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Grape Pomace as a Promising Antimicrobial Alternative in Feed: A Critical Review

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ABSTRACT: Antimicrobial resistance is among the most urgent global challenges facing sustainable animal production systems. The use of antibiotics as growth promoters and for infectious disease prevention in intensive animal-farming practices has translated into the selection and spread of antimicrobial resistance genes in an unprecedented fashion. Several multiresistant bacterial strains have been isolated from food-producing animals, thus constituting an alarming food-safety issue. Many industrial byproducts with potential antimicrobial properties are currently being investigated to identify empirical and affordable solutions/alternatives that can potentially be used in feed for animals. Grape pomace is among such byproducts that gained the attention as a result of its low cost, abundance, and, most importantly, its bioactive and antibacterial properties. This review discusses the recently reported studies with regard to exploring the use of grape pomace (and its extracts) in animal production to control pathogens, along with the promotion of beneficial bacterial species in the gut to ultimately alleviate antibacterial resistance. The review further summarizes realistic expectations connected with grape pomace usage and lists the still-to-be-addressed concerns about its application in animal agriculture.

KEYWORDS: *grape pomace, extracts, antimicrobial, alternatives, feed*

1. INTRODUCTION

The 2016 United Nation's meeting[1] clearly demonstrated that antimicrobial resistance (AMR) has become among the biggest public health and socio-economic threats around the world. In many societies and countries, developed and developing alike, the appearance of antimicrobial-resistant bacterial strains endangers their physical and social wellbeing through longer illnesses and more deaths.[2,3] The issue of increasing AMR is attributed to the fact that not enough new antibiotics are being developed as a result of the high costs involved in a lengthy approval process coupled with low economical returns for the responsible pharmaceutical companies.[4] These two factors have contributed to a less-than-optimum replacement of older or ineffective antibiotics.

2. AMR IN AGRICULTURE

Among the fundamental factors that contributed to the widespread of antibiotic resistance globally, is the extensive and uncontrolled use in animal farming. The use of many wide-spectrum antibiotics as growth promoters and prophylactic agents has contributed to the rise of antimicrobial-resistant bacterial strains.[5] In conventional production, antibiotics are used in feed to control clinical and subclinical diseases, such as necrotic enteritis (NE) (caused by *Clostridium perfringens*) in broilers, respiratory infections (as a result of pathogens, such as *Mannheimia hemolytica*, *Pasteurella multocida*, and *Histophilus somni*) in beef cattle, and mastitis (involving several pathogenic bacteria, including *Escherichia coli*, *Klebsiella* ssp., and *Staphylococcus* ssp.) in dairy cows, resulting in enhanced performance and economic benefits. For example, NE costs the poultry industry close to $2 billion/year worldwide,[6] and antibiotic-resistant strains of *C. perfringens* from animal origin have been on the continuous rise.[7,8] The increasing AMR in animals and its potential threat to human health led to the ban of using antibiotics as growth promoters in livestock production by the European Union in 2006 and to the restriction of antibiotic use in animals in both Canada and the United States of America. In 2018, the European Parliament approved more restrictions on the use of antimicrobials in healthy livestock. The new legislation, which is expected to become law by 2022, will ban the use of antibiotics that are important in human medicine for animals while prohibiting the use of any antimicrobials in livestock without a prescription (http://www.europarl.europa.eu/news/en/press-room/20181018IPR16526/meps-back-plans-to-halt-spread-of-drug-resistance-from-animals-to-humans). To reduce antibiotic use in Canadian agriculture, farmers nowadays need a prescription to obtain veterinary antibiotics for their livestock under the new federal regulations. This regulated and more responsible use of antibiotics in animal production is intended mainly to preserve antibiotic effectiveness and minimize the development and spread of antimicrobial resistance (https://www.canada.ca/en/public-health/services/antibiotic-antimicrobial-resistance/animals/actions/responsible-use-antimicrobials.html). Accordingly, the Chicken Farmers of Canada (CFC) eliminated the use of category I and II antibiotics, while the use of category III antibiotics will be eliminated completely by the end of 2020 (www.chicken.ca).

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Every year, several million people are affected worldwide by food-borne illnesses caused by pathogens, such as *C. perfringens*, *Campylobacter* ssp., and *Salmonella*. Colonization of farm animals by these pathogens, specifically non-typhoidal *Salmonella* (NTS) serovars, presents a significant food safety dilemma for the poultry industry.[9,10] More importantly, multi-AMR pathogenic bacterial strains have been isolated from both humans and animals making the problem more complicated and deeply worrisome. The rise of antibiotic resistance in bacteria (such as the β-lactam-resistant Gram-negative Enterobacteriaceae and the macrolide−lincosamide−streptogramin B in Gram-positive bacteria) is of particular concern.[11−13] If nothing is done, it has been estimated that AMR could cause more deaths than cancer with a pinnacle global economic damage, similar to that of the 2008 financial crisis, by 2050.[14,15] As a result of the aforementioned reasons, the use of cost-effective strategies in the production of antibiotic-free animals with improved health and productivity will provide the industry with alternative solutions while increasing the public trust in antibiotic use policies as well as decreasing problems associated with AMR pathogens. Despite having many regulatory agencies recognizing the importance of withdrawing and banning antibiotics from use other than clinical applications,[16,17] the narrow profitability margins in addition to the high costs of production within the animal/feed industries are among the top reasons for producers to be reluctant of embracing a complete antibiotic ban in animal feed.[17]

3. ANTIMICROBIAL ACTIVITY OF GRAPE POMACE AND EXTRACTS

Among the best "would be solutions" for AMR is identifying alternative products that are cost-efficient, possess acceptable potency, and originate from natural sources to achieve maximum acceptance by consumers. Wastes of fruit industries (such as pomaces and seeds) have all of the above qualifying factors as a result of their high bioactive (polyphenolic) content. This review will focus solely on grape pomace, the antimicrobial activity of its bioactive compounds, and the relationship between the phenolic compounds (with regard to grape species and cultivars) and the exhibited activity.

In terms of classification, grapes belong to the genus *Vitis* and different species and cultivars/varieties exist. The *Vitis vinifera* species include wine grapes, of which Merlot and Chardonnay are examples of red and white cultivars/varieties, respectively. This species is also used for table grapes and juice production. Concord grapes are popular cultivars/varieties of grapes used for table (fresh eating), juice, and preserves. Concord grapes belong to the *Vitis labrusca* species, while muscadine grapes belong to the *Vitis rotundifolia* species.

Grape byproducts from wine production (composed of the skin, stem, and seed fractions) are key sources of pomace, resulting from the industrial processing of grapes. Converting grapes into juice and jelly also results in the generation of grape pomace. During wine production, approximately 25% of the grape weight ends up as waste,[18] constituting a rich economical yet underutilized source of active phytochemicals. For example, close to 22 000−23 000 tons of grape pomace resulting from wine manufacturing are produced each year in British Columbia and Ontario (Canada) alone.[18−20] A part of these pomaces are composted and reintroduced back into vineyards.

Throughout the years, the antimicrobial activities of grape pomace and extracts and their inhibitory activity on multiple pathogenic microorganisms have been investigated. The reported outcomes indicate varying but promising results with regard to the efficacy and used-dosages as discussed below.

