.025
C16:0 (palmitic)	23.24^{b}	23.41^{a,b}	24.19^{a}	24.00^{a,b}	0.319	23.49	23.93	0.233
C17:0 (heptadecanoic)	0.23	0.21	0.25	0.23	0.018	0.23	0.23	0.012
C18:0 (stearic)	12.66	12.69	12.94	13.11	0.354	12.95	12.75	0.258
C20:0 (eicosanoic)	0.14	0.15	0.14	0.14	0.006	0.14	0.15	0.004
C16:1 (palmitoleic)	3.18	3.07	3.10	2.96	0.124	2.94	3.22	0.090
C17:1 (heptadecenoic)	0.30^{a}	0.24^{b}	0.26^{a,b}	0.28^{a,b}	0.014	0.27	0.26	0.010
C18:1n-7 (vaccenic)	4.15	4.05	4.05	3.91	0.101	3.92^{b}	4.17^{a}	0.074
C18:1n-9 (oleic)	38.88	37.27	39.03	37.48	0.651	37.54^{b}	38.78^{a}	0.475
C20:1 (eicosenoic)	0.62	0.64	0.67	0.66	0.033	0.62	0.68	0.023
C18:2n-6 (linoleic)	9.45	9.70	8.38	9.37	0.514	9.80^{a}	8.65^{b}	0.374
C18:3n-3 (α-linolenic)	0.49^{b}	1.85^{a}	1.62^{a}	1.84^{a}	0.086	1.53	1.37	0.062
C18:3n-6 (γ-linolenic)	0.22^{a}	0.19^{a,b}	0.16^{b}	0.19^{a,b}	0.014	0.20	0.18	0.010
C20:2n-6 (eicosadienoic)	0.23	0.24	0.21	0.24	0.010	0.24^{a}	0.22^{b}	0.007
C20:3n-3 (eicosatrienoic)	0.08^{b}	0.22^{a}	0.20^{a}	0.22^{a}	0.010	0.19	0.17	0.007
C20:4n-6 (arachidonic)	3.54^{a}	2.86^{a,b}	2.03^{c}	2.37^{b,c}	0.252	2.96	2.44	0.184
C20:5n-3 (eicosapentaenoic)	0.14^{c}	0.58^{a}	0.40^{b}	0.48^{a,b}	0.046	0.44	0.36	0.033
C22:4n-6 (docosatetraenoic)	0.53^{a}	0.31^{b}	0.24^{b}	0.28^{b}	0.029	0.37	0.31	0.021
C22:5n-3 (docosapentaenoic)	0.45^{b}	0.80^{a}	0.59^{b,c}	0.69^{a,c}	0.067	0.69	0.57	0.049
C22:6n-3 (docosahexaenoic)	0.09^{ab}	0.10^{a}	0.07^{b}	0.08^{a,b}	0.009	0.10^{a}	0.07^{b}	0.006
Total saturated	37.65	37.88	38.99	38.95	0.648	38.19	38.55	0.472
Total monounsaturated	47.13	45.27	47.11	45.29	0.778	45.29^{b}	47.11^{a}	0.567
Total polyunsaturated	15.22^{ab}	16.85^{a}	13.90^{b}	15.76^{a,b}	0.965	16.52^{a}	14.34^{b}	0.703
Total n-6	13.97^{a}	13.30^{c}	11.02^{b}	12.45^{a,b,c}	0.789	13.57^{a}	11.80^{b}	0.575
Total n-3	1.25^{c}	3.55^{a}	2.88^{b}	3.31^{a,b}	0.198	2.95	2.54	0.145
n-6:n-3 ratio	11.39^{a}	3.73^{b}	3.95^{b}	3.79^{b}	0.142	5.74	5.70	0.103
Atherogenic index AI	0.45	0.46	0.48	0.48	0.012	0.46	0.48	0.009
Thrombogenic index TI	1.05^{a}	0.91^{b}	1.00^{a,b}	0.95^{a,b}	0.035	0.96	0.10	0.026

C: control group; L: experimental group with 5% of extruded linseed; LSA: experimental group with 5% of extruded linseed, 200 ppm vitamin E + 0.21 ppm selenium; LNA: experimental group with 5% of extruded linseed and vegetal extract 5 g/kg of feed (3.00 g of grape skin extract + 2.00 g of oregano extract).
SEM: standard error of mean values.
Atherogenic index, AI = [C12:0 + (4 × C14:0) + C16:0] / [n-6 PUFA + n-3 PUFA + MUFA] (Ulbricht and Southgate, 1991).
Thrombogenic index, TI = [C14:0 + C16:0 + C18:0] / [(0.5 × MUFA) + (0.5 × n-6 PUFA) + (n-3 PUFA / n-6 PUFA)] (Ulbricht and Southgate, 1991).
^{a,b}Different letters in the same line indicate statistically different mean values ($P < 0.05$).

antioxidants. Absence of further improvement with the addition of natural antioxidants may be due to the low quantity used in this study, based on the quantities used in human diets.

Further research should investigate the effects of higher levels of natural antioxidants, added to n-3 PUFA-enriched diets, on pork quality under different storage conditions and cooking methods.

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Changes in physicochemical characteristics, polyphenolics, and amino acids of wax apple cider vinegar during prolonged storage

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PAPER

Abstract

Several quality attributes of wax apple cider vinegar (WACV) were determined every 30 days for six months at ambient temperature. Acetic acid fermentation significantly increased the acetic acid content in WACV. The lightness and yellowness gradually decreased, whereas redness increased during storage. Density, viscosity, and pH of WACV continuously retarded, and total acidity, volatile acidity, electrical conductivity, and total nitrogen content increased during storage. Phenolic and flavonoid contents and antioxidant potentials of WACV were affected by storage. Various amino acids and volatile compounds were observed in WACV during storage. Throughout the storage period, the microbial growth in WACV was considerably low.

Keywords: wax apple cider vinegar, functional properties, storage, quality

Introduction

Fruit processing plays a significant role in controlling the quality loss of numerous fruits and vegetables and generates various unique products with added economic value. Wax apple is a fruit susceptible to damage from handling, physiological, and physicochemical changes, which adversely influence its quality and value (Techakanon and Sirimuangmoon, 2020; Venkatachalam *et al*., 2018). They are one of the unique fruits in peninsular Thailand. Their special characteristics include apple-like crispness, watery sweetness, low pungency, and have a bit of rose-like aroma. They are available in different colors, from green to dark red. Usually, a vibrant red color indicates readiness for consumption. Wax apple fruit has a different names depending on the region; common names include rose apple, water apple, wax jambus, lianwu, chomphu, and jamalac. Furthermore, wax apple contains lots of phytochemicals that can possess potent antioxidant activities with health benefits. They are consumed mainly as whole fruit or in fresh-cut form. To date, no products or alternatives are developed nor under development on wax apples. This study initiated developing a wax apple cider (WAC). Cider is a fermented alcoholic (1–12% alcohol by volume (ABV)) beverage produced by a specific cider yeast. Ciders are popular in Western countries, and their consumption is also increasing in southeast Asia (Wood and Anderson, 2006). WAC possesses low acidity and has improved minerals, increased essential amino acids, phytochemicals, and antioxidants (Techakanon and Venkatachalam, 2021).

Typically, alcoholic beverages are highly resistant against spoilage and most pathogenic microorganisms. The cider is slightly unstable because of its rich sugar and low alcohol content, depending on the fruits and preprocessing conditions. Several traditional and modern techniques have already been used in various ciders to improve their

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quality and characteristics. An alternative approach is to proceed to acetic acid fermentation or conversion of cider into cider vinegar (Lea, 1989). Among the various processed foods, cider vinegar could be a new product suitable for small or large-scale production and a valuable extension to Thailand's food industries.

