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Biomedical, Health Beneficial & Nutritionally Enhanced Plants

Genome-wide association studies of antimicrobial activity in global sorghum

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Abstract

Sorghum [Sorghum bicolor (L.) Moench] is a common feed grain globally with vast genetic and phytochemical diversity that may provide numerous health benefits, including its aptitude as an antimicrobial feed grain. This study highlights the antimicrobial potential of a collection of 384 diverse sorghum accessions against two prominent foodborne pathogens, *Clostridium perfringens* and *Salmonella enterica*. Following extensive screening, we determined that sorghum grain extract is more efficient at inhibiting *C. perfringens* than *S. enterica*. Antimicrobial activity observed against *C. perfringens* was not significantly correlated with either total phenols ($r = 0.12$) or tannin concentration ($r = 0.12$). Moreover, we mapped loci associated with antimicrobial activity to *C. perfringens* that are independent of loci associated with total phenols and tannins. The two most significant associations were determined to have an epistatic interaction and 20 candidate genes were identified. By sequence homology studies, we found the potential functions of these candidates to include plant stress response (*Sobic.002G083600*) and phenol metabolism regulation (*Sobic.010G222600).* Additionally, we noted no relationship between antimicrobial activity and either grain yield or composition. These results highlight significant heritable variation of antimicrobial activity in sorghum that may be useful for breeding to improve its value as a feed source by incorporating grain-based antibiotics in animal production.

1 | INTRODUCTION

Animal agriculture contributes to the global public health concern of antibiotic resistance, as the industry uses 73% of the global distribution of clinical antibiotics to treat bacterial infection in animals raised for food (Mathew, Cissell, & Liamthong, 2007; White, Zhao, Simjee, Wagner, & McDermott, 2002; Van Boeckel et al., 2019). Facilities with poor sanitation and loose veterinary regulation enable the spread of harmful pathogens and are overcome with copious use of antibiotics (Van Boeckel et al., 2019). *Clostridium perfringens* and *Salmonella enterica* are two prominent foodborne pathogens that regularly threaten the poultry industry

Abbreviations: BLUPs, best linear unbiased predictors; GAE, gallic acid equivalent; GWAS, genome-wide association study; LD, linkage disequilibrium; MIC, minimum inhibition concentration; NIRS, near-infrared spectroscopy; SAP, sorghum association panel; SNP, single nucleotide polymorphism; VIF, variance inflation factor.

and are traditionally treated with antibiotics. *C. perfringens* causes inflammation in the gut of broilers, accounting for 1% of losses a day (Van Immerseel et al., 2005), whereas *S. enterica* is a threat to both human and animal health, as it repeatedly contaminates animal products (Swartz, 2002; White et al., 2002). Prolific spread of these pathogens impairs the efficiency of the farm and, if untreated, are detrimental to farmers. Antibiotic usage in agriculture could be reduced by implementing alternative solutions, such as incorporating feed products with antimicrobial properties into animal rations (Gyawali & Ibrahim, 2014).

Sorghum [*Sorghum bicolor* (L.) Moench] is a cereal grain used most commonly for feed, food, forage, and bioenergy. Though its uses are diverse, sorghum grain is economically prominent as a staple food supply in Africa and Asia but is most prevalently used as a feed grain for livestock in the United States. Sorghum was first domesticated in the Horn of Africa, where subsequent migration events and adaptation of early domesticates led to the establishment of the five major genetically distinct races within the sorghum species (Brown, Myles, & Kresovich, 2011; Harlan & De Wet, 1972). The racial structure within sorghum largely contributes to the genetic and phenotypic diversity within the species; however, because of its photoperiod sensitivity (it requires a short daylength to flower and produce grain), much of the available germplasm in the US National Plant Germplasm System has yet to be exploited for crop improvement in temperate regions. For instance, nutritional and health-related traits have traditionally been underexploited in breeding programs, although the genetic potential may exist. To identify the genes that underlie these quantitative traits, the Sorghum Association Panel (SAP) is a genetic resource designed to be used for association mapping studies. The SAP has been created from a diverse collection of accessions that represents the five major cultivated races, geographic centers of diversity, and important US breeding lines (Casa et al., 2008).

Sorghum grains are phytochemically rich with phenols, which have beneficial antioxidant (Herald, Gadgil, & Tilley, 2012), anticancer (Hargrove, Greenspan, Hartle, & Dowd, 2011), and anti-inflammatory properties (Burdette et al., 2010; Rhodes & Kresovich, 2016). Phenols are ubiquitously found throughout the plant kingdom as secondary metabolites produced in response to biotic and abiotic stresses. Because of their wide range in structural differences and diversity within the class, phenols contribute to several physiological processes and traits. One of the more prominent phenolic-based traits studied in plants is the antimicrobial effect (Cowan, 1999; Nitiema, Savadogo, Simpore, Dianou, & Traore, 2012; Alzoreky & Nakahara, 2003; Kil et al., 2009). Plant phenols exhibit antimicrobial activity against a number of bacteria, both Gram-negative and Gram-positive, and maintain these inhibitory effects in vitro (Nitiema et al., 2012). Furthermore, antimicrobial activity against foodborne pathogens has previously been observed in sorghum through evaluation of metabolite extractions (Kil et al., 2009). However, these tests were limited to a small collection of genotypes that were not representative of global diversity; the underlying genetics and mechanisms of antimicrobial activity were not considered.

Core Ideas

- Heritable variation exists for breeding for antimicrobial activity in sorghum grain.
- Antimicrobial activity did not only correlate with phenol & tannin accumulation.
- Antimicrobial activity did not negatively impact yield or other traits of the grain.
- Twenty candidate genes were identified that may regulate antimicrobial activity.

Importantly, not all phenol subclasses may be beneficial for use as a feed grain. Tannins are a broad class of phenols, divided into three subclasses, that are recognized for their antimicrobial effects (Scalbert, 1991). Specifically, the subclass of proanthocyanidins, or condensed tannins, is the most prominent in sorghum grain. Condensed tannins localized in the testa, a layer of tissue that is located between the pericarp and endosperm of a grain, can, along with other polyphenols, give pigment to the testa layer (Earp & Rooney, 1982). The presence of condensed tannins in sorghum grain is modulated by two loci, B_1 and B_2 (Dykes, Rooney, Waniska, & Rooney, 2005), whose underlying genes have now been identified as *Tannin1* (*Tan1*) and *Tannin2*, respectively (Wu et al., 2012, 2019).

Though tannins are found throughout the plant kingdom and are readily available antimicrobial agents, tannins are not a solution to the current problem of antibiotic resistance. Tannins bind to a variety of nutrients and impair digestion, thus reducing the bioavailability of essential nutrients and nutrient efficiency (Chung, Wong, Wei, Huang, & Lin, 1998). For this reason, tannins have largely been eliminated in common cereal grains such as wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), and rice (*Oryza sativa* L.). Sorghum, however, has maintained nontannin and tannin cultivars over time because the tannin types grown by African (Wu et al., 2019) and South American farmers provided protection against severe bird predation. Despite recent findings that suggest tannins in feed grain may replace antibiotics in poultry production as growth promoters (Huang, Liu, Zhao, Hu, & Wang, 2018; Redondo, Chacana, Dominguez, & Fernandez Miyakawa, 2014), the threshold at which tannins provide beneficial versus adverse effects for the animal are still unknown and therefore breeding for low tannin cultivars remains a priority.