3.1. Inhibition of Bacterial Growth. The antimicrobial activity of grape pomace extracts with and without resistant starch (as a prebiotic supplement) was evaluated most recently. The obtained results showed that a concentration of 1600 ppm of grape pomace extracts coupled with 400 ppm of resistant starch was active against *Streptococcus* spp. growth.[21] Similarly, extracts prepared from grape seeds (rich in proanthocyanidins) showed the ability to rapidly inactivate *Listeria monocytogenes* and *Listeria innocua* through the instant permeabilization and clumping of bacterial cells.[22] Moreover, the synergic interactions between grape pomace extracts and many readily used antibiotics against multiple clinical isolates of *Staphylococcus aureus* and *E. coli* were evaluated using the checkerboard approach.[23] Numerous pure phenolic compounds (identified in the extract by reverse-phase high-performance liquid chromatography) were also included in the above assays. The results showed that certain components of the extract (quercetin, gallic acid, protocatechuic acid, and luteolin) in combination with different classes of antibiotics (β-lactam, quinolone, fluoroquinolone, tetracycline, and amphenicol) synergically inhibited the growth of *S. aureus* and *E. coli*.[23]

In another study, grape-stems extracts were assessed for their antimicrobial activity based on their total phenol, *ortho*-diphenol, and flavonoid contents using disc diffusion assays. All of the polyphenolic extracts (10 μL of 195 mg/mL polyphenolic extracts in 10% dimethyl sulfoxide) induced growth inhibition of selected Gram-positive (*L. monocytogenes*, *S. aureus*, and *Enterococcus faecalis*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria.[24]

Extracts of grape skins obtained from organically or conventionally grown Riesling grapes (*V. vinifera* var. Riesling) were tested using well diffusion assays to assess their activity against *L. monocytogenes*, *S. aureus*, *Enterococcus faecium*, and *E. faecalis* in addition to *Salmonella enterica* serovar Typhimurium and *E. coli*. Both extracts (organic and conventional) showed similar levels of activity against all Gram-positive bacteria, except for *L. monocytogenes*, which was slightly more sensitive to extracts from organic grape skins as a result of their higher quercetin levels. However, both extracts were unable to inhibit the growth of the tested Gram-negative bacteria (*E. coli* and *Salmonella*).[25] Using a Malthus apparatus, the antimicrobial activity of extracts of grape seeds was evaluated.[26] With the tracking of conductance changes (as a response to bacterial metabolites accumulation in the liquid growth medium), the results showed the ability of grape seed extracts at a minimum concentration of 0.261% to inhibit the growth of *L. monocytogenes*.[26] In another study, the extracts of Merlot grape (*V. vinifera* var. Merlot) pomace were able to inhibit *E. coli*, *Morganella morganii*, *P. aeruginosa*, *E. faecalis*, *L. monocytogenes*, methicillin-resistant *S. aureus*, and methicillin-susceptible *S. aureus* at varying concentrations (5−20 mg/mL).[27]

V. rotundifolia (muscadine) pomace was also found effective against some anaerobic digestive pathogens, including *C. perfringens*,[28] the causative agents of NE in chicken. Levels up to 2% of pomace significantly lowered lesion scores in broiler chickens without affecting the final weight of birds. Moreover, grape pomace phenolics included at 5% of feed with hydrolyzing enzymes (carbohydrase enzyme complex and tannase at 500 ppm) displayed a measurable antimicrobial effect against *C. perfringens* while improving the antioxidant capacity of birds.[29] Other studies reported similar outcomes.[30]

Grapes (and their byproducts) also showed varying inhibitory activities against other bacterial species, including but not limited to *Helicobacter pylori*,[31,32] *Enterobacter sakazakii*,[33] *Streptococcus sanguis*,[34] and *Cronobacter sakazakii*.[35] Furthermore, polyphenolic compounds extracted from red wine and grape pomace showed anti-adherence activities against multiple species, including *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus rhamnosus*, *Actinomyces viscosus*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*.[36] Similar results were also reached by Hannig et al.[37]

Overall, many studies have documented the capability of total grape extracts or a particular functional fraction/compound isolated from grapes/pomace/seeds/skins (such as resveratrol for example) in controlling the bacterial growth.[38−40] Table 1 lists more examples of key studies that investigated the antimicrobial activity of grape pomace extracts in recent years.

3.2. Inhibition of Fungal Growth. Several fungal species, including *Aspergillus*, *Penicillium*, and *Fusarium*, are well-known for their secretion of different mycotoxins. It has been reported that aflatoxins produced by *Aspergillus parasiticus* and *Aspergillus flavus* are among the major contaminants of common feed ingredients used in poultry feed[41] and were detected in all broiler rations, including the starter, grower, and finisher rations.[41] Secreted mycotoxins affect liver metabolism in addition to epithelial membrane integrity through the inhibition of protein synthesis in the enterocytes, leading to a significant decrease of nutrient uptake and growth performance and, hence, substantial economic losses to livestock operations/production.

A limited number of studies investigated the inhibitory activity of grape pomace extracts against fungi. A crude phenolic extract obtained from a mixture of Chilean grape varieties (including Cabernet Sauvignon, Carmènere, and Syrah) that was further fractionated using hexane, chloroform, or ethyl acetate was recently tested.[42] Phenolics in the hexane and chloroform fractions showed the highest inhibitory effect against the mycelial growth of the phytopathogenic fungus *Botrytis cinerea*, with a half maximal inhibitory concentration (IC_{50}) value of 40 ppm. Quercetin was found in almost all of the studied fractions.[42] Similar results were obtained and correlated with the total anthocyanin content of grape pomace of different Chilean grape varieties (*V. vinifera* var. Cabernet Sauvignon, Carménère, and Syrah). The major compound found within these active fractions was malvidin-3-*O*-glucoside.[43]

Other work looking at reducing fungal populations in wheat using grape pomace extracts highlighted the ability of such extracts to efficiently inhibit the growth of *Penicillium verrrucosum* but indicated less effectiveness against the *Aspergillus* genera. The same pomace extracts also affected (at varying degrees) the growth of other fungi, including *Rhizopus microsporus*, *Fusarium graminearum*, *Alternaria infectoria*, and *Cladosporium herbarum*.[44] Furthermore, the ability of pomace to inhibit growth of *Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii* in addition to *Candida albicans*, *Candida krusei*, and *Candida parapsilosis* has also been reported.[45−52]

Table 1. Extraction Mediums, Screening Protocols, and Range of Concentrations of Some Key Grape Pomace/Extract Studies

extraction medium	screening method	solvent	control	range of concentrations	harmonized range (mg/mL)	reference
acetone/water/acetic acid (90:9.5:0.5)	disc diffusion assay	methanol	absolute methanol	1−20%	1−200 mg/mL	156
acetone/water/acetic acid (90:9.5:0.5)	agar well diffusion method	methanol	absolute methanol	1−20%	1−200 mg/mL	134
70% methanol	disc diffusion assay	distilled water	distilled water	290−450 μg/disc	14.5−22.5 mg/mL	71
preheated deionized water	shaking Petri dishes	bacterial culture/broth	bacterial culture/broth	98−99.7%	films	157
methanol/ethanol/water (50:25:25%, v/v/v)	broth micro- and macrodilution	tryptic soy broth		62.5−500 μg/mL	0.062.5−0.5 mg/mL	82
methanol/water (50:50, v/v) and acetone/water (70:30, v/v)	agar well diffusion method	DMSO	DMSO	10−182.13 mg/mL	10−182.13 mg/mL	154
1% HCl/methanol (v/v)	disc diffusion assay	DMSO	DMSO	300−3000 μg/mL	0.3−3 mg/mL	23
passing through columns with adsorbent resins that retain polyphenols before elution with 4% NaOH and passing through cationic resins	broth microdilution method	phosphate-buffered saline		3.9−2000 μg/mL	0.0039−2 mg/mL	158
70% acetone/0.1% HCl/29.9% water (v/v/v)	broth macrodilution	tryptic soy and brain heart infusion broths	tryptic soy and brain heart infusion broths	1−15%	10−150 mg/mL	81
acetone/water/acetic acid (90:9.5:0.5) and methanol/water/acetic acid (90:9.5:0.5).	melted nutrient agar	propylene glycol	propylene glycol	250−1500 ppm	0.25−1.5 mg/mL	159
1% HCl/methanol (v/v)	microdilution	methanol		62.5−500 μg/mL	0.062.5−0.5 mg/mL	160
pure CO_2/ethanol (co-solvent)	agar well diffusion method and microdilution	10% DMSO in water	10% DMSO in culture broth	20 mg/mL	20 mg/mL	47

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3.3. Inhibition of Protozoal Growth. As mentioned above, improved growth performance parameters, such as bodyweight gains and feed conversion ratios, are vital indicative parameters for food-producing animal industries. Intestinal diseases (such as coccidiosis) can significantly impair these parameters and negatively impact livestock productivity. Thus, developing alternative control strategies of intestinal diseases is urgently needed for both livestock production and public health interests alike. While coccidiosis is typically induced by protozoa parasites belonging to the genus *Eimeria*, including *Eimeria acervulina*, *Eimeria tenella*, and *Eimeria maxima*,[53] several protozoa within the rumen of domestic and wild ruminants have been described for their involvement in host metabolism and the digestion of plant materials.[54]