Cider vinegar is a newly developed product with several health benefits and has extensive culinary applications. Typically, vinegar is the result of failed cider fermentation or poor cider storage conditions and are produced by aerobic fermentation with the acetic acid bacteria, wherein ethanol gets converted into acetic acid. The microorganism is a crucial factor for controlling the fermentation and the product's quality and functional properties (Mas *et al*., 2014). Wax apple cider vinegar (WACV) is obtained by the double fermentation of alcohol and acetic acid. Initially, apple juice is subjected to alcoholic fermentation to produce the required acetic acid (Dabija and Hatnean, 2014). Later, acetic acid fermentation takes place in two steps. In the first step, ethanol is oxidized to acetaldehyde by alcohol dehydrogenase. Then in the second step, acetaldehyde is oxidized to acetic acid by aldehyde dehydrogenase (Raspor and Goranovic, 2008). The reaction is exothermic, thus increasing the temperature in the medium. The acetic acid can be further oxidized to carbon dioxide when the ethanol concentration is limited in the tricarboxylic cycle, an unwanted process in vinegar production (Johnston and Gaas, 2006).

Cider vinegar is an amazingly versatile cooking ingredient. It adds a tangy taste to many drinks and deepens the flavors of various foods. It is important to note that vinegar is a significant ingredient in most condiments. Mayonnaise and tomato ketchup account for more than 10% of the vinegar production in America. Cider vinegar benefits include many external that include soothing sunburns and insect bites, shiny hair, and dandruff treatment. Cider vinegar has functional therapeutic properties, such as antioxidative, antibacterial activity, promoting recovery from exhaustion, and regulating blood pressure and blood glucose (Johnston and Gaas, 2006; Verzelloni *et al*., 2007). With increasing interest in the potential health effects of cider vinegar worldwide, there have been many reports confirming the antioxidative activity of various kinds of cider vinegars (Budak *et al*., 2014). Phenolic compounds play an essential role in vinegar's antioxidative activity (Shimoji *et al*., 2002; Verzelloni *et al*., 2007; Zhao *et al*., 2018). Several fruits, particularly apple, perry, pear, pome, and orange, are used to produce cider and vinegar products (Techakanon and Sirimuangmoon, 2020). However, no prior research is attempted to produce cider vinegar from wax apples. Also, no study about the stability of such products during prolonged storage is available. Therefore, the present study aimed to explore the possibilities of converting the WAC into WACV and to extensively study the stability of various chemical components, including phenolics, amino acids, volatile compounds, and antioxidant properties in WACV under prolonged storage.

Material and methods

Materials and reagents

Fully matured wax apples (*Syzygium agueum Alston* cv. *Taaptimjan*) were purchased from a commercial orchard in Surat Thani province, in southern Thailand. The cider yeast strain (*Saccharomyces bayanus*) was purchased from FERMENTIS (France). *Acetobacter estunensis* for vinegar production was obtained from Thailand Bioresources Research Center, Thailand. Folin-Ciocalteu reagent, 2,4,6-Tri (2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate (ABTS), ethylene diamine tetraacetic acid (EDTA), trichloroacetic acid (TCA), L-ascorbic acid, chlorogenic acid, gallic acid, caffeic acid, ferulic acid, vanillic acid, catechin, quercetin, cyanidin-3-o-glucoside, cyanidin-3-o-rutinoside, and amino acid standard solution were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol, methanol, and acetic acid were purchased of analytical grade from J. T. Baker (Phillipsburg, NJ, USA). Potato dextrose agar and plate count agar were purchased from HiMedia Laboratory (Mumbai, India).

Wax apple juice extraction

Matured wax apples of uniform size, color, total soluble solids (10-14 °brix), and free from any apparent damage or disease were selected for cider production. The procured fruits were taken to the laboratory within eight hours. Fruits were cleaned of dust with tap water and then washed again with distilled water. Later, the fruits were thoroughly juiced using a food processor in a cold environment, roughly strained and fermented to produce cider.

Cider and cider vinegar preparation

Wax apple juice (6 L) was fermented to cider in a fermenter by previously followed method (Venkatachalam *et al*., 2018). Filtered cider samples proceeded to vinegar production. The cider (4.7% alcohol) sample (10 L) was transferred to a sterile fermenter and inoculated with bacterial culture, specifically *Acetobacter estunensis,* in a ratio of 10:1. The fermenter was placed in the dark at ambient temperature and was continuously aerated by an electric aeration pump. The acidification was continued

for 30 days. Every 24 hours, samples were collected and tested for acetic acid content and fermentation efficiency (Figure 1). After fermentation, cider vinegar was filtered to get rid of the slime. Then, the filtered samples were racked in brown bottles for 6 months to study their stability under ambient storage conditions. Every month, samples were measured for various quality criteria.

Quality Analysis

Physicochemical analysis

The color coordinates lightness (L*), redness (a*), and yellowness (b*) were measured for WAC using a HunterLab Colorimeter. Electrical conductivity was measured using an electrical conductivity meter, and the results were expressed in mS/cm^2. The vinegar sample's density was measured using a hydrometer, and the results were expressed in g/cm^3. The sample's viscosity was analyzed using a Brookfield viscometer, and the results were expressed in cP. The pH of the cider vinegar was measured using a pH meter. The fermentation efficiency of cider vinegar during fermentation was measured following the method of Kaur *et al.* (2011). The results are expressed as percentages. The total nitrogen content in cider vinegar was determined by the Kjeldahl method following AOAC (2000). The results are expressed in g/L. The ethanolic and acetic acid contents in cider before acetic acid fermentation and during the fermentation were analyzed using the chromatographic method proposed by Dias *et al.* (2016). The results are expressed as percentages. The cider vinegar's total acidity and volatile acidity were measured using the method of Kaur *et al.* (2011). The results are expressed as percentages.

Phytochemicals, phenolic compounds, and antioxidant activities

Phytochemical analysis

For total phenolic content (Alberti *et al.,* 2014), 100 µL of WACV, 8.4 mL of distilled water, and 500 µL of Folin-Ciocalteu reagent were added to a test tube. Later, 1 mL of sodium carbonate (20%) was added and mixed well. Then, the reaction mixture was incubated at room temperature for 30 minutes and was measured using a spectrophotometer at 720 nm. The absorbance was compared with the calibration curve made using gallic acid (10–100 µg/ mL; the coefficient of determination (R^2) = 0.9970; $P < 0.0001$). The results were expressed as µg gallic acid equivalent (GAE)/mL. For total flavonoid content (Alberti et al., 2014), 250 µL of WACV was placed in the test tube, and 2.72 mL of 30% ethanol and 120 µL of 0.5 mol/L sodium nitrite were added, mixed, and incubated for 5 minutes. Later, 120 µL of 0.3 mol/L aluminum chloride was added to the mixture, followed by 800 µL of 1 mol/L sodium hydroxide, and mixed well. Then the reaction mixture was measured at 510 nm using a spectrophotometer. The absorbance was compared with the calibration curve made using catechin (10–100 µg/ mL; $R^2 = 0.9960$; $P < 0.0001$). The results were expressed as µg catechin equivalent (CE)/mL. For ascorbic acid (AsA; Bavisetty and Venkatachalam, 2021), 1 mL of WACV and 24 mL of 0.4% oxalic acid were mixed in a flask. After that, the reaction mixture was titrated against the 0.04% aqueous sodium dichlorophenolindophenol solution to obtain the first pink shade (endpoint). Then, the ascorbic acid in WACV was calculated based on the method of Kabasakalis *et al.* (2000). The results were expressed as µg AsA/ mL.

