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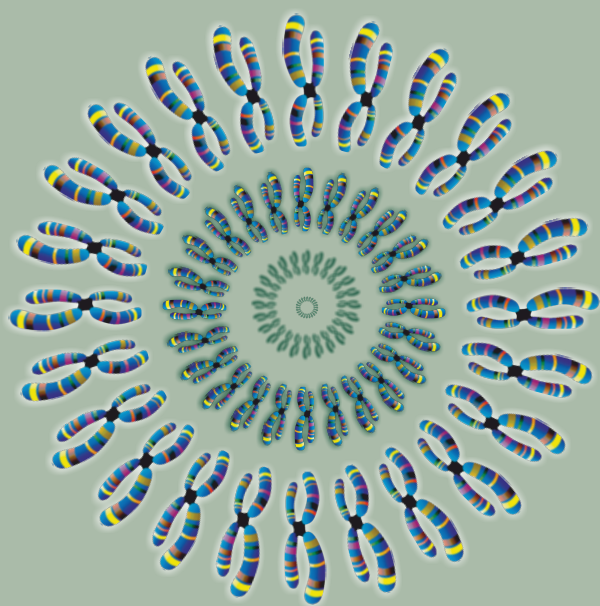

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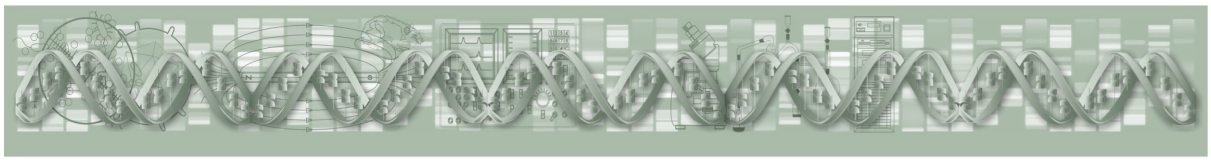

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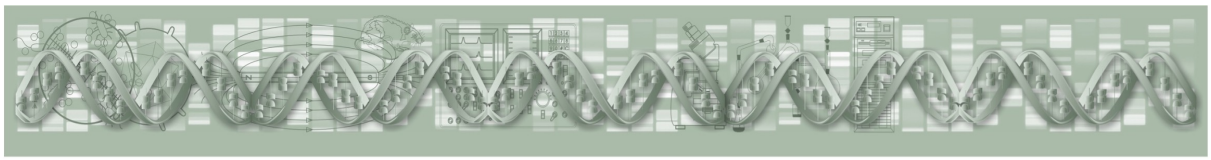

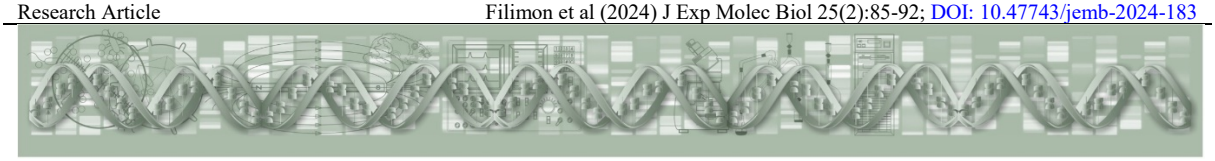

STUDIES ON THE CARBON CATABOLITE REPRESSION IN LACTIC ACID BACTERIA ISOLATED FROM WINE

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Abstract

In wine, lactic acid bacteria (LAB) are responsible for the bioconversion of malic acid to lactic acid, malolactic fermentation that mainly aims at reducing wine acidity. Two LAB strains isolated from the red wine microbiota (*Oenococcus oeni* 13-7 and *Lactobacillus plantarum* R1-1), were tested for their ability to exhibit the carbon catabolite repression (CCR) mechanism, that allows the rapid use of certain carbohydrates, over other carbon sources. Bacterial cells were inoculated in 0.1 M glycine buffer (pH 3.5), incubated at 30°C, with different carbohydrates (45 mM) and malic acid (45 mM). For both strains, the presence of glucose significantly inhibited malic acid metabolization (−60%), a similar effect being observed for galactose, mannose and maltose. The highest rate of malic acid conversion was shown in fructose/malate medium. Obtained results showed that malolactic strains can control the utilization of carbon sources via CCR, further studies being necessary to elucidate the mechanisms underlying this process.
Keywords: carbohydrate metabolism, *Lactobacillus plantarum*, malolactic fermentation, *Oenococcus oeni*, wine

Introduction

Malolactic fermentation (MLF) is defined as the enzymatic bioconversion of malic acid to lactic acid, a process performed by lactic acid bacteria (LAB), that aims at the reduction of wine acidity and to enhance the aromatic profile (Filimon et al. 2022). Performed in a controlled manner, with selected starter cultures, MLF has a significant influence on wine quality: balances the acidity, slightly increases the pH, ensure the biological stability of the wine by avoiding subsequent uncontrolled fermentations, and improves the aroma and taste of the wine, increasing its complexity (Capozzi et al. 2021, Lerm et al. 2010).
LAB species involved in the winemaking process belongs to the *Lactobacillus*, *Leuconostoc*, *Oenococcus*, and *Pediococcus* genera. For conducting a controlled MLF, the starter cultures are obtained from strains belonging mainly to two species: *Oenococcus oeni* and *Lactobacillus plantarum* (Filimon 2023). To be used as starter cultures, after isolation from indigenous microbiota LAB isolates are subjected to various screening procedures, testing of the yield of malic acid bioconversion and the ability to produce undesirable or even toxic by-products (acetic acid, ethyl carbamate, biogenic amines, acetoin or diacetyl, acrolein, mannitol).
LAB are Gram-positive, catalase-negative, immobile and non-sporulated, anaerobic microorganisms, tolerant to high concentrations of acids, that assimilate carbohydrates both in the homofermentative and heterofermentative pathways (De Vos et al. 2009). Malolactic

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bacteria exhibit a chemoorganotrophic metabolism, requiring media rich in nutrients and fermentable sugars, presenting a high phenotypic variability (Coelho et al. 2022).
Carbon catabolite repression (CCR) is a mechanism utilized by various species of bacteria and fungi to accommodate changes in the environment, allowing for the rapid use of certain substrates like glucose over other carbon sources (Nair and Sarma 2021). CCR has a universal function in the regulatory system, which ensures efficient utilization of preferred carbon sources and prevents the activation of unnecessary metabolic pathways to save energy (Vinuselvi et al. 2012). For example, *E. coli* in a medium with a mixture of glucose and lactose uses the glucose completely first, then stops growing while the genes for degradation of lactose are induced, a phenomenon that enable bacteria to make a hierarchical choice between different sources of carbon (Plumbridge 2009). Also, catabolite repression is an important process for biotechnological applications. In winemaking, there are concerns that the MLF can be delayed, slowed down or even blocked in wines with high concentrations of specific carbohydrates (semi-sweet and sweet wines). Reidler (1967) showed that in the absence of carbohydrates, the biological conversion of malic acid was not possible. The main sugars in wine are glucose and fructose, LAB species being able to use both carbon sources (Cibrario et al. 2016). Also, most LAB are able to use other monosaccharides present in wine in lower concentrations (arabinose, mannose, galactose, xylose etc.), as well as polysaccharides or glycosylated compounds (Déléris-Bou and Krieger-Weber 2014). The carbohydrates metabolism is the main way of obtaining the energy necessary for LAB growth and development, fructose being preferentially used, compared to glucose. According to Nonomura (1983), the lack of fructose does not always allow the growth of LAB cells, although there were some cases when pyruvic acid, pentoses, ascorbic acid or cysteine can replace fructose. Also, was reported that MLF can be inhibited in wines where the sum of glucose and fructose is less than 0.2 g/L (Krieger 2005). Studies on malate-carbohydrate co-fermentation suggested that malate metabolism significantly influences carbohydrate metabolism (Henick-Kling 1993), but the opposite has also been reported (Salou et al. 1991). Moreover, Miranda et al. (1997) showed that carbohydrate-malate co-fermentation seems to depend largely on the strain involved. Considering these aspects, the purpose of the study was to highlight the CCR phenomenon in indigenous LAB strains responsible for the malolactic fermentation of wines. For a more rigorous control of the bioconversion process, is necessary to understand the interactions that occur in the competitive use of different carbon sources present in wine.

Materials and Methods

Tested LAB strains were isolated from red wines (Merlot, Cabernet Sauvignon, Arcaş), obtained by classic winemaking technology (grape crushing and destemming) at the Research - Development Station for Viticulture and Winemaking Iasi, NE of Romania. After preliminary selection, identification and characterization, the two strains R1-1 *Oenococcus oeni* and 13-7 *Lactobacilus plantarum* were preserved in De Man-Rogosa-Sharpe (MRS) broth medium supplemented with 30% glycerol, at −20 °C, a procedure that ensured their stability of during storage (minimum 6 months) (Filimon et al. 2022). Bacterial isolates were subsequently inoculated in FT80 broth medium (Cavin et al., 1989), at a cell density of 10^8 CFU/mL, and incubated in anaerobiosis (GENbag anaerobic®; BioMérieux, France), 72 hours, at 28°C. The bacterial cell biomass was separated by centrifugation (4000 rpm, 15 min.), washed twice in physiological serum and inoculated (25 mg dry biomass/mL) in 0.1 M glycine buffer solution (pH 3.5), supplemented with 45 mM malic acid and 45 mM of each tested carbohydrate (glucose, fructose, galactose, mannose, ribose and maltose), at 30°C, for 72 hours, according to the protocol presented by Miranda *et al*. (1997). Glycine is one of the 20 proteinogenic amino acids, used as nitrogen source. To highlight the CCR sensibility, the ability of the LAB strains

to metabolize malic acid was tested in the presence of different concentrations of glucose: 1, 5, 10, 30, 45 and 50 mM.

Monitoring of the MLF process was performed by thin layer chromatography (TLC), using cellulose plates 20×20 cm (Merck, Germany) and a mixture of solvents as mobile phase: n-butanol : distilled water : acetic acid : bromophenol blue (100:20:20:0.1 v/v/v/w). For the quantitative determination of malic acid was used the enzyme kit produced by Biosystems, Spain (Ref. 12803). Malic acid generates NADH when transformed by L-malate dehydrogenase, its concentration (g/L) being measured spectrophotometrically at 340 nm.

The reported data are mean values obtained in three independent experiments (n=3), with standard deviation (±). Analysis of variance ANOVA test (XLSTAT 2021.5.1 for Microsoft® Excel) was initiated to investigate significant differences between data, *p*-values ≤ 0.05 were considered significant. The method used to discriminate among the means was Tukey's test at 95% confidence level.

Results and discussions

Bacteria use CCR through different mechanisms to achieve different physiological goals required to their survivability and development (Nair and Sarma 2021). The pH of the incubation medium was low (3.5), similar to wine, due to the fact that at low pH malic acid is metabolized at a higher rate, while carbohydrate metabolism proceeds very slow (Firme et al. 1994). During the fermentation process, the increase in pH induced by the metabolism of malate allows the subsequent utilization of carbohydrates.

After 72 hours, the presence of malic and lactic acids was assessed by thin layer chromatography (TLC), the corresponding spots being observed (Figure 1). The spots of malic acid were visible on the plate, indicating its partial conversion.

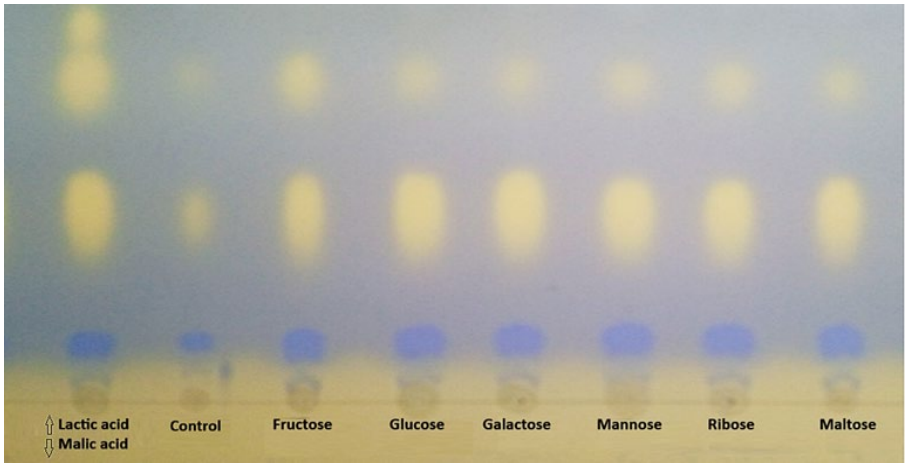

Figure 1. TLC chromatogram of the 0.1 M glycine buffer solution (pH 3.5) inoculated with *Oenococcus oeni* 13-7, supplemented with malic acid (45 mM) and different carbohydrates (45 mM) (72 h, 30°C). Control sample represent the glycine buffer solution (pH 3.5) with malic acid, without carbohydrate.

The retention factors (Rf), calculated as the ratio between the migration distance of each compound (to the center of the spot) and the total migration distance of the solvent (7.5 cm), were 0.48 for malic acid, respectively, 0.75 for lactic acid.

The experimental results obtained indicated that for both LAB species the presence of glucose in the medium at concentrations of 45 mM significantly inhibited the malolactic bioconversion. Residual malic acid in the presence of glucose was between 4.32-4.39 g/L from the initial quantity of 6.0 g/L (45 mM), while in the case of fructose were determined the lowest

residual amounts of malic acid for both strains (1.03-1.10 g/L) (Table 1). For both galactose and mannose, the concentration of malic acid determined in the medium after the incubation period varied non-significantly from 4.05±0.31 to 4.22±0.22 g/L. Excluding glucose and galactose, *O. oeni* strain showed a more pronounced CCR phenomenon compared to *L. plantarum.*

Table1. Residual malic acid (g/L) determined in the glycine buffer solution with carbohydrates

Species/ strain code	Carbohydrates						
	Control	Fructose	Glucose	Galactose	Mannose	Ribose	Maltose
O. oeni 13-7	1.55±0.12^{b}	1.03±0.09^{d}	4.39±0.19^{a}	4.22±0.22^{a}	4.05±0.31^{a}	1.90±0.24^{bc}	4.13±0.21^{a}
L. p. R1-1	1.59±0.16^{b}	1.10±0.15^{d}	4.32±0.14^{a}	4.16±0.19^{a}	4.09±0.41^{a}	2.01±0.14^{c}	4.17±0.19^{a}

The disaccharide maltose showed an effect similar to glucose. Although several studies showed that some LAB strains are unable to metabolize maltose (De Vos et al. 2009), this sugar can be used totally or partially by some strains of *O. oeni* or *L. plantarum* as a source of carbon and energy (Izquierdo et al. 2004). Cibrario et al. (2016) reported that from 41 *O. oeni* strains more than 75% were able to use glucose, ribose and mannose; fructose and L-arabinose were used by about half the strains, while 25% of the strains were able to use maltose.
However, for both LAB species, the presence of glucose in the medium reduced malic acid bioconversion by about 45% compared to the control sample (without carbohydrates). A negative effect on malic acid bioconversion, similar to glucose, was showed in the case of the hexoses galactose and mannose, the percentage of malic acid degraded being about 33% (Figure 2). A lower inhibition of the malolactic process was observed in the case of the pentose ribose, the malic acid being metabolized in a proportion of 68.57% by the strain 13-7 *O. oeni* and 66.74% by the bacterial strains R1-1 *L. plantarum*.

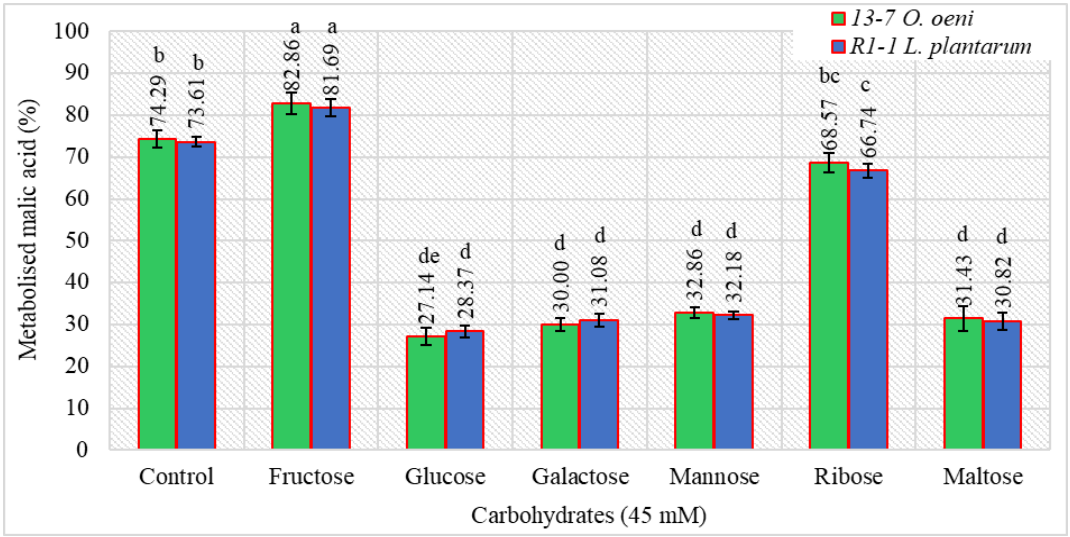

Figure 2. The effect of various carbon sources on the malolactic activity of indigenous LAB strains. Data represent the mean values (n=3), while error bars indicate standard deviation (±). Different letters within the same figure indicate significant differences in Tukey test ($p \leq 0.05$).

The highest rate of malic acid metabolization was registered in the medium with 45 mM fructose (>80%), the percentage of malic acid consumed being higher compared to the control by up to 10%, for both bacterial strains. According to Maicas et al. (1999), glucose is used as the carbon and energy source by all strains of *O. oeni*, but was reported that fructose is the most rapidly and efficiently metabolized sugar. The use of fructose as an electron acceptor has usually been seen as beneficial for most LAB strains. Also, should be mentioned that non-

significant differences were found between the two analyzed strains regarding the CCR process, their behavior being similar in the experimental conditions.
Because glucose induced the lowest rates of malic acid metabolization, the second part of the experiment aimed at highlighting the capacity of the indigenous LAB strains to metabolize malic acid at different concentrations of glucose (1, 5, 10, 30, 45 and 50 mM) (Figure 3).

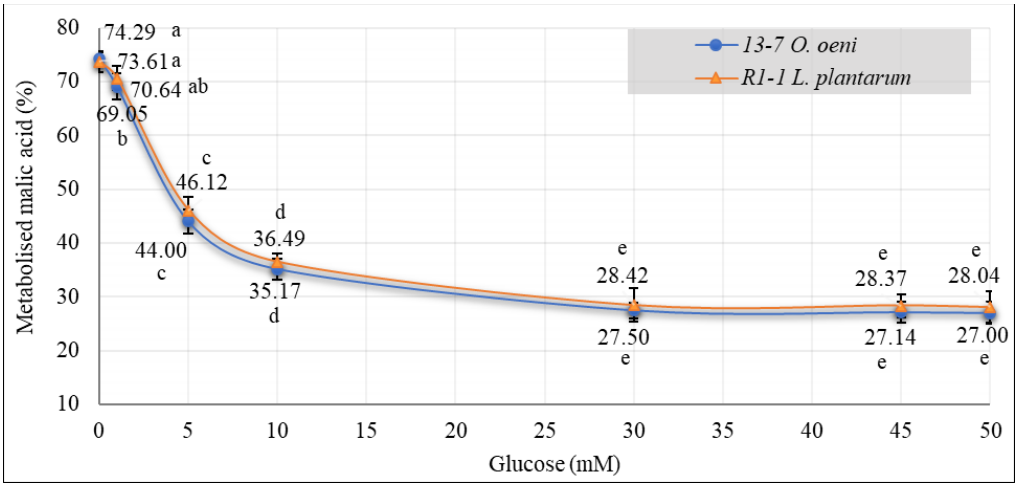

Figure 3. Effect of glucose concentration on malic acid metabolism by indigenous LAB strains. Data represent the mean values (n=3), while error bars indicate standard deviation (±). Different letters indicate significant differences in Tukey test ($p \leq 0.05$).

For both bacterial strains, a glucose concentration of 1 mM in the medium reduced the amount of malic acid metabolized with an average value of 7% compared to the control variant (without carbohydrates). Increasing the glucose concentration to 5 and 10 mM, resulted in a corresponding decrease in the amount of malic acid transformed by the LAB strains by 37 and 52%, respectively. At glucose concentrations above 30 mM, the inhibition of malic acid metabolism was very high, the percentage of malic acid metabolized being between 27 and 28%, lower by about 62% compared to the control variant. It should be noted that the *O. oeni* 13-7 strain showed the lowest values of malate bioconversion in the presence of glucose regardless of its concentration.
The results obtained are consistent with those reported by Miranda et al. (1997), except that the CCR phenomenon in the case of the studied bacterial strains 13-7 and R1-1 was not of the same intensity. Previous research showed that for *O. oeni* strains a concentration of 2 mM glucose in the medium inhibited the malolactic fermentation by up to 50%, while concentrations of 5 mM determined an inhibitory effect of about 70% (Miranda et al. 1997).

Conclusions

The indigenous malolactic bacteria strains isolated from wine microbiota (*Oenococcus oeni* 13-7 and *Lactobacillus plantarum* R1-1), showed a high ability to exhibit the carbon catabolite repression mechanism. Cultivated in glycine buffer medium with different carbohydrates (hexoses and pentoses) and malic acid, the strains reduced the malolactic bioconversion process by up to 60% in the presence of glucose, a similar effect being observed for galactose, mannose or maltose. The highest rate of malic acid metabolization was shown in the presence of fructose. At glucose concentrations above 30 mM, the inhibition of malic acid metabolism was very high, the percentage of malic acid metabolized being between 27 and 28%, which means by up to 62% lower compared to the control variant. However, *Oenococcus oeni* strain showed lower values of malic acid bioconversion in the presence of glucose regardless of concentration. The

experimental results indicate that the addition of fructose in the medium may cancel the repression of malic acid metabolism, further studies being necessary to elucidate the mechanisms underlying this process.

Acknowledgments: This research was funded by the Academy of Agricultural and Forestry Sciences "Gheorghe Ionescu-Sisești", research project no. 3311/08.12.2022.

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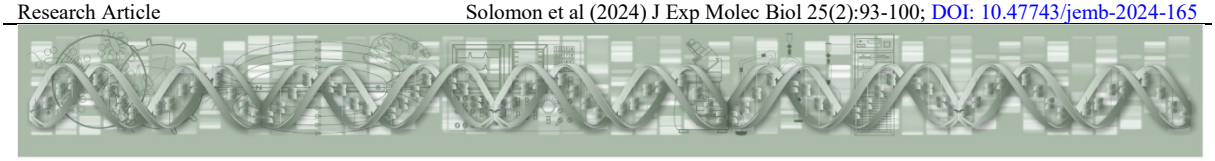

PERFORMANCE, HAEMATOLOGY, AND SERUM BIOCHEMISTRY OF RABBIT BUCKS FED SUPPLEMENTAL LEVELS OF *SACCHAROMYCES CEREVISIAE*

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Abstract

This study was carried out to investigate the effect of Saccharomyces cerevisiae on the growth performance, haematological and serum biochemical indices of rabbit bucks. Eighteen (18) bucks were used for the study. They were assigned to three treatment groups with 6 bucks per treatment, replicated 3 times in a completely randomized design for twenty-eight (28) days. The bucks were allowed one week of acclimatization before the commencement of the study, during which they were fed commercial growers diets and forages. The bucks were fed a formulated ration supplemented with S. cerevisiae at 0.0, 0.3, and 0.6g, respectively, and were coded T1, T2, and T3, respectively. T1served as control. Growth parameters, haematological indices, and serum chemistry were measured in the course of the study. The data collected were subjected to analysis of variance (ANOVA) using SPSS version 21, and means were separated using Duncan of the same software. The results revealed that S. cerevisae did not have an effect ($p > 0.05$) on the growth parameters in the rabbit bucks. This study revealed that Saccharomyces cerevisiae did not have a significant effect ($p > 0.05$) on the packed cell volume (PCV) of bucks in the study, as T1, T2, and T3 all had similar ($p > 0.05$) PCV values of 39.00, 39.00, and 41.00% respectively. S. cerevisiae influenced ($p < 0.05$) serum enzymes but did not have a significant effect ($p > 0.05$) on total protein, blood urea, Alanine transaminase (ALT), creatinine, and glucose. In conclusion, dietary supplementation of S. cerevisiae at 0.60% did not have a significant effect on growth parameter or most haematological and serum biochemical indices.

Keywords: Yeast, rabbit bucks, *Saccharomyces cerevisiae*, probiotics, growth promoters

Introduction

The ban on the use of antibiotics as growth promoters in the EU has led to investigating different natural feed additives to replace dietary antibiotics (Mahrose et al. 2019). It is well known that high levels of antibiotics have been used in livestock and poultry production as growth promoters and for disease prophylaxis. However, a major problem with this practice is the occurrence of antibiotic residues in meat because of the wide use of antibiotics as well as antibiotic resistance in both humans and animals. It therefore, becomes imperative to search for new safe alternatives for improving growth performance, health and disease control in animals. Probiotics (bacterial and yeast cultures) are non-pathogenic microbial adjuncts, that have been used as feed supplements and also as growth promoters, improving the immune system of animals by promoting the composition and microbial balance in their guts (Besseboua and Ayad 2021). The probiotic characteristics of S*accharomyces cerevisiae* have been documented

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(Belhassen et al. 2016, Abd el-aziz et al. 2021, Besseboua and Ayad 2021, Adli et al. 2023). *Saccharomyces cerevisiae*, also known as baker's yeast, is rich in proteins, vitamins, and minerals (Piskur et al. 2006). The choice for S*accharomyces cerevisiae* is based on availability in the study region, since all research is expected to address local problem(s).
However, there is a paucity of empirical studies on the effects of *S. cerevisiae* on rabbit bucks. Thus, this study was designed to evaluate the effect of *S, cerevisiae* on growth performance, haematological profile, and serum biochemical indices in rabbit bucks.

Materials and Methods

Experimental Site

The study was carried out at the Rabbitry Unit of the Teaching and Research Farm of the Department of Animal *Sc*ience, University of Uyo, Uyo, Akwa Ibom State. Uyo is located at $5^0$2'N and $7^0$55'E, with a mean annual temperature of between 26 ^{0}C and 28 ^{0}C, while the mean annual rainfall ranges from 2000 mm to 3000 mm (Solomon and Udoh 2017).

Experimental Design

The study adopted a completely randomized design (CRD). Eighteen mongrel bucks, aged 20 - 24 weeks, were randomly assigned to three treatment groups. Each treatment was further replicated three times, with two bucks per replicate. The total period of the experiment was 28 days (4 weeks).

Animal and Management

The eighteen bucks were housed individually in a three–tier wooden hutch according to their treatments and managed under standard husbandry conditions with an ad libitum supply of feed and water. Other routine management practices were carried out in accordance with the best animal welfare principles.

Experimental Diet

The bucks were fed a formulated ration with the proportionate inclusion of *S. cerevisiae* in treatments two and three at 0.3 and 0.6 g, respectively, while treatment one, which served as the control, was not supplemented with *S. cerevisiae*. The diets were coded as T1, T2, and T3, respectively.

Data Collection

Growth Performance

To evaluate the growth performance of these bucks, their initial weights were recorded at the beginning of the experiment and thereafter on a weekly basis for the 4 weeks of the study. Other parameters evaluated, namely total and daily feed intake, total and daily weight gains, and feed conversion ratio (FCR).

Serum and Haematological parameters

Upon termination of the experiment, blood samples for biochemical and haematological analysis (2 mL each) were collected via the external ear veins of the bucks from each replicated. The samples for haematological analysis were collected into sample bottles containing ethylene diamine tetra acetic acid (EDTA) to prevent coagulation, while those for serum biochemistry were collected in plain sample bottles without EDTA. The blood samples were subjected to laboratory examinations using an auto analyser (Sysmex kx-21n). The serum blood samples were kept in a slope position until they were separated through centrifugation at 1000 rpm for 20 minutes, followed by cooling in a deep freezer. All samples were then taken to the laboratory within two hours after collection for analysis. The haematological parameters evaluated were packed cell volume (PCV), red blood cells (RBC), white blood cells (WBC), haemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), lymphocytes, eosinophils,

monocytes, neutrophils, and basophils, while serum biochemical indices measured were total protein, urea, creatinine, aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine amino transaminase (ALT), and glucose. Olorede et al. (1996) described the clinical routine procedures that were used to determine the serum biochemical indices.

Table 1. Composition of Experimental Grower Diet

Ingredient (%)	**T1 (0.00g *S. cerevisiae*)**	**T2 (0.3g *S. cerevisiae*)**	**T3 (0.6g *S. cerevisiae*)**
Maize	35.50	35.50	35.50
SBM	19.00	19.00	19.00
Wheat offal	26.00	26.00	26.00
PKC	15.50	15.50	15.50
Bone meal	3.00	3.00	3.00
Premix	0.25	0.25	0.25
Common Salt	0.25	0.25	0.25
Lysine	0.25	0.25	0.25
Methionine	0.25	0.25	0.25
**S. cerevisiae*	0.00	0.30	0.60
Total	**100**	**100**	**100**
Calculated Proximate Composition			
Crude Protein	17.11	17.11	17.11
Crude Fibre	6.65	6.65	6.65
Ether Extract	6.72	6.72	6.72
Calcium	1.15	1.15	1.15
Phosphorus	0.74	0.74	0.74
Lysine	1.00	1.00	1.00
ME	2644.20	2644.20	2644.20

Vitamin Premix supplied the following per kg of diet: vitamin A 10,000 i.u., vitamin D3 12,000 i.u., vitamin E 20,000 i.u., vitamin K 2.5 mg, thiamin 2.0mg, riboflavin 3.0mg, **S. cerevisiae*: *Saccharomyces cerevisae*; SBM: Soybean Meal; PKC: Palm Kernel Cake; ME: Metabolizable Energy

Statistical Analysis:

The data collected were subjected to analysis of variance (ANOVA) using SPSS version 21, and means were separated using Duncan of the same software to separate significantly different means.

Results

Growth performance of rabbit bucks fed diets containing dietary levels of *Saccharomyces cerevisiae*

The results on the growth performance of rabbit bucks fed diets containing dietary levels of *S. cerevisae* are presented in table 2. The results revealed that *S. cerevisae* did not exhibit an effect ($p > 0.05$) on the growth parameters of the rabbit bucks. The final weight was similar ($p > 0.05$) in the study but was not significantly increased in T3. The addition of *S. cerevisae* to the bucks' diets had no effect on total and daily weight gains, but it caused non-significant increases in groups treated with *S. cerevisae* compared to the non-treated group. The values obtained in the current investigation study were 1772.67, 1778.67, and 1819 g for T1, T2, and T3, respectively, for final weights, and 389.33, 395.00 and 444.00 g for T1, T2, and T3, respectively, for the total

weight gain. Although there was no significant difference in the total and daily feed intake, rabbit bucks on T3 diets had an insignificantly ($p > 0.05$) lower feed intake. Bucks in T3 presented total feed intake of 2787.67 g, while T1 and T2 had total feed intake values of 3095.33 and 3204.00 g respectively. A non-significantly higher daily feed intake was observed in T2 ($p > 0.05$). Furthermore, better ($p > 0.05$) feed conversion in T3-treated bucks was noticed, but it remains insignificant ($p > 0.05$). FCR values in the study were 8.27 (T1), 8.12 (T2), and 6.41 (T3).

Table 2. Growth performance of rabbit bucks fed diets containing dietary levels of *Saccharomyces cerevisiae*

Parameters	T1	T2	T3	SEM
Initial weight (g)	1383.33	1383.67	1375.33	65.96
Final weight (g)	1772.67	1778.67	1819.33	71.64
Total weight gain (g)	389.33	395.00	444.00	32.31
Daily weight gain (g)	13.90	14.11	15.86	1.15
Feed intake (g)	3095.33	3204.00	2787.67	142.49
Daily feed intake (g)	110.55	114.43	99.56	5.09
Feed Conversion Ratio	8.27	8.12	6.41	0.46

SEM is the standard error of mean; means without letters were not significant ($p > 0.05$)

Haematological indices of rabbit bucks fed diets containing dietary levels of *Saccharomyces cerevisiae*

The dietary inclusion of *Saccharomyces cerevisiae* in the diets of rabbit bucks, as shown in Table 3, significantly affected white blood cells (WBC) ($p < 0.05$), platelets, and neutrophils in the bucks. However, other haematological parameters were not influenced by dietary levels of *Saccharomyces cerevisiae.* This study revealed that *Saccharomyces cerevisiae* did not have an impact in a significant manner on PCV ($p>0.05$), as T1, T2, and, T3 all had similar ($p>0.05$) PCV values of 39.00, 39.00, and,41.00%, respectively.

A higher WBC was observed with inclusion of *Saccharomyces cerevisiae* in the diets of the bucks when compared with those without *Saccharomyces cerevisiae* in their diets (T1) ($p < 0.05$). Bucks fed T3 and T2 had WBC values 5.23 and 5.17 $\times 10^9$/dL, respectively, while bucks on T1 diet had a WBC value of 3.80 $\times 10^9$/dL. Significantly higher ($p<0.05$) platelet was observed in bucks fed T2 in the study, while similar ($p>0.05$) lower values were recorded in T1 and T3, respectively. S*accharomyces cerevisiae* did not have an effect on RBC, as the values recorded were statistically similar ($p > 0.05$). The MCV, MCH, and MCHC were all similar ($p > 0.05$) across dietary treatment groups. Haemoglobin was not impacted by S*accharomyces cerevisiae* in the study, as the values observed were 13.27, 13.10, and 13.38 g/dL, respectively, for T1, T2 and T3. Similarly, lymphocytes, eosinophils, basophils, and monocytes all showed no significant alteration ($p>0.05$) with inclusion of S*accharomyces cerevisiae* in the bucks' diets. However, neutrophils were substantially increased ($p<0.05$) in bucks fed the T1 diet compared to those fed T2 and T3 diets respectively.

Table 3. Haematological indices of rabbit bucks fed diets containing dietary levels of *Saccharomyces cerevisiae*

Parameters	T1	T2	T3	SEM
Packed Cell Volume (%)	39.00	39.00	41.00	0.69
White Blood Cells ($\times 10^9$/dL)	3.80^b	5.17^a	5.23^a	0.29
Platelet	174.67^b	339.67^a	221.67^{ab}	30.61
Red Blood Cells ($\times 10^{12}$/L)	5.87	5.77	5.97	0.18
MCV (fl)	66.67	68.67	70.00	1.45

MCH (pg)	23.00	23.67	23.00	0.32
MCHC (%)	34.00	33.67	33.67	0.15
Haemoglobin (g/dL)	13.27	13.10	13.38	0.28
Lymphocytes (%)	47.00	59.00	59.00	2.71
Eosinophil (%)	2.67	2.67	2.00	0.53
Monocytes (%)	2.00	2.00	1.67	0.11
Neutrophils (%)	48.67^{a}	35.67^{b}	37.67^{ab}	2.67
Basophil (%)	0.00	1.00	0.00	0.17

MCV: mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; SEM: Standard error of means; Means with different superscripts are significant ($p<0.05$).

Serum biochemistry of rabbit bucks fed diets containing dietary levels of *Saccharomyces cerevisiae*

The result on serum biochemical indices of male rabbits fed different levels of dietary *S. cerevisiae* are presented in table 3. The present study showed that *S. cerevisiae* influenced serum enzymes ($P<0.05$), but did not have any significant effect on total protein, blood urea, ALT, creatinine and glucose ($p > 0.05$). The values of total protein observed in the study were 60.67, 56.00, and 60.67 g/dL for T1, T2, and T3, respectively. Similar urea values were recorded for all treatments in the study ($p > 0.05$). Lower aspartate aminotransferase (AST) and alanine alkaline phosphatase (ALP) were detected when adding *S. cerevisiae* to the bucks diets ($p < 0.05$). AST was higher (168.67 µ/L) in the control group (T1), while bucks on T2 diet presented the least AST value. Alkaline phosphatase was higher in bucks' diet that did not contain *S. cerevisiae.* Alanine aminotransaminase (ALT) did not show any significant statistical variation with dietary inclusion of *S. cerevisiae* in the bucks' diets ($p > 0.05$). Creatinine values were 154.00, 119.33, and 133.00 mg/dL for T1, T2, and T3, respectively. Glucose values were also similar across diets ($P>0.05$). The values obtained in the study were 5.60, 5.55, and 6.30 g/dL for T1, T2, and T3, respectively.

Table 4. Serum biochemistry of rabbit bucks fed diets containing dietary levels of *Saccharomyces cerevisiae*

Parameters	**T1**	**T2**	**T3**	**SEM**
Total protein (g/dL)	60.67	56.00	60.67	1.03
Urea (mmol/L)	7.90	7.30	7.40	0.18
AST (µ/L)	168.67^{a}	91.67^{b}	112.00^{ab}	15.00
ALP (µ/L)	31.00^{a}	24.67^{b}	26.00^{ab}	1.13
ALT (µ/L)	46.67	37.67	37.00	3.15
Creatinine (mg/dL)	154.00	119.33	133.00	8.17
Glucose (g/dL)	5.60	5.55	6.30	0.21

AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; ALT: Alanine transaminase; SEM: Standard error of means; Means with different super Scripts are significant ($p<0.05$).

Discussions

Growth performance of rabbit bucks fed diets containing dietary levels of *Saccharomyces cerevisiae*

The reported findings in this study showed that the diet with *S. cerevisiae* did not affect the weight of the animals. The reported findings are not in line with those of Besseboua and Ayad (2021), who noticed a decrease in the animals' weight. The insignificant effect of *S. cerevisiae* on the growth parameters in rabbit bucks confirmed earlier studies by Seyidoglu and Galip

(2014) and Belhassen et al. (2016), who reported no significant differences in the growth performance of rabbits fed diets supplemented with *S. cerevisiae*. Kimsé et al. (2012), in their investigation, noted that yeast did not affect final body weight, daily weight gain, or feed intake in New Zealand rabbits. Abdel-Aziz et al. (2021) also demonstrated insignificant improvement in the growth performance of mice administered *S. cerevisiae*. However, Saied et al. (2011), and Onwurah and Okejim (2014), reported significant improvements with *S. cerevisiae* supplementation in broiler chicks and broiler chickens, respectively. These variations may be attributed to the species of animals, age, dose, and environmental conditions.

Haematological indices of rabbit bucks fed diets containing dietary levels of *Saccharomyces cerevisiae*

The WBCs, platelets, and neutrophils of rabbit bucks fed diets supplemented with *S. cerevisiae* were significantly different in the study as compared to the control group, while other haematological parameters remained the same. This observation agrees with the reports of Seyidoglu and Galip (2014) and Belhassen et al. (2016), who noted that *S. cerevisiae* in the diets of rabbits had no effect on some haematological parameters, although they detected a slight increase in (PCV) and, haemoglobin (HGB) concentrations in rabbits supplemented with yeast, which differ with this study. The significant effect of *S. cerevisiae* supplementation in bucks' diets on WBC in the current study supports the reports of Paryad et al. (2008) and Mulatu et al. (2019), who recorded significantly elevated WBCs in their studies, which differs from the reported findings of Besseboua and Ayad (2021), for the established parameters. Mulatu et al. (2019) revealed that WBCs, packed cell volume (PCV) and haemoglobin (HGB) were higher in chickens fed diets containing *S. cerevisiae*. Elghandour et al. (2019) noticed that yeast-fed rabbits had more WBCs, and LCTs, compared to the rabbits fed the control diet. The significant increase in the WBC, platelets, and decrease in neutrophils suggests improved immunity, better clotting factors, and reduced parasitism in the bucks, supporting the earlier report of Kazuun and Kazuun (2019), who reported that probiotics have immune-stimulatory effects.

Serum biochemistry of rabbit bucks fed diets containing dietary levels of *Saccharomyces cerevisiae*

Total protein, urea, ALT, creatinine, and glucose were not influenced by *Saccharomyces cerevisiae* supplementation in the bucks' diets in the current study. Serum total protein is a marker of the synthetic function of the liver and a valuable guide to assess the severity of liver damage (Osigwe et al. (2017). The similarity, therefore, in the study suggests no liver damage in the bucks. Low or high total protein is an indication of liver disorders and malnutrition (Augustine et al. 2020). The insignificant effect of *S. cerevisiae* on ALT, urea, and creatinine in this study is similar to the findings of Abd El-aziz et al. (2021). Similar results were demonstrated by Seyidoglu and Galip (2014), who supplemented 3 g of yeast per kg in rabbit diet, and Ozsoy and Yalcin (2011) with Saccharomyces *cerevisiae* in broiler turkey. The reduction in AST and ALP may be triggered by the presence of *S. cerevisiae* in the bucks' diet. According to Alagbe and Adegbite (2019), serum enzyme values are triggered by the presence of anti-nutrients or toxic substances in the feed of animals. Raised ALT and AST have been identified by Yin and Tong (2014) to be biomarkers of hepatocellular damage, which is induced by these enzymes leaking into the blood stream. Hence, the results in this present investigation indicated that the hepatic functions of the bucks were not compromised in the course of the study.

Conclusions

The present findings showed that dietary supplementation of *S. cerevisiae* at 0.60% did not exhibit a significant effect on growth parameters or most haematological and serum biochemical indices in rabbit bucks, therefore implies that the inclusion of *S. cerevisiae* in the rabbit bucks diet may not be necessary since it has very minimal effect growth and most blood parameters add to the total cost of production.

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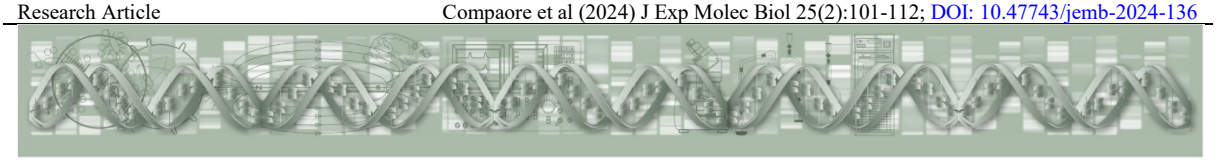

LINKAGE BETWEEN ACE2 GENE POLYMORPHISMS AND SARS-COV-2 INFECTION IN BURKINA FASO, SUB-SAHARAN AFRICA

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Abstract

The *ACE2* gene polymorphisms (rs143936283, rs146676783, and rs4646116) in infected and noninfected persons by SARS-CoV-2 in Burkina Faso. Our cross-sectional study population comprised 137 SARS-CoV-2 infected persons and 181 non-infected persons. Three *ACE2* gene polymorphisms, rs143936283, rs146676783, and rs4646116, were genotyped using the real-time PCR standard TaqMan allelic discrimination technique. The association between SARS-CoV-2 infection and the polymorphisms were evaluated by a binary logistic regression. There was no association between the polymorphisms rs143936283 rs4646116 haplotypes, and SARS-CoV-2 infection in our study population. However, in the female population, the heterozygous genotype CT of rs146676783 increased by two and half the risk (OR=2.58 95%CI (1.2-5.48), p= 0.014) of being infected by SARS-CoV-2. Additionally, carrying the homozygous minor allele (genotype TT) of rs146676783 increased by more than five and half the risk (OR=5.57 95%CI (1.64-18.78), p=0.006) of being infected by SARS-CoV-2 among females. This study showed that the *ACE2* gene variant rs146676783 was associated with an increased risk of being infected by SARS-CoV-2 in females, suggesting a need for further investigation to contribute to a better understanding of the African COVID-19 enigma**.**
Keywords: SARS-CoV-2, ACE2, polymorphism, haplotypes, Burkina Faso

Introduction

The corona disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has severely challenged the global health system worldwide. Africa and particularly Burkina Faso, was not an exception (Abdou Azaque Zoure 2022). In Burkina Faso, the first case of COVID-19 was recorded early March 2020 (Savadogo 2021, Ouattara et al. 2023). High seroprevalence of SARS-CoV-2 was recorded in Burkina Faso but mainly in asymptomatic patients, as shown by the studies of (Sagna et al. 2022, Struck et al. 2022) compared to Western countries with higher symptomatic cases and higher deaths. This discrepancy could be due to genetic and environmental factors. The genetic elements implied in SARS-CoV-2 infections are ACE2, cellular transmembrane serine protease two

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(TMPRSS2), and endosomal/lysosomal cysteine proteases cathepsin B and L (CTSB) (Trougakos et al. 2021).

Studies have shown that the *angiotensin-converting enzyme 2 (ACE2*) gene is on the X-chromosome (Srivastava et al. 2020, Zhou et al. 2020). The main entry of the virus into human cells is through the angiotensin-converting enzyme (ACE) receptor two, which exists in two (2) forms, a full-length m*ACE2,* and a s*ACE2*, a soluble form found in circulation (Nelson-Sathi et al. 2022, Scialo et al. 2020). The spike (s) protein on the SARS-CoV-2 envelope *ACE2* found on human cell membranes, consisting of a transmembrane anchor and an extracellular domain. The protein S of SARS-CoV-2 is cleaved into S1 and S2, and the interaction of the complex S1 protein/receptor is crucial for the virus to infect a host cell (Batlle et al. 2020, Samavati and Uhal 2020). The severity of the COVID-19 disease could be alleviated by a decreased level of *ACE2*. Moreover, cardiovascular homeostasis and electrolyte balance, as well as lung injury protection, are sustained by ACE2 (Yang et al. 2020). *ACE2* gene variants have been shown to have a functional role in binding the SARS-CoV-2 spike protein (Cruz et al. 2021, Hussain et al. 2020). Of which rs143936283 and rs4646116, two of the missense single nucleotide variation (SNV) have a higher susceptibility to SARS-CoV-2 infection (Hussain et al. 2020). The SNV rs4646116 had a population allele frequency (AF) of 0.012 in Ashkenazi Jewish, 0.005 in European, 0.0033 in American, and 0.0008 in Asian populations based on the Genome Aggregation Database (gnomAD) (Suryamohan et al. 2021). The SNV rs146676783 is one of the missense SNVs whose allele frequency is 0.0001 in European and African people based on the Gnom AD exomes data and is thought to lessen SARS-CoV-2 infection susceptibility (Wang et al. 2020). African countries such as Burkina Faso have had low hospitalized and death related to COVID-19 (JHU 2023). Furthermore, data on *ACE2* gene polymorphism associated with SARS-Cov-2 infection in Africa is scarce. Our study aimed to evaluate the *ACE2* gene single nucleotide polymorphisms (SNPs), rs143936283, rs146676783, and rs4646116 among infected or noninfected persons by SARS-CoV-2 in Burkina Faso.

Material and Methods

Study subjects

It was a cross-sectional study between August and November 2022 in Ouagadougou, the capital of Burkina Faso. The study included 137 positive and 181 negative cases. The positive and negatives cases were tested by real-time PCR using the Quant Studio 5 (Applied Biosystems). All samples were tested for antibodies against SARS-CoV2 by ELISA using the WANTAI SARS-CoV-2 Ab ELISA kit (Wantai SARS-CoV-2 Diagnostics). This study included nasal or oropharyngeal and blood samples collected from non-vaccinated persons between November 2020 and December 2021 from the Biomedical Research laboratory of the "Institut de Recherche en Sciences de la Sante) and the Pietro Annigoni Biomolecular Research Center (CERBA).

Screening and genotyping of the selected Single Nucleotide Polymorphisms (SNPs)

According to the manufacturer's protocol, the "PureLink™ Genomic DNA" mini kit (Invitrogen) was used to extract genomic DNA from the nasopharyngeal swabs. The SNP were selected from the PubMed database literature and presented in Table 1. The genotyping of the selected polymorphism of the *ACE2* gene was performed by the standard allelic discrimination technique using TaqMan® probes based on the 5'exonuclease activity of DNA polymerase by real-time PCR on Quantstudio 5 (Applied Biosystems). The amplification program was as follows a pre-amplification step at 60 °C for 30 seconds, denaturation at 95°C for 10 minutes followed by 40 hybridization cycles at 95 °C for 15 seconds, 40 elongation cycles at 60 °C for 1 minute, and a final elongation of 60 °C for 30 seconds.

The Haploview software 4.1 ensured that our selected SNPs (rs143936283, rs146676783, and rs4646116) minor allele frequencies were ≥ 1.

Ethical Considerations

The protocol approval for the study was obtained from the Ethics Committee for Health Research in Burkina Faso (number 2022-02-035). The patients' samples were handled with anonymity by using a codification system.

Data Processing and Analysis

Data were entered using Excel 2016 software. These data were then analyzed using Statistical Package for Social Sciences (SPSS) version 25.0.0.0. Frequencies were used to classify variables. The chi-square test was used to compare the difference between the distributions. Since the *ACE2* gene is located on chromosome X, the distribution of the selected SNPs is presented as positive and negative SARS-CoV-2 groups stratified by gender. Binary logistic regression tests were then determined by adjusting the odds ratio (OR) with age. We also analyzed overdominant, dominant, and recessive models to evaluate the inheritance model. Haplotype analysis was done using Haploview 4.1 software. The difference was statistically significant for $p \leq 0.05$.

Tabel 1. Marker information

Gene	SNP rs#	Position	Database Alleles	Reference Allele	Amino Acid change
Ace2, Chr X	rs143936283	15581305	T>C	T	E329G
	rs146676783	15600803	C>T	C	E37K
	rs4646116	15600835	T>C	T	K26R

Results

Study population

Samples from 137 individuals infected with SARS-CoV-2 and 181 not infected were successfully genotyped.

The mean age of our study population was 34.30 ± 14.93 years. Male participants were more represented than females, with a sex ratio (M/F) of 1.10. The age group of 25-40 years was the most represented in both SARS-CoV-2-infected and uninfected groups. Furthermore, age seems to be linked to SARS-CoV-2 infection status $p=0.051$ (Table 2).

Table 2. Socio-demographic data

Characteristics	Negative cases n (%)	Positive cases n (%)	*p-value*
Sex			
Female	88 (48.6)	63 (46)	*0.652*
Male	93 (51.4)	74 (54)	
Total	181 (56.9)	137 (43.1)	
Age group (years)			
<24	50 (27.6)	31 (22.6)	
25-40	79 (43.6)	66 (48.2)	
41-55	36 (19.9)	17 (12.4)	*0.051*
55>	16 (8.8)	23 (16.8)	
Total	181 (59.1)	137 (40.9)	

Association between Age by sex and SARS-CoV-2 infection status

Table 3 shows that age groups among women are not related to the SARS-CoV-2 infection status. Among males, subjects aged over 55 had three (3) an increased risk compared to patients younger than 24 but it was not statistically significant [OR=3.08; 95%CI (0.925-10.25); *p*=0.067].

Table 3. Association between Age, sex, and SARS-CoV-2 infection status

Age groups	Negative n (%)	Positive n (%)	OR	95% CI	p-value
Female					
<24	22 (25)	11 (17.5)		**Reference**	
25-40	38 (43.2)	29 (46)	1.526	0.64-3.64	0.34
41-55	17 (19.3)	11 (17.5)	1.294	0.45-3.69	0.63
55>	11 (12.5)	12 (19)	2.18	0.73-6.5	0.16
Male					
<24	28 (30)	20 (27)		**Reference**	
25-40	41 (44.1)	37 (50)	1.263	0.61-2.61	0.53
41-55	19 (20.4)	6 (8.1)	0.442	0.15-1.30	0.14
55>	5 (5.4)	11 (14.9)	3.08	0.925-10.25	0.067

OR: odds ratio, **CI:** confidence interval

Comparison of ACE2 gene polymorphism between cases and controls

The polymorphism rs143936283 and rs4646116 have both TT as the normal homozygous genotype and TC as the heterozygous genotype, while rs146676783 have CC as the normal homozygous genotype and CT has the heterozygous genotypes.

The overall minor allele frequency (MAF) of rs143936283 was C = 0.311, the MAF of rs146676783 was T = 0.307, and that of rs4646116 was C = 0.247 in our study population n. Table 4 displayed the allele frequency distribution of these three single nucleotide polymorphisms of the *ACE2* gene in cases and control groups. It also shows clearly that one (1) polymorphism, rs146676783, is related to the infection SARS-CoV-2.

Table 4. Allele frequency of *ACE2* gene polymorphism between SARS-CoV-2 infected and noninfected in a population from Burkina Faso

SNPs	Negative n (%)	Positive n (%)	n (%)	χ^2	*p-value*
		rs143936283			
T	231 (63.8)	184 (67.2)	415 (65.3)	0.768	*0.381*
C	131 (36.2)	90 (32.8)	221 (34.7)		
		rs146676783			
C	277 (76.5)	177 (64.60)	454 (71.4)	10.85	***0.001***
T	85 (23.5)	97 (35.40)	182 (28.6)		
		rs4646116			
T	299 (82.6)	236 (86.1)	535 (84.1)	1.459	*0.227*
C	63 (17.4)	38 (13.9)	101 (15.9)		

Association between ACE2 gene polymorphisms and infection by SARS-CoV-2 in male patients

Table 5 shows that in terms of allele frequencies of each *ACE2* SNP studied, and there is no statistical difference between the two groups. Furthermore, genotypically-wise, there was also no statistical significance for SARS-CoV-2 infection. As shown in Table 2, age is a factor that might be related to SARS-CoV-2 disease among males. The association between *ACE2* gene polymorphism and SARS-CoV-2 infection odds ratio was adjusted for age to avoid its influence, and there was no statistical significance.

Table 5. Allelic frequency and association of *ACE2* gene polymorphisms and infection by SARS-CoV-2 in male patients

SNPs	Negative, n (%)	Positive, n (%)	OR (95% CI)	*p-value*
		rs143936283		
T	138 (74.2)	108 (73.0)	**Reference**	-
C	48 (25.8)	40 (27.0)	1.065 (0.683 – 1.737)	*0.801*
		rs146676783		
C	144 (77.4)	108 (73.0)	**Reference**	**-**
T	42 (22.6)	40 (27.0)	1.270 (0.770 – 2.093)	*0.349*
		rs4646116		
T	168 (90.3)	140 (94.6)	**Reference**	-
C	18 (9.7)	8 (5.4)	.833 (0.225-1.264)	*0.153*

Association between ACE2 gene polymorphisms and infection by SARS-CoV-2 in female patients

The genotypic distributions of rs146676783 and rs4646116 were consistent with the Hardy-Weinberg equilibrium law, and the *p-values* were 0.978 and 0.457, while rs143936283 was not consistent with the *p-value* was 0.027. When comparing the case and control groups in female for the 3 SNPs in Table 6, the minor Allele T of rs146676783 seem to increase by more than two (2) times the risk of being infected by SARS-CoV-2 among females, and it was statistically significant ($p < 0.001$). Regarding genotyping frequency, when the odds ratio was adjusted for age, the CT of rs146676783 increased by two and half the risk of being infected by SARS-CoV-2 [OR=2.582; 95%CI (1.216-5.485); $p=0.014$]. Additionally, genotype TT of rs146676783 increased by almost more than five and half times the risk of being infected by SARS-CoV-2 among females [OR=5.557; 95%CI (1.644-18.785); $p=0.006$].

Table 6. Genotype and allele frequencies of three genetic polymorphisms among negative patients and SARS-CoV-2 infected female patients

Genotype	Negative, n (%)	Positive, n (%)	OR (95% CI)	*p-value*
		rs143936283		
TT	20 (22.7)	20 (31.7)	**Reference**	-
TC	53 (60.2)	36 (57.1)	0.658 (0.203-2.132)	*0.485*
CC	15 (17.0)	7 (11.1)	0.883 (0.391-1.995)	*0.765*
C	83 (47.2)	50 (39.7)	0.737 (0.464-1.172)	*0.197*
		rs146676783		

CC	50 (56.8)	18 (28.6)	**Reference**	-
CT	33 (37.5)	33 (52.4)	**2.582 (1.216-5.485)**	***0.014***
TT	5 (5.7)	12 (19.0)	**5.557 (1.644-18.785)**	***0.006***
T	43 (24.4)	57 (45.2)	**2.55 (1.56-4.176)**	***< 0.001***
		rs4646116		
TT	49 (55.7)	34 (54.0)	**Reference**	-
TC	33 (37.5)	28 (44.4)	1.166 (.564-2.410)	*0.678*
CC	6 (6.8)	1(1.6)	0.356 (.039-3.219)	*0.358*
C	45 (25.6)	30 (23.8)	0.91 (.535-1.548)	*0.727*

TT: *Homozygous genotype of* ***T*** *alleles;* ***CT :*** *Heterozygous genotype of* ***C*** *and* ***T*** *alleles,* ***TC:*** *Heterozygous genotype of* ***T*** *and* ***C*** *alleles.****OR:*** *odds ratio,* ***CI****: confidence interval*

We then analyzed the risk of association between female patients' genotype and SARS-CoV-2 infection by looking at the over-dominant, dominant, and recessive inheritance models (Table 7). The odds ratio shows statistical differences for the dominant [OR=3.289; 95%CI (1.649-6.561); *p=0.001*] and recessive [OR=3.906; 95%CI (1.300-11.734); *p=0.015*] models for rs146676783.

Table 7. Association between 3 single loci and SARS-CoV-2 infection Status, Based on Overdominant, Dominant, and Recessive Models

Markers	Models	OR	95% CI	*p-Value*
rs143936283	TT&CC (referent) vs TC, Overdominant	0.881	0.457-1.698	*0.704*
	TT*vs*.TC &CC, Dominant	0.632	0.305-1.31	*0.217*
	CC *vs*. TT &TC, Recessive	0.608	0.232-1.693	*0.311*
rs146676783	CC&TT (referent) vs CT, Overdominant	1.833	0.951-3.534	*0.07*
	CC *vs*. CT & TT, Dominant	**3.289**	**1.649-6.561**	***0.001***
	CC & CT *vs*. TT Recessive	**3.906**	**1.300-11.734**	***0.015***
rs4646116	TT&CC (referent) vs TC Overdominant	1.333	0.690-2.575	*0.392*
	TT *vs*. TC & CC, Dominant	1.072	0.560-2.053	*0.835*
	TT & TC *vs* CC, Recessive	0.220	0.026-1.878	*0.167*

TT: *Homozygous genotype of* ***T*** *alleles;* ***CT:*** *Heterozygous genotype of* ***C*** *and* ***T*** *alleles,* ***TC:*** *Heterozygous genotype of* ***T*** *and* ***C*** *alleles.****OR:*** *odds ratio,* ***CI****: confidence interval*

Linkage Disequilibrium and Haplotype Analysis

As the three (3) SNVs are on chromosome X, linkage disequilibrium and haplotype analysis were carried out; results are shown in the supplementary file, implying that rs143936283 and rs146676783 might fall in the same linkage area in male subjects. The 3 SNPs were in linkage disequilibrium. The haplotype analysis displayed that rs143936283 and rs146676783 of the *ACE2* gene define a block of 19 Kb. Furthermore, three (3) sorts of haplotype (combination frequency >5%) are yielded from this analysis, among which the "T-C" had the higher frequency, 67.7%, followed by "T-T" (24.6%) and "C-C" (7.8%) in male patients. There was no association between SARS-CoV-2 infection and *ACE2* gene SNP haplotypes in males. The haplotype analysis was also conducted in the female population and did not yield a haplotype.

Discussions

To our knowledge, this is one of the first studies addressing the association of *ACE2* gene polymorphisms (rs143936283, rs146676783, and rs4646116) with SARS-CoV-2 infection. The mean age of our study population was 34.30 ± 14.93 years, which is coherent with the other studies carried out in Burkina Faso (Compaore et al. 2016) but different from the mean age in studies from China which were between 49 and 55 years (Abdou Azaque Zoure 2022). This difference could be due to the critical youth proportion in Burkina Faso (INSD 2022). Our study results show that age seems to be a risk factor for SARS-CoV-2 infection, especially among males. Age-wise, our study showed that males over 55 years had three (3) times an increased risk of being infected by SARS-CoV-2 compared to those who were younger than 24 years. This observation is consistent with the literature showing that older people are more likely to be infected by SARS-CoV-2 (Farshbafnadi et al. 2021).

ACE2 expression in plasma is linked to SARS-CoV-2 infection (Scialo et al. 2020). However, we could not carry out that experiment, but we concentrated on three single nucleotide variations (rs143936283, rs146676783, and rs4646116). The minor allele frequencies of these SNP in our population differed from those of western or Asian descent (Darbani 2020). Our study shows that *ACE2* gene polymorphism rs146676783 is linked with SARS-CoV-2 infection, and it was statistically significant with $p=0.001$. To further understand the link between *ACE2* gene rs143936283, rs146676783, and rs4646116, we stratified the alleles and genotype frequencies by gender. Only the T allele of rs146676783 had a higher frequency in females infected with SARS-CoV-2 compared to those who were not. The carriers of the T alleles of the SNP rs146676783 had about 2.55 times the risk of being infected by SARS-CoV-2 compared to the carriers of the reference allele C and $p<0.001$.

Regarding rs146676783 genotypes, the female carriers of the CT genotype, when adjusted by age, had 2.58 times the risk of being infected by SARS-CoV-2, and the carriers of genotype TT had an odds ratio adjusted by 5.56. Inheritance model analysis showed that a dominance model transmits the T allele in females as one copy of T is enough to modify the risk. These results differ from that found in the literature, especially on the population of Asia descent, as rs146676783 or E37K is thought to reduce the binding of the S protein to the ACE2 receptor (Wang et al. 2020). We found no association between SARS-CoV-2 infection and the SNPs rs143936283 and rs4646116. However, authors have found decreased and increased SARS-Cov2 S protein binding in populations of Asian and western descent (Darbani 2020).

These differences could be due to genetic variation, as there are population-based differences between these variants (Mahmood et al. 2022; Sarangarajan et al. 2021; Srivastava et al. 2020). The haplotype analysis only yielded haplotypes among males but not in females. The latter could be due to the relatively low numbers of females after stratification, which did not allow for obtaining accuracy. It could also be due to the gender-specificity of *ACE2*. ACE2, a Renin-Angiotensin System (RAS) member, might have its gene regulated by estrogen (Wu et al. 2018). Some studies have found that ACE2 might play more prominent roles in females than males, while others found the opposite (Mohana et al. 2012, Ahmed A. Suleiman 2021, Chen et al. 2021). The location of the *ACE2* gene on the Xp22 site of chromosome X is said to have genes to prevent X chromosome inactivation and could lead to differences between genders (Carrel and Willard 2005, Talebizadeh et al. 2006, Berletch et al. 2011). The impact of the *ACE2* gene was found to be different between genders, according to several studies (Patel et al. 2012, Meng et al. 2015, Wu et al. 2018). Unfortunately, we could not gather clinical data on the COVID-19 disease and its complication related to our study subjects to further analyze their relation to *ACE2* gene SNVs studied. Additionally, a larger sample size is also needed to validate our findings.

Conclusions

This study showed that the *ACE2* gene variant rs146676783 was associated with an increased risk of being infected by SARS-CoV-2 in females in Burkina Faso. Gender-based studies are necessary to investigate the association between *ACE2* gene polymorphisms. However, polymorphisms of rs4646116 and rs143936283 showed no association with the occurrence of infection. Their expression would contribute to further understanding of why Burkina Faso and many other countries of the African continent had fewer COVID-19 cases. The proposed studies could potentially be used to develop supportive therapy for COVID-19 patients.

Abbreviations

Ab	**:**	Antibody
ACE2	**:**	angiotensin-converting enzyme receptor 2
COVID-19	**:**	Coronavirus disease 2019
ELISA	**:**	Enzyme Linked ImmunoSorbent Assay
RT-PCR	**:**	Reverse transcriptase polymerase chain reaction
SARS-CoV-2	**:**	severe acute respiratory syndrome coronavirus 2

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Declarations

Conflict of interests:
The authors declare that there are no conflicts of interest**.**

Ethics statement
The study was approved by the ethics committee for health research of Burkina Faso (CERS) (approval n° 2022-02-035). Informed Consent was obtained from the participants/parents/legal guardians in the study, for the subsequent use of the collected samples. The research has been performed in accordance with the Declaration of Helsinki. The patients' samples were handled with anonymity by using a codification system**.**

Consent for publication
Not applicable.

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Author contributions:
TRC, IS, LT, WFD, HGO, and JS contributed to the design and implementation of the study. TRC, SZ, STS, AAZ, AKO, RK, VS, CD, ARN, OO, ZS, DK collected the data and performed laboratory testing. TRC, IS, SL, OHG, ATY, AAZ, TS performed the analysis and interpretation of data. TRC, IS, LT, and AKO drafted the manuscript. All authors commented on the manuscript. All the authors reviewed and approved the final manuscript to be published.

Data availability statement:
The data sets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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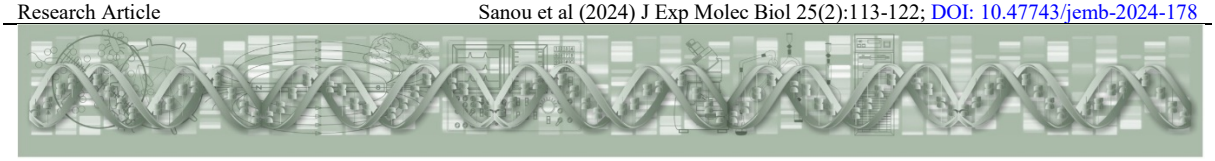

INHIBITORY ACTIVITY AGAINST α-AMYLASE AND GLUCOSE ADSORPTION CAPACITY OF THE AQUEOUS DECOCTATE OF *CHAMAECRISTA NIGRICANS* (VAHL) GREENE

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Abstract

Diabetes management involves preventing its risk factors. Inhibition of glucosidases and adsorption of excess free glucose are approaches to the prevention of postprandial hyperglycemia. The objective of the present study was to evaluate the antioxidant activity, glucose adsorption capacity, and α-amylase inhibitory activity *in vitro* of the aqueous extract of *Chamaecrista nigricans*. Determination of phenolic compounds content was performed using the Folin-Ciocalteu reagent and the aluminum chloride method was used for total flavonoids one. The glucose oxidase peroxidase kit was used to determine the adsorption capacity of glucose while the 3,5-dinitrosalicylic acid method was used to assess the inhibitory activity against α-amylase. Levels ranging from 33.87 ± 2.48 mg GAE/100 mg dry extract (DE) for total phenolic compounds and 1.98 ± 0.51 mg QE/100 mg DE for total flavonoids were observed. The adsorption capacity was correlated with the glucose concentration of the solution ($r = 0.95$) and was up to 36.61 µmol/g DE for a glucose concentration of 30 mM. The extract from the November collection was most active against α-amylase with $IC50 = 0.17$ mg DE/mL. Observations confirm the traditional use of this species as a preventive measure in recipes for the treatment of diabetes. This data provides a basis for future pharmaceutical prospecting.
Keywords: Diabetes, phenolic compounds, adsorption, α-amylase, *Chamaecrista nigricans*

Introduction

The management of hyperglycemia is crucial in the therapeutic management of diabetes mellitus because it can very quickly be fatal for the patient. Postprandial hyperglycemia is partly related to gastrointestinal factors, including intestinal absorption of glucose, which derive in part from the catalytic activities of glucosidases (Paquot & Scheen 2006). Indeed, the action of these enzymes releases monosaccharides from polysaccharides rich foods that are absorbed at the intestinal level. Thus, adsorbents and intestinal glucosidase inhibitors are potential drugs sought in the management of type 2 diabetes (Puls et al. 1977). Intestinal α-glucosidase inhibitors indirectly reduce glucose absorption, thereby preventing or mitigating postprandial

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hyperglycemia. On the one hand, phenolic compounds are well known to have glucosidase inhibitory activities (Tadera et al. 2006). On the other hand, polysaccharides and other secondary metabolites slow down postprandial hyperglycemia by adsorbing glucose already released by the action of enzymes and thus making it unavailable for intestinal absorption (Ahmed et al. 2011). In addition, antioxidants provide protection against metabolic disorders such as in the case of diabetes (Nimse & Pal 2015) where they are used in the management of stress. These compounds have the ability to slow down or prevent the oxidation of some molecules, most often either by the transfer of hydrogen atoms or by the transfer of electrons (Prior et al. 2005). Nowadays, plants are sources of natural compounds with multiple properties and which are often said to have fewer harmful side effects than their synthetic analogues.

Chamaecrista nigricans is a plant species in the family of Fabaceae-Cesalpinoideae. Many traditional uses of this plant have been reported and several scientific studies have mentioned a number of its very interesting properties. Thus, studies have shown its use against fungal and bacterial germs involved in dermatoses, its anti-inflammatory potential (Mogode 2005, Ayo et al. 2007, Eyang Esseng 2007), its anthelmintic properties (Oba et al. 2016) and its anti-plasmodial activity (Fatima et al. 2017). The phytochemistry of this species reveals the presence of anthraquinones such as emodin (1,6,8-trihydroxy-3-methyl-anthraquinone), chrysophanol and physcion. It also contains compounds such as 1,2-benzenedicarboxylic acid diisooctyl ester, 4-C-methyl-myo-inositol, n-hexadecanoic acid, 2-methyl-butanoic acid and octadecanoic acid. Besides coumarins, anthracenosides, flavonoids, mucilages, sterols and triterpenes, cardiotonic heterosides and leucoanthocyanins are found in its extracts. In addition to the above, other isolated molecules of this species are emic acid, luteolin (flavonoïd), hydroxyestranic acid ethyl ester, citreorosein (Ayo et al. 2007, Mogode 2005, Tangavelou et al. 2018).

Until now, few studies have focused on the anti-diabetic properties of this plant. In addition, our previous work has shown that *C. nigricans* extract is a potential inhibitor of α-amylase enzymatic activity, with quite significant antioxidant activity(Sanou et al. 2021). In addition, *C. nigricans* is a potential source of phenolic compounds (Ayo 2010). These compounds are known to be involved in antioxidant activity and enzyme inhibition (Tadera et al. 2006, Kang et al. 2014). However, the bioactive compound content of a plant may very well vary depending on the season and the locality of harvest (Ouédraogo et al. 2022) and may miss out on an interesting activity if the timing of harvest is not appropriate.

The objective of the present study was to evaluate the antioxidant activity, glucose adsorption capacity and *in vitro* inhibitory activity against α-amylase of the aqueous extract of *Chamaecrista nigricans* and at the same time to make a comparative study of these activities for samples from three different months of harvest.

Materials and methods

Plant material

The plant material consisted of the leaves of *Chamaecrista nigricans* (Vahl) Greene. The samples were harvested during the months of October, November and December 2020 in the Classified Forest of Dindéresso about twenty kilometers from the city of Bobo-Dioulasso, Houet province in Burkina Faso. They have been authenticated and deposited in the herbarium of the Floristic Center of the Nazi BONI University under the number UNB-957.

Chemicals

All reagents and chemicals used were of analytical grade. Absolute methanol were procured from Chem-Lab (Belgium). Absolute anhydrous ethanol, ferric chloride, sodium carbonate, trichloroacetic, starch soluble and potassium hexacyanoferrate acid were procured from Carlo Erba (France). L-(+)-ascorbic acid, Gallic acid, 3,5-dinitrosalicylic acid, quercetin and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate acid, were purchased from Sigma-Aldrich-

Chemical (China). Folin-Ciocalteu reagent were obtained with MERCK KGaA, HC9059050I, Germany, and 2-2-diphenyl-1-picrylhydrazyl were purchased from Thermo Fisher, P19F002, Germany. A ELISA microplate reader (Biobase, BK-EL10C, MBY10C22040496, China) and centrifuge (Hettich Mikro 220R, Germany) were also used. A hot plate (701546-Economy hot plate, 1500 W; 230 V) were used for boiling.

Compound Extraction

The method used by Ranilla et al. (2007) was chosen to perform the extraction of the compounds according to the modifications of Sanou et al. (2023). In short, 5 g of dry matter was boiled for 30 min in 100 mL of distilled water. After filtration using Whatman No. 1 paper, the filtrate was adjusted to 100 mL with distilled water. Then, the solution was centrifuged (6530 rpm; 4°C) for 30 min. Part of the supernatant was used for the Fe^{3+} reduction test and the other part dried at 55°C for the other tests such as DPPH$^{\bullet}$ Radical Sweeping Activity, $ABTS^{+\bullet}$ discoloration, glucose adsorption capacity and α-amylase inhibition test.

Determination of total phenolic content

The total phenolic content was carried out according to the method of Kwon et al. (2006). Thus, a mixture was formed from 0.25 mL of extract, 0.25 mL of 95% ethanol and 1.25 mL of distilled water. To this mixture, 0.125 mL of Folin-Ciocalteu 50% reagent was added. After 5 min of incubation, 0.25 mL of Na_2CO_3 (5%) was added to the reaction medium and the whole was again incubated for 60 min. Optical density (OD) was read at 725 nm against a blank. The phenolic content of each sample was determined from the equation ($y = 40.798x + 0.0094$; R^2=0.998) of the gallic acid standard curve (0.00625 - 0.2 mg/mL).

Results were expressed as milligrams of gallic acid equivalent per gram of dry extract (mg GAE/100 mg DE) and the average of five readings was used.

Determination of total flavonoid content

Flavonoid testing was performed according to the protocol of Gokhan & Abdurrahman (2014). Briefly, 100 µL of 2% aluminum trichloride was added to 100 µL of extract and the mixture incubated for 15 min. The OD reading was made at 430 nm.

Iron III Reduction Activity

The ability of extracts to reduce iron was evaluated according to the method used by Ouédraogo et al. (2022). Thus, 0.25 mL of extract was mixed with 0.625 mL of phosphate buffer (0.2 M, pH 6.6) and 0.625 mL of aqueous solution of potassium hexacyanoferrate [$K_3Fe(CN)_6$] 1%. An incubation was made for 30 min at 50°C. Then, 0.625 mL of 10% trichloroacetic acid was added and the mixture was centrifuged at 3000 rpm and 4°C for 10 min. Next, 0.625 mL of the supernatant was mixed with 0.625 mL of distilled water and 0.125 mL of a freshly prepared 0.1% iron trichloride solution. Optical density were read at 700 nm and ascorbic acid (0.00625 - 0.2 µg/mL) was used as a standard ($y = 6.149x + 0.245$; $R2 = 0.99$). The average of five readings was expressed as µmol ascorbic acid equivalent per gram dry matter (µmol AAE)/g DM).

DPPH radical scavenging activity

The DPPH$^{\bullet}$ radical scanning ability of the extracts was evaluated according to the method used by Kwon *et al.* (2006). Thus, a range of extract concentration (5 - 100 µg/mL) was prepared in ethanol. Then, 375 µL of extract was mixed with 750 µL of the DPPH$^{\bullet}$ solution. The initial optical density (OD_0) of DPPH$^{\bullet}$ solution was 0.78 ± 0.03. The mixture was incubated in the dark for 15 min at laboratory temperature. The optical density was then read using a spectrophotometer (1100A) at 517 nm against a control. The tests were carried out three times. The percent inhibition (%I) of the DPPH$^{\bullet}$ radical was calculated from the formula below:

$$\%I = (OD_{control} - OD_{test}) \times 100 / OD_{control}$$

Where: $OD_{control}$: Control optical density at 517 nm wavelength

OD_{test}: optical density of the sample at the wavelength of 517 nm

The IC_{50}s corresponding to the value of the extracts that neutralized 50% of the initial DPPH$^{\bullet}$ free radicals were determined with the software GraphPad_Prisme_9.2.0.332x64.

ABTS$^{\bullet+}$ radical discoloration Test

The ability of the extracts to remove ABTS$^{+\bullet}$ radicals was evaluated by the standard method of discoloring the ABTS$^{+\bullet}$ solution. To do this, 10 µL of extract at 1 mg/mL was added to 990 µL of ABTS$^{+\bullet}$ solution and incubated in the dark for 15 min. Optical density was read at 405 nm against a blank and with control. The experiment was performed in triplicata and the percentage of inhibition (%I) of ABTS$^{+\bullet}$ radicals was calculated from the following formula:

$$\%I = (OD_{control} - OD_{test}) \, x \, 100 / \, OD_{control}$$

With:

$OD_{control}$: Control absorbance at 405 nm wavelength

OD_{test}: Absorbance of the sample at the wavelength of 405 nm

Glucose Adsorption Capacity

Glucose adsorption capacity was assessed using the glucose oxidase peroxidase kit method (Rehman *et al.*, 2018) with modifications. Thus, each extract was tested on glucose solutions at 5 ; 10 ; 15 ; 20 and 30 mM. To make control, each extract was prepared under the same conditions without glucose. The different mixtures were then incubated for 6 h at laboratory temperature (29 ± 0.5°C). After incubation, each solution was centrifuged (4800 rpm,10 min). The GOD kit was used to determine the glucose concentration of the supernagent at time zero and at the end of incubation using a Mindray BA-88A UV-Visible spectrophotometer.

The average of four readings was used to calculate the amount of adsorbed glucose expressed as micromoles of glucose per gram of dry extract (µmol/g DE) using the formula below:

$$\text{Glucose bound} = \frac{(G_1 - G_6) * Volume\ of\ the\ solution}{Dry\ extrait\ mass}$$

G_1: concentration of glucose at the initial time

G_6: glucose concentration after 6 h of incubation

Inhibitory activity against α-amylase

The enzyme was extracted from a Megamylase® tablet (Sanou et al. 2023). Next, a 2U α-amylase extract solution was prepared for the quantitative inhibition assay. But, a qualitative test of the α-amylase solution was performed before on 1% starch using lugol.

The modified indirect colorimetric method of 3,5-dinitrosalicylic acid (DNA) (Adewale et al. 2006) was used to carry out the quantitative test. Thus, a mixture was constituted with 7 mL of 1% starch, 2 mL of PBS pH = 6.9, 1 mL of extract and 100 µL of enzymatic solution. After incubation at room temperature (29 ± 0.5°C) for 1 h, 1.5 mL was removed from the reaction mixture and 1 mL of DNA was added to. That solution was incubated for 5 min at 100°C and then cooled down by adding 7 mL of distilled water. OD readings was made at 540 nm with an ELISA plate reader (Biobase Biodustry (Shandong) CO., LTD, BK-L10C) against one blank where the α-amylase extract was replaced by buffer. For the control, the extract volume was replaced by buffer. The results were expressed in inhibition rates calculated as follows:

$$\%I = (Abs_{control} - Abs_{test}) \, x \, 100 / \, Abs_{control}$$

Where:

$Abs_{control}$: Control absorbance at 540 nm

Abs_{test}: Absorbance of the sample at 540 nm

Statistical analysis

The data were processed and analysed with the software GraphPad_Prisme_9.2.0.332x64. The analysis of variance of the different parameters and the multiple comparison test of Tukey at the 5% threshold were performed. A Pearson correlation coefficient was calculated between the parameters. The charts were generated using Microsoft Excel.

Results

Total phenolic content and antioxidant activity

The phenolic content of the extracts shows a variation depending on the month of harvest of the sample. Indeed, the highest content with is about 33,87 ± 2,48 mg GAE/100 mg DE was observed with the extract from the December collection (CNDD) and the lowest with the November extract (CNDN). This last content was about 23,88 ± 2,80 mg GAE/100 mg DE (Table 1). A correlation of r = 0.76 was observed between the total phenolic and total flavonoid content of the three extracts (Table 2).

The inhibition concentration of half of the $DPPH^{\bullet}$ radicals was determined for all extracts. The results showed that the extract activity of the October collection is the highest. The lowest activity was observed with the extract from the December collection (Table 1). These values were 24,72 ± 2,17 µg GAE/mL and 51,05 ± 4,13 µg GAE/mL respectively for CNDO and CNDD. The correlation study between total phenolic content and the activity against free radical yielded a coefficient of 0.85 (Table 2).

The results of the discoloration activity of ABTS radicals are presented as inhibition rate. Of the three extracts, none was able to discolor $ABTS^{+\bullet}$ by 50% with an initial control absorbance of 0.89±0.01 at the evaluated concentration (1 mg ES/mL). However, the extract from the October collection showed the highest percentage of inhibition (45.91%) compared to 12.66% for the extract from the November collection (Table 1). A strong correlation was observed between this activity against $ABTS^{+\bullet}$ and flavonoid content (r = 0.98) (Table 2).

Iron reduction power was evaluated with liquid extracts immediately after extraction. The extract from the November collection (CNDN) showed the greatest metal iron reduction activity, about 2444 ± 74,44 µmol AAE/g DM despite the lower total flavonoid content in contrast to the other two extracts (Table 1). This observation is supported by the negative correlation coefficient (r = – 0.92) found between iron reduction activity and flavonoid content (Table 2).

Table 1. Total phenolic content and antioxidant activity of aqueous extracts from *C. nigricans* leaves

Compounds/activity	CNDO	CNDN	CNDD	*p-value*
Total phenolic content (mg GAE/100mg DE)	27,15 ± 4,31^{ab}	23,88 ± 2,80^{a}	33,87 ± 2,48^{b}	0,0257
Total flavonoid content (mg QE/100 mgES)	1,95 ± 0,15^{a}	0,66 ± 0,31^{b}	1,98 ± 0,51^{a}	0,006
$DPPH^{\bullet}$ IC_{50} (µg GAE/mL)	24,72 ± 2,17^{a}	30,92 ± 1,39^{a}	51,05 ± 4,13^{b}	<0,0001
$ABTS^{+\bullet}$ inhibition rate (%)	45,91 ± 1,69^{a}	12,66 ± 1,07^{b}	39,88 ± 1,33^{c}	<0,0001
FRAP (µmol AAE/g DM)	1544 ± 29,39^{a}	2444 ± 74,44^{b}	1894 ± 72,95^{c}	<0,0001

Extract from the October (CNDO) November (CNDN) and December (CNDD) collection; DE: dry extract; QE: quercetin equivalent; GAE: gallic acid equivalent; AAE: Ascorbic Acid Equivalent. Values that have the same letter in the same line are not significantly different.

Table 2. Pearson's correlation coefficient between phenolic content and antioxidant activity

Parameters	Total phenolics	Total flavonoids
Flavonoids content	0,76	-
DPPH• (CI_{50})	0,85	0,31
$ABTS^{+\bullet}$	0,63	0,98
FRAP	-0,44	-0,92

Glucose adsorption

The October collection sample showed the highest *in vitro* adsorption capacity for almost all glucose test concentrations (figure 1). Its adsorption capacity is strongly correlated with glucose concentration (r = 0.95). Similarly, the extract from the November collection come in second place with a Pearson correlation coefficient of 0.97 (Table 3). The analysis showed that there is a high positive correlation between the DPPH anti-free radical activity of the extracts and their adsorption capacity at high glucose concentrations (15 mM, 20 mM, 30 mM) in contrast to the activity against $ABTS^{+\bullet}$ (Table 4).

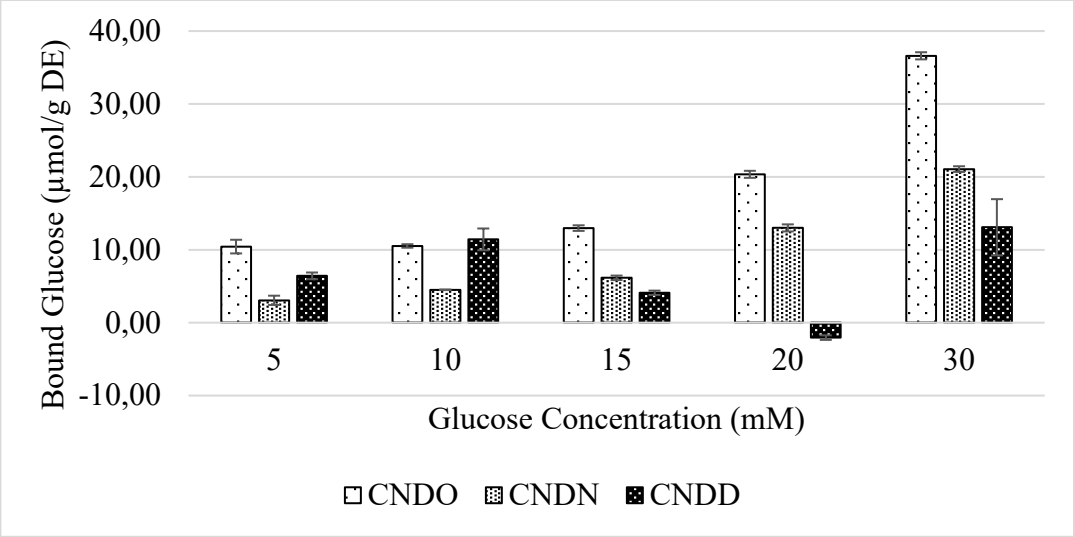

Figure 1. Glucose adsorption capacity of *Chamaecrista nigricans* extracts collected in October (CNDO), November (CNDN) and December (CNDD) at the Dindéresso site.

Table 3. Pearson's correlation coefficient between the change in the concentration of glucose solution and the adsorption capacity of aqueous extracts of *C. nigricans* leaves

Extract	Correlation coefficient
CNDO	0,95
CNDN	0,97
CNDD	0,14

CNDO: excerpt from the October collection; CNDN: excerpt from the November collection; CNDD: excerpt from the December collection

Table 4. Pearson correlation coefficient between glucose adsorption capacity and antioxidant activity of extracts from the October, November and December collection of *Chamaecrista nigricans* as a function of changes in the glucose concentration of the solution

Glucose solution concentration (mM)	5	10	15	20	30
$r_{(CI50\ DPPH\bullet)}$	-0,27	0,40	-0,83	-0,99	-0,89
$r_{(ABTS+\bullet)}$	0,92	0,96	0,45	-0,02	0,35
$r_{(FRAP)}$	-0,98	-0,87	-0,64	-0,20	-0,55

Inhibitory activity against α-amylase

Chamaecrista nigricans extracts showed different levels of inhibition of α-amylase activity depending on the month of collection. The extracts from the October and November collections were more active compared to the December one with IC_{50s} of 0.23 and 0.17 mg DE/mL, respectively (Table 5). The December collection extract, exhibited stimulatory rather than inhibitory activity with negative inhibition rates for all concentrations tested (Figure 2).

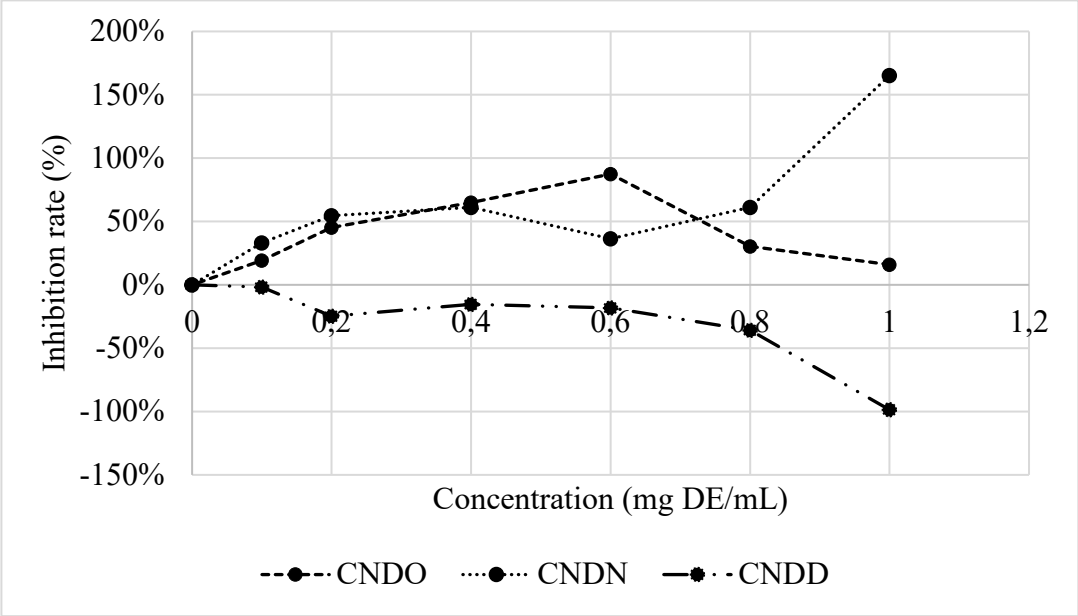

Figure 2. Effect of *Chamaecrista nigricans* leaf extracts on the activity of α-amylase enzyme extract at 2 U/mL.
CNDO: extract from the October collection; CNDN: extract from the November collection; CNDD: extract from the December collection; DE: Dry extract.

Table 5. IC_{50} value of the inhibitory activity of *Chamaecrista nigricans* leaf extracts against α-amylase (2 U/mL)

Extrait	IC_{50} α-amylase (mg DE/mL)
CNDO	0,23
CNDN	0,17
CNDD	ND

CNDO: extract from the October collection; CNDN: extract from the November collection; CNDD: extract from the December collection; DE: dry extract; ND: Not determined

Discussions

The purpose of this study was to investigate potential preventive ingredients for type 2 diabetes. To this end, we evaluated the antioxidant activity, the *in vitro* glucose adsorption capacity of extracts of this species and their effect on α-amylase activity. The determination of secondary metabolite compounds in plant extracts often largely explains their biological activities. Phenolic compounds in general and flavonoids in particular are renowned for their antioxidant potential. The structure of these natural compounds and their reactivity with free radicals give them a real power to neutralize or stabilize these radicals, thus limiting their damage to the body (Prior et al. 2005). Several types of radicals are generated during metabolism and also in response to any kind of aggression or stress. Thus, some radicals can be stabilized by proton and/or electron transfer such as $DPPH^{\bullet}$, $ABTS^{+\bullet}$ (Prior et al. 2005). This electron transfer also reduces some oxidants in redox reactions which are very important in metabolism. As a result, *C. nigricans* leaf extracts exhibited notable antioxidant activity through the DPPH, ABTS, and iron reduction methods. However, this activity did not correlate positively with the total flavonoid content of the different months extracts. This shows a difference in trend from studies of some authors (Kwon et al. 2006, Laoufi et al. 2017, Sanou et al. 2023) who found an apparent positive correlation between antioxidant activity and phenolic content in medicinal plant extracts. Nevertheless, this state of affairs is understandable, especially since the structure of certain flavonoid molecules, and therefore the flavonoid profile of an extract, is likely to influence its antioxidant activity. Indeed, the number of hydroxyl groups and their position influences the antioxidant activity of a molecule. Indeed, the plant contains compounds with therapeutic potential such as anthraquinone derivatives, including 1,6,8-trihydroxy-3-methyl anthraquinone, also known as emodin, emic acid, citreorosein and liteolin (Ayo 2010). Other classes of secondary metabolites present in crude extracts have good antioxidant potential and are therefore thought to be responsible for the different activities of the extract (Skerget et al. 2005). These compounds are also a factor in the response to the antioxidant activity of these plants in their living environment (Skerget et al. 2005).

In addition, the extract from the December collection with the highest phenolic content was found to be less active on α-amylase than those from the October and November collection. Phenolic compound, and particularly flavonoids, have α-amylase inhibitory activity that depends on their profile (Tadera et al. 2006, Kwon et al. 2008, Kang et al. 2014). Flavonoids are likely to establish hydrogen bonds with the functional amino acid groups of the enzyme's active site. These interactions can inhibit reactions between α-amylase and starch, thereby inhibiting starch digestion (Ng et al. 2015). As with phenolic compound in general, the structure of flavonoids and the number and position of their hydroxyl groups are determining factors for enzymatic inhibition. Thus, the inhibitory activity increases with the number of these groups in the B cycle (Tadera et al. 2006).

Besides, the aqueous decocts of *C. nigricans* leaf extracts showed an interesting *in vitro* glucose adsorption capacity. The ability of *C. nigricans* extracts to adsorb glucose is a property that could be very beneficial in the therapeutic management of postprandial hyperglycemia and therefore diabetes mellitus. The season is one of the factors of variability in the bioactive composition of samples, and therefore in activity (Ouédraogo et al. 2022). Among the extracts from the three harvesting months, the one from the October collection is the most active for *in vitro* glucose adsorption. Dietary fibres, such as non-starch polysaccharides, are known to have glucose adsorbent properties (Ahmed et al. 2011). This potential presented by these extracts can therefore be explained by the probable presence of these polysaccharides, as reported in the extracts of Cassia species (Ayo 2010). In addition, other compounds such as phenolic compounds may well have this ability because of the possible interactions between them and

simple sugars. A concerted property of partial inhibition of carbohydrate degradation by α-amylase supported by adsorption of residual monosaccharides could induce a considerable reduction in postprandial hyperglycemia. Still and all, at the intestinal level, it is α-glucosidase that releases most of the monosaccharides that can be absorbed by the body. Thus, a study of the inhibitory activity of this species extracts against this *α*-glucosidase will be an asset in the search for natural antidiabetic drugs from *C. nigricans*.

Conclusions

In order to find potential therapeutic agents for type 2 diabetes, research has turned to medicinal plants which remain a valid source of bioactive compounds. During the present study, we were interested in studying the glucose adsorption capacity and the inhibitory activity of aqueous extracts from different months of *Chamaecrista nigricans* in order to highlight its antidiabetic potential. The results obtained showed a potential for glucose adsorption and significant inhibition against α-amylase. These activities may truly have a concerted action for the reduction of postprandial hyperglycemia. This work is therefore very encouraging. To complete this study, we are considering further *in vitro* and *in vivo* investigations. Also, splitting will be necessary to determine the most active stable fractions.

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