OPEN ACCESS

African Journal of Biotechnology

August 2021
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

About AJB

The African Journal of Biotechnology (AJB) is a peer reviewed journal which commenced publication in 2002. AJB publishes articles from all areas of biotechnology including medical and pharmaceutical biotechnology, molecular diagnostics, applied biochemistry, industrial microbiology, molecular biology, bioinformatics, genomics and proteomics, transcriptomics and genome editing, food and agricultural technologies, and metabolic engineering. Manuscripts on economic and ethical issues relating to biotechnology research are also considered.

Indexing

CAB Abstracts, CABI's Global Health Database, Chemical Abstracts (CAS Source Index) Dimensions Database, Google Scholar, Matrix of Information for The Analysis of Journals (MIAR), Microsoft Academic, Research Gate

Open Access Policy

Open Access is a publication model that enables the dissemination of research articles to the global community without restriction through the internet. All articles published under open access can be accessed by anyone with internet connection.

The African Journals of Biotechnology is an Open Access journal. Abstracts and full texts of all articles published in this journal are freely accessible to everyone immediately after publication without any form of restriction.

Article License

All articles published by African Journal of Biotechnology are licensed under the Creative Commons Attribution 4.0 International License. This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited. Citation should include the article DOI. The article license is displayed on the abstract page the following statement:

This article is published under the terms of the Creative Commons Attribution License 4.0
Please refer to https://creativecommons.org/licenses/by/4.0/legalcode for details
about Creative Commons Attribution License 4.0

Memberships and Standards

Academic Journals strongly supports the Open Access initiative. Abstracts and full texts of all articles published by Academic Journals are freely accessible to everyone immediately after publication.

All articles published by Academic Journals are licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0). This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited.

Crossref is an association of scholarly publishers that developed Digital Object Identification (DOI) system for the unique identification published materials. Academic Journals is a member of Crossref and uses the DOI system. All articles published by Academic Journals are issued DOI.

Similarity Check powered by iThenticate is an initiative started by CrossRef to help its members actively engage in efforts to prevent scholarly and professional plagiarism. Academic Journals is a member of Similarity Check.

CrossRef Cited-by Linking (formerly Forward Linking) is a service that allows you to discover how your publications are being cited and to incorporate that information into your online publication platform. Academic Journals is a member of CrossRef Cited-by.

Academic Journals is a member of the International Digital Publishing Forum (IDPF). The IDPF is the global trade and standards organization dedicated to the development and promotion of electronic publishing and content consumption.

Contact

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.org

Academic Journals
73023 Victoria Island, Lagos, Nigeria
ICEA Building, 17th Floor,
Kenyatta Avenue, Nairobi, Kenya.

Editor-in-Chief

Editorial Board Members

Table of Content

Vol. 20(8), pp.318-324, August, 2021
DOI: 10.5897/AJB2021.17339
Article Number: FB7A53567489
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Phytochemical and *in-vitro* antioxidant properties of ethyl acetate leave extract of *Dryopteris dilatata* on Wistar rats

Akpotu Ajirioghene[1], Ani Celestine[2*], Agiopu Toby[6], Okeke Adaobi[3], Agu Francis[3], Ugwuchukwu Nnaemeka[4], Ugwudike Patrick[5], Eze Chukwuka[5], Uzoigwe Jide[3], Anyaeji Pamela[3] and Ghasi Samuel[1]

[1]Department of Pharmacology and Therapeutics, College of Medicine, University of Nigeria, Enugu Campus, Enugu State, Nigeria.
[2]Department of Human Physiology, College of Medicine, Enugu State University of Science and Technology, Parklane Enugu, Enugu State, Nigeria.
[3]Department of Physiology, College of Medicine, University of Nigeria, Enugu Campus, Enugu State, Nigeria.
[4]Department of Medicine, University of Benin Teaching Hospital, Benin−City, Edo State, Nigeria.
[5]Medical Biochemistry Department, College of Medicine, Enugu State University of Science and Technology, Parklane Enugu, Enugu State, Nigeria.
[6]Child Health Department, University of Benin Teaching Hospital, Benin−City, Edo State, Nigeria.

Received 8 March, 2021; Accepted 30 July, 2021

Medicinal plants contain natural bio-active constituents in various parts called photochemical that are of therapeutic importance. This research was undertaken to determine the phytochemical constituents and *in-vitro* antioxidant activities of ethyl acetate extract of *Dryopteris dilatata* leaves (DDL). Phytochemical analysis was determined on the plant samples following established protocols. The antioxidant property of *Dryopteris dilatata* leaves was evaluated using ferric reducing antioxidant power (FRAP), superoxide scavenging radical activity (SSRA) and hydroxyl radical activity (OH) assay. The results of the phytochemical analysis revealed the presence of the following phenols, terpenoid, alkaloids, flavonoids, tannins, cardiac glycosides and saponins, while steroids, carbohydrates, proteins, amino acids and phlobotannins were below detectable levels. DDL leaves produced significant (P<0.05) levels of ferric reducing antioxidant power (FRAP), superoxide scavenging radical activity (SSRA) and hydroxyl radical activity (OH) in a concentration dependent manner compared to the reference antioxidants, ascorbic acid and manitol; therefore, the extract could serve as free radical scavenger, acting as primary antioxidants. Based on the phytoconstituents and antioxidant activities, it could be concluded that *D. dilatata* could be of great value in the management of hyperglycemia, hyperlipidemia, and cancer, among other diseases that could be caused as a result of oxidative stress. This calls for further exploration of its bioactive compound.

Key words: Phytochemicals, *Dryopteris dilatata*, *in-vitro* antioxidant and, oxidative stress.

INTRODUCTION

Since ancient time, herbal remedies have been a natural source of medicines that have been implicated traditionally in the prevention and treatment of a wide range of human diseases in many parts of the world,

where cost and several undesired side effects has limited the prospect of some conventional medicines (Priyanga et al., 2014). These medicinal plants contain natural bioactive constituents in their various parts known as the phytochemical which include carbohydrates, phenols, flavonoids, steroids, tapenoids, alkaloids, tannins compounds. There are endogenous metabolites that can contribute to pharmacological properties of plants (Sowmya et al., 2015). These metabolites are found in abundant quantity in plants. Several of these plants are still used by various over 80% ethnic groups worldwide for the management of several diseases such as dysentery, asthmatic attacks, malaria ,skin diseases (Manandhar, 1998), whose effectiveness have been shown to be relatively nontoxic, free from underlying side effects and safe (Iniaghe et al., 2008). Medicinal plants with numerous active ingredients have bioactive and chemical entities with various pharmacological activities such as anticancer, antibacterial, analgesic, anti-inflammatory, antitumor, antiviral and other activities

Free radicals are produced naturally in cells during stress or respiration and from external sources such as smoking, radiation, alcohol, viral toxins and bacteria. Protection of the living systems against deleterious effect caused by oxidative stress is done by antioxidant defense mechanism (Jayachitra and Krithiga, 2010). Imbalance between reactive species generation, other free radicals and the antioxidant activity of living systems, results in oxidative stress. Excessive oxidative stress results in the loss of the functions of the cells producing a lot of structural damages such as lipid, proteins and DNA (Hiransai et al., 2016), resulting in the pathogenesis of several chronic and metabolic diseases such as cardiovascular diseases, cancer, aging, diabetes and metabolic syndrome (Maehre et al., 2015). Adverse effects such as carcinogenicity leading to restrictions in the use of synthetic antioxidants has increased the search for foods and plants with naturally occurring antioxidants potentials with the capability to eradicate or neutralize the harmful effect of free radicals in cells and tissues (Patel et al., 2010). This has revealed the importance of medicinal plants to prevent and control chronic diseases associated with oxidative stress through their antioxidant potentials.

Dryopteris dilatata (Broad buckler fern) is a medicinal plant that is found within the *Dryopteridaceae* family and can grow to about 120 cm and 90 cm in height and width respectively. This plant has dark green tripinnate fronds, with the ribs covered in brown scales (Rünk et al., 2012). *D. dilata* is known as Okpomie by the Olomoro ethnic group in Isoko South Local Government Area, Delta State, Nigeria. The plant is distributed throughout tropical region of Nigeria and people from Olomoro ethnic region in Isoko South Local Government Area of Delta State make concoctions of this plant when diabetic and their conditions usually get ameriolated using the plant. It has also been documented by Brown et al., (2011) that the leaves and roots are used for medicinal purposes such as management and treatment of dandruff and worm expeller According to Ajiriogehne et al., (2018), *D. dilatata* leaves contain phytochemicals such as phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids, and alkaloids. Hence this study was undertaken to determine the phytochemicals and *in-vitro* antioxidant properties of ethyl acetate extract of *D. dilatata* leaves on Wistar rats.

MATERIALS AND METHODS

Equipment/Instruments

Spectrophotometer 20D, Laboratory Incubator DNP-9082, Centrifuge 80-2. Electric water baths (model DK 420) were products of Techmel and Techmel USA and Electronic weighing balance by Metlar, China. Refrigerator was by Haier Thermocool, Micropipettes (REMI) (100-1000 µl), porcelain mortar and pestle, stop watch, dissecting set, measuring cylinders, beakers, spatula, syringe, filter paper, micropipette, and test-tubes

Chemicals/reagents and manufacturers

Disodium hydrogen phosphate, Sodium dihydrogen phosphate, Adrenalin by Loba Chemie Mumbai, India. Hydrogen chloride, Sodium carbonate, Trichloroacetic acid Thiobarbituric acid, Sodium hydroxide pellet, Sodium chloride. Sodium chloride Alanine aminotransferase Assay kits, Aspartate aminotransferase Assay kits. Alkaline phosphatase Assay kits, Triglyceride Assay kits, Total Cholesterol Assay kits, High Density Lipoprotein Assay kits , Urea kits, Randox laboratory Ltd, UK .Creatinine kits. Randox Laboratory Ltd, UK with cover, rotary evaporator, oven, beaker (20 ml, 50 ml and 500 ml).

Plant collection, identification and authentication

D. dilatata leaves were harvested from a wide growing habitat at Olomoro Community in Isoko South Local Government Area of Delta State, Nigeria. The samples were identified and authenticated at Forestry Research Institute of Nigeria, Ibadan, Oyo State and was assigned a herbarium number: FHI 1100338.

Phytochemical analysis

Qualitative analysis of the phytochemicals

The extract of *D. dilatata* was subjected to different phytochemical analysis using standard methods of Harborne (1973) to investigate

*Corresponding author. E-mail: anicelestine2006@gmail.com. Tel: +2348034607689, +2348159416345.

the availability of bioactive substances as follows.

Test for proteins: This was done using Millon's test as follows: 2 ml of Million's reagent was mixed with one gram of the crude extract and the presence of white precipitate which turned red upon gentle heating confirmed the presence of protein.

Test for carbohydrates: This was also tested using Iodine test procedure as stated: 2 ml of iodine solution was mixed with One gram of crude extract of *D. dilatata* leaves and the presence of a dark blue or purple coloration indicated the presence of the carbohydrate.

Test for phenols and tannins: 2 ml of 2% solution of $FeCl_3$ was mixed with one gram of crude extract of *D. dilatata* leaves and a blue-green coloration indicated the presence of phenols and tannins.

Test for flavonoids: This was an alkaline reagent test which was performed as stated: 2 ml of 2% solution of NaOH was mixed with one gram of crude extract of *D. dilatata* leaves and the formation of an intense yellow color which later turned colorless on addition of few drops of diluted acid indicated the presence of flavonoids.

Test for phytosterol: A solution of alcoholic potassium hydroxide was refluxed with one gram of *D. dilatata* leaves extract till complete saponification occurred. The mixture was extracted after dilution with a solution of ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol, The residue was dissolved in few drops of diluted acetic acid. Later, 3 ml of acetic anhydride was added followed by few drops of concentrated H_2SO_4. Appearance of bluish green coloration indicated the presence of phytosterol.

Test for triterpenoids: Ten milligram (10 mg) of the *D. dilatata* leave extract was dissolved in 1 ml of chloroform, This was followed by the addition of 1 ml of acetic anhydride followed by 2 ml of concentrated H_2SO_4. The formation of reddish violet color indicated the presence of triterpenoids.

Test for phlobatannins: 2 ml of 1% HCl was mixed with one gram of the powdered *D. dilatata* leave and the mixture was boiled. The formation of a red precipitate was an indication for the presence of phlobatannins.

Test for saponins: 5 ml of distilled water was mixed with one gram of the crude extract of *D. dilatata* leaves in a test tube, and this was shaken vigorously. The formation of stable foam showed the presence of saponins.

Test for glycosides: Keller-Kilani test for glycosides was performed as stated: 2 ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. was mixed with one gram of the crude extract of *D. dilatata* and the mixture was poured into another test tube containing 2 ml of concentrated H_2SO_4. The appearance of a brown ring at the interphase indicated the presence of cardiac glycosides.

Test for steroid: 2 ml of chloroform concentrated with H_2SO_4 was mixed with one gram of the crude extract of *D. dilatata*. A red coloration that formed in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing the crude extract with 2 ml of chloroform. Then, 2 ml of each of concentrated H_2SO_4 and acetic acid was poured into the mixture. The development of a greenish coloration indicated the presence of steroids.

Test for terpenoids: 2 ml of chloroform was mixed with one gram of the crude extract of *D. dilatata* leaves and was evaporated to dryness. Then, 2 ml of concentrated H_2SO_4 was added and heated for 2 min and a greyish coloration indicated the presence of terpenoid.

Test for alkaloids: 2 ml of 1% HCl was mixed with one gram of the crude extract of *D. dilatata* leaves and heated gently, after which Mayers and Wagner's reagents were added to the mixture. Turbidity of the resulting precipitate indicated the presence of alkaloids (Harborne, 1973).

In vitro antioxidant activities of *D. dilatata* leaves

Assay hydroxyl radical scavenging assay

The assay was performed using standard methods (Salah et al., 1995). The fundamental principle that governs the assay deals with the 2-deoxyribose degradation product quantification by condensation with TBA. Furthermore, the Fe^{3+}-ascorbate-EDTA-H_2O_2 system, also known as the Fenton reaction has helped to generate the hydroxyl radical.

The reaction mixture is contained in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM); ascorbic acid (100 µM) and various concentrations (0–200 µg/ml) of the plant extract. The reaction mixture remained incubated at 37°C for 1 h; post incubation, 0.5 ml of the reaction mixture was mixed with 1 ml 2.8% TCA in addition to 1 ml of 1% aqueous TBA. The solution was subjected to incubation for 15 min at 90°C to produce coloration. Thereafter, the solution was allowed to cool and the absorbance was determined at 532 nm against a suitable blank solution. All tests were conducted six times. As a classical •OH scavenger, Mannitol served as a positive control. Evaluation of inhibition percent was done using the ensuing equation:

$$\% \text{ of inhibition} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 = control absorbance and A_1 = absorbance in the presence of the samples and standard.

Superoxide radical scavenging assay

Based on NBT reduction, this experiment was conducted in line with a previously reported technique (Salah et al., 1995). After the non-enzymatic PMS/NADH system produces superoxide radicals, nitro blue tetrazolium are then reduced into a purple-colored formazan by these radicals. The 1 ml reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and different sample solution concentrations (0–50 µg/ml). The reaction mixture was subjected to incubation for 5 min at room temperature; thereafter, the absorbance was taken at 562 nm against an appropriate blank solution. All tests were conducted six times with quercetin used as positive control. Further, percent inhibition of superoxide anion generation was estimated with the aid of the ensuing formula:

$$\% \text{ of inhibition} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 = Absorbance of the control; A_1 = Absorbance in the presence of the samples and standard.

Measurement of ferric reducing power

This was done according the method of Oyaizu with slight medication to determine the ferric reducing power of ethyl acetate extract of *D. dilatata* leaf. Various concentrations (0–1.0 mg/ml) of

Table 1. Qualitative phytochemical screening.

Phytochemicals	Ethyl acetate extract mg/100 ml
Phenols	++
Steroids	-
Tarpenoids	+
Carbohydrates	-
Proteins	-
Amino acids	-
Alkaloids	+++
Phlobotannins	-
Cardiac glycosides	+
Flavonoids	++
Tannins	+++

-Absent, + trace, ++ moderately present, +++abundantly present.

Table 2. Result of the ferric reducing antioxidant power.

Concentration (ug/ml)	Ethyl acetate	Ascorbic acid
250	61.18±0.07	70.05±0.07
125	51.35±0.33	60.22±0.33
63.5	45.81±0.58	54.68±0.58
31.5	36.29±0.06	45.16±0.06
15.25	19.52±0.56	28.38±0.55
7.625	2.68±.17	4.55±0.17
3.825	0.09±.07	1.62±0.27

Values are expressed as mean ± SEM. ANOVA followed by Post Hoc (LSD) multiple range tests. Values not sharing a common superscript differ significantly at P<0.05.

extract (0.5 ml) were mixed with 0.5-ml phosphate buffer (pH 6.6) and 0.5 ml 0.1% potassium hexacyanoferrate. The solution was subjected to incubation for 20 min in a water bath at 50°C; thereafter 0.5 ml of trichloroacetic acid (10%) was added to end the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water plus a further 0.1 ml $FeCl_3$ solution (0.01%). After leaving the reaction mixture at room temperature for 10 min, absorbance will be determined at 700 nm against an appropriate blank solution. All tests were conducted six times. A higher absorbance of the reaction mixture is an indication of greater reducing power. In this approach, butylated hydroxyltoluene (BHT) was used as a positive control.

Statistical analysis

Data collected were presented as Mean ± SEM (standard error of mean). Results were analysed using one-way analysis of variance (ANOVA), followed by post Hoc Fisher's test (LSD) for multiple comparison and $P < 0.05$ were considered statistically significant.

RESULTS

The preliminary phytochemical analysis (Table 1) revealed the presence of phenols, terpenoids, alkaloid, cardiac glycoside, flavonoids, tannins and saponins in ethyl acetate extract of the leaves of *D. dilatata*, and the absence of phlobotannins, amino acids, steroids, carbohydrates and proteins.

Ferric reducing power of ethyl acetate leaf extract of *D. dilatata* showed the ferric reducing capacity of the plant extract. The reducing power of ethyl acetate extract was seen in a concentration-dependent manner at 250 ug/ml where there is maximum value, and decreases as the concentration reduces for ethyl acetate which has significant ferric reducing power compared to the reference compound ascorbic acid (Table 2).

Hydroxyl radical scavenging capacity of ethyl acetate leaf extract of *D. dilatata* leaves was seen in concentration-dependent manner with the maximum inhibition at 125 ug/ml and declined as the concentration reduces compared to the reference antioxidants manitol (Table 3).

Superoxide scavenging radical capacity of ethyl acetate leaf extract of *D. dilatata* was seen in concentration-dependent manner with maximum scavenging activity

Table 3. Result of the hydroxyl radical.

