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Bioactive compounds from fermented *Vernonia amygdalina* leaf: Potent antibiotics against multidrug-resistant *Escherichia coli* and *Salmonella typhi*

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Running title: Fermentation promotes the potential for antibiotics in Bitter-leaf extracts.

Abstract

Antibiotic resistance microorganisms (ARMs), especially gram-negative bacteria, are a global health threat. The impact of fermentation on phytochemicals is numerous, and exploring this potential is the front line of drug development. The study investigates the role of fermentation in modifying *V. amygdalina* leaf secondary metabolites as a potent antibiotic against *Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhi*. The results of this work showed that the fermentation process increased the content of lycopene, flavonoid, and carotenoid compounds but decreased chlorophyll, soluble protein, and phenol. Pearson's correlation *heatmap* showed a strong correlation between microbial activities and secondary metabolic changes. The methanolic extract of fermented *V. amygdalina* leaf pulp (VALP) showed significant antioxidant and anti-inflammatory activities. The GCMS and FTIR outputs showed unique compounds and structural modifications at different intervals of the fermentation period. *In-silico* analyses showed that 4,6-Cholestadien-3 β -ol – a compound that is unique to the 9th day of fermentation was responsible for the inhibition of the gram-negative bacteria via multiple non-ionic interactions with key catalytic site residues and higher binding affinity to OmpC, OmpF, and RNase P than ampicillin. Therefore, this steroid-derived compound may open a new pipeline into the development of multi-target antibiotics against a broad-spectrum multidrug-resistant gram-negative bacterial.

Keywords: Antibiotics-resistant, *E. coli*, *B. subtilis*, *S. typhi*, anti-inflammatory, *V. Amygdalina*, antioxidants

1 Introduction

Antibiotic resistance microorganisms (ARMs) pose a significant health threat, causing 5 million deaths worldwide in 2019 [1]. In the USA, ARMs cause 2.8 million infections and 35,000 deaths annually. Despite WHO measures, ARM deaths have risen by 700,000 annually and are projected to be 10 million by 2050 [2]. Sub-Saharan Africa, including Nigeria, is experiencing alarmingly high ARM indices, with 99 deaths per 100,000 people [3].

The increased mortality rate in Gram-negative bacteria (GNB) is attributed to inadequate awareness, wrong diagnoses, misuse, abuse, and poor disposition of non-biodegradable antibiotics [1,4]. The WHO 2017 report highlights that GNB occupies a significant portion of the list of multidrug-resistant (MDR) pathogens, with Enterobacteriaceae like *Klebsiella spp.*, *Enterobacter spp.*, and *Escherichia coli* not exempted [4,5]. The MDR features of GNB are attributed to their unique triple-layered envelope, which has multiple defence mechanisms, including outer-membrane porins, β -lactamases, aminoglycosides modifying enzymes, and plasmid-borne antibiotic-resistance genes. The origins of MDR gene expression in bacteria are natural and acquired, with GNB exploring the latter due to its spontaneous mutation rate and resistance to β -lactams and tetracycline [1,4–7]. As a result, developing potent antibiotics against GNB is tailored to inhibit the efficiency of the outer membrane machinery like Omps without ignoring the suppression of its gene expression via ribonuclease inactivation.

Plants, including *Vernonia amygdalina*, can resist localised stress from both abiotic and biotic sources, including microbial infections, due to their ability to synthesise phytochemicals. *V. amygdalina*, a bitter leaf, has antioxidant, anti-inflammatory, antidiabetic, and antibiotic potentials [8,9]. In southern Nigeria, bitter leaf juice has been used to treat enteric disorders like bacterial infection and deworming [10]. Despite its anti-antibiotic roles and acceptability in this region, ARMs-related infection incidences and mortality are alarming, according to the WHO 2022 report [2,3].

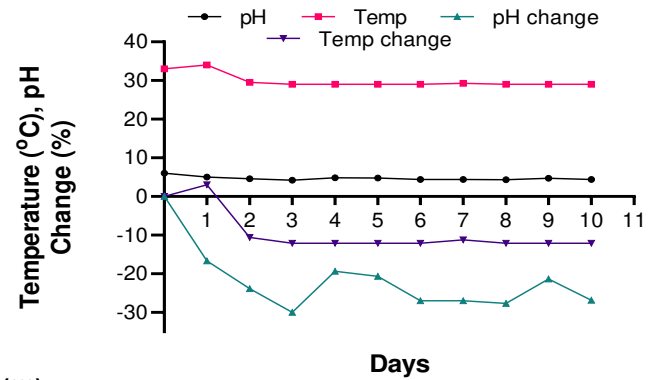
Fermentation, a microbe-driven biological process, enhances plant product chemical constituents, phytochemical profile, and health benefits, providing biodegradable, environmentally friendly compounds, particularly antibiotics [11–14]. The study aimed to investigate the impact of fermentation on the phytochemicals, antioxidants, and antibiotic properties of *V. amygdalina* against antibiotics-resistant GNB, using an in-vitro assay and an in-silico method to study the possible mechanism of interaction between promising metabolites and the bacteria antibiotics target-proteins.

2 Results

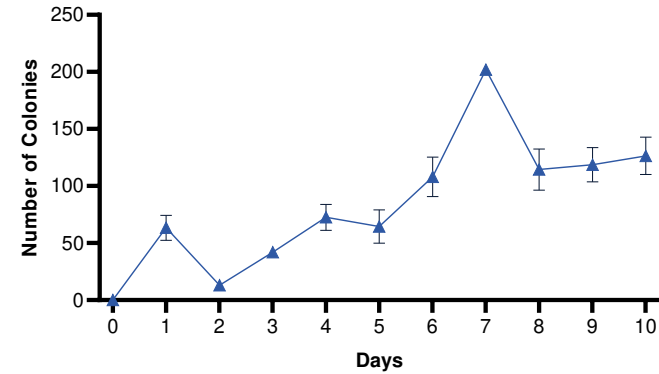
2.1 Effect of fermentation on the physicochemical changes in *V. amygdalina* leaf pulp (VALP) methanolic extract

Figure 1a (i - iii) shows the effect of pH, temperature and fermentation time on the microbial load and physiochemical constituents of *V. amygdalina* leaf pulp (VALP) methanolic extract. Figure 1b (i - v) shows the Pearson correlation *heatmap* of microbial load, physiochemical changes, and bioactivities of the fermented VALP (f-VALP) methanol extract. In Figure 1a (i), the percentage change in the temperature rose by 5 % within the first 24 hours but dropped by 15 % twenty-four later. A steady temperature was noted from day 3 to the terminal point of the fermentation period (day 10). The pH change from day 0 to day 3 consistently decreased to 30 %; from days 4 to 10, the pH change rose and dropped within 10 %. Figure 1a(ii) showed that the microbial load of the fermented sample increased by 70 folds within the first 24 hours and then decreased by about 50 units at day 2. A steady increase of an average of 30 colonies per day was noted from day 3 to day 10, except at day 7, which had a sharp increase. In Figure 1a(iii), chlorophyll, phenols, total protein, and ascorbic acid decreased significantly within the first 24 hours, while flavonoids and lycopene increased within the same period. Between days 1 and 6, the phytochemicals were not significantly altered except chlorophyll and lycopene, which decreased and increased. Between days 7 and 10, the phenols and ascorbic acid contents increased significantly while other phytochemicals decreased.

(i)



(ii)



(iii)

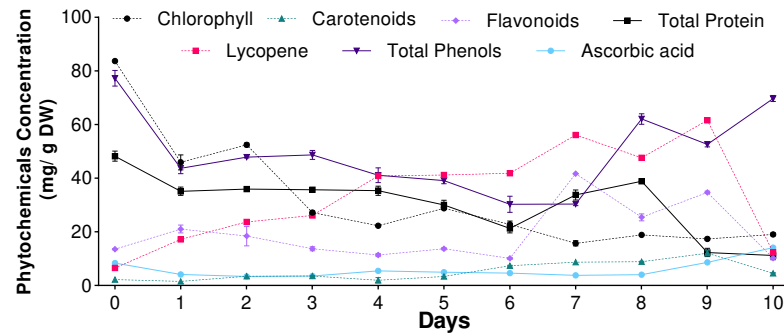
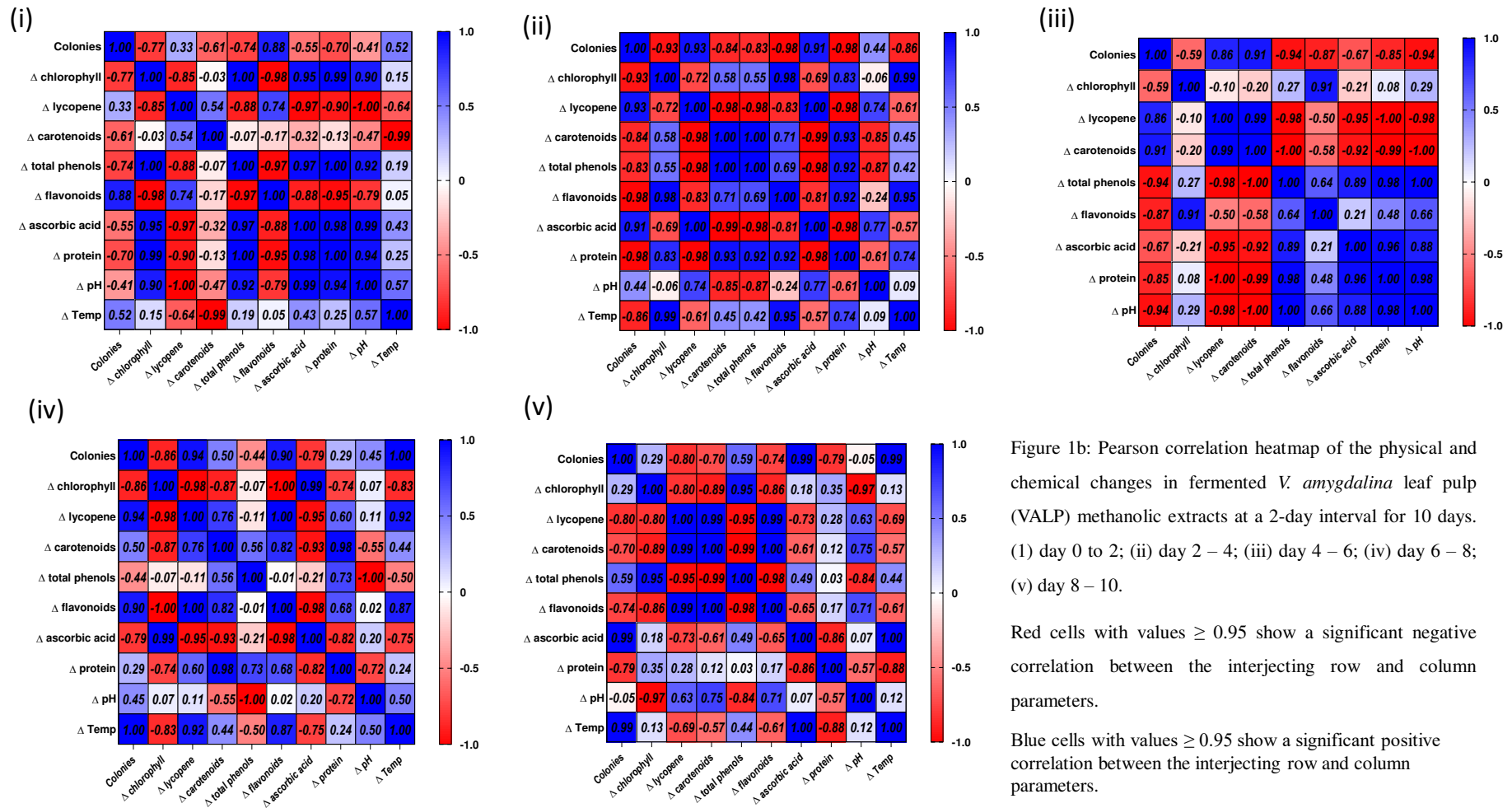


Figure 1a: Effect of fermentation period on the phytochemical profiles of *V. amygdalina* leaf pulp (VALP). (i) temperature, pH, temperature change (%) and pH change per day; (ii) bacterial colonies count per day; (iii) Chlorophyll, carotenoids, flavonoids, total protein, lycopene, phenols and ascorbic acid contents of fermented VALP for 10 days.