Phenolic compound analysis

A sample of WACV (2 mL) was mixed with 4 mL of 100% methanol and vortexed at high speed for 1 minute, incubated at 60°C for 15 minutes, and was centrifuged at 8160 g for 20 minutes at an ambient temperature. Later, the supernatant was collected and filtered through a 0.22 µm nylon syringe filter (Waters, Milford, MA, USA). High-performance liquid chromatography (HPLC) was performed with the collected filtrate to analyze the phenolics (Alberti *et al.*, 2014). The phenolic compounds mainly gallic acid, chlorogenic acid, caffeic acid, ferulic acid, vanillic acid, cyanidin-3-o-glucoside, and cyanidin-3-o-rutinoside; and the flavonols such as catechin and quercetin were identified and quantified in the WACV samples. The standard calibration samples for the listed phenolic compounds in this study were prepared at seven concentrations (10–70 µg/mL; $R^2 \geq 0.998$; $P < 0.0001$). The retention time and ultraviolet spectra of the standards were used to identify and quantify the phenolic compounds in the samples. The results were expressed in µg/mL.

Antioxidant activities

For DPPH radical scavenging assay (Brand-Williams *et al.*, 1995), 100 µL of WACV and 3.9 mL of 60 µmol/L DPPH were mixed well in a test tube, the reaction mixture was incubated for 30 min in the dark at an ambient temperature and was measured at 515 nm using a spectrophotometer. The results were expressed as a percentage of DPPH radical scavenging ability.

For ABTS radical cation scavenging assay, 100 µL of WACV and 100 µL ABTS reagent (as described in Lee *et al.*, 2015) in a 96 well microplate incubated for 6 minutes at room temperature. Later, the sample was measured at 734 nm using a microplate reader. The results were expressed as a percentage of ABTS radical scavenging ability.

For ferric reducing antioxidant potential assay (FRAP), 100 µL of WACV was mixed with 3 mL of FRAP reagent

(as described in Alberti *et al.*, 2014). The reaction mixture was incubated for 20 minutes for a blue-colored complex development and was measured at 593 nm using a spectrophotometer. The absorbance of sample was compared with the calibration curve made for ferrous ion (Fe^{2+};10–100 mmol/mL; $R^2 = 0.0997$; $P < 0.0001$). The results were recorded in mmol Fe^{2+} equivalent ($Fe^{2+}E$) /mL.

For hydroxyl radical scavenging assay (Halliwell *et al.*, 1987), 1 mL of WACV was added to a test tube containing 1 mL of ferrous ammonium sulfate (0.13%– 0.26%) (EDTA), 0.5 mL of 0.018% EDTA, 1 mL of 0.85% dimethyl sulfoxide, and 0.22% ascorbic acid, mixed well, and incubated in a water bath at 90°C for 10 min. Later, 1 mL of ice-cold and 3 mL of Nash reagent wax were added to the reaction mixture and incubated at room temperature for 15 minutes for yellow color development. Finally, the reaction mixture was measured at 412 nm using a spectrophotometer, and the outcomes were expressed as a percentage of hydroxyl radical scavenging ability.

Amino acid profile

About 3 mL sample of WACV was mixed well with 2 mL of 0.25 mmol/L norleucine and centrifuged at 13,000 g for 20 minutes at 4°C. The supernatant was collected and filtered through a nylon syringe filter (0.2 µm, Waters) and was used to identify the amino acids by the chromatographic method proposed by Alberti *et al.* (2016). The amino acids were derivatized using a Waters AccQ Tag™ reagent kit (flask 1: 200 mmol/L borate buffer, pH 8.8; flask 2A: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; and flask 2B: acetonitrile). Then the identification and quantification were accomplished by HPLC using the AccQ Tag reagent kit methodology with a Pico-Tag column (4 mm, 3.9 × 150 mm). The identified amino acids in WACV were quantified and were reported in mg/100 mL.

Analysis of volatile compounds

The volatile compounds in the WACV were extracted and analyzed by gas chromatography-mass spectroscopy (GC-MS; Agilent GC: 6890, with a 7683B autosampler, Agilent Technologies, Palo Alto, CA) using the static headspace method proposed by Pietrowski *et al.* (2012) with some modifications. The capillary column (Phenomenex column with 30 m in length with 25 mm internal diameter and 0.25 µm thick ZBWAX film) was attached directly to the Agilent GC. The analysis conditions were programmed with an initial temperature of 40°C for 5 minutes and then increased to 10°C/min to 300°C with an isothermal state of 10 minutes. The sample injector port temperature was set to 250°C; the transfer line was set to 290°C, splitless. About 1.5 mL sample was injected into GC via an automatic injector with a column flow rate of 1 mL/min. LECO Pegasus 4D mass spectrophotometer was used to record the electronic ionization source at –70 eV. The solvent delay was set to 9 minutes, acquisition rate to 10 spectra/second, the ion source temperature to 250°C, the mass range was 50–1000 amu, detector voltage 1800 V, and the scan time was 1.5 seconds. The data was recorded in total ion chromatogram mode. The Agilent ChemStation software was adopted to Identify the volatile compound name, chemical formula, and % peak area of unknown compounds and were compared with the known data from the GC-MS library (NIST mass spectral library).

Microbial analysis

WACV was examined each week for microbiological counts. Plate count agar (PCA) determined the total counts for aerobic microbes. Triplicate serial dilution in peptone water of each sample bag was prepared, 1 mL from each dilution were aseptically inoculated on a Petri dish with 20 mL of sterilized agar and mixed thoroughly. Upon agar solidification, the Petri dishes were inverted and incubated for 48 h at 30°C. Colonies were counted and are reported as log10 CFU/mL (American Public Health Association, 1978).

Yeast and mold counts were determined using acidified (pH 3.5) potato dextrose agar. Dilutions and samples were prepared as described in the PCA method. The sterilized agar's pH was adjusted to 3.5, with sterilized 10% (W/V) tartaric acid and poured on Petri dishes, mixed thoroughly, inverted, and incubated for two days at 30°C. Later, colonies were counted and reported as log10 CFU/mL (Beever and Bollard, 1970). *Escherichia coli* (*E. coli*) was determined using Petrifilm *E. coli* Count Plate. Onto the center of the bottom film, 1 mL of sample was added by lifting the top film and was slowly rolled down onto the sample to prevent air bubble entrapment. The plate was left undisturbed for one minute to permit solidification of the gel and then incubated at 37°C for two days. After incubation, the colonies were counted and reported as log10 CFU/mL (AOAC, 2002).

Statistical analysis

All the experiments were carried out in triplicates. The data were presented as the mean and standard error of the mean (SEM), one-way analysis of variance studied the differences during the storage. Duncan's multiple range test identified the significant differences between the means at $P < 0.05$. SPSS v6 for Windows (SPSS, Chicago, IL, USA) was used to run all the statistical analyses.

Results and discussion

Acetic acid production and fermentation efficiency

WAC was studied for acetic acid production to understand the acetic acid bacteria's efficiency and the cider medium's suitability for acetic acid production for up to 29 days (Figure 1). The fermentation was ceased at 29 days, as no significant changes in acetic acid levels were noted after 20 days. During the continuous acetic acid fermentation of WAC, a gradual increase in acetic acid content was noted. In the initial stages, a gradual increase in the production was recorded on day 11 of the fermentation, an increase in the pace of acetic acid production was noted until day 21. Later it was found to be almost constant till day 29.