A number of studies have documented the positive effect of grape pomace extracts/powders on lowering rumen protozoal populations upon feed supplementation. Incorporating grape pomace powder at 2% of dry matter intake in dairy steer rations significantly decreased protozoal populations in the treated animals in comparison to the control group ($p < 0.05$).[55] Similar outcomes were also reported for swamp buffaloes[56] and sheep.[57] Muscadine (*V. rotundifolia*) pomace was reported lately to enhance the primary resistance of chickens against coccidiosis and NE. At 2% levels incorporated in the diet, birds showed significantly lower lesion scores as a result of *E. acervulina*, *E. maxima*, and *E. tenella* infections after 7 days of challenge. Moreover, the incorporated dietary pomace (at 0.5−2.0% levels) significantly reduced the overall mortality of animals.[28]

The effect of grape pomace polyphenols on regulating the parasite burdens of animals was examined recently through an experimental infection model using the enteric nematode *Ascaris suum* in pigs. Upon the assessment of parasite establishment, the bioactive-compound-supplemented diet significantly increased the numbers of eosinophils induced by *A. suum* within the gastrointestinal tract. A similar increase in gene expression activities related to host defense mechanism(s) within the jejunal mucosa of infected animals was also observed as a result of the supplementation. These increases collectively reflect an overall positive modulation by grape polyphenols of the response of the host's response toward the induced helminth infection.[58]

3.4. Antiviral Activity. A number of viral immunosuppressive infections are known to have detrimental effects on livestock production, often as a result of increased susceptibility to secondary infections. Among these are the infectious bursal disease virus (IBDV, Gumboro disease) affecting 3−6-week-old broiler chickens and the Newcastle disease virus.[59] Both diseases continue to cause important economic losses in poultry farming worldwide.

Among the earliest studies that reported the ability of grape pomace bioactive compounds to inhibit viral spread was the study published by Konowalchuk and Speirs.[60] The study demonstrated that infusions and extracts of table grapes [*V. vinifera* (green seedless, red and blue seedless cultivars) and *V. labrusca* (Concord cultivar)] inactivated poliovirus particles. The responsible agent(s) behind this inactivation were found in the skin of the grape. Recently, antibody titers against Newcastle disease viruses at 42 days were reported to be higher in chickens fed a diet containing grape pomace in comparison to control birds.[61] Similarly, Pinot meunier pomace extracts at a concentration of 1 mg/mL provided protection against influenza A viruses.[62] Likewise, a polyphenol-based grape extract was suggested to reduce total adenovirus type 5 virion numbers and to irreversibly inhibit virus replication.[63] The effect of grape juice pH on its antiviral activity was also investigated using rotavirus as a model enteric virus system. The collected results indicated a pH-dependent loss of viral capsid integrity. Moreover, grape proanthocyanidins displayed the greatest antirotavirus capabilities in suspensions with pH values close to 6.7.[64] The ability of Suosuo grapes (*Vitis vinifer* L.) to control the hepatitis B virus (HBV) was investigated recently by tracking viral antigen production within cellular secretion, HBsAg and HBeAg levels, and quantifying HBV DNA levels released into the culture supernatant. A combination treatment of total triterpene, total flavonoids, and total polysaccharides obtained from the above grapes effectively suppressed the secretion of HBsAg and HBeAg (indicators of virus reproduction and liver damage in the HepG2.2.15 cell line) as well as HBV DNA release.[65] Similar results were reported for the effect of grape seed extracts on the hepatitis C virus (HCV), connecting the ability of pomace to inhibit viruses to its ability to inhibit viral replication in addition to suppressing HCV-elevated cyclooxygenase-2 expression levels.[66] Furthermore, a recent study reported the ability of grape seed proanthocyanidins to significantly inhibit the replication of respiratory syncytial viruses in human airway epithelial cells with doses close to 5−10 μg/mL.[67]

Finally, the potential of grape seed extracts to be used for water disinfection and virus inactivation was explored most recently. The incorporation of grape seed extracts in water led to a 2.5 $\log_{10}$ reduction of the human adenovirus type 5 within 120 min. One additional benefit of using grape extracts in similar disinfections is the minimal observed interference of naturally present organic matter with the noticed antiviral activity of the extracts, unlike other commercial chemical disinfectants available on the market.[68]

4. FACTORS AFFECTING ANTIMICROBIAL ACTIVITY OF GRAPE POMACE AND EXTRACTS

Literature reporting on the antimicrobial activities of grape pomace indicates a tentative correlation between the phenolic content of the pomace and its antimicrobial and antioxidant capacities.[69−71] The notion that factors which influence the overall phenolic content of pomace also influence both its antimicrobial and antioxidant functionalities is becoming widely recognized.

Polyphenols in general are organic compounds characterized by many phenol structures attached to hydroxyl groups and classified into functional groups based on the number of phenol structures and their bonding properties. They are divided into 13 classes,[72] with the major groups being phenolic acids, flavonoids, stilbenes, and lignans.[73] Flavonoids are characterized by two aromatic rings bound by three carbon atoms forming an oxygenated heterocycle and grouped into six classes, including flavonols, flavones, flavanones, isoflavanones, flavanols (also called flavan-3-ols), and anthocyanidins. Similarly, phenolic acids are conventionally grouped into two classes: hydroxybenzoic and hydroxycinnamic acids.[74] Specific factors that influence polyphenol levels in pomace are provided in sections 4.1−4.3.

4.1. Grape Cultivar. Grape pomace is considered a rich source of a variety of polyphenols, with the main polyphenolic compounds being phenolic acids and alcohols, flavan-3-ols, and flavonol.[75] While the composition profile of white grape extracts in general is not significantly different from those of

Table 2. Total Phenolic Content Obtained with Varying Extraction Solvents As Reported in Some Recent Studies

extraction solvent	species	cultivar	total phenolic content	reference
50% ethanol	*V. vinifera*	Cabernet (red)	228.4 mg of GAE/g of extract	161
90% methanol/1% HCl	*V. vinifera*	Aidani (white)	107.12 mg of GAE/g of extract	162
methanol/1% HCl	*V. vinifera*	Cabernet Sauvignon (red)	26.3% gallic acid	23
90% methanol/1% HCl	*V. vinifera*	Asyrtiko (white)	465.3 mg of GAE/g of extract	162
50% methanol/25% ethanol	*Vitis* interspecies hybrid	Baco Noir (red)	51.5 g of GAE/100 g of extract	82
ethyl acetate	*V. vinifera*	Bangalore Blue (red)	27.9% GAE	90
methanol	*V. vinifera*	Bangalore Blue (red)	35.7% GAE	90
water	*V. vinifera*	Bangalore Blue (red)	6.1% GAE	90
50% methanol/25% ethanol	*V. vinifera*	Cabarnet Franc (red)	46.9 g of GAE/100 g of extract	82
80% acetone	*V. vinifera*	Cabernet Franc (red)	153.8 mg of GAE/g of extract	69
methanol/1% HCl	*V. vinifera*	Cabernet Sauvignon (red)	9.61 mg of GAE/g of extract	163
ethanol/1% HCl	*V. vinifera*	Cabernet Sauvignon (red)	168.6 mg of GAE/g of extract	164
80% acetone	hybrid	Chambourcin (red)	92 mg of GAE/g of extract	69
90% acetone/0.5% HAC	*V. vinifera*	Emir (white)	68.77 mg of GAE/g of extract	134
methanol/1% HCl	*V. vinifera*	Frappato (red)	3.75 mg of GAE/g of extract	163
ethanol/1% HCl	*V. vinifera*	Frappato (red)	323.1 mg of GAE/g of extract	164
90% acetone/0.5% HAC	*V. vinifera*	Kaleck Karasi (red)	96.25 mg of GAE/g of extract	134
90% methanol/1% HCl	*V. vinifera*	Mandilaria (red)	207.79 mg of GAE/g of extract	162
70% acetone/0.1% HCl	*V. vinifera*	Merlot (red)	40.98 mg of GAE/g of extract	81
40% ethanol	*V. vinifera*	Merlot (red)	73.588 mg/g of extract	27
50% ethanol	*V. vinifera*	Merlot (red)	254.6 mg of GAE/g of extract	161
methanol/1% HCl	*V. vinifera*	Nerello Cappuccio (red)	45.27 mg of GAE/g of extract	163
methanol/1% HCl	*V. vinifera*	Nerello Mascalese (red)	9.1 mg of GAE/g of extract	163
ethanol/1% HCl	*V. vinifera*	Nerello Mascalese (red)	397.7 mg of GAE/g of extract	164
methanol/1% HCl	*V. vinifera*	Nero d'Avola (red)	28.7 mg of GAE/g of extract	163
ethanol/1% HCl	*V. vinifera*	Nero d'Avola (red)	224.6 mg of GAE/g of extract	164
80% acetone	*V. rotundifolia*	Noble (red)	114 mg of GAE/g of extract	95
not included	*V. rotundifolia*	Noble (red)	34.1 mg of GAE/g of extract	165
50% methanol/25% ethanol	*Vitis* interspecies hybrid	Noiret (red)	41.1 g of GAE/100 g of extract	82
50% ethanol	*V. vinifera*	Petit Verdot (red)	204.9 mg of GAE/g of extract	161
50% acetone	*V. vinifera*	Pinot Meunier (red)	77.5 mg of GAE/g of extract	79
70% acetone/0.1% HCl	*V. vinifera*	Pinot Noir (red)	67.74 mg of GAE/g of extract	81
50% methanol/25% ethanol	*V. vinifera*	Pinot Noir (red)	69.8 g of GAE/100 g of extract	82
50% acetone	*V. vinifera*	Pinot Noir (red)	148.3 mg of GAE/g of extract	79
60% methanol	*V. vinifera*	Reisling (white)	31.27 μmol of GAE/g of extract	25
50% ethanol	*V. vinifera*	Syrah (red)	186.3 mg of GAE/g of extract	161
50% ethanol	*V. vinifera*	Tempranillo (red)	177.1 mg of GAE/g of extract	161
80% ethanol	*V. vinifera*	Tempranillo (red)	69.3 mg of GAE/g of extract	166
50% ethanol	*V. vinifera*	Tintilla (red)	321.6 mg of GAE/g of extract	161
80% ethanol	*V. vinifera*	Touriga Franca (red)	100.1 mg of GAE/g of extract	166
80% ethanol	*V. vinifera*	Touriga Nacional (red)	131.7 mg of GAE/g of extract	166
80% acetone	hybrid	Vidal Blanc (white)	55.5 mg of GAE/g of extract	69
80% acetone	*V. vinifera*	Viognier (white)	99.1 mg of GAE/g of extract	69
90% methanol/1% HCl	*V. vinifera*	Voidomato (red)	376.71 mg of GAE/g of extract	162