Concentration (ug/ml)	Ethyl acetate	Manitol
250	4.22±0.09	9.20±0.24
125	68.51±0.29	86.92±0.38
62.5	43.05±0.75	68.80±.42
31.25	33.95±0.53	54.26±0.89
15.625	28.91±0.49	38.38±0.29
7.825	20.49±0.07	26.61±0.18
3.906	14.17±0.14	14.97±0.50

Values are expressed as mean ±SEM. ANOVA followed by PostHoc (LSD) multiple range tests. Values not sharing a common superscript differ significantly at P<0.05.

Table 4. Superoxide scavenging radical activity.

Concentration (ug/ml)	Ethyl acetate	Ascorbic acid
250	59.7651±.34073	72.53±0.34
125	45.1510±.55675	67.94±0.56
62.5	38.4635±.82301	61.23±0.82
31.25	25.8133±.51445	48.58±0.51
15.825	14.29301±.09923	37.06±0.09
7.825	9.3670±.14411	19.37±0.14
3.906	6.5899±.10600	6.59±0.11

Values are expressed as mean ± SEM. ANOVA followed by PostHoc (LSD) multiple range tests. Values not sharing a common superscript differ significantly at P<0.05

observed at 250 ug/ml concentration which declined as the concentration reduces compared to the reference antioxidant ascorbic acid (Table 4).

DISCUSSION

Bioactive substance found in plants provides biological systems the ability to defend against degenerative and life threatening disorders (Tripoli et al., 2007). The phytochemical investigation of ethyl acetate extract of *D. dilatata* extract revealed the presence of phenols, terpenoid, alkaloid, cardiac glycoside, flavonoids, tannins and saponins in ethyl acetate extract of the of *D. dilatata* leaves and the absence of phlobotannins, amino acids, steroids, carbohydrates and proteins. Terpenoid and cardiac glycosides were present in trace, phenols, flavonoids and saponins were moderately present while alkaloids and tannins were abundantly present.

The findings of these phytochemicals are similar to the findings of Mordi et al. (2016) and Ajirioghene et al., (2018). Some of these phytochemical constituents are known to produce a wide range of pharmacological activities, as medicinal plants contain different phytoconstituents possessing biological activities with a wide range of therapeutic index (Nagaraj et al., 2016); such bioactive compounds that are found in this study like phenols, terpenoids, alkaloid, cardiac glycoside, flavonoids, tannins and saponins are associated with several biological activity such as antioxidant and lipid lowering capacity (Chimaobi et al., 2019). Ability of the ethyl acetate extract of *D. dilatata* leaf to reduces Fe^{3+} to Fe^{2+} suggest that it donates electron which signifies it reducing power. This shows the antioxidant activity of plant extract as reported in other medicinal plants studies (Sahreen et al., 2010). In the present study, ethyl acetate extracts of *D. dilatata* compared to the standard compound reveals its electron donating capacity, showing its antioxidant potential as a medicinal plant against free radicals (Reenu et al*.,* 2015).

The highly reactivity of hydroxyl radical that is being continuously formed in a process of reduction of oxygen to water which causes lipid peroxidation is evident in its deleterious effect in cells and organs of the body (Dizdaroglu and Jaruga, 2012). Ethyl acetate extracts of *D. dilatata* inhibited the generation of reactive species of hydroxyl radical at different inhibition values in different concentrations as reported in other medicinal plant studies (Treml and ˇSmejkal, 2016). In the present study, the ethyl acetate extract derived from *D. dilatata* leaves

showed a significant superoxide scavenging radical activity. Superoxide radical is one of the strongest reactive oxygen species among the free radicals (Gabriele et al., 2017). It is generated in living systems through incomplete metabolism of oxygen which causes damage of cells components and organs (Pizzino et al., 2017). This harmful effect to cellular components can be prevented by removing superoxide radicals (Ighodaro and Akinloye 2018). The result from the present study indicates that the scavenging activity of superoxide of extract compared to the reference antioxidant increased as the concentration increased which reveals the potency of the extract to scavenge for superoxide radical and reverse its deleterious effect to cells and organs of living systems. This indicates that *D. dilatata* could be efficacious (Lipinsk, 2011), and could be useful in ailments such as chronic pain, inflammation and Crohn's disease, cancer (murine and cell line research models).

Conclusion

The results of this study have shown that the ethyl acetate leaf extract of *D. dilatata* contains considerable amounts of bioactive compounds, with phenols, alkaloids, flavonoids, and tannins abundantly present, which possess high antioxidant and free radicals scavenging activities. The *in vitro* antioxidants activity of the *D. dilatata* leaf showed that the plant contain significant quantity of hydroxyl radical, super oxide and ferric reducing power activity compared to the reference antioxidant compound ascorbic acid and manitol and could be a source of natural antioxidants that ameliorate the induction and progression of oxidative stress.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors sincerely appreciate the effort of the Chief Laboratory Technologist/Lecturer/ Research Director (Mr. Ani Celestine O.) who ensured the success of this article.

REFERENCES

Ajirioghene A, Ani C, Nworgu C, Pamela O, Uzoma I, Uzoigwe J, Mercy A, Ogochukwu N, Onwujekwe O (2018). Antidiabetic and anti-hyperlipidemic effects of ethanolic extract of Dryopteris dilatata leaves. Journal of Diabetes and Endocrinology 9(3):20-27.

Brown C, Tedla FM, Brar A, Browne R (2011). Hypertension in chronic disease: Navigating the Evidence. International Journal of Hypertension doi: 10.4061/2011/132405.

Chimaobi JO, Adamu JA, Aminu I, Abdullahi AI, Godwin OI, Tajudeen AO, Mohammed SS (2019). Methanol-Extract/Fractions of Dacryodes edulis Leaves Ameliorate Hyperglycemia and Associated Oxidative Stress in Streptozotocin-Induced Diabetic Wistar Rats Journal of Evidence-Based Integrative Medicine DOI: 10.1177/2515690X19843832

Dizdaroglu M, Jaruga P (2012). Mechanisms of free radical-induced damage to DNA. Free Radical Research 46(4):382-419.

Gabriele P, Natasha I, Mariapaola C, Giovanni P, Federica M, Vincenzo A, Francesco S, Domenica A, Alessandra B (2017). Oxidative Stress: Harms and Benefits for Human Health. Oxidative Medicine and Cellular Longevity doi: 10.1155/2017/8416763

Harborne JB (1973). Phytochemical methods. Second edition, Chapman and Hall Ltd., London 288 p.

Hiransai P, Tangpong J, Kumbuar C, Hoonheang N, Rodpech O, Sangsuk P, Kajklangdon U, Inkaow W (2016). Anti-nitric oxide production, antiproliferation and antioxidant effects of the aqueous extract from *Tithonia diversifolia*, Asian Pacific Journal of Tropical Biomedicine 6(11):950-956.

Ighodaro OM, Akinloye OA (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria Journal of Medicine 54(4):287-293.

Iniaghe OM, Malomo SO, Adebayo JO (2008). Hepatoprotective effect of the aqueous extract of leaves of *Acalypha racemosa* in carbon tetrachloride treated rats. Journal of Medicinal Plants Research 2(10):301-305.

Jayachitra A, Krithiga N (2010). Study on antioxidant property in selected medicinal plant extract. International Journal of Medicinal and Aromatic Plants 2(3):495-500.

Lipinsk B (2011). Hydroxyl Radical and Its Scavengers in Health and Disease. Oxidative Medicine and Cellular Longevity https://doi.org/10.1155/2011/809696.

Maehre HK, Jensen IJ, Elvevoll EO, Eilertsen KE (2015). ω-3 Fatty Acids and Cardiovascular Diseases: Effects, Mechanisms and Dietary Relevance. International Journal of Molecular Sciences 16(9):22636-22661.

Manandhar NP (1998). Native phytotherapy among the Raute tribes of Dadeldhura district, Nepal. Journal of Ethnopharmacology 60(3):199-206.

Mordi JC, Lawrence EO, Chiedozie O (2016). queous Leaf Extract of Dryopteris dilatata on STZ--Induced Diabetic Wistar Rats with Associated Hyperlipidemic Ameliorating Property. Journal of Dental and Medical Sciences 15(6):97-104.

Nagaraj S, Suriyamoorthy P, Subrhamanian H, Kanakasabapathi D (2016). Phytochemical analysis, Anti inflammatory activity, *in vitro* antidiabetic activity and GC-MS profile of *Erythrina variegata* L. bark. Journal of Applied Pharmaceutical Science 6(7):147-155.

Patel VR, Patel PR, Kajal SS (2010). Antioxidant activity of some selected medicinal plants in western region of India. Advances in Biological Research 4(1):23-26.

Pizzino G, Irrera N, Cucinotta M, Pallio G, Mannino F, Arcoraci V, Squadrito F, Altavilla D, Bitto A (2017). Oxidative Stress: Harms and Benefits for Human Health. Oxidative Medicine and Cellular Longevity doi: 10.1155/2017/8416763.

Priyanga S, Hemmalakshmi S, Devaki K (2014). Comparative phytochemical investigation of leaf, stem, flower and seed extracts of *Macrotyloma uniflorum* L. Indo American Journal of Pharmaceutical Research 4(11):5415-5420.

Reenu J, Azeez S, Bhageerathy C (2015). *In vitro* antioxidant potential in sequential extracts of *Curcuma caesia* Roxb, Indian Journal of Pharmaceutical Sciences 77(1):41-48.

Rünk Kai, Zobel Martin, Zobel Kristjan (2012). Biological Flora of the British Isles: *Dryopteris carthusiana*, *D. dilatata* and *D. expansa*. Journal of Ecology 100(4):1039-1063.

Sahreen S, Khan MR, Khan RA (2010). Evaluation of antioxidant activities of various solvent extracts of *Carissa opaca* fruits. Food Chemistry 122 (4):1205-1211.

Salah N, Miller NJ, Paganga G, Tijburg GP, Bolwell, Rice-Evans C (1995). Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Archives of Biochemistry and Biophysics 322(2):339-346.

Sowmya S, Chella PP, Anusooriya P, Vidya B, Pratibha P (2015). *In vitro* Antioxidant Activity, In vivo Skin Irritation Studies and HPTLC Analysis of *Cayratia trifolia* (L.) Domin. International Journal of Toxicological and Pharmacological Research 7(1):1-9.

Treml J, Šmejkal K (2016). Flavonoids as potent scavengers of hydroxyl radicals. Comprehensive reviews in food science and food safety 15(4):720-738.
Tripoli E, Guardia ML, Giammanco S, Majo D, Giammanco M (2007) Citrus flavonoids: molecular structure, biological activity and nutritional properties: a review. Food Chemistry 104(2):466-479.

Vol. 20(8), pp.325-334, August, 2021
DOI: 10.5897/AJB2021.17358
Article Number: 8C2EB9B67491
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Physiological and biochemical traits, antioxidant compounds and some physico-chemical factors of *Spirulina platensis* cultivation as influenced by *Moringa oleifera* leaves extract culture medium enriched with sodium bicarbonate and kanwa

Wamba Fotsop Oscar[1], Magwell Rodrigue Pierre Fils[2,3], Tchoffo Djoudjeu Kennedy[2], Bahoya Joseph Aime[4], Minyaka Emile[2], Tavea Marie-Frédéric[2], Lehman Léopold Gustave[5] and Taffouo Victor Désiré[1*]

[1]Laboratory of Plant Biology, Faculty of Science, University of Douala, P. O. Box 24157 Douala, Cameroon.
[2]Biochemistry Laboratory, Faculty of Science, University of Douala, P. O. Box 24157 Douala, Cameroon.
[3]Institute of Agricultural Research for Development (IRAD), Nko'olong Station, P. O. Box 219 Kribi, Cameroon.
[4]Jay P. Johnson Biotechnology Laboratory, Institute of Agricultural Research for Development, IRAD Ekona Centre, P. M. B. 25, Buea, Cameroon.
[5]Laboratory of Animal Biology and Physiology, Faculty of Science, University of Douala. P. O. Box 24157 Douala, Cameroon.

Received 13 May, 2021; Accepted 30 July, 2021

The prospects for using *Moringa oleifera* leaves extract (MLE) supplemented with different concentrations of kanwa or sodium bicarbonate ($NaHCO_3$) as a low-cost alternative growth medium of *Spirulina platensis* were evaluated in a small-scale outdoor cultivation system. The present study was aimed to evaluate the potential of MLE growth medium enriched with different concentrations (4 or 8 g L^{-1}) of kanwa and $NaHCO_3$ on growth, chlorophyll content, biochemical characteristics, antioxidant compounds and some physico-chemical factors. Jourdan's standard medium was taken as control. The results showed that the growth parameters such as cell population, biomass dry weight, cell productivity and specific growth rate were positively affected in MLE cultivation medium enriched with kanwa or $NaHCO_3$ at different concentration levels. The addition of urea, kanwa or $NaHCO_3$ in MLE cultivation medium at different concentration levels increased significantly ($p< 0.05$) the protein content, the peroxidase and polyphenol oxidase activity, the conductivity, pH, total dissolved solids and salinity from 20 to 25 days of cultivation whereas a decrease in carbohydrate and phenol content was recorded during all the period of the experimentation. The highest values of growth parameters were notably in MLE medium supplemented with urea and kanwa at 8 g L^{-1}. The MLE medium enriched with urea and kanwa at 8 g L^{-1} was shown to be an appropriate growth medium that can be used as a low-cost alternative growth medium for commercial cultivation of *S. platensis*.

Key words: Antioxidant compounds, biochemical traits, growth, *Moringa oleifera* leaves extract, physico-chemical factors, *Spirulina platensis*.

INTRODUCTION

Microalgae is in high demand in many biotechnology sectors such as bioremediation, biofuels, biofertilizers (Kosamia et al., 2020; Markou et al., 2021), cosmetics, biomedicals (Mesquita et al., 2017; Mellou et al., 2019; Wen et al., 2020), aquaculture (Shah et al., 2018) and animal and human nutrition (Molino et al., 2018) because of the biological and commercial value of its products. In this respect, *Spirulina platensis* is one of the most promising microalgae (Lupatini et al., 2017). It represents the most abundant and common photosynthetic, filamentous, multicellular and microscopic microalgae in the tropics and subtropics (Nyabuto et al., 2015). *S. platensis* is commercially produced because of its high digestibility and interesting protein content (46-71%) of the dry weight of the algae, as well as high amounts of essential fatty acids and amino acids, vitamins, pigments (phycobiliproteins and carotenoids) and polysaccharides (Zhu et al., 2018; Corrêa and Teixeira, 2021). This cyanobacterium has gained in importance and international demand not only for its nutritional and therapeutic properties but also for its applications in human and animal nutrition, therapeutics and diagnostics (Panjiar et al., 2017; Ama Moor et al., 2020). It is generally recognized as safe from the US Food and Drug Administration and considered as the most complete food for the future by the Food and Agriculture Organization of the United Nations (Goulamabasse, 2018; Branyikova and Lucakova, 2020).

The growth and the biochemical composition of the biomass produced by S. platensis depend on many factors, the most important of which are temperature, nutrient availability and light (Madkour et al., 2012; Soni et al., 2019). The temperature of the culture medium of *S. platensis* was positively influenced by the addition of $FeSO_4.7H_2O$ and NaCl to Jourdan's medium, while $MgSO_4.7H_2O$, $CO(NH_2)_2$ and $NaHCO_3$ lead to a decrease of the temperature from 31.66 to 25.90°C (Ndjouondo et al., 2017). Production of spirulina with reduced costs is necessary when considering large-scale cultivation for industrial purposes. The cost of nutrients and availability are considered the second major factors influencing the cost of spirulina biomass production after harvesting (Vonshak, 1997). Zarrouk's medium has successfully served as the standard medium for *S. platensis* culture for many years (Zarrouk, 1966). Consequently, many media synthetic have been developed such as CFTIR medium (Venkataraman et al., 1995), OFERR medium (Singh, 2006) and Jourdan medium (Jourdan, 2013). However, they are expensive, require rapidly depleting minerals, and not readily available. The change of the nutrients in Jourdan's medium has the potential to produce a large scale biomass of *S. platensis* and could be suitable for its optimal growth culture conditions that could be beneficial for human's health. According to Ndjouondo et al. (2017), the dry weight, the specific growth rate and the cell productivity of *S. platensis* were positively influenced by the addition of $FeSO_4.7H_2O$, NaCl, $MgSO_4.7H_2O$, $CO(NH_2)_2$ and $NaHCO_3$ to Jourdan's medium at 0.01, 2.5, 0.1, 0.02 and 4 g L^{-1}, respectively. Nutrients such as phosphorus, nitrogen, calcium, potassium and iron present in agro-industrial effluents and vegetables can be used to increase microalgae growth. A number of green algal species have been shown to be able to utilize carbonates such as Na_2CO_3 and $NaHCO_3$ for cell growth (Emma et al., 2000).

Kanwa, a type of salt, is formed when salt water from a sea or lake evaporates and leaves behind colorful crystals of sodium chloride. It is also called halite or rock salt. Rock salts offer numerous health benefits, such as treating colds and coughs, as well as aiding digestion and contain various levels of trace minerals, such as manganese, copper, iron, and zinc (Nafee et al., 2013). Rock salt contains natural impurities having calcium sulfate ($CaSO_4$) and potassium chloride (KCl) as impurities. It is found in deposits of rock salt, brines, saline lakes, marshes, seawater and saline earth (Nafee et al., 2013).

Plants subject to stress conditions produce cytotoxic activated oxygen that can seriously disrupt normal metabolism, through oxidative damage of lipids, proteins, and nucleic acids (Abbaspour, 2012). In response to stress, plants activate powerful antioxidant systems, both enzymatic (e.g., SOD, POD, catalase, glutathione reductase) and non-enzymatic (flavonoid, PC, carotenoids, vitamins C and E) (Ashraf, 2009; Kahrizi et al., 2012). According to Kasote et al. (2015) and Mostafa et al. (2016), this increase of PPO and POD activity could be correlated to a decrease in oxidative stress and reactive oxygen derivatives produced during photosynthesis and to the high content of phenols which would act as antioxidant by producing an enzymatic substrate to alleviate the harmful effects of reactive oxygen species.

The increase of some physico-chemical parameters such as salinity and total dissolved solids in *S. spirulina* cultivation media had being previously explained by the presence of electrically charged atoms which increase with the evaporation of water in media and to the change

*Corresponding author. E-mail: dtaffouo@yahoo.com.

of the other variables of the culture media due to uptake of nutrients brought by the different concentrations of $NaHCO_3$ in MLE media or by the increase in alkalinity and concentration of dissolved ionic salts resulting from MLE media (Mutanda et al., 2014; Rusydi, 2018). Soni et al. (2019) reported that for flourishing and optimal growth, temperature for *S. platensis* is between 30 and 35°C and pH value between 8.5 and 10.5.