Development of nutritionally efficient natural food products with antimicrobial activity may combat the continuing rise in antibiotic resistance in animal agriculture. The genetic and metabolite diversity maintained in sorghum germplasm provides support that it may be a promising candidate for this use. Therefore, the goals of this study were: (a) to characterize antimicrobial activity across a globally diverse collection of sorghum germplasm, and (b) to identify the genetic basis associated with antimicrobial activity that is unrelated to the antinutritional effects of tannins. From these experiments, we found significant variation of antimicrobial activity that exists in sorghum grain independent of antinutritional components such as tannins and total phenols. This research also highlights that antimicrobial effects did not have a negative impact on yield or grain macro- and micronutrient composition. Twenty potential candidate genes were identified through the results of the genome-wide association studies, which may regulate antimicrobial activity, further supporting sorghum's potential as an antimicrobial feed grain.

2 | MATERIALS AND METHODS

2.1 | Plant material and field design

The plant material evaluated was a subset of 384 accessions, representing the SAP (Casa et al., 2008; Boyles et al. 2016) (https://www.ars-grin.gov/npgs/, accessed 7 Dec. 2020). Materials were grown and sampled during the 2017 field season. The SAP was planted in a randomized complete block design with two replications at the Clemson University Pee Dee Research and Education Center in Florence, SC. Plots contained two rows, each 6.1 m in length and spaced 0.726 m apart, with an average planting density of approximately 62,350 plants ha^{-1}. Blocking decisions were based on both maturity and plant height, with the full details described in Sapkota et al., 2020. Fields were irrigated when needed and adequate nutrients were supplied to minimize abiotic stress. In detail, variable rates of N, P, and K fertilizer applications were applied prior to planting on the basis of soil samples, followed by an application of 93 kg ha^{-1} of N 35 d after planting. Bicep II Magnum (*S*-metolachlor + atrazine)(Syngenta, Calgary, AB, Canada) was applied prior to planting at 3.5 L ha^{-1}. Atrazine was subsequently applied at 4.7 L ha^{-1} after emergence. To control the sugarcane aphid population, a single application of 0.5 L ha^{-1} of Sivanto Prime (Bayer CropScience, Calgary, AB, Canada) occurred 60 d after planting (Sapkota et al., 2020). Grain was collected from the primary panicle when the plant reached physiological maturity. Harvesting grain from the secondary panicles, located on the tillers of the sorghum plant, was avoided to prevent the confounding effects of maturity on grain composition. Harvested panicles were dried for 10 to 14 days in an electric dryer to a constant weight and subsequently threshed with a BT-14 belt thresher (Almaco, Nevada, IA). Maximum forced air was used when threshing to remove all glumes, foreign plant debris, and poorly filled or damaged grains.

2.2 | Compositional analysis

Compositional data were collected from each genotype by near-infrared spectroscopy (NIRS), performed with a DA7250 NIR analyzer (Perten Instruments, Springfield, IL). Dried and threshed grained (as described in Section 2.1) was ground to a particle size of 1 mm with a Cyclotec sample mill (FOSS, Hillerod, Denmark) and used to evenly fill a 43-ml Teflon dish. Ground samples, as opposed to whole-kernel samples, have been reported to get the most efficient measurements (De Alencar Figueiredo et al., 2010).

The Teflon dish containing the ground sample was gradually rotated during NIRS analysis for accurate sampling. NIRS data were recorded for 29 compositional traits for each sample, including key macronutrients such as starch, protein, and crude fat (ether extracted lipids). A full list of compositional traits can be found in Supplemental File S1. Trait calibrations were previously established in a subset of 100 samples in the SAP (Boyles et al., 2017). Harvested panicles were air-dried to a constant moisture and hand-threshed. The dried ground samples were sent to Dairyland Laboratories, Inc. (Arcadia, WI) and the Quality Assurance Laboratory in Murphy-Brown LLC (Warshaw, NC) for wet chemistry. Calibration curves were established with a DA7250 NIR analyzer (Perten Instuments).

2.3 | Extraction of metabolites

Following NIRS, metabolites were extracted from ground sorghum via an acetone extraction method described in Herald et al. (2012). For each sample, the metabolites were extracted by adding 10 ml of 70% acetone to 0.5 g of representative ground grain, which was agitated for 2 h. Samples were stored at –20 ˚C overnight. The next day, samples were centrifuged at 2,970 × *g* for 10 mi at 4 ˚C, then the supernatant was transferred to new tubes. An additional round of extraction was performed on the existing tissue by adding another 10 ml of 70% acetone. Samples were agitated for 10 min and centrifuged (2,970 × *g* for 10 min at 4 ˚C). This supernatant was collected and combined with the previously collected supernatant. Acetone was removed from the extract via nitrogen evaporation with a 96-well microtiter (Microvaps, Fisher Scientific; Pittsburgh, PA). For long-term storage, extracts were resuspended in 1 ml of dimethyl sulfoxide and stored in the dark at –20 ˚C.

2.4 | Quantification of polyphenols and tannins

Total phenols were quantified via the Folin–Ciocalteu assay (Singleton, Orthofer, & Lamuela-Raventos, 1999). A standard curve was established with gallic acid concentrations ranging from 12.5 to 400 µg ml in 70% acetone, following the protocol outlined in Rhodes, Gadgil, Perumal, Tesso, and Herald (2017). In individual wells of a 96-well plate, 75 µl of deionized water, 25 µl of Folin–Ciocalteu reagent (diluted 1:1 with deionized water) was mixed with either 25 µl of the extract, the standard, or 70% acetone, and left for 6 min for the reaction to complete. Subsequently, 100 µl of 7.5% sodium carbonate was added to each well and mixed; the wells were then covered and left in the dark for 90 min. Absorbance at 765 nm was measured with a Synergy H1 Multi-Mode Microplate Reader (BioTek Instruments, Inc.; Winooski, VT). Twenty-five microliters of 70% acetone was used as a control. Total phenol concentrations are reported in [gallic acid equivalent (GAE) g^{-1}] based on the dry weight.

Tannin data were kindly provided by Dr. Davina Rhodes (Rhodes et al., 2014). To generate these data, the SAP was planted and harvested at Clemson University Pee Dee Research and Education Center in Florence, SC, in 2013 and 2014, under the same field conditions and management described in Section 2.1. Tannin concentrations were collected via NIRS, for which 20 g of whole-grain samples were scanned with a FOSS XDS spectrometer (FOSS North America, Eden Prairie, MN) at a wavelength range from 400 to 2,500 nm. Each sample was measured in duplicate, based on dry weight, the reported tannin concentration (mg Catechin equivalent g^{-1}) represented the mean of duplicates. The calibrations curves, software, and spectrometer used were all previously described in Dykes, Hoffmann, Portillo-Rodriguez, Rooney, and Rooney (2014).

Average tannin concentration, for each year was used in a *t*-test and Pearson's correlation. A *t*-test was performed to determine if the 2 yr of tannin data were statistically different. Previous studies have found that the tannin trait had a high broad-sense heritability estimate ($H^2 = 0.80$).