red grape cultivars, extracts from white grape pomace typically contain lower concentrations of polyphenols and, particularly, contain little to no anthocyanins, which are responsible for the red pigmentation in grapes.[76] Surprisingly, both Yıldırım et al.[77] and Alonso et al.[78] reported lower overall concentrations of polyphenolic compounds in selected red cultivars compared to white cultivars. Additionally, differences in white and red wine grape pomaces can be attributed to variations in their fruit vinification processes. Red grape seeds and skins are usually fermented, while white grape seeds and skins are not, hence leaving behind higher levels of extractable sugars within the white grape pomace.[79] Despite this, its possible to obtain higher phenolic extract yields from white grape pomace using 80% acetone.[80]

Furthermore, the distribution and concentration of total polyphenolic compounds as well as specific polyphenolic constituents have been found to vary considerably across *V. vinifera* cultivars. Tseng et al.[81] reported high concentrations of total phenolics and flavonols in Pinot Noir pomace extracts, while Merlot pomace extracts showed higher anthocyanin concentrations only. In accordance with the above fact, Pinot Noir extracts also exhibited better antiradical scavenging and antimicrobial activities than Merlot extracts. On the other hand, higher phenolic contents and greater antimicrobial activities in Merlot extracts than Syrah extracts have been reported by Oliveira et al.[47] Thimothe et al.[82] evaluated the polyphenolic concentrations of extracts prepared from the following cultivars: Pinot Noir, Cabernet Franc, Baco Noir, and Noiret. Pinot Noir had the highest levels of phenolics and flavanols but the lowest levels of anthocyanins, while Noiret had the highest levels of anthocyanins. Differences between these cultivars were also observed by tracking specific polyphenolic constituents. No procyanidin B1 was detected in Baco Noir, while of all cultivars, Noiret showed the highest amounts of this compound. Additionally, Noiret was the only cultivar in which no gallic acid was detected. Table 2 compares the total phenolic content obtained from various cultivars and reported in the literature in recent years.

Gene expression profiling of teinturier grape cultivars (red fleshed with red skin berries) in comparison to those of Cabarnet Sauvignon (clear/white fleshed with red skin) cultivars revealed the cellular factors behind the aforementioned variations of the phenolic content in different grape cultivars and indicated that at least 10 metabolic pathways were involved in grape total polyphenol synthesis and catabolism.[83] The authors reported that 13 genes related to the biosynthesis and transport of phenolics were upregulated in red-fleshed berries (with a more than 3.0-fold increase) in comparison to Cabernet Sauvignon. In particular, the expression of genes, such as *MybA1* (associated with anthocyanin accumulation in red flesh), *GST* (associated with transport of anthocyanins, proanthocyanidins, and some other phenolic compounds), and *COMT* (associated with accumulation of ferrulic acid), increased by more than 6.6-fold within the red-fleshed berries. These expression levels correlated with higher measured concentrations of total anthocyanins, phenols, flavonoids, and proanthocyanidins in red fleshed berries (and their wines) compared to those of Cabarnet Sauvignon, resulting in an increased overall antioxidant capacity. Similarly, gene expression analysis of *Vvufgt* (encoding the CFGT enzyme responsible for catalyzing the glycosylation of anthocyanidins) and *VvmybA1* (encoding a MTB-type transcription factor responsible for regulating expression of *Vvufgt* and other structure-related genes during ripening) showed that the Pink Globe cultivar, which usually has significantly lower levels of anthocyanins (particularly monomeric anthocyanins) had significantly lower *VvmybA1* expression levels (measured close to the 50% veraison stage), leading to lower *Vvufgt* expression levels and decreased anthocyanin biosynthesis than its somaclonal variant.[84]

4.2. Environmental Factors. As reflected in Table 2, differences in the total phenolic content can also be observed even within the same cultivar, suggesting that climate, soil conditions, geographical location, cultivation techniques, harvesting dates, and exposure to diseases all affect the composition of wine grapes.[85,86] Kammemer et al.[87] compared the polyphenolic content of extracts of grape pomace obtained from 2001 and 2002 vintages of nine different cultivars. The pomaces were obtained from the same winery and processed in the same manner to rule out any other interfering factors. The anthocyanin content of 2001 vintage extracts ranged from 4745 to 131 868 mg of total anthocyanins/kg of extracts, while the 2002 vintages contained up to 38% less, ranging from 2744 to 50 616 mg of total anthocyanins/kg of extracts. Similarly, differences in the anthocyanin profiles of Syrah pomace of the same vintage from two different locations were observed.[88]

4.3. Extraction Conditions. Extraction conditions influence the polyphenolic content of grape pomace extracts.[62] Solvents, acidity, and dry matter concentrations are important parameters to consider when optimizing polyphenolic yields which ultimately influence antimicrobial applications.

4.3.1. Solvent Type. In general and for products intended for the utilization in the food/feed industry, either water or hydroalcoholic solvents are preferred for the extraction purposes of polyphenols.[89] Multiple studies have analyzed the efficiency of various solvents with respect to the achieved yields of polyphenolics. Methanol has been reported to yield extracts containing ∼1.28 times polyphenols and nearly 6 times higher total phenolic content than similar extracts obtained by either ethyl acetate or water, respectively.[90]

A comparison between the extraction efficiency of 70% ethanol and water found that 70% ethanol yielded higher levels of total phenols, tannins, and anthocyanins than water.[88] Cheng et al.[79] compared the effects of using 50% acetone, 50% methanol, and 50% ethanol as extraction solvents. The 50% methanol resulted in extracting more catechins (and their derivatives) but led to the poorest levels of total phenolics. Both 50% acetone and 50% ethanol yielded larger amounts of flavanols, while 50% acetone generated extracts with the richest levels of phenolic compounds and the highest total phenolic content in general. The efficiency of water, methanol, and ethanol at extracting anthocyanins was compared in another study.[91] Methanol was the best solvent, followed by ethanol, while water was the least efficient. Guerrero et al.[92] found that absolute ethanol resulted in poorer yields of polyphenols than pure water at high-temperature extractions. The effect of increasing ethanol concentrations (from 28.5 to 85.5%) in the extraction solution was also tested.[93] The study outcomes showed that the 57% mixture yielded the highest total polyphenolic content, while 85.5% ethanol yielded the highest levels of anthocyanins. It should be noted that this review has only discussed extractions using conventional organic solvents. The use of different extraction techniques to obtain polyphenols from grape pomace, such as pressurized hot water extraction, supercritical fluid extraction, ultrasound-assisted extraction, and microwave-assisted extraction, and the use of industrial enzymes and/or fermentations to facilitate extractability could be the subject of another review.