The appropriate organic waste collected from digested sago starch (Miah et al., 2000), molasses (Andrade and Costa, 2007), rice mill effluents (Usharani et al., 2012), palm oil empty fruits bunches (Suharyanto et al., 2014), and digested rotten apple (Mia et al., 2019) were also used as growth media for *S. platensis* culture. Thus, for the mass production of *S. platensis* particularly in developing countries as Cameroon, there is a need to find an effective, cheaper and readily available alternative cultivation media.

Moringa *(Moringa oleifera* Lam.) is a highly valued plant, distributed in many countries of the tropics and subtropics. It has a high nutritional value and an impressive range of medicinal and industrial purposes (Khalafalla et al., 2010; Adebayo et al., 2011; Moyo et al., 2011). Moreover, Moringa leaves extract (MLE) has received enormous attention from the scientific community because of its rich source in hormones, antioxidants, vitamins and minerals such as iron, calcium and potassium as well as vitamins and macronutrients which have plant growth-promoting capabilities and often applied as exogenous plant growth enhancers (Rady et al., 2013; Yasmeen et al., 2013; Khan et al., 2017a, b). Thus, MLE contains appreciable amounts of macro and micronutrients and readily available and cost-effective feed to substitute inorganic fertilizers and support good *S. platensis* growth. To the best of our knowledge, few information is so far available for the use of MLE as *S. platensis* growth media.

The present study was aimed to evaluate the effect of MLE medium supplemented with different concentrations of kanwa or bicarbonate on the growth, chlorophyll content, biochemical characteristics, antioxidant compounds and some physico-chemical factors of *S. platensis* in order to define the optimal growth cultivation conditions.

MATERIALS AND METHODS

Plant material and growth medium

The cyanobacterium *S. platensis* strain used in this study was obtained from the culture pond of SAGRIC Common Initiative Group Farm, Douala-Cameroon. *S. platensis* was grown on Jourdan's modified medium consisting of (per liter): 8 g $NaHCO_3$, 5 g NaCl, 3 g $Na_2CO_3.10H_2O$, 2 g KNO_3, 0.15 g $MgSO_4.7H_2O$, 0.12 g $(NH_4)_2HPO_4$, 0.05 g $CO(CH_2)_2$, 0.02 g $FeSO_4.7H_2O$ and 0.02 g $CaCl_2$ and maintained at pH 8 by use of dilute 0.2 M NaOH solution. Jourdan's medium was taken as control. For the five experimental groups, the *M. oleifera* leaves extracts (MLE) used as growth medium, was supplemented with 0.05 g L^{-1} of urea (U) and 4 or 8 g L^{-1} of kanwa (NaCl: K) or sodium bicarbonate ($NaHCO_3$: B), respectively as follow: MLE+U; MLE+U+B4; MLE+U+K4, MLE+U+B8 or MLE+U+K8. The depth of the culture in the open concrete tanks was 15 cm with 15 L of MLE. For preparation of *M. oleifera* leaves extracts (MLE), the young moringa leaves were collected from a mature moringa tree from SAGRIC Common Initiative Group Farm. An amount of 40.0 g of young moringa leaves was suspended in 1.0 L of distilled water for 7 days. The suspension was stirred using a homogenizer to help maximize the amount of the extract. The solution was then sieved and filtered through a 30-mesh Tylor net. The extract diluted with distilled water at a 1:8 ratio (v/v) was used as MLE medium. The algae *S. platensis* cells were inoculated at a concentration of 15% (V inoculation/V media). The initial pH of all culture media was adjusted to 8 with 0.2 M NaOH before addition of *S. platensis* cells. Cultures were carried out in 25-L open concrete tanks under daylight in a greenhouse for 25 days. Growth and maintenance of the culture was done at 30 ± 2°C under 12/12 h light-dark cycles. Cultures were agitated by aeration at a flux of 20 L/h provided by a diaphragm pump. Samples were collected every 5 days for assessment of the cyanobacteria growth as well as estimation of biochemical components status. All experiments were carried out with three replicates.

Growth and productivity parameters determination

S. platensis cell populations were determined by direct microscopic counting method described by Usharani et al. (2012). The number of filaments was evaluated using a light microscope (Cyscope® HP, Sysmex-Partec, Japan).

For dry weight concentrations measurement, homogenous suspension of *S. platensis* sample *(*200 ml) was filtered through Whatman no. 1 filter paper and oven dried at 50°C for 48 h. The dry filter containing *S. platensis* dry weight was cooled and weighed. The difference between the initial and final weight was taken as dry weight of *S. platensis* biomass.

The cell productivity of *S. platensis* was calculated according to the formula described by Jarisoa (2005):

$$P = X_2 - X_1 / t_2 - t_1$$

Where X_2 and X_1 represent the biomass concentrations at the times t_2 and t_1.

The specific growth rate (μ) was calculated as follows (Göksan et al., 2007):

$$\mu = \ln X_2 - \ln X_1 / t_2 - t_1$$

where X_2 and X_1 represent the biomass concentrations at the times t_2 and t_1 respectively.

Biochemical characteristics determination

Total soluble carbohydrates were estimated by phenol-sulphuric acid method (Dubois et al., 1956). 1 g of plant fresh materials was weighed and digested by hot ethanol 80% two times, each time by 5 mL ethanol and then filtered by Whatman No. 2. Filter paper and the extracts were diluted by distilled water to the volume of 50 mL. 1 ml for each sample was placed in the test tube and then 1 mL

Table 1. Variation of cell population, biomass dry weight, cell productivity and specific growth rate of *S. platensis* of Moringa leaf extract media under different concentrations of kanwa or $NaHCO_3$.

Cultivation media	Treatment	Cell population (cp mL^{-1})	Dry weight (mg)	Cell productivity (g L^{-1} day^{-1})	Specific growth rate (cell day^{-1})
Jourdan medium	Control	90037 ± 2363^a	1.01 ± 0.42^a	0.25 ± 0.002^a	0.028 ± 0.009^a
	MLE+U	20783 ± 1258^e	0.40 ± 0.26^d	- 0.04 ± 0.003^d	- 0.09 ± 0.004^d
	MLE+U+B4	47848 ± 725^d	0.64 ± 0.62^c	0.053 ± 0.008^c	0.010 ± 0.005^c
Moringa leaf extract	MLE+U+K4	61296 ± 793^c	0.74 ± 0.40^b	0.14 ± 0.002^b	0.015 ± 0.006^b
	MLE+U+B8	63778 ± 2422^c	0.79 ± 0.74^b	0.15 ± 0.007^b	0.018 ± 0.006^b
	MLE+U+K8	67825 ± 815^b	0.86 ± 0.02^b	0.16 ± 0.006^b	0.022 ± 0.005^a

Data are mean ± standard error (n = 5) . MLE: *Moringa oleifera* leaves extract, U: urea, K: kanwa (NaCl), B: $NaHCO_3$.. Means followed by the same letter in the same column are not significantly different ($p < 0.05$) as determined by Fisher LSD test.

phenol solution added. The procedure was followed by adding 5 mL of sulphuric acid by shaking well. The yellow-orange colour was pipetted off and the wavelength was read in 490 nm by spectrophotometer (Pharmaspec UV-1700 model). The amount of carbohydrates was presented from the glucose standard curve.

Total soluble protein content was measured according to the method described by Bradford (1976) using bovine serum albumin (BSA) as a protein standard. Fresh leaf samples (100 mg) were homogenized with 4 mL Na-Phosphate buffer (pH 7.2) and then centrifuged at 13000 g for 4.5 min at 4°C. 1 ml of supernatant is added to the Bradford reagent (5 mL) and the mixture was incubated thereafter in the dark for 15 min. Then, it was pipetted in spectrophotometer cuvettes and absorbance was measured at 595 nm using a UV spectrophotometer (PG instruments T60).

Chlorophyll content determination

Total leaf chlorophyll (CHL) of plants was extracted in 80% (v/v) aqueous acetone and absorption was measured in spectrophotometer (Thermospertronic Heλios β) at 645 and 663 nm (Arnon, 1949). CHL was calculated using the formula:

Total leaf chlorophyll = (20.2 x D645 + 8.02 x D663) x (50/1000) x 100/5) x ½

Where, D: Absorbance

Antioxidant compounds determination

The activity of peroxidase (POD) and polyphenol oxidase (PPO) were determined according to Thorpe and Gaspar (1978) and Van Kammen and Broumer (1964) methods, respectively. For the assay of POD and PPO, a fresh *S. platensis* sample was extracted in 10 mL potassium phosphate buffer (50 mM, pH 6.0). The homogenate was subsequently centrifuged (6000 g, 30 min at 4°C) and the supernatant was collected. The pellet was re-suspended in the same buffer centrifuged under the same conditions as previously. The second supernatant was added to the first to obtain extract which was used for PPO and peroxidase POD activity. POD activity was determined by measuring the oxidation of guaiacol and the increase in absorbance at 420 nm was recorded in 3 min. PPO activity was assayed by measuring the decomposition of H_2O_2 by following the decline in its absorbance at 330 nm for 30 s. The activity was defined as Unit/µg of proteins contents.

The phenol content (PC) was determined according to the method described by Singleton and Rossi (1965). Ethanol extracts (0.2 mL) were added to 1.6 mL of H_2O and 0.5 mL of Folin-Ciocalteu reagent and incubated at 25°C for 10 min. Afterwards, 1 mL of a 7.5% solution of Na_2CO_3 was added to each sample and left at 40°C for 20 min in a water bath, with intermittent shaking. The absorbance of the sample was recorded at 760 nm. The calibration curve was performed with gallic acid and the results were expressed as mg of gallic acid equivalents per g of dry weight.

Physico-chemical parameters determination

The conductivity, temperature, pH, total dissolved solids and salinity of media were measured according to the methods described by Rodier et al. (2009). The physico-chemical parameters were recorded daily using multi-parameters (HI 98130, HANNA Instruments, Rhodes Island, USA).

Statistical analysis

The data obtained were represented as the mean ± standard error. All of the statistical analyses were conducted using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). The one-way analysis of variance (ANOVA) with Duncan's Multiple Range Tests was used to compare differences between treatment means when significant F values were observed at $p < 0.05$.

RESULTS AND DISCUSSION

Growth parameters

Growth of *S. platensis* was expressed in terms of cell population (CP), biomass dry weight (DW), cell productivity (CPr) and specific growth rate (SGR) (Table 1). The highest values of CP (90037 ± 2363 cp mL^{-1}), DW (1.01 ± 0.42 mg/L), CPr (0.25 ± 0.002 g L^{-1} day^{-1}) and SGR (0.028 ± 0.009 cell day^{-1}) were recorded in the Jourdan medium (control) compared to all other treatments comprising moringa leaf extract (MLE) during all the period of experimentation (Table 1). The highest values of CP (67825 ± 815 cp mL^{-1}), DW (0.86 ± 0.02 mg L^{-1}), CPr (0.16 ± 0.006) and SGR (0.022 ± 0.005) registered in MLE enriched with urea and kanwa at 8 g L^{-}

^{1} were also significantly ($p < 0.05$) higher than those grown in other MLE media compared to control (Table 1). Kanwa, a type of salt, is formed when salt water from a sea or lake evaporates and leaves behind colorful crystals of sodium chloride. According to Nafee et al., (2013), it also contains natural impurities such as calcium sulfate, potassium chloride and various levels of trace minerals, such as manganese, copper, iron, and zinc. Moreover, MLE has received enormous attention from the scientific community because of its rich source in hormones, antioxidants, vitamins and minerals such as iron, calcium and potassium as well as vitamins and macronutrients which have plant growth-promoting capabilities and often applied as exogenous plant growth enhancers (Rady et al., 2013; Yasmeen et al., 2013; Khan et al., 2017a, b). In this study, the CP, DW, CPr and SGR were significantly ($p < 0.05$) increased when the MLE cultivation medium was enriched with $NaHCO_3$ or kanwa compared to those supplemented only with urea (Table 1). These results could be explained by the fact that the supply of MLE cultivation medium $NaHCO_3$ or kanwa can improve water quality and increase the quality of *S. platensis* growth and it will also cause good removal of turbidity in the cultivation media so that the light penetration increases will improve photosynthesis and production of *S. platensis* (Ogbonna and Chukukwu, 2018; Silva et al., 2020). This may be also due to uptake of nutrients (carbohydrates and ash) and minerals (Na, K, Ca, Mg) brought by MLE and supplemented $NaHCO_3$ or kanwa which increase cell growth and the metabolism of carbon in the photosynthetic activity of *S. platensis* (Nweze and Nwafor, 2014a,b). A number of green algal species have been shown to be able to utilize carbonates such as Na_2CO_3 and $NaHCO_3$ for cell growth (Emma et al., 2000). Large-scale production of *S. platensis* biomass is essentially a complex process involving a large number of variables and for their successful growth; the nutrient sources and the temperature needs to be conditioned to meet as many of the essential requirements of the organism (Ndjouondo et al., 2017). On the other hand, the values of CP (20783 ± 1258 cp mL^{-1}) and DW (0.40 ± 0.26 mg L^{-1}) and those of CPr (- 0.04 ± 0.003) and SGR (- 0.09 ± 0.004) of *S. platensis* were negatively affected by MLE supplemented only by urea (Table 1). These low values of CP, DW, CPr and SGR were influenced by nutrients found in *S. platensis* cultivation media (Magwell, 2017).

Biochemical characteristics

In this study, the presence of kanwa or $NaHCO_3$ in MLE cultivation medium of *S. platensis* at different concentration levels resulted in a significant ($p < 0.05$) increase in proteins content compared to Jourdan standard medium (control) except in the MLE medium supplemented only with urea while a significant ($P < 0.05$) decrease in carbohydrate and phenol contents was recorded during all the period of experimentation (Figure 1b and c). The results showed that the MLE medium enriched with urea and kanwa (8 g L^{-1}) was significantly ($P < 0.05$) higher than other treatments. This could be related to the high amount of nutrients in the medium such as colorful crystals of sodium chloride and natural impurities having calcium sulfate and potassium chloride (Nafee et al., 2013). The effect of the MLE 'supplemented with urea and kanwa could be also explained by the increase of nitrogen assimilation due to the high amount of inorganic carbon provided by $NaHCO_3$ in the medium. A number of green algal species have been shown to be able to utilize carbonates such as Na_2CO_3 and $NaHCO_3$ for cell growth (Emma et al., 2000). In this study, the carbohydrate content remains very low compared to the protein content (Figure 1a and b). Depraetere et al., (2015) reports that when the amount of nitrogen is high or excessive, it would lead to carbohydrate hydrolysis. The present study also revealed significant ($P<0.05$) decrease in MLE medium supplemented only with urea for all the treatments compared to Jourdan standard medium (Figure 1a, b and c). This effect of urea could be attributed to the low concentration used for this experiment (50 mg L^{-1}). According to Rangel-Yagui et al. (2004), the best cellular growth for *S. platensis* was observed with 500 mg L^{-1} of urea at a light intensity of 5600 lux.

Chlorophyll content

The enrichment of MLE cultivation medium with urea, kanwa or $NaHCO_3$ at different concentration levels led to a significant decrease ($p< 0.05$) in chlorophyll content during all the cultivation period of *S. platensis* compared to Jourdan standard medium (Figure 2). This depressive effect may be attributed to salt-induced weakening of protein-pigment-lipid complex or increased chlorophyllase (Strogonov, 1970). The significant ($P< 0.05$) decrease of chlorophyll content could be also due to low (50 mg L^{-1}) concentration of urea supply. According to Rangel-Yagui et al. (2004), the highest concentration of chlorophyll in the biomass was observed with 500 mg L^{-1} at a light intensity of 1400 lux.

Antioxidant compounds

In the present study, antioxydant compounds of *S. platensis* were expressed in terms of polyphenol oxidase (PPO), peroxidase (POD) and phenol content (PC) (Table 2). The highest values of PPO (6.02 ± 0.044 UE µg-1) and POD (0.63 ± 0.03 UE µg-1) were recorded in the medium which contains MLE enriched with urea

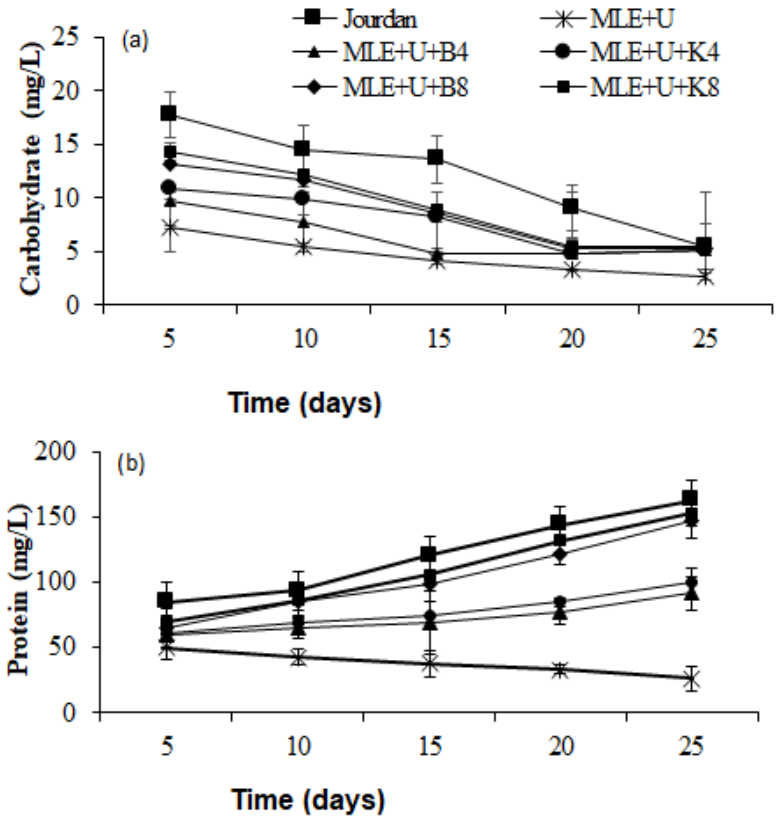

Figure 1. Variation of some biochemical characteristics in Moringa leaf extract (MLE) in response to kanwa or $NaHCO_3$ at different concentration levels. (a) Carbohydrate, (b) proteins. Data are Mean ± standard error (n = 5). Mean followed by the same letter are not significantly different ($p < 0.05$) as determined by Fisher LSD test. Bars indicate standard error.