Concerning acetic acid production, the ethanol content in WAC gradually decreased during the formation of acetic acid in the WAC. A similar finding was reported by Štornik *et al*. (2016) in a study of apple cider vinegar production. Kocher *et al*. (2006) reported that the rapid conversion of ethanol into acetic acid by acetic acid bacteria is facilitated by aeration, stirring, and heating. At the end of the fermentation and before bottling for storage and/or maturation for six months, WACV had about 2.3% acetic acid. On the other hand, the ethanol content decreased from 4.8% to 2.1% as it was used up by the bacteria to produce acetic acid during the fermentation.

The fermentation efficiency (FE) tended to increase with fermentation time (Figure 1). Though a continuous boost in FE was observed during the fermentation, the level of acetic acid was stable after 21 days because of the nature of the acetic acid-producing bacteria. Joshi *et al*. (2016) reported that some acetic acid-producing bacterial strains could over-oxidize acetic acid into carbon-di-oxide and water, which could halt the acetic acid production in the WAC during the fermentation. Moruno *et al*. (1993) reported three main factors that could adversely affect the production of acetic acid during fermentation: (1) the genetics of the yeast strain, (2) the presence of polyphenols, especially catechin and anthocyanins, and (3) the presence of unsaturated fatty acids.

Physicochemical properties

WACV was bright yellowish. During the vinegar-making process, the usual pinkish-yellow WAC wholly turned yellow (Figure 2A). Furthermore, the color changes were consistent during the prolonged storage of WACV (the yellowness increased during storage). On the other hand, the lightness representing luminosity and redness, with negative values representing the darkness, tended to decrease throughout the storage period without many fluctuations in the results. The vinegar samples' color changes could be caused by maturation, with the degradation of anthocyanin residues in WACV and potential effects from acidification. Mas *et al*. (2014) reported that polyphenolic compounds are responsible for the color and astringency of vinegar. Tarazona-Diaz and Aguayo (2013) observed the reduced redness in the fruit juice during acidification, which agreed with the present study as the pH of WACV increased during storage (Figure 2C). The density decreased steadily, along with viscosity

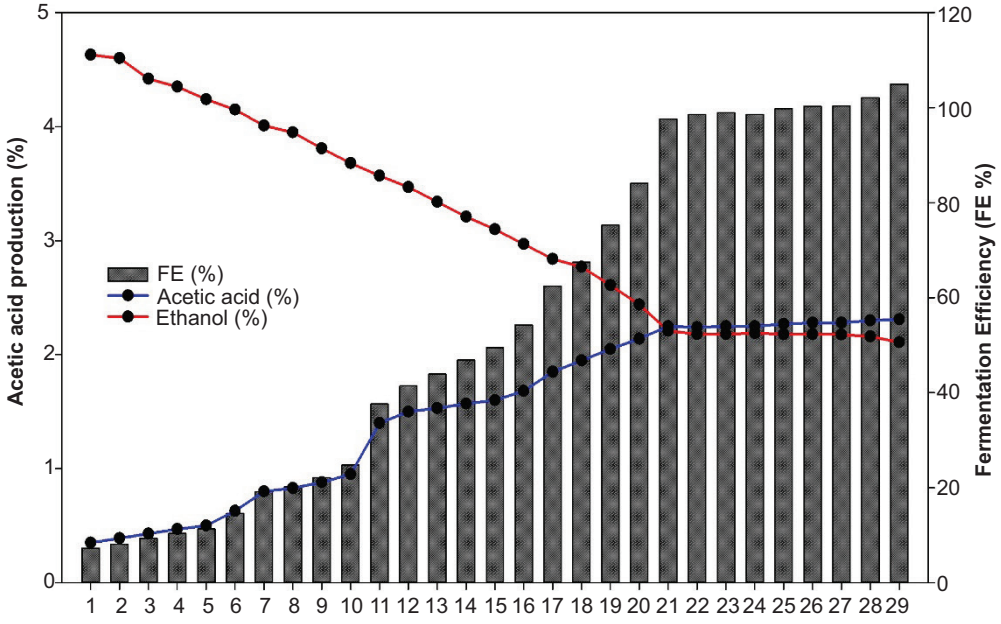

Figure 1. Acetic acid production and fermentation efficiency of wax apple cider.

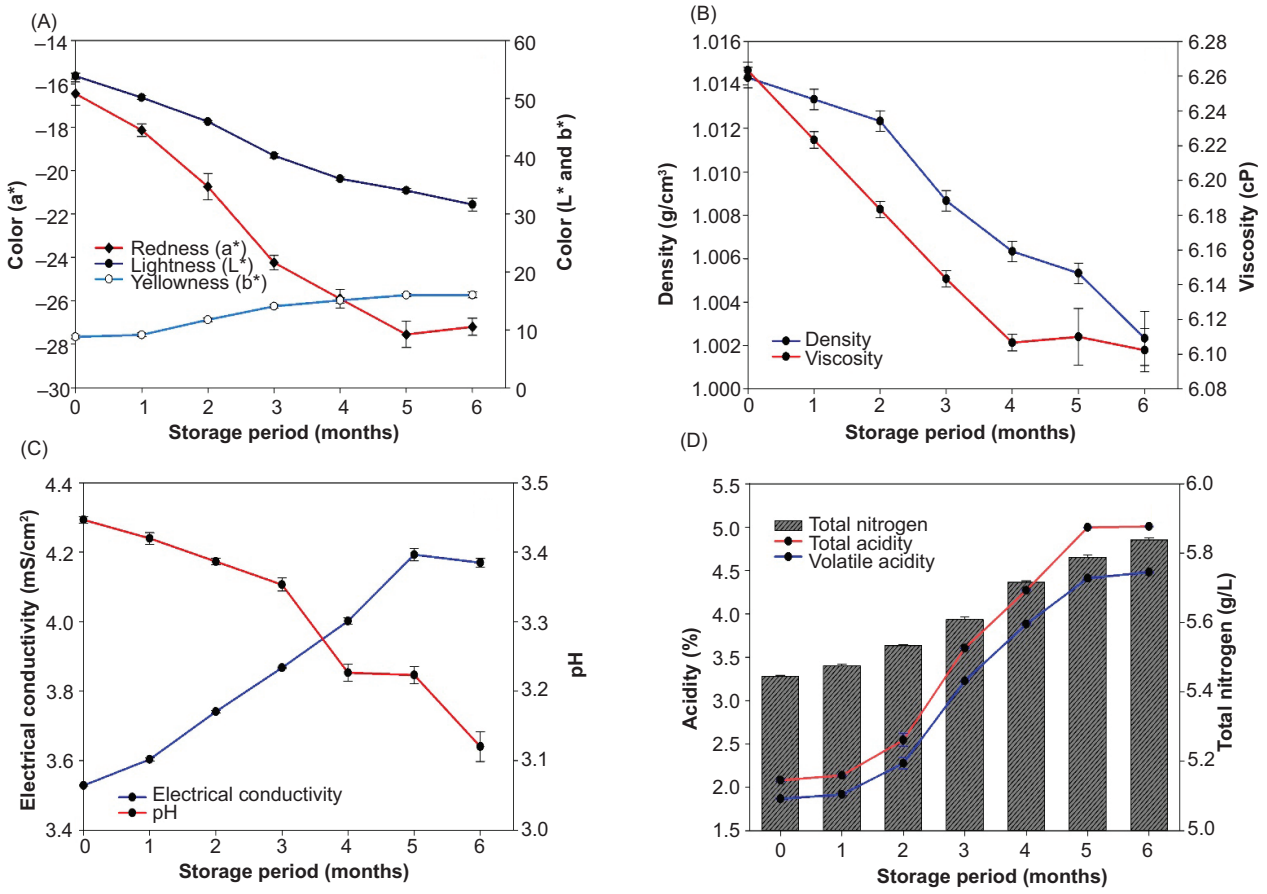

Figure 2. Physiochemical qualities of wax apple cider vinegar during the storage period.

throughout the storage of WACV (Figure 2B). The vinegar density is linked to acetic acid density directly in case it has been distilled.