4.3.2. Extraction pH. The pH value of used solvents/extraction mixtures also influences the final concentration of obtained polyphenols. In general, acidification increases the levels of anthocyanins in final extracts. In one study, anthocyanin recovery levels increased when hydrochloric acid was paired with ethanol, while citric acid utilization worked optimally when coupled with methanol. In a parallel fashion, acetic acid worked best with water.[91] However, while anthocyanin levels are improved at lower pH levels, the acidification adversely affects other polyphenols. The acidification of extraction solvents (with 0.1% hydrochloric acid, 1% acetic acid, and/or 1% tartaric acid) increased anthocyanin overall yields in one study; however, it decreased the yields of flavanols, flavonoids, proanthocyanidins, and phenols.[93] Similarly, increasing the pH value by the addition of 0.01, 0.02, or 0.04% SO_2 resulted in higher flavanol levels while decreasing all other polyphenols. In addition to considering its influence on yields, sustainability and safety factors may also be taken into account when choosing solvent parameters. Because ethanol is a biosolvent, it may be given preference over other organic solvents, while some countries may only allow for the use of water as an extraction medium.[89,93] Likewise, organic acids may be preferred over the use of hydrochloric acid (corrosive agent).[91]

4.3.3. Drying and Storage Conditions. Conditions during the initial drying of pomace and storage may also affect the

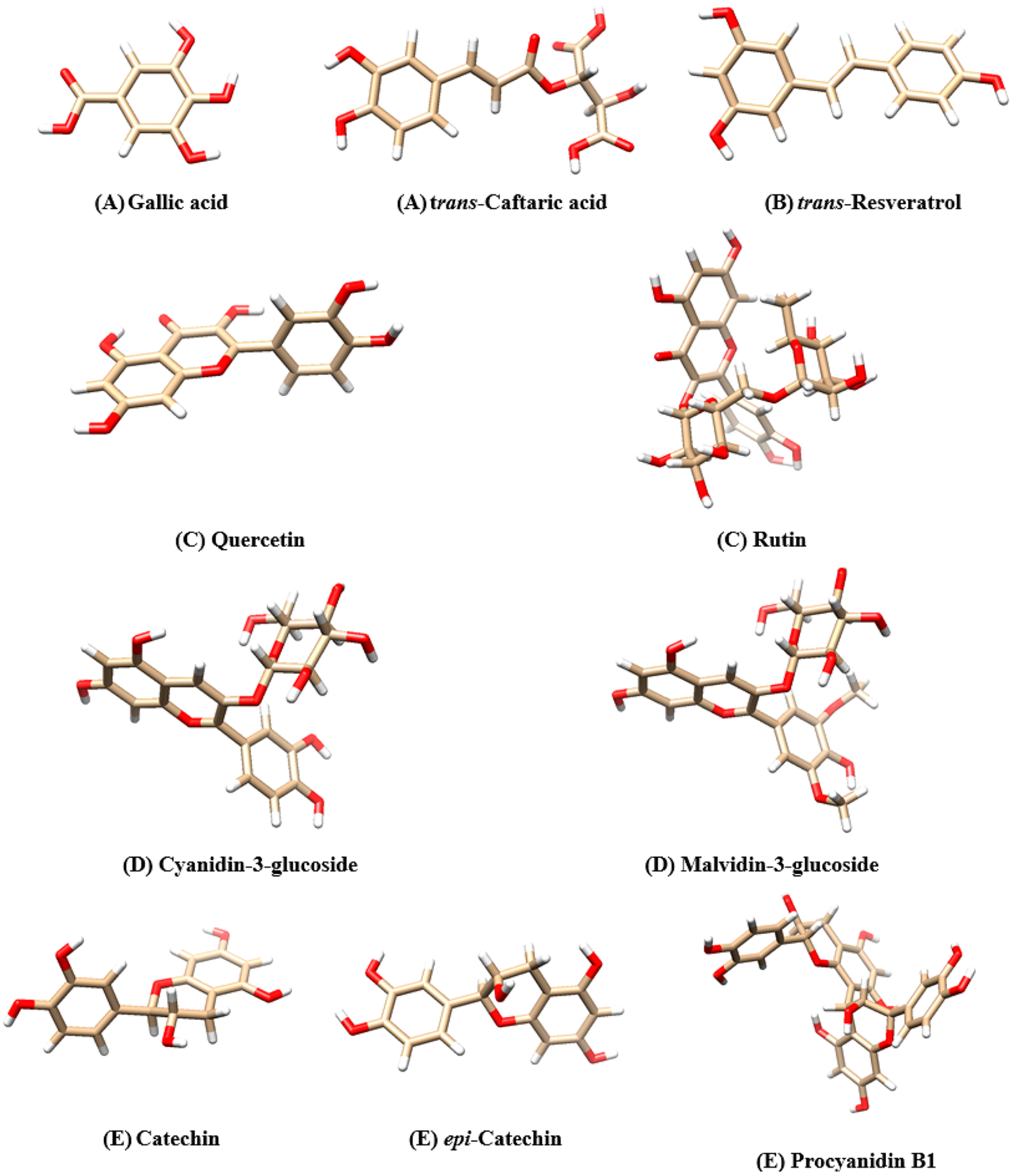

Figure 1. Chemical structure of some active compounds identified in grape pomace and extracts with possible antimicrobial activities: (A) phenolic acids, (B) stilbenes, (C) flavonols, (D) anthocyanins, and (E) flavanols.

stability and concentrations of final polyphenols. Larraui et al.[94] reported that both freeze drying and heat drying at 60 °C did not result in any significant losses of polyphenols, while drying at 100 and 140 °C both resulted in decreasing concentrations of total extractable polyphenols and condensed tannins as well as decreasing the overall antioxidant activity, rendering these conditions of extraction not suitable for commercial uses. Vashisth et al.[95] found that lyophilizing the pomace for 14−16 h retained the best levels of polyphenols in muscandine pomace samples. Vacuum belt drying for 60−90 min at combinations of temperatures varying between 60 and 120 °C retained acceptable overall levels, while air drying at 70−80 °C for 180−240 min significantly caused the degradation of the tested samples. Similarly, Chamorro et al.[96] found that furnace heating and autoclaving at 100 °C both caused a significant degradation of grape seed extracts (procyanidins and catechins alike) as well as inducing browning (although this was not found in the pomace itself likely as a result of the different forms of polyphenols present in pomace in comparison to pure extracts). Tseng et al.[81] showed that 25 °C air drying and freeze drying both produced extracts with higher concentrations of phenolics, anthocyanins, flavonols, and better antiradical scavenging abilities compared to 40 °C vacuum or conventional oven drying. However, the later methods were much more feasible and still yielded sufficient polyphenolic contents and antioxidant capacities to be used in commercial applications. The team also found that 16 weeks of storage at 15 °C significantly decreased anthocyanins and the total phenolic content as well as decreased the antioxidant and antimicrobial activities, although the flavonol contents slightly increased with time.[81]

Acidity during storage also affects the stability of anthocyanins. Fossen et al.[97] found that, under aqueous conditions, anthocyanin cyanidin-3-glucoside was stable for 60 days at pH 1.0−3.1, but at pH 4.0−9.0, it degraded significantly when stored at 10 °C. At 23 °C, it was significantly less stable regardless of the pH value, although more stable at pH 1.0−3.1. The more polymerized anthocyanin, petunidin, was more stable than cyanindin-3-glucoside but followed the same general trend of degradation influenced by storage conditions.

