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Preface

We would like to present, with great pleasure, the inaugural volume-4, Issue-7, July 2018, of a scholarly journal, *International Journal of Environmental & Agriculture Research*. This journal is part of the AD Publications series *in the field of Environmental & Agriculture Research Development*, and is devoted to the gamut of Environmental & Agriculture issues, from theoretical aspects to application-dependent studies and the validation of emerging technologies.

This journal was envisioned and founded to represent the growing needs of Environmental & Agriculture as an emerging and increasingly vital field, now widely recognized as an integral part of scientific and technical investigations. Its mission is to become a voice of the Environmental & Agriculture community, addressing researchers and practitioners in below areas

Environmental Research:

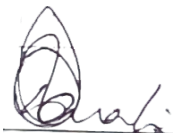
Environmental science and regulation, Ecotoxicology, Environmental health issues, Atmosphere and climate, Terrestric ecosystems, Aquatic ecosystems, Energy and environment, Marine research, Biodiversity, Pharmaceuticals in the environment, Genetically modified organisms, Biotechnology, Risk assessment, Environment society, Agricultural engineering, Animal science, Agronomy, including plant science, theoretical production ecology, horticulture, plant, breeding, plant fertilization, soil science and all field related to Environmental Research.

Agriculture Research:

Agriculture, Biological engineering, including genetic engineering, microbiology, Environmental impacts of agriculture, forestry, Food science, Husbandry, Irrigation and water management, Land use, Waste management and all fields related to Agriculture.

Each article in this issue provides an example of a concrete industrial application or a case study of the presented methodology to amplify the impact of the contribution. We are very thankful to everybody within that community who supported the idea of creating a new Research with *IJOEAR*. We are certain that this issue will be followed by many others, reporting new developments in the Environment and Agriculture Research Science field. This issue would not have been possible without the great support of the Reviewer, Editorial Board members and also with our Advisory Board Members, and we would like to express our sincere thanks to all of them. We would also like to express our gratitude to the editorial staff of AD Publications, who supported us at every stage of the project. It is our hope that this fine collection of articles will be a valuable resource for *IJOEAR* readers and will stimulate further research into the vibrant area of Environmental & Agriculture Research.

Mukesh Arora
(Editor-in Chief)



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Sustainable Natural Resource Utilisation	Management of the Environment
Agricultural Management Practices	Agricultural Technology
Natural Resources	Basic Horticulture
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Potential of Mealybugs Infestation, *Planococcus* spp. (Hemiptera: Pseudococcidae), in an Agroforestry System in Coffee Crops

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Abstract— *The association of tree species to coffee plantations is a common practice in coffee crops, and studies must be performed to establish the effects of these associations. Pests such as the citrus mealybug,* Planococcus citri *(Risso), and the pacificus mealybug,* Planococcus minor *(Maskell) (Hemiptera: Pseudococcidae), can host in several plants and should be studied in relation to this integration. The aim of this study was to evaluate the potential of associated trees to be a source of infestation for coffee crops. The treatments consisted of acrocarpus (*Acrocarpus fraxinifolius*), African mahogany (*Khaya ivorensis*), teak plants (*Tectona grandis*) and macadamia (*Macadamia *sp.), as well as the coffee tree* Coffea Arabica *cv. Mundo Novo. Food preference was studied in laboratory through the test of free choice. Mortality, development and reproduction were also evaluated on each host. Attractiveness of these plants towards the coffee tree was tested by means of an olfactometer, whereby the scale insects were exposed for 15 minutes to the odors of these plants. Both scales settled in all tested plants but the trees did not appear to be suitable hosts. High mortality was found on trees. These scales showed no olfactory preference between the coffee tree and the other tested species and teak leaves had even a repellent effect. It is concluded that acrocarpus, mahogany, macadamia and teak are not potential sources of infestation of mealybugs to the coffee tree, and by consequence they do not represent a threat to the crop.*

Keywords—Planococcus citri, Planococcus minor, Biology, Food Preference, Olfatometry.

I. INTRODUCTION

The use of arboreal species with economic and environmental values can add value to the coffee activity. However, this association requires a detailed knowledge since trees can be a source and refuge of pests and/or the modified environmental conditions, as shading, can affect the incidence of phytophagous arthropods. On the other hand, a diverse agrosystem may have positive impacts where the natural enemies can find refuge, additional food as nectar and pollen, and extra preys, increasing the natural control of pests on coffee (Venzon *et al*., 2014; Tomazella, 2016).

Among these insects, the mealybugs are considered key pests, especially the citrus mealybug, *Planococcus citri* (Risso), and the pacificus mealybug, *Planococcus minor* (Maskell), (Pseudococcidae), which constitute a threat to the coffee plants since they attack flower buds and fruits causing heavy fruit drop (Santa-Cecilia & Souza, 2014). In spite of the diversity of plants that colonize (Williams & Granara de Willink, 1992), these mealybugs may show a certain preference for a host or to have their development and reproduction favored in certain plants due to their nutritional quality.

The insect host selection and recognition process includes several steps, such as habitat and host location, host acceptance and feeding and/or breeding (Le Rü *et al*., 1995b). For this, olfactory, visual, gustatory and tactile stimuli are used, as well as the humidity and intensity of the environment light (Heard, 2000; Powell *et al*., 2006).

Several species of scale insets exploit a limited number of plants, however, they may occasionally occur in other hosts even being not suitable for their development. Mealybugs of the genus *Planococcus*, *Phenacoccus* and other scale insects have sensilla in the antenna with contact and olfactory functions (Salama, 1971; Koteja, 1980; Le Rü *et al*., 1995b; Calatayud & Le Rü, 2006) and it has been hypothesized that they use these structures for the host selection. However, the efficiency to use these sensory organs is limited due to the fact they are apterous (females and males in the first instars) with reduced mobility. Some authors even consider olfactory stimuli of limited value for host location by insects of the order Hemiptera and, such stimuli, would act only at short distances (Backus, 1988). The infestation of new plants would be mainly a passive process, circumscribed to neighboring plants, dispersion by wind, tools used by men or the use of infested plants coming from nurseries. This would result in localized infestations.

In contrast to olfactory stimuli as a mechanism to locate a host, other stimuli may exert some action in the selection (or rejection) of a plant (Le Rü *et al*., 1995b). Olfactory and contact chemoreceptors are present at the apical end of the labium of mealybugs and can be used in the host selection by detecting the stimuli at the leaf surface (Le Rü *et al*., 1995b; Calatayud & Le Rü, 2006). These stimuli may be more important than the olfactory ones present in the antenna. Contact chemoreceptors seem to be used by sucking insects as already verified for aphids. However, it is still under discussion the true role played by volatiles as stimuli for host location in homopteran insects (Powell *et al*., 2006). The host selection by tasting the plant contents through the gustative sensilla present in the cibarium cavity of the alimentary canal is determinant in other sucking insects (Powell *et al*., 2006) and we expect to be similar in mealybugs. Unfortunately to our knowledge there is not studies regarding the presence of gustatory sensilla in the cibarium of mealybugs but we can assume to be similar to other sucking insects.

All these factors may influence the host selection process, however, food preference and plant quality, reflected in the nutritional value, will finally influence the reproduction and the capacity to host the insect.

Thus, the objective of this work was to determine if tree species used in association with coffee plants are potential hosts of two species of mealybugs and by consequence representing a threat to the crop. These studies will ensure a better understanding of the interaction of mealybugs and arboreal species in shaded coffee plantations. The following hypotheses were tested: (a) *P. minor* and *P. citri* mealybugs exhibit dietary preference for coffee plants and have, in this host, better conditions for their development and reproduction; (b) both mealybugs can reproduce in the tested tree species; (c) both mealybugs show olfactory preference for certain plants.

II. MATERIAL AND METHODS

2.1 Mealybugs

Planococcus minor were originally collected in cocoa (*Theobroma cacao*) cv. Comum and *P. citri* in coffee (*Coffea arabica*) cv. Mundo Novo. Both species were reared in laboratory on pumpkins (*Cucurbita maxima* L.) cv. Cabotchá. They were kept in a room, inside wooden cages at 25 ± 2°C and 70 ± 10 RH and in total scotophase.

2.2 Plants

The treatments were constituted by the trees acrocarpus (*Acrocarpus fraxinifolius*), African mahogany (*Khaya ivorensis*), teak (*Tectona grandis*) and macadamia (*Macadamia* sp.). Tree leaves were compared with coffee *C. arabica* cv. Mundo Novo. Tree species were chosen based on the system already implemented in a farm located in Santo Antônio do Amparo, MG, where they are already used for shading coffee plants.

2.3 Food Preference

A free choice test was used to evaluate the preference. Mealybugs were exposed to foliar sections of coffee and a tree in pair comparisons. Leaf sections, with the abaxial side up, were placed on agar (1%) inside Petri dishes of 15 cm diameter. Three foliar sections of each plant were placed alternately and equidistantly, forming a circle. It was used five replicates of each combination and for each species, in a randomized complete block experimental design.

Insects were fasted during one hour before using in the experiment. Fifteen second instars of each species were placed on a circle of filter paper fixed in the center of each plate. These containers were immediately sealed with a plastic film and kept at a room temperature of 25 ± 1 °C and 70 ± 10% RH. The whole set up was covered with black cloth to avoid possible phototropic effect. The evaluations were carried out at 24, 48 and 72 hours counting the number of insects present in each leaf, which was considered as a choice related to food preference. Mealybugs found outside the leaves were not counted.

2.4 Development

A 4-cm diameter leaf section of each tested vegetable was placed inside a 5-cm diameter Petri dish containing a 5 mm layer of agar (1%). Ninety individual first instars of 24 hours-old were collected from the rearing material and placed on the leaf section.

The plates were sealed with plastic film, and dried leaves were replaced when necessary. The plates were placed in room at 25 ± 1 °C and 70 ± 10% RH and total scotophase. The development was followed until emergence of the adults. Mating was assured by isolating a male, already inside the cocoon, and one female in a Petri dish with a plant section inside.

The evaluations were performed daily, recording the duration of the nymphal stage, mortality and the number of viable eggs (according to the hatched nymphs). Ovipositing females were considered as fertile. The experimental design was a completely randomized block design considering one insect as the experimental unit. Initially 90 first instars were used to follow the development but those not found during evaluations were discarded from analysis. Thus, the number of replicates for each treatment is that indicated in the Tables 1 and 2.

2.5 Olfactory response

A four branched olfactory device was used to evaluate the response of both mealybugs to the volatiles emitted by the trees face to those of coffee (Vet *et al*., 1983). The source of odors originated from freshly leaves kept inside a 400 cc glass container. Air flux was calibrated to 1200 mL/min so each branch received 300 mL/min of air. Coffee and tree odors occupied one branch each while purified air occupied the two other branches. They were positioned at random in each branch of the olfactometer. Individual mealybug of third instar was exposed for 15 minutes to the odors of the hosts testing 30 insects for each combination. A choice for an odor was defined when the insect surpassed a mark located at 2 cm from the releasing point toward a branch.

The residence time in each branch was recorded by means of the software JWatcher vs 1.0. After 10 tests, the leaves were replaced and the olfatometer, washed with detergent, water and ethanol 70%. The tests were conducted in an environment without any visual interference.

2.6 Data analysis

Data from the choice test was analyzed by means of the Chi-Square (χ^2) test considering the observed and expected frequencies. Data from nymphal mortality was analyzed by the Chi square test (χ^2). The duration of nymphal stage was only analyzed for *P. citri* by using the Student Test with data transformed to $\sqrt{x}$, because the high mortality impeded to make more than one comparison. For the same reason no statistical analysis was possible to compare the number of viable eggs.

The Chi-square test (α=0.05) was used for pair comparisons of the final choice. Means of the total time in each branch was submitted to Analysis of Variance and were compared by the Tukey test ($p \leq 0.05$), with data transformed in $\arcsin\sqrt{x}/100$. The number of nymphs that did not respond (undecided) and remained in the neutral zone of the olfactometer were recorded but not considered for analysis.

III. RESULTS AND DISCUSSION

3.1 Food Preference

Some food preference was found in the choice test between the offered hosts (Figs. 1-4). *Planococcus citri* showed preference for coffee face to teak and macadamia. Mealybugs abandoned these hosts after 24 and 48 hours, a sufficient time to taste the phloem sap suggesting a repellent effect. Coffee, mahogany and acrocarpus were equally preferred. *Planococcus minor* also avoided teak and macadamia and settled on coffee. Mahogany was equally preferred face to coffee and acrocarpus showed to be very attractive to this mealybug.

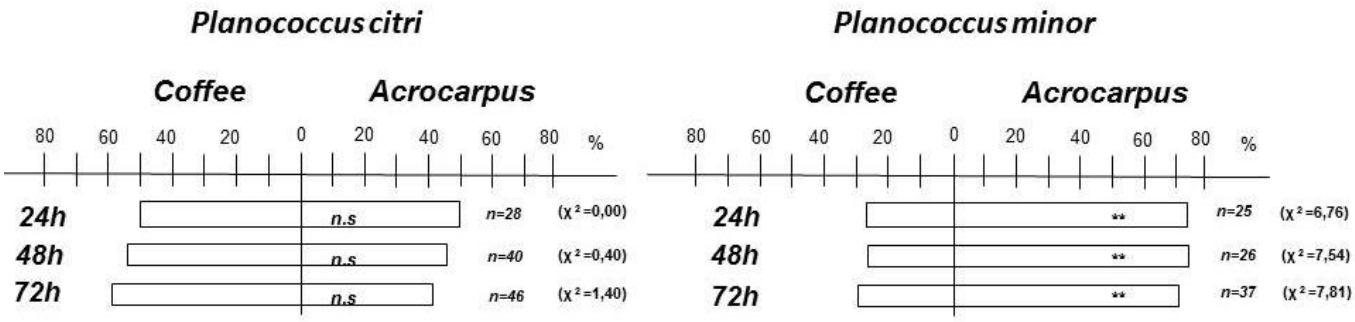

FIG. 1. Free-choice test. Differences according to the χ^2 test with 1 d.f. at 5% (χ^2 value= 3.84) (n = number of insects with choice).

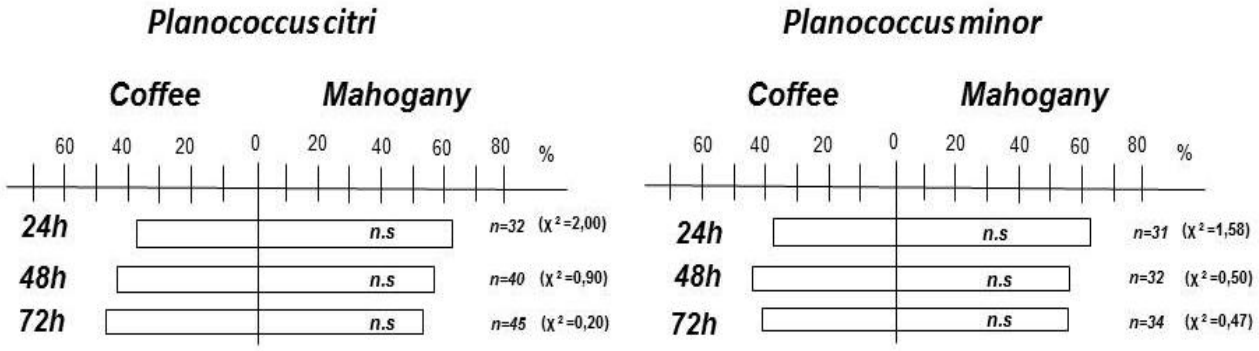

FIG. 2. Free-choice test. Differences according to the χ^2 test with 1 d.f. at 5% (χ^2 value= 3.84) (n = number of insects with choice).

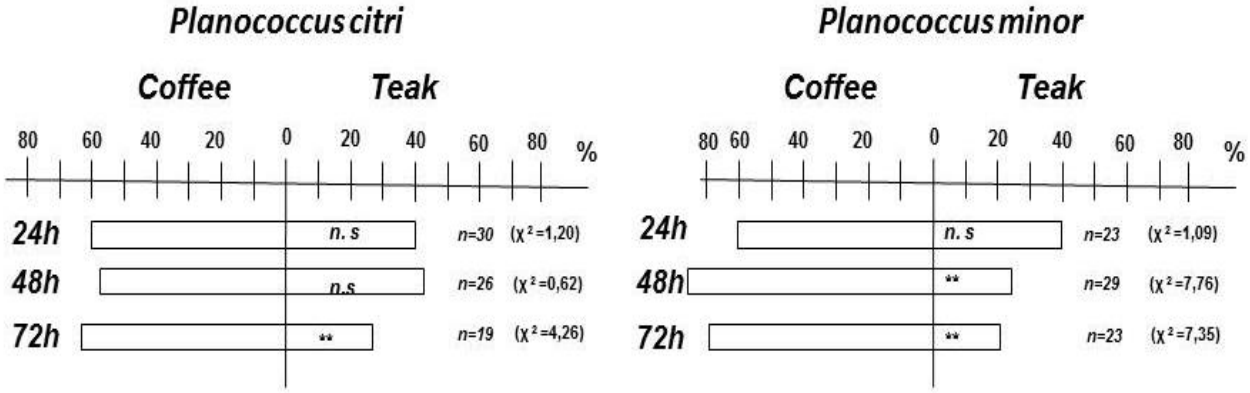

FIG. 3. Free-choice test. Differences according to the χ^2 test with 1 d.f. at 5% (χ^2 value= 3.84) (n = number of insects with choice).

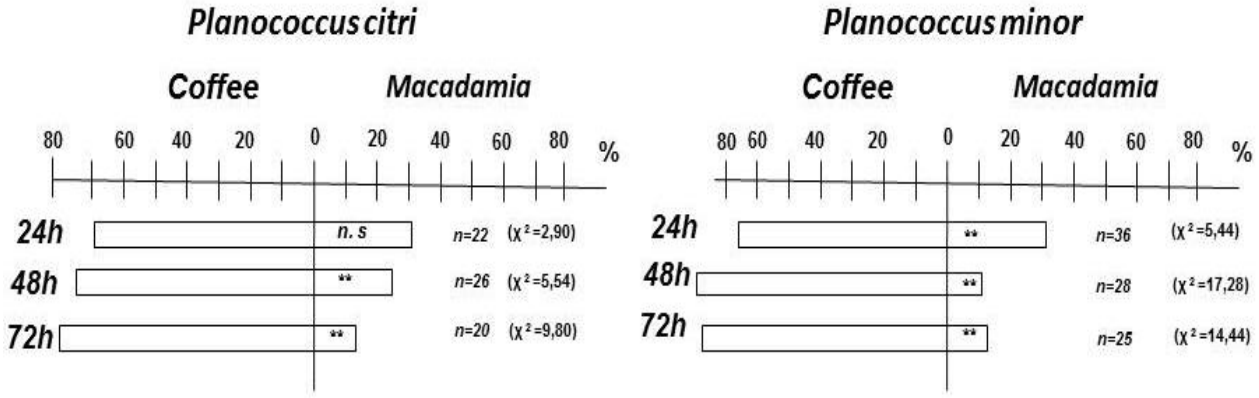

FIG. 4. Free-choice test. Differences according to the χ^2 test with 1 d.f. at 5% (χ^2 value= 3.84) (n = number of insects with choice).

3.2 Development

A high mortality was found in *P. citri* and *P. minor* in all tested trees, excepting in coffee, with values above 75% (Tables 1 and 2). The low number of emerged females impeded to evaluate other biological parameters related to the effect of the substrate (host).

TABLE 1
DEVELOPMENT OF *Planococcus citri* IN DIFFERENT HOSTS (25±1°C, 70±10% RH, total scotophase).

Host	Nymph mortality (%)	Nymph period of females (days)	Number of viable eggs/fertile female
Coffee *Coffea arabica*	50.0 b (n=46)	24.0±1.1 b (n=23)	39.4±10.6(1) (n=8)
Acrocarpus *Acrocarpus fraxinifolius*	75.8 b (n=33)	32.3±0.8 a (n=8)	18.0±0.0(1) (n=1)
Mahogany *Khaya ivorensis*	98.3 a (n=58)	22.0±0.0 (1) (n=1)	(1)
Teak *Tectona grandis*	100.0 a (n=41)	0.0 (1)	(1)
Macadamia *Macadamia* sp.	96.0 a (n=50)	19.0±0.0 (1) (n=2)	(1)
p value	≤ 0.001 (χ^2)	≤ 0.001 (Student) (2)	

(1) Not considered for statistical analysis; (2) Means followed by the same letter in the column are not different according to the Chi square (χ^2) and Student Test. Data transformed to √x; n = number of insects.

TABLE 2
DEVELOPMENT OF *Planococcus minor* IN DIFFERENT HOSTS (25±1°C, 70±10% RH, total scotophase).

Host	Nymph mortality (%)	Nymph period of females (days)	Number of viable eggs/fertile female
Coffee *Coffea arabica*	48.8 b (n=41)	24.0±1.1 (1) (n=21)	28.4 ± 13.6(1) (n=5)
Acrocarpus *Acrocarpus fraxinifolius*	97.2 a (n=36)	35.0±0.0 (1) (n=1)	(1)
Mahogany *Khaya ivorensis*	92.7 a (n=67)	16.4±1.4 (1) (n=5)	(1)
Teak *Tectona grandis*	100.0 a (n=53)	0.0 (1)	(1)
Macadamia *Macadamia* sp.	100.0 a (n=43)	0.0 (1)	(1)
p value	≤ 0.001 (χ^2)		

(1) Not considered for statistical analysis; (2) Means followed by the same letter in the column are not different according to the Chi square (χ^2). Data transformed to √x; n = number of insects.

3.3 Olfactory response

3.3.1 Response to coffee plants and acrocarpus

Coffee, acrocarpus and clean air hosted similar number of mealybugs. So, the supposed volatile compounds emitted by acrocarpus or coffee trees were neither attractive nor repellent for both mealybug species (Table 3). The total residence time of *P. citri* in each branch was similar. *Planococcus minor* stayed for similar time in both host branches and little longer in blank air.

TABLE 3
FINAL CHOICE 3rd INSTARS OF *Planococcus citri* AND *Planococcus minor* IN OLFACTOMETER (4 BRANCHES) (N=30) (15 MINUTES).

Insect	Olfactory response			Combination		
	Coffee (branch 1)	Acrocarpus (branch 2)	Clean air (branches 3 & 4)	Without response	1 *vs* 2	(1+2) *vs* (3+4)
P. citri	6	8	10	6 (20%)	0.6 n.s	0.7 n.s
P. minor	5	8	16	1 (3.3%)	0.8 n.s	0.3 n.s

Differences according to the Chi square test (χ^2) (α=0.05), (N= number of insects).

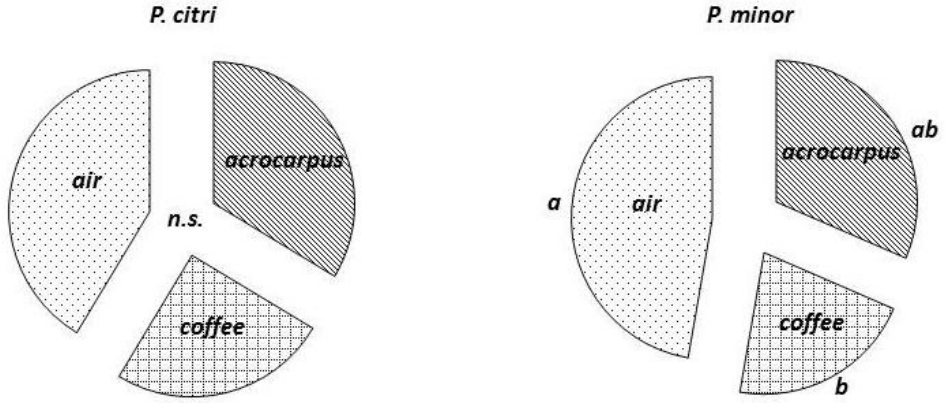

FIG. 5. Mean relative time (%) spent in each field in an olfactometer by 3rd instar nymphs of *P. citri* and *P. minor* exposed to three odors. ANOVA values: p=0.207, n=27 and p=0.026, n=30, respectively. Data transformed to arcsin √x/100. Means followed by the same letter are not different according to Anova followed by the Test of Tukey; n.s = no significant.

3.3.2 Response to coffee plants and mahogany

Planococcus citri nymphs showed no preference for the offered odors, while those of *P. minor* showed olfactory preference for mahogany face to coffee. Air was not more attractive than plant odors (Table 4). Nymphs of *P. citri* remained longer in clean air. Nymphs of *P. minor* remained longer in the air and mahogany, and shorter period in coffee (Fig. 6).

TABLE 4
FINAL CHOICE 3rd INSTARS OF *Planococcus citri* AND *Planococcus minor* IN OLFACTOMETER (4 BRANCHES) (N=30) (15 MINUTES).

Insect	Olfactory response			Combination		
	Coffee (branch 1)	Mahogany (branch 2)	Clean air (branches 3 & 4)	Without response	1 *vs* 2	(1+2) *vs* (3+4)
P. citri	6	6	16	2 (6.7%)	0.3 n.s	0.6 n.s
P. minor	0	8	15	7 (23.3%)	**6.6***	2.1 n.s

Differences according to the Chi square test (χ^2) (α=0.05), (N= number of insects).

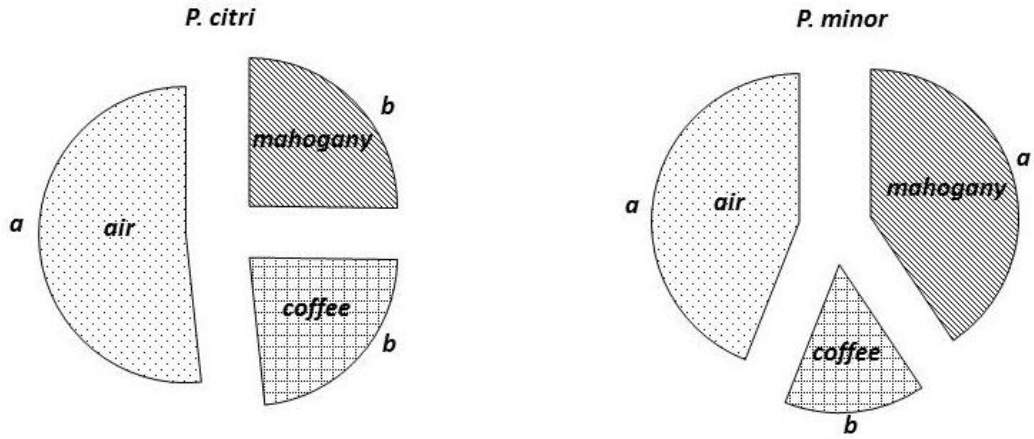

FIG. 6. Mean relative time (%) spent in each field in an olfactometer by 3rd instar nymphs of *P. citri* and *P. minor* exposed to three odors. ANOVA values: p=0.005, n=28 and p=0.002, n=30, respectively. Data transformed to arcsin √x/100. Means followed by the same letter are not different according to Anova followed by the Test of Tukey.

3.3.3 Response to coffee plants and teak

Teak appeared to have a repellent effect only for *P. citri* since insects were attracted to coffee and air (Table 5), but the permanency time was similar in all braches (Fig. 7).

TABLE 5
FINAL CHOICE 3rd INSTARS OF *Planococcus citri* AND *Planococcus minor* IN OLFACTOMETER (4 BRANCHES) (N=30) (15 MINUTES).

Insect	Olfactory response			Combination		
	Coffee (branch 1)	Teak (branch 2)	Clean air (branches 3 & 4)	Without response	1 *vs* 2	(1+2) *vs* (3+4)
P. citri	11	4	8	7 (23.3%)	**5.3 ***	2.1 n.s
P. minor	7	7	14	2 (6.7%)	0.0 n.s	0.0 n.s

Differences according to the Chi square test (χ^2) (α=0.05), (N= number of insects).

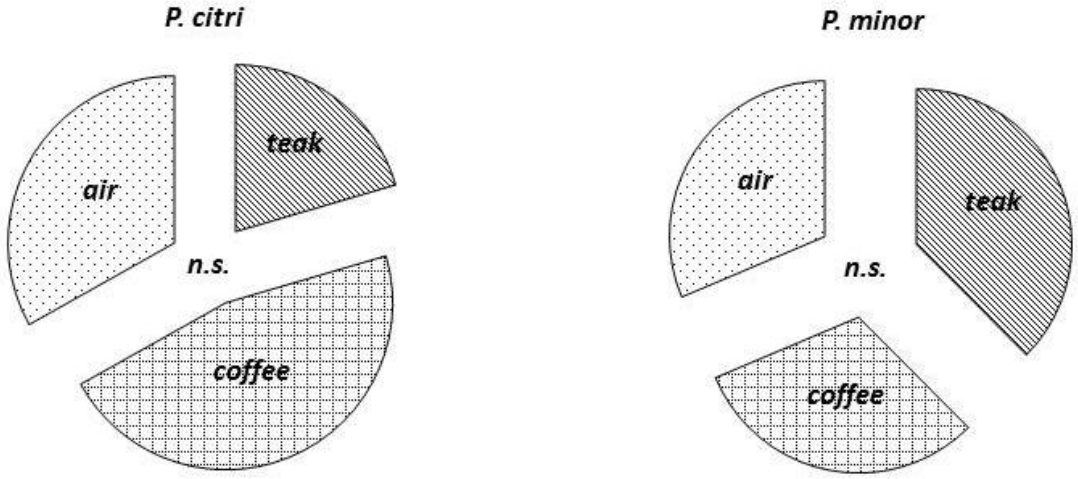

FIG. 7. Mean relative time (%) spent in each field in an olfactometer by 3rd instar nymphs of *P. citri* and *P. minor* exposed to three odors. ANOVA values: p=0.063, n=22 and p=0.775, n=30, respectively. Data transformed to arcsin √x/100. Means followed by the same letter are not different according to Anova followed by the Test of Tukey; n.s = no significant.

3.3.4 Response to coffee plants and macadamia

Coffee and macadamia odors had no effect on any of the mealybugs, which were equally distributed in olfactometer branches (Table 6). The permanency time inside each branch neither showed differences between odors (Fig. 8).

TABLE 6
FINAL CHOICE 3rd INSTARS OF *Planococcus citri* AND *Planococcus minor* IN OLFACTOMETER (4 BRANCHES) (N=30) (15 MINUTES).

Insect	Olfactory response			Combination		
	Coffee (branch 1)	Macadamia (branch 2)	Clean air (branches 3 & 4)	Without response	1 *vs* 2	(1+2) *vs* (3+4)
P. citri	6	6	9	9 (30%)	0.2 n.s	0.4 n.s
P. minor	6	7	11	6 (20%)	0.2 n.s	0.2 n.s

Differences according to the Chi square test (χ^2) (α= 0.05), (N= number of insects).

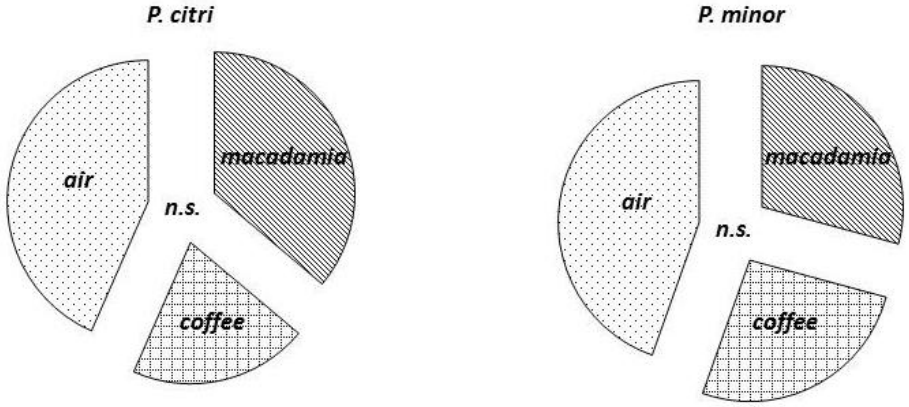

FIG. 8. Mean relative time (%) spent in each field in an olfactometer by 3rd instar nymphs of *P. citri* and *P. minor* exposed to three odors. ANOVA values: p=0.169, n=22 and p=0.137, n=25, respectively. Data transformed to arcsin √x/100. Means followed by the same letter are not different according to Anova followed by the Test of Tukey; n.s = no significant.

If we accept that tasting plant contents (cell or phloem sap) by ingesting plant fluids should be the main mechanism for plant selection, the free choice test should give a good insight about plant suitability. Mealybugs take a long time before reaching the phloem and ingest phloem sap (Santa-Cecilia *et al*., 2013), so plant exposing to these insects should last long period, 72 hours in this test, to get reliable results.

This study showed that, despite of the mealybugs were able to settle in the tested trees, there are different responses when compared with coffee plants. Settling or feeding in a plant does not mean that the plant is adequate and can support an insect colony. Plant nutrients can be suboptimal for reproduction (Le Rü *et al*., 1995a) and restrains colony size. Our data indicated that neither acrocarpus nor mahogany have a repellent effect for mealybugs but they seem to be poor hosts due to the high mortality. Data from the olfactometer are in agreement with these results.

Teak did not appear to be a good host in all tests. Data from the choice test showed a repellent effect and rearing on leaves showed a high mortality.

Plant selection process is a sequence of steps involving different environmental and plant stimulus. The olfactory response is one of these steps. All responses are related to the degree of adaptation of the insect to the host (Moura *et al*., 1991). Despite the reports about the presence of olfactory receptors in the mealybug antenna (Salama, 1971; Koteja, 1980; Le Rü *et al*., 1995a; Calatayud & Le Rü, 2006), we ignore the role they play in plant selection since the nymphs and adult females are apterous with little option to search and select a host.

Both tested mealybug species are able to colonize diverse plants since they are polyphagous. Macadamia has been reported as host for *P. citri*, and macadamia and teak for *P. minor* (García Morales *et al*., 2016). However, in the study presented here they did not appear as acceptable hosts for these mealybugs.

Cacao plants are usually colonized by *P. minor* and in less extension for *P. citri* suggesting the former should be more selective. Our results did not showed difference between species although cacao was not tested in this study.

This study showed that the tested trees, usually associated to coffee crops, are not suitable hosts for both species of mealybugs and they would not be source of infestation for coffee crops. It should be noted that this study was performed in laboratory, under controlled conditions, and field conditions could change the mealybug behavior according to environmental conditions.

IV. CONCLUSION

Acrocarpus, mahogany, teak and macadamia are not suitable hosts for *P. citri* and *P. minor* and they should not be source of infestation when associated to coffee crops.

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Water productivity and yield of Paddy Rice cultivation under AWD irrigation management in Pingtung, southern Taiwan

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Abstract— *Decreasing water input while maintaining grain yield remains a challenge for World to produce rice sustainably. In recent years, the Alternate wetting and Drying Irrigation(AWD)has been developed toward Asian' farmers. However, the gap observed is the low assessment of its performances, particularly in Taiwan.*

The aim of this study is to determine whether AWDI management could maintain grain yield with reduced water input.

AWD approach experiment field was conducted in National Pingtung University, in Southern Taiwan. A two leaves old rice seedling, TAINAN11 was arranged in a randomized complete block design with five water treatments: AWD_{2cm}, AWD_{3cm}, $AWD_{3cm/w}$, AWD_{4cm} and AWD_{5cm} with respectively 2cm and 3 cm water depth monitoring by soil hairline cracks; 3cm, 4 cm, and 5cm weekly irrigation. The irrigation regimes were done by AWD technics. The plant water status was assessed through soil water content. Crop growth, grain yield, and water productivity were measured.

The results showed that grain yields under AWD_{3cm}, AWD_{2cm} and $AWD_{3cm/w}$ presented the high yield and irrigation water productivity about 0.211, 0.208 and 0.205 respectively. The AWD_{4cm} and AWD_{5cm} despite the high-water depth presented low yield with respectively 3081 Kg/ha and 2604 Kg/ha.

The results confirmed also that AWD3cm and more precisely $AWD_{3cm/w}$ could obtain comparable grain yield close to farmers practices with fewer irrigations. These findings suggested that AWD with 3 cm water depth (AWD_{3cm} and $AWD_{3cm/w}$) could be used for water-saving while maintaining grain yield in paddy rice production.

Keywords—Grain yield, Water productivity, Alternate Wetting and Drying, AWD.

I. INTRODUCTION

The 2017's edition of The State of Food Security and Nutrition in the World reveled that in 2016, the number of undernourished people in the world increased to an estimated 815 million, up from 777 million in 2015 but still down from about 900 million in the year 2000[1]. It's well known that rice (*Oryza sativa L.*) is a staple food for nearly half of the world's seven billion people particularly in Asia, and this alimentary need must be satisfy by doubling the present production over 2030 [2].

The fresh water, one the indispensable input in rice production, is a finite resource which is faced to the increasing large demand of Agriculture. The per capita available water resources in Asia are expected to decline by 15-54 % compared with 1990 [3] and already 12 million hectares of south Asia's irrigated rice are at risk of severe water shortage. In Taiwan were rice is a very important and valuable crop, with a total yield of more than 1.73 million tons[4], the possibilities of sustainable production are welcome. Indeed, Rice is grown from February to July, and August to December [5], but according to Kuo andal., (2006) [6] the amount of water available for agricultural use has recently become critical.

Agriculture is now faced with the challenge of securely delivering sufficient food to meet the projected demands of population growth and overcoming issues such as climate change and water scarcity through sustainable agricultural intensification ([7]. This poses major challenges for scientists, extension workers, and farmers.

Numerous technologies have been developed with the aim to reduce water use and help the farmers in rice production with higher water productivity ([8] and [9]). One of these technologies is the Alternate Wetting and Drying (AWD) which has been developed since the 1970s [10]- [9]. The concept of AWD technology is based on the fact that rice high yields can be obtained by just providing the only need water to the crop.

In 2015 and 2016 a field experiment was conducted in southern of Taiwan by KIMA andal., (2015) [11] and Pascual andal., (2016) [12] respectively, to determine the most suitable ponded water depth for enhancing water saving in paddy rice irrigation. The firsts found that lowest water reduced yield component between 15-32%. They mentioned that weekly application of 3cm water depth combined with rainfall improved AWD effectiveness and yielded the highest beneficial water productivity with less yield expenses. The seconds showed that the highest total water productivity, (0.75 kg/m^3) and irrigation water productivity (1.40 Kg/m^3) was achieved in T_2 cm. They also found that weekly application of T_4 cm ponded water depth produced the lowest yield reduction (1.57%) and grain production loss (0.06 kg), having no significant impact on yield loss compared to T_5 cm.

Due to some uncertainties from the two previous studies, in 2017, Kissou and al.,[13] conducted a similar experiment during the dry season. Their results shown that 3 cm water depth gave the best results in terms of water saving, high yield, but some uncertainties underlined were the closeness of these results with the 4 cm water depth. Therefore, the present experiment was conducted applying the AWDto more clarify these findings.

The objective of this study was to determine whether the AWD irrigation management could maintain grain yield with less water use. It was specifically expected to determine the water input (quantity and frequency) and the water productivity;and its effect on agronomic traits and grain yield.

II. MATERIALS AND METHODS

2.1 Study area description and field experiment

The experimentation was conductedfrom 21st November 2017 to 20th of May 2018, in the irrigation experimental field of the National Pingtung University of Science and Technology (NPUST) in Southern of Taiwanlocated at 71 m above sea level; at 22.39° (N) latitude and 34.95° (E) longitude. Previous analysis of the soil physical properties[11]-[12], concluded that the textural class of the soil in the experimental field was loamy soil(27% of sand and 24% of clay).

The experimental field was a randomized complete block design with four replications and five water treatmentsAWD_2, AWD_3, $AWD_{3cm/w}$, AWD_4 and AWD_5. Irrigation water depths were applied at 2 cm, 3 cm (biweekly) and 3 cm, 4 cm and 5cm (per week) representing respectively treatments. Each plot was 4 m long and 1.5 m wide with total area of 6 m^2 and 0.3 m hardpain. The spacing between blocks was 1 m. Water depth was kept constant and the soil hairline cracks occurring in the concerned plotswere monitored.The experimental site is equipped with irrigation pipes and drainage systems.

Two leaves age (25 days-old) seedlingsof the Tainan 11 (TN11) varietywere manually transplanted on 5th December 2017. Seedling was transplanted, lined up with 25 cm hills spacing between rows and 25 cm between hills (75 hills per plot). Each hill received one seedling. Plants gap's filling was done with the same seedlings, one week after transplanting due to young plant attacks by snails and insects in some plots of the blocks 2 and 3.

To ensure plants nutrients need, some fertilizers was applied. The Organic Fertilizer (NPKS) with a ratio of 5-2.5-2.5-81, was applied at the rate of 270Kg/ha as basal fertilizer before rice transplanting. Chemical fertilizer N-P2O5-K2O at the ratio of 15-15-15-4-50 was applied once during the development stage at the dose of 270 kg/ha.Urea was applied in split doses at the rate of 150 kg/ha.Liquide containing Probiotic bacteria were applied on 17, 21, 70, and 105 DAT at a concentration of $12*10^8$ cells.Weed management were done manually at 20, 40, and 76DAT.

The frequency of irrigation was initiated according to the irrigation interval of each treatment. The use of previous approach [11], allowed to precise the desired water depth to reach the amount of each plot need as shown in the equation 1 below :

$$R = A \times h \times 10\text{^}3 \quad [1]$$

Where ***R*** is the amount of irrigation water (liters) for a desired depth above the soil surface (m); ***A*** is the surface area of the plot (m^2), and ***h*** is the desired water depth above the soil surface.

The irrigation water was applied using a water pipe, after measured its discharge. The duration of irrigation to reach the required water depth corresponding to each treatment was computed through the following equation 2 :

$$Q \times t = d \times A \quad [2]$$

Q is the flexible link discharge expressed in liter per second (l/s); ***t*** is the set time of irrigation (second); ***d*** is the depth of irrigation water applied (millimeters) and ***A*** is the area irrigated (m^2).

Irrigation water depth in the field was kept to 50 mm during the first 2 weeks (16 days) after transplanting (from 6th to 21stDecember 2017), all plots were irrigated daily applying (50 mm) per day to maintain soil near saturation and facilitate the seedling rooting (semi-tillering stage). During the panicle initiation (from 1st April), irrigation date of all the plots were reduce one day due to the higher water demand by crop and the increase temperature during this period.

2.2 Parameters Observed

2.2.1 Leaves Chlorophyll Content

The Chlorophyll meter (model SPAD-502, MINOLTA, Japan) provides an easy, quickly, safe and low-cost method as an approach supported by[14]. At 60 days after transplanting, three randomized samples in plots were selected for their chlorophyll content measurement. Five uppermost fully expanded leaves were randomly selected from each sampled hill to analyze the variability of leaves chlorophyll amount treatments. The collection area on each leaf is located between 40% and 70% along the length from the leaf base and three different points for readings were done and the average data recorded. Analysis of leaves sampling patterns follows the approach of [15].

2.2.2 Leaf Area Index measurement

The total leaf area of a rice population is a factor closely related to grain production because the total leaf area at flowering greatly affects the amount of photosynthates available to the panicle [16].

LAI was measured using the approach of LAI with leaves not removed from plants.The two hills x two hill sample approach was applied. The tillers for each sample hill in each plot is counted, thus, for each measurement session, the length and maximum width of each leaf on the middle tiller was measured and the area of each leaf based on the length-width method (Tilahun-Tadesse andal., 2013; Yoshida, 1981) was computed as equation 3 and equation 4 below:

$$Leaf\ area\ (cm^2) = L \times W \times K \quad [3]$$

Where L is leaf length, W is maximum width of the leaf and K is a correction factor of 0.75

Then the LAI was obtained by:

$$LAI = \frac{sum\ of\ the\ leaf\ area\ of\ all\ leaves}{ground\ \ area\ cover\ by\ 4\ hills} \quad [4]$$

FIGURE 1 : Chlorophyll meter (SPAD-50MINOLTA)

FIGURE 2 : View of the plant growth measurement

2.3 Yield and Water productivity assessment

At harvest, ten panicles randomly sampled from hills of the plot center square meter were cut at the base, separated from the straw and grain handly threshed. The amount of grain collected was dried at 14% humidity. Marketable yield was gotten after unfilled spikelets were separated using a seed blower for 2 mm.

Productivity is a ratio between a unit of output (yield) and input (water). The total amount of water applied (Twu) is the sumof irrigation water (Irw) and rain water (Rw). Thus,Total water productivity (TWP), Irrigation water productivity (IWP) and Rain water productivity (RWP) were calculated through thefollowing equationsof [17] :

$$TWP = \frac{Yact}{TWU} \quad [5]$$

$$IWP = \frac{Yact}{Irw} \quad [6]$$

$$RWP = \frac{Yact}{Rw} \quad [7]$$

TWP, IWP and RWP are expressed in $kg.m^{-3}$. Y_{act} is the actual markable yield (Kg. ha^{-1}), Twu, Irw and Rw are expressed in m^3. ha^{-1}.

2.4 Statistical analysis of Data

Field Observation data were compiled using Microsoft Excel software.The collecting data of each sample was analyzed separately using Statistical Analysis System (SAS) to evaluate the variance of treatment effects. The significance of the treatment effect was determined using F-test. When ANOVA indicated that there was a significant difference, multiple comparisons of means were performed using the Least Significant Difference method (LSD) at 0.05 probability level. The student's t-test was employed to test the significance of difference between the two water management treatments.

III. RESULTS AND DISCUSSIONS

3.1 Climate characteristic during the experimental period

Data collected from the Agro-Meteorological station of the NPUST and computed gave some pieces of information about the weather evolution during the cropping period. The Figure 3 below, shows the occurrence of rainfalls from December 2017 to May 2018, considered as dry season in Taiwan. However, the repartition of the rainfall was irregular during the cropping period. The maximum cumulative rainfall was 85.4 mm with a minimum of 4.5 mm. The effective rainfalls represented around 11% of the water applied to treatment (11.84% of AWD_2, 10.36 of AWD_3, 12.03% of $AWD_{3/w}$, 11.02% of AWD_4 and 10.17% of AWD_5) during the cycle of production. The average temperature was high with 27.79 and 19.7 °Crespectively for maximum and minimum.

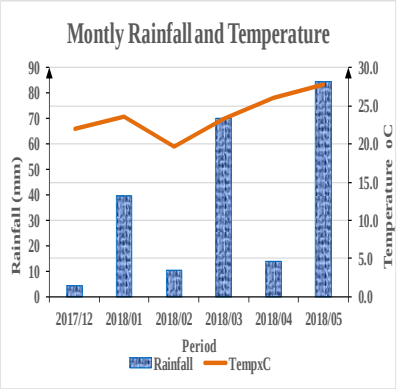

FIGURE 3 : Evolution of the monthly rainfall and temperature during the rice cropping

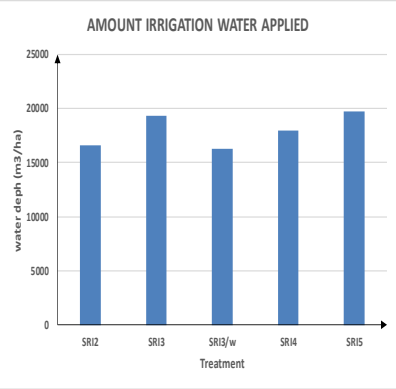

FIGURE 4 : View of the amount of irrigation water applied in AWD rice crop

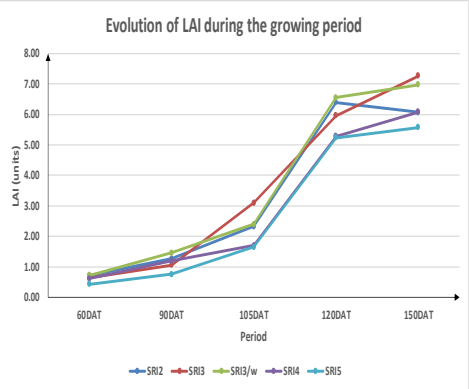

FIGURE 5 : Evolution of Leaf Area Index during the from 60 DAT to 150 DAT

These values hid the real daily influence of temperature during the growing period. Indeed, in 2018 low temperatures were recorded and the minimum reached 6.8°C. These low temperatures might affect the rice plants growth at this period.

3.2 AWD irrigation managementand water used efficiency

3.2.1 Irrigation applied and total water received

The principle of the irrigation is to supply water required by rice plant to satisfy its need and compensate the water lost due to evapotranspiration. Each of the five treatments received the irrigation water following the planning of irrigation. The Fig. 4 above presented the amount of water consumed (irrigation and rainfall) during the survey.

The irrigation by AWD technic for treatments started at 17 DAT, due to the interval of water application and reduction of the quantity, some hairline cracks appeared on soil and help for monitoring visually the interaction soil-plantduring all growing stages of rice plants.

3.2.2 LAI response to irrigation

Leaves are plants organs consideredas the regulators of plants face to climate factors in particular due to their stomates functions. In this survey, water was applied for each treatment and the leaves morphological status was observed

through the leaf area index. The Fig. 5 above presents the evolution of the leaf area index from the tillering to the maturity stage.

The LAI increase during the plant growth stages. At maturity (150 DAT), rice leave area was maximum and the treatment AWD_3 presented the highest surface, followed by $AWD_{3/w}$, AWD_4, AWD_2, and AWD_5. The water treatment was a factor that influenced these values. Indeed, the ANOVA test of the variance shown that there is no significant difference between treatment $AWD_{3/w}$, AWD_3, and AWD_2; also, AWD_4, and AWD_5 are not significantly different.

3.2.3 Effect of water application on plant chlorophyll content

During the present study, chlorophyll content was measured to monitor rice plant health status and his response to the AWD water application.The measurement results shown that chlorophyll content varied with vegetation growth of the plant. During the cropping season, chlorophyll increased from 60 DAT to 120 DAT and the values stagnated or decreased in the late phase. The comparison of different stages by t-Test using the LSD (at p= 0.8787) also revealed that AWD_4, AWD_5, and $AWD_{3/w}$ presented the same means from 105 DAT until maturity (44.96, 44.69 and 44.44 respectively); but the first and second stages are significantly different.

The analysis of the means value through ANOVA showed that there is no difference between AWD_2, AWD_3, and AWD_4. $AWD_{3/w}$ presented a significant difference from the three and also different from AWD_5.

TABLE 1
DUNCAN'S MULTIPLE RANGE TEST FOR CHLOROPHYLL

Duncan Grouping		Mean	N	TRT
	A	43.3535	20	$AWD_{3/w}$
B	A	42.8050	20	AWD_4
B	A	42.6135	20	AWD_2
B	A	42.4050	20	AWD_3
B		42.0620	20	AWD_5

These means comparison to show that plant under water treatment tend to use the resources from soil to express his potential and this is not easily detectable without analysis of the chlorophyll.

Previous studies underlined that the water stress in AWD did not change the relationship between leaf N and SPAD readings and the SPAD values can contribute to the prediction of leaf N of rice under AWD.[18] supported that apart from irrigation treatment, the PAD value ranged to 38 could be used as the critical value for fertilizer application. This approach could offer to the farmer location-specific critical information ideal and time for assisting decision makers in monitoring their crops and managing farming activities to achieve maximum production.

3.2.4 Effect of irrigation water on yield and water productivity

The application of AWD irrigation in the different plots leads to the yield that the range is presented in the Table 2 below. The table contains the assessment of the water productivities calculated based on the approach of [17].

TABLE 2
WATER PRODUCTIVITY

Treatment	Yield (Kg. ha^{-1})	Irrigation Water ($m^3.ha^{-1}$)	Effective Rain Water ($m^3.ha^{-1}$)	TWP ($Kg.m^{-3}$)	RWP ($Kg.m^{-3}$)	IWP ($Kg.m^{-3}$)
AWD_2	3448	16600	2230	0.183	1.546	0.208
AWD_3	4072	19300	2230	0.189	1.826	0.211
$AWD_{3/w}$	3340	16300	2230	0.180	1.498	0.205
AWD_4	3081	18000	2230	0.152	1.382	0.171
AWD_5	2604	19700	2230	0.119	1.168	0.132

Where TWP, IWP, and RWP arerespectively the total, irrigation, and rainwater productivities.

The results showed that even if the grain yields are important, they were relatively low than expected. This situation may partially imputable to the climate parameters as low temperature during the tillering, but also to other parameters as reseeding after nails and insect's attacks and others no identify. The results showed that the low grain yield was borne

by the plots which received the lowest water treatment. Kima*andal.*,[11], followed by Pascual and Wang[12]observed that the lowest water treatment resulted in the lowest values of panicle weight. The AWD with 2cm contradicted these results but can be explained by the fact that water application frequency is short (biweekly irrigation).

IV. CONCLUSION

The present survey was conducted during a dry season with the aim to improve the AWD capacities as water-saver for yield improvement. Five irrigation treatment were design and realized in NPUST irrigation experiment station.

The applying of twice-weekly irrigation water depth of about 2 cm and 3cm and the monitoring of the soil hairline cracks as an indicator of water status known in farmer practice, showed good results. The yield result was good correlated to the water treatment with a probability ofabout 0.825. The treatment $AWD_{3/w}$, a weekly single irrigation underlined as the optimum irrigation depth by previous studies, presented some results closed to AWD_2 and AWD_3 with high grain yield.

The marketable yield obtained tend to be low than those presented in other studies. The possible effects of the climatic parameters throughout the rice cycle may explain this yield value. From nursery to seedling and tillering ages, rice plant suffered about temperature fluctuation, specifically the cold. The daily variation of temperature largely influenced the growth of rice plants leading to lengthening of rice reproduction cycle and then a more consumption of water and other resources. One of the consequence is also the reduction of the water productivity even if the rate found was positive (0.208 to 0.132 respectively from SR_{2cm} to SR_{5cm}).

Despite of the weather influence, the irrigation management ensured a good productivity of yield and water. The IWP and the RWP registered an average of 0.208 and 1.623 respectively for AWD_2, AWD_3, and $AWD_{3/w}$. AWD_4, and AWD_5 have an average of 0.152 and 1.275 of irrigation and rainwater productivity.

Regarding to the yield performance and the water used efficiency, rice grown with AWD methods reveals a lot of opportunities in rice farming system. Under drought condition, adaptation capacity of rice plant can give good yields and water productivity. The results of this study open some perspectives forricecultivation performing on different periods.

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Influence of Baobab Leaf Enrichment on the Physicochemical, Sensory and Nutritional Characteristics of Plantain/Cashew kernels Composite Flours

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Abstract**—** *The purpose of this study is to determine the nutritional value and sensory acceptance of Baobab leaf enriched plantain- cashew kernel meal. Composite flours formulated from plantain and cashew almond were enriched with baobab leaf powder at substitution levels of 10%, 15% and 20%. The biochemical composition, minerals, vitamin C, antioxidant activity, sensory properties and nutritional parameters of the enriched composite flours were measured. The addition of baobab leaf powder evidenced significant ($p < 0.05$) increase in protein, fiber, vitamin C, main mineral elements, total polyphenols contents and antioxidant activity; but dropped the lipids and carbohydrates contents. With sensory evaluation, cashew-almond-based composite meal formulas substituted for 10% by baobab leaf powder showed a similar overall sensory acceptance to non-enriched cashew-nut kernels. In addition, the ingestion of these meal formulas by the young rats was favorable to their growth. Thus, diets incorporating 10% baobab leaves are more suitable for consumption and growth of young rats.*

Keywords— Flour, plantain-cashew nut, enrichment, Baobab, leaves.

I. INTRODUCTION

Vitamin and mineral deficiencies are among the major nutritional problems affecting populations, especially those with low incomes. Even in developed countries, the micronutrient deficit is not negligible since the affected population sometimes exceeds 30% (Gallan *et al*., 2005). Such nutritional deficiencies cause serious health concerns, specifically among vulnerable people such as children and pregnant and lactating women (FAO/WHO, 1994; FAO/WHO, 1992). Worldwide, half of pregnant women and one-third of under five children suffer from various degrees of anemia due to iron deficiency (UNACC/SCN 1997; Frossard *et al*., 2000). Strategies for addressing these nutritional deficiencies include pure protein supplementation and dietary approaches including the fortification of staple foods with functional foods from the traditional foods often richer in micronutrients (Gibson *et al*., 2000, West, 2002). Improving public health through food biofortification has thus the advantage in promoting natural resources such as highly widespread fruits and vegetables that are generally available even in developing countries.

Fruits and vegetables are products of great importance in terms of food and health. They have many natural flavors and contain nutritional and functional compounds such as carbohydrates, proteins, vitamins, minerals, dietary fiber, antioxidants and other bioactive substances (FAO/WHO, 1992; Traore *et al*., 2015; UNACC/SCN, 1997). These products are part of the Africa's vast reservoir of natural forest resources products exploitable for the well-being of people (Honfo *et al*., 2007; Magdi *et al*., 2004). Thus, the plant species *Adansonia digitata* L. (Malvaceae) locally known as "baobab" is among the vegetables commonly used in Africa. It is a hundred-year life span plant providing multiple uses for populations. Indeed, the different parts of the plant are widely used as food and medicine (Sidibe & Williams, 2002). By the season, fresh and/or dried leaves are important source of protein and minerals for families using this plant as a staple (Sidibe & Williams, 2002). Previous studies highlighted the nutritional features of this fresh leafy vegetable (Oulai *et al*., 2014). As every leafy vegetable, it contains higher levels of magnesium, potassium, calcium, iron and records a significant amount of protein (FAO, 2012; Oulai *et al*., 2014). The micronutrient richness of leafy vegetables is therefore an advantage usable for the biofortification of staple foods during specific periods and diets such as growth, breastfeeding and weaning characterized by increased needs and

requiring adaptation of food contributions. The development of composite food formulas enriched with leafy vegetables is an application to meet nutritional needs. In this sense, formulations based on starchy foods enriched in vegetables such as cowpea beans and moringa leaflets have been successfully investigated by Mahan *et al*. (2016a). However, there are scanty data regarding the baobab leaf vegetable in improving the nutritional value of local starchy products used during weaning. Earlier reports have highlighted the nutritional limits of plantain/cashew kernels composites flours (Fofana *et al*., 2017). The purpose of this study is to evaluate the effects of baobab leaf enrichment on the nutritive, sensory and nutritional value of composite flours produced on plantain and cashew kernel basis for use as weaning foods.

II. MATERIAL AND METHODS

2.1 Biological material

The study was conducted on composite flours made from banana plantain (*Musa paradisiaca* sp) and cashew kernels (*Anacardium occidentale* L.). Fresh leaves of baobab (*Adansonia digitata* L.) were used for the enrichment of composite flours. Two infantile commercial flours, namely infantile flour of Bledine® (ETB) and infantile flour of Farinor® (ETF), were used as a reference.

2.1.1 Production of plantain / cashew kernel composite flours

The raw flours were obtained after treatment of ripening plantain at stage 4 and cashew nuts according to the methods of Fofana *et al*. (2017). Then, two composite flours were processed by substitution of part of the plantain flour by the cashew kernel flour. Indeed, a composite flour A was worked by incorporation of 15% unfermented cashew kernel flour, and a composite flour B was filled by incorporation of 10% fermented cashew kernel flour. Each formulation was thoroughly blended, and then divided into 250 g fractions in polyethylene plastics and kept at room temperature till analyses.

2.1.2 Production of baobab leaf powder

The leaves powder from *A. digitata* L. was produced according to the processing reported by Soro *et al*. (2014). The collected leaves were disinfected in a diluted sodium hypochlorite solution (1/1000) for 15 min and thoroughly rinsed with tap water. Thereafter, the leaves were sunny drained for 2 hours (30 ± 5 ° C), immersed in a boiling water bath for 15 min, dripped, and sunny dried for 48 hours resulting in an unvarious weight. Afterwards, the dried leaves were ground using a grinder with a mesh size of 200 µm. The resulted powder was batched in 250 g, hermetically packaged into polyethylene plastic bags each, and then stored at room temperature till incorporation into the plantain/cashew kernels composite flours.

2.1.3 Formulation of enriched composite flours: plantain- cashew kernel -baobab

Formulas enriched with plantain-almond cashew-baobab were obtained by incorporating various ratios of baobab powder into the plantain-almond cashew meals. Thus, samples of plantain + 15% unfermented cashew kernel and plantain + 10% fermented cashew kernel flour were substituted with 10%, 15% and 20% baobab leaf powder weight. So, three enriched flours were formulated from each plantain-cashew composite flour: A10BP, A15BP, and A20BP (from composite flour A), and B10BP, B15BP, and B20BP (from composite flour B). Each final formulation was thoroughly blended, divided into 250 g fractions in polyethylene plastic bags, and stored at room temperature until analysis.

2.2 Physico-chemical analyzes of flours

The physicochemical investigation of the enriched composite formulations consisted in determination of their contents in moisture, macronutrients (proteins, lipids, carbohydrates, and fibre) and micronutrients (minerals, vitamin C, total phenols and antioxidant activity). Thus, the moisture content was determined by drying 5 g of each sample in an oven (Memmert 854 Schwabach, Germany) at 105 ° C for 24 h (AOAC, 1995).

The crude protein content was determined according to the Kjeldahl method using a Kjeltec 8400 (FOSS, Sweden) unit of the analyzer and the resulting nitrogen (% N) allowed the deduction of the percentage of crude protein (% P) using the relation: % P =% N x 6.25 (AOAC, 1995).

The lipid content was determined by extraction using hexane as extraction solvent and a Soxhlet device (Unid Tecator, System HT2 1045, Sweden) (AOAC, 1995). For the crude fibres, 1 g of flour sample was first digested with 1.25 N sulfuric acid and 1.25 N sodium hydroxide. The insoluble residue obtained was washed with hot water and dried in an oven

(Memmert 854 Schwabach, Germany) at 105 ° C for 24 h. The dried residue was then incinerated into a muffle furnace (Nabertherm GmbH Babnhofstrasse 20, 28865 Lilienthal / Bremen, Germany) at 600 ° C for 6 h and weighed for the determination of the crude fiber content. The total carbohydrate content and energy value were estimated from the following formulas provided by AOAC (1995):

Total Carbohydrate Content (%) = 100 - (% moisture +% protein +% fat +% ash)

Total Energy Value (kcal/100 g) = (% of protein * 4) + (% of carbohydrates * 4) + (% of fat * 9)

The flours ash was estimated by incineration of 5 g of each sample in a muffle furnace (Nabertherm GmbH Babnhofstrasse 20, 28865 Lilienthal / Bremen, Germany) at 600 ° C for 6 h (AOAC, 1995), and the resulting ash allowed the minerals evaluation using Pelkin Elmer type of atomic absorption spectrometry (PE 3110, Norwalk USA) according to the AOAC method (1995).

Vitamin C was quantified from the samples by extraction using metaphosphoric and acetic acids and 2,6-dichlorophenol indophenol solutions against a standard vitamin C solution (Poncracz *et al*., 1971). The total polyphenols were extracted according to the AOAC method (Christensen, 1974) using Folin Ciocalteus reagent. The full antioxidant activity of the studied flours was assessed on the anti-radical activity basis, according to the method reported by Choi (2002) and deriving from the bleaching of a DPPH reagent by antioxidant components previously extracted using methanol.

2.3 Sensory analyzes of dishes derived from enriched composite flours

2.3.1 Preparation of the porridges

From each sample, 50 g of flour was added with 275 ml of tap water and then prepared accordingly to indications from both selected reference flours. The porridges were gently cooked for 10 min and table sugar was thereafter added at a rate of 6%. They were then cooled at room temperature before being dished up for analysis about the sensory acceptance and description against both infant flours taken as controls.

2.3.2 Porridges analysis for sensory acceptance

The full acceptance of the porridges prepared from the enriched composite flours was estimated by a group of 60 tasters selected for their availability and regardless of gender and age (18 to 35 years). Twenty (20) ml of porridge samples, identified by individual 3-digit codes, were simultaneously presented to each panelist in a random order. The pleasure perceived by the panelist after tasting each sample was expressed with a quantitative mention on a 9-points hedonic rating scale, ranged from 1 (when the sample is extremely unpleasant) to 9 for the extreme pleasance (Meilgaard *et al*., 1999).

2.3.3 Quantitative sensory description of the porridges

Descriptive sensory analysis of porridges consisted in quantifying appropriate descriptors, such as odor, flavor, consistency, color, and texture. The tests were achieved according to a category scale. The enriched composite meal flours and the commercial infant flours were presented to a jury of 15 people duly trained to sensory analysis methodology (AFNOR, 1984). The porridge samples were then 3-digits coded and presented simultaneously in a random order to quantify their sensory parameters (Meilgaard *et al*., 1999).

2.4 Animal experimentation

2.4.1 Animals and housing

Wistar rats with mean weight of 60 g and at the growth stage were housed in individual metabolism cages. These cages were built with racks and bottles for providing feed and water to animals.

2.4.2 Distribution of animals

Three batches of 7 rats were submitted to a particular diet each resulting from the formulations successfully enjoyed by tasters after the sensory acceptance analysis. Animal experimentation was achieved according to the method reported by previous authors (Adrian *et al*., 1991; Meité *et al*., 2008).

The investigation ran over sixteen days including an adaptation period of two days where animals were fed pellets (standard food) and a 14-day growth period.

2.4.3 Implementation of the experimentation

The experimental animal room temperature was 26 °C, with moisture between 70% and 80%. The food formulas retained at the end of the sensory analysis were distributed *ad libitum* to animals one a day between 8 AM and 10 AM in the form of mash. Drinking water was also provided at will and renewed every day. The animals were weighed at the beginning of the experiment and then at two-day intervals; the last weighing took place at the end of the experiment.

2.4.4 Expression of the nutritional parameters of the studied flours

The chemical analyzes performed regarding the nutritional parameters are consistent with those defined by AOAC (1975), namely the total ingested dry matter (TIDM) which is the total quantity of feed dry matter ingested during the experimentation period, the animals weight gain (WG), the food efficiency coefficient (FEC), the total ingested protein (TIP), and the protein efficiency coefficient (PEC). These parameters are worked with the following mathematical expressions:

TIDM (g) = Total feed dry matter provided – remaining dry matter after the experimentation period

WG (g) = Animal Final weight after experimentation – animal initial weight before experimentation

FEC = WG / IDM

TIP (g) = IDM * % Crude Protein

Protein Efficiency Ratio (PER) = GP / TIP

2.5 Statistical analysis

The data recovered were treated using the Statistical Program for Social Sciences software (SPSS 7.1, SPSS for Windows, USA). The statistical analysis consisted in evaluating the variability of the parameters through a one-way analysis of variance (ANOVA-1) at 5% statistical significance. The enrichment rate was the ANOVA criterion. The averages were classified using the Duncan post-ANOVA test.

III. RESULTS

Table 1 shows the physicochemical composition of the various flours. The moisture content of the flours oscillates between 5.66 ± 0.11% for the plantain flour enriched with 15% unfermented cashew kernel flour (composite flour A) and 4.63 ± 0.60% for the Baobab leaf raw powder. The protein, ash and fibre contents of baobab powder are measured at respective means of 16.90, 9.24, and 29.83 g/100 g. The results attest that incorporation of Baobab raw powder into the composite flours A and B resulted in a successful significant ($P<0.05$) increase in the final protein, ash, and fiber contents. The protein contents of the formulated flours range from 10.11 g/100 g (A10PB) to 12.54 g/100 g (B20PB), while the ash contents vary from 3.15 g/100 g (B10PB) to 5.26 g/100 g (A20PB). These amounts are over that of the ETB control sample (3.33 g/100 g). The fiber contents of composite flour A increase significantly from 1.40 g/100 g to 5.66 g/100 g with 20% incorporation of baobab leaf powder (A20PB). For the composite flour B, the 20% incorporation of Baobab powder increases the fiber content from 0.84 g/100 g to 4.16 g/100 g.

The baobab leaf powder mean content in total fat and carbohydrates are 2.48 g/100 g and 66.75 g/100 g, respectively. Unlike proteins, ashes and fibers, the substitution of proportions of composite flours with baobab powder leads to a significant decrease ($P<0.05$) in fat and carbohydrates levels, respectively from 4.92 g/100 g and 79.62 g/100 g to 3.87 g/100 g and 72.70 g/100 g for the composite flour A and from 3.69 g/100 g and 79.85 g/100 g to 2.99 g/100 g and 75.57 g/100 g for the composite flour B. In addition, Table 1 shows that the overall formulated flours are lower in fat (2.99 to 4.15 g/100 g) than the control infant flour (6.01 g/100 g). Regarding carbohydrates, the experimental infant flours (72.70 g/100 g and 78.59 g/100g) are as provided as the control infant flour (72.81 g/100 g). The results also show that baobab powder has a low energy value (281.42 kcal/100 g) compared to the flours produced. Thus, enrichment with baobab leads to a significant decrease in the energy value of the flours according to the rate of incorporation. The mean energy values of the formulated flours range from 375.35 to 385.85 kcal/100 g, which are statistically lower ($P<0.05$) than that of the ETB control sample (400.09 kcal/100 g).

TABLE 1
CHEMICAL PROPERTIES OF COMPOSITE FLOURS PROCESSED FROM PLANTAIN, CASHEW KERNELS AND FORTIFIED WITH BAOBAB LEAVES POWDER

Flours	Moisture (%)	Protein (mg/100g)	Fat (mg/100g)	Carbohydrate (mg/100g)	Fiber (mg/100g)	Ash (mg/100g)	Energy (kcal/100g)
PB	4.63±0.60^{f}	16.90±0.32^{a}	2.48±0.65^{i}	66.75±0.50^{h}	29.83±0.28^{a}	9.24±0.57^{a}	281.42±1.06^{g}
A	5.26±0.05^{a}	8.03±0.3^{h}	4.92±0.02^{b}	79.62±0.04^{a}	1.40±0.03^{g}	2.15±0.01^{e}	394.92±0.40^{b}
B	5.66±0.11^{f}	8.75±0.22^{g}	3.69±0.29^{f}	79.85±0.12^{a}	0.84±0.64^{h}	2.04±0.23^{e}	387.63±0.51^{c}
A10PB	4.83±0.79^{c}	10.11±0.20^{f}	4.15±0.51^{c}	76.49±0.95^{c}	3.41±0.38^{e}	3.27±0.57^{d}	385.85±0.80^{cd}
A15PB	4.40±0.51^{d}	11.04±0.10^{e}	3.95±0.34^{d}	74.90±0.45^{f}	4.83±0.28^{c}	4.37±0.32^{c}	380.23±2.28^{e}
A20PB	4.21±0.17^{e}	12.42±0.25^{c}	3.87±0.46^{e}	72.70±0.76^{g}	5.66±0.28^{b}	5.26±0.69^{b}	379.45±2.33^{e}
B10PB	4.90±0.42^{b}	11.16±0.98^{e}	3.42±0.25^{g}	77.59±0.15^{b}	2.50±0.57^{f}	3.15±0.00^{d}	383.79±2.68^{d}
B15PB	4.77±0.37^{c}	11.90±0.98^{d}	3.03±0.35^{h}	76.32±0.66^{d}	3.33±0.36^{e}	4.26±0.57^{c}	379.31±2.61^{e}
B20PB	4.33±0.23^{d}	12.54±0.17^{c}	2.99±0.28^{h}	75.57±0.50^{ef}	4.16±0.57^{d}	4.86±0.76^{c}	375.35±2.43^{f}
ETB	4.16±0.23^{e}	13.69±0.33^{b}	6.01±0.02^{a}	72.81±0.28^{g}	2.29±0.05^{f}	3.33±0.57^{d}	400.09±1.44^{a}

Values are casted with average ± standard deviation per parameter. In columns, values with different letters are statistically different at statistical probability value of 5%. ***PB****: Baobab raw leaf powder;* ***A****: Plantain flour with 15% unfermented cashew kernel flour;* ***B****: Plantain flour with 10% fermented cashew kernel flour;* ***A10PB***, ***A15PB***, ***A20PB****: Flour A enriched with respective 10%, 15%, and 20% Baobab leaf powder;* ***B10PB***, ***B15PB***, ***B20PB****: Flour B enriched with respective 10%, 15%, and 20% Baobab leaf powder;* ***ETB****: Control Infant flour from Bledine.*

Table 2 displays the mineral content of the flours investigated. These minerals consist of Na, K, Ca, Mg, Fe and Zn. The incorporation of baobab leaf powder results in significant contribution ($P<0.05$) for the mineral interest of the composite enriched flours. Thus, potassium is the major mineral, with contents ranging between 384.37 (B10PB) and 539.76 mg/100 g (A20PB). However, the ETB control flour provides higher potassium content (626.17 mg/100 g) compared to the formulated composite flours. Calcium contents range from 149.21 mg/100 g (B10PB) to 281.59 mg/100 g (A20PB) and iron levels range from 6.62 mg/100 g (A10PB) to 9.80 mg/100 (A20PB), whereas they are respectively lower than 30 mg/100 g and 3 mg/100 in non-enriched composite flours. Also, with at least 15% incorporation of baobab powder, the formulated composite flours have iron contents (8.02 to 9.80 mg/100 g) comparable to the ETB control (8.92 mg/100 g). However, the ETB control flour has higher calcium content (695.33 mg/100 g) than the baobab fortified composite formulations. The magnesium and zinc contents of the composite flours are respectively between 93.49 mg/100 g (B10PB) and 113.35 mg/100 g (A20PB) and between 1.88 mg/100 g (B10PB) and 3.01 mg/100 g (A20PB). The tests show a greater presence of magnesium in the formulated flours, compared to the ETB control flour (81.91 mg/100 g). Also, the fortified flours have zinc levels (1.88 to 3.01 mg/100 g) close to that of the ETB control flour (2.39 mg/100 g). With the sodium content, the averages range from 8.21 mg/100 g (B10PB) to 13.45 mg/100 g (A20PB), which are statistically lower ($P<0.05$) compared to ETB control flour (115.49 mg/100 g).

In addition, enrichment with baobab leaves powder resulted in an increase in the vitamin C content of the composite flours formulated according to the incorporation rate (Table 3). Indeed, the vitamin C contents of the flours vary from 4.03 mg/100 g (A10PB) to 7.13 mg/100 g (B20PB) whereas without baobab powder they are worth hardly 2.5 mg/100 g. However, fortified flours have significantly ($p<0.05$) lower vitamin C than the control infant flour (45.13 mg/100 g). With the polyphenols compounds, Table 3 also indicates a significant ($p<0.05$) increase with the addition of baobab powder in the composite flours. The total polyphenols contents are between 630.45 mg GAE/100 g DM (B10PB) and 809.84 mg GAE/100 g DM (A20PB). The overall flours formulations have more significant polyphenols compounds compared to the control flour

sample. The antioxidant activity of composite flours (43.22% for A and 42.38% for B) is strengthened by the baobab powder. The fortified flours thus provide an antioxidant activity between 66.42% (B10PB) and 79.55% (A20PB), higher compared to the control flour (50.81%).

TABLE 2
MINERAL CONTENTS (mg/100 g) OF COMPOSITE FLOURS PROCESSED FROM PLANTAIN AND CASHEW KERNELS (FERMENTED AND UNFERMENTED), AND FORTIFIED WITH BAOBAB LEAVES POWDER

Flours	K	Ca	Mg	Na	Fe	Zn
PB	1672.54±1.10^{a}	1375.25±1.51^{a}	213.93±1.10^{a}	35.41±0.45^{b}	38.05±0.53^{a}	10.22±0.74^{a}
A	273.54±1.65^{i}	25.24±0.60^{f}	85.31±0.51^{def}	6.95±0.57^{ef}	2.78±0.02^{f}	0.69±0.07^{d}
B	248.68±0.90^{j}	21.48±0.55^{F}	78.27±0.63^{f}	5.89±0.05^{f}	2.60±0.01^{f}	0.55±0.06^{d}
A10PB	406.49±1.51^{g}	151.92±1.08^{e}	99.59±0.53^{cd}	11.86±0.57^{cd}	6.62±0.63^{e}	2.08±0.63^{bc}
A15PB	469.46±0.46^{e}	212.23±1.21^{d}	106.25±0.40^{bcd}	12.65±0.32^{cd}	8.54±0.06^{cd}	2.50±0.17^{bc}
A20PB	539.76±1.03^{c}	281.59±1.73^{c}	113.35±0.78^{b}	13.45±0.21^{c}	9.80±0.08^{b}	3.01±0.32^{b}
B10PB	384.37±1.06^{h}	149.21±1.00^{e}	93.49±0.40^{cde}	8.21±0.48^{ef}	6.43±0.57^{e}	1.88±0.26^{c}
B15PB	449.26±1.49^{f}	210.44±1.51^{d}	100.93±0.79^{bc}	9.20±0.35^{def}	8.02±0.15^{d}	2.38±0.95^{bc}
B20PB	516.81±1.58^{d}	276.96±1.63^{c}	107.72±1.02^{bc}	10.20±0.62^{cde}	9.64±0.05^{b}	2.90±0.69^{b}
ETB	626.17±0.51^{b}	695.33±1.15^{b}	81.91±0.62^{ef}	115.49±0.64^{a}	8.92±0.60^{c}	2.39±0.01^{bc}

TABLE 3
CONTENTS IN POLYPHENOLS COMPOUNDS, VITAMIN C, AND ANTIOXIDANT ACTIVITY OF THE STUDIED COMPOSITE FLOURS

Flours	Total polyphenols (mg GAE/100g DM)	Vitamin C (mg/100g)	Antioxydant activity (%)
PB	1003.07±0.59^{a}	22.35±0.26^{b}	85.33±0.05^{a}
A	387.01±0.60^{e}	1.29±0.44^{h}	43.22±0.51^{g}
B	388.65±0.71^{e}	2.58±0.44^{g}	42.38±1.29^{g}
A10PB	637.61±0.00^{d}	4.03±0.56^{h}	66.62±1.10^{e}
A15PB	742.66±0.00^{bc}	5.29±0.74^{e}	72.78±0.68^{c}
A20PB	809.84±0.98^{b}	6.06±0.61^{d}	79.55±0.37^{b}
B10PB	630.45±0.00^{d}	5.58±0.25^{de}	66.42±0.45^{e}
B15PB	697.34±1.06^{cd}	6.10±0.49^{d}	70.47±0.02^{d}
B20PB	765.56±0.83^{bc}	7.13±0.45^{c}	79.27±0.62^{b}
ETB	173.87±0.60^{f}	45.13±0.98^{a}	50.81±1.14^{f}

Values are casted with average ± standard deviation per parameter. In columns, values with different letters are statistically different at 5% significance. ***PB****: Baobab raw leaf powder;* ***A****: Plantain flour with 15% unfermented cashew kernel flour;* ***B****: Plantain flour with 10% fermented cashew kernel flour;* ***A10PB***, ***A15PB***, ***A20PB****: Flour A enriched with respective 10%, 15%, and 20% Baobab leaf powder;* ***B10PB***, ***B15PB***, ***B20PB****: Flour B enriched with respective 10%, 15%, and 20% Baobab leaf powder;* ***ETB****: Control Infant flour from Bledine;* ***GAE****: gallic acid equivalent;* ***DM****: dry matter.*

From the sensory analysis, the overall porridges acceptances are shown in Table 4. The formulations are variously appreciated. Thus, the enrichment with baobab powder decreases the full acceptance of the porridges the more the incorporation rate. Flours supplemented with 10% baobab leaf powder (A10PB and B10PB) record acceptance rating of 6.96/9 and 6.67/9 close to that of commercial flour ETB (7.28/9), and are the most preferred in both batches of composites flours formulations A and B. With the same rate of baobab incorporation, the slurries containing unfermented cashew meal (A) is statistically more enjoyed than the porridge with fermented cashew kernel flour.

The descriptive sensory parameters are exhibited by the sensory profiles drawn in Figures 1 and 2. The flours enrichment with baobab powder generates new sensory parameters (green color, bitter taste and glutinous aspect) which are globally not appreciated by the tasters. These descriptors are perceived accordingly to the rate of incorporation of the baobab leaf powder. Thus, the green appearance is rated from the composite porridges formulated between 3.68/9 (B10PB) and 5.25/9 (A20PB), while control flours (ETB and ETF) do not reveal any obvious green appearance (1.00/ 9). Regarding the flavors, the bitterness of the porridges is rated between 2.58/9 (A10PB) and 5.37/9 (B20PB); which is relatively lower compared to the sweet flavor perceived with indices of 6.18/9 (B20PB) to 6.75/9 (A10PB). The sticky texture is also mentioned from the formulations having baobab leaf powder, with scores ranging from 3.06/9 (B10PB) to 6.06/9 (A20PB). In addition, the more the rate of incorporation of baobab powder, the more the aroma and smooth texture are decreasing. The smooth texture of enriched composite porridges is rated with indices ranging from 6.82/9 (A10PB) to 4.81/9 (B20PB). The aroma records a sustained decrease in the perception degree from the composite flour A with scores dropping from 6.43/9 (A10PB) to 5.62/9 (A20PB), whereas the scores recorded from the flour B formulations range from 6.88/9 (B10PB) to 4.75/9 (B20PB). On the other hand, the incorporation of the baobab leaf powder accentuates the fluidity of the formulated meal mixes. The fluid texture is perceived with indices increasing from 6.95/9 to 7.42/9 and 6.88/9 to 7.13/9 for the respective enriched formulations deriving from flour A and flour B, according to the incorporation rate.

TABLE 4
SENSORY ACCEPTANCE OF PLANTAIN/CASHEW KERNELS COMPOSITE FLOURS PORRIDGES ENRICHED WITH BAOBAB LEAVES POWDER

Porridges	Overall sensory acceptance (/9)
A	7.07 ± 1.51^{c}
B	6.98 ± 1.46^{c}
A10PB	6.96 ± 0.93^{c}
A15PB	6.25 ± 1.54^{e}
A20PB	5.17 ± 0.68^{g}
B10PB	6.67 ± 1.43^{d}
B15PB	5.81 ± 1.45^{f}
B20PB	5.05 ± 0.56^{h}
ETB	7.93 ± 0.26^{a}
ETF	7.28 ± 0.39^{b}

Averages± standard deviations with different superscripts are statistically different at 5% significance. ***A****: Plantain flour with 15% unfermented cashew kernel flour;* ***B****: Plantain flour with 10% fermented cashew kernel flour;* ***A10PB****,* ***A15PB****,* ***A20PB****: Flour A enriched with respective 10%, 15%, and 20% Baobab leaf powder;* ***B10PB****,* ***B15PB****,* ***B20PB****: Flour B enriched with respective 10%, 15%, and 20% Baobab leaf powder;* ***ETB, ETF:*** *Control Infant flours from Bledine and Farinor.*

Since the porridges A10PB and B10PB are more enjoyed par tasters, they were used for the nutritional *in-vivo* analysis with rats. The nutritional values of the various diets provided to animals are recorded in Table 5. Rats fed with the ETB control diet have a more significant daily consumption (9.01 g/day) than those fed respectively with A10PB diets (5.16 g/day) and B10PB (5.89 g/day). The A10PB and B10PB diets provide the same daily amounts of total dry matter ingested. The highest levels of protein intake are achieved with the ETB control diet (1.22 g/day); and both A10PB and B10PB investigated diets result in similar amounts of protein ingestion (0.52 g/day and 0.65 g/day, respectively). All animals fed with the different diets gain weight. Animals fed at the A10PB and B10PB diets display respective mean weight gains of 0.68 g/day and 0.79 g/day. The rats with the highest growths are those subjected to ETB control diets (2.07 g/ day).

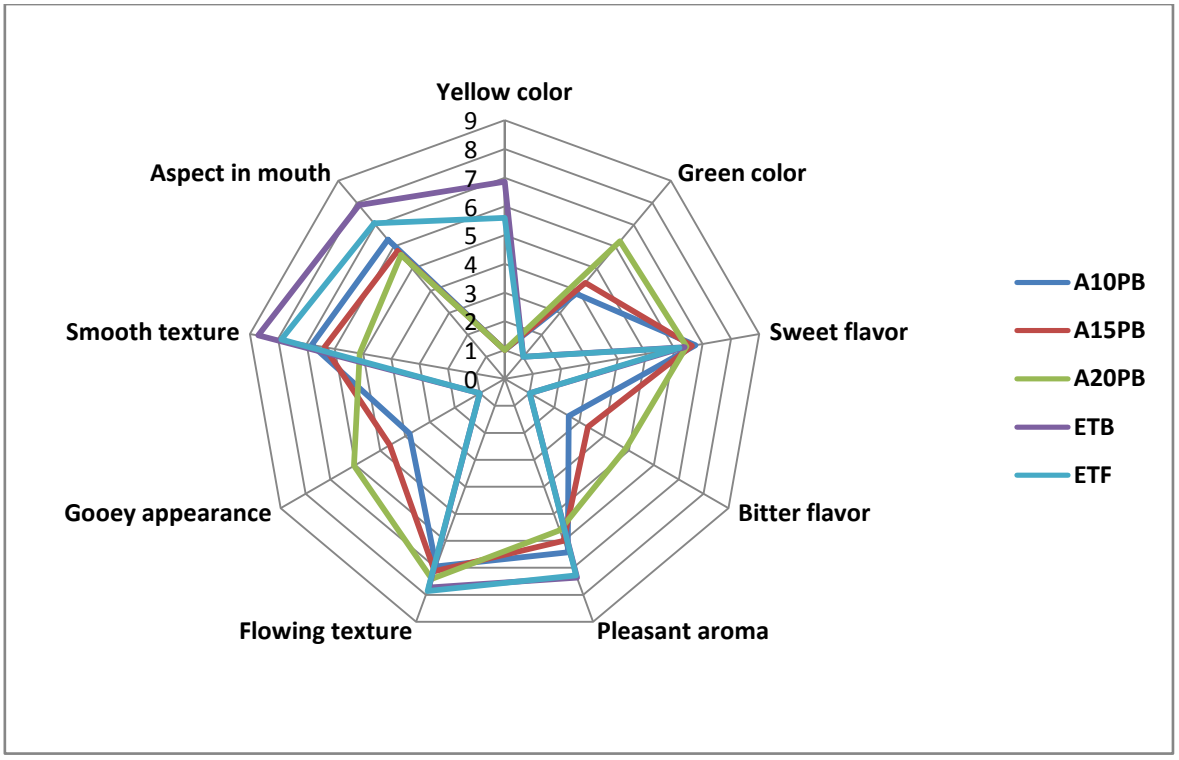

FIGURE 1: Sensory profiles of plantain/unfermented cashew kernels composite porridges enriched of baobab leaves powder and control flours

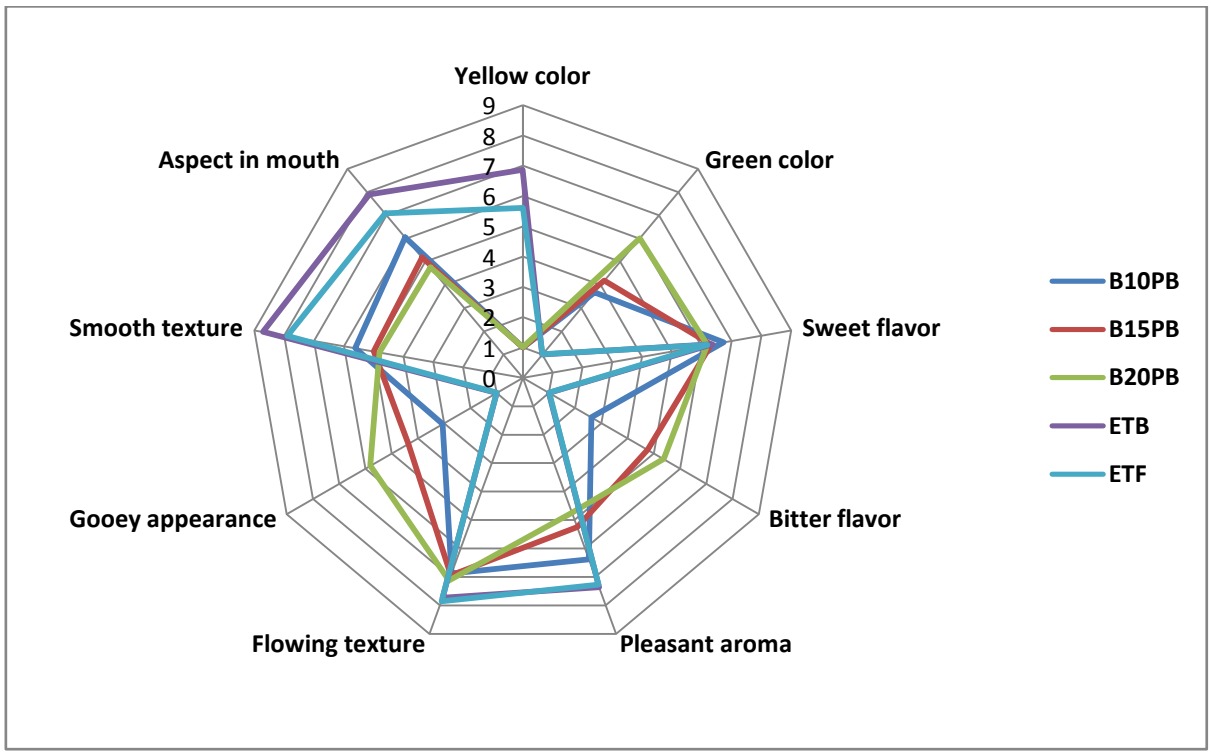

FIGURE 2: Sensory profiles of plantain/fermented cashew kernels composite porridges enriched of baobab leaves powder and control flours

A10PB, ***A15PB***, ***A20PB***: *Flour A enriched with respective 10%, 15%, and 20% Baobab leaf powder;* ***B10PB***, ***B15PB***, ***B20PB***: *Flour B enriched with respective 10%, 15%, and 20% Baobab leaf powder;* ***ETB, ETF:*** *Control Infant flours from Bledine and Farinor.*

The highest food efficiency coefficient (FEC) values are recorded from rats fed with the ETB diet (0.23). These values are significant ($p<0.05$) than those provided from fed diets A10PB (0.13) and B10PB (0.13). Similar to the FEC, the ETB control diet has the most protein efficiency coefficient (1.69).

TABLE 5
NUTRITIONAL VALUE OF PLANTAIN / CASHEW KERNEL COMPOSITE FLOURS ENRICHED WITH BAOBAB LEAVES POWDER

Parameters	ETB	A10PB	B10PB
TIDM (g)	126.14±2.01[a]	72.26±1.00[c]	82.46±1.04[b]
DIDM (g/day)	9.01±0.50[a]	5.16±0.11[b]	5.89±0.50[b]
TIP (g)	17.15±0.99[a]	7.30±0.20[c]	9.16±0.56[b]
DIP (g/day)	1.22±0.21[a]	0.52±0.50[b]	0.65±0.53[b]
FWG (g)	29.08±1.01[a]	9.58±0.59[b]	11.12±0.22[b]
DWG (g/day)	2.07±0.29[a]	0.68±0.58[b]	0.79±0.30[b]
FEC	0.23±0.35[a]	0.13±0.70[b]	0.13±0.97[b]
PER	1.69±1.92[a]	1.31±1.04[b]	1.21±1.07[c]

Per column, the averages ± standard deviations with different superscripts are statistically different at 5% significance. TIDM: total ingested dry matter, DIDM: daily ingested dry matter; TIP, total ingested protein; DIP, daily ingested protein; FWG: full weight gain; DWG: daily weight gain; FEC: food efficiency coefficient; PER: protein efficiency ratio.

***ETB:** Control Infant flour from Bledine; **A10PB, B10PB**: Respective Flours A and B enriched with 10% Baobab leaf powder.*

IV. DISCUSSION

The moisture of the flours studied is at the same range as the maximum limit of 5% required for the good preservation of infant flours (FAO/WHO, 2006). Indeed, the low moisture content of food products reduces the enzymatic and biochemical activities of the degradation microorganisms; with the advantage of preventing the deterioration of these products during storage (Kikafunda, 2006).

The incorporation of dried baobab leaf powder into composite flours A and B has significantly increased the protein, fibre and mineral contents in the formulated flours. These data strengthen previous studies considering baobab leaves as a natural reserve of minerals and proteins (Sidibé *et al*., 2002, FAO, 2012, Oulai *et al*., 2014). Regarding the protein content, the incorporation of baobab into composite flours A and B succeeded in infant flours with protein contents in accordance with the proteins amounts of 11-21 g/100g recommended by the FAO/WHO (2008) for weaning foods. These results are comparable to those obtained from the enrichment of *Ogi*, a corn and sorghum-based porridge in Nigeria, with moringa (*Moringa oleifera* L.) worked by Abioye and Aka (2015). Proteins being among the most important nutrients required in weaning foods, the high protein values observed in the current formulations are favorable for their valorization in the diet of young children. Thus, they could complete traditional foods mainly consisted of cereal and tuber porridges and causing protein-energy disorders among children weaned at low ages in developing countries (Anigo *et al*., 2009).

The dried baobab leaf powder used revealed fibre content (29.83g/100g) comparable to the value of 28.6 g/100 g reported by the FAO (2012). After enrichment, the fibre contents of the formulated flours comply with the international standard (≤ 5 g/100g) provided by FAO/WHO (2006) for complementary foods. Fibres regulate intestinal transit and capture some of the lipids and carbohydrates molecules, helping thereby the regulation of blood sugar levels and preventing the cholesterol excess (Ponka *et al*., 2016). They also have a positive effect against overweight and metabolic diseases due to their high degree of saturation (Henauer and Frei, 2008); and facilitate the hydration of feces (AFSSA, 2002).

The reduction of carbohydrate content in the formulated flours could logically be attributed to the lower presence of these molecules in the baobab powder, since the enrichment technique consisted in substituting a proportion of the composite flour by the baobab powder. Decreasing carbohydrate content, especially in starch, may reduce the water absorption from flours. Such a factor is advantageously used during preparation of the concentrate-based flours in dry matter (flour) to obtain a suitable fluidity for children. In addition, the decrease in lipid content is in accordance with many studies reporting that leafy vegetables are fat-free food resources (Ejoh *et al*., 1996). The lipid contents of overall formulated flours remain below the maximal limit of 8% admitted for the weaning foods (FAO/WHO, 2006). The low lipid content in foods could succeed in increasing their self-life by decreasing the rancidity ability.

The substitution of flour with baobab powder also resulted in a reduction of the energy value of the formulas obtained. Nevertheless, the energy values of the flours formulated in this work (375.35 to 385.85 kcal/100 g) are greater than the values of about 300 kcal/100 g resulting from the composite formulations worked by Mahan *et al*. (2016a) during enrichment trials of the flour deriving from young shoots tubers of Borassus aethiopum Mart with moringa leaves and cowpea beans. The energy values recorded from baobab-enriched flours are within the recommended range (344.4 to 473.81 kcal/100 g) for infant flours (Lutter, 2003); which is therefore favorable to the use of these flours against protein-energy malnutrition.

Moreover, the incorporation of baobab leaf powder increased the ash content of plantain/cashew nut flour, resulting in improvement of their mineral richness since minerals are the main ash components. The great minerals presence in baobab leaves has been highlighted by other works (FAO, 2012, Oulai *et al*., 2014) concluding on a good source of macroelements (potassium, calcium, magnesium) and oligoelements (iron, zinc, copper) for such a vegetable. The levels of potassium, magnesium and iron in baobab-enriched flours fill the recommendations of FAO/WHO (2006), even though their calcium and zinc contents remain below the standards. The food mineral nutrients are essential for the full metabolism in the body (Sowoola *et al*., 2002).

The ascorbic acid contents ran from 2.58 to 7.13 mg/100g, but remained significantly lower than the recommended value of 30 mg/100g (FAO/WHO, 2006). Ascorbic acid is an essential cofactor in various biological reactions and an antioxidant in the aqueous phase (Naziroglu and Butterworth, 2005). It participates in the absorption of iron in the gut and is necessary for the formation of collagen, the main protein of connective tissue that protects various organs (Shiriki *et al*., 2014). As a result, the consumption of fruit and vegetable juices, rich in this vitamin, in addition to the porridge is imperative for the well-being of children and infants.

The antioxidant properties of the methanolic extracts from the flours studied, although variable, forecast on the inhibitory ability of these flours against free radicals. The antioxidative ability of the composite flours is strengthened by the baobab leaf powder may because of its higher total polyphenol content (Oulai *et al*., 2014). Polyphenol compounds are important antioxidants known as protectors of biological macromolecules against degradation. Thus, they effectively fight against aging and the occurrence of cancer cells (Scalbert *et al*., 2005, Xia *et al*., 2011). Moreover, the affinity of polyphenols for free radicals is advantageous against the oxidation of low-density lipoproteins cholesterol (LDL-Cholesterol), which could conclude in the prevention of cardiovascular diseases (Kayodé *et al*., 2012). This antioxidant property is thus beneficial and helpful against carcinogenesis (Hooper and Cassidy, 2006). The regular consumption of these formulated flours as baby food-based could strengthen children's health.

The sensory evaluation showed a significant ($p<0.05$) influence of baobab fortification on the overall acceptance of the plantain-almond cashew composites meals (A and B). The maximum incorporation rate of 10% leafy vegetables is consistent with the work of Abioye and Aka (2015) when incorporating moringa leaf powder. Pleasant flavor and aspect are fundamental parameters in the appreciation of the porridge, especially from the children. Indeed, the sweet taste is an important parameter of habitual appreciation of the porridge. In addition, studies also indicate that taste for sweet foods is acquired as early as the birth (Nicklaus *et al*., 2005). Thus, the porridges of composite flours A10PB and B10PB, whose bitter taste is weakly expressed, are considered pleasant by overall consumers. The fair appreciation of the other porridges prepared could be resulted from the significant bitterness provided by the incorporation rates beyond 10% baobab leaf powder since baobab leaves displayed higher content of tannins, bitterness based compounds. These results are in agreement with those obtained by Mahan *et al*. (2016b), who showed that the bitterness is as strengthened as the porridges are incorporated with higher rate of moringa leaf powder. For the porridges texture, the glutinous aspect observed is due to the presence in the baobab leaves of mucilages which are hydrosoluble complex polysaccharides and lead to highly viscous solutions (Kerharo *et al*., 1974). The decrease of the aroma and taste of the porridges the more the incorporation rate are consistent with the work of Abioye and Aka (2015) and Ijarotimi and Oluwalana (2013). Thereby, the incorporation of natural aroma, natural flavor enhancers, and food grade oil could promoting infant formulas A10PB and B10PB as food support against child malnutrition. Such forecasting is as plausible as the ETB control commercial flour, which contains flavor and aroma, records a higher sensory acceptance and consumption compared to the formulated flours. These observations are consistent with the reports of Serna-Saldivar *et al*. (1999) who mentioned higher consumption values obtained by animals fed with casein diet (milk protein) compared to those receiving diets containing fortified breads with soy flour. The superiority of the rats' protein digestive use from the ETB control diet is explained by the good quality of the milk proteins. Indeed, these proteins have a good balance in essential amino acids with higher digestibility trait (Apfelbaum *et al*., 2004). However, despite the relative lower consumption index of the A10PB and B10PB formulations compared to the ETB control, they succeed in significant contribution in weight gain. These results confirm the nutritional and sensory quality recorded during biochemical and sensory tests of flours.

V. CONCLUSION

The results obtained in this study clearly indicate the nutritional benefits from the use of baobab leaf powder to enrich the plantain- cashew kernel composite meal. Among the substitution rates, the incorporation of 20% of baobab leaf powder into the composite flours of the plantain- cashew kernel meal improves significantly the protein, fiber, mineral and phytochemical

contents of the formulated flours, but formulations with 10% substitution by baobab powder are more enjoyed as the non-enriched samples. They provide acceptable indicators of growth and nutritional use for rats; which parameters are extrapolable to the human race. Exploitation of plantain-cashew nut meal enriched with 10% baobab leaf powder could therefore be recommended in nutrition programs to deal with children malnutrition concerns.

DISCLOSURE OF INTEREST

The authors state that they have no competing interests.

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Nutritional and Physiological Effects of Gradual Fish Replacement by Volvacea Volvaria Powder in Growing Rats

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***Abstract*—** *This work aimed to evaluate the effect of substitution of fish proteins by powder of Volvariella volvacea, an edible mushroom of Côte d'Ivoire, in the growing rats. The gradual substitution of fish proteins by mushroom proteins leads to a decrease in the growth performance of rats. With 75 % and 100 % mushroom in diets, body weight gain, food efficiency and protein efficiency ratio are negative. These diets do not affect the average weight of some organs (heart, liver and spleen), except for the kidney average weight that increases as mushroom levels increase in diets. Likewise, the average weight of abdominal fat decreases and disappears as the mushroom incorporation rate increases. The incorporation of mushroom powder into the control diet provokes changes in the mean value of some serum metabolites and electrolytes. The popular belief that states that mushrooms proteins can substitute meat proteins is not valid. Mushrooms can be proposed as dietetic regime for obese people.*

***Keywords*— Rats, Volvariella volvacea, growth, metabolites.**

I. INTRODUCTION

Mushrooms are part of Non-Timber Forest Products (NTFPs). They have long time played an important role in the survival of both rural and urban populations in Africa [1]. Furthermore, edible mushroom are rich in nutrients such as proteins [2] with a good proportion of essential amino acids and minerals [3, 4, 5]. They can be consumed in addition to cereals to meet the protein needs of poor populations [5]. [6] have argued that *Agrocybe chaxingu* may be a food source for protein enrichment and can therefore be classified as high protein foods for both humans and livestock. [7] stated that edible mushroom proteins are affordable and less expensive and can be consumed as a supplement or alternative to fish or meat. This work is carried out in order to study the performance of fish proteins replacement by the proteins of *Volvariella volvacea,* a widely known edible mushroom in Côte d'Ivoire.

II. MATERIAL AND METHODS

2.1 Animals

Growing male rats (*Rattus norvegicus*, *Muridae*, L.1753) of Wistar strain, aging between 50 and 60 days, weighing between 60 g and 70 g , were raised at the Laboratory of Nutrition and Pharmacology of the University Félix HOUPHOUET-BOIGNY. The experiment lasted 15 days, including three days of adaptation. The total number of animals used was 24 at the rate of 6 rats per group. They were housed in individual metabolism cages [8] with 12 hours of light and 12 hours of darkness. Animals received tap water *ad libitum*, and were fed every day, between 7 am and 8 am, and weighed every three days. The feed were weighed and served daily; the weight of the refused feed was used to determine the feed ingested. A scale from Denver (Germany), was used to determine weight of rats and feed (rats and feed).

2.2 Dietary treatments

After weaning, the animals were fed with pellets for rabbits manufactured by "IVOGRAIN" (Abidjan). For three days, the rats were all subjected to a unique diet, based on fish meal, in order to accustom them to experimental semi-synthetic diets. The different diets were prepared according to [9], with modifications (Table I). A total of 5 diets were tested during this experiment. A control diet (F) based on fish meal and four other diets containing different rates of *Volvariella volvacea* powder (25 %, 50 %, 75 % and 100 %) were formulated to provide 10 % of proteins to the rats. The preparation of diets consisted of mixing the different ingredients in a "Moulinex" brande blender (France), according to the proportions mentioned in the table 1. These ingredients were then transferred into a saucepan, and after homogenization in 1 L of water, the liquid mixture was then subjected to baking, on an electric stove, marked "IKAMAG" (Germany), until it was set in bulk. This feed was placed on a plate and stored in a refrigerator (4 °C). The preparation was renewed every 4 days. The dry matter of the feed was determined on 5 g of feed sample at 104 °C for 4 hours growth parameters were measured according to mathematical formulas (Table II). Dry matter intake (DMI) and average weight gain (AWG) were estimated by day and by rat.

TABLE 1
COMPOSITION OF DIETS

Ingrédients	Diet treatments (1 kg of dry matter)				
	F	25 %	50 %	75 %	100 %
Fish powder (g)	140.26	105.20	70.13	35.07	0
Volvariella volvacea powder (g)	0	209.03	418.06	627.09	836.12
Cornstarch (g)	784.74	620.77	446.81	281.84	78.88
Sugar (g)	9	9	9	9	9
Premix (g)	1	1	1	1	1
Agar agar (g)	5	5	5	5	5
Sunflower oil (ml)	50	50	50	50	50
Water (ml)	1000	1000	1000	1000	1000

*Protein level in diets: 10.00%; 25%, 50%, 75%, 100%: different protein inclusion rates of **Volvariella volvacea**; Energy F: 4072.078 kcal / kg; Energy 25%: 3790.6915 kcal / kg; Energy 50%: 3313.30199 kcal / kg; Energy 75%: 2871.91549 kcal / kg; Energy 100%: 2122.526 kcal / kg ; F: fish-based diet;*

Source: [9] Garcin et al. (1984).

TABLE 2
EXPRESSION OF NUTRITIONAL PARAMETERS

Nutritionnal parameters	Mathematical Expressions
Feed intake (FI) (g)	Feed given – Feed refused
Material moisture content (MMC) %	[(Fresh Material - Dry Matter) / Fresh Material] x 100
Dry matter ratio (DM) %	100 – MMC
Dry matter intake (DMI/) (g)	FI x DM
Protein intake (PI) (g)	TPI x % protein of diet
Average weight gain (AWG) (g)	Final weight – Initial weight
Feed efficiency (FE)	AWG / TDMI
Protein efficiency (PE)	AWG / TPI

2.3 Sampling of organs and dosage of serum metabolites

At the end of the experiment, all animals were subjected to a 16 hours fasting. Then, they were sacrificed after anesthesia with ethyl urethane (20 %), the next day, in the morning. Blood was collected in dry tubes. These tubes were centrifuged in a refrigerated centrifuge (4 °C). Serum collected was used for the metabolites and electrolytes assays, with a HITACHI 902 autoanalyzer (Roche, Japan). Later on, a longitudinal laparatomy was made on the rats, to isolate heart, liver, kidneys, Spleen and abdominal fat for biometry.

2.4 Expression and statistical analysis of results

The results presented here are in tabular forms and figures. Statistica version 7.1 was used for statistical analysis. The analysis of the variances (ANOVA), followed by the Newman-Keuls test (at the level of 5 %), was used respectively for the comparison of several means. The means are followed by their standard deviations. Two means are significantly different if the probability arising from the statistical tests is less than or equal to 0.05 ($P \leq 0.05$). Otherwise, these differences are not significant ($P > 0.05$). The letters a, b, c, d, e, etc., in super script, follow the means contrasts from Newman-Keuls tests in the tables. Means ± STD with different small letters within a row are significantly different ($P \leq 0.05$).

III. RESULTS

3.1 Effects of mushrooms on growth characteristics of rats

The evolution of rat growth in this experiment is illustrated in Figure 1. It's shown that, as the rate of incorporation of mushroom increased (25 %, 50 %, 75 % and 100 %), the growth of mushroom-fed rats decreased, compared to that of fish-fed rats (diet F). The average values of the growth characteristics of the rats are shown in Table III. As the mushroom incorporation rate in the control diet increased (25 %, 50 %, 75 %, and 100 %), final weight, ingested dry matter (IDM),

ingested protein values (IP), body weight gain (BWG) decreased in rats fed on these diets. The food efficiency (FE) and the protein efficiency ratio (PE) decrease proportionally. At 75 % of the mushroom incorporation rate, the FE and PE values were negative. Similarly, for 75 % of incorporation the average values of FE and PE were negative. For each nutritional characteristic, there were significant differences ($p \leq 0.05$) between the rats subjected to the five diets.

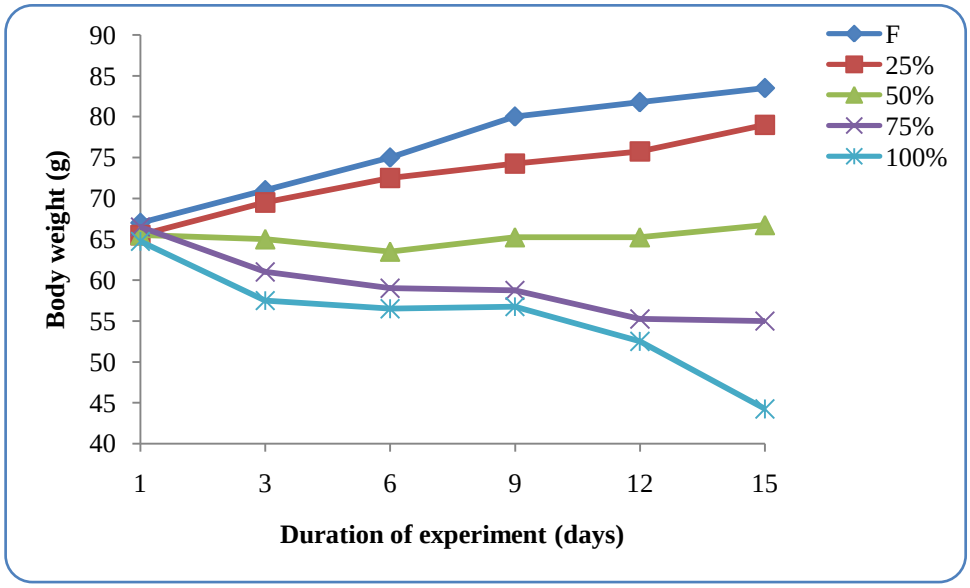

FIGURE 1: Growth of rats based on mushroom incorporation rate in the control diet

n = 6): Number of rats per treatment. ANOVA followed by the Newman-Keuls multiple comparison test at the 5 %. F: fish-based diet; 25%, 50%, 75%, 100%: different protein inclusion rates of ***Volvariella volvacea****.*

TABLE 3

AVERAGE VALUE OF GROWTH CHARACTERISTICS OF RATS

Parameters	Diet treatments				
	F (n=6)	**25 % (n=6)**	**50 % (n=6)**	**75 % (n=6)**	**100 % (n=6)**
Initial weight (g)	67.00±9.66^{a}	65.50±7.54^{a}	65.50±5,97^{a}	66.50±3.41^{a}	64.75±2.36^{a}
final weight (g)	85.50±6.95^{d}	79.00±8.98^{d}	66.75±7.45^{c}	55.00±4.83^{b}	44.25±3.09^{a}
DMI (g)	8.50	7.36	6.27	5.25	4.09
IP (g)	0.85	0.73	0.62	0.52	0.40
AWG (g)	1.10±0.40^{d}	0.90±0.17^{d}	0.08±0.11^{c}	-0.76±0.17^{b}	-1.36±0.31^{a}
FE	0.03±0.01^{d}	0.03±0.00^{d}	0.00±0.00^{c}	-0.03±0.00^{b}	-0.08±0.01^{a}
PE	0.32±0.12^{d}	0.30±0.05^{d}	0.03±0.04^{c}	-0.36±0.08^{b}	-0.83±0.18^{a}

(n): Number of rats per treatment. ANOVA, followed by the Newman-Keuls multiple comparison test at the 5%. On the same line, Means ± STD followed by different small letters within a row are significantly different (P≤0.05). F: fish-based diet; 25%, 50%, 75%, 100%: different inclusion rates of ***Volvariella volvacea*** *proteins. ; DMI: dry matter ingested per day per rat; PI: protein ingested per day per rat; AWG: Average weight gain per day per rat; FE: food efficiency; PE: protein efficiency coefficient.*

3.2 Effects of mushrooms on organs weight

The observation in Table IV indicates that, regardless of the rate of incorporation of mushroom in the control diet (fish), there is no significant difference ($p > 0.05$) between the mean weight of hearts and livers of the mushroom consuming rats, and those of the control fish fed rats. Table IV also shows that the average kidney weight increased significantly ($p \leq 0.05$) as the rate of mushroom incorporation increased in rat diets. In contrast, mean abdominal fat weights of rats were significantly reduced ($p \leq 0.05$) as the rate of mushroom incorporation increased in diets.

TABLE 4
EFFECTS OF EDIBLE MUSHROOM ON MEAN ORGAN WEIGHTS IN RATS

Parameters (% BW)	Diet treatments				
	F (n=6)	25 % (n=6)	50 % (n=6)	75 % (n=6)	100 % (n=6)
Heart	0,49±0,09	0,48±0,04[a]	0,65±0,25[a]	0,52±0,03[a]	0,65±0,10
Liver	3,90±0,54[a]	4,49±0,45[a]	4,13±0,18[a]	4,07±0,24[a]	3,72±0,10a
Kidneys	0,78±0,04[a]	0,88±0,08[b]	0,99±0,03[c]	1,03±0,01[c]	1,29±0,05[d]
Spleen	0,23±0,04[a]	0,25±0,04[a]	0,26±0,03[a]	0,25±0,04[a]	0,24±0,03[a]
Abdominal fat	1,24±0,29[c]	0,77±0,53[b]	0,28±0,30[a]	0,00±0,00[a]	0,00±0,00[a]

*(n = 6): Number of rats per treatment. ANOVA, followed by the Newman-Keuls multiple comparison test at the 5 %. On the same line, Means ± STD followed by different small letters within a row are significantly different (P ≤ 0.05). F: fish-based diet; 25 %, 50 %, 75 %, 100 %: different inclusion rates of **Volvariella volvacea** proteins; PC: body weight.*

3.3 Effects of mushrooms on the level of serum metabolites

Table V shows that the intake of 75 % of mushroom proteins in the diet of the rats causes a significant increase ($p \leq 0.05$) in the mean value of serum creatinine compared with rats that consume fish powder (P). Enrichment of rat diets with 25 % and 50 % resulted in a significant ($p \leq 0.05$) increase in mean uric acid values in rats compared with fish-fed rats. At 75 % and 100 % incorporation of mushroom into the control diet (fish), there was a significant decrease ($p \leq 0.05$) in the uric acid value in the rats. Incorporation of 25 % of mushroom proteins in the control diet (fish) resulted in a significant decrease ($p \leq 0.05$) in the mean value of total and conjugated bilirubins. Likewise, with 100 % mushroom protein, the average value of conjugated bilirubin decreases. Still in Table V, it is noted that the averages values of glucose, triglycerides, total proteins, total cholesterol, HDL-cholesterol, LDL-cholesterol and urea are not different ($p > 0.05$) from a batch of rats to another.

TABLE 5
EFFECTS OF MUSHROOM ON SERUM METABOLITES IN RATS

Parameters	Diet treatments (1 kg of dry matter)				
	F (n=6)	25 % (n=6)	50 % (n=6)	75 % (n=6)	100 % (n=6)
Glucose (g/L)	0.73±0.10[a]	0.67±0.03[a]	0.64±0.02[a]	0.69±0.04[a]	0.70±0.02[a]
Triglycérides (g/L)	0.40±0.10[a]	0.43±0.11[a]	0.68±0.21[a]	0.60±0.32[a]	0.46±0.08[a]
Total proteines (g/L)	68.75±5.73[a]	65.75±3.59[a]	70.75±4.34[a]	73.75±10.87[a]	68.00±4.08[a]
Tota-Cholesterol (g/L)	1.42±0.21[a]	1.64±0.24[a]	1.75±0.16[a]	1.62±0.31[a]	1.50±0.12[a]
HDL-Cholestérol (g/L)	0.32±0.06[a]	0.35±0.08[a]	0.34±0.07[a]	0.34±0.07[a]	0.31±0.03[a]
LDL-Cholestérol (g/L)	1.02±0.15[a]	1.20±0.19[a]	1.25±0.15[a]	1.14±0.21[a]	1.10±0.14[a]
Urea (g/L)	0.14±0.04[a]	0.25±0.01[a]	0.26±0.18[a]	0.45±0.24[a]	0.26±0.26[a]
Creatinin (mg/L)	6.75±0.95[a]	9.50±0.57[ab]	9.50±3.78[ab]	14.25±4.34[b]	9.50±1.29[ab]
Uric acid (mg/L)	37.25±7.41[a]	39.00±8.86[a]	42.50±5.44[b]	33.85±14.59[b]	35.25±5.05[b]
Total Bilirubines (mg/L)	14.00±3.55[c]	6.75±0.95[a]	11.25±2.50[bc]	15.25±1.70[c]	8.50±1.29[ab]
Conjugated bilirubin (mg/L)	3.47±0.62[bc]	1.36±0.07[a]	2.85±0.47[b]	4.15±0.86[c]	1.40±0.40[a]

*(n = 6): Number of rats per treatment. ANOVA, followed by the Newman-Keuls multiple comparison test at the 5 %. On the same line, Means ± STD followed by different small letters within a row are significantly different (P ≤ 0.05). F: fish-based diet; 25 %, 50 %, 75 %, 100 %: different inclusion rates of **Volvariella volvacea** proteins.*

3.4 Effects of mushroom on the activity of serum enzymes

In Table VI, there is no significant difference ($p > 0.05$) between the mean values of the activity of enzymes such as alkaline phosphatase (ALP), aspartate amino transferase (ASAT), alanine amino transferase (ALAT) and gamma glutamyl transpeptidase (γGT) of rats belonging to the five groups of rats (0 %, 25 %, 50 %, 75 % and 100 %).

TABLE 6
EFFECTS OF EDIBLE MUSHROOM ON THE MEAN ACTIVITY VALUE OF SERUM ENZYMES IN RATS

Parameters (UI/l)	Diet treatments				
	F (n=6)	25 % (n=6)	50 % (n=6)	75 % (n=6)	100 % (n=6)
ALP	188.50±91.54[a]	120.50±10.21[a]	137.75±40.72[a]	135.25±20.27[a]	132.25±24.17[a]
ASAT	51.75±55.45[a]	38.00±15.25[a]	34.75±22.91[a]	45.25±19.31[a]	41.50±9.53[a]
ALAT	36.50±26.60[a]	39.00±19.61[a]	42.00±15.53[a]	48.25±18.73[a]	40.25±9.74
γGT	49.25±24.25[ab]	33.50±11.67[a]	25.75±7.18[b]	34.75±7.27[b]	50.75±9.32

*(n = 6): Number of rats per treatment. ANOVA, followed by the Newman-Keuls multiple comparison test at the 5 %. On the same line, Means ± STD followed by different small letters within a row are significantly different (P≤0.05). F: fish-based diet; 25 %, 50 %, 75 %, 100 %: different inclusion rates of **Volvariella volvacea** proteins*

3.5 Effects of mushroom on the level of serum electrolytes

The mean values of the serum electrolytes (phosphorus, calcium, magnesium, iron, sodium, potassium) are shown in Table VII. Similarly, the incorporation of 25 % and 50 % mushroom protein levels resulted in a significant increase ($p \leq 0.05$) in mean serum of iron compared with 75 % in fish-fed rats and 100 %. Finally, the different incorporation rates (25 %, 50 %, 75 % and 100 %) of the mushroom do not affect ($p > 0.05$) the different mean serum values of the rats (P^{5+}, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and P^{5+}/Ca^{2+}).

TABLE 7
EFFECTS OF MUSHROOM ON SERUM ELECTROLYTES OF RATS

Parameters	Diet treatments				
	F (n=6)	25 % (n=6)	50 % (n=6)	75 % (n=6)	100 % (n=6)
P^{5+} (mg/L)	44.75±4.27[a]	46.25±6.39[a]	44.50±3.69[a]	48.00±2.94[a]	46.25±3.77[a]
Ca^{2+} (mg/L)	91.25±2.87[a]	91.25±5.12[a]	93.00±2.44[a]	92.25±4.34[a]	91.00±3.74[a]
Mg^{2+} (mg/L)	20.00±0.81[a]	20.75±0.95[a]	22.75±1.25[a]	21.50±1.29[a]	21.50±2.64[a]
Fe^{2+} (mg/L)	1.20±0.09[a]	1.61±0.31[b]	1.71±0.28[b]	1.19±0.11[a]	1.27±0.10[a]
Na^{+} (mEq/L)	139,25±2,62[a]	137,50±2,08[a]	139,50±3,10[a]	138,50±3,00[a]	140,25±1,70[a]
K^{+} (mEq/L)	4.17±0.30[a]	3.90±0.14[a]	4.30±0.60[a]	4.40±0.77[a]	3.87±0.09[a]
Ca^{2+}/P^{5+}	2.05±0.16[a]	1.99±0.25[a]	2.09±0.15[a]	1.92±0.16[a]	1.97±0.15[a]

*(n = 6): Number of rats per treatment. ANOVA, followed by the Newman-Keuls multiple comparison test at the 5%. On the same line, Means ± STD followed by different small letters within a row are significantly different (P≤0.05). F: fish-based diet; 25%, 50%, 75%, 100%: different inclusion rates of **Volvariella volvacea** proteins.*

IV. DISCUSSION

Evaluation of growth parameters such as final weight, ingested dry matter, ingested protein, body weight gain, feed efficiency and protein efficiency ratio shows that at the end of 15 days of experimentation, there is a significant difference between the control rats fed on fish and the rats that consumed supplemented mushroom diets. The performance of diets decrease when the rate of incorporation of mushroom (*Volvariella volvacea*) increase. Ingested dry matter and body weight gain decrease as the rate of incorporation of the mushroom increase. This decrease of intake diet resulted in a decrease of the average value of the proteins ingested. The dietary efficiency ratio and the protein efficiency ratio of the diets decrease significantly to reach negative values for diets with 75 % and 100 % incorporation of the mushroom. The dietary energy contribution decrease when the rate of insertion of mushrooms increase. This decline reflect in a decline of growth, due to an inefficiency of mushroom proteins to replace fish proteins [10]. Also, the increasing tannin of mushroom intake by rats would have inhibited protein efficiency [11, 12]. The gradual increase of the amount of mushroom in the diets of rats provokes an increase in the content of these diets in tannins and polyphenols [13, 14, 15, 16].

The average weight of the kidneys of the rats increase as the rate of insertion of the mushroom increase, and conversely, the average weight of rats abdominal fat is lower than that of rats consuming fish. This decrease in abdominal fat goes to a complete disappearance of abdominal fat at 75 % and 100 % incorporation rate of mushroom. The increase in the weight of the kidneys of rats is due to hyperactivity of the kidneys and the presence of antinutrients. On the other hand, the decrease of abdominal fat accumulation, or even its total disappearance in rats when the rate of incorporation of the mushroom increases energy would be the consequence of the low dietary energy intake of diets containing the mushroom.

The incorporation of mushroom provokes an increase in the serum mean value of uric acid and a decrease in the mean serum value of total and conjugated bilirubins. The decrease of the mean serum concentration of total and conjugated bilirubin is due to the presence of compounds such as flavonoids which, through their antioxidant effect, could play a protective role on liver cells [17, 18]. The increase of serum uric acid concentration think to be due to the catabolism of purine bases or nucleic acids. The increase of serum uric acid and creatinine would indicate renal failure in these rats [19]. The mean value of serum enzymes activity is not affected by the different treatments. The consumption of diets supplemented with the mushroom caused an increase in the average serum iron value. This lowering of the average iron value can be due to the disturbance of blood cell metabolism. The ratio of calcium serum to phosphoremia is close to 2. This ratio is an indicator of phosphocalcic metabolism. It would thus translate a good metabolism of these two minerals by hormones (parathyroid hormone and calcitonin). Nephrons would provide good control of plasma calcium and phosphorus levels [20, 21].

V. CONCLUSION

From this study, it's concluded that mushrooms consumption provoked low feed ingestion and consequently low growth. The results indicated that edible mushroom proteins cannot support the growth of rats. So, the popular belief that states that mushrooms proteins can substitute meat proteins is not valid. Mushroom-based diets may have possible negative effects on heart, kidneys and liver function. The absence of abdominal fat suggests that mushrooms can be proposed as dietetic regime for obese people. However, no serious affection of these edible compounds is noted on blood parameters.

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Effects of supplementation on the mycelial ergosterol content of *Agaricus bisporus* grown on media formulated with olive oil subproducts.

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***Abstract*—** Supplementation is one of the most relevant procedures for mushroom growth modulation. Little is known about the influence of supplementation on the concentrations of metabolites with potential health benefits. Experiments on compost require long cultivation times. Similar composition has been detected in fruiting bodies and mycelia. Therefore, the mycelial composition can be assumed to be similar to that of the fruiting bodies. This study examines the effect of supplementing a minimal defined growth medium with components derived from olive oil industry subproducts on *A. bisporus* mycelial composition, primarily ergosterol, when grown on minimal defined liquid and solid media in an attempt to obtain a higher concentration of ergosterol (pro-vitamin D2).

A. bisporus supplemented with alperujo meal (ALPM) and olive leaf meal (OLM) led to higher ergosterol content than that of the fungi grown in non-supplemented media (5.64±0.47, 6.60±0.86 and 4.08±0.53 mg/g p.s. in MDLm and 5.36±0.39, 6.79±0.41 and 4.22±0.43 mg/g p.s. in MDSm). Western blotting was used to validate the cultivation results. Three proteins (ERG2, ERG6, and EGR11) involved in the ergosterol biosynthetic pathway were significantly upregulated, indicating the importance of supplementation to ergosterol biosynthesis.

This report represents the first comprehensive study on the protein expression profiling of supplementation studies directed to improve metabolites with potential health benefits in *A. bisporus*. It provides new insights and a better understanding of the development of cultivation processes directed to increase ergosterol biosynthesis. These results could be used to obtain mycelia with higher vitamin D2 content after irradiation with UVB light.

***Keywords*— *Agaricus bisporus*, cultivation medium supplementation, ergosterol, olive leaf meal, two-phase pomace.**

I. INTRODUCTION

Until the last decades of the XX century, the growth of mushrooms on an industrial scale was based more on an art of cultivation than on scientific knowledge. It is known that mushroom growth is influenced by the composition of the growth media [1]. Supplementation is one of the most relevant of the different procedures used to modulate mushroom growth. This practice consists of adding specific substances to compost, during composting to increase nutrient availability and/or active substances, which, when consumed, improve performance and/or quality and probably increase the production of metabolites with health benefits. This technique was introduced in the 1960s [2]; [3]; [4], and from a practical point of view, some important aspects should be considered prior to its application, such as the types of nutrients required, the most suitable application time and economic costs and profits [5]. Most of the relatively few studies published on this topic focused on the productivity of the process. These processes result in yields that generally increase by 10–20% and occasionally by more [6], but little is known about the influence of supplementation on the concentration of metabolites with health benefits, such as ergosterol, ergothioneine, glucans, and chitin. Among the different raw materials that can be used for these purposes are agroindustrial subproducts such as cereal grain brands and meals, oilseed meals, cottonseed meal, and peanut oil and its derivatives, which contain varying amounts of the basic nutritional biomolecules, including carbohydrates, proteins, and lipids. In this study, we examined two agroindustrial subproducts of the olive oil industry that are highly abundant in southern Spain: two-phase pomace or alperujo (ALP) and olive leaf (OL), and its derivatives: alperujo meal (ALPM), olive leaf meal (OLM) and olive leaf hydroalcoholic extract (OLHAE).

Experiments leading to fructification (mushrooms) with edible fungi grown on compost require long cultivation times, and since the same components and metabolites with health benefits have been detected in the fruiting bodies and the mycelia

[1], the mycelial composition can be assumed to be similar to that of the fruiting bodies. Therefore, in this study, we examined the effect of supplementing minimal defined growth medium with different compounds derived from olive oil industry subproducts, ALPM, OLM and OLHAE, on *Agaricus bisporus* (*A. bisporus*) mycelial composition, primarily ergosterol. *A. bisporus* was grown on liquid and solid media as a preliminary step to the study on fruiting bodies after growth on compost supplemented with the same supplement, in an attempt to obtain mushrooms with higher ergosterol (pro-vitamin D2) concentration.

II. MATERIAL AND METHOD

2.1 Mushroom

The mushroom species used in this study was *A. bisporus* (J.E Lange) Imbach strain CBS 57166, obtained from Colección Española de Cultivos Tipo (CECT), Universidad de Valencia (Spain). The mycelia were grown and maintained on potato dextrose agar (PDA) plates at 28ºC. *A. bisporus* was maintained by replication onto new media once a week to keep the mycelia actively growing.

2.2 Agroindustrial subproducts and chemicals

The raw materials or agroindustrial subproducts and the supplements used in this study are shown in **Table-1**. ALP, the solid liquid waste or two-phase pomace generated by the two-phase method of olive oil extraction [7] and OL from Arbequina olive trees were supplied by the Instituto de la Grasa of Seville (Spain). ALPM, OLM and OLHAE were prepared in our laboratory using standard procedures. Briefly, ALP was dried by recirculating air at 50ºC in a drying tunnel until the weight was constant, and dried ALP was milled in a cutting mill (Retsch SM 100, Haan, Germany) and sieved through a 0.25 mm mesh to obtain the ALPM. Similarly, the OL was dried and ground and contained OLM.

2.3 Cultivation media

A. bisporus was grown in two types of cultivation media: minimal defined liquid medium (MDLm) and minimal defined solid medium (MDSm). The composition of MDLm was as follows: 0.5 g/L dextrose, 2.5 g/L potato extract, 0.45 g/L urea, $MgSO_4 \cdot 7\ H_2O$, 590 mg/L, $CaCl_2 \cdot 2\ H_2O$, 602 mg/L and 20 mg/L of mineral solution, pH 5.6. The mineral solution composition was as follows: $FeSO_4 \cdot 7\ H_2O$, 250 mg/L, $MnSO_4 \cdot H_2O$, 80 mg/L, $ZnSO_4 \cdot 7\ H_2O$, 70 mg/L, and $CoCl_2 \cdot 6\ H_2O$ 100 mg/L. The composition of the MDSm was similar but contained 15 g/L of agar. The cultivation media supplemented with ALPM, OLM and OLHAE used in this study is shown in **Table-3** and **Table-4**. Cultivation in MDLm was conducted in 500 mL flasks with 200 mL culture media at 28ºC, pH 5.6 and 120 rpm in a shaker for 7 days. Five millilitre samples were removed every day and analysed for biomass and ergosterol. Cultivation in MDSm was carried out in Petri dishes of 9 cm in diameter containing 20 mL culture media at 28ºC and pH 5.6 in a cultivation oven for 7 days. Samples (one Petri dish) were removed each day and analysed for biomass and ergosterol. The physical and chemical characteristics of the supplements used in this study are shown in **Table-2**.

TABLE 1
RAW MATERIALS AND SUPPLEMENTS USED ON MUSHROOM (*A. BISPORUS*) GROWTH MEDIA FORMULATION.

Agroindustrial subproduct	Supplement
Alperujo (ALP)	Alperujo meal (ALPM)
Olive leaf (OL)	Olive leaf meal (OLM)
Olive leaf (OL)	Olive leaf hydroalcoholic extract(OLHAE)

TABLE 2
PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE SUPPLEMENTS ASSAYED IN THIS STUDY.

	ALP	OLM	OLHAE
Dry matter[&] (%)	88.7±3.8	93.2±1.1	22.3±3.7
Moisture* (%)	11.3±3.8	6.8±1.1	77.7±3.7
Ash* (%, d.w)	11.4±1.2	9.4±3.1	5.2±0.8
Organic matter[&] (%, d.w.)	88.6±1.2	90.6±3.1	94.8±0.8
Total-N* (%, d.w.)	1.5±0.2	1.9±0.3	3.4±0.4
Protein[&] (N_t x 5.5) (%, d.w.)	8.3±0.9	10.5±1.3	18.7±1.8
Carbohydrates* (%, d.w.)	50.2±3.6	46.6±4.2	59.4±3.8
Lignin (%, d.w.)	21.7±3.2	17.2±1.9	-
Fat* (%, d.w.)	6.8±0.8	5.3±0.6	13.8±1.6
Others[&] (%, d.w.)	1.6±	3.8±	2.9±
Polyphenols (mg/g, d.w.)*	12.4±3.7	23.7±3.6	240.1±15.1
C/N*	9.7±2.4	15.3±3.1	4.1±0.7
pH*	5.7±0.3	6.9±0.2	7.1±0.2

**Experimental data are the mean of at least three experiments; [&]calculated data.*

2.4 Proximate composition of supplements

The moisture content was measured using a moisture analyser (Mettler Toledo, Barcelona, Spain). The total nitrogen content was determined using an AOAC 920.87 semi-micro Kjeldahl method [8] with a conversion factor of 5.5 to transform nitrogen into protein. The ash content was determined using the direct ashing method of AOAC [9]. The fat content was measured using an automated Soxhlet extraction apparatus (Soxtec™ 2050 FOSS, Hillerød, Denmark). Fat was extracted from 3 g of sample with 100 mL of petroleum ether at 90 °C for 180 min, and the defatted sample was collected and dried under nitrogen gas.

2.5 Carbohydrate determinations

The total carbohydrate content of the mycelial samples was determined using the phenol-sulfuric acid method [10], adapted to microplate analysis. Briefly, 1 mg samples were mixed with 1 mL of MilliQ water and stirred for 2 min. Twenty-five microlitres of the mixtures were added to a 96-well plate along with 25 µL of 5% phenol solution (w/v) and 125 µL of concentrated H_2SO_4. The plate was sealed and incubated in a water bath at 80 ºC for 30 min. Sample absorbance was determined using a microplate reader (Biorad Model 680) at 595 nm. A standard curve of D-glucose (0.03 to 1.0 mg/mL) was used for quantification.

2.6 Total phenol content

The total phenol concentration was determined using the Folin-Ciocalteu method according to standard procedures [11]. Gallic acid was used as the standard for quantification.

2.7 Soluble protein determination

Total soluble protein concentration was determined using the Bradford method according to standard procedures using bovine serum albumin as the standard [12].

2.8 Ergosterol determination

2.8.1 Sample extraction

The extraction method used is a modification of the direct hexane extraction method proposed by Shao et al. [13]. Briefly, 2.5 mg of mushroom powder sample was vortexed with 6 mL of hexane for 2 min, centrifuged at 4000 rpm for 15 min, and the supernatant was transferred to a 25 ml beaker. The mushroom residue was then extracted twice using 6 mL hexane each time.

The three hexane phases obtained were pooled and dried under a nitrogen stream. The extract was dissolved in 1.5 mL of methanol. This solution was homogenised in an ultrasonic bath and then filtered through 0.22 µm PVDF filters (VWR, Spain) before chromatographic analysis. To carry out the internal standard (I.S.) calibration method, adequate volumes (µL) of 100 mg L^{-1} standard solution of vitamin D3 was added to the mushroom powder before extraction, so that the final concentration of the I.S. was 1 mg L^{-1}.

2.8.2 Liquid chromatography-mass spectrometry analysis

The analysis of ergosterol and ergocalciferol, using cholecalciferol as an internal standard, was performed in an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to a Xevo G2S QTOF mass spectrometer (Waters, Micromass, Manchester, UK) with a dual electro spray chemical ionisation (ESCI) in positive mode. The LC separation was performed using a conditioned autosampler at 5ºC and a Synergi Hydro-RP column (100 mm x 3.00 mm id, 2.5 µm particle size), (Phenomenex, CA, USA), thermostated at 35ºC. The mobile phase consisted of aqueous formic acid solution 0.1% (solvent A) and methanol (solvent B). The gradient elution starts at 1% A, maintaining these conditions for 4 min and then, increasing % A from 1% to 10% in 1 min maintaining conditions for 5 min at a flow rate of 0.5 mL min^{-1}. The injection volume was 5 µL. To avoid cross-contamination, pre and post-inject washes were conducted. MS conditions were as follows: nebulisation gas (nitrogen) 700 l/h, cone gas (nitrogen) 29 L h^{-1}, desolvation temperature 500 ºC, source temperature 150 ºC, corona current was set at 5 mA and sample cone voltage at 40 V. MS spectra were collected in a continuum between 50 and 750 Da with a scan time of 1.55. Ergosterol, ergocalciferol and cholecalciferol were identified using retention times of 9.13 min for ergosterol; 4.68 min for ergocalciferol and 5.06 min for cholecalciferol, as well as the accurate mass of each compound (379.3377 for ergosterol; 397.3462 for ergocalciferol and 385.3463 for cholecalciferol).

2.9 Western blot analysis

Total soluble protein was resolved on 12.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) as described by Laemmli [14] and electro-transferred to nitrocellulose membranes. Western blot analysis was performed as described by Haid and Suissa [15]. Key enzymes related to ergosterol biosynthesis were detected using rabbit antisera as the primary antibodies directed against ERG2, ERG4, ERG6 and ERG11 (**Figure 1**). The primary antibodies were detected using peroxidase-conjugated goat anti-rabbit IgG as a second antibody and SuperSignal® West Pico Chemiluminescent substrate solution.

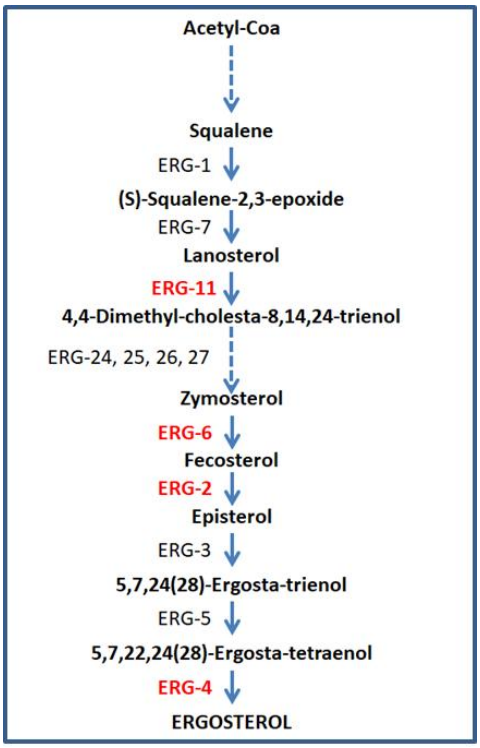

FIGURE 1: REPRESENTATIVE ENZYMES IMPLICATED IN ERGOSTEROL BIOSYNTHESIS IN FUNGI.

(ERG1 − squalene epoxidase; ERG2 − sterol C-8 isomerase; ERG3 − sterol C-5 desaturase; ERG4 − sterol C-24 reductase; ERG5 − sterol C-22 desaturase; ERG6 − sterol C-24 methyltransferase; ERG7 − lanosterol synthase; ERG11 (CYP51) − lanosterol C-14 demethylase; ERG24 − sterol C-14 reductase; ERG25 − sterol C-4 methyl oxidase; ERG26 − sterol C-3 dehydrogenase (C4-decarboxylase); ERG27 − sterol C-3 ketoreductase).

2.10 Statistical analysis

The results are presented as the mean value ± standard deviation (SD). Statistical analyses were performed using a 2-way analysis of variance (ANOVA) with a subsequent Bonferroni post hoc test for pairwise comparisons between various combinations of two groups. A *p*-value of < 0.05 was considered statistically significant. Statistical analyses were performed using the SPSS 16.0 software.

III. RESULTS AND DISCUSSION

3.1 Preparation and characterisation of supplements

The first objective of this study was to collect enough raw materials (agroindustrial subproducts) to obtain supplements for all experiments planned. These included growth on liquid- and solid-minimal medium and, in the future, in compost. Using this strategy reduced the variability in the raw material composition, which can occur when subproducts obtained at different periods of times were used. The agroindustrial subproducts used in this study were ALP and OL, and the supplements derived from them were ALPM, OLM and OLHAE (**Table-1**). These subproducts were selected because they are highly abundant in southern Spain, cheap, and their composition can influence the growth of *A. bisporus* [16]. Although available seasonally, further processing can provide enough supplements to use for a long period of time, such as an entire year. For example, ALPM and OLM can be processed to a stable dry form or into a liquid form or syrup, such as OLHAE, that remains stable for long periods.

The physical and chemical characteristics of the supplements used in this study are shown in **Table-2**. As these results show, the properties and composition of the supplements vary from each other in their stabilised forms, while ALPM and OLM are solids (meals), and OLHAE is liquid. Thus, its composition depends on the components of the raw materials (ALP and OL). Stabilisation is required to keep these supplements from degrading for one year. Therefore, the water content was reduced by air drying and constitutes the primary cost of the process.

ALP is a subproduct from the olive oil industry, generated by oil centrifugation, with a high water content of approximately 60%. After drying, the moisture is reduced to 11.3±3.8%. The dry matter that constitutes 88.7±3.8% of the ALPM is comprised primarily of carbohydrates (50.2±3.6%), lignin (21.7±3.2%), proteins (8.3±0.9%), fat (6.8±0.8%) and ash (11.4±1.2%). OL provides similar results. The fresh leaves contain approximately 15% of water, and after air drying and milling, it can be stabilised as OLM with a moisture content of 6.8±1.1%. The dry matter that constitutes 93.2±1.1% of the OLM is comprised primarily of carbohydrates (46.6±4.2%), lignin (17.2±1.9%), proteins (10.5±1.3%), fat (5.3±0.6%) and ash (9.4±3.1%). OL milling to a fine powder (OLM) allows the hydroalcoholic extraction of bioactive compounds. The extract that occurs after ten-fold concentration by vacuum is a syrup containing a high water content (77.7±3.7%) and, consequently, a lower percentage of dry matter (22.3±3.7%), comprised primarily of carbohydrates (59.4±3.8%), proteins (18.7±1.8%), fat (13.8±1.6%) and ash (8.8±2.3%).

The analysis of the organic matter shows that the level in ALPM (88.6±1.2%) is lower than that in OLM and OLHAE (90.6±3.1% and 94.8±0.8%, respectively) due to its higher ash content.

The fat contents of the three supplements are 6.8±0.8%, 5.3±0.6% and 13.8±1.6% in ALPM, OLM and OLHAE, respectively. The relatively low fat content in ALP (6.8±0.8%) can be explained because developing fats is the primary objective during olive oil production, and minimal fat loss is desired during the oil recovery process. OLM shows the lowest fat content (5.3±0.6%) since the fat content is low in the OL. However, the highest lipid concentration (13.8±1.6%) was found in the OLHAE, since a lipophilic solvent (ethanol) was used in the hydroalcoholic extraction medium.

The protein contents in the ALPM, OLM and OLHAE supplements are 8.3±0.9%, 10.5±1.3% and 18.7±1.8, respectively. These values were obtained by multiplying the respective total nitrogen (N_t) value by the more convenient conversion factor 5.5 for vegetable proteins, rather than by 6.25, which is appropriated for animal proteins [17].

The carbohydrate content was 50.2±3.6%, 46.6±4.2% and 59.4±3.8% in ALPM, OLM and OLHAE, respectively. The primary carbohydrates present in the supplements contain cellulose and hemicellulose with a low concentration of low molecular weight saccharides. The lignin content was 21.7±3.2% and 17.2±1.9% in ALPM and OLM, respectively. OLHAE, due to the nature of its preparation process, does not contain lignin.

The C/N ratio is an important factor for mycelial growth, and fungi grow well when its values are approximately 10 to 12 [18]. Thus, our supplements result in the following values: 9.7±3.4%, 15.3±3.1% and 4.1±1.3% for ALPM, OLM and

OLHAE, respectively. Supplementation with OLHAE could explain the faster growth and higher productivity that *A. bisporus* shows in media supplemented with OLM and ALPM (data not shown). However, the objective of the supplementation in this study was not nutritionally related but instead examined the utilisation of stress-inducing factors that could potentiate the increase in metabolites such as ergosterol and ergothioneine. Thus, substances such as polyphenols and other non-identified metabolites present in the supplements could be of special interest for those purposes. The concentration of polyphenols in the supplements varies from 12.4±3.7 mg/g, 23.7±3.6 mg/g and 240.1±15.1 mg/g in ALPM, OLM and OLHAE, respectively. The higher value observed in OLHAE is related to the extraction and concentration processes that facilitate the extraction of such types of compounds. As we will demonstrate, a relatively high polyphenol content, as shown in ALPM and OLM, induces ergosterol biosynthesis; however, very high content, as is the case with OLHAE, could be less effective, probably due to its action as pro-oxidants rather than as antioxidants [19].

3.2 Effects of supplementation on *A. bisporus* growth and mycelial composition

The modification of MDLm and MDSm was conducted once the supplements had been prepared and characterised (see **Tables-3** and **-4**). Although the primary objective of this study was to examine the effect on the biosynthesis of some components with health benefits, such as ergosterol, the precursor of vitamin D2, and ergothioneine, the effects on biomass production were also considered.

Methods for increasing the productivity of any one metabolite of interest, such as ergosterol, are generally based on two approaches: i) engineering metabolic pathways [20], and ii) utilising a physiological approach to exploit knowledge about the physiology of the mushroom strain and its response to changing environmental conditions [21]. Since the genetics and biochemistry of *A. bisporus* have not been fully developed, we used the physiological approach to study the effect of a group of supplements derived from the olive oil industry (ALPM, OLM and OLHAE) on the synthesis of ergosterol, in an attempt to obtain *A. bisporus* with higher ergosterol concentration.

A. bisporus mycelia were growth on MDLm supplemented with different amounts of ALPM, OLM and OLHAE as shown in **Table-3** at pH 5.5, 28ºC and gentle agitation (120 rpm) for 7 days. As observed from these results, supplementation with ALPM, OLM and OLHAE at 0.1%, 0.5%, 1% and 2% significantly increased the biomass and ergosterol content compared to the non-supplemented control culture. Although the increase in the biomass was more significant in ALPM and OLM, probably due to their higher C/N rates of 9.7±2.4 and 15.3±3.1, with an increased amount of supplementation, the ergosterol concentration reaches its maximum at 1% supplementation. A higher concentration does not lead to the production of more ergosterol, and it reached a plateau at 1% supplementation. Therefore, we selected 1% supplementation for further studies.

TABLE 3
***A. BISPORUS* GROWTH ON MDLM SUPPLEMENTED WITH DIFFERENT SUBSTRATES AND CONCENTRATIONS.**

Culture media	Biomass (mg/L)	Ergosterol (mg/g, d.w.)	n
MDLm (control)	7.8±0.6	4.08±0.53	4
MDLm +ALPM (0.1%)	9.7±0.7	4.86±0.93	4
MDLm + ALPM (0.5%)	11.9±1.1	5.61±0.89	6
MDLm +ALPM (1.0%)	12.2±0.9	5.64±0.47	6
MDLm +ALPM (2.0%)	12.7±1.4	5.59±0.35	4
MDLm + OLM (0.1%)	10.2±0,8	5.14±0.53	4
MDLm + OLM (0.5%)	12.1±1.3	6.23±0.47	6
MDLm + OLM (1.0%)	12.6±0.5	6.60±0.86	6
MDLm + OLM (2.0%)	12.9±2.1	6.62±0.56	4
MDLm + OLHAE (0.1%)	8.8±0,9	4.92±1.33	4
MDLm + OLHAE (0.5%)	9.3±1.2	5.31±0.61	4
MDLm + OLHAE (1.0%)	9.6±1.1	5.87±0.91	4
MDLm + OLHAE (2.0%)	9.9±2.2	5.85±0.78	4

A. bisporus mycelia were grown on MDSm supplemented with different amounts of ALPM, OLM and OLHAE, as shown in **Table-4**, at pH 5.5, 28ºC without agitation and in darkness for 7 days. The results of this experiment were similar. These results show that supplementation with ALPM, OLM and OLHAE at 0.1%, 0.5%, 1% and 2% significantly increase the biomass and ergosterol content in comparison with the non-supplemented control culture. In this case, the biomass also increases more significantly in ALPM and OLM than in OLHAE, probably due to its higher C/N rate, 9.7±2.4 and 15.3±3.1, respectively, which increased with the degree of supplementation. Ergosterol concentration reaches its maximum at 1% supplementation, and a higher concentration of supplements did not result in higher ergosterol content, which reached a plateau at 1% supplementation. Therefore, in this case, we also selected 1% supplementation for further studies.

The expression of several enzymes implicated in ergosterol biosynthesis was studied to investigate the reason for the observed increase in ergosterol biosynthesis and/or accumulation in *A. bisporus* grown in media supplemented with OLM and ALP. For this purpose, we prepared antibodies against four of the key enzymes (**Figure 1**) implicated in ergosterol biosynthesis, ERG2, ERG4, ERG6 and ERG11, to use for a Western blot analysis. Due to the difficulty of purifying these enzymes implicated in the biosynthesis of this phytosterol, we choose a procedure to produce the antibodies based on antibody preparations against synthetic peptides with high immunogenicity [22] towards the different enzymes.

TABLE 4
***A. BISPORUS* GROWTH ON MDSM SUPPLEMENTED WITH DIFFERENT SUBSTRATES AND CONCENTRATIONS.**

Culture media	Biomass (mg/L)	Ergosterol (mg/g, d.w.)	n
MDSm (control)	6.6±0.4	4.22±0.43	3
MDSm +ALPM (0.1%)	8.7±0.8	5.07±0.71	4
MDSm + ALPM (0.5%)	8.9±0.6	5.21±0.33	4
MDSm +ALPM (1.0%)	9.2±0.2	5.36±0.39	4
MDSm +ALPM (2.0%)	9.5±0.5	5.42±0.41	3
MDSm + OLM (0.1%)	8.6±0.6	5.22±0.34	4
MDSm + OLM (0.5%)	8.9±0.5	6.45±0.55	4
MDSm + OLM (1.0%)	9.4±0.7	6.79±0.42	4
MDSm + OLM (2.0%)	9.7±0.3	6.83±0.65	3
MDSm + OLHAE (0.1%)	7.1±0.2	4.53±0.81	4
MDSm + OLHAE (0.5%)	7.3±0.3	4.72±0.65	4
MDSm + OLHAE (1.0%)	7.6±0.5	4.93±1.01	4
MDSm + OLHAE (2.0%)	7.8±0.4	5.13±0.32	3

Our results show that all of the antibodies produced recognised the synthetic peptide by a dot analysis, even at a level of 5 ng (**Figure 2**). However, when we assayed the antibodies against the *A. bisporus* enzymes, only three of them recognised any protein using Western blot analysis, anti-ERG4, anti-ERG6 and anti-ERG11, (Figure-3), probably due to the localisation of the immunogenic peptides at the molecular surface. In contrast, the selected anti-ERG4 peptide could be occulted in the interior of the molecule and thus not recognised by anti-ERG-2.

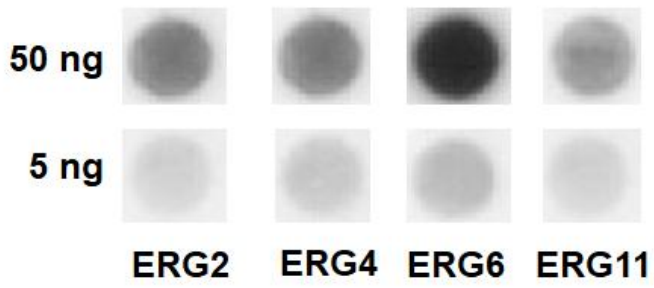

FIGURE 2: DOT ANALYSIS USING 5 AND 50 NG OF SYNTHETIC PEPTIDES AND DETECTION BY THEIR ANTIBODIES (ANTI-ERG2, ANTI-ERG4, ANTI-ERG6 AND ANTI-ERG11).

Western blot analysis also showed that ERG6 (C-24 sterol methyltransferase) and ERG11 (cytochrome P450 lanosterol C-14α-demethylase) were expressed at a higher level than in the control (2.75-fold and 3.52-fold, respectively) in all three supplemented culture media assayed, both in MDLm (**Figure-3**) and MDSm (data not shown) than ERG2 (sterol C-8 isomerase). Although the ERG2 expression level was 1.85-fold higher than that in the control, it is lower than that observed for the other two enzymes analysed. From these results, we concluded that at least three proteins (ERG2, ERG6 and ERG11) involved in the ergosterol biosynthesis pathway were significantly upregulated, indicating the importance of activating ergosterol biosynthesis by supplementation with ALPM, OLM and OLHAE.

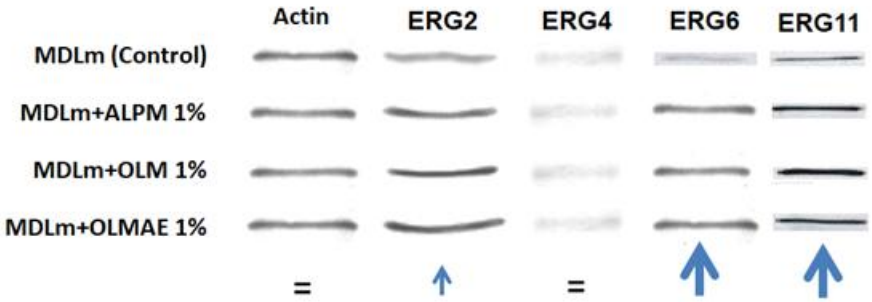

FIGURE 3: WESTERN BLOT ANALYSIS OF *A. BISPORUS* KEY ENZYMES (ERG2, ERG4, ERG6 AND ERG11) IMPLICATED IN ERGOSTEROL BIOSYNTHESIS

IV. CONCLUSION

From these results, we can conclude that supplementation with ALPM, OLM and OLHAE upregulate some of the key enzymes implicated in ergosterol biosynthesis (ERG2, ERG6 and ERG11), but we do not know what compound is responsible for this upregulation and the subsequent increase in ergosterol production. Therefore, more research is necessary to increase our knowledge in this field. To the best of our knowledge, this report represents the first comprehensive study on the protein expression profiling of supplementation studies directed to improve the production of metabolites with potential health benefits. It provides new insights into a better understanding of the development of cultivation processes directed to increase ergosterol biosynthesis, which is a prerequisite for obtaining mushrooms with high vitamin D2 levels, sufficient for a daily dose (15 µg) in one portion (100 g w/w.).

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