2.5 | Disc diffusion antimicrobial assay

Antimicrobial susceptibility testing for sorghum grain extracts against *C. perfringens* (CP#6; Miller, Skinner, Sulakvelidze, Mathis, & Hofacre, 2010) and *S. enterica* (ATCC 30661) was performed via a disc diffusion assay following Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). Samples were prepared by saturating cotton discs 6 mm in diameter (Becton, Dickenson and Company) with 20 µl of extracts, twice, allowing sufficient time for the discs to dry in between saturations. All samples were prepared in triplicate. Each pathogen was appropriately prepared for the assay by following the optimal culturing methods described by Chen and Jiang (2017) for *S. enterica* and Dharmasena and Jiang (2018) for *C. perfringens*. Cultures of each pathogen were washed twice and resuspended in 0.85% sterile saline to and optical density of 0.5. Mueller-Hinton (*S. enterica*) and Brucella blood (*C. perfringens*) agar plates were inoculated and streaked to ensure an evenly distributed lawn of growth. Six saturated discs were placed equidistantly on the surface of the plate, ensuring the disc lay completely flat. Two additional discs were included in each assay plate: a positive control with 30 µg per disc of kanamycin (*S. enterica*) or tetracycline (*C. perfringens*), and a disc saturated with 30 µg per disc of dimethyl sulfoxide as a negative control. Plates were inverted and incubated at 35 °C for 16 to 18 h (*S. enterica*) or 24 h (*C. perfringens*). The diameter was measured to the nearest tenth of a millimeter for the zone of inhibition. The zone of inhibition for each sample was compared with the inhibition with a standard microorganism, *Escherichia coli* (ATCC 25922). Samples that had clear inhibition zones or zones with a diameter greater than 7 mm (Figure 1d) were considered to have a strong effect. However, samples that showed detectable inhibition but maintained some bacterial growth in the inhibition zone were classified as having a weak effect (Figure 1a–c).

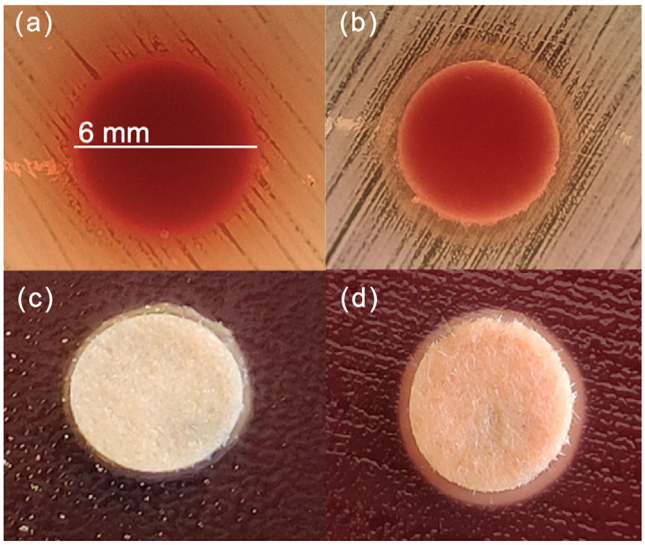

FIGURE 1 Range of antimicrobial activity observed in the disc diffusion assay with a 6-mm cotton disc. (a) and (b) show weak antimicrobial activity against S. *enterica*. Although some inhibition can be visually observed, there is still growth in the inhibition zone, making it difficult to measure. (c) is an example of weak antimicrobial activity against *C. perfringens* and (d) shows strong antimicrobial activity against *C. perfringens*, which is identified as having clear inhibition zone boundaries or an inhibition zone greater than 7 mm

2.6 | Minimum inhibitory concentration assay

To confirm the results of the disc-diffusion assay, all sorghum extracts were tested by with the microbroth dilution method following Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standard Institute, 2012). Briefly, Brucella broth and Mueller–Hinton broth were inoculated with *C. perfringens* and *S. enterica*, respectively, and incubated at 37 ˚C with anaerobic shaking (*C. perfringens*) or aerobic shaking (*S. enterica*) to establish logarithmic growth (Chen & Jiang, 2017; Dharmasena & Jiang, 2018). Following incubation, each culture was pelleted by centrifugation (3,500 × *g* for 5 min) and resuspended in 0.85% sterile saline solution to an optical density of 0.1 measured at 625 nm. Samples were tested in triplicate in 96-well microplates, yielding final bacterial concentrations of 5 × 105 colony-forming units per ml, and incubated overnight at 37 ˚C. Following incubation, the optical density of each well was determined with a μQuant microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT) at 625 nm. *Escherichia coli* (ATCC 25922) and *Clostridium difficile* (ATCC 700057) were used as quality control strains for *S. enterica* serotype *enteritidis* and *C. perfringens*, respectively. Minimum inhibition concentration (MIC) was defined as the lowest concentration needed to completely inhibit growth compared with the controls.

2.7 | Genomic analysis

The genotypes of the SAP were collected by genotype-by-sequencing (Morris et al., 2013; Boyles et al., 2016). Raw sequence reads were aligned to the most current sorghum reference genome (BTx623 Version 3.1; https://phytozome.jgi.doe.gov, accessed 3 Dec. 2020) with the Burrow–Wheelers aligner (Li & Durbin, 2010). Single nucleotide polymorphism (SNP) calling was done via the TASSEL Version 5.0 pipeline (Glaubitz et al., 2014) and subsequent imputation was performed with the FILLINFindHaplotypesPlugin and FILLINImputationPlugin in TASSEL (Swarts et al., 2014). In total 484,799 SNPs were generated. The SNPs were filtered for minor allele frequency (>0.05), missing data (0.30), and Hardy–Weinberg equilibrium (0) with VCFtools (Danecek et al., 2011). Subsequently, the marker set was pruned with PLINK (Purcell et al., 2007). The PLINK pruning method is based on the variance inflation factor (VIF), which recursively removes SNPs above the VIF threshold (VIF = 2) within a sliding 50-SNP window, shifting steps every five SNPs. The VIF is defined as 1 $(1 - R^2)^{-1}$, with R^2 being the multiple correlation coefficient for the SNP being tested against all other SNPs. Therefore, the VIF accounts for the multicollinearity in the linear regression (Purcell et al., 2007). Filtering and pruning resulted in 99,126 SNP markers that were used for association mapping analysis.