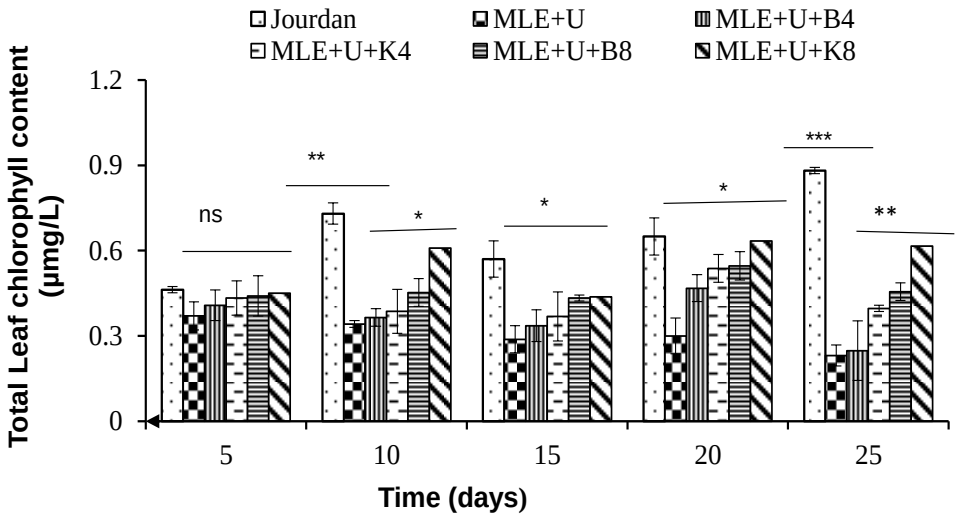

Figure 2. Variation of total leaf chlorophyll content in Moringa leaf extract (MLE) in response to kanwa or $NaHCO_3$ at different concentration levels. Data are Mean ± standard error (n = 5). Mean followed by the same letter are not significantly different ($p < 0.05$) as determined by Fisher LSD test. Bars indicate standard error.

Table 2. Variation of antioxidant compounds of *S. platensis* in response to kanwa or $NaHCO_3$ at different concentration levels.

Media	Treatment	PPO (10^{-3}×UE µg^{-1})	POD (10^{-3}× UE µg^{-1})	PC (mg l^{-1})
Jourdan medium	Control	2.00 ± 0.024^{d}	0.03 ± 0.03^{d}	2.09 ± 0.07a
MLE	MLE+U	6.02 ± 0.044^{a}	0.63 ± 0.03^{a}	1.00 ± 0.01e
	MLE+U+B4	4.07 ± 0.002^{b}	0.27 ± 0.02^{b}	1.55 ± 0.21d
	MLE+U+K4	3.33 ± 0.012^{c}	0.26 ± 0.01^{bc}	1.63 ± 0.25c
	MLE+U+B8	2.30 ± 0.018^{cd}	0.18 ± 0.02^{cd}	1.80 ± 0.21bc
	MLE+U+K8	2.23 ± 0.010^{cd}	0.17 ± 0.02^{cd}	1.88 ± 0.11b

Data are mean ± standard error (n = 5). MLE: *Moringa oleifera* leaves extracts, U: urea, K: kanwa (NaCl), B: $NaHCO_3$, POD: peroxidase and PPO: polyphenol oxidase, PC: phenol content. Means followed by the same letter in the same column are not significantly different ($p < 0.05$) as determined by Fisher LSD test.

compared to Jourdan standard medium (control) which showed the lowest values (2.00 ± 0.024 UE µg^{-1}) and (0.03 ± 0.03 UE µg^{-}1), respectively (Table 2). On the other hand, the highest value of PC was noted in Jourdan standard medium (2.09 ± 0.07 mg L^{-1}) while the lowest value was observed in MLE cultivation medium supplemented only with urea (1.00 ± 0.010 mg L^{-1}) (Table 2). In response to stress, plants activate powerful antioxidant systems, both enzymatic (e.g., SOD, POD, catalase, glutathione reductase) and non-enzymatic (flavonoid, PC, carotenoids, vitamins C and E) (Ashraf, 2009; Kahrizi et al., 2012). The activity of antioxidant enzymes (PPO and POD) was notably increased with the cultivation period of *S. platensis* in all MLE medium supplemented with different concentrations of kanwa or $NaHCO_3$ compared to Jourdan standard medium (Table 2). The use of MLE based medium supplemented with a high concentration of kanwa or $NaHCO_3$ may cause the increasing trend of the amount of PPO and POD activity. According to Kasote et al. (2015) and Mostafa et al. (2016), this increase in enzymatic activity could be correlated to a decrease in oxidative stress and reactive oxygen derivatives produced during photosynthesis and to the high content of phenols which would act as antioxidant by producing an enzymatic substrate to alleviate the harmful effects of reactive oxygen species.

Physico-chemical parameters

In this study, some physico-chemical parameters (conductivity, pH, total dissolved solids and salinity) of *S. spirulina* cultivation media led to a significant ($p < 0.05$) increase in MLE media enriched with different concentrations of urea, kanwa or $NaHCO_3$ from 20 to 25 days of cultivation compared to Jourdan medium and others treatments (Figure 3a, c, d and e). This increase of salinity and total dissolved solids could be explained by increase with the evaporation of water in media and to the change of the other variables of the culture media due to uptake of nutrients brought by the different concentrations of kanwa or $NaHCO_3$ in MLE media **(**Rusydi, 2018). This progressive increase in total dissolved solids and salinity could be explained by the increase in alkalinity and concentration of dissolved ionic salts resulting from MLE media with different concentration of kanwa or $NaHCO_3$ following the evaporation of water from the media as reported by Mutanda et al. (2014). The temperature of *S. platensis* cultivation media varied significantly ($p < 0.05$) between 29 and 31°C and the pH between 8 and 10 with the highest value of temperature (31°C) recorded in MLE medium supplemented with urea and Kanwa at 8 g L^{-1} and the lowest (29°C) was noted in Jourdan standard medium (Figure 3b). The results obtained are in agreement with those reported by Soni et al. (2019) which indicated that for flourishing and optimal growth, temperature for *S. platensis* is between 30 and 35°C and pH value between 8.5 and 10.5.

Conclusion

In general, the results of this study showed that the growth parameters such as cell population, biomass dry weight, cell productivity and specific growth rate were positively affected in MLE cultivation medium enriched with kanwa or $NaHCO_3$ at different concentration levels. This result may be due to uptake of nutrients (carbohydrates and ash) and minerals (Na, K, Ca, Mg) brought by MLE and supplemented $NaHCO_3$ or kanwa which increase cell growth and the metabolism of carbon in the photosynthetic activity of *S. platensis*. The addition of kanwa or $NaHCO_3$ in MLE cultivation medium at different concentration levels increased significantly the presence of electrically charged atoms which physico-chemical parameters (conductivity, pH, total dissolved solids and salinity) from 20 to 25 days of cultivation, the protein content and antioxidant enzymes (PPO and POD) activity whereas a decrease in carbohydrate content was

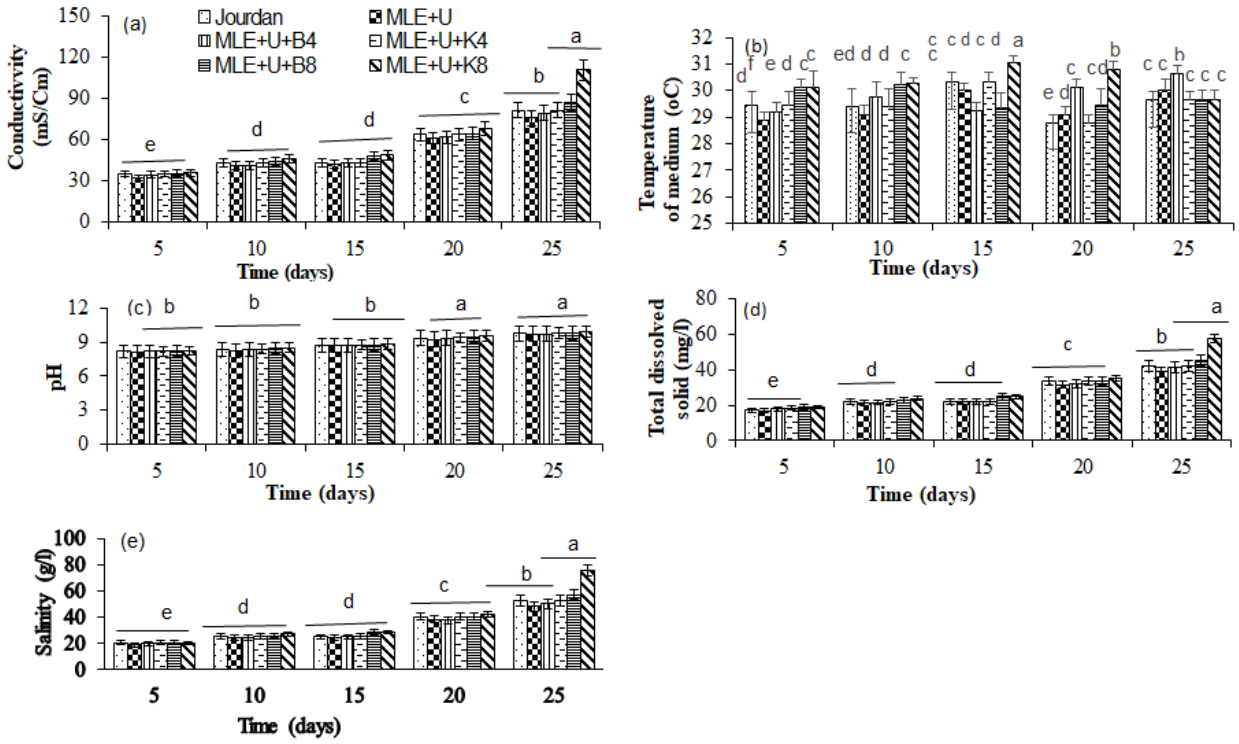

Figure 3. Variation of some physico-chemical parameters in Moringa leaf extract (MLE) in response to kanwa or $NaHCO_3$ at different concentration levels. (a) Conductivity, (b) Temperature, (c) pH, (d) Total dissolved solid, (e) Salinity. Data are Mean ± standard error (n = 5). Mean followed by the same letter are not significantly different ($p < 0.05$) as determined by Fisher LSD test. Bars indicate standard error.

recorded during all the period of the experimentation. This increase in enzymatic activity could be correlated to a decrease in oxidative stress and reactive oxygen derivatives produced during photosynthesis and to the high content of phenols which would act as antioxidant by producing an enzymatic substrate to alleviate the harmful effects of reactive oxygen species. This study draws attention to a good view on MLE as a cheaper and easily available organic fertilizer that need to be enriched for the culture of *S. platensis*. Thus, further investigations should be done in the commercial cultivation of *S. platensis* using MLE in agriculture, food industry, cosmetics, pharmaceutics and medicine.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

ACKNOWLEDGEMENT

The authors thank the SAGRIC Common Initiative Group (CIG) Farm, Douala-Cameroon who graciously provided us with the *Spirulina* strain.

REFERENCES

Abbaspour H (2012). Effects of salt stress on lipid peroxidation, antioxidative enzymes and proline accumulation in pistachio plants. Journal of Medicinal Plant Research 6(3):526-529.

Adebayo AG, Akintoye HA, Olufolaji OO, Aina MT, Olatunji MT, Shokalu AO (2011). Assessment of organic amendments on vegetative development and nutrient uptake of *Moringa oleifera* Lam. in the nursery. Asian Journal of Plant Sciences 10(1):74-79.

Ama Moor VJ, Pieme CA, Nkeck JR, Nya Biapa CP, Ikomey MG, Kouanfack C, Okomo Assoumou MC, Ngogang J (2020). *Spirulina platensis* enhances immune status, inflammatory and oxidative markers of HIV patients on antiretroviral therapy in Cameroon. Research Square pp. 1602-6494.

Andrade MR, Costa JAV (2007). Mixotrophic cultivation of microalga *Spirulina platensis* using molasses as organic substrate. Aquaculture 264(1-4):130-134.

Arnon DI (1949). Copper enzymes in isolated chloroplasts. Polyhenoloxidases in *Beta vulgaris.* Plant Physiology 24(1):1-15.

Ashraf M (2009). Biotechnological approach of improving plant salt tolerance using antioxidants as markers. Biotechnology Advances 27(1):84-93

Bradford M (1976). A rapid and sensitive method for the quantitative of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72(1-2):248-254.

Branyikova I, Lucakova S (2020). Technical and physiological aspects of microalgae cultivation and productivity-*spirulina* as a promising and feasible choice. Organic Agriculture 11(2):269-276.

Corrêa PS, Teixeira CMLL (2021). Polyhydroxyalkanoates and pigments coproduction by *Arthrospira* (*Spirulina*) *platensis* cultivated in crude glycerol. Journal of Applied Phycology 33:1487-1500.

Depraetere O, Deschoenmaeker F, Badri H, Monsieurs P, Foubert I, Leys N, Wattiez R, Muylaert K (2015). Trade-off between growth and carbohydrate accumulation in nutrient-limited *Arthrospira sp*. PCC 8005 studied by integrating transcriptomic and proteomic approaches. PLoS ONE 10(7):e0132461.

Dubois M, Gilles KA, Hamil JK, Rebers PA, Smith F (1956). Colorimetric method for determination of sugars and related substances. Analytical Biochemistry 28(3):350-356.

Emma Huertas I, Colman B, Espie GS, Lubian LM (2000). Active transport of CO_2 by three species of marine microalgae. Journal of Phycology 36(2):314-320.

Göksan T, Zekeriyaoglu A, Ak I (2007). The growth of *Spirulina platensis* in different culture systems under greenhouse condition. Turkish Journal of Biology 31(1):47-52.

Goulamabasse R (2018). *Spirulina*: Therapeutic activities and its interest in the fight against malnutrition in Madagascar, Ph.D Thesis, UFR pharmaceutical and biological sciences, University of Lille 135 p.

Jarisoa T (2005). Adaptation of *Spirulina* from southern Madagascar to seawater culture. Development of production structures at the village level. Ph.D, Thesis University of Toliara, Madagascar, 129p.

Jourdan JP (2013). Grow your *Spirulina*. Manual of artisanal culture for the production of *Spirulina*. Antenna Technology, 143p.

Kahrizi S, Sedghi M, Sofalian O (2012). Effect of salt stress on proline and activity of antioxidant enzymes in ten durum wheat cultivars. Annals of Biological Research 3(8):3870-3874.

Kasote DM, Katyare SS, Hegde MV, Bae H (2015). Significance of antioxidant potential of plants and its relevance to therapeutic applications. International Journal of Biological Sciences 11(8):982-991.

Khalafalla MM, Abdellatef E, Dafalla HM, Nassrallah AA, Aboul-Enein KM, Lightfoot DA, Eldeeb FE, El-Shemy HA (2010). Active principle from *Moringa oleifera* Lam. leaves effective against two leukemais and a hepatocarcinoma. African Journal of Biotechnology 9(49):8467-8471.

Khan S, Basra SMA, Afzal I, Wahid A (2017a). Screening of moringa landraces for leaf extract as biostimulant in wheat. International Journal of Agriculture and Biology 19:999-1006.

Khan S, Basra SMA, Afzal I, Nawaz M, Rehman HU (2017b). Growth promoting potential of fresh and stored *Moringa oleifera* leaf extracts in improving seedling vigor, growth and productivity of wheat crop. Environmental Science and Pollution Research 24:7601-27612.

Kosamia NM, Samavi M, Uprety BK, Rakshit SK (2020). Valorization of biodiesel by product crude glycerol for the production of bioenergy and biochemicals. Catalysts 10(6):609.

Lupatini AL, Colla LM, Canan C, Colla E (2017). Potential application of microalga *Spirulina platensis* as a protein source. Journal of the Science of Food and Agriculture 97(3):724-732.

Madkour F, Abd El-Wahab K, Hodam S (2012). Production and nutritive value of *Spirulina platensis* in reduced cost media. The Egyptian Journal of Aquatic Research 38(1):51-57.

Magwell PFR (2017). Influence of sulphate salts on growth performance and biochemical analysis of *Spirulina platensis*. Master thesis, University of Douala. Douala-Cameroon, 111p.

Markou G, Diamantis A, Arapoglou D, Mitrogiannis D, Gonzalez-Fernandez CUA (2021). Growing *Spirulina* (*Arthrospira platensis*) in seawater supplemented with digestate: Trade-offs between increased salinity, nutrient and light availability. Biochemical Engineering Journal 165:107815.

Mellou F, Varvaresou A, Papageorgiou S (2019). Renewable sources: applications in personal care formulations. International Journal of Cosmetic Science 41(6):517-525.

Mesquita SDS, Teixeira CMLL, Servulo EFC (2017). Carotenoids: properties, applications and market. Revista Virtual de Quimica 9:672-688.

Miah MS, Phang SM, Chu WL, Hashim MA (2000). *Spirulina* culture in digested sago starch factory wastewater. Journal of Applied Phycology 12(3):395-400.

Mia ML, Habib MAB, Hoque N, Islam MS (2019). A study on growth performance of *Spirulina platensis* in different concentrations of rotten apple as a carbon source. International Journal of Excellence Innovation and Development 2(1):29-40.

Molino A, Iovine A, Casella P, Mehariya S, Chianese S, Cerbone A, Rimauro J, Musmarra D (2018). Microalgae characterization for consolidated and new application in human food, animal feed and nutraceuticals. International Journal of Environmental Research and Public Health 15(11):2436. doi: 10.3390/ijerph15112436.

Mostafa MS, Yassin EM, Michele PN (2016). Role of pH on antioxidants production by *Spirulina* (*Arthrospira*) *platensis*. Brazilian Journal of Microbiology 47(2):298-304.

Moyo B, Masika PJ, Hugo A, Muchenje V (2011). Nutritional characterization of Moringa (*Moringa oleifera* Lam.) leaves. African Journal of Biotechnology 10(60):12925-12933.

Mutanda T, Ranjith RK, Bux F (2014). Physico-chemical and biotic factors influencing microalgal seed culture propagation for inoculation of a large scale raceway pond. African Journal of Biotechnology 13(35):3607-3616.

Nafee M, Nazish K, Shah R, Adila B (2013). Analysis of rock and sea salts for various essentials and inorganic elements. Journal of Science and Technology University of Peshawar 37(1):9-20.

Ndjouondo JP, Dibong SD, Wamba FO, Taffouo VD (2017). Growth, productivity and some physico-chemical factors of *Spirulina platensis* cultivation as influenced by nutrients change. International Journal of Botany 13(2):67-74.

Nweze NO, Nwafor FI (2014a). Phytochemical, proximate and mineral composition of Leaf extracts of *Moringa oleifera* Lam. from Nsukka, South-Eastern Nigeria. IOSR Journal of Pharmacy and Biological Sciences 9(1):99-103.

Nweze NO, Nwafor FI (2014b). Promoting increased *Chlorella sorokiniana* Shih and Krauss (*Chlorophyta*) biomass production using *Moringa oleifera* Lam. leaf extracts. African Journal of Biotechnology 13(53):4720-4725.