5. SUGGESTED MODES OF ACTION FOR ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES

As mentioned earlier, numerous studies correlate the antimicrobial activity of grape pomace with its antioxidant capacity[69−71] as a result of the fact that many of the grape polyphenols involved in the latter functionality could also be involved in the former functionality. This review briefly mentions the mechanism(s) behind the antioxidant capacity of grape phenolics because our focus is directed rather toward the antimicrobial activity. The reader is referred to some recent in-depth reviews that cover the antioxidant potential of grape pomace/extracts/phenolics more thoroughly.[98−100]

5.1. Antioxidant Activity. The molecular structure of polyphenols make them ideal antioxidants as a result of their abilities to act as electron or hydrogen donors, form stable radical intermediates, and chelate transition metals. The *ortho* 3′,4′-dihydroxy moiety in the B ring and the *meta* 5,7-dihydroxy arrangements in the A ring (present in certain polyphenols) are both important in particular to the above-noted antioxidant activity. Similarly, the 2,3-double bond in combination with the 4-keto group and the 3-hydroxyl group in the C ring and the *o*-diphenolic groups in the 3′,4′-dihydroxy positions in the B ring in addition to the ketol structures (4-keto, 3-hydroxy or 4-keto, and 5-hydroxy in the C ring) of flavonols are all fundamental contributors to the noted ability of chelating transition metals.[101]

In general, there seems to be a strong correlation between the polyphenolic compounds content in final extracts and the resulting antioxidant activity, despite the activity being found to correlate far more strongly with the total phenolic content than with one specific compound.[78] However, certain polyphenolic compounds appear to make a greater contribution to the noticed antioxidant capacity of the extracts. Arnous et al.[102] found strong overall correlation with flavanols, moderate correlation with phenolic compounds, and a minor correlation with anthocyanins. Similarly, Alonso et al.[78] found a strong correlation between the total phenolic content and antioxidant activity, as did Yıldırım et al.[77] In contrast, González-Paramás et al.[103] attempted to correlate the flavanol content in grape extracts with the measured antioxidant activity and found only a weak correlation. While procyanidin B1 is considered as the major contributor;[104,105] gallic acid, catechin, and epicatechin have also been found to contribute significantly[78] to the studied antioxidant capacity. In conclusion, while the total polyphenolic concentration is considered the most influential factor, the specific composition of extracts is considered as pivotal as well. Maier et al.[104] found that flavonoid fractions of grape seed extracts having an even distribution of compounds and more complex overall profiles had better antioxidant activities, hence confirming that a balanced mixture of various phenolic compounds is needed for enhanced functional activities of pomace extracts.

5.2. Antimicrobial Activity. The exact mechanism(s) behind the inhibitory effect of grape pomace extracts/constituents against microorganisms is not yet clear, partially as a result of its chemical complexity and composition (tannins, anthocyanins, catechins, procyanidins, flavonol glycosides, phenolic acids, stilbenes, etc.).[106] Figure 1 shows the chemical structure of 10 possible antimicrobial compounds found in grape pomace extracts, yet other compounds remain to be identified. Furthermore, the distribution of active antimicrobial compounds among pomace fractions is another factor to consider because concentrations vary significantly in grape skin, grape seeds, and mixtures (pomace). For example, (+)-catechin was reported with concentrations ranging between 7.5 and 13 mg/kg in grape skin and between 21 and 270 mg/kg in seeds. Similarly, (−)-epicatechin was reported with a concentration range of 3.6−47.5 mg/kg in skin and 38−223 mg/kg in seeds, while quercetin was reported with a concentration range of 40−1043 mg/kg in skin and 11.7−1009 mg/kg in seeds. In a parallel fashion, myricetin was reported with concentrations of 40−1043 mg/kg in skin and 11.7−1009 mg/kg in seeds, while rutin existed in 3.6−47.5 mg/kg in skin and 38−223 mg/kg in seeds. Other chemicals that vary in concentrations are kaempferol, with a reported range of 3.6−47.5 mg/kg in skin and 38−223 mg/kg in seeds; gallic acid, with a reported range of 3.6−47.5 mg/kg in skin and 38−223 mg/kg in seeds; ellagic acid, with a reported range of 3.6−47.5 mg/kg in skin and 38−223 mg/kg in seeds; syringic acid, with a reported range of 3.6−47.5 mg/kg in skin and 38−223 mg/kg in seeds; caffeic acid, with reported concentrations of 3.6−47.5 mg/kg in skin and 38−223 mg/kg in seeds, and ferulic acid, with reported concentrations of 2 mg/kg in skin and 2 mg/kg in seeds.[107] Compounds that are still under study include caftaric acid, quercetin 3-glucuronide, *trans*-resveratrol, delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, malvidin 3-*O*-glucoside, delphinidin 3-*O*-acetylglucoside, petunidin 3-*O*-acetylglucoside, peonidin 3-*O*-acetylglucoside, malvidin 3-*O*-acetylglucoside, cyanidin 3-*O*-*p*-coumaroylglucoside, petunidin 3-*O*-*p*-coumaroylglucoside, peonidin 3-*O*-*p*-coumaroylglucoside, and malvidin 3-*O*-*p*-coumaroylglucoside.

Nevertheless, the mode of action of grape pomace is becoming more understood as more compounds are identified and purified from grape pomace with promising *in vitro* and *in vivo* antimicrobial activities.[108]

The inhibitory effect of grape phenolics on the activity of many enzymes/proteins (including those from bacterial origin) is well-documented.[109] For example, the influence of grape phenolics on the activity of lysozyme (from egg white) was reported many years ago.[110] Lysozyme, with its muramidase activity, is routinely used in winemaking to control lactic acid bacteria, but this enzyme is generally less active in red grapes than in white grapes possibly as a result of the high content of polyphenols in red cultivars.[111] Other enzymes, including trypsin, were also reported to interact with grape phenolics (especially chlorogenic acid, ferulic acid, gallic acid, quercetin, rutin, and isoquercetin),[112] α-lactalbumin,[113] and soy glycinin.[114] Likewise, grape tannins influence bacterial growth by inhibiting microbial enzymes (peroxidase, pectinases, lactase, cellulases, glycosyltransferase, and xylanases).[115] In general, the non-covalent binding of phenolics to proteins induces changes in their tertiary structure, while the secondary structure remains intact,[114] leading to the precipitation of the involved enzymes.[116]

Grape flavonoids were also suggested to form complexes with proteins through either covalent or hydrogen bonding, affecting microbial adhesions[117] and/or cell envelope transport

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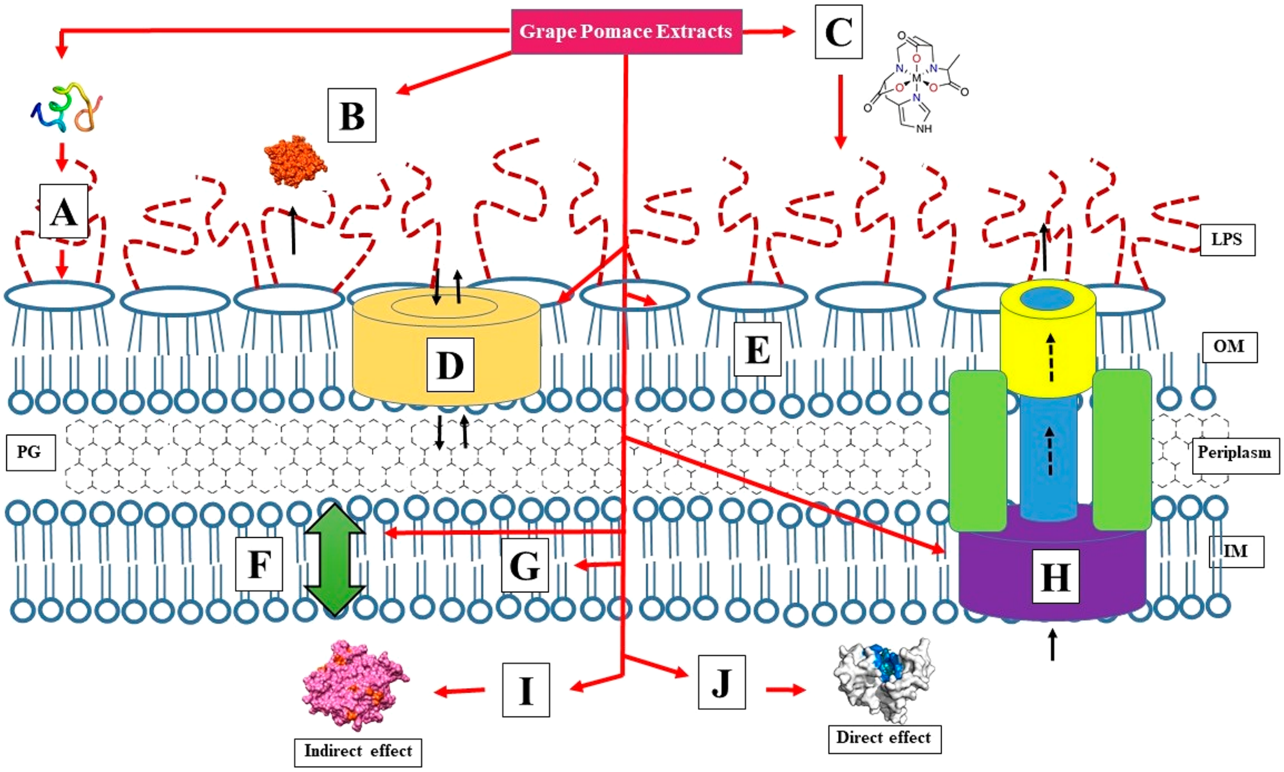