The present study shows that density decreased with storage time, indicating that organic and inorganic substances in WACV could lose their binding potential and sediment at the bottom of the bottle, consequently affecting density and viscosity. The electrical conductivity of WACV (Figure 2C) shows a linear pattern with storage time. During the study period, it increased from 3.58 to 4.11 mS/cm^2. At the end of the study, the conductivity of the samples was slightly lowered. The pH of WACV steadily changed to acidic during prolonged storage under ambient temperature (Figure 2C). The pH was acidic from the beginning (3.44) and gradually decreased to 3.22 at the end of storage. A decrease in pH is the direct influence of organic acid's presence in the samples. In this study, a continuous increase of acid content (total acidity and volatile acidity) in WACV during the storage was observed (Figure 2D), which agreed with the study of Vithlani and Patel (2010). Although there is a significant change in the pH and organic acid level in the WACV, it still stayed in the cider vinegar range recommended by the codex (Ho *et al*., 2017). The total acidity was slightly higher in WACV than in volatile acidity. The increased acidity and decreased pH in the samples indicate that prolonged storage tended to increase the acidification of WACV. Typically, the volatile acidity represents the odorous fatty acids in vinegar, mainly acetic acid. Chidi *et al.* (2018) and Jackson (2008) reported that the acetic acid level in vinegar increase during aging. Furthermore, the total nitrogen content in the WACV showed a steady increase during storage (Figure 2D).

Phenolic contents and antioxidant activities

Phytochemicals of WACV are shown in Figure 3(A). Among the various phytochemicals in the WACV, the total phenolic content (TPC) was the most abundant, followed by total flavonoids (TFC) and AsA. Prolonged storage significantly affected the phytochemicals in the samples. TPC and TFC contents steadily increased in WACV during prolonged storage, whereas AsA steadily decreased. Various phenolic acids and flavonoids in WACV were quantified, and the results are presented in Figure 3(B). Phenolic acids such as gallic

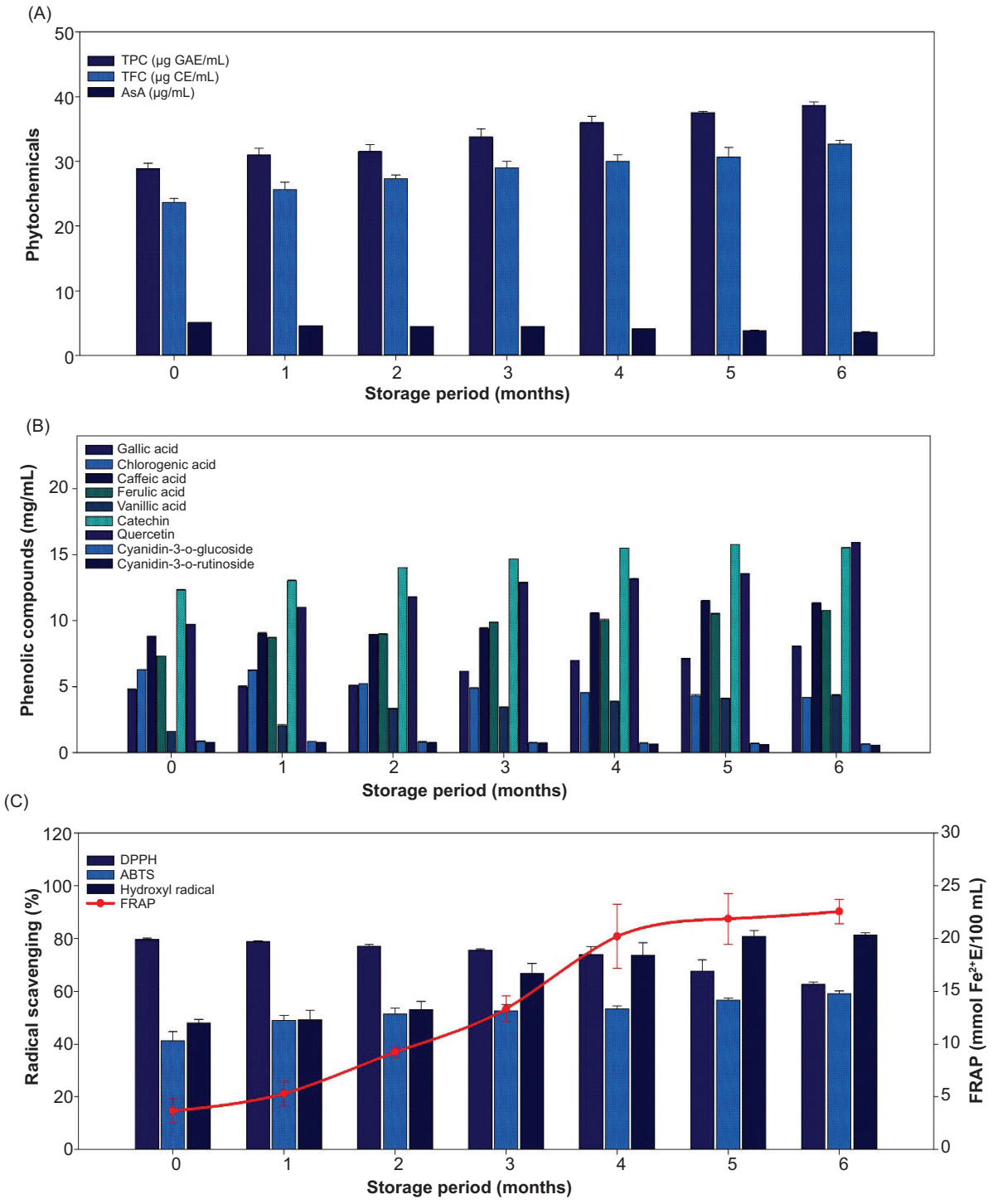

Figure 3. Phytochemicals, phenolic compounds, and antioxidant activities of wax apple cider vinegar during the storage period.

acid, chlorogenic acid, caffeic acid, ferulic acid, and vanillic acid were observed in WACV. Among the various phenolics, caffeic acid, ferulic acid, and gallic acid were the highest in WACV ($P < 0.05$). Chlorogenic acid tended to decrease throughout the storage period. Vanillic acid gradually increased in WACV until four months and was constant later. However, flavonoids such as catechin and quercetin concentrations highest among the various phenolic compounds. During storage, catechin and quercetin steadily increased in WACA, whereas the lesser components cyanidin-3-o-glucoside and cyanidin-3-o-rutinosidecontinuously decreased.

Hornedo-Ortega *et al*. (2017) found that anthocyanin content in the vinegar was more susceptible than in the cider.