The Pearson's correlation *heatmap* between the microbial colonies, temperature, pH and phytochemicals of the *f*-VALP was presented in Figure 1b (i - v); each figure depicts the correlations of the variables mentioned earlier at an interval of two days. The association between two variables are considered to be positively or negatively correlated, provided such associations have a p-value ≥ 0.95 or ≤ -0.95 , respectively; this value corresponds to the 95% level of confidence in probability.

At days 0 – 2 (Fig 1b(i)), no correlation was observed between the microbial proliferation, phytochemicals, pH and temperature changes. In Fig. 1b(ii) (days 2 – 4), the microbial proliferation rate was negatively correlated with the changes in flavonoids and protein. Furthermore, on days 4 - 6 (Fig. 1b(iii)), the change in the flavonoids, protein contents, and temperature was negatively associated with the bacterial proliferation rate. On days 8 – 10 (Fig. 1h), changes in ascorbic acid and temperature were the two parameters that showed a positive association with bacterial growth rate.



2.2 Effect of fermentation on the in-vitro antioxidant and anti-inflammatory profile of *V. amygdalina* leaf pulp (VALP) methanolic extract

The bioactivities assessment of the *f*-VALP methanolic extract was presented in Figure 2a, and Figure 2b (i - v) showed Pearson's correlation *heatmap* between the phytochemicals and bioactivities (antioxidants and antiinflammation).

In Figure 2a, the IC₅₀ values of the VALP methanolic extract against DPPH, protein denaturation and lipid peroxidation were significantly lowered at day 2 (48 hours fermentation). In contrast, the IC₅₀ value against hydrogen peroxide increased within the same period. The free radicals scavenging capacity of the fermented sample was not significantly altered from day 3 to day 8 except for the protein denaturation. The VALP methanolic extract, on day 9, had the lowest IC₅₀ against DPPH, hydrogen peroxide, protein denaturation, and lipid peroxidation. Compared to the ascorbic acid, the IC₅₀ value of the unfermented and *f*-VALP methanol extract was higher against hydrogen peroxide and lipid peroxidation, while other free radicals scavenging and protein stabilisation IC₅₀ values were lower. A comparison between days 0 – 8, 10 and diclofenac showed that diclofenac had a lower IC₅₀ value in protein stabilisation; however, day 9 showed a lower IC₅₀ value than the diclofenac.

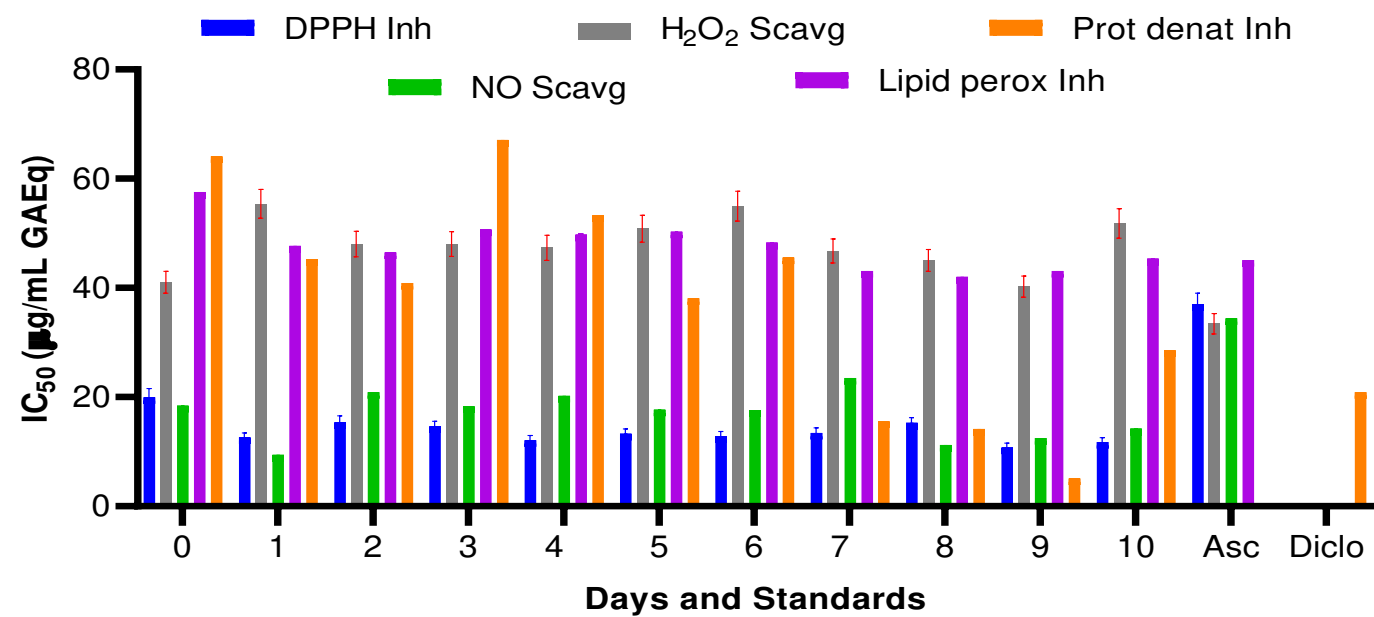


Figure 2a: Effect of fermentation period on the *in-vitro* antioxidant and anti-inflammatory properties of methanolic extract of *V. amygdalina* leaf pulp (VALP).

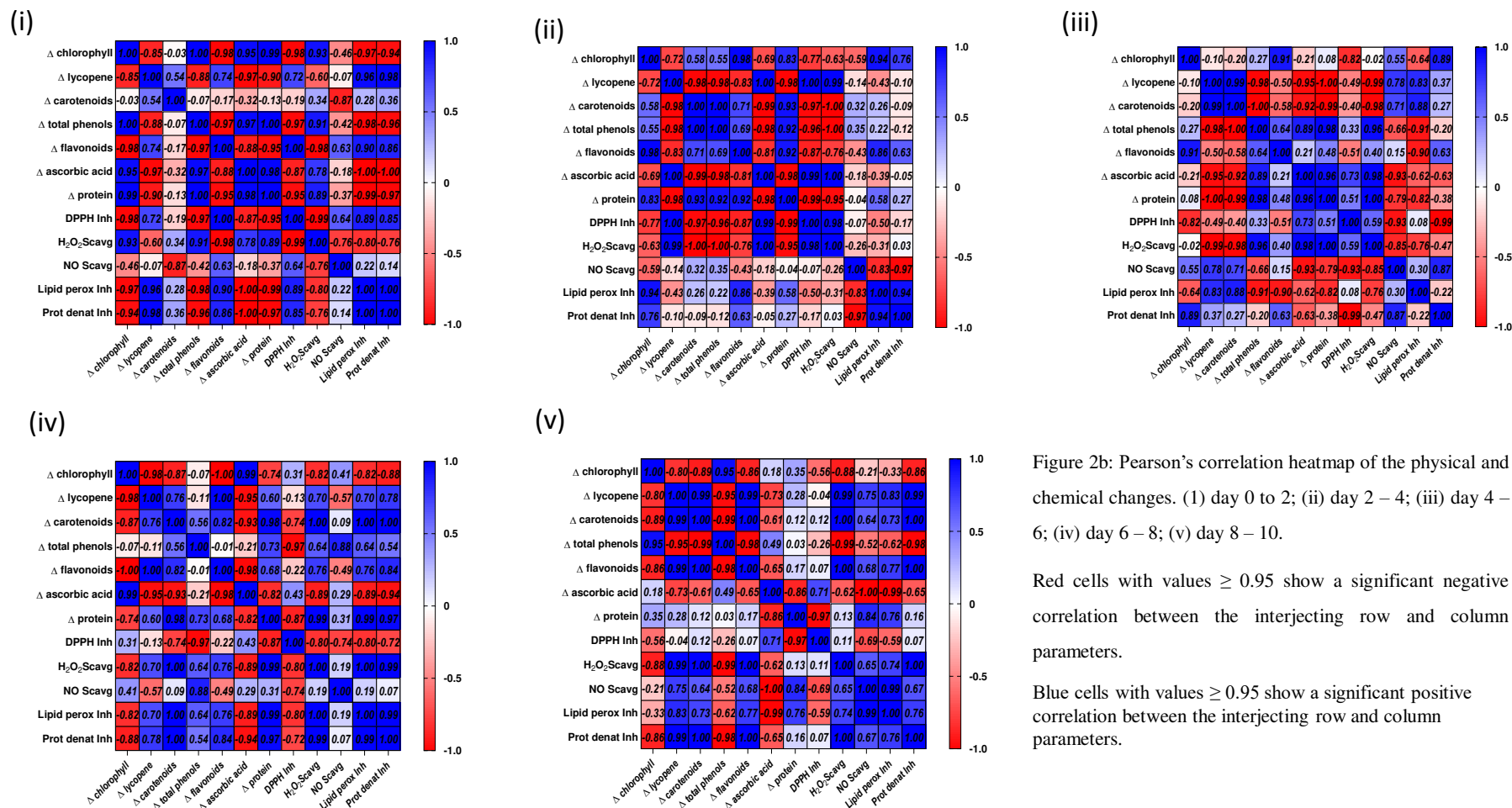
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121 In Figure 2b(i), Pearson's correlation *heatmap* showed that changes in chlorophyll content are
122 negatively correlated to DPPH, lipid peroxidation and protein denaturation inhibition (days 0 -
123 2). Meanwhile, the shift in lycopene content positively correlates with lipid peroxidation and
124 protein denaturation inhibition within the same period. No significant association between the
125 carotenoid change, the antioxidants and the anti-inflammatory parameters investigated were
126 recorded during the first 48 hours. The phenol change was negatively correlated with DPPH,
127 lipid peroxidation and protein denaturation inhibition within the same period. The change in
128 the flavonoid content was positively associated with the DPPH inhibition, although this change
129 was negatively associated with hydrogen peroxide inhibition. Also, the changes in ascorbic
130 acid and protein contents were negatively correlated with lipid peroxidation and protein
131 denaturation inhibition levels, and the change in protein content was negatively correlated with
132 hydrogen peroxide inhibition.