GEMMA Version 0.98 (Zhou & Stephens, 2014) software was used to perform the genome-wide association study (GWAS) on antimicrobial activity against *C. perfringens* by implementing a univariate linear mixed model, which takes population stratification and sample structure into account. To genetically confirm antimicrobial activity's independence from *Tan1*, best linear unbiased predictors (BLUPs) were calculated for tannin concentrations and used as a covariate in a univariate linear mixed model. The use of BLUPs instead of mean values accounts for environmental variation in the tannin measurements across the years (Section 2.4). Manhattan and quantile–quantile plots were generated by R software CMplot (https://github.com/YinLiLin/R-CMplot, accessed 3 Dec. 2020). The Bonferroni-corrected significance threshold (0.05 ∝ 99,126 SNPs = 5.04×10^{-7}) was used to determine significant associations ($\alpha = .05$) in the Manhattan plot. Linkage disequilibrium (LD) was calculated locally within 1 MB of significantly associated SNPs in PLINK. Linkage disequilibrium was considered to decay at $r^2 < 0.1$. Genes found within local LD of each SNP were identified with a custom script and considered as potential candidate genes for antimicrobial activity in sorghum grain. Broad-sense heritability was calculated by the R package 'Heritability' (Kruijer et al., 2015). Marker-based narrow-sense heritability (h^2) was calculated from the relatedness matrix generated by GEMMA, which used the same SNPs as the GWAS. All scripts used for analysis are located at https://github.com/lkshiel/ACRE (accessed 3 Dec. 2020)

2.8 | Epistatic interaction analysis

We performed SNP–SNP interaction tests on significant SNPs identified from the antimicrobial activity GWAS. PLINK files generated for the LD analysis were used to test each SNP pair for epistasis via the following linear regression model implemented with PLINK's –epistasis command and –set parameter:

$$Y = \beta_0 + \beta_1 g_A + \beta_2 g_B + \beta_3 g_A g_B,$$

where Y represents the antimicrobial activity as measured in the disc diffusion assay, g_A and g_B are allele counts for each inspected variant pair, and the β coefficients 0 to 3 represent the intercept, the effect of g_A, the effect of g_B, and the epistatic interaction between the variant pair, respectively. the frequencies of the two locus genotypes were also calculated by PLINK's *twolocus function*. Analysis was performed on unimputed data so as to not skew the frequency of a particular genotype.

2.9 | Statistical analysis

Pearson's correlation coefficient was determined for all relationships investigated in this study. Statistical significance was determined for *p*-values less than .05. Categorical values such as 'weak' were given a numerical value of 3.55, following the method described in Billard and Diday (2000). This numerical value was determined by averaging the lowest measured inhibition zone (0 mm) and the lowest 'strong' value (7.1 mm). Folin–Ciocalteu samples that were measured above 420 GAE g^{-1} were capped at 420 GAE g^{-1} for analysis. The *t*-test was performed by the Python package *pingouin* (Vallat, 2018). Tables were generated with the Python package *pandas* (McKinney, 2010) and figures were generated with the python packages *seaborn* and *matplotlib* (Hunter, 2007; Waskom et al., 2014).

3 | RESULTS

3.1 | Antimicrobial activity of sorghum grain extracts

Approximately half of the tested germplasm (188 accessions) were found to demonstrate antimicrobial activity, ranging from weak to strong effect (Supplemental File S2). Weak activity was defined as having an indistinguishable inhibition zone while still showing inhibition, whereas a strong effect was defined as having a clear inhibition zone or a zone with a diameter greater than 7 mm. Through the disc diffusion assay, we identified 103 accessions that had antimicrobial activity against *C. perfringens*, 37 of which had a strong effect with inhibition zones that ranged from 6.88 to 9.45 mm in diameter. Assays tested on *S. enterica* showed 119 accessions with antimicrobial activity; however, only a weak effect was observed across those genotypes.

3.2 | Minimum inhibitory concentration analysis

Minimum inhibitory concentrations were determined for a subset of 12 accessions that were selected on the basis of a range of activity levels observed in the disc diffusion assay, including both high and low extremes. The average MIC values for *C. perfringens* were 0.911 mg ml^{-1} for the first replicate and 1.37 mg ml^{-1} for the second. However, the average MIC values for *S. enterica* were reported as 32.8 mg ml^{-1} in the first replication and 24.8 mg ml^{-1} in the second (Table 1). The MIC values observed confirm the results of the disc diffusion assay. We expected extracts with strong antimicrobial activity to require a smaller concentration to inhibit growth than extracts with weaker or no activity. The MIC analysis of *S. enterica* revealed instances with high MIC values but relatively strong antimicrobial activity. We would expect that genotypes demonstrating antimicrobial activity would have lower MIC values than genotypes that displayed no activity. High MIC values that were found to have weak antimicrobial activity in the disc diffusion assay could be attributed to the diffusion patterns of the extract on the agar media, which impacts the role of these compounds in evaluations of antimicrobial potential (Alzoreky & Nakahara, 2003). However, because the initial characterization of *S. enterica* inhibition did not provide reliable measurements of antimicrobial activity and because antimicrobial activity against *S. enterica* was found to be correlated with unfavorable traits (Supplemental Figure S1, Supplemental Figure S2, Supplemental Table S1), data regarding *S. enterica* assays were subsequently excluded from further analyses used to identify useful germplasm for crop improvement.

3.3 | Quantification of total phenol concentration

Phenol concentrations were measured for each sample via a Folin–Ciocalteu phenolic assay. It was found that across samples, total phenol concentration (GAE g^{-1}) was highly variable across the diverse sorghum accessions, ranging from 5.38 to 420 GAE g^{-1}, where 420 GAE g^{-1} was the highest value the instrument could measure on the basis of established standards (Supplemental File S3). Total phenol concentrations across field replications were found to be highly correlated ($r = 0.82$) regardless of soil differences, pest pressure, and weather events. This is consistent with previous reports, as total phenols were previously found to be highly heritable (broad-sense heritability = 0.82) (Pfeiffer & Rooney, 2016).

3.4 | Effects of total phenols and tannin concentration on food-borne pathogens

The relationship between total phenol concentration and inhibition zone size from the disc diffusion assay was investigated to determine if higher total concentrations of phenols were correlated with greater antimicrobial activity. Samples showing positive inhibition of *C. perfringens* had striking variation in total phenol concentration, with a range of 6.91 to >420 GAE g^{-1}. As a result, *C. perfringens* inhibition was not significantly correlated ($r = –0.12$) with total phenol concentration at the .01 significance level (Figure 2a).

The results of the *t*-test showed that the 2 yr of tannin data were not statistically different (*p*-value = .34) and therefore provide support for using the 2013 and 2014 tannin data

TABLE 1 Minimum inhibitory concentrations (MIC) for *C. perfringens* and *S. enterica*

Accession	*C. perfringens*				*S. enterica*			
	MICr1[a]	MICr2	IZr1	Izr2	MICr1	MICr2	Izr1	Izr2
	mg ml^{-1}		mm		mg ml^{-1}		mm	
PI607931	0.2	0.08	0	0	25.16	20.16	0	0
PI629059	1.14	0.23	0	0	36.35	29.49	0	0
PI576376	0.14	0.16	weak	weak	18.16	ND	0	0
PI597957	0.3	0.16	weak	8.6	18.91	20.16	0	0
PI533979	0.39	0.14	8.2	weak	49.92	9.27	weak	weak
PI576393	0.13	0.14	8.4	weak	8.56	17.59	weak	weak
PI533869	0.19	0.19	8.1	8.4	11.91	11.99	0	0
PI656003	0.18	0.11	weak	weak	46.1	58.28	weak	weak
PI656035	0.13	0.13	weak	weak	16.21	16.58	0	0
PI655995	0.1	0.09	8.53	9.45	50.71	44.29	weak	weak
PI642998	0.05	0.06	7.4	8.05	26.3	7.1	weak	weak
PI641836	7.5	12.54	7.9	weak	14.99	12.54	weak	weak

[a]MICr1, minimum inhibitory concentration: replication 1; MICr2, minimum inhibitory concentration: replication 2; Izr1, inhibition zone: replication 1; Izr2, inhibition zone: replication 2; ND, not detectable.