Figure 2. Suggested mechanisms of action of multiple active antimicrobial compounds identified in grape pomace against Gram-positive bacteria (IM, inner membrane; OM, outer membrane; PG, peptidoglycan; and LPS, lipopolysaccharide). Some of these mechanisms act in synergy for maximum antibacterial effects: (A) short oligopeptides that disrupt cellular membranes, (B) inactivation of bacterial exoenzymes, (C) chelating essential metals (iron, etc.)/nutrient deprivation (especially tannins), (D) interference with porin functionalities, (E) permeabilization, rupture, and disintegration of outer membranes, (F) reduction of the pH gradient across the cell membrane and inhibition of proton exchangers, (G) cytoplasmic membrane destabilization, (H) inhibition of multicomponent efflux transporters/multidrug resistance (MDR) pumps (especially flavonoids), (I) hydroxyl radical (OH)/H_2O_2 generation and, hence, inactivating bacterial enzymes/proteins (indirect effect), and (J) inactivating bacterial cytoplasmic proteins/enzymes directly through binding/precipitation.

proteins.[118,119] Klančnik et al.[117] provided evidence about the ability of Pinot Noir grape skin and seed extracts to inhibit *Campylobacter jejuni* cell adhesion to abiotic and biotic surfaces.

Again, although the mechanism of action to describe the overall antimicrobial activity of grape pomace is not fully understood, the implication and magnitude of the above suggested mechanism/phenomenon describing the ability of grape phenolics to destabilize sensitive bacterial enzyme/protein complexes through the above routes deserve to be noted.

Furthermore, the noticed ability of plant/grape polyphenols to inactivate a wide array of bacterial enzymes (rather than a few selected bacterial enzymes) is in fact advantageous because it theoretically minimizes the chances of the bacteria to develop resistance through functional mutations (the upregulation of endogenous bacterial enzymes through a single nucleotide polymorphism within the gene promoter region for example). Moreover, its seems that protein/polyphenol interactions are isoform/variant-dependent. A conceptual model was developed in the past,[120] suggesting that each protein molecule has a fixed number of polyphenol binding sites. This might explain the observed diverse behavior of proteins/enzymes with regard to their polyphenol interactions reported in nature. It also explains how certain proteins/enzymes are more susceptible for the polyphenolic inactivation/precipitation, while others (including laccases, grindamyl pectinase, celluclast, and cellulases[121] used to enhance total phenol recoveries from plant pomaces for example) are less susceptible for such inactivation.

Another mode of action of grape phenolics against bacteria involves their ability to act on bacterial cell walls. Plant phenolics are known to act on cell wall components and/or to inhibit cell wall biosynthesis in Gram-positive bacteria,[122,123] in addition to disintegrating Gram-negative bacteria outer membranes.[124] According to Nohynek and others,[125] the exposure of *Salmonella* cells to phenolics (extracted from cloudberry and raspberry) resulted in an increase in 1-*N*-phenyl-naphthylamine uptake and lipopolysaccharide release, two indicators of cell membrane permeabilization.

A third inhibitory mechanism seems to be connected with condensed tannin capacity to deprive bacteria of necessary nutrients and metals, such as iron[126] essential for bacterial growth.[115] Berry phenolics could chelate and remove divalent cations (necessary for outer membrane stabilization) from Gram-negative bacteria.[127,128]

Furthermore and notwithstanding the established link between the total polar and phenolic compounds[108] in grape with the overall antimicrobial functionality, water-soluble fractions are also believed to exert an inhibitory activity.[33] Malic and tartaric acids were among the compounds involved in the reported antimicrobial activity against selected bacterial species.[33] Moreover, the existence of multiple natural oligopeptides in grapes that hinder bacterial growth was predicted in the past, but no experimental confirmation was presented.[129] Only one oligopeptide (77 amino acids in length) with known antifungal capabilities was reported in the literature.[130] It has been suggested that such oligopeptides act on bacterial cell membranes[131] through their high affinity toward membranes, attributable in part to their amphipathic nature/net positive charges.[132]

Recently, an interesting new mechanism of bacterial inactivation through the pro-oxidative activity of polyphenols was suggested. Accordingly, the oxidation of polyphenols (such as catechins) coupled with the reduction of dissolved oxygen leads to hydrogen peroxide (H_2O_2) molecule generation, which later inhibits bacterial growth.[133] Moreover, the above oxidation step can be exogenously augmented through photoirradiations to generate highly reactive hydroxyl radicals (−OH), hence exerting greater bactericidal activities.[133] Figure 2 summarizes most of the reported modes of action associated with grape pomace against bacteria.

Despite generally shared mechanism(s) of action, the effect of grape pomace extracts varies among bacterial genera and species. Some of the sensitive pathogenic species toward grape pomace extracts that were reported are *E. coli* O157:H7 and *S. aureus*.[134] Interestingly, total polyphenolics tend to be more potent against Gram-positive species in comparison to Gram-negative species. This can possibly be attributed the composition of bacterial cell walls and their higher lipid content in addition to the presence of porins in Gram-negative species.

As for fungal, viral, and protozoal hosts, the inactivation mechanism(s) are not clear and studies are critically needed to address the existing gap of knowledge. A recent study highlighted the ability of resveratrol tetramers as potent inhibitors of hepatitis C virus helicase[135] as a possible route of controlling virus spread. Similarly, condensed tannins have shown the ability to inhibit parasites *in vitro*. Flavan-3-ols and their galloyl derivatives dose-dependently inhibited the development of *Trichostrongylus colubriformis* larvae *in vitro*. Galloyl derivatives of flavan-3-ols were able to at least partially inhibit egg hatching at 100 μg/mL, while a 100% inhibition was achieved at 1000 μg/mL, with epigallocatechin gallate being the most effective derivative.[136] While *in vitro* results have repeatedly shown promising outcomes, the *in vivo* results are not conclusive. The effect of a grape seed condensed tannin extract was modest against *T. colubriformis* infections in sheep, showing an 11% drop in egg count and 18% drop in the number of adult *T. colubriformis*.[137]

However, the incorporation of grape seed proanthocyanidin extracts into feed in a different study showed a significant reduction of the severity of cecal lesions and mortality rates in chickens after induced experimental infections with *Eimeria tenella* while increasing animal body weight gains.[138]

6. EMPIRICAL USE OF GRAPE POMACE IN ANIMAL FEEDING

The earlier research progress (conducted mainly *in vitro*) started most recently to translate into more refined *in vivo* observations in regard to grape pomace usage. In an experimental Sprague−Dawley rat model, grape pomace (up to 20.7%) was shown to not negatively influence the overall growth performance but rather to lower blood triglycerides in addition to decreasing the very low-density lipoproteins (VLDLs) while slightly increasing the high-density lipoprotein (HDL) and low-density lipoprotein (LDL) fractions,[139] reflecting a positive effect on blood lipids/lipoproteins.

In farm animals, whole grape pomace (not extracts) was used lately as a feed additive for weaned piglets.[140] Data from the above study showed a significant increase in the tissue/cellular antioxidant capacity [judged by elevated glutathione (GSH) levels, higher H_2O_2 decomposition activities, and an enhanced overall antioxidant capacity] and decreased oxidative stresses in grape-pomace-fed piglets. Furthermore, this experimental diet was found to enhance the growth of probiotic and lactic acid bacteria while inhibiting the growth of pathogenic bacterial species, including *C. jejuni* and the Enterobacteriacae group in animal gut.[140] The grape pomace treatment in the above studies showed a dual positive effect through either enhancing the antioxidant capacity of animal tissues or inhibiting the growth of pathogens.