133 Figure 2b(ii) (day 2 - 4) revealed that change in the lycopene content was positively correlated
134 with the changes in the DPPH and hydrogen peroxide inhibition; however, a negative
135 correlation was noted between the changes in carotenoids, phenols, flavonoids, protein
136 contents, and hydrogen peroxide and DPPH inhibition level. Meanwhile, the change in ascorbic
137 acid content was positively correlated with DPPH and hydrogen peroxide inhibition.

138 Between days 4 and 6 (Fig. 2b (iii)), lycopene and carotenoid changes were negatively
139 associated with hydrogen peroxide inhibition levels. Also, phenols and flavonoid changes were
140 negatively correlated with lipid peroxidation inhibition level, although phenols and protein
141 changes correlated with hydrogen peroxide inhibition. In (days 6 – 8), the carotenoids and
142 protein content changes were positively associated with hydrogen peroxide inhibition, lipid
143 peroxidation and protein denaturation inhibition (Figure 2b(iv)).

144 In Fig 2b (v) (days 8 – 10), carotenoids, lycopene and flavonoid content changes were
145 positively associated with hydrogen peroxide and protein denaturation inhibition rate. On the
146 other hand, change in phenols was negatively correlated with nitric oxide scavenging and
147 protein denaturation inhibition. Similarly, changes in ascorbic acid content negatively
148 correlated with nitric oxide scavenging and lipid peroxidation inhibition.



150

151 **2.3 Effect of fermentation on the antibiotic activity of *V. amygdalina* leaf pulp (VALP)**
152 **methanolic extract against *E. coli*, *B. subtilis* and *S. typhi***

153 In Figure 3, the effect of the fermentation period on the antibiotic properties of VALP-
154 methanolic extract via *in-vitro* antibiotics susceptibility test against three multidrug-resistant
155 gram-negative bacteria (*E. coli*, *B. subtilis* and *S. typhi*) was presented. The minimum
156 inhibition concentration (MIC) of the *f*-VALP methanolic extract against *E. coli* reduced as the
157 fermentation period progressed except on days 3 and 5, when it increased and decreased,
158 respectively. Although the MIC value of VALP methanolic extract was the lowest,
159 fermentation did not alter its inhibitory action against *B. subtilis*. The inhibitory action of
160 VALP methanol extract against *Salmonella typhi* was not recorded until day 4 of fermentation.
161 This inhibitory activity was further observed on days 5 – 8, 9 and 10.

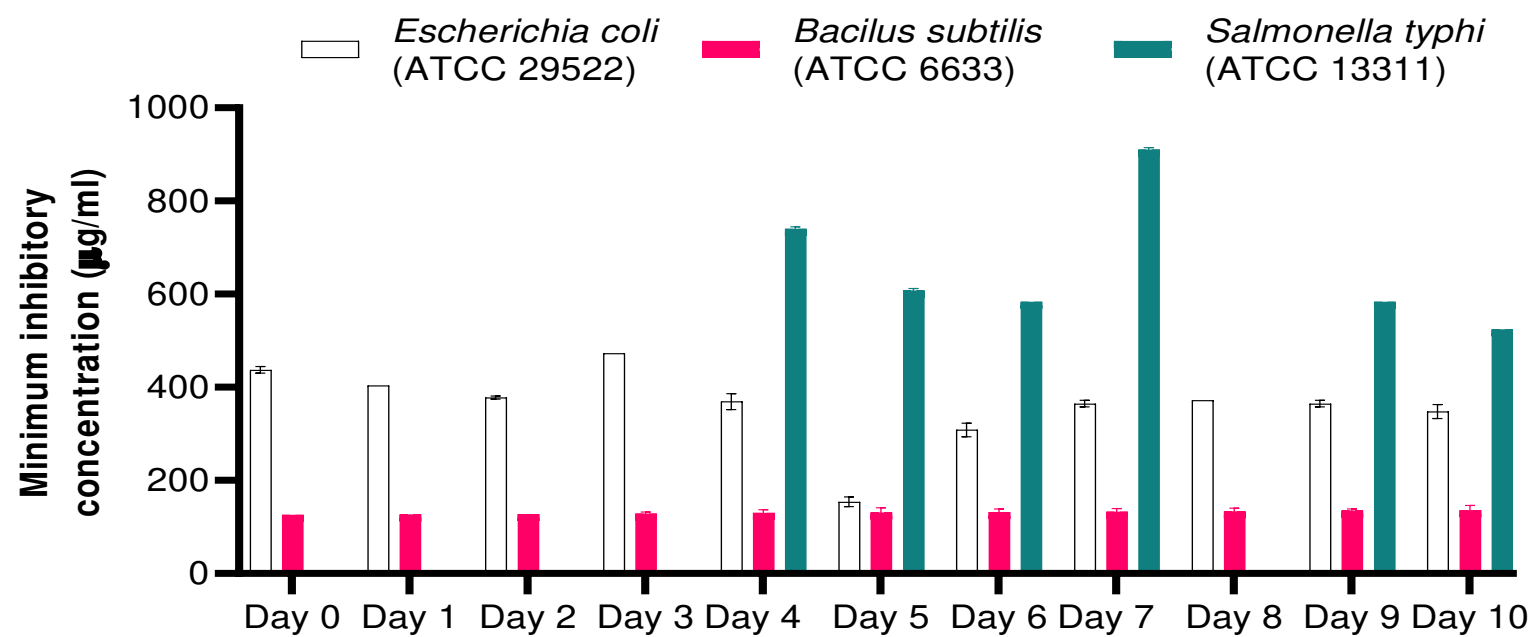


Figure 3: *In-vitro* minimum inhibitory concentration potential of fermented *V. amygdalina* leaf pulp (f-VALP) methanolic extracts against *E. coli*, *B. subtilis* and *S. typhi*.

2.4 Morphological and biochemical identification of bacteria present in fermented *V. amygdalina* leaf pulp (VALP)

The data presented in Table 1 are the morphology and biochemical characteristics of the seven bacteria isolated from the *f*-VALP sample. Five of the seven isolates were small, cocci, gram-positive bacteria, while the other two were rod-like, large and gram-negative morphologically (Table 1a). In Table 1b, the biochemical identification tests showed that two of the seven isolates were negative for MSA (mannitol salt agar); four isolates were yellow, and one had a creamy colour to MSA. All the isolates were pink in MCA (MacConkey agar) and negative in SSA (salmonella shigella agar), DRA (DeMan-Rogosa agar) and CA (cetrimide agar) tests. Two of the seven isolates showed brick colour in the eosin methylene blue agar. Hence, those two (isolates 2 and 3) are probably *Klebsiella spp.*, while four isolates (1, 4, 5 and 7) are suspected to be *Staphylococcus spp.* While isolate 6 is probably *Enterococcus spp.*

Table 1c presents the biochemical characteristics of enzymatic activities and carbon source metabolism. Six isolates were positive for the catalase, citrate and urease test, while *Enterococcus spp.* was negative. All the isolates were negative for oxidase, indole, hydrogen sulfide, and motility tests. Only isolates 2 and 3 (*Klebsiella spp.*) were positive for the gas production test. Sugar fermentation tests showed that the isolates were positive for glucose, maltose, dextrose, fructose and fructose fermentation, while *Staphylococcus spp.* (isolates 4, 5 and 7) did not ferment cellulose, and isolates 1, 4, 5 and 7 (*Staphylococcus spp.*) could not ferment lactose.

Table 1: Morphology and Biochemical Characteristics of bacteria isolated from fermented *Vernonia Amygdalina* leaf

Table 1a: Morphology of isolates from fermented *V. amygdalina*

Isolates	1	2	3	4	5	6	7
Size	Small	Large	Large	Small	Small	Small	Small
Colour	Creamy	Deep pink	Deep pink	Creamy	Creamy	Gray	Creamy
Edges	Round	Round	Round	Round	Round	Round	Round
Clarity	Translucent	Opaque	Opaque	Translucent	Translucent	Opaque	Translucent

Elevation	Raised	Raised	Raised	Raised	Raised	Smooth	Raised
Gram-stain	+ve	-ve	-ve	+ve	+ve	+ve	+ve
Shape	Cocci	Rod	Rod	Cocci	Cocci	Cocci	Cocci

Table 1b: Biochemistry of isolates from fermented *V. amygdalina*

Isolates	1	2	3	4	5	6	7
Mannitol Salt Agar	Yellow	NG	NG	Yellow	Yellow	Creamy	Yellow
MacConkey Agar	Light pink	Deep pink	Deep pink	Light pink	Light pink	Pink	Light pink
Salmonella Shigella Agar	NG	NG	NG	NG	NG	NG	NG
DeMann-Rogosa Agar	NG	NG	NG	NG	NG	NG	NG
Cetrimide Agar	NG	NG	NG	NG	NG	NG	NG
Eosin Methylene Blue Agar	NG	Brick-red	Brick-red	NG	NG	NG	NG
Probable bacteria	<i>Staphylococcus spp.</i>	<i>Klebsiella spp.</i>	<i>Klebsiella spp.</i>	<i>Staphylococcus spp.</i>	<i>Staphylococcus spp.</i>	<i>Enterococcus spp.</i>	<i>Staphylococcus spp.</i>

Key: NG - No growth

Table 1c: Biochemical test of isolates from fermented *V. amygdalina*

Bacteria	<i>Staphylococcus spp.</i>	<i>Klebsiella spp.</i>	<i>Klebsiella spp.</i>	<i>Staphylococcus spp.</i>	<i>Staphylococcus spp.</i>	<i>Enterococcus spp.</i>	<i>Staphylococcus spp.</i>
Catalase	+ve	+ve	+ve	+ve	+ve	-ve	+ve
Oxidase	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Indole	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Citrate	+ve	+ve	+ve	+ve	+ve	-ve	+ve
Urease	+ve	+ve	+ve	+ve	+ve	-ve	+ve
H ₂ S	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Motility	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Test							
Gas	-ve	+ve	+ve	-ve	-ve	-ve	-ve
Production							
Glucose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Cellulose	+ve	+ve	+ve	-ve	-ve	+ve	-ve
Maltose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Dextrose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Fructose	+ve	+ve	+ve	+ve	+ve	-ve	+ve
Sucrose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Lactose	-ve	+ve	+ve	-ve	-ve	+ve	-ve

Note: +ve (positive); -ve (negative)

2.5 Effect of fermentation on the FTIR and GCSM characterised *V. amygdalina* leaf pulp (VALP) methanolic extract

The FTIR spectra of the *f*-VALP (Days 1 - 10) and *unf*-VALP (Day 0) methanolic extracts are presented in Fig 4a. To further understand metabolite patterns in the fermented sample and their relatedness, an FTIR exploratory approach was adopted (Fig 4b). The unique regions of the FTIR fingerprint for carbohydrates (1200 - 950 cm⁻¹) [29,30], lipids (3100 - 2800 cm⁻¹ combined with 1800 - 700 cm⁻¹) [31,32], proteins (1700 - 1500 cm⁻¹) [33,34] and phenolics (3400 - 3200 cm⁻¹) [35,36] were explored for each of the macromolecules. The hierarchy component analysis (HCA) *heatmap* in Figures 4b (i – iv) showed the unfermented (day 0) and fermented (days 1 - 10). In Fig 4b (i), the carbohydrate hierarchy of the days 0, 2, 3, 4, 6 and 7 (A, C, D, E, G, H and I) are closely associated, while the other closely related carbohydrate group is days 1, 5, 9 and 10 (B, F, J and K). In the lipids hierarchy *heatmap* group (Fig 4b (ii)), days 0 – 2, 4 and 10 (A, B, C and E) were closely related, while days 3, 5, 6 – 9 (D, F, G and J) belong to the second hierarchy. Figure 4b(iii) showed that protein hierarchy relatedness of days 0, 1 and 4 (A, B and E) was higher, while days 2, 6 – 9 and 10. (C, G, H, I,

203 J and K) are closely related. Days 3 and 5 (D and F) appear outliers in this hierarchy relatedness.
204 The phenols hierarchy (Fig 4b (iv)) relatedness gives two distinct groups: days 0, 1, 3, 4 and 5
205 (A, B, D, E and F) are closely related, while days 2, 6 – 10 (C, G, H, I, J and K) are closely
206 related. The HCA *heatmap* of the fatty acids, protein and phenols grouping showed that two
207 significant groups could be deduced as unfermented and fermented bitter leaf extracts.
208 However, days 3 (D) and 5 (F) appear more closely related in the three biomolecules HCA
209 *heatmap* and appear as outliers in the protein HCA *heatmap*; thus, these two fermentation days
210 were categorised as a distinct group and formed groups 3

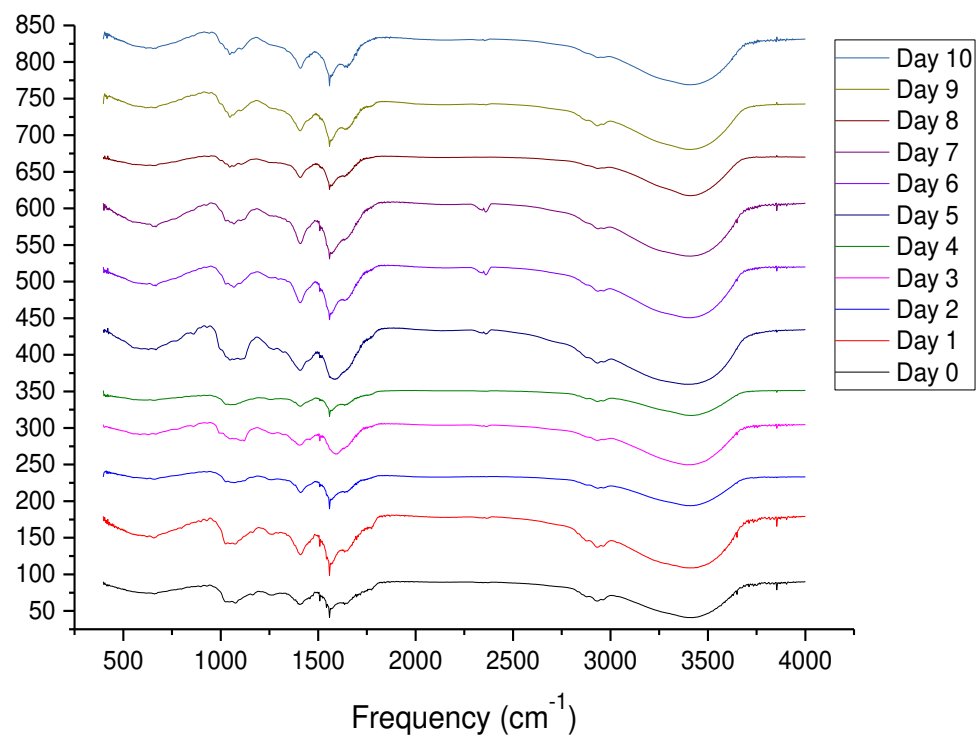
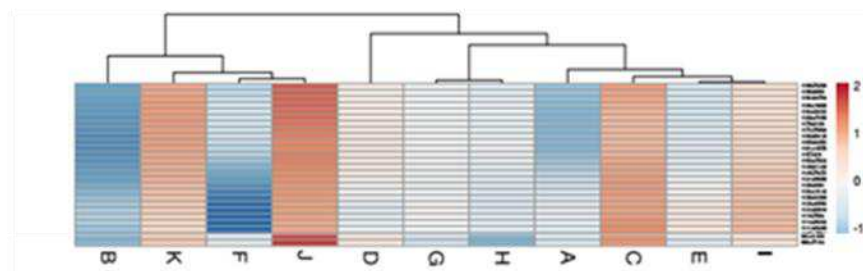


Figure 4a: Fourier transformer infrared Spectra of unfermented (day 0) and fermented (days 1 to 10) *V. amygdalina* leaf pulp (VALP) methanolic extracts.

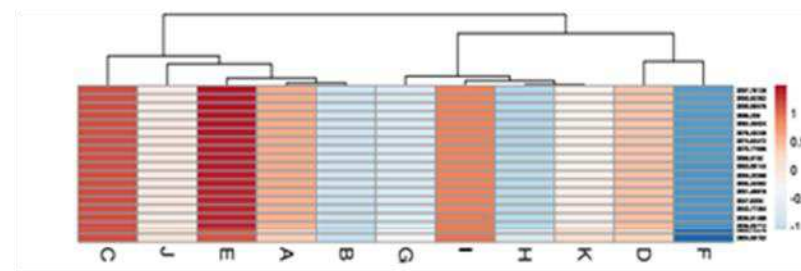
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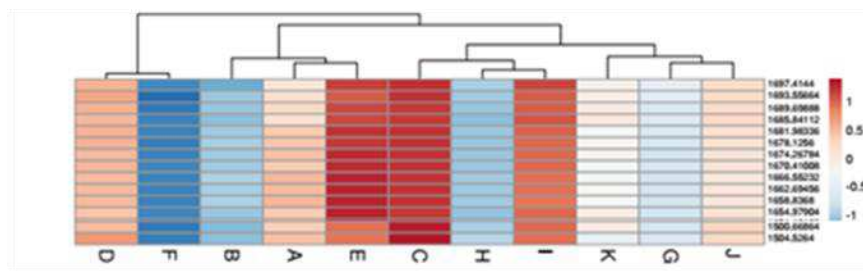
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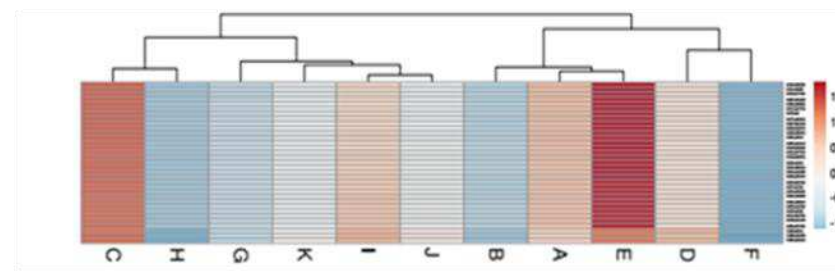
(i) 1,200–850 cm^{-1}



(ii) 3100–2800 cm^{-1}



(iii) 1700–1500 cm^{-1}



(iv) 1700–1500 cm^{-1}

Figure 4b: Fourier transform infrared Spectra of unfermented (day 0) and fermented (days 1 to 10) *V. amygdalina* leaf pulp (VALP) methanolic extracts. (i) The unique regions of the FTIR fingerprint for carbohydrates (1200 - 950 cm^{-1}) [30,31]; (ii) The unique regions of the FTIR fingerprint for lipids (3100 - 2800 cm^{-1} combined with 1800 - 700 cm^{-1}) [32,33]; (iii) proteins (1700 - 1500 cm^{-1}) [34,35]; (iv) The unique regions of the FTIR fingerprint for phenolics (3400 - 3200 cm^{-1}) [36,37].

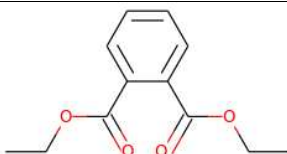
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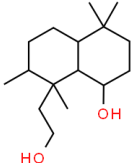
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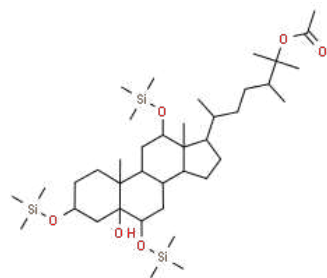
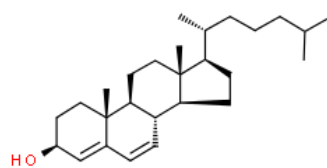
219 Table 2 presents the GCMS-identified compounds in unfermented and *f*-VALP methanolic
220 extracts (Suppl Fig. 1 - 3). Based on the relatedness of the HCA *heatmap observations*, a
221 *representative of each of the 3 groups was used for GCMS analysis, and the representatives were*
222 day 0 (unfermented), day 3 (mid-fermentation time) and day 9 (terminal fermentation). To
223 further study the dynamics of the effect of fermentation on the phytochemicals, retention time
224 (RT) was used to group the compounds; hence, nine retention times were generated. The GCMS
225 identified compounds on day 0 were 4 identified; on day 3, 6 compounds were identified, and 4
226 compounds on day 9. Only Diethyl phthalate was the compound common to day 0
227 (unfermented), 3 and 9. In addition, methyl N-hydroxybenzenecarboximidate was identified on
228 days 0 and 3. 1-Tetradecanol and 15,17,19-Nonacosatriynoic acid were unique to day 0 only; 1-
229 Heptadecanol, 1, 2-Diethoxyethane, Ergostane-5,25-diol, 3,6,12-tris[(trimethylsilyl)oxy]-, 25-
230 acetate and Benzenepropanoic acid, 3,5-bis(1,1-dimethyl ethyl)-4-hydroxy-, octadecyl ester
231 were the unique compounds present in the day 3 only. While, day 9 has 2,2-Dimethylbutane, 8-
232 (2-Hydroxyethyl)-4,4,7,8-tetramethyldecahydro-1-naphthalenol and 4,6-Cholestadien-3 β -ol as
233 it unique compounds.

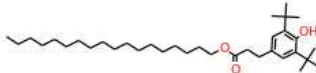
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235 Table 2: Compounds identified from Gas Chromatography-Mass Spectrometry analysis of the fermented and unf-VALP methanolic extract.