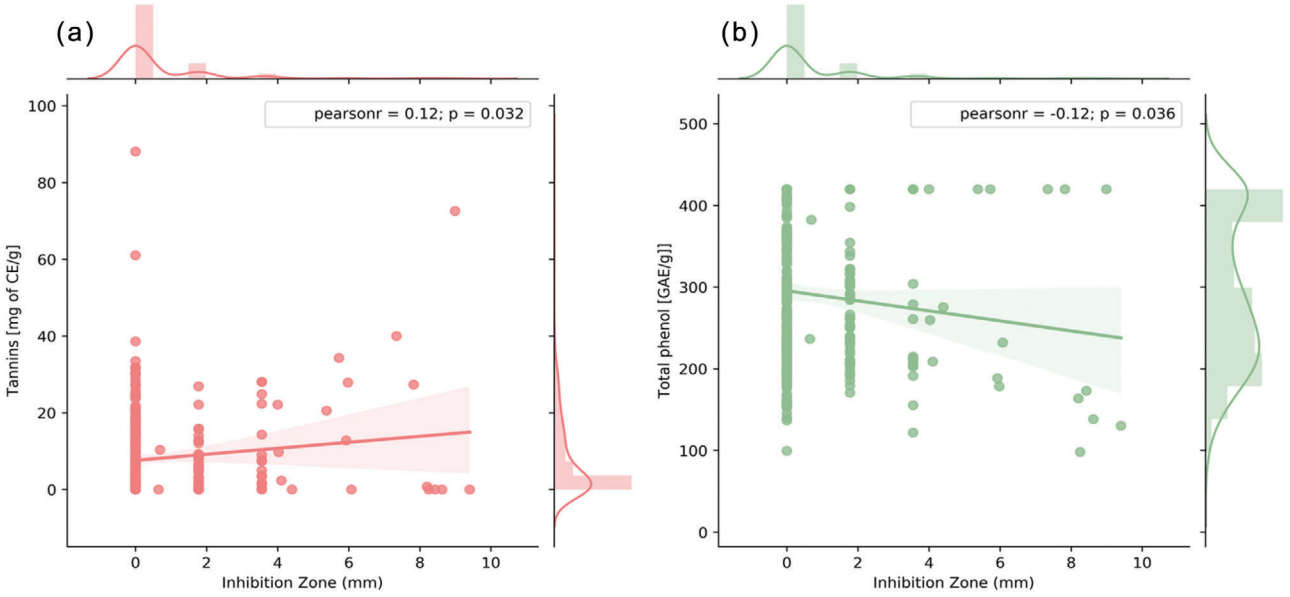

FIGURE 2 Relationships between *C. perfringens* inhibition zone (mm) and (a) total phenol concentration ($r = 0.12$; $p = .36$) and (b) tannin concentration ($r = 0.12$; $p = .032$)

TABLE 2 Summary of *C. perfringens* samples regarding testa and antimicrobial activity

	No activity	Weak activity	Strong activity
Unpigmented testa	299	51	19
Pigmented testa	227	32	15

for our analysis with grain extracts from 2017 (Supplemental Table S2).

Additionally, the correlations between the average inhibition zone diameter (mm) for each accession and average tannin measurement across years were calculated. The inhibition zone diameter did not have a significant correlation ($r = 0.12$) with tannin measurements at the $\alpha = .01$ significance level (Figure 2b).

3.5 | Identification of antimicrobial germplasm with unpigmented testa

Testa presence was previously identified across the SAP by cutting a thin layer of the pericarp from each seed and examining testa pigmentation under a dissecting microscope (Rhodes et al., 2014). Pigmented testa may be indicative of the presence of condensed tannins; therefore, investigation of the relationship between antimicrobial activity and pigmented testa is warranted. There were no observable differences between the distributions of accessions with unpigmented testa and pigmented testa across the *C. perfringens* inhibition zones (Supplemental Figure S3). The similar distributions and the low correlation between inhibition zone and tannin concentration suggests that antimicrobial activity could be achieved in the absence of tannins for *C. perfringens* (Table 2).

3.6 | Selection of suitable accessions with antimicrobial activity

To identify germplasm that maintain antimicrobial activity without condensed tannins, we rigorously filtered genotypes by phenotypic values. First, from the list of accessions that demonstrated antimicrobial activity, only the accessions that maintained strong antimicrobial activity across both replicates were considered. Next, accessions with detectable tannins were eliminated from consideration. Filtering resulted in five accessions characterized as having strong antimicrobial activity and little or no detectable tannin concentration (Table 3). Key agronomic traits, such as plant height, days to maturity, and 1000-grain weight, were also evaluated to determine if the agronomic and compositional phenotypes of the identified accessions were undesirable for plant breeding. The agronomic and compositional traits for the accessions in the SAP were reported by Sapkota et al. (2020).

3.7 | Compositional and yield data of the grain sorghum accessions

Near-infrared spectroscopy was used to measure 29 compositional traits spanning prevalent macronutrients across the 384 accessions with two replications. The quantitative variation

TABLE 3 Accessions with antimicrobial properties and their associated agronomic traits

PI	Common name	IZ[a]	PH	DTM	TGW
		mm	cm		g
PI533869	Msumbji SB 117	8.25	129	104	19.26
PI533871	M 1	8.43	118	101	29.78
PI533948	Nebraska 6350	8.63	79	96	27.72
PI534115	Akwu	8.20	78	113	31.20
PI597957	SC1158	6.08	76	105	22.65
Mean[b]			113	106	23.34

[a]IZ, inhibition zones, reported as averages between replicates; PH, plant height; DTM, days to maturity; TGW, 1000-grain weight.
[b]Phenotype value across the entire sorghum accession panel.

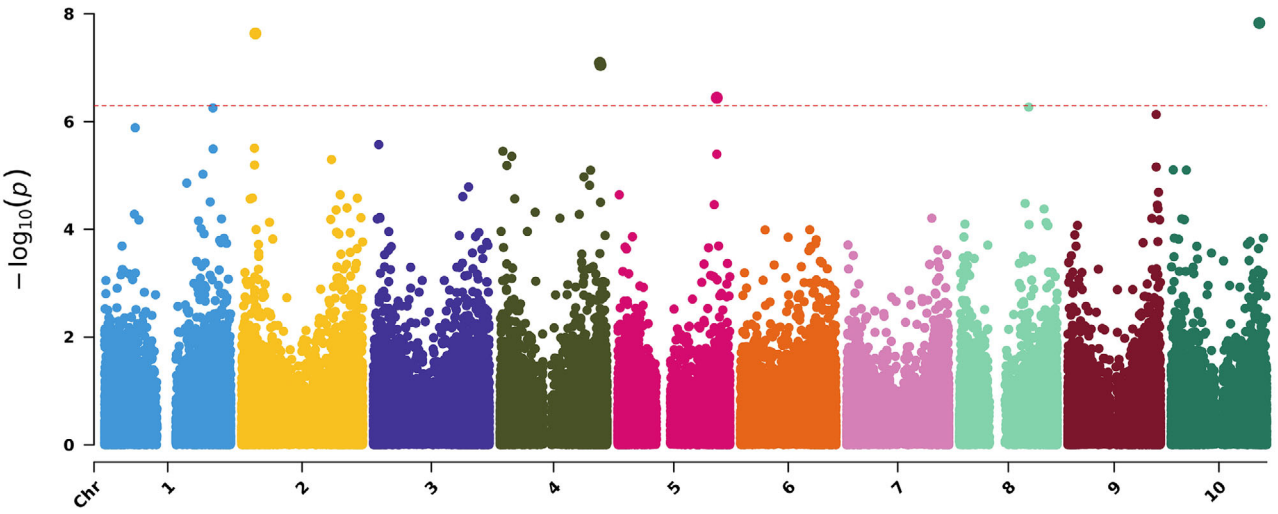

FIGURE 3 Genome-wide association study of antimicrobial activity in sorghum grain. A linear mixed model was used for association analysis off 99,126 single nucleotide polymorphism markers. The *y*-axis ($-\log_{10}$ *p*-values) is plotted against the position on the chromosome (*x*-axis). The dashed line indicates the Bonferroni significance threshold. Regions with $-\log_{10}$ *p*-values above the threshold (dotted line) are candidates