Anthocyanin (91%) continuously decreased in the acetic acid fermentation versus alcoholic fermentation (19%). Figure 3(C) presents the radical scavenging activity of WACV during the prolonged storage period. Among the various scavenging activities, DPPH activity in WACV was higher at the beginning of the storage. For the first four months of storage, minimal changes in the scavenging activity of WACV was noted. When the storage reached between 5 and 6 months, the DPPH activity significantly decreased. Instead, hydroxyl and ABTS radical scavenging activities gradually increased throughout the storage. WACV showed a higher potential of scavenging hydroxyl radicals than ABTS radicals. Davies *et al*. (2017) have found that the AsA content was the primary contributor of the DPPH activity in vinegar. A reduction of AsA in the vinegar during storage was probably the cause of chemical changes by the bio-oxidative process. Furthermore, the ABTS activity in the vinegar could be influenced by the synergic effect of phenolics, ascorbic acids, and some amino acids (Campodonico *et al*., 1998). Overall, all types of radical scavenging activities tested in WACV were maintained for at least 40% of the initial level during the storage. However, the FRAP of WACV was minimal and steadily increased throughout the storage ($P < 0.05$). FRAP activity is strongly correlated with the polyphenolics present in a food product, especially in beverages, which agreed with the finding of Schlesier *et al*. (2002).

Amino acid profile

Table 1 shows the amino acid content in WACV during the prolonged storage period. About 18 amino acids were observed in WACV during storage. The storage period significantly affected the levels of amino acids in WACV. Glutamic acid, hydroxyproline, histidine, lysine, methionine, proline, serine, threonine, tyrosine, valine, and glutamine levels increased in WACV during the storage. However, alanine, arginine, aspartic acid, glycine, isoleucine, leucine, and phenylalanine levels decreased. Alanine, histidine, isoleucine, leucine, and valine were found in very minimal contents in WACV. Acetic acid bacteria could metabolize the amino acids from cider vinegar, particularly alanine, arginine, glycine, and leucine (Valero *et al*., 2005; Wang *et al*., 2015). The storage period could limit the suitable conditions for the bacteria to survive and reproduce. Hence, it catalyzed the available resources from the vinegar to sustain. Arginine, lysine, methionine, proline, threonine, and glutamine were the primary amino acids found in the WACV. The other listed amino acids in Table 1 were found at moderate levels. In our previous study (Venkatachalam *et al*., 2018), we observed 23 amino acids in the WAC.

The present study shows that acetic acid fermentation significantly influenced the amino acid contents. Cysteine, hydroxylysine, tryptophan, asparagine, and glutamine, were not detected in the WACV. Furthermore, Ardö (2006) reported that the aminotransferase enzyme in vinegar could catabolize amino acids (aspartic acid, isoleucine, leucine, and phenylalanine) for flavor formation (buttery, malty, fruity, sweaty, floral, chemical, and fecal).

Volatile profile

The volatile profile of WACV during prolonged storage is presented in Table 2. Normally, volatile compounds of fermented beverages are by-products of the catabolic processing of amino acids by microorganisms. The volatile profile of WACV was significantly influenced by acetic acid fermentation and by prolonged storage. About 31 volatile flavor compounds were observed in WACV. The present study showed that cider vinegar exhibited complex flavors, including strawberry, apple, raspberry, vinegar, acetic acid, fruity, wine, plastic, sweet, floral, acidic, feet, gruyere cheese, fusty, ripened cheese, rancid, rose, sour, spicy, potato, burnt, floral, honey, wax, caramel, and clover, that agreed with the research of Charles *et al*., 2000. Most volatile compounds in WACV gradually decreased with storage time. Among the different volatile compounds, acid, and fatty acid-related volatile compounds were predominant, followed by esters, ketones, alkanes, phenol, and benzothiazole. Ethyl acetate and acetoin were the major volatile compounds found in the WACV, which agreed on the studies of Ubeda *et al*. (2011) and Valero *et al*. (2005) reporting that acetoin is the predominant volatile compound present in cider vinegar and tends to decrease as the vinegar ages. The other volatiles were minimal.

Volatiles that increased during storage included boranemethyl sulfide, ethyl acetate, 2, 3-butanedione, isoamyl alcohol, 3-acetoxy-2-butanone, dimethyl sulfone, butanoic acid, 2-propenoic acid, benzyl alcohol, 2-ethyl hexanoic acid, benzothiazole, phenol, 2-ethyl heptanoic acid, octanoic acid, nonanoic acid, and decanoic acid. However, 2,4,5-trimethyl-1,3-dioxolane, acetoin, 2-hydroxyethyl propanoate, 1-phenylpropane-1,2-dione, propanoic acid, 3-methyl butanoic acid, β-phenethyl acetate, hexanoic acid, 2-isopropyl-2,3-dimethylbutanoic acid, 2-ethyl-2,5-dimethylhexanoic acid, (+)-curdione and 4-ethyl-phenol decreased in WACV during the storage. Furthermore, during the storage study, some volatile compounds such as (3,4-diphenylisothiazol-5-yl)-phenylmethanone and 2-methylpropylmethyl ether

Table 1. Amino acid contents in wax apple cider vinegar during prolonged storage.

Amino acid (mg/100 mL)	Storage period (months)						
	0	1	2	3	4	5	6
Alanine	6.78 ± 0.56^{a}	6.48 ± 0.11^{a}	4.56 ± 0.74^{b}	4.01 ± 0.05^{bc}	3.25 ± 0.12^{c}	3.01 ± 0.19^{c}	1.47 ± 0.28^{d}
Arginine	779.05 ± 5.30^{a}	725.14 ± 3.51^{b}	701.29 ± 4.11^{c}	689.21 ± 0.56^{d}	678.17 ± 3.11^{e}	662.41 ± 1.27^{f}	654.39 ± 0.99^{g}
Aspartic acid	44.89 ± 0.80^{a}	43.56 ± 0.73^{ab}	42.15 ± 0.27^{b}	40.19 ± 0.69^{c}	39.87 ± 1.27^{c}	36.54 ± 0.59^{d}	34.44 ± 1.75^{e}
Glutamic acid	71.89 ± 0.43^{f}	77.89 ± 0.21^{e}	81.45 ± 0.67^{d}	83.56 ± 0.59^{cd}	84.59 ± 1.51^{c}	87.15 ± 1.21^{b}	90.16 ± 0.78^{a}
Glycine	37.89 ± 2.64^{a}	36.51 ± 1.41^{ab}	35.44 ± 2.41^{b}	35.01 ± 1.17^{b}	34.45 ± 0.89^{b}	31.87 ± 1.66^{c}	30.99 ± 0.92^{d}
Histidine	8.94 ± 1.11^{b}	9.01 ± 0.48^{b}	9.22 ± 0.95^{b}	10.41 ± 0.27^{ab}	10.78 ± 0.67^{ab}	11.01 ± 1.66^{a}	11.50 ± 1.10^{a}
Hydroxy proline	12.41 ± 0.59^{d}	13.56 ± 0.44^{c}	14.78 ± 0.62^{bc}	12.46 ± 0.79^{d}	13.89 ± 0.75^{c}	15.66 ± 0.30^{b}	16.74 ± 0.89^{a}
Isoleucine	2.77 ± 0.50^{a}	2.01 ± 0.24^{b}	1.45 ± 0.19^{c}	1.25 ± 0.12^{cd}	1.05 ± 0.14^{d}	0.89 ± 0.06^{e}	0.77 ± 0.09^{e}
Leucine	8.88 ± 0.80^{a}	8.01 ± 0.53^{ab}	7.55 ± 0.85^{b}	7.17 ± 0.82^{c}	6.89 ± 1.20^{c}	6.04 ± 0.24^{cd}	5.89 ± 0.64^{d}
Lysine	215.89 ± 18.00^{g}	235.11 ± 13.00^{f}	241.56 ± 4.00^{e}	256.78 ± 19.00^{d}	288.71 ± 7.80^{c}	301.41 ± 3.74^{b}	317.84 ± 4.56^{a}
Methionine	2561.82 ± 88.00^{g}	2571.88 ± 61.00^{f}	2586.19 ± 90.00^{e}	2601.82 ± 56.00^{d}	2657.44 ± 32.00^{c}	2715.53 ± 17.00^{b}	2723.51 ± 48.00^{a}
Phenylalanine	29.18 ± 1.00^{a}	28.56 ± 0.40^{a}	27.15 ± 2.00^{b}	24.56 ± 0.86^{c}	22.14 ± 0.44^{d}	20.15 ± 0.69^{e}	18.95 ± 0.17^{f}
Proline	288.79 ± 12.00^{g}	295.78 ± 15.00^{f}	312.41 ± 3.40^{e}	333.45 ± 7.00^{d}	345.87 ± 3.00^{c}	351.51 ± 8.50^{b}	359.87 ± 7.12^{a}
Serine	55.48 ± 8.00^{g}	57.89 ± 7.00^{f}	58.91 ± 3.60^{e}	62.15 ± 0.78^{d}	64.52 ± 1.00^{c}	67.14 ± 0.19^{b}	68.11 ± 0.91^{a}
Threonine	189.78±9.00^{g}	217.11 ± 8.40^{f}	234.56 ± 38.00^{e}	247.81 ± 14.00^{d}	251.41 ± 7.89^{c}	255.87 ± 6.41^{b}	257.49 ± 9.19^{a}
Tyrosine	10.41 ± 0.50^{d}	12.14 ± 0.90^{c}	13.21 ± 0.90^{bc}	13.56 ± 0.20^{b}	14.01 ± 0.73^{ab}	14.45 ± 0.14^{ab}	14.98 ± 0.56^{a}
Valine	1.67 ± 0.09^{e}	2.07 ± 0.06^{d}	2.45 ± 0.50^{cd}	2.78 ± 0.17^{c}	3.05 ± 0.05^{b}	3.56 ± 0.87^{a}	3.78 ± 0.51^{a}
Glutamine	1114.11 ± 94.78^{g}	1127.56 ± 27.39^{f}	1156.15 ± 61.18^{e}	1174.21 ± 24.12^{d}	1191.14 ± 17.10^{c}	1201.45 ± 2.77^{b}	1224.51 ± 17.31^{a}