S/No	Days	RT (min)	Area (%)	Height (%)	A/H	Compound name [SMILES]	Abbreviations	Structure	
1	0	5.904	30.09	47.29	1.08	Methyl N-hydroxybenzenegcarboximidate COC(=NO)c1ccccc1	MHBX		
	3	5.804	12.48	39.37	1.12				
	9		-	-	-	-	-		
	0		-	-	-	-	-		
2	3		-	-	-	2,2-Dimethylbutane CCC(C)(C)C	DMBU		
	9	6.634	6.08	6.58	2.71				
	0	12.873	46.32	29.91	2.63	Diethyl phthalate	DEPH		
	3	12.870	20.94	31.18	2.38	<chem>CCOC(=O)c1ccccc1C(=O)OCC</chem>			
9	12.869	43.32	53.94	2.36					
4	0	17.861	5.91	6.62	1.51	1-Tetradecanol CCCCCCCCCCCCCCCCO	TETD		

S/No	Days	RT (min)	Area (%)	Height (%)	A/H	Compound name [SMILES]	Abbreviations	Structure
	3	17.862	2.36	4.20	1.99	1-Heptadecanol CCCCCCCCCCCCCCCCCCO	HEPL	
	9	-	-	-	-			
	0	-	-	-	-			
	3	18.817	16.84	5.35	11.14	1,2-Diethoxyethane CCOCCOCC	DITH	
5	9	18.650	25.87	12.75	5.95	8-(2-Hydroxyethyl)-4,4,7,8-tetramethyldecahydro-1-naphthalenol CC1CCC2C(C(O)CCC2(C)C)C1(C)CCO	HTOL	
6	0	-	-	-	-			

S/No	Days	RT (min)	Area (%)	Height (%)	A/H	Compound name [SMILES]	Abbreviations	Structure
	3	19.781	6.45	4.38	5.21	Ergostane-5,25-diol, 3,6,12-tris[(trimethylsilyl)oxy]-, 25-acetate <chem>CC(=O)OC(C)(C)[C@H](C)CC[C@@H](C)[C@H]1CC[C@H]2[C@@H]3C[C@@H](O[Si](C)(C)C)[C@@]4(O)C[C@@H](O[Si](C)(C)C)CC[C@]4(C)[C@H]3C[C@@H](O[Si](C)(C)C)[C@@]21C</chem>	EGTA	
	9	19.046	16.39	17.96	2.68	4,6-Cholestadien-3β-ol <chem>CC(C)CCC[C@@H](C)[C@H]1CC[C@H]2[C@@H]3C=CC4=C[C@@H](O)CC[C@]4(C)[C@H]3CC[C@@]21C</chem>	CHDL	
7	0	22.677	17.69	16.18	1.86	15,17,19-Nonacosatriynoic acid <chem>CCCCCCCCC#CC#CC#CCCCCCCCCCCCC CC(=O)O</chem>	NTYA	

S/No	Days	RT (min)	Area (%)	Height (%)	A/H	Compound name [SMILES]	Abbreviations	Structure
3	22.461	40.94	15.52	9.34	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	CCCCCCCCCCCCCCCCCCCCCOC(=O)CCc1cc(C(C)(C)C)c(O)c(C(C)(C)C)c1	BPHE	
9	-	-	-	-	-	-	-	-

236

2.6 Summary of the *In-silico* drug-likeness screening of the GCSM identified compounds in fermented *V. amygdalina* leaf pulp (VALP) methanolic extract against *E. coli*, *B. subtilis* and *S. typhi*

The data in Table 3 depicts the structure-function relationship and drug-likeness of compounds identified in unfermented and fermented bitter leaf methanolic extracts. This study used Lipinski, Pfizer, GSK and GoldenTriangle for drug-likeness screening (Supplementary Table 1), summarised in Table 3. Hence, the drug-likeness result of the compounds showed that all the drug-likeness rules rejected two of the nine compounds.

Table 3: In-silico quantity structure-activity relationship (QSAR) and drug-likeness Screening of GCMS-identified compounds in unfermented and fermented bitter leaf methanolic extracts.

Compound name	Lipophilicity (Log Po/w (iLOGP))	Water Solubility	Pharmacokinetic s (GI absorption)	Druglikenes s
MHBX	1.72	Soluble	0.006	Accepted
DMBU	3.68	Soluble	0.015	Accepted
DEPH	2.68	Soluble	0.002	Accepted
TETD	5.80	Moderately soluble	0.003	Accepted
HEPL	7.24	Insoluble	0.004	Accepted
DITH	0.76	Soluble	0.001	Accepted
HTOL	3.61	Soluble	0.006	Accepted
EGTA	9.33	Insoluble	0.012	Rejected
CHDL	7.16	Moderately soluble	0.006	Accepted
NTYA	10.24	Insoluble	0.005	Accepted
BPHE	11.37	Moderately soluble	0.004	Rejected

2.7 Molecular docking analysis of the antibiotic activity of *V. amygdalina* leaf pulp (VALP) methanolic extract against *E. coli*, *B. subtilis* and *S. typhi*

Table 4 presents the effect of fermentation on VALP methanolic extracts as an antibiotic agent via *in-silico* protein-ligand interaction (molecular docking). Two molecular docking platforms, a standalone offline (Pyrx-Python Prescription version 0.8) and an online (CB-Dock2), were

used to promote the reliability of the work. Clinically available antibiotics (ampicillin and puromycin) were adopted as the reference ligands for the antibiotics-target proteins in the *in-vitro* antibiotics susceptibility test. The primary rule for selecting a promising compound in this study was that the binding energy values generated by the two virtual screening platforms for a ligand-protein interaction must be greater than or equal to the corresponding reference ligand-protein interaction.

The data in Table 4 revealed that 4,6-Cholestadien-3 β -ol (CHDL), which is unique to the day 9 fermentation sample, showed binding energy of -7.5 KJmol⁻¹, -6.4 KJmol⁻¹ and -7.8 KJmol⁻¹ while the reference compounds had -7.3 KJmol⁻¹, -6.3 KJmol⁻¹ and -7.2 KJmol⁻¹ to 4GCS, 1A6F and 3UU2 respectively in Pyrx. The result from CB-Dock, CHDL had -8.1, -5.9 and -7.8, while the reference compounds had -5.1, -6.4 and -7.3 to 4GCS, 1A6F and 3UU2, respectively. The results generated from the two virtual molecular docking platforms were similar for most of the compounds, while those that had varying values had differences ranging between 0.1 to 0.9 KJmol⁻¹.

Table 4: Molecular docking phytochemicals from fermented and unfermented *V. amygdalina*.

	Binding Affinity (kJ mol ⁻¹)					
	4GCS		1A6F		3UU2	
	Pyrx	CB-DOCK2	Pyrx	CB-DOCK2	Pyrx	CB-DOCK2
Antibiotics	-7.3*	-5.1*	-6.3**	-6.4**	-7.2*	-7.3*
MHBX	-5.4	-5.1	-4.6	-4.5	-5.3	-5.3
DMBU	-3.8	-3.4	-3.2	-2.9	-4.3	-3.5
DEPH	-5.4	-5.4	-4.5	-4.3	-5.5	-5.7
TETD	-4.4	-4.6	-4.0	-3.9	-4.8	-4.7
HEPL	-5.2	-4.7	-3.7	-3.8	-4.4	-5.2
DITH	-3.4	-3.3	-3.3	-3.4	-3.6	-3.4
HTOL	-6.1	-6.1	-4.8	-4.9	-6.4	-5.9
CHDL	-7.5	-8.1	-6.4	-5.9	-7.8	-7.8
NTYA	-4.4	-5.4	-5.1	-4.9	-5.4	-5.1

Known antibiotics of respective target protein: *Ampicillin;**Puromycin

Figure 5 represents the binding poses of standard antibiotics (ampicillin) and the CHDL to OmpF porin (4GCS) in the 2D, 3D receptor-ligand pose and 3D forms. The binding interactions show that both compounds bind to the same cavity within the core of the ompF porin (4GCS).

275 While ampicillin had four hydrogen bonds (HB) to four residues and eight non-hydrogen bonds
276 (non-HB), one unfavourable interaction was observed. In the 4GCS-CHDL interaction, one
277 HB and thirteen non-HBs were noted.

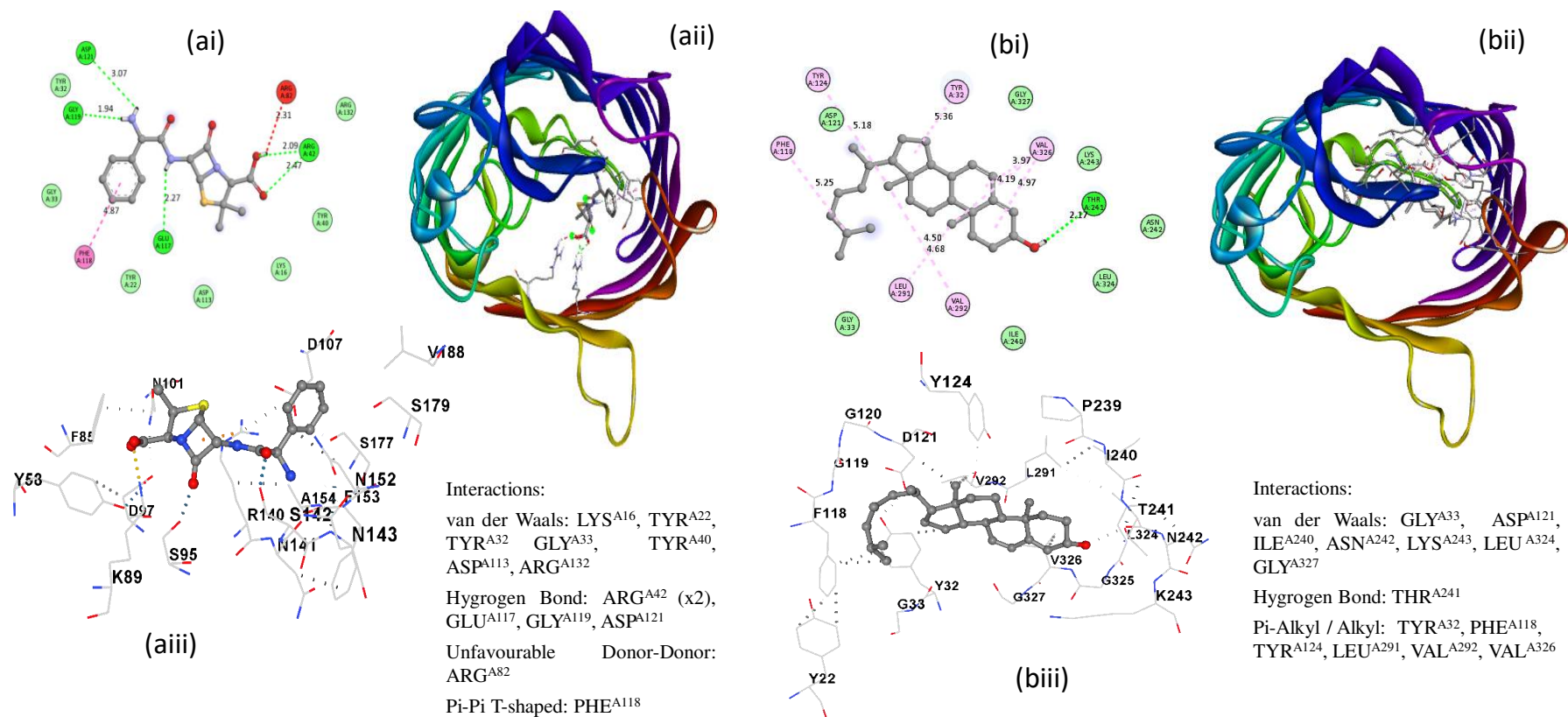


Figure 5: Binding orientation of ampicillin and the phytochemical (CHDL) with a higher binding affinity than ampicillin to (4GCS) OmpF porin of *E. coli*. (ai - iii) 2D and 3D of ampicillin interaction with 4GCS, (bi - iii) 2D and 3D of CHDL interaction with 4GCS.

279

280 Figure 6 presents the binding poses of a puromycin and the CHDL to *B. subtilis* ribonuclease
281 P protein (1Å6F) in the 2D, 3D receptor-ligand pose and 3D forms. The binding interactions
282 showed that puromycin and CHDL bind within the same cavity and shared non-HB with
283 identical residues in the cavity. While puromycin had 2 HB and 11 non-HB with 1a6f, CHDL
284 showed 1 HB and 15 non-HB interactions with the same protein.

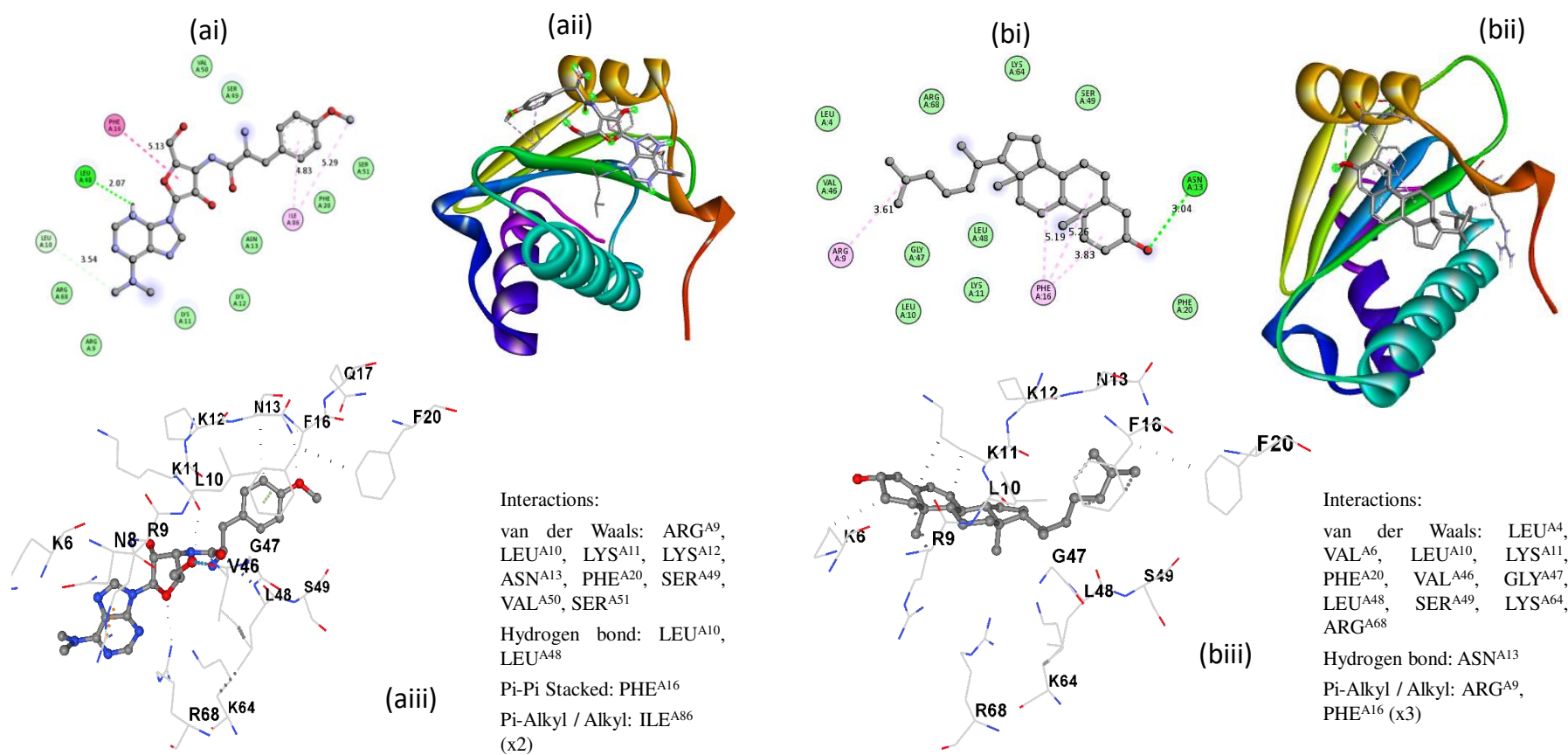


Figure 6: Binding orientation of puromycin and the phytochemical (CHDL) with higher binding affinity than puromycin to Ribonuclease P protein of *B. subtilis*. (ai- iii) 2D and 3D of puromycin interaction with 1A6F, (bi - iii) 2D and 3D of CHDL interaction with 1A6F.

286

287 Figure 7 (a and b) showed the binding poses of ampicillin and the CHDL to *S. typhi* OmpC
288 (3UU2) in the 2D, 3D receptor-ligand pose and 3D forms. The binding orientation of ampicillin
289 to the 3UU2 shows that ampicillin had 4 HB and non-HB with the residues, while CHDL had
290 15 non-HB but no HB.

3 Materials and methods

3.1 Materials

All the chemicals used in this work were analytical grades. The 1,1 -diphenyl-2 picrylhydrazyl (DPPH) was obtained from Sigma Chemical Inc. (USA), and quercetin, catechol, gallic acid, and ascorbic acid were from Merck (Germany). Other chemicals and solvents were analytical or otherwise synthetic grades.

3.2 Sample preparation and methanolic extraction

Before sunrise, the *V. amygdalina* leaves were obtained at the Redeemer's University Botanica Garden. The leaves were rinsed under running clean tap water in the biochemistry laboratory, and the water on the leaves was allowed to drain for about four hours. The leaves were mechanically homogenised with 1:1 w/v distilled water into a paste, and fermentation was initiated by exposing the paste to air for about 30 minutes in an isothermal batch-fermenting chamber. Samples from the exposed paste were collected daily from days 0 (unfermented) and from days 1 to 10 (fermented). The sample collected from each day was added to an equal volume of absolute methanol and agitated in a shaker at 150 rpm for 30 min before it was filtered with Whatman No. 1 filter paper and concentrated on the rotatory evaporator at 45 °C. The temperature and pH of the sample were monitored daily and recorded. Furthermore, colony count from the samples collected was done as soon as the samples were collected.

3.3 Phytochemical Analyses

3.3.1 Chlorophyll content estimation

Chlorophyll was estimated using the method described by Senthilkumar *et al.* [15]: 100 mg of the extract with 10 mL of 80% acetone. The mixture was centrifuged at 5000 rpm for 5 min, and the supernatant was collected. The procedure was repeated until the residue turned colourless. The volume collected was made up to 100 mL with 80% acetone, and the absorbance was read in a spectrophotometer (JENWAY 7305, Barloworld Scientific Ltd., UK) at 645 and 663 nm against a reference blank of 80% acetone. Total chlorophyll was calculated using the formula as described below:

$$\text{Total chlorophyll (mg/g)} = 20.2 (\text{Abs } 645\text{nm}) + 8.02 (\text{Abs } 663\text{nm}) - \frac{\text{Volume supernant collected}}{\text{Weight of sample} \times 1000}$$

3.3.2 Lycopene and carotenoid content determination

Lycopene and carotenoid contents were determined as described by Zakaria *et al.* [16]. The analysis was completed in darkness to prevent the photolysis of carotenoids after saponification. The sample (100 mg) was homogenised and saponified with 2.5 ml of 12% alcoholic potassium hydroxide in a water bath at 60°C for 30 minutes. The saponified extract was transferred to a separation funnel containing 10-15 ml of petroleum ether and thoroughly mixed. The aqueous layer was then transferred to another separation funnel, and the petroleum ether layer, which contained the carotenoids, was collected. This extraction process was repeated until the aqueous layer was transparent. Anhydrous sodium sulfate was added to the petroleum ether extract to remove any remaining moisture. The final volume of the petroleum ether extract was recorded. The absorbance of the yellow colour was measured in a spectrophotometer (JENWAY 7305, Barloworld Scientific Ltd., UK) at 450nm and 503nm using petroleum ether as a blank. The total carotenoids and lycopene content was determined using the appropriate formulae.

$$\text{Total carotenoids (mg/g)} = \frac{A_{450} \times \text{Volume of the sample} \times 100 \times 4}{\text{Weight of sample}}$$

$$\text{Total lycopene (mg/g)} = \frac{3.12 \times A_{450} \times \text{Volume of the sample} \times 100}{\text{Weight of sample}}$$

3.3.3 The total phenol content determination

The 500 µg/mL extracts and standard catechol solutions (0.2 - 1 mL) corresponding to 2.0 - 10.0 µg were pipetted into each tube and made up to 3.0 mL with distilled water. Then, 0.5 mL 1 N Folin-Ciocalteu reagent was added into each tube and placed in a boiling water bath for 1 min. The tubes were allowed to cool and read in a spectrophotometer at a wavelength of 650 nm. The phenolic content was subtracted from the standard curve and expressed as mg/g catechol equivalent. [17]

3.3.4 The flavonoid content determination

Briefly, 500 µL of each 1% extract solution and four varying concentrations of quercetin standard (100 µg/mL) were mixed with 4 mL of vanillin reagent (1% in 70% sulphuric acid). Each tube was heated for 15 min in a boiling water bath and allowed to cool down; the absorbance of each tube was read in a spectrophotometer at a wavelength of 340 nm. The values of flavonoids were expressed as mg/g quercetin equivalent. [18]

3.3.5 Ascorbic acid content determination

A standard (100 µg/mL of ascorbic acid) volume ranging between 200 -1000 µL and triplicate samples of 500 µL of the extracts were prepared. The volume was made up to 2000 µL with 4% trichloroacetic acid. To this mixture, 500 µL of 2,4-dinitrophenyl hydrazine reagent (DNPH) was added to each tube, followed by two drops of 10% thiourea solution. The mixture was allowed to stand for 3 hours at 37 °C. After this, 2500 µL of chilled 85% sulphuric acid was added and cooled on ice. The absorbance was read at 540 nm in a spectrophotometer (JENWAY 7305, Barloworld Scientific Ltd., UK). The ascorbic acid content was estimated from the ascorbic acid standard curve. [19]

3.3.6 Total protein estimation

As described by Lorwy *et al.* . [20], 200 to 1000 µL egg albumin standards (100 µg/mL) and 100 µL of extracts (1 %) were pipetted into test tubes and made up to 1.0 mL with distilled water. To each of the tubes, 5.0 mL of alkaline copper solution ((50: 1 (v/v) sodium carbonate (2% in 0.1N NaOH): copper sulphate (0.5% in 1% potassium sodium tartrate)) was added, thoroughly mixed and incubated for 10 min at 37 °C. Then 500 µL of 1 N Folin-Ciocalteu reagent was added, incubated for 30 min at 37 °C and read at 660 nm in a spectrophotometer.

3.4 In-vitro antioxidants analyses

3.4.1 The 1, 1-diphenyl-2-picryl hydrazyl (DPPH) spectrophotometric assay

In respective tubes, 20 µL of standards (100 – 500 µg/mL gallic acid and ascorbic acid) and extracts were added to 500 µL of 0.3 mM methanolic solution of DPPH and 480 µL of methanol. The mixture was allowed to react in the dark for 30 min at room temperature. [21] The extent of discolouration of the purple-coloured solutions was read at 518 nm in a spectrophotometer, and the radical scavenging activity was calculated as below:

$$\text{Scavenging activity \%} = 100 - \frac{\text{Abs (sample)} - \text{Abs (blank)}}{\text{Abs (blank)}} \times 100$$

The percentage inhibition was equated to gallic acid equivalent inhibition concentration from the gallic acid standard curve of percentage (%) inhibition Vs gallic acid Equivalent as reported in our previous study.

3.4.2 Hydrogen peroxide scavenging activity

A 3 mL of 67 mM H₂O₂ (2 mM in phosphate buffer) solution was added to the 40 µL of 100 µg/mL extracts. The same was repeated for the 100 µg/mL standards (gallic acid and ascorbic acid with a concentration of 20 – 100 µg/mL each). A blank solution containing phosphate buffer, without hydrogen peroxide, was prepared. The absorbance of the reaction mixture,

blank, and standards was recorded at 230 nm in a spectrophotometer and calculated using the formula below: [14]

$$\text{Hydrogen peroxide scavenging \%} = \frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}} \times 100$$

An inhibition concentration of the extract was estimated from the gallic acid concentration vs inhibition (%) standard curve [14].

3.4.3 Measurement of Nitric Oxide Scavenging Activity

The reaction was initiated by adding 2.0 mL of sodium nitroprusside (100 mM) to a mixture of 500 µL phosphate-buffered saline (0.2M, pH 7.4) and 500 µL of each of the extracts (50 mg/mL), gallic acid (100 mg/mL) and ascorbic acid (100 mg/mL) in their respective test tubes and incubated for 30 min at 25 °C. To the mixture, 500 µL Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylene diamine dihydrochloride and 2% H₃PO₄) was added and incubated at room temperature for another 30 min. Control tubes were prepared in the same manner without the extracts, and the absorbance was read at 546 nm against the reagent blank in a spectrophotometer. [22] Percentage inhibition was calculated in the formula below:

$$\text{Inhibition \%} = \frac{\text{Abs (sample)} - \text{Abs (control)}}{\text{Abs (control)}} \times 100$$

Furthermore, the inhibition concentration was estimated from the gallic acid concentration against the percentage (%) inhibition curve, and the values were reported as GAEq (gallic acid Equivalent) [14].

3.4.4 Lipid Peroxidation Inhibition Activity

The method was slightly modified; 20% of healthy rat liver was homogenised in an ice-cold KCl (0.15M). The extracts (1 mL) and the liver homogenate (1 mL) were mixed with 1 mL of a solution containing 25 mM FeSO₄, 100 mM ascorbate and 10 mM KH₂PO₄. The volume was made up to 3 mL with distilled water and incubated for 30 min at 37 °C. To 0.5 mL of the homogenate solution, 0.5 mL of 0.12 M phosphate buffer (pH 7.2) was added, followed by the addition of 1.0 mL of 10% trichloroacetic acid and 1.0 mL of 0.1M thiobarbituric acid and mixed thoroughly. The mixture was heated in a water bath at 80 °C for 20 min. The tubes were centrifuged at 1000 xg for 10 min, and the absorbance reading was taken at 535 nm against a reference blank containing all the reagents except the homogenate. The MDA equivalents of the samples were calculated using the extinction coefficient 1.56 × 10⁵ M⁻¹cm⁻¹. [23]

The inhibition concentration was equated to gallic acid equivalent from the gallic acid concentration – percentage inhibition standard curve as reported in our previous study[24].

3.4.5 Egg albumin denaturation assay

Using Chandra *et al.* [25] procedure, a mixture of 200 µL of 1% freshly prepared egg albumin solution, 2 mL of sample extract or non-steroidal anti-inflammatory drug (NSAID), and 2.8 mL of 0.2 M phosphate-buffered saline (pH 7.4) were mixed and incubated at 37 °C for 30 min, followed by heating in a water bath at 70°C for 15 min. The extract volume was replaced by distilled water in the control test tube. The reaction mixtures were read at 280 nm in a spectrophotometer. The percentage inhibition was calculated as stated below:

$$\text{Protein denaturation inhibition \%} = \frac{\text{Abs (sample)} - \text{Abs (control)}}{\text{Abs (control)}} \times 100$$

The gallic acid inhibition concentration equivalent of the extracts was interpolated from the gallic acid concentration – percentage inhibition standard curve as reported in a previous study [14].

3.5 Hierarchy Component Analysis and Heatmap

Hierarchy component analysis of Fourier transformer infrared (FTIR) spectra was analysed in fragments of spectra regions unique to respective biomolecules on a web-based tool, Clustvis: a web tool for visualising clustering of multivariate data using Principal Component Analysis and heatmap [26].

3.6 In-silico Quantitative structure-activity relationships (QSAR)

The QSAR screening was done via ADMETlab 2.0, an integrated online platform for accurate and comprehensive predictions of ADMET properties. The SMILES and 3D structure files of each of the GCMS-identified compounds were generated from the PubChem server (<https://pubchem.ncbi.nlm.nih.gov/>) and collected in a single text file and uploaded on the ADMET 2.0 virtual screening page [27].

3.7 Molecular Docking

Three antibiotic-target proteins (4GCS, 3UU2 and 1A6F) pdb file were collected from protein data bank (<https://www.rcsb.org/>) [28]

3.8 Statistical Methods

Data generated were analysed using GraphPad Prism 8.0 and OriginLab 2015 versions for Windows. The triplicate values were subjected to analysis of variance (ANOVA), comparison

between means was assessed using Turkey, and the significance level was expressed at $p < 0.05$. Pearson correlation heatmap was used to evaluate correlation coefficients.

4 Discussion

The search for effective, biodegradable antibiotics against multidrug-resistant enterobacteria has become extremely important, especially in sub-Saharan Africa, due to the increase in the pathogenic incidences caused by these bacteria. Also, the microbial transformation of plants' secondary metabolites from a well-documented antibiotic plant like *V. amygdalina* may boost the achieving the objective. To further propagate such attainment, Computer Aided Drug Discovery (CADD) techniques were explored in this work.

The results of this study revealed that changes in the temperature and pH of the *f*-VALP dropped by 10 % and 30 %, respectively, within the first 3 days of fermentation. While the temperature was steady until the terminal period, pH fluctuated at a 3-day interval during the process (Fig 1a (i)). In Fig 1a (ii), the microbial load steadily increased after 48 h of fermentation until day 6 and became static until day 10. This observation agrees with our previous study [24] and the report of Wouters *et al.* [33], which shows that *Lactobacillus* proliferation became rapid after 3 days of fermentation. Also, Qu 2023 [37] shows that microbial growth attains a stationary phase after 6 days due to environmental change. In Fig 1a(iii), fermentation significantly decreased chlorophyll, phenols, protein and ascorbic acid contents within the first 48 h but increased flavonoid and lycopene contents within the same period. On day 9, the lycopene content increased by 5 folds; flavonoid was 2 folds higher, while carotenoid and ascorbic acid contents were significantly higher than their unfermented counterparts. Ayse and Osman [38] reported an over 10-fold increase in the lycopene content of the various fermented plant composites within seven days.

To further explore the influence of the fermenter's proliferation at 48 h intervals on the physicochemical changes in VALP, Pearson's correlation *heatmap* (Fig 1b(i - v)) was adopted. The Fig. 1b (i) *heatmap* (days 0 - 2) shows that the fermenter's effects were negligible on the physicochemical change in the VALP. From days 2 to 4 (Fig. 1b (ii)), the microbial actions were responsible for the decreased flavonoids and protein contents. Hence, this suggests that the role of fermenters in *f*-VALP may not be significant until after 72 h, although it accounts for the reduction in flavonoids and protein content of the VALP. The association between the

temperature and the bacteria proliferation rate is well observed in Pearson's correlation *heatmaps* (Figures 1b (iii - v)) because it was observed that the three figures captured the proliferation stationary phase (days 6 to 10) of the fermenters. This observation agrees with the work of Frank [39], suggesting that bacterial proliferation at the stationary phase may be temperature-dependent, provided other factors are stable. In addition, the positive correlation between the ascorbic acid and fermenters' proliferation rate implies that increased ascorbic content may be propagated in *f*-VALP by fermenters' activities but not until day 8 of the fermentation process.

A comparison between the bioactivities profile of *f*-VALP and its unfermented counterpart (Fig 2a) shows that fermentation improved the antioxidants and anti-inflammatory potentials of VALP except for the H₂O₂ scavenging property, which was reduced at the onset of the fermentation but increased as the fermentation progressed upto to day 9. The H₂O₂ scavenging capacity of the *f*-VALP on day 9 was similar to the *unf*-VALP (day 0). The *f*-VALP methanolic extract of day 9 showed a relatively higher inhibitory power against heat-induced protein denaturation, lipid peroxidation and DPPH than other *f*-VALP (days 1 – 8 and 10) and *unf*-VALP. An exploratory relationship (Pearson's correlation *heatmap*) between respective bioactivities profile and phytochemical change at a 48 h interval was adopted to understand further the effect of fermentation time on VALP bioactivities (Fig. 2b (i – v)). The positive association between the increased lycopene level, lipid peroxidation and protein denaturation inhibition rate within the first 48 h of fermentation showed that lycopene might be the significant antioxidant and anti-inflammatory agent in *f*-VALP within the first 48h of fermentation because the increased anti-lipid-peroxidation and protein stabilisation is proportional to lycopene level (Fig 2b (i)). Notably, chlorophyll, phenols, ascorbic acid, and protein contents decreased within the same period, and their changes were negatively correlated with lipid peroxidation and protein denaturation inhibitions. This observation may not be unassociated with the lipophilicity of lycopene, as studies have shown that lycopene plays a key antioxidant role in the lipophilic environment of a biological system [40]. The DPPH inhibition potential recorded within 48 h of fermentation could be ascribed to an increased flavonoid content of *f*-VALP because other phytochemicals were negatively correlated with the DPPH inhibition rate. In contrast, the change in flavonoid level was positively associated.

The Pearson's correlation *heatmap* (Fig 2b (ii)) showed a correlation between chlorophyll content, inhibition of lipid peroxidation and protein denaturation between lycopene, DPPH inhibition and H₂O₂ inhibition at days 2 – 4. Also, changes in phenols, ascorbic acid and protein

content were positively correlated with the hydrogen peroxide scavenging potential changes within the same period. In Fig 2b (iii), a positive association between the change in total phenol and H₂O₂ inhibition showed that decreased total phenol level in *f*-VALP methanolic extract may have resulted in the weak H₂O₂ inhibition within days 4 - 6 because other phytoconstituents within this period increased as noted earlier in this study. In Fig 2b (iv), the change in carotenoids and protein contents was positively correlated with the increased H₂O₂ scavenging, lipid peroxidation and protein denaturation inhibitions (days 6 – 8). The reason for this may not be far-fetched as both constituents in the fermented sample increased within his period in Fig 1a (iii). On days 8 to 10 (Fig 2b (v), lycopene, carotenoid, and flavonoid levels increased and positively correlated with increased H₂O₂ scavenging and protein denaturation inhibition.

As presented in Fig 3, the MIC value of *unf*-VALP against *E. coli* was observed to be zero (0). That is, it could not inhibit the growth of the MDR bacteria, but after day 3 of fermentation, the methanolic extract showed a significant inhibition. The *E. coli* inhibition potential of the *f*-VALP improved till day 6. Both *unf*-VALP and *f*-VALP methanolic extracts inhibited *Bacillus subtilis* more significantly than the other two bacteria screened in this study. Thus, a fermentation period of up to 10 days may not alter substantially the antibiotic properties of VALP methanolic extract against MDR *Bacillus subtilis*. This outcome may not be unconnected to diethyl phthalate (Table 2), the only compound identified in fermented and unfermented VALP. According to Sandy *et al.* [41], diethyl phthalate is one of the carbon sources that *Bacillus subtilis* growth utilises; however, a concentration of 200 µg/mL or more of diethyl phthalene and dibutyl phthalate inhibits *Bacillus subtilis* growth and proliferation rate. The inhibitory effect of fermentation of VALP against *S. typhi* was not significant until day 6, and no substantial change was observed till the terminal point of the fermentation time. According to Effah-Yeboah *et al.* [42], *S. typhi* is less susceptible to *V. amygdalina* ethanolic. A comparative study by Assefa *et al.* [43] showed that *S. typhi* was poorly susceptible to water, 70% ethanolic and 80% methanolic crude extracts of *V. amygdalina* leaf. The ability of *f*-VALP (days 4 – 7, 9 and 10) to inhibit *S. typhi* recorded in this work agrees with the works of Rubinelli *et al.* [44] and Dessie *et al.* [45] that showed metabolites of fermentation inhibit both *Salmonella enteritidis* and *Salmonella typhimurium*.

Bacterial morphological and biochemical analyses in this work revealed that *Staphylococcus spp*, *Klebsiella spp* and *Enterococcus spp* were the primary bacteria responsible for the fermentation of VALP (Table 1a, 1b and 1c). Of the seven isolates collected, four isolates were

Staphylococcus spp, two isolates of *Klebsiella spp* and an isolate of *Klebsiella spp*. The 7 seven isolates were non-pathogenic because they showed negative results for SSA and non-motile, and they were multiple sugar fermenters among the four *Staphylococcus spp*. Isolates, isolate 1, was the only one that fermented cellulose. This observation agrees with the work of Flimban *et al.* [46], which showed that the genus *Staphylococcus* was the dominant cellulose-degrading bacteria that used rice straw as a carbon source. Paul *et al.* [47] isolated cellulose-digesting that *Staphylococcus saprophyticus* from termite gut.

A sub-metabolomics analysis was explored in this study to reveal the dynamics of fermentation time's effect on the phytochemicals of VALP by the FTIR technique, and a hierarchy cluster analysis *heatmap* was used to group the fermentation period. The FTIR spectra in Fig. 4a revealed that the spectra changes were gradual until day 5, which showed a distinct shift in transmittance at frequency ranges of 1000 cm⁻¹, 1400 cm⁻¹, 1600 cm⁻¹, 2,300 cm⁻¹, 2,800 cm⁻¹ and between 3,200 and 3,600 cm⁻¹. These regions of the FTIR were evaluated for their hierarchical relatedness in Fig 4b (i – iv). These regions are considered unique to different biomolecule classes [29,34,35,48]. The HCA *heatmap* showed that the soluble carbohydrates, fatty acids, protein and polyphenols constituents of *unf*-VALP and fermentation days 10 samples were distinct. On day 9, the soluble carbohydrate, protein and polyphenols were unrelated to the *unf*-VALP sample. Similarly, the extractable fatty acid, protein and polyphenols constituents of day 8 differed from day 0 (*unf*-VALP). Therefore, this suggests that by the 10 days of fermenting VALP, the soluble constituents of the fermented sample could vary substantially from the starting material. Thus, three distinct clusters were deduced as unfermented (days 0 - 2), sub-fermented (days 3 – 7) and fermented (days 8 – 10).

Gas chromatography-mass spectroscopy analysis on VALP methanolic extract was explored to identify compounds present in unfermented (day 0), sub-fermented (day 3) and fermented (day 9). The dynamics of the compound in each of these stages of fermentation showed that diethyl phthalate was identified in the three categories of the fermentation period and unfermented sample; methyl N-hydroxybenzenecarboximidate was identified in days 0 and 3 only, while other compounds identified were unique to each day.

The advent of CADD has enhanced the development of nutraceutical agents; this work explored this advancement to identify drug-like candidates and to understand the mechanism of interactions that exist between these nutraceuticals and antibiotics target-protein in the *E. coli*, *B. subtilis* and *S. typhi*. The *in-silico* solubility screening in hydrophobic and hydrophilic

solvents shows that five compounds are moderately hydrophobic and soluble in aqueous, while the others were highly hydrophobic. All the compounds possess average gastrointestinal absorption rates; however, ergostane-5,25-diol, 3,6,12-tris[(trimethylsilyl)oxyl]-25, acetate and benzene-propanoic, 3,5-bis(1,1-dimethyl ethyl) 4-hydroxy-octadecyl ester was rejected by the four drugs-rules employed in this study (Suppl. Table 1); hence they are suspected to be toxicity. It is noteworthy to emphasise that the two compounds were identified on day 3 only.

Molecular docking is considered one of the most efficient methods for understanding the interactions between a protein and small molecules. Two molecular techniques were adopted to identify the most promising drug-like candidate: Pyrx (standalone application) and CB-Dock2 (online molecular docking platform), promoting reproducibility and enhancing dual perspective evaluations and deduction. The nine compounds that passed the in-silico ADMET screening were docked. At the same time, two known antibiotics – ampicillin for OmpF porin (4GCS) and OmpC porin (3UU2) and puromycin for ribonuclease p (1A6F) were used as reference ligands. Of the nine compounds, only CHDL showed a stronger binding affinity to OmpF and OmpC porins (Table 4). The binding energy of CHDL to *B. subtilis* ribonuclease p (1A6F) was higher than the puromycin in Pyrx, while CB-Dock was slightly lower than the reference drug. This finding agrees with the study of Zhu *et al.* [38], which reported the *in-vitro* antibiotics potential of CHDL extracted from a marine organism against *E. coli* and some other enterobacteria. The CHDL -1A6F binding affinity in Pyrx was greater (-6.4 KJ mol^{-1}) than the reference drug (-6.3 KJ mol^{-1}); however, the CB-Dock value of the same ligand-protein interaction (-5.9 KJ mol^{-1}) was lower than the puromycin-1A6F (-6.4 KJ mol^{-1}). Interestingly, the *in-vitro* antibiotics result in this work showed that *B. sibilities* were significantly inhibited, and the fermentation process did not influence the inhibition rate (refer: Table 4). Hence, it may be suggested that the *in-vitro* *B. subtilis* inhibition recorded in this study might be driven by a collection of compounds that show weak binding affinities to 1A6F.

Elucidation of the interactions between a ligand and residue of a polypeptide chain creates an exploratory complementary understanding of molecular docking. This study identified CHDL as the lead drug-like candidate, and its interaction with respective protein residues was presented in Fig. 5 - 7.

Gram-negative bacteria rely on their outer membrane porins (OmpA, OmpC, OmpF, OmpW, and OmpX) for adaptation and survival against environmental assaults, including antibiotics. Omcs are a vital target for antibiotic development against MDR bacteria, as they play crucial

605 roles in adaptation mechanisms [49]. CHDL bind to OmpF at the active site 1, the same cavity
606 as ampicillin (Fig. 5). The ligand-recaptor pose (Fig, 5bii) of CHDL showed that it was more
607 embedded within the channel of OmpF. The pi-Alkyl bond in CHDL - PHE^{A118} of OmpF is
608 suspected to enhance the affinity of the compound to the protein because this interaction
609 appears as an unfavourable repulsive force in ampicillin-OmpF residue, which is likely to
610 weaken the HB of ARG^{A42} to ampicillin.

611 OmpC roles in antibiotic resistance across several classes of antibiotics are well documented,
612 unlike OmpF, which is more related to amide-containing antibiotics. OmpF is a porin that
613 transfers small-molecule antibiotics and other hydrophilic molecules [49].

614 Ribonuclease P (RNase P) as a molecular target in developing and screening antibiotics is well
615 documented due to their essential roles as endonuclease in tRNA biogenesis. Also, the
616 differences in bacterial RNase P and eukaryotic-RNase characteristics make it an ideal target
617 [50]. Aminoglycosides-derived antibiotics like puromycin inhibition mechanism are reported
618 to be solely dependent on their ionisation strength, which displaces Mg²⁺ in the catalytic site
619 of RNase P, and this may account for several van der Waal forces and HBs it shared with
620 Leu^{A10} and Leu^{A48} (Fig. 6ai and iii). Though its roles have several downsides, like pH-
621 dependency and acting as RNase activators at low doses, bulky ligands are proposed to give a
622 higher inhibition potential [50]. CHDL, on the other hand (Fig 6bi - iii), is an aromatic
623 compound which mimics puromycin in its kind of molecular interactions with 1A6F catalytic
624 site residues as it exhibits sixteen van der Waal interactions with the identical residues as
625 puromycin and addition; it shows four pi-alkyl interactions Arg^{A9} and Phe^{A16}. Hence, the
626 hydrophobicity of CHDL is suspected to possess similar inhibitory potential that is likely to
627 be pH-independent.

628 Ampicillin – OmpC interaction shows that the antibiotic shares HB with Lys^{A16}, Leu^{A107},
629 Glu^{A108} and Gln^{A356} and pi-anion with Gln^{A109} (Fig. 7ai - iii), CHDL on the other hand, shared
630 van der Waal forces with Gln^{A55}, Val^{A75}, Phe^{A77}, Asn^{A93}, Asp^{A99}, Ser^{A102}, Val^{A106}, Arg^{A132},
631 Lys^{A224}, and Tyr^{A231}. Although ampicillin and CHDL interact with similar residues, their
632 poses and positions were slightly different, as noted in Fig. 7aii and Fig. 7bii. According to
633 Elmeri *et al.* [51], one of the Omp antibiotic-resistant mechanisms of GBN, like E. coli, is the
634 salt-bridge formation by duplication of negatively charged amino acid residues within the
635 region containing residues Gln^{A55} and Lys^{A224}. Hence, the ability of CHDL to occupy this

region on the OmpC and its hydrophobicity is suspected to disrupt the salt-bridge formation, possibly accounting for the inhibition mechanism recorded in our *in-vitro* assay.

5 Conclusion

The study investigates the role of fermentation in modifying *V. amygdalina* leaf secondary metabolites as a potent antibiotic against *E. coli*, *B. subtilis*, and *S. typhi*. The study found that fermentation increased the content of lycopene, flavonoid, and carotenoid compounds but decreased chlorophyll, protein, and phenol. Pearson's correlation heatmap showed a strong correlation between microbial activities and the alteration in secondary metabolites from day 8 to the terminal point of fermentation. The methanolic extract of *f*-VALP on day 9 showed significant antioxidant and antiinflammation activities. The study also identified non-pathogenic *Staphylococcus* spp, *Klebsiella* spp, and *Enterococcus* spp as primary fermenters. In-silico ADMET and molecular dockings identified the lead compound CHDL (4,6-Cholestadien-3 β -ol) as responsible for the inhibition of the gram-negative bacteria via multiple interactions and comparatively higher binding affinity to OmpC, OmpF, and RNase P than ampicillin. The hydrophobicity of this steroid is likely to be crucial to its antibiotic potency against porins and ionisation-dependent functional outmembrane channels.

Supplementary information

The details of the gas chromatography mass spectra and the im-silico ADMET screening data were presented in supplementary Figures 1 to 3 and supplementary Table 1.

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