in the grain's compositional traits is evident in the descriptive statistics of each trait (Supplemental Table S3). Most importantly, the compositional data generated establish that grain macro- and micronutrient composition were not compromised by the presence of antimicrobial activity. No significant relationships were found between individual compositional traits and antimicrobial activity (Supplemental Table S3). Additionally, to examine any potential tradeoffs resulting from antimicrobial activity on grain yield components, we compared antimicrobial activity of grains to the grain number per panicle, 1000-grain weight, and grain yield per primary panicle (Sapkota et al., 2020). No significant correlation was observed between antimicrobial activity against *C. perfringens* and any of the grain yield component traits (Supplemental Table S4), suggesting that the presence of antimicrobial activity does not compromise yield. Additionally, we looked at the geographical and racial distribution of genotypes exhibiting antimicrobial activity against *C. perfringens* to identify any potential correlation with antimicrobial activity, though no relevant statistical inferences could be made concerning the role of race and/or origin.

3.8 | Genome-wide association studies

To investigate the underlying genetics of antimicrobial activity against *C. perfringens* in sorghum grain, three GWAS were conducted. First, a univariate linear mixed model was used to map antimicrobial activity against *C. perfringens* (Figure 3, Supplemental Figure S4). However, since the effect of *Tan1* is so strong and may impact the GWAS, tannin BLUPs were used as a covariate for a second association analysis (Figure 4, Supplemental Figure S5). Comparisons between the two antimicrobial GWAS show that by adding tannin as a covariate to the model, the same four SNPs on chromosomes 2, 4, and 10 were found to be significant across both models

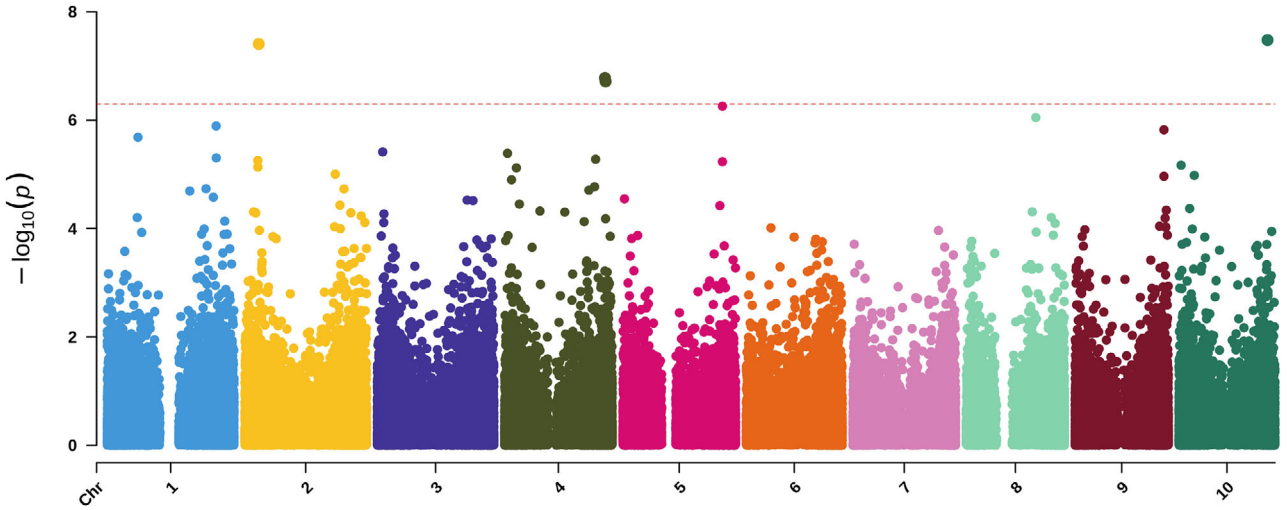

FIGURE 4 Genome-wide association study for antimicrobial activity with tannin as a covariate. Manhattan plot of the association analysis of 99,126 single nucleotide polymorphism markers. The *y*-axis ($-\log_{10}$ *p*-values) is plotted against the position on the chromosome (*x*-axis). The dashed line indicates the Bonferroni significance threshold

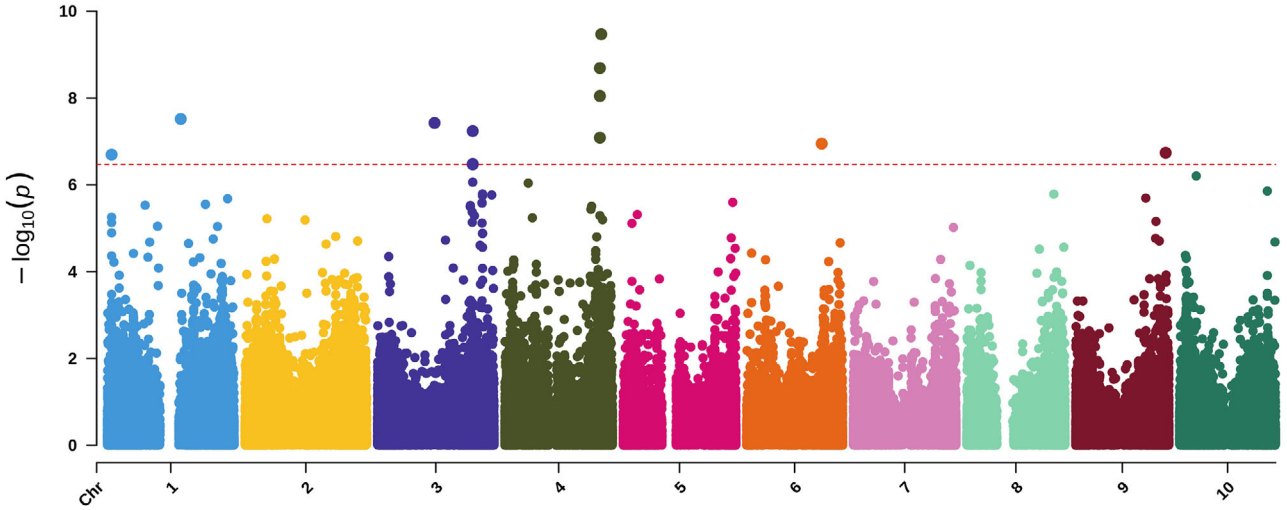

FIGURE 5 Manhattan plot based on the genome-wide association study for the tannin trait from tannin best linear unbiased predictors (BLUPs). The association analysis included 146,280 single nucleotide polymorphism markers from 367 individuals. The *y*-axis ($-\log_{10}$ *p*-values) is plotted against the position on the chromosome (*x*-axis). The dashed line indicates the Bonferroni significance threshold. The notable peak on chromosome 4 colocalizes with *Tan1* (~61–62 MB)