Nyabuto D, Cao K, Mariga A, Kibue G, He M, Wang C (2015). Growth performance and biochemical analysis of the genus *Spirulina* under different physical and chemical environmental factors. African Journal of Agricultural Research 10(36):3614-3624.

Ogbonna NC, Chukukwu EI (2018). Harvesting *Chlorella variabilis* biomass using *Moringa oleifera* seed-induced sedimentation. Journal of Advances in Biology & Biotechnology 18(4):1-11.

Panjiar N, Mishra S, Yadav AN, Verma P (2017). Functional foods from cyanobacteria. in microbial functional foods and nutraceuticals (Gupta VK, Treichel H, Shapaval V, Antonio de Oliveira L, Tuohy MG eds, 314 p.

Rady MA, Varma BC, Howladar SM (2013). Common bean (*Phaseolus vulgaris* L.) seedlings overcome NaCl stress as a result of presoaking in *Moringa oleifera* leaf extract. Scientia Horticulturae 162:63-70.

Rangel-Yagui CO, Danesi EDG, de Carvalho JCM, Sato S (2004). Chlorophyll production from *Spirulina platensis*: Cultivation with urea addition by fed-batch process. Bioresource Technology 92(2):133-141.

Rodier J, Legube B, Marlet N, Brunet R (2009). Water analysis. 9th edition DUNOD, Paris, 1579p.

Rusydi AF (2018). Correlation between conductivity and total dissolved solid in various type of water: A review. IOP Conference Series: Earth and Environmental Science 118(1):012019.

Shah MR, Lutzu GA, Alam A, Sarker P, Kabir CMA, Parsaeimehr A, Liang Y, Daroch M (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. Journal of Applied Phycology 30:197-213.

Silva DF, Speranza L, Quartaroli L, Moruzzi R, Silva GH (2020). Separation of microalgae cultivated in anaerobically digested black water using *Moringa Oleifera* Lam. seeds as coagulant. Journal of Water Process Engineering 39:2214-7144.

Singh S (2006). *Spirulina*: A Green gold mine. Paper presented at: Spirutech 2006. *Spirulina* cultivation: Potentials and prospects. Jabalpur, Madhya Pradesh.

Singleton VL, Rossi JA (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture 16(3):144-158.

Soni RA, Sudhakar K, Rana RS (2019). Comparative study on the growth performance of *Spirulina platensis* on modifying culture

media. Energy Reports 5:327-336.
Suharyanto TP, Permatasari S, Syamsu K (2014). Production of *Spirulina platensis* in continuous photobioreactor using palm oil mill effluent media. Menara Perkebunan 82(1):1-9.
Thorpe TA, Gaspar T (1978). Changes in isoperoxydase during shoot formation in tobacco callus. *In Vitro* 14(6):552-539.
Usharani G, Saranraj P, Kanchana D (2012). *In vitro* cultivation of *Spirulina platensis* using rice mill effluent. International Journal of Pharmaceutical and Biological Archives 3(6):1518-1523.
Van Kammen A, Broumer D (1964). Increase of polyphenol oxidase activity by the local virus infection in uninoculated parts of leaves. Virology 22(1):9-14.
Venkataraman LV, Bhagyalakshmi N, Ravishankar GA (1995). Commercial production of micro and macro algae problems and potentials. Indian Journal of Microbiology 35:1-19.
Vonshak A (1997). *Spirulina*: Growth, physiology and biochemistry. In: Vonshak A (Ed) *Spirulina platensis (Arthrospira)*: Physiology, Cell-Biology and Biotechnology. Taylor and Francis, London, 233p.
Wen Y, Wen P, Hu T, Linhardt RJ, Zong M, Wu H, Chen Z (2020). Encapsulation of phycocyanin by prebiotics and polysaccharides based electrospun fibers and improved colon cancer prevention effects. International Journal of Biological Macromolecules 149(2):672-681.
Yasmeen A, Basra SMA, Farooq M, Rehman H, Hussain N, Athar HR (2013). Exogenous application of moringa leaf extract modulates the antioxidant enzyme system to improve wheat performance under saline conditions. Plant Growth Regulation 69:225-233.
Zarrouk C (1966). Contribution to the study of a cyanophyceae. Influence of various physical and chemical factors on the growth and photosynthesis of *Spirulina maxima* (Setch.Et Gardner) Geitler. Ph.D Thesis. University of Paris, France 412 p.
Zhu C, Zhu H, Cheng L, Chi Z (2018). Bicarbonate-based carbon capture and algal production system on ocean with floating inflatable membrane photobioreactor. Journal of Applied Phycology 30(2):875-885.

Vol. 20(8), pp.335-339, August, 2021
DOI: 10.5897/AJB2020.17064
Article Number: E883BD267573
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Review

Reconstruction of metabolic pathways in selected bacterial and yeast strains for the production of bio-ethylene from crude glycerol: A mini review

Idan Chiyanzu

Agricultural Engineering, Agricultural Research Council, Private Bag X519, Silverton 0127, South Africa.

Received 16 January, 2020; Accepted 24 May, 2021

The role of bioengineering in industrial processes cannot be ignored, especially in circumstances where the processes are uneconomical, demand high energy, use finite resources and emit huge amounts of carbon dioxide. Ethylene is a two-carbon unsaturated hydrocarbon that is industry's most important building block for polyester fibres, plastics, and ethylene glycol. For decades, ethylene production has relied on steam-cracking without many improvements, especially on issues of environmental impact and adoption of appropriate renewable approaches. This paper discusses selected microbial pathway modifications as novel approach to developing systems that could be alternatives to conventional ethylene production systems. Bioengineering of the ethylene pathway is suggested in view of the need to meet the criteria of high efficiency, increase sustainability and ensure product qualities and quantities that can exceed the existing approach.

Key words: Ethylene, crude glycerol, tolerance, synthesis, bioengineering.

INTRODUCTION

Greenhouse gas emission associated with industrial activity is perceived as having the greatest single impact on climate change. Ethylene gas is an unsaturated hydrocarbon, which is used by industry as building block for the production of polyester fibres, plastics, and ethylene glycol production (Alshammari et al., 2016). For decades, ethylene has been produced by steam-cracking of natural gas and naphthalene, allowing huge carbon emissions into the atmosphere (estimated at over three tonnes of carbon dioxide per ton of ethylene) (Amghizar et al., 2017). Additionally, the steam-cracking route has regularly posed numerous other challenges, including non-selectiveness, high energy input and decreased economic perspectives (Haribal et al., 2018; Ren et al., 2006). For this reason, it is necessary to look into the adoption of eco-friendly methods such as conversion of alternative feedstock, including plant biomass, industrial waste and agricultural residues for improved techno-economic balance of ethylene production. It is worth mentioning that an abundance of renewable sources of feedstock also exist, such as crude glycerol, bioethanol, carbon dioxide, and lignocellulose, that can be tapped

E-mail: Chiyanzul@arc.agric.za Tel: +27 (0) 12 842 4044. Fax: +27 (0) 12 804 0753.

(ACS, 2016).

This paper's focus is on highlighting the importance of the biological sources of ethylene and placing emphasis on bioengineering as a potential approach to improve volumetric yields, reduce carbon dioxide emissions and decrease cost. Finally, it might present the opportunity of discovering biodiesel-derived crude glycerol as a relevant and abundant raw material that could potentially be used for bioengineered microbial systems in efforts to reduce production costs.

PLANT AND MICROBIAL METABOLIC PATHWAY STUDIES

Ethylene synthesis is a naturally occurring process in higher plants and many microbial classes that belong to bacteria and fungi (Ballester et al., 2020). Ethylene occurs as a hormone that regulates growth and stress responses in plants and is understood to be a metabolic product of many fungi and bacteria that live with plants. A known number of biological pathways for ethylene biosynthesis exists, which exploit one of three distinct routes, depending on the host organism. In one pathway, ethylene can be produced via an intermediate molecule called 2-oxoglutarate, while in the other pathways, ethylene is produced from a precursor called methionine MET, as seen in Figure 1 (Fukuda et al., 1993).

For example, the biosynthesis of ethylene in higher plants from amino acid MET involves two key enzymes, including ACC (1-aminocyclopropane-1carboxylic acid) synthase and oxidase, in a series of sequences; MET → S-adenosylmethionine (SAM) → 1-aminocyclopropane-1 -carboxylic acid (ACC) → ethylene. The enzyme AdoMet synthetase initiates the catalytic conversion of MET to SAM with one ATP molecule expended per synthesis. In the next step, ACC synthase (ACS) catalyses the conversion of SAM to ACC and methylthioadenosin. While the methylthioadenosin is recycled to produce SAM through the Yang cycle, ACC oxidase degrades ACC into ethylene, CO_2 and HCN. Most literature suggests that ethylene biosynthesis must be regulated at the level of ACC-oxidase, which also acts as a rate-limiting step (Van de Poel et al., 2011).

In several microbial species such as fungal and bacterial ethylene producers, ethylene is produced from methionine *via* 2-keto-4-methyl-thiobutyric acid (KMBA) or through an intermediate called 2-oxoglutarate by an ethylene-forming enzyme (*efe*) (Eckert et al., 2014; Ogawa et al., 1990). In the former pathway, KMBA is oxidized to ethylene gas by a complex reaction involving NADH (Figure 1). In the 2-oxoglutarate-dependent pathway, a key enzyme (*efe*) deoxygenates 2-oxoglutarate to produce one molecule of ethylene and three molecules of carbon dioxide. In the sub-reaction, both 2-oxoglutarate and L-arginine are mono-oxygenated to yield succinate plus carbon dioxide and L-hydroxy arginine, respectively, the latter being further transformed to guanidine and L-δ-1-pyrroline-5-carboxylate (Pattyn et al., 2020).

HETEROLOGOUS EXPRESSION SYSTEM STUDIES

Heterologous gene expression is one of the most promising technologies found to deliver higher protein yields. For example, heterologous gene expression of nitrile hydratase in *Escherichia coli* (*E. coli*) offers rapid growth, high yields and economic production of recombinant products (Chiyanzu et al., 2010). Over the past two decades, a large number of researchers have focused on the expression of genes that encode *efe* for ethylene biosynthesis. The first research involved a purified cell-free extract of *Pseudomonas syringae pv*. *phaseolicola* PK2 (Nagahama et al., 1991) being expressed in a range of host systems. The first increased ethylene in a heterologous system was reported in heterologous systems involving *E. coli* in JM109 and DH5α strains where a 73-fold higher yield than the wild type was found (Ishihara et al., 1991). Other heterologous systems reported thus far also include *Saccharomyces cerevisiae* (Nagahama et al. 1991), *Pseudomonas putida* (Ishihara, 1995), *Trichoderma viride* (Pirkov et al., 2008), *Trichoderma reesei* (Ishihara et al., 1996), tobacco (Tao et al., 2008), cyanobacteria (Chen et al., 2010), yeast (Araki et al., 2000) and *Synechocystis* sp. PCC68037 (Sakai et al., 1997).

Among all the heterologous systems for production of ethylene, the *P. putida* system incorporated with the pBBR1MCS2 vector has been the best, with 489 mg ethylene produced in 24 h, equivalent to a conversion rate of 21.7 mg ethylene g ($^{-1}$) substrate (Hamilton et al., 1991). Despite all the benefits, heterologous gene expression can be time-consuming and costly. Recently, gene synthesis has emerged as a new application of genetic engineering and is quickly becoming a cornerstone of modern molecular biology (Guerrero et al., 2012). The technique offers an opportunity to design a construct of choice from the start, typically tailored for the final products. So far constructs have been designed in many ways, usually with the aim of disrupting a selected gene to prevent transcription of a functional protein (a knock-off) or by mutating the gene (a knock-in). A classic functional gene construct consists of a promoter region, gene-coding region and terminator region. In addition, a few gene constructs may contain special sequences including an enhancer, silencer, or reporter sequences, depending on the nature of the desired product.

Use of crude glycerol as a raw material in biosynthesis

Crude glycerol is one of the world's most widely abundant

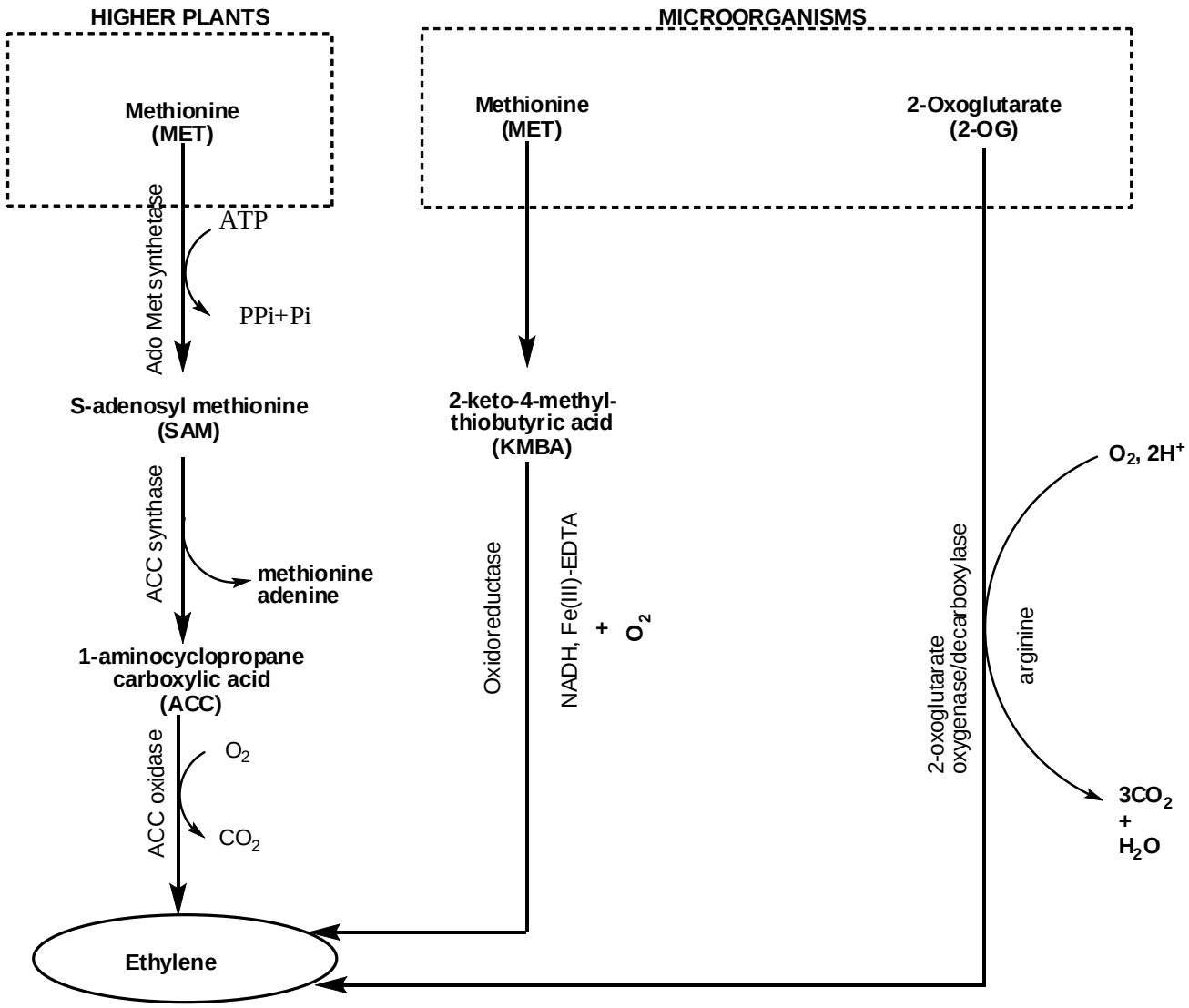

Figure 1. Plants and microbial biosynthesis pathways of ethylene involving substrates, enzymes, and intermediary metabolism. Source: Modified from Fukuda et al. (1993).

waste products that can be further exploited as an alternative precursor for the production of ethylene. The first report on the possible conversion of glycerol to ethanol, and finally ethylene, was published in literature by Zhang and Yu (2013). However, the initial step involves fermentation of glycerol to ethanol and then the alcohol is dehydrated in the presence of a vapour phase of two-catalyst systems in support of phosphoric acid and activated alumina. The use of a two-step process is lengthy and expensive in terms of catalysts. In this review, crude glycerol is suggested for direct conversion to ethylene, as this approach would be more economically feasible. Usually, this type of approach will be sustainable for the management of crude glycerol in an environmentally friendly manner, especially for the biodiesel industry.