The incorporation of grape pomace in lactating ewe rations and its effect on meat quality and fat composition of their suckling lambs were studied most recently. Churra ewes were fed rations containing two levels of grape pomace, while their lambs were nourished exclusively by suckling until they were slaughtered. Dietary grape pomace did not generate any adverse effects on the inspected carcasses in regard to meat quality parameters compared to the control group. Moreover, the grape pomace treatment improved the water-holding capacity of the meat without affecting its fatty acid profile.[141] Similarly, supplementing lamb diets with sun-dried grape pomace was recently investigated. Dohne Merino lambs were fed varying levels of pomace (5−20%) for 42 days, and the growth, carcass, and meat physicochemical quality parameters were tracked. Meat quality traits were not affected negatively by grape pomace inclusion, while the gross profit was influenced by this diet with an optimum inclusion level at 12.2% at the expense of wheat bran middlings and/or oat bran.[142] Matching results were also reported by others, with beneficial effects on the redox status, fecal microbiota, carcass traits, and meat fatty acid composition of the involved lambs.[143−145] Altogether, these studies indicated better meat quality (in addition to the harnessed antimicrobial benefits) when grape pomace was incorporated into animal rations on a regular base.

Reis et al.[146] investigated the addition of grape pomace flour to the diet of laying hens to reduce heat stress and enhance egg quality. Levels spanning the 1−3% range were used for 35 consecutive days. Overall, the grape pomace treatment exerted positive effects on hens' performance and the quality of eggs. More particularly, it enhanced feed intake of heat-stressed chickens and maintained the physiochemical composition of fresh eggs. The total antioxidant capacity against peroxyl radicals (as well as superoxide dismutase and glutathione peroxidase activities) was higher in the serum of hens that received grape pomace flour in comparison to the control group. Comparably, the effect of grape pomace on the growth, apparent total tract digestibility of nutrients, blood profile, and meat quality in broilers was evaluated.[147] Broiler chicks were randomly allotted to dietary treatments spanning 5−10 g of grape pomace/kg of feed for 28 days. Body weight gains, feed intake, feed conversion ratios, and nutrient digestibility remained unaffected during the entire period, while both cholesterol levels (serum) and thiobarbituric acid reactive substances (in breast meat) decreased within the supplement groups. Surprisingly, the meat color (redness) was decreased, leading to a paler final product at the end of the feeding trial. Grape pomace ability to decrease the oxidative stress of blood and vital organ tissues while improving the redox status (at least in broilers) was also confirmed in another study.[148] The impact of grape pomace phenolics on broiler chick intestinal microflora was recently examined.[149] Analyzing the dietary supplementation of grape pomace extracts (at 60 g/kg of feed) indicated that the overall performance of experimental birds was not affected except that increased enterococcal populations and decreasing *Clostridia* counts were observed in the ileal content of grape-pomace-fed birds. Furthermore, the analysis of cecal digesta showed higher counts of *E. coli*, *Lactobacillus*, *Enterococcus*, and *Clostridia* in the grape-pomace-treated group than those in other groups. The study also concluded that the biodiversity and presence of phenol-degrading bacteria as well as unidentified and uncultured organisms increased in animals fed grape-pomace-supplemented diets.[149] The above studies suggest that grape pomace can be used (up to a certain level) in poultry feed to enhance production outcomes without any associated negative effects on the chicken meat/egg quality.

In dairy cows, the inclusion of grape pomace (15%) in feed was reported to improve the overall normal blood metabolite profile with no negative influence on average levels of milk fat, protein, and caseins.[150] Grape pomace in these studies significantly increased lactose and β-lactoglobulin levels in milk without affecting α-lactalbumin or albumin levels. Another study scrutinized the influence of grape pomace supplementation on rumen microbiome in dairy cattle.[151] Holstein-Friesian calves that were feed grape pomace and copper sulfate for 75 days showed an altered rumen diversity/microbiome with certain microbial taxa [such as an uncultured Bacteroidales UCG-001 genus and operational taxonomic units (OTUs) from genus *Sarcina*], increasing in abundance within pomace-fed calves compared to the controls. *Ruminiclostridium* and *Eubacterium* spp. (with functions related to the degradation of grape pomace constituents, especially flavonoids or xyloglucan) were also enriched by the grape pomace feeding. More interestingly, the lipopolysaccharide biosynthetic pathway was inhibited in the supplemented groups as a result of the antimicrobial effects of grape pomace.

The form of how the pomace is served to animals seems initially to affect some production parameters. Most recently, the ensiling of grape pomace (through 4 weeks of fermentation using a *Lactobacillus buchneri* inoculum) introduced positive changes in rumen fermentation outcomes while reducing methane generation in addition to inducing compositional changes in the fibrous fraction of feed during the above process.[152] Reduced methane levels are among the most appealing outcomes of using pomace silage within the animal industry. In the above study, the reduction of methane partially correlated with increases in total volatile fatty acids (enhanced propionate proportions) and/or considerable adjustment of rumen microbial composition (lower numbers of methanogenic archaea and higher numbers of fibrolytic bacteria). Collectively the above studies confirmed that dietary polyphenol-rich grape products can modify the gut intestinal microflora and increase its biodiversity with a positive influence on animal productivity.[140,143−145,149,151] The above studies collectively indicate the possibility of refining applications of grape pomace bioactive compounds in feeding animals. However, it should be recognized that there are differences between pomaces as a result of cultivars, implemented extraction procedures, and used solvents, which influence the composition of each active fraction and its overall functional properties. The standardization and optimization of such extraction methods/fractions should be further investigated separately for best empirical outcomes of each feed application.

7. FUTURE OPPORTUNITIES AND CHALLENGES

Many screening methods, including well and disc diffusion assays, have been used to investigate antimicrobial activities of grape pomace extracts (Table 1). The lack of a universal protocol (used solvents, media preparations, microbial loads, etc.) needs to be addressed to make the results more comparable.

Moreover, among the top challenges is the solubility of grape extracts, which can be a barrier for their optimum usage as antimicrobial alternatives. The above challenge dictates the use of novel approaches to test and confirm the antimicrobial activity of grape pomace extracts in non-aqueous solvents, such as absolute methanol/ethanol, hexane, and dimethyl sulfoxide (DMSO).[38,134] These solvents need to be taken into account, with their utilization (if any) optimized properly in feed applications. Non-conventional fluorescence-based protocols could also serve as alternatives to track the inhibitory effect of pomace in liquid cultures[71,153] or where the dark color of pomace extracts interferes with the measured optical density.

Attention is needed when extrapolating inhibitory doses from laboratory settings to actual farming applications because each application needs to be separately optimized. This is true in the light of some recent studies that showed a stimulatory effect of grape pomace polyphenols on the growth of selected bacterial species (*Lactobacillus acidophilus* in this case).[154] It is exciting to see that grape pomace can inhibit many foodborne pathogens and promote the growth of beneficial bacteria, such as lactic acid bacteria. The ability of grape pomace extracts, at specific doses, to also promote the survival of some pathogens should not be merely neglected but fully investigated and further scrutinized. In short, more collaborative research efforts are granted on the basis of the above observation to refine efficient doses in each targeted agricultural and/or farming application.

A controversy also exists about risks and toxicities associated with the high and continuous consumption/usage of polyphenolics.[155] This issue should not be of any major concern in the short-living production animals (such as broilers and beef cattle) nor for the human consumption of their meat products because any carryover effects are close to impossible as a result of the low intestinal absorption of grape pomace polyphenols. More studies are needed to check the adverse effects (if any) on long-term production animals, such as dairy cows and egg-laying hens.

In short, promising bactericidal and bacteriostatic activities have been reported by different research teams around the world regarding grape pomace and its extracts. These activities coupled with the already documented other beneficial effects of grape flavonoids on animal and human health (including the reduction of risks related to chronic conditions) establish a solid ground to use the pomace/extracts in every feasible mean. Identifying and optimizing the empirical conditions of grape pomace large-scale utilization as an antimicrobial feed additive is far away from completion, and more basic and applied research is needed. The literature has provided encouraging results to argue for the validity and pursuance of such approaches.

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Notes

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