(Supplemental Table S5). The only difference was the singular association on chromosome 5 in the tannin covariate model, which failed to pass the Bonferroni-corrected significance threshold. To confirm that there was no statistical difference between the antimicrobial activity GWAS and tannin covariate GWAS, we performed a *t*-test comparing each SNP's Wald *p*-values from both GWAS. The *t*-test showed that the two models were not statistically different (Supplemental Table S6; *p*-value = .227), and concerns regarding the effect of *Tan1* were disregarded. Additionally, tannin BLUPs were mapped as a genetic control and used to distinguish tannin peaks from novel peaks associated with antimicrobial activity (Figure 5, Supplemental Figure S6). The tannin GWAS contained a peak on chromosome 4, residing at ~62.3 MB, approximately 1 kB from the start position of *Tan1 (Sobic.004G280800*; Wu et al., 2012) (Figure 5). Significant associations found on chromosome 4 in the antimicrobial activity GWAS were located at ~64 MB and were not found in LD with *Tan1*. The associations on chromosome 2 (~ 8.9 MB) and chromosome 10 (~56 MB), however, are unique to antimicrobial activity and were the most significant loci.

3.9 | Epistatic interactions

Epistatic interaction was only found between two of the four SNPs, S2_8924006 and S10_56476103 (p-value = 1.93×10^{-6}), which were the two most significant SNPs in the antimicrobial activity association analysis. Joint and marginal counts and the frequencies of the two locus genotypes are shown in Supplemental Table S7. Moreover, we plotted the distributions of accessions with both, just one, and neither favorable allele in regard to inhibition zone. Accessions with missing data for at least one allele were removed; therefore, 184 accession were used for this analysis. We observed that accessions with favorable alleles at both S2_8924006 (C/C) and S10_56476103 (T/T) had the largest median inhibition zone across four accessions (Supplemental Figure S7). Accessions that had only the S10_56476103 allele maintained weak inhibition with a median inhibition zone at approximately 1 mm across six accessions, whereas accessions that had only the S2_8924006 allele still had no inhibitory effects, with the exception of two outliers across 10 accessions. However, accessions that had neither of the favorable alleles largely had no measured inhibitions across the 164 accessions, with the exception of the 12 outliers, which demonstrated a wide range of inhibition zones from 0 to 8.55 mm.

3.10 | Heritability and potential candidate genes

On the basis of local LD estimates, 20 genes were identified around significant SNPs that putatively regulated antimicrobial activity (Supplemental Table S8). Six potential candidate genes were within the LD block containing the SNP on chromosome 2. Four of the candidate genes were within local LD of S4_64038743 marker on chromosome 4; six genes were within LD of the S4_64439967 marker. The remaining four potential candidate genes were found within local LD of the marker on chromosome 10. Marker-based narrow-sense heritability was calculated to be $h^2 = 0.55$.

4 | DISCUSSION

Food-borne pathogens that infect livestock during production and post-processing impact the productivity and efficiency of the animal protein industry, as well as human health. These food-borne pathogens are frequently controlled with antibiotics which, when used in excess, may lead to the evolution of resistance in pathogen populations (McEwen & Fedorka-Cray, 2002). Antibiotic resistance is a global issue that may be reduced by supplementing natural products with antibacterial agency (Gyawali & Ibrahim, 2014). Several plant species have been identified that contain secondary metabolites demonstrating antimicrobial activity (Cowan, 1999). However, these traits have yet to be integrated into feed grain breeding programs to produce improved cultivars that would minimize antibiotic usage in animal production. Our identification of loci significantly associated with antimicrobial activity against *C. perfringens* is the first step toward the incorporation of natural health-promoting compounds for sorghum improvement.

In this study, we tested the inhibitory properties of metabolite extracts from 384 diverse accessions of sorghum grain against two prominent foodborne pathogens, *C. perfringens* and *S. enterica.* Through a combination of disc diffusion and microbroth dilution assays, 188 unique lines were identified as having antimicrobial activity, constituting half of the experimental germplasm. The significant number of accessions with varying antimicrobial capabilities shows that vast genetic potential exists for antimicrobial activity in sorghum. Additionally, the results of the disc diffusion assay, later confirmed by the MIC analysis on selected accessions, demonstrate that sorghum grain metabolite extract is a more effective antimicrobial agent against *C. perfringens* than against *S. enterica*.

The influence of total phenol concentration on the antimicrobial effect against *C. perfringens* was investigated and found to be insignificant. The apparent lack of a relationship between *C. perfringens* inhibition and total phenol concentration suggests that total phenol concentration alone cannot predict the antimicrobial effect in sorghum. However, the metabolite responsible for inhibiting *C. perfringens* may be an individual subclass of phenols, such as flavan-3-ols, which have previously been reported for their antimicrobial effect on *C. perfringens* (Daglia, 2012). Relationships between the antimicrobial effects on *C. perfringens* and the subclass of phenols could not be determined precisely from the data collected. Moreover, total phenols were extracted by acetone, a method that is more prominently used to extract flavanols and other phenols with higher molecular weight (Dai & Mumper, 2010). Therefore, our correlation measures between total phenols and antimicrobial activity may not include effects from phenols with a lower molecular weight.

Importantly, we noted that the presence of antimicrobial activity against *C. perfringens* was not significantly correlated with tannin concentration, and we subsequently identified five sorghum accessions that upheld strong antimicrobial activity against *C. perfringens* that did not have a pigmented testa and quantifiable tannins. Condensed tannins are a long-established antimicrobial; however, their negative effects on nutrient efficiency have prevented their use in crop improvement (Scalbert, 1991). In the same regard, there is often a carbon utilization tradeoff between crop yield and metabolite production (Brown, 2002). However, our analysis showed

there were no significant correlations found between antimicrobial activity and either compositional traits or yield.

This study used GWAS to identify novel genetic associations with sorghum grain antimicrobial activity against *C. perfringens*. We successfully identified significant associations that are independent of tannin and total phenols. As noted previously, antimicrobial activity has been associated with condensed tannins, which are regulated by duplicate recessive epistatic gene interaction between *Tan1* and *Tan2* located on chromosomes 4 and 2, respectively (Wu et al., 2012; Wu et al., 2019). Similarly, total phenols also have been associated with antimicrobial activity and were found to be associated with loci on chromosome 2 in Rhodes et al. (2017). However, the loci we identified on chromosomes 2 and 4 in the antimicrobial activity GWAS were different from the loci reported for *Tan1*, *Tan2*, and total phenols. Total phenol association was found at 7.5 (Rhodes et al., 2017) and *Tan2* (*Sobic.02G076600*) resides nearby at ~8.2 MB. However, the antimicrobial activity SNP was located at ~8.9 MB. Moreover, the significant associations identified on chromosome 4 were located at ~64 MB, whereas *Tan1* resides at ~62.3 MB (Wu et al., 2012). Loci significantly associated with antimicrobial activity were not found in LD with either the *Tannin* gene or total phenols, further supporting the idea that factors other than tannins or phenols facilitate antimicrobial activity in sorghum grain. Moreover, we accounted for the effect tannin might have on our association analysis by including a tannin covariate. However, the antimicrobial activity GWAS with and without tannin as a covariate were not statistically different, which supports the results from the correlation analysis in Section 3.4 and suggest that the effects of *Tan1* and *Tan2* did not impact our mapping of *C. perfringens* antimicrobial activity. Thus, we concluded that our significant loci were neither artifacts nor artificially inflated by tannin-related effects.

The two most significant associations positioned on chromosomes 2 and 10 were also determined to have an epistatic interaction. This epistatic interaction suggests that their multiplicative effects on antimicrobial activity arise from a nonlinear combination of allele presence, as shown through the results of the *twolocus function* analysis. Moreover, SNP interactions indicate that the loci may interact through intermediate loci in the metabolic pathways, which further confirms the genetic complexity of antimicrobial activity. Furthermore, mean antimicrobial activity was calculated among allele combinations, which demonstrated that accessions that were homozygous for favorable alleles at both S2_8924006 and S10_56476103 had the highest average inhibition zone (Figure 6). This provides additional evidence that S2_8924006 and S10_56476103 are important for regulating antimicrobial activity against *C. perfringens* in sorghum grain. There was substantial amount of missing data for SNP S10_56476103, preventing a meaningful interpretation of the type of interaction occurring between the two loci. The SNP markers were called from genotype-by-sequencing data, which are well-known for providing a cost-effect method for genotyping; however, because this method is dependent on the frequency of the ApeK1 recognition site, some areas of the genome receive a low depth of coverage. This may explain the abundant missing data for S10_56476103, as well as its low minor allele frequency. Better sequence coverage to fill the missing SNP calls and subsequent analysis are needed to better understand how the interaction between these two loci regulate antimicrobial activity in sorghum.

From these genomic analyses, we were able to extract several potential candidate genes. The genes have yet to be characterized; however orthologs identified by OrthoDb (Kriventseva et al. 2018) were found in corn and rice, which may provide insights into the potential function of these genes. For instance, orthologs from maize and rice suggest that *Sobic.002G083200* is a zinc transport protein. Zinc ions have been found to contribute to antimicrobial activity and are commonly added to food as a preservative for their antimicrobial effect in food packaging and material science (Espitia et al., 2012; Stanić et al., 2010). Moreover, the gene *Sobic.002G083600* was found to be an ortholog to *sid1*, which has been characterized as playing a role in inflorescence architecture in maize (Chuck, Meeley, & Hake, 2008). However, the similarity between *Sobic.002G083600* and *sid1* was caused, in part, by the presence of the AP2/ERF domain, which characterizes a family of transcription factors that have been found to be key regulators for various stress responses (Dietz, Vogel, & Viehhauser, 2010, Xie, Nolan, Jiang, & Yin, 2019, Mizoi, Shinozaki, & Yamaguchi-Shinozaki, 2012). Similarly, *Sobic.010G222600* is an ortholog to *Z. mays TRAF34*, a transcription factor that has been found to be involved in a gene regulatory network for phenolic metabolism (Yang et al. 2017). As such, *Sobic.010G222600* may also transcribe a Tumor Necrosis Factor Receptor-Associated Factor transcription factor that regulates sorghum phenolic metabolism. The study by Yang et al. (2017) also identified a myriad of transcription factor families that are responsible for regulating phenolic metabolism; therefore, the putative TRAF and AP2/ERF transcription factors may work jointly to regulate antimicrobial activitiy in sorghum.

Other potential candidate genes include two vacuolar iron transporters, which regulate and facilitate the accumulation of soluble sugars in the plant. Brenton et al. (2020) recently described a species-specific tandem duplication that resulted in the two vacuolar iron transporter genes accounting for higher sugar accumulation. Soluble sugars are known signaling modules for responses such as plant stress; specifically, sucrose has been linked to anthocyanin accumulation in *Arabidopsis thaliana* (L.) Heynh., as well as activation of pathogenesis-related genes in rice and maize (Bolouri Modhaddam & Van den Ende, 2012; Solfanelli, Poggi, Loreti,

Alpi, & Perata, 2006; Thibaud et al., 2004; Gómez-Aiza et al., 2007). Like other phenolic and flavonoid compounds, anthocyanins have antimicrobial effects (Cisowska, Wojnicz, & Hendrich, 2011). Higher sugar levels may amplify the signal for anthocyanin accumulation, resulting in antimicrobial activity. Furthermore, soluble sugars such as sucrose control the expression of cyclins in *A. thaliana* (Riou-Khamlichi, Menges, Healy, & Murray, 2000). *Sobic.004G305700* and its maize and rice orthologs contain a cyclin domain. Proteins containing cyclin domains are ubiquitous, regulating the cell cycle and, in turn, several biological processes across all life. Cyclins have been found to be involved in plant stress responses (Kitsios & Doonan, 2011). Therefore, *Sobic.004G305700* may be a cyclin involved in a plant stress response. Sugar accumulation and signaling may play a role in regulating plant hormones that are responsible for stress responses such as antimicrobial activity. Further investigation of these potential candidate genes is needed to better understand their functional roles in sorghum's antimicrobial activity. Importantly, although we were successful in mapping loci for antimicrobial activity, we emphasize that that the antimicrobial effects assayed for mapping were specific to *C. perfringens* and therefore these loci may not be applicable to represent a broader range of pathogens. Further susceptibility testing on a wider and more diverse collection of bacteria is necessary to understand the genetic architecture regulating the antimicrobial potential of sorghum grain.

5 | CONCLUSIONS

Antimicrobial activity was identified in half of the accessions within the SAP. Although the inhibitory activity of sorghum extracts was demonstrated across both food-borne pathogens in the study, it was determined that sorghum inhibits *C. perfringens* more efficiently than *S. enterica.* Further, heritability estimates show that this activity is under moderate genetic control, and strong activity for the selected accessions was found to be reliable across field replicates. Antimicrobial activity was found to be insignificantly correlated with condensed tannins and total phenols, indicating that antimicrobial activity was not entirely dependent on the accumulation of these antinutrients. Additionally, antimicrobial activity did not negatively impact yield or other compositional traits. Novel associations found in the GWAS allowed for the identification of 20 potential candidate genes that may regulate antimicrobial activity. Using orthologs identified by OrthoDB, we characterized potential candidate genes that may be transcription factors known to regulate plant abiotic stress responses and phenolic metabolism. Subsequent studies are required to elucidate and validate the roles of the candidate genes, as well as the putative metabolites that may be responsible for the observed antimicrobial activity to fully reveal the genetic and mechanistic basis of this phenotype. However, this initial phenotypic and genetic evaluation of nearly 400 diverse sorghum accessions for antimicrobial potential provides valuable information on germplasm and genetic markers to facilitate the incorporation of natural antimicrobial activity via plant breeding for use in animal agriculture, which will provide a more healthy and sustainable feed grain for animal production systems.

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CONFLICTS OF INTEREST

The authors declare that there is no conflicts of interest.

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SUPPORTING INFORMATION

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