Bioengineering of microbial pathways towards bioethylene synthesis

A metabolic redirection strategy, which would involve re-routing of the central metabolism of a selected microorganism achieved *via* gene knock-out to block undesirable metabolic pathways, can effectively change the course of carbon flow of pyruvate from organic acids in the ethylene. Based on literature studies, the three genes contained in the ethylene-forming metabolic pathway with potential for manipulation include *glycerol kinase* (*glpF*) (a transporter of glycerol across the cytoplasmic membrane), the 2-oxoglutarate dehydrogenase multi-enzyme complex (responsible for 2-oxoglutarate formation from isocitrate), and the gene encoding an *efe*. A simple flow diagram of direct

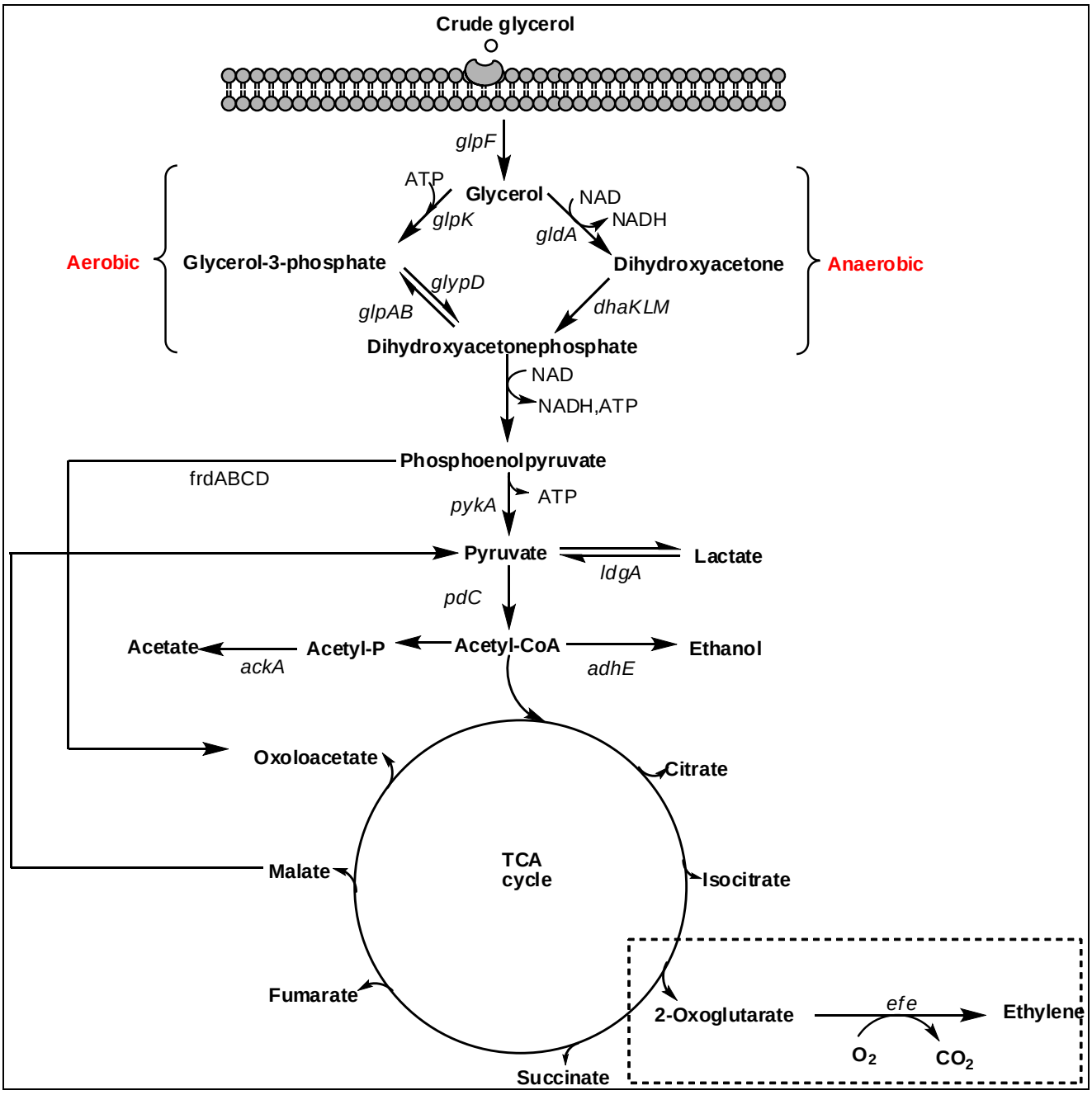

Figure 2. The scheme of events for ethylene production from crude glycerol through metabolic engineering of microbial strains. Source: Modified from Eckert et al. (2014).

conversion of crude glycerol into ethylene is presented in Figure 2. The glycerol molecule is fed through a membrane with the help of *glpF* that is mutated (or has a knock-in gene). Thereafter, it enters the microorganism metabolic pathway. As mentioned before, the bioengineering of the 2-oxoglutarate dehydrogenase multi-enzyme complex and *efe* would increase the bioconversion of crude glycerol to ethylene.

The performance of such a system can depend on several factors, including the type of expression host, expression vector, substrate concentration, and process conditions optimum pH and temperature. However, further research would be needed to determine the stability and biological activity of such transformants and systems. Studies also need to be focused on making such systems cost-effective and feasible for integration into industry production. The metabolic engineering for ethylene production may have to ponder screening a

broader range of microbial strains and vectors in order to reduce failures in the operations.

CONCLUSION

It is apparent that ethylene is one of the most important chemical precursors; it is mainly produced by steam-cracking of naphtha and natural gas. Microbial strains that utilise crude glycerol can be considered for metabolic manipulation as an alternative route for ethylene production of these strains; only impurity-tolerant ones will be economically viable for industrial application, since they will not require the purification step that adds to the cost of the process. To produce ethylene in volumes representative of or required by the industry will need both technical and economic innovations. First, screening and possible isolation of microbial strains that use crude glycerol as a sole carbon source will have to be identified. Second, accurate construction of expression vectors with designed gene inserts will limit making larger and more complex heterologous gene expression systems or constructs. However, the contaminants in crude glycerol may have an impact on the process. Previous results showed that impurities in the crude glycerol do significantly affect its bioconversion. Therefore, it is necessary to perform a rigorous screening step in the selection of strains that show tolerance to a high concentration of impurities. Overall, the use of crude glycerol in direct bioconversion of crude glycerol is an interesting opportunity, especially in view of the benefit that these microbial-based ethylene production processes can lead to localised production plants that can be set up at consumer locations without the risk and hazard associated with a large chemical-based industry.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

REFERENCES

Alshammari A, Kalevaru VN, Bagabas A, Martin A (2016). Production of ethylene and its commercial importance in the global market. Petrochemical Catalyst Materials, Processes, and Emerging Technologies. Hershey, PA: Engineering Science Re*f*erence pp. 82-115

American Chemical Society (2016). Beyond the ethylene steam cracker. Industrial Chemistry & Engineering. Available at https://www.acs.org/content/acs/en/pressroom/cutting-edge-chemistry/beyond-the-ethylene-steam-cracker.html.

Amghizar I, Vandewalle LA, Van Geem KM, Marin GB (2017). New trends in olefin production. Engineering 3(2):171-178.

Araki S, Matsuoka M, Tanaka M, Ogawa T (2000). Ethylene formation and phenotypic analysis of transgenic tobacco plants expressing a bacterial ethylene-forming enzyme. Plant & Cell Physiology 41:327-334.

Ballester AR, González-Candelas L (2020). EFE-Mediated Ethylene Synthesis Is the Major Pathway in the Citrus Postharvest Pathogen *Penicillium digitatum* during Fruit Infection. Journal of Fungi 6(3):175.

Chen X, Liang Y, Hua J, Tao L, Qin W, Chen S (2010). Overexpression of bacterial ethylene-forming enzyme gene in *Trichoderma reesei* enhanced the production of ethylene. International Journal of Biological Sciences 6(1):96-106.

Chiyanzu I, Cowan DA, Burton SG (2010). Immobilization of *Geobaccillus pallidus* RAPc8 nitrile hydratase (NHase) reduces substrate inhibition and enhances thermostability. Journal of Molecular Catalysis B: Enzymatic 63(3-4):109-115.

Eckert C, Xu W, Xiong W, Lynch S, Ungerer J, Tao L, Gill R, Maness PC, Yu J (2014). Ethylene-forming enzyme and bio ethylene production. Biotechnology Biofuels 7(1):1-11.

Fukuda H, Ogawa T, Tanase S (1993). Ethylene production by microorganisms. Advances in Microbial Physiology 35:275-306.

Guerrero F, Carbonell V, Cossu M, Correddu D, Jones PR (2012) Ethylene synthesis and regulated expression of recombinant protein in *Synechocystis sp* PCC6803. PLOS One 7(11):e50470.

Hamilton AJ, Bouzayen M, Grierson D (1991). Identification of a tomato gene for the ethylene-forming enzyme by expression in yeast. Proceedings of the National Academy of Sciences of the United States of America 88(16):7434-7437.

Haribal VP, Chena Y, Neal L, Li F (2018). Intensification of ethylene production from naphtha via a redox oxy-cracking scheme: Process simulations and analysis. Engineering 4(5):714-721.

Ishihara K, Matsuoka M, Inoue Y, Tanase S, Ogawa T, Fukuda H (1995). Overexpression and in vitro reconstitution of the ethylene-forming enzyme from *Pseudomonas syringae*. Journal of Fermentation and Bioengineering 79(3):205-211.

Ishihara K, Matsuoka M, Ogawa T, Fukuda H (1996). Ethylene production using a broad-host-range plasmid in *Pseudomonas syringae* and *Pseudomonas putida*. Journal of Fermentation and Bioengineering 82(5):509-511.

Nagahama K, Ogawa T, Fujii T, Tazaki M, Tanase S, Morino Y, Fukuda H (1991). Purification and properties of an ethylene-forming enzyme from *Pseudomonas syringae pv. phaseolicola* PK2. Journal of General Microbiology 137(10):2281-2286.

Ogawa T, Takahashi M, Fujii T, Tazaki M, Fukuda H (1990). The role of NADH: Fe (III) EDTA oxidoreductase in ethylene formation from 2-keto-4-methylthiobutyrate. Journal of Fermentation and Bioengineering 69(6):287-291.

Pattyn J, Vaughan-Hirsch J, Van de Poel B (2020). The regulation of ethylene biosynthesis: a complex multilevel control circuitry. New Phytologist 229(2):770-782.

Pirkov I, Albers E, Norbeck J, Larsson C (2008). Ethylene production by metabolic engineering of the yeast *Saccharomyces cerevisiae*. Metabolic Engineering 10(5):276-280.

Ren T, Patel M, Blok K (2006). Olefins from conventional and heavy feedstock: Energy use in steam cracking and alternative processes. Energy 31(4):425-451.

Sakai M, Ogawa T, Matsuoka M, Fukuda H (1997). Photosynthetic conversion of carbon dioxide to ethylene by the recombinant cyanobacterium, *Synechococcus* sp. PCC 7942, which harbours a gene for the ethylene-forming enzyme of *Pseudomonas syringae*. Journal of Fermentation and Bioengineering 84(5):434-443.

Tao L, Dong HJ, Chen X, Chen SF, Wang TH (2008). Expression of ethylene-forming enzyme (*EFE*) of *Pseudomonas syringae* pv. glycinea in *Trichoderma viride*. Applied Microbiology and Biotechnology 80(4):573-578.

Van de Poel B, Bulens I, Hertog MLATM, Nicolaï BM, Geeraerd AH, Oppermann Y, Sauter M, De Proft MP (2011). ACC-oxidase is the rate-limiting step in ethylene biosynthesis during postharvest storage of tomato. Acta Horticulturae, 945:429-434.

Zhang M, Yu Y (2013) Dehydration of ethanol to ethylene. Industrial & Engineering Chemistry Research 52(28):9505-9514.

Vol. 20(8), pp.340-348, August, 2021
DOI: 10.5897/AJB2021.17376
Article Number: D4F975867575
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Review

Survival rate of donkey foals: Status quo and improvement methods

Yonghui Wang, Tianqi Wang, Zhengwei Zhang and Changfa Wang*

Liaocheng Research Institute of Donkey High-Efficiency Breeding and Ecological Feeding, Liaocheng University, P. R. China.

Received 25 June 2021; Accepted 18 August 2021

The industrial improvement of donkey-hide gelatin and the development of related products, such as donkey meat, donkey milk, and donkey-hide gelatin cake, drive the upstream donkey breeding industry. However, the shortage of donkey germplasm resources seriously restricts the further development of the donkey industry. Under the biological limitations of long breeding cycle, slow breeding, low conception rate, high abortion rate, and low survival rate, lower numbers of donkey foals have become an important limiting factor in the development of this industry. This paper clarifies the ways by which the survival rate of donkey foals can be improved from five aspects: Selection and breeding of female donkeys; physiological characteristics of donkey foals; key points of nursing; prevention and control of transportation stress; and control of common diseases. These aspects are important in enhancing the economic benefits of donkey farms and breeding farmers.

Key words: Female donkeys, donkey foals, feeding, survival rate, nursing.

INTRODUCTION

Donkey breeding has a long history in China; in the heyday of the donkey industry in the country, about 12 million domestic donkeys were in stock. However, the role of donkeys in agriculture has gradually decreased because of mechanization. By 2020, the population of donkeys in the country has drastically decreased to about 2.32 million. Moreover, the shortage of germplasm resources and degradation of donkey quality hinder the development of this industry in China (China Statistical Report, 2020). The donkey breeding industry in China is gradually transitioning from the family farming model to large-scale and integrated industrial operations, which can ensure uniform product quality and stable donkey population. However, theoretical research on donkey breeding and its technical development lag far behind that of other livestock, thereby limiting its industrial development. Various technologies applied in cattle husbandry, such as artificial insemination using frozen semen, are new in donkey breeding; these technologies are worthy of promotion and application in this field (Dai et al., 2011; Yao et al., 2018).

A previous study that focused on small-scale breeders in eastern Mongolian and western Liaoning revealed that the overall reproductive period of female donkeys is 7.3 years, the average litter production is 2.8, the age of first foaling is 45.3 months, the foaling rate is 80%, and the

*Corresponding author. E-mail: wangcf1967@163.com. Tel: +86-635-8239956.

foaling interval is 500.5 days (Canisso et al., 2019; Fielding, 2005). Similar issues are experienced by large-scale donkey farms in Shandong and other areas (Deng et al., 2020). The high mortality rate of newborn donkey foals not only reduces the efficiency of donkey foal output but also requires breeders to increase the number of reserve females, thereby substantially increasing the costs and seriously affecting the economic efficiency of donkey breeding. The survival rate of foals can be improved by gaining knowledge of selection and breeding of female donkeys, understanding the physiological characteristics and key points of nursing newborn foals, taking precautions during transportation, and ensuring the prevention, treatment, and control of common diseases.

Selection and breeding of female donkeys

A female donkey reaches sexual maturity at 12 to 15 months of age. However, at this age female donkeys are not yet fully developed. Female donkeys should be mated at about the age of 2.5 years or it reaches 95% of adult body height and 70% of adult body weight as mating them too soon negatively affects their skeletal development. At this age, female donkeys are still relatively small. When they become pregnant, their fetus would be too large relative to their size. Thus, the risk of difficult delivery is high, and it will also affect their subsequent pregnancy and foaling (González et al., 2018).

Donkeys in temperate regions of China are seasonal estrus animals, with a gestation period of nearly a year. Spring (late March to early June) is the peak estrus season for donkeys and the season with the highest mating success rate (Henry et al., 1991). Despite the similar climate between spring and autumn, the distribution of donkey foal births is uneven throughout the year, and fertility peaks between March and May, during which 61.7% of the total births occur. By comparison, the percentage of births in September and October is only 1.8%, and very few foals are born in November and December. Donkeys in Shandong reproduce earlier because this region has a longer photoperiod than other areas (Deng et al., 2014). By contrast, female donkeys in northeast China, the mean age at first conception time was 33.3 months and gives birth to only a single donkey. Through selective breeding, female donkeys can maintain a relatively favorable foaling rate and foaling interval until they are 15 years old (Quaresma et al., 2014; Sprayson et al., 2008). The present review seeks to provide useful information published in the literature for the targeted training of smallholder farmers, which in turn will promote the nutritional and reproductive management of donkey populations. However, further research should explore sustainable breeding programs and examine changes in male donkey semen quality during the peak of breeding season (Allen and Wilsher, 2012).

Genetic selection for female donkeys

Multiple pregnancies are considered one of the main reasons of donkey abortion (González et al., 2018). Statistically, 72.6% of twin pregnant donkeys would experience abortion or give birth to a stillborn. Among the cases of abortion, 64.5% occurs from the third month of gestation to the time of parturition. In the remaining cases, twins are born alive. However, one of the foals is usually stunted or emaciated, a condition that prevents them from surviving for more than one month after birth (Jeffcott and Whitwell, 1973). For donkeys, the total mortality rate of newborn foals within the first month of life is close to 9%, whereas the conception rate of normal twins is only about 3%, which has a mortality rate of 40% (Quaresma et al., 2014). Thus, when a pregnancy test in equine animals performed about 16 to 20 days after artificial insemination reveals the conception of twin fetuses, one side of the fertile follicle can be artificially removed and only one can be retained (Pascoe et al., 1987). Therefore, breeding programs should progressively adopt new research methods, such as the use of genetic markers and genotyping. Moreover, breeding programs should combine the latest genetic advances with relatively stable traditional traits, such as embryo testing, to select individuals that are less prone to multiple ovulations and avoid the risk of multiple pregnancies. As new perspectives on donkey breeding and conservation, these measures serve as preventive selection tools for reducing the risks associated with pregnancy (Navas et al., 2019).

Warmblood fragile foal syndrome (WFFS), an autosomal recessive, single-gene defect, is characterized by hyperelasticity of the skin and joints, vascular lesions, and cardiac and ocular defects (Burrows, 1999). In horses, the incidence of difficult births in purebred foals with WFFS is 10% higher than that in normal and healthy foals (Ginther and Williams, 1996). It is caused by fetal malposition, and the incidence of fetal malposition at birth is higher in purebred fetuses with this disease. In most cases, the fetuses of mares with a pure WFFS mutation in the *PLOD1* gene survive the entire gestation period. The foals are usually born alive, but they do not survive long after birth (Monthoux et al., 2015). Abortion in the last months of gestation is not the main manifestation caused by the WFFS genotype. Nevertheless, abnormal fetal development in early gestation due to WFFS cannot be completely excluded as the majority of WFFS pure congeners abort before the end of gestation, indicating that the purity of the WFFS allele is always incompatible with life outside the uterus, that is, the purity of WFFS is the cause of abortion or stillbirth (Aurich et al., 2019). Skin lesions are detected in most, but not all, WFFS-pure positive foals, and the nature and extent of skin

abnormalities widely vary between foals with defects. Moreover, there are WFFS-pure positive foals with undetectable skin lesions. In the absence of skin abnormalities, the WFFS purebred condition is not considered a major factor in stillbirths. Therefore, warm-blooded stallions should be genotyped and marked for WFFS carrier status, and WFFS carrier mares should not be used as stallions in production; moreover, the presence of *PLOD1* gene mutations causing WFFS disorder in donkeys must be investigated further and confirmed (Aurich et al., 2019).

Feeding of female donkeys

Donkey milk is hailed as an elixir of immortality because of its nutritional, cosmetic, and therapeutic properties. Donkey milk has been recently used for infants whose mothers have insufficient or lacking breast milk, and for infants who are allergic to cow's milk proteins because donkey milk is the closest to breast milk in terms of lactose and protein content among all animal milks (Prasad, 2020; Swar, 2011). Donkeys have a higher digestive efficiency and a lower nutrient requirement than horses. The daily nutrient intake of nonpregnant females is 2.56 kg/316±29 kg (316±29 kg is the donkey's body weight), and late pregnant females have 31% lower nutrient intake including total amount of roughage and concentrate (1.76 kg/316±29 kg) compared to the non-pregnant (Burden, 2012), which is probably due to the lower abdominal space available for feeds because of the abdominal cavity occupied by the conceptus, membranes, and fluids. A gradual transition to a higher energy diet should be planned in the last phase of pregnancy, the transition lasted 8 days and consisted of a gradual increase in concentrate (+0.25 kg/day) (Pearson et al., 2006). Donkeys breed in temperate regions, where food is relatively abundant and of good quality. In these regions, obesity is a common disease. Thus, optimal feeding is essential to prevent this condition. During pregnancy, the forage food intake of female donkeys decreases, making them susceptible to hyperlipidemia. Hence, they should gradually shift from a normal diet to a high-energy diet in the later stages of pregnancy. Female donkeys that are pregnant over 11 months and in the prodromal period should be individually fed in separate pens. The original supplemental concentrate feed should be adjusted according to the body fat status of pregnant donkeys. The amount of feed can be appropriately reduced in the case of individuals with high body fat, whereas those with low body fat should be supplemented with an appropriate amount of nutritional concentrate feed (Martin-rosset, 2018; Salari et al., 2020).