#Date represented as mean ± standard deviation from triplicates. The different alphabet in the column indicates a significant difference.

Table 2. The volatile profile of wax apple cider vinegar during prolonged storage.

Volatile Compound	RT	Peak area % Storage period (months)						
		0	1	2	3	4	5	6
Borane-methyl sulfide	3.0629	0.47 ± 0.01^{e}	1.16 ± 0.08^{d}	1.31 ± 0.05^{c}	1.51 ± 0.01^{bc}	1.74 ± 0.04^{b}	1.78 ± 0.01^{b}	2.04 ± 0.05^{a}
Ethyl acetate	3.7655	3.38 ± 0.01^{d}	3.69 ± 0.05^{c}	3.84 ± 0.07^{bc}	4.00 ± 0.03^{b}	4.19 ± 0.00^{a}	4.19 ± 0.00^{a}	4.21 ± 0.00^{a}
2,4,5-Trimethyl-1,3-dioxolane	4.6020	0.69 ± 0.01^{a}	0.68 ± 0.07^{a}	0.61 ± 0.01^{a}	0.58 ± 0.04^{ab}	0.54 ± 0.01^{ab}	0.54 ± 0.03^{ab}	0.51 ± 0.07^{b}
2,3-Butanedione	5.1506	1.61 ± 0.02^{d}	1.66 ± 0.04^{d}	2.27 + 0.06^{c}	2.51 ± 0.04^{b}	2.57 ± 0.01^{b}	2.55 ± 0.03^{b}	2.79 ± 0.07^{a}
Isoamylalcohol	6.7146	0.61 ± 0.01^{c}	0.68 ± 0.08^{c}	0.74 ± 0.01^{b}	0.77 ± 0.01^{b}	0.73 ± 0.01^{b}	0.77 ± 0.06^{b}	0.98 ± 0.11^{a}
Acetoin	15.234	8.86 ± 0.07^{a}	8.56 ± 0.17^{ab}	8.33 ± 0.22^{b}	7.94 ± 0.10^{c}	7.94 ± 0.55^{c}	8.01 ± 0.11^{c}	7.99 ± 0.07^{c}
2-hydroxyethyl propanoate	17.572	0.76 ± 0.07^{a}	0.64 ± 0.04^{b}	0.55 ± 0.10^{c}	0.52 ± 0.08^{c}	0.47 ± 0.03^{cd}	0.43 ± 0.02^{cd}	0.41 ± 0.04^{d}
3-acetoxy-2-Butanone	18.870	0.25 ± 0.05^{b}	0.26 ± 0.05^{b}	0.30 ± 0.01^{a}	0.31 ± 0.04^{a}	0.31 ± 0.01^{a}	0.31 ± 0.09^{a}	0.32 ± 0.01^{a}
1-phenylpropane-1,2-dione	19.666	0.28 ± 0.01^{a}	0.28 ± 0.03^{a}	0.20 ± 0.00^{b}	0.17 ± 0.01^{c}	0.17 ± 0.00^{c}	0.13 ± 0.01^{d}	0.11 ± 0.01^{d}
Propanoic acid	21.021	0.30 ± 0.03^{b}	0.37 ± 0.01^{a}	0.33 ± 0.02^{ab}	0.33 ± 0.06^{ab}	0.32 ± 0.07^{ab}	0.19 ± 0.00^{c}	0.17 ± 0.02^{c}
(3,4-diphenylisothiazol-5-yl)-phenylmethanone	21.949	0.09 ± 0.00^{b}	0.15 ± 0.01^{a}	0.16 ± 0.02^{a}	ND	ND	ND	ND
2-methyl propanoic acid	22.096	1.17 ± 0.08^{a}	1.18 ± 0.00^{a}	1.15 ± 0.00^{a}	1.10 ± 0.00^{b}	1.05 ± 0.00^{b}	1.07 ± 0.00^{b}	1.05 ± 0.00^{b}
2-methylpropylmethyl ether	22.481	0.09 ± 0.00^{NS}	0.08 ± 0.00^{NS}	0.08 ± 0.00^{NS}	ND	ND	ND	ND
Dimethyl sulfone	22.596	0.12 ± 0.00^{d}	0.23 ± 0.01^{c}	0.24 ± 0.04^{c}	0.32 ± 0.02^{b}	0.35 ± 0.04^{ab}	0.37 ± 0.02^{a}	0.37 ± 0.03^{a}
Butanoic acid	23.265	0.20 ± 0.03^{NS}	0.20 ± 0.01^{NS}	0.20 ± 0.04^{NS}	0.21 ± 0.01^{NS}	0.21 ± 0.01^{NS}	0.22 ± 0.01^{NS}	0.22 ± 0.00^{NS}
2-Propenoic acid	23.389	0.21 ± 0.00d	0.33 ± 0.02c	0.43 ± 0.08b	0.47 ± 0.02b	0.48 ± 0.04b	0.55 ± 0.08a	0.57 ± 0.02a
3-methyl butanoic acid	23.763	4.43 ± 0.07^{a}	4.36 ± 0.08^{ab}	4.22 ± 0.05^{b}	4.12 ± 0.09^{b}	3.97 ± 0.10^{c}	4.03 ± 0.08^{c}	4.01 ± 0.12^{c}
β-Phenethyl acetate	24.369	0.40 ± 0.01^{a}	0.38 ± 0.03^{a}	0.36 ± 0.04^{ab}	0.35 ± 0.07^{ab}	0.33 ± 0.02^{ab}	0.27 ± 0.06^{b}	0.25 ± 0.01^{b}
Hexanoic acid	25.525	1.34 ± 0.01^{a}	1.34 ± 0.00^{a}	1.33 ± 0.00^{a}	1.30 ± 0.00^{ab}	1.30 ± 0.00^{ab}	1.26 ± 0.00^{b}	1.24 ± 0.00^{b}
Benzyl alcohol	26.141	0.07 ± 0.00^{NS}	0.07 ± 0.00^{NS}	0.08 ± 0.00^{NS}	0.08 ± 0.00^{NS}	0.09 ± 0.00^{NS}	0.09 ± 0.00^{NS}	0.10 ± 0.00^{NS}
2-ethyl hexanoic acid	26.917	0.09 ± 0.00^{b}	0.09 ± 0.00^{b}	0.14 ± 0.00^{ab}	0.14 ± 0.00^{ab}	0.14 ± 0.00^{ab}	0.14 ± 0.00^{ab}	0.19 ± 0.00^{a}
Benzothiazole	27.077	0.32 ± 0.01^{b}	0.36 ± 0.04^{ab}	0.36 ± 0.07^{ab}	0.38 ± 0.01^{ab}	0.40 ± 0.04^{ab}	0.43 ± 0.01^{a}	0.45 ± 0.03^{a}
Phenol	27.422	0.34 ± 0.03^{b}	0.38 ± 0.01^{ab}	0.40 ± 0.02^{a}	0.40 ± 0.01^{a}	0.40 ± 0.01^{a}	0.41 ± 0.01^{a}	0.43 ± 0.01^{a}
2-ethyl heptanoic acid	27.590	0.13 ± 0.01^{b}	0.14 ± 0.00^{b}	0.14 ± 0.01^{b}	0.15 ± 0.00^{b}	0.18 ± 0.00^{a}	0.18 ± 0.00^{a}	0.18 ± 0.01^{a}
2-isopropyl-2,3-dimethylbutanoic acid	27.6261	0.19 ± 0.02^{a}	0.19 ± 0.00^{a}	0.18 ± 0.02^{a}	0.16 ± 0.00^{a}	0.14 ± 0.00^{b}	0.14 ± 0.00^{b}	0.14 ± 0.00^{b}
2-ethyl-2,5-dimethylhexanoic acid	27.683	0.18 ± 0.00^{a}	0.17 ± 0.00^{a}	0.16 ± 0.00^{a}	0.14 ± 0.00^{ab}	0.12 ± 0.00^{ab}	0.13 ± 0.00^{ab}	0.10 ± 0.00^{b}
Octanoic acid	27.805	0.13 ± 0.01^{d}	0.77 ± 0.00^{c}	0.97 ± 0.01^{b}	1.04 ± 0.00^{ab}	1.08 ± 0.06^{ab}	1.14 ± 0.09^{a}	1.18 ± 0.02^{a}
(+)-Curdione	27.902	0.37 ± 0.04^{a}	0.37 ± 0.01^{a}	0.37 ± 0.04^{a}	0.36 ± 0.02a	0.34 ± 0.04^{ab}	0.34 ± 0.06^{ab}	0.27 ± 0.07^{b}
Nonanoic acid	28.186	0.11 ± 0.00^{c}	0.13 ± 0.02^{b}	0.13 ± 0.00^{b}	0.13 ± 0.01^{b}	0.13 ± 0.00^{b}	0.14 ± 0.00^{ab}	0.16 ± 0.01^{a}
4-ethylphenol	29.2275	0.22 ± 0.01^{a}	0.22 ± 0.00^{a}	0.21 ± 0.04^{a}	0.20 ± 0.00^{b}	0.20 ± 0.01^{b}	0.20 ± 0.00^{b}	0.16 ± 0.01^{c}
Decanoic acid	30.4926	0.35 ± 0.05^{d}	0.54 ± 0.04^{c}	0.58 ± 0.04^{c}	0.58 ± 0.01^{c}	0.76 ± 0.03^{a}	0.75 ± 0.04^{a}	0.70 ± 0.01^{b}

[#]Data presented as mean ± standard deviation from three replications. The different superscripts in a column indicate significant differences ($P < 0.05$).
ND, not detected; RT, means retention time; NS, nonsignificant difference.

were nondetectable in WACV after two months of storage. Volatile compounds in the vinegar could be influenced by the environmental conditions, light intensity, constituents formed during vinegar production, and aging (Valero *et al*., 2005; Chen *et al*., 2020). Ribéreau-Gayon *et al*. (2006) reported that oxygen plays a key role in the formation of volatile compounds in vinegar. Kang *et al*. (2020) observed that extended storage of fruit cider vinegar significantly decreased the alcoholic, bitterness, and sweetn flavors and significantly increased sourness and astringency flavors.

Microbial growth

Microbial growth in WACV was minimal to absent during the prolonged storage (Table 3). Total plate count showed active microbial growth in the samples at the beginning of the storage period, which could be because of the active stage of the acetic acid-producing bacteria. However, during the prolonged storage, the bacterial growth in WACV decreased because of acidity and the anaerobic conditions. Furthermore, pathogenic bacteria, particularly *E. coli*, were absent in the WACV throughout the storage. Similarly, yeast and mold also did not survive in the WACV. The results showed that WACV potently inhibited microbial growth. Yagnik *et al*. (2018) reported that cider vinegar could possess multiple antimicrobial properties against various microbial species, especially *E. coli, Staphylococcus aureus,* and *Candida albicans*, controlling the microbial growth and suppressing mononuclear cytokine and phagocytic responses. Gomez-Garcia *et al*. (2019) observed various organic acids and their antifungal effects. Their study reported that acetic acid inhibited (45.21%) fungal growth compared with other organic acids. Yang *et al*. (2016) observed that phenolics and flavonoids from vinegar exhibited higher antimicrobial activity, especially against pathogens.

Table 3. Microbial growth in wax apple cider vinegar during prolonged storage.

Storage time (months)	Total plate count (log CFU/mL) *	Yeast and mold (log CFU/mL)	*E. coli* (log CFU/mL)
0	2.4 ± 0.3[a]	ND	ND
1	2.3 ± 0.3[a]	ND	ND
2	1.8 ± 0.0[b]	ND	ND
3	<10[c]	ND	ND
4	<10[c]	ND	ND
5	<10[c]	ND	ND
6	ND	ND	ND

ND, not detected.
[#]Data presented as mean ± standard deviation from three replications. The different superscripts in a column indicate significant differences.

Conclusion

This study was the first to understand the physiochemical characteristics, flavor, and functional properties of wax apple cider vinegar during prolonged storage. Overall, the study found that the storage period or aging of WACV significantly influenced its qualities. Organic acids, polyphenolics, and flavonoids in WACV increased significantly, and consequently, the antioxidant and antimicrobial activities were higher in the WACV. The flavor profile of WACV was significantly influenced by continuous changes in the amino acid contents during storage.

Author contributions

Lekjing S conducted the experiment, performed a literature review, and complied the first draft. Venkatachalam K developed the experimental design, conducted the experiments, reviewed, revised the manuscript, and performed the final revision. All authors agreed to publish this manuscript.

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Conflicts of interest

No potential conflict of interest was reported by the authors.

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