Reproduction forecast for donkey foals

Early interventions that anticipate the birth period of a pregnant mare and preparation for foaling can be very effective in improving the physical condition of the mare and foal, especially to improve the survival rate of the foal (Amorim et al., 2019). The gestation period is longer in females that have never given birth than in females that have already produced foals (Ewert et al., 2018; Morel et al., 2002). Starting at 320 days of gestation, mammary secretions can be found on both teats of female donkeys. Mammary secretions (about 5 mL) can be collected every evening (6 pm to 9 pm) and then evaluated via calcium ion titration, calcium and magnesium strip (Ca and Mg) test, pH meter, pH strip (Amorim et al., 2019). The pH value determined by pH strips is not as accurate as that determined by a pH meter, but pH strips can be tested with only a few drops of mammary secretion (the foaling rate within 24 h is 78.5% when pH value of mammary secretion ≤7.0), making it clinically convenient. Calcium titration method is the most sensitive, but it requires large amounts of mammary secretions for accurate testing and has a certain error rate for unproductive mares. Therefore, the most recommended application is the combination of calcium titration (Ousey et al., 1989) and the use of a digital pH meter (Korosue et al., 2013). The ideal diagnostic test should be both sensitive and specific. Moreover, staffing levels, competence, and costs should be considered. This combination must be implemented to be able to obtain precise information on the time of birth and alert the attendant that the pregnant donkey should be closely monitored and prepared for birth (Dascanio, 2014).

PHYSIOLOGICAL CHARACTERISTICS AND BEHAVIOR OF DONKEY FOALS

Physiological characteristics of newborn donkey foals

The Apgar scoring system was designed by Virginia Apgar in 1952 to provide a method for assessing the condition of newborn infants at specific times after birth. This system can be used to better evaluate neonatal physical condition according to various criteria, such as skin color and appearance, pulse rate, reflex irritability, muscle tone, and respiration (Apgar, 1953). The Apgar scoring system has been introduced to veterinary medicine to assess the clinical condition of animals, such as foals, puppies, calves, and piglets, and compare them with human newborn babies to assess the survival of newborns (Knottenbelt et al., 2004; Madigan, 1997).

According to the modified Apgar scoring system, which are critical for the early detection of diseases in newborn foals (the scoring process can be completed within 5 min of foal birth), newborn foals with a score of 7/8 or 8/8 have a normal respiratory rate and body temperature, indicating that they are viable. By comparison, foals with a score of 6/8 have a lower than normal respiratory rate

and body temperature, suggesting that the low respiratory rate may be associated with respiratory abnormalities due to perinatal diseases, such as hypoxic-ischemic encephalopathy, immaturity/dysplasia, and sepsis (Bonelli et al., 2019; Madigan, 1997). Diseased donkey foals may show abnormally low respiratory rate and a substantial decrease in body temperature because of central nervous system damage or depression, and this condition may be associated with a decrease in metabolic demand. The modified Apgar scoring system has been proved a simple and effective method for assessing the viability of newborn donkey foals not only for hand feeding but also under field conditions. Nevertheless, the predictive value of the Apgar score for survival rates in the short term warrants further study (Giguère et al., 2008).

The normal heart rate, respiratory rate, and rectal body temperature of a newborn donkey foal ranges from 45 to 60 heartbeats/min, 11 to 20 min^{-1}, and 37.5 to 38.6°C, respectively. Both ears of a healthy foal turn naturally and flexibly, its eyes are energetic, its body moves are coordinated, and its back hair is fluffy and not dry. The normal first standing time after birth is 62.3±25.7 min, the first suckling time is 87.1±29.15 min, and the meconium is excreted on the day of birth.

Donkey foal behavior

The birth process and early developmental stages of all domestic mammals after parturition play a vital role in the adaptation of newborn donkey foals to life outside the womb. Like other hoofed animals, donkeys are precocious mammals because their motor and sensory systems rapidly develop after birth and the mother should provide active care for the precocious newborn foal to induce intense mutual stimulation. The mother's response is stimulated by complex movements, smells, and grunts provided by the foal, and the mother will use visual, tactile, and acoustic stimuli to induce and guide the foal's activity (Crowell-Davis, 1986). The mothers can spontaneously give birth without assistance, mostly between 10:00 p.m. and 4:00 a.m., and fetal and placental expulsion times are consistent with normal births. Foals are mostly mature, healthy, and vigorous. Furthermore, their Apgar score, weight, standing time, and first suckling time are all within the normal range. The most representative behaviors of female donkeys are "observation" and "sniffing", which account for 70% of all behaviors, and these are associated with "autogrooming" during the day and "excretion" at night (Carluccio et al., 2008, 2015; Panzani et al., 2012).

In precocious animals, the neonatal period is considered a stage of intense interaction between the mother and the infant, interactions between the mother and the foal are slightly greater at night than during the day, a finding that may reflect a behavior that has been preserved from a pre-evolutionary state (Barber and Crowell-Davis, 1994; French, 1998). In the wild, nighttime is very dangerous for newborn donkey foals, and interactions with their mothers help ensure their safety and keep them calm. After birth, maternal licking increases the neuro excitability of the foal and promotes rapid motor development. Jennies provides nutrition, thermoregulation, passive immune transfer, and protection, as well as appropriate stimulation, education, and socialization for the foal. In the special social structure of donkeys, the binary relationship represents the sole and fundamental social institution. The description of neonatal donkey dichotomous behavior provides useful information for behavioral studies of early "follower" species and provides first-hand information for future behavioral descriptions of donkeys (Mazzatenta et al., 2019).

Nursing of newborn donkey foal

In winter and early spring, special attention should be given to the warmth of newborn donkey foals. Straws should be used to ensure that the foals are well rested as their thermoregulatory center is not yet fully developed. The immunity of their body decreases in the cold environment, leading to diseases caused by pathogenic invasion. When a newborn donkey foal is separated from its mother, the umbilical cord must be disinfected well to prevent infection. The correct method for severing the umbilical cord (mostly via freehand umbilical cord severing) must be adopted. Freehand umbilical cord severing quickly dries up and is not easily infected. A 5% tincture of iodine should be used to disinfect the severed end of the umbilical cord to effectively avoid many associated diseases, such as umbilitis. The midwife should observe the condition of the donkey foal after birth. The concentration of serum immunoglobulin in newborn foals is substantially lower than at other times, and the midwife should allow the newborn foal to receive colostrum as soon as possible. If the foal does not excrete feces in time, then a veterinarian should be called in to assist the foal, either by rectal instillation of soap and water to lubricate the intestines or by directly injecting a clyster into the end of the rectum after birth (Wang et al., 2020; Zhang, 2020).

NEWBORN DONKEY FOALS

Importance of colostrum for donkey foals

Donkey colostrum is the first donkey milk produced by the mammary glands of the mother donkey. It has specific components that are essential for the foal. Compared with cow's milk, donkey colostrum has a higher concentration of immunoglobulin type G (IgG) and

antimicrobial and immunomodulatory factors, such as lactoferrin, lactoperoxidase, lysozyme, oligosaccharides, and fat (Li et al., 2019a, b). The majority of passive transfer of immunity (FPT) failure in donkey foals are due to feeding of foals with low quality colostrum, delayed lactation, or lack of IgG absorption. Colostrum can be rated as of excellent quality if the IgG concentration is higher than 80 mg/mL, good if the IgG concentration is between 50 and 80 mg/mL, fair if the IgG concentration is between 28 and 50 mg/mL, and poor when the IgG concentration is below 28 mg/mL (McCue, 2014). Healthy foals usually suckle colostrum from their dams within 2 to 3 h of birth. For foals that do not successfully feed on colostrum within 2 h of birth, feeders should squeeze out the colostrum and feed it to them. During the first 12 to 24 h of life, the small intestines of donkey foals are still permeable to macromolecules, including immunoglobulins, and approximately 50% of the ingested immunoglobulins is absorbed during the first 12 h of life. By comparison, between 12 and 18 h of life, only 28% of the ingested immunoglobulins is absorbed and enters the bloodstream. After 18 h of life, the absorption efficiency continues to decrease until 36 h after birth, at which point the intestines are no longer permeable (McKenzie, 2018). The degree of passive transfer of immunity to donkey foals can be adequately evaluated by quantifying serum IgG concentrations. The circulating transfer of immune IgG from colostrum to the donkey foal is considered adequate when the serum IgG concentration is greater than 8 mg/mL. A partial failure of colostrum immune transfer is considered when the IgG concentration is between 4 and 8 mg/mL. Finally, a complete failure of colostrum immune transfer is considered when the IgG concentration is less than 4 mg/mL (Hines, 2003). Colostrum has specific antimicrobial and anti-inflammatory properties that protect donkey foals from diseases and also help to maintain a good growth rate in the first months of life. Healthy foals are more likely to gain weight than those suffering from defects in immune passive transfer. Thus, colostrum quality and the degree of immune passive transfer must be evaluated (Turini et al., 2020).

Feeding of newborn donkey foals

Supplemental feeding is required one month after birth and must be done with appropriate concentrated feed and adjusted according to the foal's appetite, the amount of its mother's milk and its health. If the foal slowly eats, then it must be individually fed. For foals with a small body size, the number of times it is fed should be increased to make it grow healthy. In addition, attention should be paid to the amount of water the foal drinks, and a water trough can be placed in the feeding pen to prevent water shortage. For 1 to 2-month-old foals, careful management is required. If the number of foals exceeds 50, then they must be separated from the females and kept in separate pens with dedicated care to prevent other females from trampling and chewing on the foals (Salari et al., 2020).

A milkless foal is a donkey foal whose mother died after giving birth or was unable to nurse after giving birth due to insufficient milk. The best way to feed milkless foals is to find a substitute mother donkey or by giving a milk substitute, which is usually made from cow and goat milk. Donkey milk has a lower fat content than cow and goat milk. When supplementing a milkless foal, the upper fat layer should be removed and diluted with water at a ratio of 1:1. A little amount of sugar should be added to make it a nutritional product close to donkey milk. Avoid feeding pure milk, pure goat's milk, or unblended cow's and goat's milk as they can cause indigestion and dehydration in newborn donkey foals, even death in serious cases (Palo et al., 2018). As the foal grows up, it needs more nutrients. Mares lose a lot of nutrients due to lactation, which will have a certain impact on the health of both the mares and their foals. As the foal grows faster in the first year after weaning, the feed should contain 1/3 of concentrate feed, which should be gradually increased with age. The amount of concentrate feed added should reach the average level given to adult donkeys when the foals reach sexual maturity at the age of 1.5 to 2 years (Martin-Rosset, 2018). The amount of concentrate feed to give to male donkey foals must be increased by 15 to 20%, and the concentrate feed should contain 30% protein feed (Wang et al., 2020).

PREVENTION AND CONTROL OF STRESS IN DONKEY FOALS DURING TRANSPORTATION

Different regions in China have different levels of economic development, environmental conditions, and breeding habits. In the south of the Changjiang River, the temperatures and humidity are high in the summer. Thus, the stock of donkeys in this area is very small, but the consumption of donkey meat is large. By comparison, there are more donkeys in stock in the north and west of this area as forage is abundant. Hence, donkeys from the north are transported to the south and from the west to the east. Long-distance transportation of donkey foals for over 10 h increased mortality and morbidity by 1 to 3% and 10 to 15%, respectively (Zhao et al., 2020). Thus, the stimulation of pregnant females and foals must be reduced during long-distance transportation to reduce abortion rate of pregnant females and improve their survival rate of foals (Liu et al., 2016).

A study that examined postpartum mares and 5-day-old foals in a trailer during a 20 km road transport experiment indicated that cortisol concentrations in both mares and foals increased after transport, with a more pronounced and sustained increase in foals than in mares. Moreover, the heart rate of both mares and foals increased during transport, with a less pronounced increase in foals than in

mares. Heart rate variability (HRV) in mares was found to increase during transport. HRV increased in mares but remained unchanged in foals. In addition, both the deceleration and the acceleration of the vehicle during transport were found to cause transient changes in the heart rate of donkeys. The recommended loading density for equine loaded in groups is $y = (54.837) \times W^{0.325}$, where y = density in kg/m^2 and W = average animal weight in kilograms (Whiting, 1999). Overcrowding (According to Scientific Opinion Concerning the Welfare of Animals during Transport, space allowance for equine should be given in terms of kg/m^2 instead of m^2/animal) during transport can also negatively affect donkeys. Various factors during transport, such as violent driving, excessive transport time, provision of food and water, presence or lack of anti-skid equipment for the vehicle and the loading and unloading platform, fit of the platform to the edge of the vehicle, enclosure of the loading and unloading platform, and many other factors, can affect the abortion rate of female donkeys and the mortality rate of donkey foals (Melchert et al., 2020).

Transport stress syndrome refers to a syndrome in which the immunity of the organism is reduced by long-distance trafficking and various stressors. Biological factors, especially pathogens, take advantage of this situation to cause respiratory and digestive system diseases and even systemic pathological reactions. Given that donkeys have a strong tolerance, the disease is already at an advanced stage when symptoms are detected. Weight loss, morbidity, and mortality due to long-distance transportation can be reduced by adding a “nutrition soup” (mainly includes oral rehydration salt, electrolytic multivitamin, vitamin C soluble powder, glucose powder, and other ingredients) to the water and let donkeys drink it before and after transportation. This step was experimentally proved to improve the biochemical index, weight, morbidity, and mortality of donkeys (Liang et al., 2020).

The transport vehicle should be meticulously disinfected before and after transportation. The donkeys being transported in the same vehicle should come from the same farm to prevent spread of diseases. During transportation, the route should be flat as much as possible. Sharp braking or sudden acceleration must be avoided during driving. The front part of the donkey’s body has a stronger weight-bearing capacity than the back part. The head of the donkey at standing direction should be oriented backward to maintain good body balance. Temperature also has a great impact on donkey foals during transport. Large differences in temperature result in greater weight loss in donkeys. Thus, donkeys should be transported at the right temperature (In the summer, transportation should take place during the cooler hours of the day or at night; while in the winter, transportation should occur during the warmer hours of the day such as midday) as much as possible to reduce morbidity (Schmidt et al., 2010; Tadich et al., 2015).

PREVENTION AND CONTROL OF COMMON DISEASES FOR DONKEY FOALS

Vaccination and deworming in donkey foals

Vaccinations are a safe and reliable method of stimulating the immune system, the system that will help your donkey fight infection and disease. They may not always prevent diseases, but they can help to significantly reduce the symptoms. We recommend that donkeys are vaccinated against Equine Influenza (Flu) and Tetanus (Cullinane and Newton, 2013; Mumford et al., 1994). However, due to the high price of equine influenza and tetanus vaccines, few donkey farms in China receive equine influenza vaccination at present. A primary course of injections must be given. These are then followed by booster vaccinations that keep the vaccinations topped up. The intervals between the injections of the primary course and boosters vary according to the type of vaccine and the disease that they protect against according to the vaccine used and the risk of disease initial course of vaccination and frequency of boosters are different (Archer et al., 2021).

Worms live in donkey’s body, draining their nutrients and sometimes causing damage to the various organs that they can be found in. Donkeys that are heavily affected by worms can fail to gain or maintain weight and may be in poor condition. However, even without these signs it is important to follow a worm control program (Matthews and Burden, 2013). Donkeys are affected by strongyles (Roundworm) and tapeworm, just like horses, but there are two other significant parasites: Lungworm and Fluke. A ‘zero tolerance’ approach should be taken to Lungworm as once established eradication can be a lengthy process (Dixon et al., 1995); donkeys grazing wet, marshy paddocks are at risk of liver fluke (Villa-Mancera and Reynoso-Palomar, 2020), and may be at particular risk if grazed with other infected livestock (e.g. sheep or cattle). As a useful method to prevent and control parasites, faecal worm egg count test (FWEC) records the number and type of worm eggs seen in a sample of your donkey’s dung. The count indicates whether your donkey is shedding a high (over 200 eggs per gram (EPG)) number of eggs in its dung (Lester and Matthews, 2014).

Enterococcus in donkey foals

Enterococcus species are a group of Gram-positive diplococcus bacteria that are emerging pathogens for sepsis in humans and animals. Over the past three decades, the prevalence of *Enterococcus* as an emerging pathogen causing sepsis in donkey foals has increased, with *E. faecalis* and *E. faecium* being the most common enterococci (Adams et al., 2016; Theelen et al., 2014). A comparative study of donkey foals with sepsis caused by

other bacterial diseases revealed that foals are more likely to be infected with *Enterococcus* through the digestive tract and umbilicus and urinary tract, similar to human infections. Enterococcal isolates are multidrug resistant and foals have a low survival rate after infection. The mortality rate for donkey foals that tested positive for enterococci is 52.1%, well outside the range of medically reported mortality rates for humans (23-50%) but slightly lower compared with that for human newborns, which have a 61% mortality rate for enterococci positivity (Billington et al., 2014; Lopes et al., 2005). Veterinarians should be aware of the relationship between enterococcal infections and umbilical infections as early as possible. Moreover, they should recognize the potential for multidrug resistance among these isolates, and they should help donkey foals choose antimicrobial drugs or specific phage preparation at an early stage. This measure emphasizes the necessity and importance of targeted use of antimicrobial drugs. The findings of previous studies supported the use of ampicillin as the initial clinical antimicrobial choice for enterococci, but chloramphenicol is a better choice when culturing enterococci in the laboratory (Willis et al., 2019).

Pneumonia in donkey foals

Respiratory disease is a major cause of mortality in donkey foals. *Rhodococcus hoagii* and *Prescotella equi* are Gram-positive parthenogenic intracellular microorganisms that cause pneumonia in foals between 1 and 6 months of age (Giguère, 2017; Giguère et al., 2011; Huber et al., 2018). Distinguishing pneumonia caused by *E. equi* from other pathogens is difficult because their external manifestations are very similar (Giguère et al., 2011). Diagnostic accuracy in the early stages of infection is critical because donkey foals tend to respond poorly to the antibiotics commonly used to treat other types of bacterial pneumonia; moreover, the use of appropriate antimicrobial agents can substantially improve the success of treating equine erythrococcal infections (Attili et al., 2006). Bacteriological culture or amplification of the Vap A gene from tracheobronchial aspirates (TBA) by PCR is a reliable method for establishing the diagnosis of equine erythrococcal pneumonia. However, TBA was abandoned by veterinarians because of the invasive nature of the technique, the disapproval of farm owners, and the costs and risks associated with the procedure. Moreover, bacterial cultures take up to 72 h to yield accurate identification results (Cohen, 2014). Bronchoalveolar lavage (BALF) is commonly used as a diagnostic test for equine respiratory diseases to diagnose noninfectious inflammatory diseases in mature horses, such as equine asthma and exercise pulmonary hemorrhage, as well as to treat pneumonia in donkey foals. Compared with TBA, the BALF method is simpler, can be performed without endoscopic guidance, less invasive, and more acceptable to farm owner. Furthermore, the analysis of BALF cellular components is instantaneous and does not require a specific laboratory. By examining the cells in the bronchoalveolar lavage fluid of donkey foals with pneumonia caused by other bacteria, possible differences can be identified and detected to help veterinarians in clinical settings to achieve an early diagnosis (Cowell and Tyler, 2002; Hostetter et al., 2017; Vitale et al., 2019).

Donkey foals born in winter and spring have an extremely high risk of pneumonia. Owing to the cold weather and the lack of vitamins in the mare's feed, the foals are negatively affected, resulting in poor resistance and a weaker constitution. The incidence is also substantially higher during sudden changes in weather. A sick donkey foal will show various symptoms, such as mental depression, runny nose, shortness of breath, and elevated body temperature. Therefore, female donkeys should be fed more vitamins and mineral-rich feed during pregnancy. Moreover, the donkeys must be kept warm in winter and spring by keeping the barn warm and dry. The staff of the farm should carefully observe the donkey foals for any abnormalities. For sick foals an immediate diagnosis should be made and treatment implemented early. Donkey foals during the treatment period should be well taken into care to ensure sufficient nutritional intake for recovery.

CONCLUSION

The survival rate of donkey foals has been gradually increasing because of the progress made in research and epidemic prevention measures. This literature review elaborated on the measures to improve the survival rate of donkey foals from five aspects: selection and breeding of female donkeys, physiological characteristics of donkey foals, key points of nursing donkey foals, prevention and control of stress of donkey foals during transportation, and prevention and control of common diseases of donkey foals. These aspects may help the donkey breeding industry in China to gradually transition from a one-family scattered breeding model to a large-scale and intensive operation. The development of new technologies and the adoption of scientifically effective measures to improve the survival rate of donkey foals can reduce breeding costs and improve the economic benefits of farm households.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank Mr. Yujia Wang of Northeastern

University (Boston) for providing guidance in the literature review and offering critical comments prior to submitting this manuscript for peer review. This research was funded by the National Natural Science Foundation of China (grant no. 31671287), Well-bred Program of Shandong Province (grant no. 2017LZGC020), Taishan Leading Industry Talents-Agricultural Science of Shandong Province (grant no. LJNY201713), Shandong Province Modern Agricultural Technology System Donkey Industrial Innovation Team (grant no. SDAIT-27), and Research on Donkey Pregnancy Improvement (grant no. K20LC0901).

REFERENCES

Adams D, Eberly MD, Goudie A, Nylund CM (2016). Rising Vancomycin-Resistant Enterococcus Infections in Hospitalized Children in the United States. Hospital Pediatrics 6(7):404-411.

Allen W, Wilsher S (2012). The influence of mare numbers, ejaculation frequency and month on the fertility of Thoroughbred stallions. Equine Veterinary Journal 44(5):535-541.

Amorim MDd, Montanholi Y, Morrison M, Rodriguez ML, Card C (2019). Comparison of Foaling Prediction Technologies in Periparturient Standardbred Mares. Journal of Equine Veterinary Science 77:86-92.

Apgar V (1953). A proposal for a new method of evaluation of the newborn infant. Current researches in Anesthesia and Analgesia. 32(4):260-267.

Archer D, Sullivan RJE, Rickards K (2021). Emergency Management for Donkeys and Mules. The Veterinary clinics of North America. Equine Practice 37(2):495-513.

Attili AR, Kennerman E, Takai S, Or ME, Marenzoni ML, Torun S, Pieramati C, Kayar A, Golcu E, Parkan Ç, Yilmaz Z, Gonul R, Valente C, Cuteri V (2006). Seroepidemiological survey of Rhodoccocus equi infection in asymptomatic horses from Bursa, Izmir and Istanbul provinces, Turkey. Comparative Immunology, Microbiology and Infectious Diseases 29(5):323-333.

Aurich C, Müller-Herbst S, Reineking W, Mueller E, Wohlsein P, Gunreben B, Aurich J (2019). Characterization of abortion, stillbirth and non-viable foals homozygous for the Warmblood Fragile Foal Syndrome. Animal Reproduction Science 211:106-202.

Billington E, Phang S, Gregson D, Pitout J, Ross T, Church D, Laupland K, Parkins M (2014). Incidence, risk factors, and outcomes for Enterococcus spp. blood stream infections: a population-based study. International Society for Infectious Diseases 26:76-82.

Bonelli F, Nocera I, Conte G, Panzani D, Sgorbini M (2019). Relation between Apgar scoring and physical parameters in 44 newborn Amiata donkey foals at birth. Theriogenology 142(15):310-314.

Burden F (2012). Practical feeding and condition scoring for donkeys and mules. Equine Veterinary Education 24(11):589-596.

Burrows (1999). The molecular genetics of the Ehler“ Danlos syndrome. Clinical and Experimental Dermatology 24(2):99-106.

Canisso IF, Panzani D, Miró J, Ellerbrock R (2019). Key Aspects of Donkey and Mule Reproduction. The Veterinary clinics of North America. Equine Practice 35(3):607-642.

Carluccio A, Amicis ID, Panzani S, Tosi U, Faustini M, Veronesi MC (2008). Electrolytes changes in mammary secretions before foaling in jennies. Reproduction in Domestic Animals 43(2):162-165.

Carluccio A, Gloria A, Veronesi M, Amicis ID, Noto F, Contri A (2015). Factors affecting pregnancy length and phases of parturition in Martina Franca jennies. Theriogenology 84(4):650-655.

China Statistical Report (2020). China's donkey inventory, slaughter and import and export analysis in 2020 (In Chinese).

Cohen ND (2014). Rhodococcus equi Foal Pneumonia. Veterinary Clinics of North America: Equine Practice 30(3):609-622.

Cowell R, Tyler R (2002). Diagnostic cytology and hematology of the horse. 2nd Edition.

Crowell-Davis S (1986). Spatial relations between mares and foals of the Welsh pony (*Equus caballus*). Animal Behaviour 34:1007-1015.

Cullinane A, Newton JR (2013). Equine influenza--a global perspective Veterinary Microbiology 167(1-2):205-214.

Dai GY, Wang JS, Man DL, Yang PQ (2011). Survey Report on Reproductive Survival Situation of Cows in Tongliao city (In Chinese). China Cattle Science 5:73-77.

Dascanio J (2014). Foaling Alert: Vulvar Device. Equine Reproductive Procedures, pp. 259-260.

Deng L, Duan H, Zhang X, Zeng S, Wu C, Han G (2014). Advances in the Research and Application of Artificial Insemination to Equids in China: 1935–2012. Journal of Equine Veterinary Science 34(3):351-359.

Deng L, Shi S, Li J, Tang C, Liao Q, Xie P (2020). A Cross-Sectional Survey of Foaling-Related Parameters of Jennies (Equus asinus) Under Smallholder Farm Conditions in Northeast China. Journal of Equine Veterinary Science 87:102928.

Dixon PM, Railton D, McGorum B (1995). Equine pulmonary disease: a case control study of 300 referred cases. Part 2: Details of animals and of historical and clinical findings. Equine Veterinary Journal 27(6):422-427.

Ewert M, Lueders I, Böröcz J, Uphaus H, Distl O, Sieme H (2018). Determinants of gestation length in Thoroughbred mares on German stud farms. Animal Reproduction Science 191:22-33.

Fielding D (2005). Reproductive characteristics of the jenny donkey--Equus asinus: A review. Tropical Animal Health and Production. 20:161-166.

French JM (1998). Mother' offspring relationships in donkeys. Applied Animal Behaviour Science 60(2–3):253-258.

Giguère S (2017). Treatment of Infections Caused by Rhodococcus equi. The Veterinary clinics of North America. Equine Practice 33(1):67-85.

Giguère S, Cohen N, Chaffin M, Slovis N, Hondalus M, Hines S, Prescott JF (2011). Diagnosis, treatment, control, and prevention of infections caused by Rhodococcus equi in foals. Journal of Veterinary Internal Medicine 25(6):1209-1220.

Giguère S, Slade J, Sanchez LC (2008). Retrospective comparison of caffeine and doxapram for the treatment of hypercapnia in foals with hypoxic-ischemic encephalopathy. Journal of Veterinary Internal Medicine 22(2):401-405.

Ginther OJ, Williams D (1996). On-the-farm incidence and nature of equine dystocias. Journal of Equine Veterinary Science 16(4):159-164.

González F, Vidal J, McLean A, Jurado JML, Capote C, Arbulu AA, Bermejo J (2018). Modelling for the Inheritance of Endangered Equid Multiple Births and Fertility: Determining Risk Factors and Genetic Parameters in Donkeys (*Equus asinus*). Research in Veterinary Science 126:213-226.

Henry M, Mcdonnell S, Lodi L, Gastal EL (1991). Pasture mating behaviour of donkeys (*Equus asinus*) at natural and induced oestrus. Journal of Reproduction and Fertility Supplement 44:77-86.

Hines MT (2003). Chapter 12.13 - Immunodeficiencies of foals. Current Therapy in Equine Medicine (Fifth Edition), pp. 692-697.

Hostetter SJ, Clark SK, Gilbertie JM, Wiechert SA, Jones DE, Sponseller BA (2017). Age-related variation in the cellular composition of equine bronchoalveolar lavage fluid. Veterinary Clinical Pathology 46(2):344-353.

Huber L, Giguère S, Slovis N, Carter C, Barr B, Cohen N, Elam J, Erol E, Locke SJ, Phillips E, Smith JL (2018). Emergence of Resistance to Macrolides and Rifampin in Clinical Isolates of Rhodococcus equi from Foals in Central Kentucky, 1995 to 2017. Antimicrobial Agents and Chemotherapy 63(1):e01714-18.

Jeffcott L, Whitwell K (1973). Twinning as a cause of foetal and neonatal loss in the thoroughbred mare. Journal of Comparative Pathology 83(1):91-106.

Knottenbelt D, Holdstock N, Madigan J (2004). Equine neonatology: medicine and surgery (First Edition). Elsevier Health Sciences.

Korosue K, Murase H, Sato F, Ishimaru M, Kotoyori Y, Tsujimura K, & Nambo Y (2013). Comparison of pH and refractometry index with calcium concentrations in preparturient mammary gland secretions of mares. Journal of the American Veterinary Medical Association 242(2):242-8.

Lester H, Matthews J (2014). Faecal worm egg count analysis for targeting anthelmintic treatment in horses: points to consider. Equine

Veterinary Journal 46(2):139-145.
Li M, Kang S, Zheng Y, Shao JH, Zhao H, An Y, Cao G, Li Q, Yue X, Yang M (2019a). Comparative metabolomics analysis of donkey colostrum and mature milk using ultra-high-performance liquid tandem chromatography quadrupole time-of-flight mass spectrometry. Journal of Dairy Science 103(1):992-1001.
Li M, Li W, Wu J, Zheng Y, Shao JH, Li Q, Kang S, Zhang Z, Yue X, Yang M (2019b). Quantitative lipidomics reveals alterations in donkey milk lipids according to lactation. Food Chemistry. 310:125866.
Liang W, Gao W, Jiang G, Li J, Liu S, Chen W, Wang W, Wang C, Deng L, Zhao F (2020). Study on the effect of adding “nutrition soup” to drinking water on relieving transport stress in donkey (In Chinese). Heilongjiang Animal Science and Veterinary Medicine 18:114-118.
Liu G, Yuan B, Qu H, Ji C, Wang Z (2016). Analysis Report on Donkey Industry in Liaocheng City under the Mode of "Northern Breeding and Southern Feeding (In Chinese). Heilongjiang Animal Science and Veterinary Medicine 05:80-81.
Lopes MDFS, Ribeiro T, Abrantes M, Marques JJF, Tenreiro R, Crespo MT (2005). Antimicrobial resistance profiles of dairy and clinical isolates and type strains of enterococci. International Journal of Food Microbiology 103(2):191-198.
Madigan J (1997). Manual of Equine Neonatal Medicine (3rd Edition).
Martin-rosset W (2018). Donkey Nutrition and Feeding: Nutrient Requirements and Recommended Allowance“ A Review and Prospect. Journal of Equine Veterinary Science 65:75-85.
Matthews J, Burden F (2013). Common helminth infections of donkeys and their control in temperate regions. Equine Veterinary Education 25:461-467.
Mazzatenta A, Veronesi MC, Vignola G, Ponzio P, Carluccio A, Amicis ID (2019). Behavior of Martina Franca donkey breed jenny-and-foal dyad in the neonatal period. Journal of Veterinary Behavior 33:81-89.
McCue PM (2014). Colostrum Banking. In: Equine Reproductive Procedures, pp. 299-301.
McKenzie HC (2018). Chapter 20 - Disorders of Foals. Equine Internal Medicine (4th Edition), pp. 1365-1459.
Melchert M, Nagel C, Aurich C, Aurich J (2020). Transport-related stress in five-day-old foals and their dams. Journal of Veterinary Behavior Clinical Applications and Research 39:86-89.
Monthoux C, Brot SD, Jackson M, Bleul U, Walter J (2015). Skin malformations in a neonatal foal tested homozygous positive for Warmblood Fragile Foal Syndrome. BMC Veterinary Research 31:11-12.
Morel MCDD, Newcombe J, Holland SJ (2002). Factors affecting gestation length in the Thoroughbred mare. Animal Reproduction Science 74(3-4):175-185.
Mumford J, Jessett D, Rollinson E, Hannant D, Draper M (1994). Duration of protective efficacy of equine influenza immunostimulating complex/tetanus vaccines. Veterinary Record 134:158-162.
Navas FJ, Jordana J, McLean A, León JM, Barba C, Arando A, Delgado JV (2019). Modelling for the inheritance of multiple births and fertility in endangered equids: Determining risk factors and genetic parameters in donkeys (Equus asinus). Research in Veterinary Science 126:213-226.
Ousey JC, Delclaux M, Rossdale PD (1989). Evaluation of three strip tests for measuring electrolytes in mares' pre-partum mammary secretions and for predicting parturition. Equine Veterinary Journal 21(3):196-200.
Palo PD, Maggiolino A, Albenzio M, Casalino E, Neglia G, Centoducati G, Tateo A (2018). Survey of biochemical and oxidative profile in donkey foals suckled with one natural and one semi-artificial technique. PLoS ONE 13(6):e0198774.
Panzani S, Carluccio A, Probo M, Faustini M, Kindahl H, Veronesi MC (2012). Comparative study on 15-ketodihydro-PGF(2α) plasma concentrations in newborn horses, donkeys and calves. Reproduction in Domestic Animals 47(1):82-86.
Pascoe DR, Pascoe RR, Hughes JP, Stabenfeldt G, Kindahl H (1987). Management of twin conceptuses by manual embryonic reduction: comparison of two techniques and three hormone treatments. American Journal of Veterinary Research 48(11):1594-1599.
Pearson R, Archibald R, Muirhead R (2006). A comparison of the effect of forage type and level of feeding on the digestibility and gastrointestinal mean retention time of dry forages given to cattle, sheep, ponies and donkeys. British Journal of Nutrition 95:88-98.
Prasad B (2020). Nutritional and Health Benefits of Donkey Milk. Food Science and Nutrition 6:022-025.
Quaresma M, Martins AF, Rodrigues JB, Colaço J, Payan-Carreira R (2014). Viability analyses of an endangered donkey breed: the case of the Asinina de Miranda (*Equus asinus*). Animal Production Science 55:1184-1191.
Salari F, Licitra R, Altomonte I, Martini M (2020). Donkey Feeding During Maintenance, Pregnancy, and Lactation: Effects on Body Weight, Milk Production, and Foal Growth. Journal of Equine Veterinary Science 91:103131.
Schmidt A, Biau S, Moestl E, Becker-Birck M, Morillon B, Aurich J, Faure J, Aurich C (2010). Changes in cortisol release and heart rate variability in sport horses during long-distance road transport. Domestic Animal Endocrinology. 38(3):179-189.
Sprayson T, Duncan J, Hadrill D (2008). Chapter 13-The care of the geriatric donkey. The professional handbook of the donkey (4th edition), pp. 239-254.
Swar MO (2011). Donkey milk-based formula: A substitute for patients with cow's milk protein allergy. Sudanese Journal of Paediatrics 11(2):21-24.
Tadich T A, Leal F, Gallo C (2015). Preliminary Study on the Effects of Long Distance Road Transport on Some Blood Constituents in Recently Weaned Thoroughbred Foals. Journal of Equine Veterinary Science 35:697-699.
Theelen MJP, Wilson WD, Edman JM, Magdesian KG, Kass PH (2014). Temporal trends in prevalence of bacteria isolated from foals with sepsis: 1979–2010. Equine Veterinary Journal 46(2):169-173.
Turini L, Bonelli F, Nocera I, Battaglia F, Meucci V, Panzani D, Mele M, Sgorbini M (2020). Evaluation of jennies' colostrum: IgG concentrations and absorption in the donkey foals. A Preliminary Study 6(8):e04598
Villa-Mancera A, Reynoso-Palomar A (2020). The prevalence and risk factors of liver fluke infection in Mexican horses, donkeys and mules in tropical and temperate regions. Parasitology Research 119(11):3699-3703.
Vitale V, Sgorbini M, Cuteri V, Preziuso S, Attili A, Bonelli F (2019). Cytological Findings in Bronchoalveolar Lavage Fluid of Foals With Pneumonia Caused by Rhodococcus equi and Other Bacteria. Journal of Equine Veterinary Science 79: 9-12.
Wang CF, Sun, Y, Yang C (2020). Dezhou Donkey (In Chinese). China Agriculture Press.
Whiting T (1999). Maximum loading density of loose horses. Canadian Journal of Animal Science 79:115-118.
Willis AT, Magdesian KG, Byrne B, Edman J (2019). Enterococcus infections in foals. Veterinary Journal 248:42-47.
Yao R, Song Y, Zhang R (2018). The Management System of Beef Cattle Breeding based on the Data Record of Whole Industry Chain. Proceedings of Science Volume 300 - Information Science and Cloud Computing (ISCC 2017) - Session IV: Communication Analysis. https://pos.sissa.it/300/051/
Zhang F (2020). Donkey foal umbilical cord infection etiology and prevention methods. The Chinese Livestock and Poultry Breeding 5:97-97.
Zhao F, Jiang G, Ji C, Zhang Z, Gao W, Feng P, Li H, Li M, Liu H, Liu G, Magalhaes HB, Li J (2020). Effects of long-distance transportation on blood constituents and composition of the nasal microbiota in healthy donkeys. BMC Veterinary Research 16(1):338.

Related Journals: