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MODIFICATION OF GROWTH AND YIELD OF THE LEAFY VEGETABLE UNDER PHOSPHOR-CONVERTED LIGHT-EMITTING DIODE*

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Key words: phosphor converted-LEDs, leafy vegetable, ground biomass, R:B LED, Far-red radiation, yield.

Abstract

Leafy vegetable are desirable crops for daily consumption due to their nutritive value. This study aims to illustrate that the commercial phosphor converted-LEDs (pcH-LED) could improve the yield of the leafy greens in ground biomass and their morphological response of leaf and root. The experimental setup were set under three treatments which are pcH-LED, R:B LED and Fluorescent in the same environment. The results indicated that the Far-red radiation from pcH--LED can have positive effects on crop quality. It could promote the highest fresh weight, perfect leaf size, and nice leaf color of the Butterhead lettuce, Cos lettuce, Red oak and Green oak lettuce. The pcH-LED and R:B could produce the same quantity in leaf number, leaf length and root number, but R:B show negative effect to Cos lettuce resulting in shorter leaf and tiny size. Based on the growth and yield of leafy vegetable for indoor plantation, the pcH-LED is recommended to be a light source.

Introduction

Red (600–700 nm) and blue (400–500 nm) light are very interesting wavelengths for the growth and development of plants. Many previous studies have shown that plants can grow normally in combination with R and B radiation. This is especially the case for leafy vegetables where when exposed to a combination of R and B light, higher yield of the vegetable was observed than leafy vegetables under sole R (red) or sole B (blue)

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light (LI et al. 2017, SAITO et al. 2010). Moreover, the combination of R and B could promote the net photosynthesis and chlorophyll content of the plants (HE et al. 2017, LI et al. 2017).

The mixed-color method has been used to construct the R:B LED light for the horticultural applications. R and B LEDs can be mixed and assembled on the aluminum PCB (print circuit board). Subsequently, they can be installed on the heat sink. The LED drivers and the cooling system are also needed in the set up. Consequently, the PWM technique could be provided to control the level of the photon flux density.

Nowadays, the LED chip with phosphor converted (pc) technology was applied for the development of LED for horticultural purposes. These pc-LEDs are known fully as phosphor converted light emitting diodes. The red phosphor coated on the blue LED chip makes it possible for the R and B photon flux to be emitted from the single chip. This light source can appropriately be applied to the indoor plantation (LI et al. 2017).

The pc-LEDs has been commercialized, for example: LUXEON SunPlus (Lumileds) which is a full spectrum white-based horticulture light (white plus red light) from SAMSUNG and CREE. The pc-LEDs could produce the light spectrum to cover the photosynthetic active radiation (PAR) in the wavelength of 380–780 nm (MCCREE 1972). This is important because the FR light(701–780nm) could promote the highest fresh weight, dry weight, stem length, leaf length and leaf width of the lettuce (MICKENS et al. 2018, LI and KUBOTA 2009). The additional of far--red radiation has promotive effects on leaf expansion, which increases radiation capture and can increase the dry mass gain (PARK and RUNKLE 2017, 2018, ZOU et al. 2019).

The advantages of pc-LEDs are multifold. Firstly, pc-LEDs are easy to assemble. In some models, the LED driver circuit in the chip is included. PC-LEDs can be directly connected to AC power source and can be easily dimmed by using the simple AC voltage-dimmer. Therefore, this saves the time and human cost for specific equipment and installation. This study present a growth study of leafy green vegetables with the use of commercial pc-LEDs for horticultural purposes in comparison to a custom-built direct emission R:B LEDs. The goal is to determine whether the commercial pc-LEDs could improve the yield of the Butterhead lettuce, Cos lettuce (baby Cos), Red oak lettuce and Green oak lettuce. The above ground biomass and the morphological structure of their leaf and root were also investigated.

Material and Methods

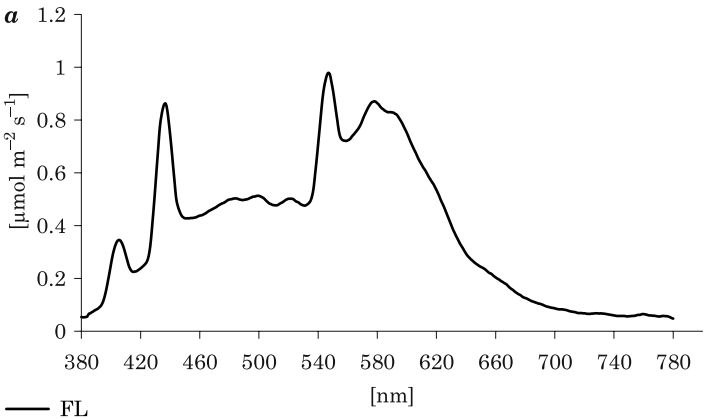
Plant and Growth Conditions

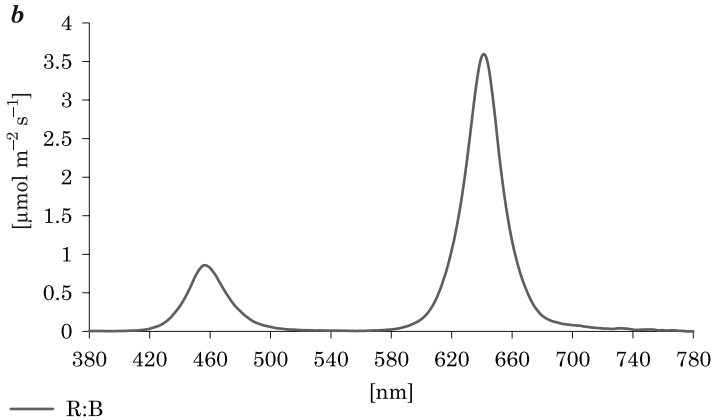
There are four different vegetable seeds used in this study: Butterhead lettuce (*Lactuca sativa var. capitata*), Cos lettuce (*Lactuca sativa var. longifolia,* L. *romana*), Red Oak *lettuce* (*Lactuca sativa var. crispa* L.), and Green Oak lettuce (*Lactuca sativa var. crispa* L.) (Chia Tai Co., ltd, Thailand). Firstly, after incubating the seeds at 4°C on moistened sponge for 5 days, the seeds of four vegetables were germinated into plastic pots (diameter is 10 cm). One seed was planted per one pot. Each pot contains loamy soil, compost, paddy husk charcoals, and coconut dust in the same quantity and was placed in the growth chamber (60 cm × 60 cm × 180 cm). The growth chamber is placed in the temperature control room. The temperature is maintained at 29/25°C (day/night) and the humidity is at 55% to 75% (day/night). There is one control group and two experimental groups. Each group consists of 12 pots per tray: 3 pots of Butterhead, 3 pots of Cos lettuce, 3 pots of Red oak, and 3 pots of Green oak lettuce. Twenty five milliliters of tap water was supplied to each pot once a day in the morning. The plants were irradiated with three treatments with different spectral of the light that is described in the next topic. Finally, the crops are harvested at 40 days after sowing (DAS).

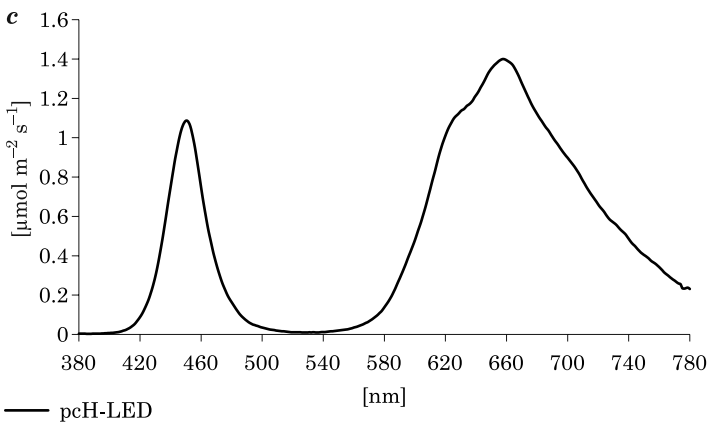
Treatments and LED Lighting System

The PPFD value presented to all experiments is at 150±3 μmol $m^{-2}s^{-1}$ with the light and dark hour of 14/10 h. The harvesting time is 40 DAS. The experimental designs are as follows:

FL light. Experiment 1: The FL light is a custom-made with the light area of 60 cm × 60 cm, consists of six 18 W, 2600 lm of warm white (Phillips Thailand). Six of FL was connected in parallel with 220 V 50 Hz AC power source, the total power is 108 W. The spectrum distribution of the FL is as show in Figure 1*a*. The FL was supplied on the top of the growing tray. The testing results of the spectrum distribution of FL determined that the percentage of B: G: R is about 31: 47: 22 and the photon flux of UV and FR were 2 and 5 μmol $m^{-2}s^{-1}$, R/B =1, R/FR = 6 (Fig. 1*d*).







d

	FL		R:B		pcH-LED	
	PPFD	% PAR	PPFD	%PAR	PPFD	%PAR
UV (380–399 nm)	2	1	0	1	0	0
B (400–399 nm)	45	31	54	19	35	23
G (500–599 nm)	69	47	3	2	8	5
R (600–700 nm)	32	22	93	79	110	72
PAR (400–700 nm)	147	100	152	100	153	100
FR (701–780 nm)	5		2		42	
R/B	1		2		3	
R/FR	6		38		3	
DLI	12.6		13.1		13.3	

Fig. 1. Spectral distribution of the light treatment: *a* – the 1st experiment group (FL); *b* – the 2nd experiment group (R:B); *c* – the 3rd experiment group (pcH-LED); *d* – comparison of the percentage of the distribution spectrum of FL, R:B and pcH-LED in PAR range (400–700 nm), UV (380–399 nm), and FR (701–780 nm) measured by a spectroradiometer "Lighting Passport Pro Essence"

R:B artificial light. Experiment 2: This study used a custom-made R:B LED light. The input voltage is 220 V 50 Hz and two 24 V 100 W of LED drivers to drive the group of R and B LED. The dimension is 27.5 cm × 16.5 cm. The R:B LED consists of two colors of narrow band spectral LED and the number was 42 of 645 nm red(R) LEDs, and 28 of 446 nm of blue (B) LEDs. The total number of LED is 70 with the total power of 200 W. The spectrum distribution of the R:B LED artificial light is as shown in Figure 1*b*. The testing results of the spectrum distribution of R:B indicated that the percentage of B: G: R is about 19: 2: 79. There is zero UV and the FR is about 2 μmol $m^{-2}s^{-1}$, R/B = 2, R/FR = 38 (Fig. 1*d*).

pcH-LED artificial light. Experiment 3: The author applied the phosphor converted LED for horticultural (pcH-LED) purposes with YXO--GLC-8001, Dimension: 78 mm × 44 mm × 1.6 mm, the spectrum range is 380–840 nm, LED chips from Bridgelux: 20W, (Shenzhen Yuxinou Technology Co., Ltd, Guangdong, China). The author designed and assembled the prototype of the pcH-LED artificial light. It consists of 6 of LED connected in parallel. The total power is 120 watts, installed on the aluminum heat sink with the dimension of 27.5 cm × 27.5 cm × 1.5 cm. The supply voltage is 220 V 50 Hz with dimming control. Moreover, the supply voltage could be applied to the LED chip without the need of an external LED driver. The spectrum distribution of the pcH-LED is as shown in Figure 1*c*. The testing results of light quality concluded the percentage of B: G: R at 23: 5: 72. No UV radiation was detected but the FR photon flux is about 41 μmol $m^{-2}s^{-1}$, R/B = 3, R/FR = 3 (Fig. 1*d*).

Measurements

The PPFD spectrum distribution of all light sources was measured by the spectroradiometer from Lighting Passport Pro Essence (Asensetek Incorporation, Taiwan). The growth of leafy vegetable was recorded at the 40^{th} day of the treatment. The yield of the leafy vegetable was investigated along with the fresh weight [g] which is measured by a digital weight scale 0.01 g to 500 g (TWK, China). The leaf number, leaf length, root number, and the root length were measured by a digital venire caliper 0–200 mm (Mitutoyo Crop., Kanagawa, Japan). The morphological investigation were described and compared. This was conducted by the observation of the plant color, tip burn and the plant shape.

Data Collection and Analysis

There are three treatment groups of the plants. Each group consists of 3 Butterhead lettuces, 3 Cos lettuces, 3 Red oak lettuces, and 3 Green oak lettuces. The plants population in each group is 12 (n = 12). Plants sample will be taken from the plants of each group for the investigation of the leaf number, leaf length, root number, root length, and the fresh weight. The results were submitted to analysis of variance in an IBM SPSS statistics software package. Tukey's test at the significance level of 0.05 was run to evaluate the significance of differences.

Results and Discussion

Fresh Weight

Figure 2*a* shows that the average fresh weight of Butterhead lettuce under pcH-LED was highest at 12.78 g, and 11.18 g under the R:B. The Butterhead lettuce under FL shows the lowest fresh weight at about 2.47 g. There is no significant difference ($p > 0.05$) of average fresh weight under all difference light sources.

The Cos lettuce under pcH-LED indicated the highest fresh weight of 18.75 g, and is significantly different ($p \leqslant 0.05$) from under R:B and FL. However, the average fresh weight of Cos lettuce under R:B (1.88 g) and under FL (3.86 g) are not significantly different ($p > 0.05$).

The fresh weight of red oak lettuce under pcH-LED was indicated the highest at 20.13 g, but not significantly different ($a < 0.05$) from under the R:B (12.02 g). The fresh weight of red oak under FL was lowest at 2.75 g and is significantly different ($p \leqslant 0.05$) by red oak under pcH-LED, but not significantly different ($p > 0.05$) from R:B.

The fresh weight of the Green oak lettuce was indicated the highest at 16.42 g under pcH-LED, and 11.40 gm under R:B. The lowest fresh weight is under FL at 6.27 g. However, there are not significantly different ($p > 0.05$) from other treatments.

From this experiment, the fresh weight of the Butterhead, Cos lettuce, Red oak and Green oak lettuce was highest under pcH-LED, and the fresh weight of Butterhead, red oak and green oak was lowest under FL treatment. This could be because the pcH-LED could exhibit the highest quantum yield of the FR is about 41 μmol $m^{-2}s^{-1}$, while FL and R:B LED produce very low FR radiation. Accordance to study of MICKENS et al. (2018) report that the FR light (700–800 nm) could increase the highest fresh mass [g] and shoot diameter of the lettuce. Confirm with the study of

ZHANG et al. (2019) R:B with FR (735 nm) with 43 µmol $m^{-2}s^{-1}$ altered leaf area and total biomass of Tomato plants when compare to R:B LED without FR. Similar to the report of ZOU et al. (2019) who have shown that red: blue light (7:1) with FR 50 µmol $m^{-2}s^{-1}$during day could increase the total biomass of the lettuce 'Tiberius' by 39% than that of red: blue (7:1) light without FR. Relative to the experimental result of MENG and RUNKLE (2019) who reported that the shoot fresh weight [g] and dry weight of lettuces 'Rex' and 'Rouxai' under R:B = 1 at 180 µmol m^{-2} s^{-1} with adding FR of 30 and 70 µmol m^{-2} s^{-1} increased more than the weights under R:B without FR; moreover, if FR is increased, the shoot fresh weight and dry weights will be linearly increased as well.

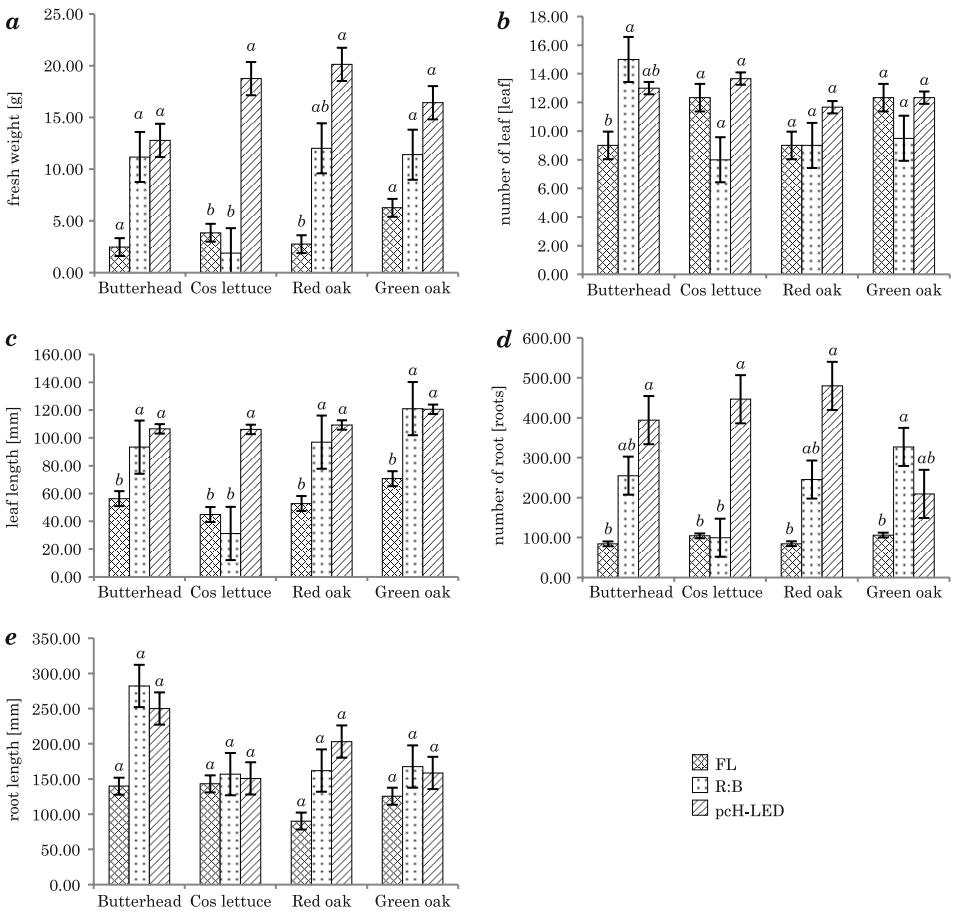


Fig. 2. Effect of three different types of the light source on: *a* – the fresh weight; *b* – number of leaf; *c* – leaf length; *d* – number of root; *e* – root length of the Butterhead lettuce, Cos lettuce, Red oak and Green oak lettuce

Leaf Number and Leaf Length

The number of leaf under all treatments is as shown in Figure 2*c*. The average leaf number of Butterhead lettuce under R:B was highest at 15 leaves, 13 leaves under the pcH-LED, and 9 leaves under FL. The leaf number of Butterhead lettuce under R:B is not significantly different ($p > 0.05$) from pcH-LED, but showed significant difference ($p \leqslant 0.05$) from the leaf number under FL.

The Cos lettuce under pcH-LED indicated that the highest number of leaf is at 13.66, under FL is 12.33 leaves, and lowest at 8 leaves from the R:B treatment. Although, the leaf number of the Cos lettuce are different, but the statistical results is not significantly different ($p > 0.05$) from all treatments. The number of leaf of red oak lettuce under pcH-LED was indicated the highest at 11.66, but showed as not significantly different ($p > 0.05$) under the R:B (9.00), and under FL (9.00). The Green oak lettuce under pcH-LED and FL resulted in the highest number of leaf equally at 12.33, but only 9.50 leaves when under FR. The statistical results are not significantly different ($p > 0.05$) from other treatments.

From this experiment, the number of leaf of the Cos lettuce, Red oak and Green oak lettuce were not significantly different ($p > 0.05$) under all treatments. This has been shown to be similar to the study of SAITO et al. (2010) where the leaf number of the Green-oak lettuce under R:B and FL are not different statistically (LI and KUBOTA 2009). But the leaf number of the Butterhead lettuce was lowest under FL and showed significant difference from other treatments.

The leaf length under all treatments is as shown in Figure 2*b*. The average leaf length of Butterhead lettuce under pcH-LED was highest at 106.51 mm, 93.35 mm under the R:B, and 56.32 mm of leaf length is under the FL treatment. The leaf length of Butterhead lettuce under FL was lowest and it showed significant difference ($p \leqslant 0.05$) from under pcH-LED and R:B.

The Cos lettuce under pcH-LED indicated the highest leaf length of 106.10 mm. This result is the significantly different ($p \leqslant 0.05$) from the leaf length that was under R:B (31.27 mm) and under FL (44.89 mm). The Cos lettuce under R:B indicated the lowest leaf length when compared to the other treatments. The leaf length of Red oak lettuce under pcH-LED was indicated the highest as 109.28 mm, and not significantly different ($p > 0.05$) from the R:B (96.94 mm). The leaf length of Red oak lettuce under FL is lowest of 52.80 mm and shows the significant difference ($p \leqslant 0.05$) from other treatments. The leaf length of the Green oak lettuce under R:B was indicated the highest at 121.05 mm, but showed no signifi-

cant difference ($p > 0.05$) from pcH-LED (120.57 mm). The leaf length of Green oak lettuce under FL is lowest at 70.67 mm and shows the significant difference ($p \leqslant 0.05$) from pcH-LED and R:B treatments.

From this experiment, the author concluded that the pcH-LED and R:B could promote the leaf length of the Butterhead lettuce, Red oak and Green oak lettuce better than from the FL treatments. This finding can also be confirmed by LI and KUBOTA (2009) who reported that the leaf length and leaf width significantly increased by 44% and 15%, respectively, with supplemental FR light when compared to white light, and the report of ZHANG et al. (2019) describe the Tomato seedling plants under R:B with FR (735 nm) as 43 μmol $m^{-2}s^{-1}$ altered leaf area when compare to R:B LED without FR. This is in agreement with the results of ZOU et al. (2019) who reported that the lettuce under red: blue light with FR 50 μmol m^{-2} s^{-1} provided a higher leaf area than the lettuce under red: blue light without FR. The adding of FR at 30 and 70 μmol m^{-2} s^{-1} on the RB = 1 at 180 and 360 μmol m^{-2} s^{-1} will increase the leaf length [cm] but not in leaf number of the lettuces 'Rex' and 'Rouxai' when compared to not adding FR (MENG and RUNKLE 2019).

Root Number and Root Length

The number of roots of the leafy vegetable under all treatments is shown in Figure 2*d*. The average root number of Butterhead lettuce under pcH-LED was the highest at 394.00 roots, 255.00 root under the R:B, and 89.33 roots of the Butterhead lettuce under the FL. The root number of Butterhead lettuce under FL was the lowest and shows significant difference ($p \leqslant 0.05$) under pcH-LED, but did not show a significant difference ($p \leqslant 0.05$) under the R:B.

The Cos lettuce under pcH-LED indicated the highest root number of 446.33 roots and shows a significant difference ($p \leqslant 0.05$) from those under R:B (99.66 roots) and under FL (104.33 roots). The Cos lettuce under R:B was shown to have the lowest root number when compared to the others but does not show a significant difference ($p > 0.05$) with the number of roots under the FL. The root number of the Red oak lettuce under pcH-LED was indicated as the highest at 479.66 roots, but did not show a significant difference ($p > 0.05$) from the root number under the R:B (245.33 roots). The root number of Red oak lettuce under FL is the lowest at 84.66 roots and shows a significant difference ($p \leqslant 0.05$) under the pcH-LED treatments. The root number of the Green oak lettuce under R:B was the highest at 327.00 roots, and did not show a significant difference from the root number under the pcH-LED (209.33 roots). The root number

of Green oak lettuce under FL is the lowest at 106.33 roots and shows a significant difference ($p \leqslant 0.05$) under pcH-LED.

From this experiment, it is possible to conclude that the effects of pcH-LED could promote the root number of the Butterhead lettuce, Cos lettuce, and Red oak lettuce. On the other hand, the R:B could promote the root number of the Green oak lettuce better than from the other treatments. According to the study of ZOU et al. (2019) who found that the effect of red: blue (7:1) light with FR (740 nm) of PPFD 50 μmol m^{-2} s^{-1} during day and at the end of the day has significantly increase the shoot/root ratio of lettuce 'Tiberius' than under red: blue (7:1) light without FR.

Figure 2*e* shows the root length of the leafy vegetable under all treatments. The average root length of the Butterhead lettuce under R:B was the highest at 282.15 mm, the root length of 250.15 mm was observed for under the pcH-LED, and 139.79 mm was obtained as the root length under the FL treatment. The root length under R:B, pcH-LED and FL were not significantly different ($p > 0.05$).

The Cos lettuce under R:B was observed to have the highest root length of 156.95 mm. The root length under pcH-LED is about 150.89 mm and 143.10 mm of the root length under FL. However, the root length under R:B, pcH-LED and FL did not show a significant difference ($p > 0.05$). The root length of the Red oak lettuce under pcH-LED was the highest at 203.22 mm, but did not show a significant difference between the root number of the Red oak under the R:B (162.02 mm), and under FL (90.26 mm). The root length of the Green oak lettuce under R:B was the highest at 167.72 mm, but did not show a significant difference ($p > 0.05$) from the root length of the Green oak under the pcH-LED (158.60 mm), and under the FL (125.60 mm).

The author can conclude that the different light treatments (pcH-LED, R:B and FL) could not have affected the root length of the Butterhead lettuce, Cos lettuce, Red oak, and Green oak lettuce. However, there are a few study of the lettuce roots and development under FR radiation, this is an interesting point for study in the future.

Morphological Observation

Under FL. Figure 3 shows the morphological response of the 4 types of leafy vegetable in this experiment at harvest time (40 DAS) under different supplemental lights. There are obvious morphological changes under pcH-LED, FL, and R:B. For example, the Butterhead lettuce (Fig. 3*a*) under FL appeared to have a short and narrow leaf. The fresh weight was also the lowest when compared to the other supplemental light.

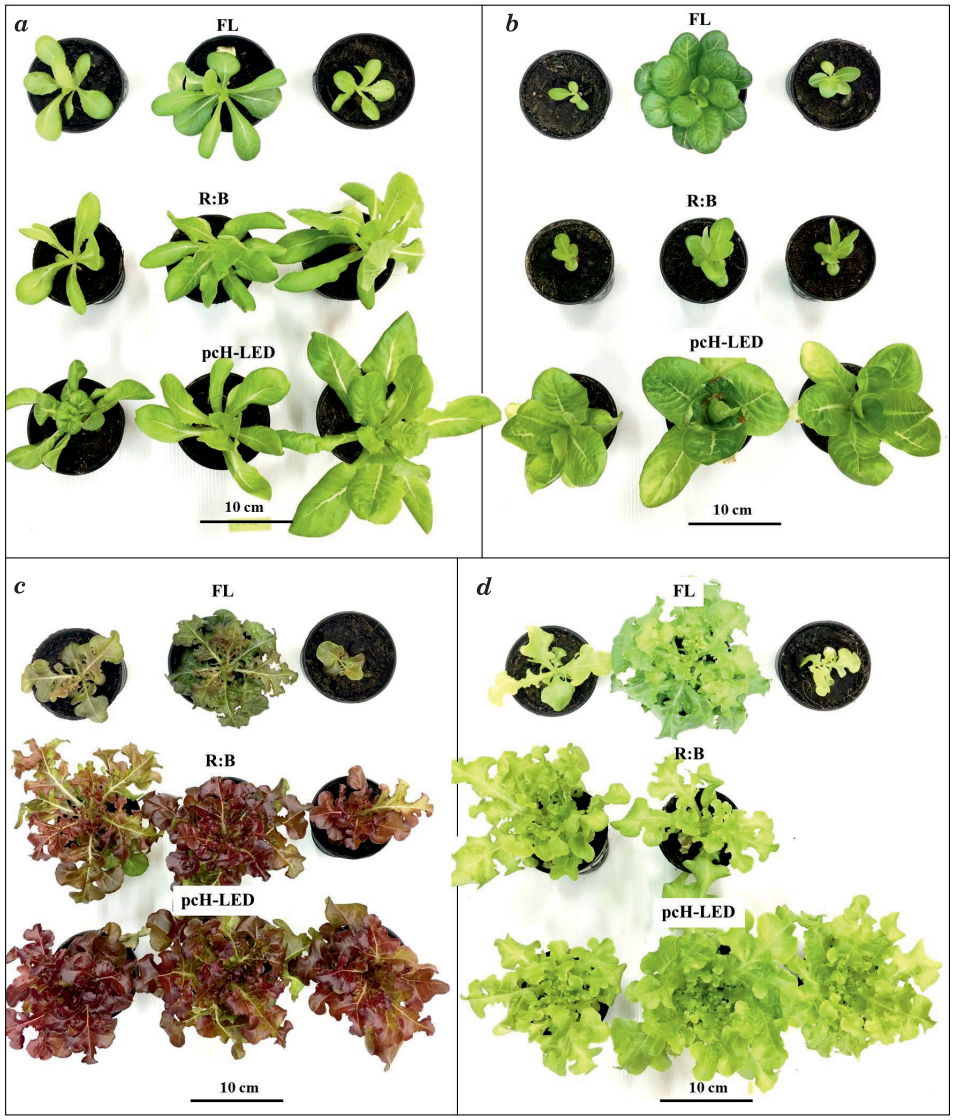


Fig. 3. Comparison of the four leafy vegetables at 40 days on land biomass under FL, R:B LED and pcH-LED: *a* – Butterhead lettuce; *b* – Cos lettuce; *c* – Red oak lettuce; *d* – Green oak lettuce

The Cos lettuce (Fig. 3*b*) growth and development under FL resulted in compact and short leaf which looks like a baby leaf. The average fresh weight is low, but has a normal leaf color. However, the Cos lettuce at the center pot shows a high number of leaves, but the leaf length was shorter than the Cos lettuce under pcH-LED. The Red oak lettuce under FL has compact leaves that looked severely dwarfed. The leaf color did not show

the red color, but moss green color (Fig. 3*c*). Green oak lettuce under FL shows the same results as the Red oak lettuce, but they contain normal green leaf color (Fig. 3*d*).

The observations were in accordance to the report of CHEN et al. (2014) where the fresh weight of green oak leaf lettuce under warm white FL shows a significant difference ($p \leqslant 0.05$) from the fresh weight of the lettuce under FL + red LED light.

According to the report of SANOUBAR et al. (2018) refers to the study of the seedling of some aromatic plants such as *Artemisia absinthium*, *Artemisia vulgaris*, *Atriplex halimus*, and more, in total of nine species under LED mixed between red (655 nm) and blue (456 nm) versus fluorescent warm-white light (5300 K) in a growth chamber. The PPFD is 150 μmol m^{-2} s^{-1}, with an experiment period of 20 days. Both growth chambers were set at 22 ± 1 °C, 70% air humidity and 16/8 h light/dark period. At the end of the day, the shoot's fresh weight and length are as short and small as those obtained under fluorescent lights, but those under red/blue LED indicate significant differences.

The spectrum of the FL light revealed too much green light (69 μmol $m^{-2}s^{-1}$) when compared to the amount of green light from other light sources within this study. On the other hand, it is found that the FL light gives out blue light that is no different than what is seen from the other light sources used within this study and the lowest amount of red light (Fig. 1*a*, *d*). With that said, the FL light then does not fully support plant development; the plants grown under FL light in this experiment did not show very good development when compared to those grown under R:B and pcH-LED (R:B+FR). Previous studies report the effects of green light which tend to reverse the processes established by red and/or blue light. In this way, green light may be informing the plant of photosynthetically unfavorable conditions (FOLTA and MARUHNICH 2007). Therefore, when the PAR spectrum was compared, it can be seen that the green light does not contribute to photosynthesis, but red and blue light will induce higher photosynthesis efficiency.

In accordance to the report of UENO and KAWAMITSU (2017), it was confirmed that the lettuce under red and blue light in the plant factory Okinawa showed the highest yield than lettuce under FL light and white LED light. Therefore, indoor agriculture favors light with mainly blue and red spectrums.

In conclusion the warm white FL is not appropriate for the application to grown the Butterhead lettuce, Cos lettuce, Red oak and Green oak lettuce, because the results obtained showed the lack of response to the shape and color of the leafy vegetable growth and development.

Under R:B. It is well known LEDs now offer controllable sources of light that can selectively and quantitatively provide specific wavelengths for indoor horticultural. Previous studies have demonstrated that the combination of red (600–700 nm) and blue light (400–500 nm) is an effective lighting source for plant growth (BIAN et al. 2015). The morphological observation results is in Figure 3*a* shows the images of the Butterhead lettuce at harvest time (40 DAS) under different supplemental lights. The Butterhead lettuce under R:B indicated that the leaf number and leaf length is higher than under FL. The fresh weight of Butterhead lettuce under R:B was lower than from under pcH-LED. But the Butterhead lettuce in all samples looked dwarfed with very short leaf and tiny size (Fig. 3*b*). However, the R:B promotes the leaf and fresh weight of the Red oak and Green oak lettuce (Fig. 3*d*). But the color of the leaf of the Red oak lettuce under R:B appeared light red in some leaves (Fig. 3*c*). However, in this study indicated that the R:B is not appropriate for the growth and development of the Cos lettuce.

In this study, the author provided R:B ratio at about 79:19 which showed good morphological and biomass of Butterhead lettuce, Red oak and Green oak lettuce. Similar to MICKENS et al. (2018) report, the red pak choi showed the greatest yield (biomass), leaf area, and relative anthocyanin accumulation under a spectrum provided by R:B (R75% B25%) LEDs. The findings suggest that for pak choi, partitioning more biomass into leaf expansion over petiole elongation had a higher influence on the overall yield. This was better than red pak choi under white, white-red, white--green and white-far-red LED at the same level of PPFD and in the same light hours. However, PAR spectrum of the sun light contains around 31% of B light. It appears that around 30% may be the maximum blue light percentage to produce plant biomass efficiently and keep appearance quality in an acceptable range (YING et al. 2020). In summary, R and B light is a major common light for indoor plant production such as lettuce, cabbage, kale, arugula and mustard, microgreens, broccoli, cucumber and so on. Each species responds to the different radiation of R and B ratio, and growth well in different level of PPFD.

Under pcH-LED. In vegetable horticulture, biomass production and product quality are important features that determine the quality of the crop yield. These features are directly correlated to photosynthesis efficiency: biomass production is dependent on the quantity of the active radiation obtained by the leaves, whereas the product quantity is dependent on the wavelengths of the light used in photosynthesis. Therefore, manipulating the blue-red light and R:FR ratio of the light can lead to an

improvement of the biomass production and product quality, which then translates to better crop yield (MAINARD et al. 2016).

In this experiment on Figure 3*a* shows the growth and development of the Butterhead lettuce at 40 DAS under FL, R:B, and pcH-LED (R:B+FR radiation). The results clearly indicated that the Butterhead lettuce under pcH-LED was perfectly beneficial. The pcH-LED could promote the increase in fresh weight, leaf size, and normal leaf color. The effect of pcH-LED that was supplemented to the Cos lettuce, Red oak and Green oak lettuce (Fig. 3*b*, 3*c*, 3*d*) shows the same results to the Butterhead lettuce. Consequently, the pcH-LED could be applied to be an artificial light for the indoor cultivation. pcH-LED could promote the good quality of leafy vegetable such as having perfect leaf size, big plants, good shape and nice leaf color of the Butterhead lettuce, Cos lettuce, Red oak and Green oak lettuce. This is because the pcH-LED could produce the FR light at a high PPFD (41 μmol m^{-2} s^{-1}) when compared to the R:B and FL. Based on literature, the light spectrum of FR range (700–800 nm) can boost lettuce yields, fresh weight, dry weight and leaf number (LI and KUBOTA 2009). Some of previous study was show that the Tomato seedling plants under R:B with FR (735 nm) at 43 μmol $m^{-2}s^{-1}$ altered leaf area and total biomass more that tomato seedling under the R:B LED without FR (ZHANG et al. 2019). Adding 110 μmol $m^{-2}s^{-1}$ of FR light (735 nm) after supplement the red: blue (ratio 76:24) and white light immediately increased the quantum yield of photosystem II (ΦPSII) of lettuce (*Lactuca sativa*) by an average of 6.5 and 3.6% under red: blue and warm-white light, respectively (ZHEN and VAN IERSEL 2017). The acclimation process of plant morphology triggered by additional FR light plays a pivotal role for improving the production of indoor cultivated lettuce, and the enhanced production by additional FR light cannot be achieved by adding similar amount of red and blue light (ZOU et al. 2019). Including FR in a light spectrum increased plant size and photosynthesis (PARK and RUNKLE 2017). These confirm that the FR light is needed for the photosynthesis efficiency of lettuce. Usually, plants grown under light with efficient photosynthesis could produce good quality and high yield of 4 leafy vegetable in this study.

Conclusion

Based on growth and yield of leafy vegetable for indoor plantation, we recommend the pcH-LED to be a light source. The yields obtained under pcH-LED treatment indicates that the phosphor conversion could generated the combination of R and B with high amount of FR spectral that can

have the positive effects on crop quality. It could promote the highest yield of leafy vegetable such as highest fresh weight and root number, perfect leaf size, good shape and nice leaf color of the Butterhead lettuce, Cos lettuce, Red oak and Green oak lettuce. The pcH-LED and R:B could produce the same quantity in leaf number, leaf length and root number, but the R:B light shows negative effect to Cos lettuce resulting in short leaf and tiny size. Therefore, the growth was substantially affected by the combination of R and B lights, with and without FR. The presence of both lighting spectrums is essential for expanding and elevating the lettuce quality. On a large scale, this technology could improve the commercial greenhouse production while helping farmers achieve maximum products.

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EFFECTS OF PALM KERNEL MEAL-BASED DIET WITH OR WITHOUT ENZYME SUPPLEMENTATION ON GROWTH PERFORMANCE, ECONOMIC BENEFITS AND VILLI MORPHOMETRY OF WEANED PIGS

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Key words: weaned pigs, growth performance, enzyme, villi morphometry, economic indices, palm kernel meal.

Abstract

This study aimed to assess the effects of palm kernel meal (PKM) based diet with or without enzyme supplementation on growth response, cost benefits and villi morphometry of weaned pigs. Forty weaned male pigs with an initial weight of 7.85 ± 0.31 kg (Large white x Landrace) were divided into four dietary groups. The control diet (*A*) consist of only PKM based diet. In contrast, the three other PKM based diets *B*, *C* and *D* were supplemented with increasing doses of commercial feed enzyme (polyzyme) at an inclusion level of 0.1%, 0.2% and 0.3% respectively. The PKM had 91.58% dry matter, 15.75% crude protein, 21.42% crude fibre, 12.23% ether extract, 1.40% ash, 40.78% nitrogen-free extract and 3,030 kcal/kg metabolizable energy. The feed cost per kg ranges from ₦102.11 in diet *A* to ₦108.71 in diet *D* with a linear increase across dietary groups. The villi height was higher in those fed diets *A* than those fed diets *B–D*. In conclusion, polyzyme supplementation up to 0.30% does not improve weight gain and profit margin but reduced villi height. Therefore increased polyzyme use should be further researched in a 55% PKM based diet.

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Introduction

The use of palm kernel meal (PKM) in mono-gastric animals such as poultry and swine is limited due to the low activity of fibre digestive enzymes in their gastrointestinal tract (SHARMILA et al. 2014). Non-starch polysaccharides (NSPs) are complex carbohydrates with the sophisticated cell wall, other than starches found in food. They form the major part of dietary fibre and can be measured more precisely than total dietary fibre. Examples include cellulose, pectins, glucans, arabinans, arabinogalactans, galactans, mannans, and galactomannans. Non-starch Polysaccharides, being a carbohydrate, are a potential energy source and are indigestible by monogastric animals. Their fibrousness can result in reduced nutrient digestibility, increased feed conversion ratio and ultimately decreased animal performance (WENK 2001, NOBLET and LE GOFF 2001), although this is determined by fibre properties (LINDBERG 2014). Palm kernel cake (PKC) consist mainly of mannans, cellulose and xylans (WING-KEONG and KAI-KAI CHONG 2002) with mannans dominating its significant portion of NSP. It contains about 78% of mannans with β-mannan amounting up to 32.5% (OLADOKUN et al. 2016).

Numerous researches have been conducted to develop various means of increasing the nutritional contents of fibrous feedstock to reduce and or eliminate the constraints of utilizing them in mono-gastric diets. Physical, chemical, biological, or combination of these treatment methods have all been used in achieving this target (SHARMILA et al. 2014). However, the chemical and natural treatments of PKC seem to be more potent and improve the nutritive values of PKC in diets. One form of biological treatment used in improving the nutritive value of PKC in diets involves supplementation of the diets with exogenous enzymes that can breakdown the cell wall of NSPs present in PKC and liberate the nutrients entrapped within the cell wall. The treatment will enable the nutrients to be easily accessible to the animal for absorption, thereby enhancing their digestibility.

In recent time, there have been collective efforts tailored towards limiting the negative effect of dietary NSP in mono-gastric diets and improve the nutritive value of feedstuffs through the use of exogenous enzymes (SEKONI et al. 2008). The digestion of non-starch polysaccharides (NSPs) of the cell wall of PKC can be enhanced with the use of enzyme supplementation in monogastric diets (SOBOTKA et al. 2011). However, studies show that the supplementation of exogenous enzymes in diets containing PKC could improve its nutritive quality, and make it more available for animal use, especially poultry and swine (SEKONI et al. 2008, CHONG et al. 2008). Nevertheless, the use of a cocktail of enzymes is limited.

This study aimed to appraise the outcome of palm kernel meal-based diet with or without polyzme enzyme supplementation on growth response, cost benefits and villi morphometry of weaned pigs.

Materials and Methods

The experiment was carried out at the Ladoke Akintola University of Technology Teaching and Research Farm Piggery Unit, Ogbomoso. Ogbomoso lies on longitude $4^{0}16^{1}$ East of the Greenwich Meridian and Latitude $8^{0}10^{1}$ North of the equator. The region has a latitude between 300 and 600 meters above sea level. The mean annual temperature is about 27^{0}C while that of average rainfall is 1247 mm. The vegetation of the study area is in the derived savannah zone (OJEDAPO et al. 2009).

Forty weaned male pigs with an initial weight of 7.85 ± 0.13 kg (Large white x Landrace) were used for this experiment which lasted for seven weeks. The pigs used were from the same piggery unit where the experiment took place. The weaned pigs selected based on sex and weight were farrowed by 6 Large white sows mated by the same Landrace boar. Concrete floor experimental pens, feed troughs and drinkers used were washed and cleaned thoroughly before the introduction of the pigs to the pen and use of other materials. They were acclimatized and fed with weaner ration of 22% CP for a week before the commencement of the experiment. The pigs were weighed individually and assigned to four (4) dietary groups based on weight with ten (10) replicates per treatment. The animals were after that weighed every week until the end of the feeding trial. Feed and water also were given *ad libitum*.

The feedstuffs and test enzyme (Polyzme, an exogenous enzyme) used for the experiment was purchased from a reputable commercial feed store in Ogbomoso. The enzyme is a cocktail of mannanase, xylanase, cellulase, glucanase, phytase, amylase, pectinase, lipase, galactosidase and protease.

Four dietary formulations of palm kernel meal (PKM) basal diet, supplemented with or without enzyme supplementation were formulated for the weaned pigs. The control diet (*A*) consist of only PKM based diet with no enzyme supplementation. In contrast, the remaining three diets were supplemented with increasing dosages of commercial feed enzyme (polyzyme) at inclusion level of 0.1% [1 g kg^{-1} feed], 0.2% and 0.3% as diets *B*, *C* and *D* respectively as displayed by Table 1.

Table 1

Percentage composition of components of experimental diets

Ingredients [%]	Diet *A* 0.0% Polyzyme	Diet *B* 0.1% Polyzyme	Diet *C* 0.2% Polyzyme	Diet *D* 0.3% Polyzyme
Maize	15.00	15.00	15.00	15.00
Fish meal	3.00	3.00	3.00	3.00
Full fat soya	9.00	9.00	9.00	9.00
Palm kernel meal	55.00	55.00	55.00	55.00
Wheat offal	15.00	15.00	15.00	15.00
Bone meal	1.50	1.50	1.50	1.50
Limestone	1.00	1.00	1.00	1.00
*Premix	0.25	0.25	0.25	0.25
Salt	0.25	0.25	0.25	0.25
Polyzyme [%]	0.00	0.10	0.20	0.30
Total	100.00	100.00	100.00	100.00
Calculated nutrients [%]				
Crude protein	19.93	19.93	19.93	19.93
Ether extract	6.35	6.35	6.35	6.35
Crude fibre	8.70	8.70	8.70	8.70
ME [MJ kg^{-1}]	11.36	11.36	11.36	11.36
Lysine	0.87	0.87	0.87	0.87
Methionine	0.40	0.40	0.40	0.40
Calcium	0.95	0.95	0.95	0.95

Explanations: ME – metabolizable energy; *premix composition: vitamin A – 12 500 000 IU; vitamin D_3 – 5 000 000 IU; vitamin E – 40 000 mg; vitamin K_3 – 2000 mg; vitamin B1 – 3000 mg; vitamin B_2 – 5500 mg; niacin – 55 000 mg; calcium pantothenate – 11 500 mg; vitamin B_6 – 5000 mg; vitamin B_{12} – 25 mg; folic acid – 1000 mg; biotin – 50 mg; choline chloride 500 000 mg; manganese – 300 000 mg; iron – 120 000 mg; zinc – 80 000 mg; copper – 8500 mg; iodine – 1500 mg; cobalt – 3000 mg; selenium – 120 mg; anti-oxidant – 120 000 mg (in every 2.5 kg package, at 2.5 kg per ton of feed)

Data were collected on growth performance indices, including feed intake, weight changes while the feed conversion ratio was calculated. Feed intake was measured individually as the differential between feed offered and feed left daily while the weight change or gain was taken weekly using a sensitive electronic scale. The feed conversion ratio was calculated as average feed intake divided by average weight gain. Economic indices were calculated as previously described (OJEDIRAN et al. 2017).

Feed cost/kg = sum (quantity of each ingredient – unit cost of each ingredient) %/100.

Feed cost per kg weight gain = feed cost/kg – total feed intake [kg]/ total weight gain.

Income per kg weight gain = selling price/kg – final weight per pig/ total weight gain [kg]

Profit per kg weight gain = income per kg weight – feed cost/kg weight gain

Economic efficiency of growth (EEG) = (profit per kg of weight gain/ feed cost per kg weight gain) · 100.

Morphometric characteristics of the jejunum, including the villus length and width, crypt depth and area, were determined. By the end of the experiment, four pigs were randomly selected from each treatment for jejunum evaluation and were starved overnight for 12 hours but allowed access to water *ad libitum*. The pigs were slaughtered by severing the jugular veins and were eviscerated to collect the jejunum portion at three different locations. The triplicate samples were fixed in 10% neutral buffered formalin labelled appropriately before further processing in an automatic tissue processor, embedded in paraffin wax and sectioned at 5 microns on a rotary microtome mounted on glass slides. The stepwise protocol for the automatic tissue processor for histological examination slide was done as previously described (SHEN et al. 2009, CARSON and CHRISTA 2009).

Only the villi considered adequate for measurements were counted. A villus is deemed to be sufficient when its base is embedded in the submucosa (10 x magnification); its body did not present any discontinuity or folds (4 x magnification), and simple columnar epithelium was present at the tip (40 x magnification). From each section, five randomly selected villi were measured in each slide per field, and five areas were used. Villi lengths, widths and cryptal depths were measured in microns (converted to cm) using top view software on the Amscope (MU900) camera.

A representative sample of the palm kernel meal used in the formulation was taken and analysed for proximate composition using the procedure of AOAC (2012). The metabolizable energy (ME) was calculated using the equation predicted by PAUZENGA (1985):

$$\text{metabolizable energy [kcal/kg]} = (37 \cdot \%\text{CP}) + (81.8 \cdot \%\text{EE}) + (35.5 \cdot \%\text{NFE})$$

The data collected in this feed trial were subjected to analysis of variance (ANOVA) in a completely randomized design using SAS (2000) software package and means was separated using Duncan multiple range test of the same package.

Results

The proximate composition of palm kernel meal (PKM) used in this study is presented in Table 2. It had 91.58% dry matter, 15.75% crude protein, 21.42% crude fibre, 12.23% ether extract, 1.40% ash, 40.78% nitrogen-free extract and 3,030.85 kcal/kg (12.67 MJ kg^{-1}) metabolizable energy.

The growth performance of weaned pigs fed PKM based diet with or without enzyme supplementation is shown in Table 3. The results indicated that average daily feed intake (ADFI) was significantly different ($P < 0.05$), however, the average initial weight, average final weight, average daily weight gain and feed conversion ratio were not significantly influenced ($P > 0.05$). ADFI reduced linearly from pigs fed diets *A* to *D* except pigs fed diet *C* that had the highest value.

Table 4 shows the economic benefits of weaner pigs fed PKM based diet with and without enzyme supplementation. The feed cost per kg was significantly different ($P < 0.05$). It ranged from ₦102.11 in diet A to ₦108.71 in diet *D* with a linear increase across dietary groups. The values obtained for the feed cost per kg weight gain, income per kg weight gain, profit per kilograms of weight gain and the economic efficiency of increase were not significantly different ($P > 0.05$).

Intestinal morphology of grower pigs fed PKC based diet with and without enzyme supplementation is as shown in Table 5. The villus height was significantly affected ($P < 0.05$) by the dietary treatments. The villi width, cryptal depth and width were not significantly different ($P > 0.05$) across the groups. The villi height was considerably higher than those fed diets *B*–*D*.

Table 2

Chemical composition of palm kernel meal (PKM)

Nutrients	Percentage composition
Dry matter	91.58
Crude protein	15.75
Crude fibre	21.42
Ash	1.40
Ether extract	12.23
Nitrogen free extract	40.78
ME [MJ kg^{-1}]	12.67

Explanations: ME – metabolizable energy

Table 3

Growth performance of weaner pigs fed PKC based diet with and without enzyme supplementation

Parameters	Diet *A* 0.0% Polyzyme	Diet *B* 0.1% Polyzyme	Diet *C* 0.2% Polyzyme	Diet *D* 0.3% Polyzyme	SEM (±)
AIW [kg]	7.81	7.80	7.98	7.89	0.31
AFW [kg]	21.56	19.76	22.04	19.78	0.81
ADWG [kg]	0.28	0.24	0.29	0.24	0.01
ADFI [kg]	0.80^{b}	0.68^{c}	0.83^{a}	0.62^{d}	0.20
FCR	3.28	2.86	3.00	2.59	0.21

Explanations: $^{a, b, c, d}$ – means with different superscripts in the same row are significantly different ($P < 0.05$); AIW – average initial weight; AFW – average final weight; ADWG – average daily weight gain; ADFI – average daily feed intake; FCR – feed conversion ratio; SEM – group standard error of mean

Table 4

Economic indices of weaner pigs fed PKC based diet with and without enzyme supplementation

Parameters	Diet *A* 0.0% Polyzyme	Diet *B* 0.1% Polyzyme	Diet *C* 0.2% Polyzyme	Diet *D* 0.3% Polyzyme	SEM (±)
FC/kg [₦]	102.11^{d}	104.31^{c}	106.51^{b}	108.71^{a}	0.58
FC/kg WG [₦]	335.00	298.59	319.70	281.58	21.00
Income/kg WG [₦]	665.12	664.85	633.96	665.42	18.76
Profit/kgWG [₦]	330.13	366.27	314.26	383.83	12.21
EEG	115.61	128.25	104.27	138.90	8.47

Explanations: $^{a, b, c, d}$ – means with different superscripts in the same row are significantly different ($P < 0.05$); FC/kg – feed cost per kilogramme; FC/kg WG – feed cost per kilogramme weight gain; EEG – economic efficiency of gain; SEM – standard error of mean; ₦ – Nigerian naira

Table 5

Villi morphometry of weaner pigs fed PKC based diet with and without enzyme supplementation

Parameters [cm]	Diet *A* 0.0% Polyzyme	Diet *B* 0.1% Polyzyme	Diet *C* 0.2% Polyzyme	Diet *D* 0.3% Polyzyme	SEM (±)
Villi height	0.32^{a}	0.26^{b}	0.23^{b}	0.25^{b}	0.01
Villi width	0.04	0.04	0.03	0.04	0.00
Cryptal depth	0.15	0.16	0.13	0.17	0.01
Cryptal width	0.03	0.03	0.02	0.03	0.00

Explanations: $^{a, b}$ – means with different superscripts in the same row are significantly different ($P < 0.05$); SEM – standard error of the mean

Discussion

The palm kernel meal used was observed to contain similar crude protein, crude fibre and moisture contents as previously reported (ADESEHINWA 2009). However, the values obtained for ash, ether extract and nitrogen-free extract were different from those indicated by the same author. The shell content of palm kernel meal, which could be as high as 10% was reported earlier (ADESEHINWA 2007) to contribute a great deal to its high fibre content. The ether extract content, however, is a function of the oil extraction method used (BOATENG et al. 2008). The metabolizable energy of PKM in this current study is similar to that reported previously (ADESEHINWA 2007), which were different from that of the typical Malaysian PKC reported by SHARMILA et al. (2014). The difference may be attributed to the predominant method of oil extraction.

In this study, polyzyme® enzyme supplementation did not affect weight gain and feed conversation ration of weaned pigs. It was also observed that the enzyme does not necessarily increase the voluntary feed intake of the animals. Still, the previous study found that Avizyme® 1300 inclusion in 100 kg of 45% Cassava peel meal (CPM) based diet improved feed utilization and performance (ADESEHINWA 2007**)**. Observation from this study is also contrary to the report that enzyme supplementation in pigs diet might improve feed conversion ratio without any significant difference in feed intake (AO et al. 2011), this author also reported that the inclusion of palm kernel meal in pig diet decreased the growth performance while carbohydrase cocktail supplementation counteracted the adverse effects caused by palm kernel meal addition in pig's diet. Another study on the response of weaned pigs to enzyme supplemented palm kernel cake in place of maize concluded that there were no differences between the total weight gain of weaned pigs on the control diet and those on diets with 40% PKC with and without enzyme supplementation (OLUFEMI and AKPODIETE 2010), this report is similar to the result of this present study, which indicated that 55% PKM in the diets of weaned pigs with and without enzyme supplementation does not adversely affect weight and feed conversation ratio.

Feed costs account for 65% to 75% of pig production expenses and significantly impact the profitability of pork producers; therefore, producing alternative sources of feed is essential (CHOI et al. 2015) because, the cost of corn, which is a significant component of animal feed, increased owing to the considerable demand for bio-ethanol and bio-fuel (DE GORTER et al. 2013). The increased feed cost per kg with increased polyzyme supplementation in this study could not be unexpected because the gross feed

composition was the same except for the enzyme quantity added. However, despite the higher cost/kg of the supplemented diets to the control diet, the feed cost per weight gain, income per kilograms of weight gain, and profit per kg weight gain and economic efficiency of gain compared favourably. The result may suggest that the polyzyme supplementation in a 55% PKM based diet may be unnecessary. Previous report showed that reduction of feed cost was not only to obtain cheaper feed but also aimed at the derivation of best production performance with the cheaper feed (ADESEHINWA 2009), this author, therefore, concluded that the efficiency with which the feed was utilized was of significant importance, this was also observed in this study.

The morphometry of the intestine (jejunum) from this study indicates that the villus heights were lower in the supplemented group than the non-supplemented group (control diet *A*). The result of villi height in this current study agrees with the report of HEDEMANN et al. (2006), who attributed such decrease in weaned pigs to high soluble NSP content. It suggests the efficacy of polyzyme in this study. Nevertheless, insoluble NSP as observed in pigs fed diet with no polyzyme according to Hedemann et al. (2006) reduce transit time through the intestine and provide substrates that modulate the gut morphometry by increasing villi height. FREIRE et al. (2003) suggested that observed differences in gut morphology is explainable by differences in digesta transit time and gut microbial activities.

Also from the histo-morphometry report is the conclusion that inclusion of 55% PKC in weaner pig's diet affects the morphometry of the intestine of the test animals and enzyme supplementation of the diets has no counteracting effects on the intestine of the animals.

Conclusions

It can be concluded that polyzyme supplementation at between 0.1–0.3% does not improve weight gain and profit margin. Nevertheless, reduced villi height. Therefore, increased polyzyme use should be further researched in a 55% PKM based diet.

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HAEMATOLOGICAL PARAMETERS, ORGAN WEIGHT AND VILLI MORPHOMETRICS OF WEANER PIGS FED BISCUIT DOUGH

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Abstract

Thirty weaner (Large white x Landrace) pigs were randomly allotted to five dietary groups of six pigs each. A maize-soybean meal-based diet served as the control (T1) while diets T2, T3, T4, and T5 had 12.5%, 25%, 37.5% and 50% biscuit dough respectively as a replacement for maize. Results showed that haematological parameters had significantly different ($p < 0.05$) quadratic responses, although, pigs fed T5 had least values for white blood cell count, haemoglobin, packed cell volume and mean corpuscular volume. Significant differences ($p < 0.05$) were also observed in the weight of liver, heart, spleen and pancreas, and also in the villi length, width and crypt depth. It can be concluded that 50% biscuit dough replacement for maize weakened the body defensive mechanism, reduced the efficiency of cellular oxygen transportation, pancreatic secretion and villi width of weaner pigs, while 37.5% biscuit dough replacement for maize improved the parameters mentioned above. It is recommended that 37.5% of biscuit dough is suitable for the replacement of maize in weaner pig's diets.

Introduction

Nutrition plays an essential role in the physiology of animals (OJEDIRAN et al. 2017a). The values of blood parameters can serve as an assessment of different physiological processes in the body: this ability of blood may be due to the fact that blood is a diagnostic tool and its analysis is

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a timely way of assessing nutritional and health status of livestock on feeding trials since ingestion of dietary components has assessable effects on blood composition (OVURU and EKWEOZOR 2004, ISAAC et al. 2013). In the same vein, haematological investigations have been explored extensively to distinguish the normal state from stress induce abnormalities which could be nutritional stress (KHAN and ZAFAR 2005). A good blood composition in animals indicate excellent performance (ISAAC et al. 2013). Blood conveys nutrients and essential materials to different parts of the body. Therefore, whatever affects the blood like nutrition will have effects on the entire body adversely or moderately alter their health, growth, maintenance and reproduction (OKE et al. 2007).

Similarly, the weight of organs can indicate the response of livestock to feed intake, the growth rate or age of the animal (OJEDIRAN et al. 2016). Previous studies shows that understanding the relationship between organ weight, and body weight will help to improve organ weight interpretation about treatment effects (PIAO et al. 2013), the authors, therefore, explained that organ weight could be the most sensitive assessment for knowing the safety of feed consumed.

Profit maximisation from the use of feed formulation with least cost is therefore the target of farmers (OJEDIRAN et al. 2017b) because feed cost accounts for about 60–76% of the total cost of running a piggery. Most farmers are interested in growth while they at times do not consider other physiological indices of these animals when opting for alternative feedstuffs. The anti-nutrients in alternative feedstuffs like *Jatropha curcas* kernel meal (OJEDIRAN et al. 2014, OLADUNJOYE et al. 2014), cassava peels (SHITU et al. 2016), with the cost of processing them and seasonal availability (OJEDIRAN et al. 2017c) have prompted research into the possibilities of using biscuit dough, an industrial waste devoid of anti-nutrients as an alternative feedstuff .

Biscuit dough is an unbaked mixture of biscuit components such as wheat flour, skimmed milk powder, vegetable fat, sugar, salt and flavour material that failed to rise and is found in substantial quantities in biscuit producing industries (SHITU et al. 2016). It could be an economical feedstuff for monogastric because the bakery does not use it for production of biscuits and it is cheaper to acquire because it is a waste product in the bakery. It has been reported to have higher metabolizable energy (ME) for swine than corn grain (NRC 1998). However, little pieces of information are available on the potential of biscuit dough as an alternative component of weaner pig diet (SHITU et al. 2016) and the physiological response of pigs fed biscuit dough is not well investigated.

This study, therefore, evaluates the haematological parameters, organ weight and villi morphometry of weaner pigs fed varying levels of biscuit dough as a replacement for maize in their diet.

Materials and Method

Experimental Location

The experiment took place at the Piggery, Unit of the Ladoke Akintola University of Technology Teaching and Research Farm, Ogbomoso, Oyo State, a derived savannah zone of Nigeria, located on latitude 18°15'N of the equator and longitude 4°5'E of the Greenwich meridian (OJEDAPO et al. 2009).

Procurement and Processing of Test Ingredients

Experimental Pigs and Their Management

The test ingredient was obtained from a biscuit factory in a pasty form. It was sun-dried to 8–9% moisture content and milled before being mixed with other feed ingredients.

Thirty (30) weaner pigs of Large White and Landrace crosses were acclimatized and fed with weaner ratio of 22% CP for a week before the commencement of the experiment. The weaner pigs were randomly allotted to five dietary groups of six weaner pigs while each pig served as a replicate. The animals had access to feed and water *ad-libitum*. The experiment took 49 days. The pigs were handled and managed following the NIH Guide for the Care and Use of Laboratory Animals NIH publication No 86–23, revised 1985 and 1991) and the ethical requirements of the United Kingdom for animal experimentation (Animals scientific procedures, Act 1986)

Experimental Diet

Five experimental diets were formulated with a crude protein content of between 20–21% and metabolizable energy ranging from 2800–3000 ME $kcal^{-1}$ kg^{-1} in a Maize-soybean meal-based diet (control) as shown in Table 1. Biscuit dough was used to replace the maize in control diet at 12.5%, 25%, 37.5% and 50% in the other diets respectively.

Table 1

Gross composition of the experimental diets

Ingredients [%]	T1	T2	T3	T4	T5
Maize	56.00	49.00	42.00	35.00	28.00
Biscuit dough	0.00	7.00	14.00	21.00	28.00
Soybean meal	25.50	24.50	23.50	21.70	19.50
Fish meal	5.50	4.00	2.80	2.00	1.00
Palm kernel cake	2.00	4.50	6.70	9.30	12.50
Fixed ingredients	11.00	11.00	11.00	11.00	11.00
Total	100.00	100.00	100.00	100.00	100.00
Calculated nutrients					
ME [kcal kg^{-1}]	3014.04	2966.49	2919.54	2873.39	2826.83
Crude protein	21.03	20.70	20.54	20.38	20.02
Ether extract	3.78	3.72	3.68	3.68	3.68
Crude fiber	3.77	4.16	4.51	4.87	5.29

Explanations: fixed ingredients – 9.00% cassava peel meal; 0.60% – limestone, 0.60% – di-calcium phosphate; 0.10% – lysine; 0.05% – methionine; 0.20% – premix; 0.50% – salt; ME – metabolizable energy

Data Collection

At the end of the experiment, three pigs were randomly selected from each treatment and were starved overnight for 12 hours but allowed access to water *ad libitum*.

Blood samples were collected with sterile needles and syringe from jugular vein into sterilized bottles containing Ethylene Diamine Tetra-acetic Acid (EDTA) which were taken to the laboratory for analysis: haematological parameters including packed cell volume (PCV), haemoglobin (Hb), erythrocyte count (RBC), leukocyte count (WBC), differential leukocyte counts, mean corpuscular value (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC), were determined using the methods described by OJEDIRAN et al. (2015).

The pigs were then sacrificed humanely and opened up for the organs which were weighed using a sensitive scale after taking the live weight of the animal.

Laboratory Procedure for Histological Processing of Villi for Its Morphometrics

Three centimetres of each pig jejunum were cut and fixed in formalin 24 h before staining for intestinal histology and were appropriately labelled. Each histological sample was dissected, fixed in10% neutral buffered formalin for further fixing before being processed in automatic tissue processor, embedded in paraffin wax and sectioned at 5 microns on a rotary microtome, and then mounted on glass slides. Staining of the slides with hematoxylin-eosin staining method was next. The villus height, villus width and crypt depth were then measured in villi per section using optical microscopy (AKPOKODJE et al. 2005, CARSON and CHRISTA 2009).

Statistical Analysis Method

Analysis of variance (ANOVA) was employed for all data collected in a completely randomized design using SAS statistical software package (SAS 2000) and means were separated using Duncan multiple range test of the same package.

Results

Haematological Parameters

Table 2 shows the haematological parameters of weaner pigs fed biscuit dough. There were significant ($p < 0.05$) differences in the parameters examined. A quadratic pattern of response was observed. The WBC count values ranged from 10.00–18.55 [$\cdot 10^3$ uL^{-1}]. T1 had the highest value (18.55), followed by T4 (17.75), T3 (15.45), T2 (14.55) while T5 (10.00) had the least. Values observed for Hb content ranged from 9.50–13.00 [g dL^{-1}]. T4 had the highest value (13.00), although not different from those fed T2 (12.95), while those fed T5 (9.50) had the least ($p < 0.05$). Pigs fed T2 had the highest value of 9.73 [$\cdot 10^6$ uL^{-1}] ($p < 0.05$) for RBC while those on T1 (8.18), T3 (8.13), T4 (8.13) and T5 (8.13) were not significantly different ($p > 0.05$) from each other. HCT had values ranging from 33.2–54.85 [%]. T2 had the highest value, while T5 (33.2) recorded the least ($p < 0.05$). MCV had values ranging from 57.0–60.75 [fL]. T1 had the highest value (60.75), T3 (59.05), T4 (58.60), T2 (57.0), and the least value from T5 (49.45) ($p < 0.05$).

MCH had values ranging from 13.45 to 14.50 [pg] which were significantly ($p < 0.05$) different, T4 (14.50) had the highest value, while T2 (13.45) had the least value. MCHC had values ranging from 22.90–28.65 [g dL^{-1}] with T5 (28.65) having the highest value and T3 (22.90) with the least value.

Treatment 5 had the highest value (90.75%) of lymphocyte, while T3 (62.60%) had the least value, while PLT count values ranging from 104.50 to 384.00 [· 10^3 uL^{-1}], showed that T2 had the highest value (384.00), T1 (342.50), T3 (135.00), T5 (124.50) and lastly T4 (104.50) which were significant difference ($p < 0.05$).

Table 2

Heamatological parameters of pigs fed graded levels of biscuit dough

Parameters	T1	T2	T3	T4	T5	SEM
WBC [·10^3 uL^{-1}]	18.55^a	14.55^d	15.45^c	17.75^b	10.00^e	0.80
Hb [g dL^{-1}]	11.84^b	12.95^a	11.05^c	13.00^a	9.50^d	0.35
RBC [·10^6 uL^{-1}]	8.18^b	9.73^a	8.13^b	8.13^b	8.13^b	0.17
HCT [%]	49.70^c	54.85^a	48.20^d	52.70^b	33.20^e	2.04
MCV [fL]	60.75^a	57.00^d	59.05^b	58.60^c	49.45^e	1.05
MCH [pg]	14.44^a	13.45^c	13.55^c	14.50^a	14.15^b	0.12
MCHC [g dL^{-1}]	23.70^c	23.65^c	22.90^d	24.70^b	28.65^a	0.55
PLT [·10^3 uL^{-1}]	342.50^b	384.00^a	135.00^c	104.50^d	124.50^c	32.55
LYMPH [%]	70.20^b	64.70^d	62.60^e	68.40^c	90.75^a	2.70

Explanations: ^{ab} means along the same row with different superscript(s) are significantly different ($p < 0.05$); WBC – white blood cell; Hb – haemoglobin; RBC – red blood cell; HCT – haematocrit; MCV – mean cell volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; PLT – platelet; LYMPH – lymphocyte

Organ Weight Expressed as a Percentage of Live Weight [%]

The organs weights expressed as a percentage of live weight of weaner pigs fed graded levels of biscuit dough (BD) are presented in Table 3. The weight of the liver, heart, spleen and pancreas expressed as a percentage of live weight of weaner pigs were significantly different ($p < 0.05$) while that of kidney and lungs were not significantly ($p > 0.05$) affected. The liver weight expressed as a percentage of live weight of weaner pigs fed T1 (2.67), T3 (2.59) and T4 (2.64) were significantly higher than that of T2 (2.39) and T5 (2.44). The heart weight expressed as percentage of live weight of weaner pigs fed T5 (0.71) was higher ($p < 0.05$) than other treatments, but that of T1 (0.51) and T4 (0.51) were comparable. Pigs fed T4 (0.17) had the highest spleen weight expressed as a percentage of live weight of weaner pigs compared with T2 (0.15) while other treatments were different ($p < 0.05$). The pancreas weight expressed as percentage of live weight of weaner pigs on T1 (0.26) was significantly higher than those fed T2–T5.

Table 3

Organ weight expressed as a percentage of live weight of weaner pigs fed graded levels of biscuit dough

Parameters [%]	T1	T2	T3	T4	T5	SEM
Liver	2.67^{a}	2.39^{b}	2.59^{a}	2.64^{a}	2.44^{b}	0.03
Kidney	0.40	0.38	0.39	0.41	0.38	0.01
Heart	0.51^{ab}	0.46^{b}	0.42^{b}	0.51^{ab}	0.71^{a}	0.36
Spleen	0.11^{c}	0.15^{ab}	0.13^{b}	0.17^{a}	0.14^{c}	0.01
Pancreas	0.26^{a}	0.20^{b}	0.20^{b}	0.17^{b}	0.14^{c}	0.01
Lungs	0.93	0.86	1.01	0.93	0.97	0.02

Explanations: *a*, *b* means along the same row with different superscript(s) are significantly different ($p < 0.05$)

Villi Morphometrics

The villi morphometry of weaner pigs fed varying levels of biscuit dough shown in Table 4. The villi lengths, villi widths, villi cryptal depths, were significantly ($P < 0.05$) influenced. Pigs fed T2 (0.078 inch) had the lowest value for villi length unlike those fed T3 (0.060 inch) which had the highest value for villi width while T4 (0.057 inches) has the highest value for villi cryptal depth compared to that fed T1 (0.048 inches), T3 (0.047) and T5 (0.051 inches).

Table 4

Villi morphometry of weaner pigs fed graded level of biscuit dough

Parameters	T1	T2	T3	T4	T5	SEM
Villi length [inch]	0.105^{a}	0.078^{b}	0.107^{a}	0.107^{a}	0.105^{a}	0.002
Villi width [inch]	0.021^{b}	0.023^{b}	0.060^{a}	0.025^{b}	0.017^{b}	0.006
Villi cryptal depth [inch]	0.048^{ab}	0.041^{b}	0.047^{ab}	0.057^{a}	0.051^{ab}	0.002

Explanations: *a*, *b* means along the same row with different superscript(s) are significantly different ($p < 0.05$)

Discussion

All the haematological parameters fall within the standard values for pigs (domestic boars) (THOM 2006, EZE et al. 2010). When these values fall within the normal range reported for the livestock, it is an indication that the diets are tolerated throughout the experiment, but when the values fall below the normal range, it is an indication of anaemia (TOGUN et al. 2007). Also, lower haematological values in pigs are thought to be due to

malnutrition. Immune status is a function of leucocytes, neutrophils and lymphocytes. Lymphocytes are known to play critical roles in the immune defence system of both man and animals (AMEEN et al. 2007), while, higher leukocyte count of the pig is thought to be due to chronic pneumonia and parasitism.

Moreover, when WBC (leucocytes); neutrophils and lymphocytes fall within the normal range, it indicates that the feed does not affect the immune system (AMEEN et al. 2007). Nevertheless, increased neutrophils: lymphocytes ratio is a good indicator of stress (MINKA and AYO 2007, ADEKOLA and DUROTOYE 2004), which could be nutritional stress (ETIM et al. 2014). Report shows an association between immune function and leucocyte (EHEBA et al. 2008), while other work observed that increase in PCV coupled with a marginal increase in RBC is indicative of more efficient erythropoiesis in the experimental animals (TOGUN et al. 2007). Also lower values of PCV and Hb imply a high level of blood dilution and low efficiency of cellular oxygen transportation (NWANBE and ELECHI 2009).

It is a common practice in feeding trials to use the weights of some internal organs like liver and kidney as indicators of toxicity (SHITU et al. 2016). Previous work showed that if there are toxic elements in the animal feed, abnormalities in weights of liver and kidney could be observed. The abnormalities will arise because of the increased metabolic rate of the organs in an attempt to reduce these toxic elements or anti-nutritional factors to nontoxic metabolites (AHAMEFULE et al. 2006). However, the reported on biscuit waste showed that biscuit waste has no anti-nutritional factor; therefore, could make a suitable replacement for maize (ADEYEMO et al. 2013). This position could be buttressed by the quadratic pattern observed by these organs except the linear decrease in the weight of pancreas as the BD increases suggesting hypo-secretion of pancreatic juice, which suggest that addition of BD to the absolute limit may reduce the production of acid by the lumen as shown in this study.

Higher villi area (length and width) could indicate a greater surface area for absorption of nutrients (OJEDIRAN et al. 2017b) which is also in line with previous observation and concluded that efficient utilization of feed could be a function of the response of the villous to the feed form (NKUKWANA 2014).

Conclusion

It can be concluded that 50% biscuit dough replacement for maize weakened the body defensive mechanism, reduced the efficiency of cellular oxygen transportation, pancreatic secretion and villi width of weaner pigs,

while 37.5% biscuit dough replacement for maize improved the parameters mentioned above. It is, therefore, recommended that 37.5% of biscuit dough is suitable for the replacement of maize in weaner pig's diets.

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USABILITY OF WHITE LUPIN AND PEA SEEDS IN THE FEEDING OF GROWING NEW ZEALAND WHITE RABBITS*

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Key words: rabbits feeding, soybean meal substitution, white lupin, pea, digestibility and nitrogen metabolism.

Abstract

The objective of the study was to determine the effect of the substitution of extracted soybean meal (SBM) with a mixture of white lupin seed (WLS) and pea seed (PS) on production results, nutrient digestibility and nitrogen retention in rabbits. The control feed mixture (SBM10 group) contained 10% SBM. In the first experimental group (SBM5), the diet contained 5% SBM, which was partially substituted with a mixture of WLS and PS. In the second experimental group (SBM0), soybean meal was completely substituted. Partial or complete replacement of SBM did not cause significant differences in body weight, feed conversion ratio, nutrients or energy digestibility, and N retention between the studied groups of rabbits. The results achieved in the experiment indicate that the partial or complete substitution of extracted SBM with WLS and PS did not have a negative effect on production results, nutrient digestibility or N retention in growing rabbits.

Introduction

Soybean meal is currently the most common source of protein in the diets of rabbits from large-scale farms. However, soybean is also used in the countries where soybean is not grown and has to be imported. To

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become independent of soybean imports, research is being conducted to replace it with other components in rabbit diets. Such components include by-products of biofuel production, mainly rapeseed meal and rapeseed cake as well as distiller's dried grains with solubles – DDGS (Gasmi-Boubaker et al. 2007, Strychalski et al. 2014b, El-Medany and El-Reffaei 2015, Gugołek et al. 2015). Products such as sunflower cake and sunflower meal are also used for replacement (Volek and Marounek 2009, 2011, Matusevicius et al. 2014). Moreover, attempts to use white lupin seed (Volek and Marounek 2009, 2011, Al-Harbi et al. 2014), pea (Seroux 1988, Castellini et al. 1991, Bonomi et al. 2003), and other plants of the *Fabaceae* family have been made (Amaefule et al. 2005, Lounaouci-Ouyed et al. 2014, Zwoliński et al. 2017). All of these products originate from plants that have been grown for years in the transitional temperate climate zone.

The above-mentioned feeds may have an adverse effect in larger amounts on animals, due to the chemical and amino acid composition being changed in relation to the initial raw material. For the grain of *Fabaceae* plants, this is a result of various anti-nutritional substances, e.g. tannins, antitrypsin factor, haemagglutinins, α-galactosides, and alkaloids (Bastianellia et al. 1998, Chilomer et al. 2010). However, when applied in moderate amounts, even when not subjected to any treatment processes, these components do not have an adverse effect on rabbits.

The objective of the current study was to determine the effect of the substitution of extracted SBM with a mixture of white lupin seed and pea seed on production results, as well as nutrient digestibility and nitrogen retention in rabbits.

Material and Methods

Experimental Factor

The experimental factor was the contribution of white lupin seed (WLS) and pea seed (PS) in pelleted feed mixtures. The chemical composition and energy value of these components and of the soybean meal (SBM) are presented in Table 1. The control feed mixture (SBM10 group) contained 10% extracted soybean meal (SBM). In the first experimental group (SBM5), the diet contained 5% SBM, which was partially substituted with a mixture of WLS and PS. In the second experimental group (SBM0), soybean meal was completely substituted. The formulation, chemical composition and energy value of feed mixtures are presented in Table 2. All rations met the nutritional requirements of growing rabbits.

Table 1

Chemical composition [%] and measured energy content [MJ] of soybean meal, white lupine seed and pea seed

Specification	SBM	WLS	PS
Dry matter	89.92	90.13	88.85
Crude ash	6.74	6.58	6.40
Crude protein	36.82	41.29	29.61
Ether extract	2.17	2.64	1.26
NDF	16.57	28.85	14.63
ADF	7.36	16.93	7.72
ADL	5.08	5.52	4.60
Lysine	2.62	1.64	1.74
Methionine + cystine	1.27	0.96	0.87
Threonine	1.64	1.48	1.09
Tryptophan	0.48	0.36	0.26
Gross energy [MJ kg^{-1}]	17.13	16.65	16.48

Explanations: SBM – soybean meal; WLS – white lupine seed; PS – pea seed; NDF – neutral detergent fibre; ADF – acid detergent fibre; ADL – acid detergent lignin

Table 2

Ingredients, chemical composition and measured energy content of feed mixtures

Specification	Group		
	SBM10	SBM5	SBM0
Ingredients [%]			
Soybean meal (37% CP)	10.0	5.0	–
White lupine seed	–	3.5	7.0
Pea seed	–	1.5	3.0
Dried alfalfa	30.0	30.0	30.0
Barley	14.5	14.5	14.5
Corn	11.0	11.0	11.0
Wheat bran	11.0	11.0	11.0
Wheat	6.0	6.0	6.0
ARBOCEL*	5.0	5.0	5.0
Beet molasses	2.0	2.0	2.0
Skimmed milk powder	2.0	2.0	2.0
Dried brewer's yeast	1.0	1.0	1.0
Calcium carbonate	1.0	1.0	1.0
Dicalcium phosphate	1.0	1.0	1.0
Mineral-vitamin premix†	1.0	1.0	1.0
NaCl	0.5	0.5	0.5

Chemical composition [%]			
Dry matter	90.6	90.6	90.6
Crude ash	5.35	5.34	5.33
Crude protein	17.02	17.06	17.11
Ether extract	2.71	2.72	2.73
NDF	26.95	27.32	27.75
ADF	14.26	14.61	14.94
ADL	3.24	3.25	3.26
Lysine	0.81	0.76	0.72
Methionine + cystine	0.73	0.71	0.70
Threonine	0.66	0.65	0.63
Tryptophan	0.18	0.17	0.16
Gross energy [MJ kg^{-1}]	16.82	16.80	16.80

Explanations: NDF – neutral detergent fibre; ADF – acid detergent fibre; ADL – acid detergent lignin. * crude fibre concentrate; † composition mineral-vitamin premix 1 kg: vit. A – 3500 000 IU; vit. D_3 – 200 000 IU; vit. E – 28 000 mg; vit. K_3 – 200 mg; vit. B_1 – 1500 mg; vit. B_2 – 2800 mg; vit. B_6 – 2800 mg; vit. B_{12} – 20 000 mg; folic acid – 200 mg; niacin – 10 000 mg; biotin – 200 000 mg; calcium pantothenate – 7000 mg; choline – 30 000 mg; Fe – 17 000 mg; Zn – 2000 mg; Mn – 1000 mg; Cu (copper sulfate x $5H_2O$, 24.5%) – 800 mg; Co – 1000 mg; I – 100 mg; methionine – 150 g; Ca – 150 g; P – 100 g

Animals and Treatments

The animals were New Zealand White (NZW) rabbits. For the experiment, 48 rabbits were selected from 8 litters. They were divided into three groups which were similar in terms of origin, proportion of sexes and body weight. The experiment was carried out in August-October and started when rabbits were weaned at 35 days of age (average body weight – 918.37±13.82 g) and terminated when they reached 91 days of age. The rabbits were kept in a closed pavilion, in wire-net flat deck cages (0.5 × 0.6 × 0.4 m; 1 animal each), and were fed *ad libitum* pelleted diets. They were kept under standard conditions: temperature of 19–22°C and relative air humidity of 60–75%, intensive ventilation of rooms, and regulated photoperiod (16-h lighting and 8-h darkness). Digestibility and balance tests were performed on 3 males and 3 females randomly selected from each group, aged from 42 to 62 days. The animals were placed individually in digestibility-balance cages adjusted for quantitative collection of faeces and urine. The 10-day period of exact experiment was preceded by a 10-day preliminary period, which allowed the rabbits to adapt to new environmental conditions. The animals were fed once a day, like the other animals in the production experiment, each time receiving 200 g of a pel-

leted feed mixture. The rabbits also had free access to drinking water. The animal protocol was carried out in accordance with EU Directive 2010/63/EU for animal experiments (OJEU 2010). The research was also conducted in accordance with the provisions of the Lithuanian legal acts.

The rabbits were weighed on an electronic scale on days 35 and 91. The data allowed calculating daily body weight gains (BWG) of the rabbits and the feed conversion ratio (FCR) [feed intake (g) / BWG (g)]. During the digestibility and balance testing, non-ingested feed residues and excreted faeces were collected every day and weighed with an accuracy of 1 g. The collected faeces were frozen and samples of faeces and feed mixtures were then dried and ground. Urine was preserved with 20% sulphuric acid to allow calculating the total volume of collected urine at the end of the experiment. The prepared samples were subjected to chemical composition and energetic value determination.

The balance method used in studies of this type enabled calculating digestibility coefficients of nutrients and energy as well as nitrogen (N) retention. Digestibility of nutrients (DN) was calculated from the following formula:

$$DN\ [\%] = (a - b)/a \cdot 100$$

where:
a – the nutrient content in a feed mixture
b – in faeces (PEREZ et al. 1995).

At the end of the production trial, after 24-hour fasting, the animals were weighed and killed according to the accepted recommendations for euthanasia of experimental animals (rabbits were stunned and bled, and the whole procedure took about 2 min.). After the slaughter, the animals were skinned and eviscerated. After cooling the carcasses (for 24 h, at 4°C), tissue samples (n = 16) were taken for chemical analyses, and dressing percentage (DP; n = 16) was calculated as follows:

DP [%] = chilled carcass weight without head and giblets [kg] live weight [kg] · 100%.

In addition, the percentage contents of the primal cuts: forepart, loin and hind part, were calculated in the carcass. The carcasses were divided into the head (cut through the craniovertebral joint), the fore part (cut between the 7th and 8th thoracic vertebrae), the loin (cut between the 6th and 7th thoracic vertebrae) and the hind part (carcass section remaining after separation of the loin from the front, comprising the hindquarters and hind limbs) (BLASCO and OUHAYOUN 1996).

Chemical Analyses

All of the analyses were performed on duplicate samples.

The nutrient content of feed was determined by standard methods (AOAC 2006). Dry matter content was determined in a laboratory drier, at 103ºC. Crude ash content was estimated by sample mineralization in a muffle furnace at 600ºC. Total nitrogen content was determined by the Kjeldahl method, in the FOSS TECATOR Kjeltec 2200 Auto Distillation Unit. Ether extract content was estimated by the Soxhlet method, in the FOSS SOXTEC SYSTEM 2043. NDF (neutral detergent fibre), ADF (acid detergent fibre) and ADL (acid detergent lignin) were estimated in the FOSS TECATOR Fibertec 2010 System. NDF was determined according to the procedure proposed by VAN SOEST et al. (1991). ADF and ADL were determined according to procedures of AOAC (2006). The amino acid levels in diets were determined using the Biochrom 20 plus amino acid analyser and Biochrom amino acid analysis reagents (Biochrom Ltd., Cambridge, England). Gross energy content was determined using a bomb calorimeter (IKA® C2000 basic, Germany).

Statistical Analysis

The data are expressed as means ± standard error of the mean (SEM) and the results were processed statistically using a least-squares analysis in GLM procedures. For comparison of data,

$$\text{the } Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha_i\beta_j + \varepsilon_{ijk} \text{ model was used}$$

where:
μ – the general mean
α_i – the effect of diet
β_j – the effect of sex
$\alpha_i\beta_j$ – the interaction effect between diet and sex
ε_{ijk} – the random error.

Since the analyses did not reveal significant effects of sex or significant interactions between fixed effects, they are not reported in the tables. All calculations were made with Statistica (STATSOFT, INC. 2010) software.

Results

Table 3 shows data concerning measurements of rabbit body weights, calculated mean body weight gains, feed conversion ratio and the car-

cass characteristics. There were no intergroup differences in either the initial or in the final body weight of the animals (which on day 91 of age ranged between 2478.6 g in SBM0 group and 2514.4 g in SBM5 group).

Table 3

Growth performance and carcass characteristics of rabbits (mean ± SEM)

Specification	Group		
	SBM10	SBM5	SBM0
BW at 35 days [g]	918.2±8.03	920.2±6.57	916.7±6.82
BW at 91 days [g]	2493.4±108.54	2514.4±120.71	2478.6±129.70
Daily BWG [g d^{-1}]	28.13±3.97	28.48±4.13	27.89±4.32
FCR [g g^{-1} gain]	3.74±0.05	3.69±0.03	3.49±0.04
DP [%]	**45.73±0.32^{b}**	46.27±0.41	**47.15±0.41^{a}**
Forepart [%]	36.39±0.17	35.62±0.16	35.46±0.18
Loin [%]	26.33±0.14	27.22±0.13	26.91±0.13
Hind part [%]	37.28±0.17	37.16±0.15	37.63±0.16
DM [%] in loin	28.17±0.36	27.85±0.29	28.00±0.32
CP [%] in loin	22.54±0.19	22.34±0.20	22.28±0.20
EE [%] in loin	4.63±0.42	4.68±0.37	4.70±0.37

Explanations: SEM – standard error of the mean; n = 16 per group; BW – body weight; BWG – body weight gains; FCR – feed conversion ratio; DP – dressing percentage; DM – dry matter; CP – crude protein; EE – ether extract.

$^{a, b}$ Values with different superscripts are significantly different at $P < 0.05$

Minor statistically insignificant differences were also recorded for daily BWG. The SBM0 group was characterised by the smallest gains (27.89 g d^{-1}), while the SBM5 group showed the greatest gains (28.48 g d^{-1}). In addition, no significant differences in FCR were recorded. However, it should be noted that rabbits in the SBM0 group consumed an average of 3.49 g feed per 1 g of body weight gain while SBM5 rabbits consumed 3.69 g g^{-1} gain and rabbits in the SBM10 control group consumed 3.74 g g^{-1} gain. Significant differences were also noted in DP between the SBM10 group (45.73%) and SBM0 (47.15%), while for rabbits in the SBM5 group this parameter amounted to 46.27%. However, there were no differences in the share of primal cuts between groups. Rabbits in the SBM10 group were also characterised by the greatest share of the forepart, rabbits fed feed including 5% portion of soybean meal exhibited the greatest share of loin (SBM5 – 27.22%), while rabbits in SBM0 group were characterised by the greatest share of the hind part (37.63%). DM, CP and EE contents were similar in all groups and were in the following ranges: 27.85–28.17% of DM, 22.28–22.54% of CP and 4.63–4.70% of EE.

Table 4 shows the results of tests for nutrient digestibility in rabbits. No intergroup differences were noted in DM, CP and EE digestibility. NDF digestibility proved to be statistically significantly better in the SBM10

Table 4

Nutrient and energy digestibility [%] in rabbits (mean ± SEM)

Specification	SBM10	SBM5	SBM0
Dry matter	69.72±2.72	69.21±3.28	68.88±3.47
Crude protein	69.74±4.38	69.93±3.84	70.07±5.39
Ether extract	88.52±3.29	87.81±3.42	87.15±3.27
NDF	**51.63±1.82**^{a}	**47.17±1.28**^{b}	**45.84±2.09**^{b}
ADF	25.79±1.00	24.83±1.82	24.90±2.24
ADL	23.71±1.28	23.70±1.70	23.89±2.19
Gross energy [MJ kg^{-1}]	75.74±2.62	75.23±2.85	74.82±2.71

Explanations: SEM – standard error of the mean; n = 6 per group; $^{a, b}$ values with different superscripts are significantly different at $P < 0.0$

control group (51.63%) than in the groups fed a mixture of white lupin and pea seeds: SBM5 – 47.17% and SBM0 – 45.84%. However, there were no significant intergroup differences in the digestibility of other fibre fractions, ADF or ADL. The total gross energy digestibility in particular groups was as follows: 75.74% in SBM10 group, 75.23% in SBM5 group, and 74.82% in SBM0 group; however, these differences were not statistically significant.

Per day, the rabbits absorbed from 2.53 g d^{-1} (SBM0 group) through 2.60 g d^{-1} (SBM5 group) to 2.63 g d^{-1} of nitrogen (Table 5). Since the animals that absorbed the most of this element also excreted the most of it in faeces and urine, nitrogen retention was very similar in all groups and amounted to 1.42 g d^{-1} in both SBM10 and SBM5 groups and 1.43 g d^{-1} in the SBM0

Table 5

Daily balance and retention of nitrogen in rabbits (mean ± SEM)

Specification	SBM10	SBM5	SBM0
N intake [g d^{-1}]	2.63±0.06	2.60±0.06	2.53±0.04
Faecal N excretion [g d^{-1}]	0.68±0.03	0.66±0.03	0.62±0.03
N digested [g d^{-1}]	1.95±0.05	1.94±0.06	1.91±0.04
Urinary N excretion [g d^{-1}]	**0.56**±0.03^{b}	0.52±0.02	**0.48**±0.02^{a}
N retention [g d^{-1}]	1.42±0.05	1.42±0.05	1.43±0.04
as % of N intake	**53.99**±1.74^{b}	54.62±1.81	**56.52**±1.69^{a}
as % of N digested	**72.82**±2.16^{b}	73.20±2.37	**74.87**±2.13^{a}

Explanations: SEM – standard error of the mean; n = 6 per group; $^{a, b}$ values with different superscripts are significantly different at $P < 0.05$

dietary group. Nitrogen retention for the nitrogen taken with the feed proved to be the lowest in SBM10 group (53.99%) and the highest in rabbits in the SBM0 group (56.52%), and the difference was significant. A similar relationship was also observed in nitrogen retention-to-digested nitrogen. This parameter was significantly lower in the SBM10 group (72.82%) than in the SBM0 group (74.87%).

Discussion

In the current experiment, it was found that partial or complete replacement of soybean meal with the white lupin seed and pea seed did not cause significant differences in body weight between the studied groups of rabbits or changes in feed efficiency. In diet balancing, the same proportions of other components in all dietary groups, such as alfalfa, barley, corn, wheat bran and wheat, were all successfully maintained, which rendered the results more reliable. However, it should be noted that although the control diet contained the most limiting exogenous amino acids: lysine, methionine, cystine, threonine and tryptophan (Table 2), this had no effect on protein content in the rabbit loin (Table 3).

The obtained content of dry matter, protein and ether extract in meat is characteristic of broiler rabbits. Chełmińska and Kowalska (2013) observed a similar content of dry matter and protein as in the current study and a slightly lower fat content in the meat of NZW rabbits. In addition, the range of DP obtained in the current study is consistent with the findings of Daszkiewicz et al. (2012) and Chełmińska and Kowalska (2013). It should be noted that, with the increase in WLS and PS in the diet of rabbits, their DP increased, and the difference between the SBM10 and SBM0 groups turned out to be statistically significant. Similarly, Volek and Marounek (2009) reported a higher DP value in rabbits fed a WLS diet compared to rabbits fed a diet with SBM or sunflower meal. On the other hand, no similar phenomena were noted by Uhlířová et al. (2015) and Volek and Marounek (2011).

The BW levels of NZW rabbits in the current experiment were slightly higher than those obtained in previous studies. However, the BWG levels of the rabbits investigated by Chełmińska and Kowalska (2013) were similar to the current study. Similar BWG levels in NZW rabbits, although calculated for the age range of 30–80 days, were reported by Cardinali et al. (2015). It should also be noted that the rabbit body weights and BWG may differ subject to breed. In the authors' previous experiment, Californian rabbits achieved body weights of 2291–2371 g at age 98 days,

with BWG of 24.6–26.2 g (STRYCHALSKI et al. 2014a). Currently, commercially bred hybrid rabbits may exceed 3000 g on day 84 of age (DÄNICKE et al. 2004, GUGOŁEK et al. 2015).

VOLEK and MAROUNEK (2009) showed that the addition of 15% WLS to the rabbits' feed did not affect weight gain or FCR as compared to animals fed diets containing SBM. PS was added to rations for rabbits by SEROUX (1988), CASTELLINI et al. (1991), and BONOMI et al. (2003). They showed that pea meal might account for up to 30% of feed ration with no negative effect on production results. BONOMI et al. (2003) suggested that pea meal is a valuable feed and that it is possible to use it as a soybean substitute. On the other hand, LOUNAOUCI-OUYED et al. (2014) demonstrated that the 30% addition of peas had no effect on growth or feed consumption, but increased the conversion of feed per 1 kg of gain as compared with a feed containing SBM.

In other publications regarding the substitution of SBM with other feeds, the authors explain that the poorer production results may be due to the poorer amino acid composition of the experimental diets (STEIN et al. 2006, YANG et al. 2010, YOUSSEF et al. 2012). However, no such relationships occurred in the experiment concerned (Table 3).

The obtained results provided in Table 4 should be considered typical for animals aged 40–60 days. Neither partial nor complete substitution of SBM with lupin and pea seeds contributed to the statistically significant differences in the nutrients or energy digestibility between the groups. VOLEK and MAROUNEK (2009) and VOLEK et al. (2013) demonstrated that the addition of 15% WL seed and 5% WL grain hulls to the ration had no effect on nutrient digestibility. LOUNAOUCI-OUYED et al. (2014) found that the gross energy and crude protein digestibility coefficients were similar to those in the groups fed diets containing SBM and field bean (*Vicia faba* L.) and were lower in the group fed feed with 30% pea seed added.

The coefficients of CP digestibility were similar to those obtained in studies by TŮMOVÁ et al. (2003), ZITA et al. (2007), and STRYCHALSKI et al. (2014a). A higher level of protein digestibility (exceeding 80%) was noted by STRYCHALSKI et al. (2014b) and GUGOŁEK et al. (2015) in groups of animals characterized by higher productivity.

Fat (EE) is relatively well-digested by animals. The level of 87–88.5% obtained in the current study is consistent with the levels obtained by several other authors (TŮMOVÁ et al. 2003, ONDRUŠKA et al. 2010, STRYCHALSKI et al. 2014b, GUGOŁEK et al. 2015). However, FU-CHANG et al. (2004) obtained a lower digestibility of EE, i.e. from 70.1% to 73.6%.

The reported digestibility levels of NDF fractions, ADF and ADL all exhibit certain differentiation in study results. Most often, the results for

the digestibility of NDF fall within the 40–50% range (VOLEK and MAROUNEK 2011, STRYCHALSKI et al. 2014b, GUGOŁEK et al. 2015). Other authors have reported results falling below 40% (VOLEK and MAROUNEK 2009, STRYCHALSKI et al. 2014a). ADF is converted by rabbits at a level of 25–32%, and ADL is converted at a level over 10% (VOLEK and MAROUNEK 2009, STRYCHALSKI et al. 2014b, GUGOŁEK et al. 2015).

The level of energy digestibility should also be considered typical for rabbits fed complete feed. In some studies, the level was slightly higher; e.g. in a study by LAKABI-IOUALITENE et al. (2008), it ranged from 71% to 76%; in a study by STRYCHALSKI et al. (2014b), from 78% to 83%; while in a study by GUGOŁEK et al. (2015) it was from 80% to 84%. A slightly lower level of digestibility was noted by VOLEK and MAROUNEK (2009) – from 66% to 69%, and by VOLEK et al. (2013).

Daily nitrogen intake was insignificantly higher in the control group SBM10 than in the experimental groups SBM5 and SBM0 (by 1.15% and 3.95% respectively, Table 5). Faecal and urinary excretions were also higher in this group. Consequently, final N retention was also very similar in all three groups (0.70%). However, it should not be disregarded that N retention, with respect to N intake and N digested, was significantly higher in the SBM0 group than in the SBM10 group. Therefore, it seems that an important achievement of this experiment is that rabbits fed without SBM were characterized by higher DP (Table 3), although their daily N intake was slightly lower than that of control rabbits, and this is related with the fact that these rabbits excreted significantly less N in urine. In general, rabbits belonging to SBM0 group wasted less N compared to their SBM10 counterparts. Further research may investigate whether dietary replacement of SBM by WLS and PS reduce phosphorus excretions from rabbits.

Levels of N retention with respect to N uptake and digestion were found to be average (within lower limits) in all groups. A higher retention level was noted in other studies (MASHAMAITE et al. 2009, STRYCHALSKI et al. 2014b, GUGOŁEK et al. 2015). The results obtained by those authors should be associated with the higher productivity of the rabbits they tested. However, results lower than the current results were presented by STRYCHALSKI et al. (2014a) in Californian and Flemish Giant rabbits aged 70 days (after a period of intense growth).

In conclusion, the results achieved in the current experiment indicate that the partial or complete substitution of extracted SBM with 3.5–7% of WLS, and 1.5-3% of PS did not have a negative effect on production results, nutrient digestibility or N retention in growing rabbits.

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REGENERATION POTENTIALS OF THE TREE SPECIES AT BC 32/4 IN SAKPONBA FOREST RESERVE, EDO STATE, NIGERIA

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Key words: tree species, basal area, tree volume, regeneration potential and forest management.

Abstract

A good knowledge of the quantity, quality and regeneration potentials of the available resource provide a guide for rotation, decision making and good management planning, which are essential for sustainable forest management. Consequently, a study of the tree regeneration potential was carried out in the forest reserve The main objective of this study was to assess regeneration potential of BC 32/4 in Sakponba Forest Reserve Edo State Nigeria. Four blocks were sampled using systematic sampling technique. Each block consist of 16 samples from which 6 samples were randomly selected for the study and 6 sub plots laid for both adult and juvenile trees. In the 24 sample plots laid, 613 adult trees and 985 juvenile trees were encountered. *Gossweilerodendron balsamiferum*, *Funtumia elastica*, *Hylodendron gabunense, Pentaclethra macrophylla*, *Cordia millenii* sp. *Drypetes chevalieri*, *Strombosia postulata* and *Alfizia idiantifolia* families had the highest number representatives in the sample while *Funtumia elastica, Strombosia postulata* and *Gossweilerodendron balsamiferum* had the highest regeneration average basal area and volume per hectare of 2.9 m^2 and 1522 m^3 respectively. Regeneration potential indices were calculated for all species encountered. The species with regeneration potential indices greater than one (> 1) were considered not threatened in the community while species which had regeneration potential less than (< 1) were considered threatened in the community. It was recommended that sustainable harvesting of the tree species should be practised in order to sustain the regeneration potential of the reserve.

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Introduction

Tropical rain forest has an outstanding natural vegetation type being the chief source of tropical hardwood for sawn timber, veneer, nuts, gums, resins, drugs and other useful plant products and animal protein (NWOBOSHI 1982). It constitutes the largest single forest biome of the world (GOWER et al. 2003). According to AKACHUKWU (1997), the tropical rain forest in Nigeria harbours over 560 trees species which attain a height of at least 12 m and a girth of 60 cm. In Nigeria, natural forest particularly rainforest are major reserves of wood resources for meeting the growing demand for wood. Apart from the growing knowledge of tropical rain forests in the Western scientific tradition, it must not be forgotten that within the tropics man has lived closed to nature and the intimate contact with tropical forests for millennia. The forests yielded all the products needed for his life, and he learned how to grow crops on inherently infertile rain forest soils, by shifting agriculture, moving the fields every 2 or 3 years and allowing forest regrowth to restore fertility. This practice which is referred to as shifting cultivation is still in existence today though minimal (WHITMORE 1998).

Tropical forests like all other forest formations are renewable, that is, they can be regenerated. This is however, only true when the forests are well managed. The problem of the tropical foresters is therefore not only how to make best use of existing natural forest resources but also how to perpetuate the productivity of the forests currently being exploited. The solution is made more difficult by the very structure, reproduction pattern and differential growing habits of the component trees and their responses to various degrees of disturbance. Consequently, one of the main considerations is the silvicultural systems to use in their management. HIGMAN et al. (2000), stated that the adoption of an appropriate regeneration method for any particular tree species depends very much on the manager's knowledge of the regeneration potentials of the species in the area. Regeneration is the process of renewal, restoration and growth that makes ecosystems resilient to natural fluctuation or events that cause damage or extinction. Ecosystems can be regenerative and every species is capable of regeneration.

Following a disturbance such as fire or pest outbreak in a forest, species will occupy, compete for space and establish themselves in the newly opened habitat and the new growth seedlings is known as regeneration. The regeneration potential of species is therefore the capability of the species to regenerate. A regeneration potential index less than one ($R_p < 1$) indicates a threatened status. This study therefore involves the assess-

ment of Sakponba Forest Reserve in the rainforest ecological zone of Edo State Nigeria to ascertain its regeneration potential with a view to providing information that would enhance its sustainable management.

Materials and Methods

Sakponba Forest Reserve is situated in the humid tropical rainforest zone of Nigeria. It lies on latitude 60°4'N and longitude 5°32'E. The forest reserve is located in Orhionmwon Local Government Area of Edo State. Sakponba Forest Reserve is divided into two main areas by River Jamieson, Area BC 29 and BC 32/4. It is gridded into 175 compartments. Out of these,101 are located in BC 29 and 75 in BC 32/4 (ISIKHUEMEN 1998). The geologic timescale is of tertiary age of the post middle colian period called the Benin Sands (OGUNTALA 1980). The topsoil is fine sandy loamy, reddish in color and less than 30 cm in depth. Down the profile at depths greater than 30 cm, the soil becomes coarser and darker reddish Chroma and becomes brick red as the depth increases (OGUNTALA 1980). The mean annual rainfall is 2162 mm. The wettest period is between July and September while the driest is between December and January.

Systematic sampling techniques was adopted for data collections and it involved four (4) blocks in the forest reserve. Each block consisted of 200 m × 200 m along the base line. Every block consists of sixteen (16) sample plots of 50 m × 50 m. Six (6) sample plots were randomly selected in each block. Each block therefore comprised six (6) sample plots. Implying that an area of 60,000 m^2 (6 ha) was assessed for the adult trees while 2,400 m^2 (0.24 ha) was assessed for tree regeneration in the reserve. However, only trees whose diameter at the breast height are less than 20 cm was regarded as juvenile trees (regeneration) – Figure 1. **Diameter girth tape** was used to measure diameter at breast height and diameter at the base while the **Spiegel relaskop** was used to measure the diameter at the middle, top and total height of the adult trees in each sample plot.

Fig. 1. Pictures of the study area

Data Analysis

All tree species recorded in the field enumerated were scored in their respective families according to the documentation of KEAY (1989).

Equation 1 was used to obtain the number of each tree species represented in the respective size-classes.

$$N = n_1 + n_2 + \ldots\ldots\ldots\ldots + n_{24} \ldots\ldots\ldots\ldots \quad (1)$$

where:
N – total number of individuals of each species in the representative size-classes
$n_{1\_}n_{24}$ – number of individual of each species in sample plot 1-24

The numbers of juvenile trees of each species from all the sample plots were added together, using equation 1, to obtain the total regeneration of each species in the sample.

Basal Area Estimation

The basal area of each tree was calculated using eq. (2):

$$\mathrm{BA} = \frac{\pi D^2}{4} \quad (2)$$

where:
BA – basal area [m^2]
π – constant (3.142)
D – diameter at breast height

Volume Estimation

The volume of each tree was estimated using the Newton's equation.

$$V = h\left(\frac{\mathrm{Ab} + 4\mathrm{Am} + \mathrm{At}}{6}\right) \quad (3)$$

where:
V – volume
h – height
Ab – diameter at the base
Am – diameter at the middle
At – diameter at the top

The basal area and volumes of the individual trees in all the sampling plots were properly sorted into their respective diameter classes. The basal

area and volumes of tree in each size class were summed together using equation 4 and 5 respectively, to obtain the total basal area and volumes for each size class in all the sample plots.

$$B = \Sigma B \tag{4}$$

where:
B – the basal area of all the trees in each size class at the sample plot level
ΣB – summation of basal area of individual trees in each size class.

$$V = \Sigma V \tag{5}$$

where:
V – the volume of the trees in each size class at the sample plot level.
ΣV – summation of volume of individual tree in each class.

The total basal area and volumes of trees in each size class from all the sample plots were further summed together and divided by 4 (the number of hectares enumerated), using equation 6 and 7 respectively, to obtain the average basal area and the volumes of trees per hectares for each size class. These data showed the distribution of basal area and volumes among the various size classes.

$$\mathrm{AB} = \frac{\Sigma B}{4} \tag{6}$$

where:
AB – average basal area of the trees per hectare in each size class
ΣB – summation of the basal areas of trees in each size class in the respective sample plot.

$$\mathrm{AV} = \frac{\Sigma \mathrm{V}}{4} \tag{7}$$

where:
AV – average volume of trees per hectare in each size class
ΣV – summation of the volume of trees in each size class in the respective sample plot.

Measure of population density and productivity of the study area were obtained by adding together the average numbers of trees, basal area and volumes per hectare from all the size classes to obtain the number of trees, basal areas and volume per hectare respectively (equation 8 and 9).

$$\mathrm{TAB} = \mathrm{AB}_1 + \mathrm{AB}_2 + \quad + \mathrm{AB}_9 \tag{8}$$

where:
TAB – total average basal area of trees per hectare
AB_1–AB_9 – the average basal area of trees per hectare, for size classes 1–10

$$TAV = AV_1 + AV_2 + \quad + AV_9 \tag{9}$$

where:
TAV – total average volume of trees per hectare
AV_1–AV_9 – the average volume of trees per hectare, for size classes 1–9.

The total number of juvenile trees (regeneration) of each species in the sample were extrapolated using equation 10 to obtain the regeneration of each species per hectare

where:
R – the regeneration of each species per hectare
H – one hectare (10,000 m^2) of the forest
N – number of juvenile trees of each species in the sample
A – the total area sampled for the juvenile trees (2,400 m^2).

The regeneration potential of each species were estimated with the formula:

$$R_p = \frac{ni \cdot ri}{N} \tag{10}$$

where:
R_p – natural regeneration potential index
ni – number of individual adult species per hectare
ri – number of regeneration or juveniles of species per hectare
N – total numbers of adult trees.

Results

A total of 44 species of both adult and juvenile trees belonging to 40 families were considered. *Caesalpiniodeae* and *Olacaceeae* families had the highest numbers of juvenile trees each represented by 75 juvenile individual in the sampled area. They were followed by *Samyolaceae* family which was represented by 54 juvenile individuals. These were followed by *Mimosoideae* and *Rubaceae* families which were represented by 50 juvenile individuals while others such as *Annonaceae, Meliaceae Caesalpinioideae, Tiliaceae*, *Rubiaceae* and *Simaroubaceae* families had no juvenile individual. All other families had between 4 and 75 juvenile representatives in the sample. In the regeneration category (Table 1) 40 out of 44 species encountered in the study were represented by their juveniles. A total of 985 juvenile trees (trees with < 20 cm in dbh) were identified and counted in all the 24 sub plots. *Funtumia elastca* (75) and *Strombosia*

pustulates (75) and *Homalum letestuii* (54) *Pentaclethra macrophylla* (50) *Rothmannia hispida* (50) were the five top most species represented in the regeneration category. On the other hand, *Anemidium mannii, Synsepalum stipulatum, Pycnanthus angolensis and Anonidium mannii* had the least number of juvenile representatives of four each in the sample.

Table 1

Juvenile trees regeneration

Species	Number of sample plot	Number per hectare
Tabernaemontana pachysiphon	8	33
Anthonotha macrophylla	8	33
Pycnanthus angolensis	4	17
Anonidium mannii	4	17
Brenania brieyi	12	50
Hannoa klaineana	8	33
Synsepalum stipulatum	4	17
Omphalocarpum procerum	8	33
Sphenocentrum jollyanium	8	33
Zanthoxylium zanthoxyloides	8	33
Ficus exasperate	12	50
Millettia aboensis	12	50
Nauclea diderrichii	8	33
Harungana madagascariensis	20	83
Cordia millenii	8	33
Milicia excelsia	8	33
Pycnanthus angolensis	12	50
Allanblackia floribunda	16	67
Musanga cecropioides	16	67
Cylicodiscus gabunensis	20	83
Piptadeniastrum africanum	16	67
Sterculiaa tragacantha	25	104
Maesopsis eminii	29	121
Trilepisium madagascariense	37	154

Celtis zenkeri	29	121
Ricinodenchron heudelotii	29	121
Trichilia heudelotii	29	121
Pansinsytalia macroceras	45	188
Anonidium mannii	37	154
Hylodendron gabunense	29	121
Guarea cedrata	49	205
Pentaclethra macrophylla	50	208
Rothmannia hispida	50	208
Homalum letestuii	54	225
Albizia idiantifolia	45	188
Drypetes chevalieri	37	154
Gossweilerodendron balsamiferum	41	171
Funtumia elastic	75	313
Strombosia postulate	75	313

Estimates of the regeneration per hectare revealed that *Funtumia elastica* had the highest regeneration of 313 juvenile trees per hectare followed by *Strombosia postulata* with 313 juvenile trees per hectare. Others were *Hylodendron gabunense* (121/ha), *Anonidium manni* (171/ha), *Guarea cedrata* (205/ha and *Rothmannia hispida* (208/ha).

Other tree species in the forest reserve had less than 100 juvenile trees per hectare. The forest therefore had an estimated population of 1736 adult and juvenile trees with 1634 juvenile trees and 102 adult trees that constituted about 94% of the population of juvenile per hectare while 6% of the population belonged to adult trees category.

The regeneration potential indices of all the species are presented in Table 2. The table showed that the regeneration potential indices of 11 (27%) species were greater than one (> 1), with *Strombosia postulate* and *Funtumia elastica* topping the list with 3.99 each. *Gossweilerodendron balsomiferum* (2.18). The remaining 30 (73%) species each had a regeneration potential index of less than one (< 1), while each of the 15 (37%) out of the 30 species, had a regeneration potential index of zero (0.00).

Table 2

Regeneration potentials of the species

Species	R_p
Enanta chlorantha	0.00
Entandrophragma angolense	0.00
Tabameamontana Pachysipton	0.00
Anthonotha macrophylla	0.00
Khaya ivorensis	0.00
Pentaclethra macrophylla	0.00
Duboscia viridiflora	0.00
Pycnanthus angolensis	0.00
Brenania brieyi	0.00
Anonidium mannii	0.00
Hannoa klaineana	0.00
Brenania brieyi	0.00
Hannoa klaineana	0.00
Synsepalum stipulatum	0.04
Omphalocarpum procerum	0.09
Sphenocentrum jollyanium	0.09
Zanthoxylium zanthoxyloides	0.09
Ficus exasperate	0.13
Millettia aboensis	0.13
Nauclea chiderrichii	0.17
Harungana madagascariensis	0.21
Cordia millenii	0.26
Milicia excelsia	0.26
Pycnanthus angolensis	0.26
Allanlackia floribunda	0.34
Musanga cecropioides	0.34
Cylicodiscus gabunensis	0.43
Piptadeniastrum africanum	0.51
Sterculiaa tragacantha	0.53
Maesopsis eminii	0.62
Trilepisium madagascariense	0.79

Celtis zenkeri	0.93
Ricinodenchron heudelotii	0.93
Trichilia heudelotii	0.93
Pansinsytalia macroceras	0.96
Anonidium mannii	1.18
Hylodendron gabunense	1.23
Guarea cedrata	1.44
Pentaclethra macrophylla	1.60
Rothmannia hispida	1.60
Homalum letestuii	1.72
Albizia idiantifolia	1.91
Drypetes chevalieri	1.97
Gossweilerodendron balsamiferum	2.18
Funtumia elastic	3.19
Strombosia postulate	3.99
$R_p < 1.0$ implies threatened status	

The 44 trees enumerated, had 1634 (94%) represented by their young ones (juvenile). However, 33 (73.3%) of the species were represented by both the adult and juvenile trees (Table 3, Table 4).

Table 3

List of species represented with either juvenile or adult trees

Species	Estimated regeneration/ha	Number of adult trees/ha
Enanta chlorantha	-	1
Entandrophragma angolense	-	1
Tabamagmontana Pachysipton	33	< 1
Anthonotha macrophylla	-	1
Khaya ivoriensis	-	1
Pentaclethra macrophylla	-	1
Duboscia viridiflora	-	1
Pycnanthus angolensis	17	< 1
Brenania brieyi	50-	< 1
Anonidium mannii	17	< 1
Hannoa klaineana	33	< 1

Synsepalum stipulatum	17	1
Omphalocarpum procerum	33	1
Sphenocentrum jollyanium	33	1
Zanthoxylium zanthoxyloides	33	1
Ficus exasperata	50	1
Millettia aboensis	50	1
Nauclea diderrichii	33	2
Harungana madagascariensis	83	1
Cordia Millenii	33	3
Milicia excelsia	33	3
Pycnanthus angolensis	50	2
Allanblackia floribunda	67	2
Musanga cecropioides	67	2
Cylicodiscus gabunensis	83	2
Piptadeniastrum africanum	67	3
Sterculiaa tragacantha	104	2
Maesopsis eminii	121	2
Trilepisium madagascariense	154	2
Celtis zenkeri	121	3
Ricinodenchron heudelotii	121	3
Trichilia heudelotii	121	3
Pansinsytalia macroceras	188	2
Anonidium mannii	154	3
Hylodendron gabunense	121	4
Guarea cedrata	205	4
Pentaclethra macrophylla	208	3
Rothmannia hispida	208	3
Homalum letestuii	225	3
Albizia idiantifolia	188	4
Drypetes chevalieri	154	5
Gossweilerodendron balsamiferum	171	5
Funtumia elastica	313	4
Strombosia pustulate	313	5

Table 4

List of species represented by both juvenile and adult trees

Species	Estimated regeneration/ha	Number of adult trees/ha
Synsepalum stipulatum	17	1
Omphalocarpum procerum	33	1
Sphenocentrum jollyanium	33	1
Zanthoxylium zanthoxyloides	33	1
Ficus exasperata	50	1
Millettia aboensis	50	1
Nauclea diderrichii	33	2
Harungana madagascariensis	83	1
Cordia millenii	33	3
Milicia excelsia	33	3
Pycnanthus angolensis	50	2
Allanblackia floribunda	67	2
Musanga cecropioides	67	2
Cylicodiscus gabunensis	83	2
Piptadeniastrum africanum	67	3
Sterculiaa tragacantha	104	2
Maesopsis eminii	121	2
Trilepisium madagascariense	154	2
Celtis zenkeri	121	3
Ricinodenchron heudelotii	121	3
Trichilia heudelotii	121	3
Pansinsytalia macroceras	188	2
Anonidium mannii	154	3
Hylodendron gabunense	121	4
Guarea cedrata	205	4
Pentaclethra macrophylla	208	3
Rothmannia hispida	208	3
Homalum letestuii	225	3
Albizia idiantifolia	188	4
Drypetes chevalieri	154	5
Gossweilerodendron balsamiferum	171	5
Funtumia elastica	313	4
Strombosia pustulata	313	5

The 613 adult trees enumerated in the sample plots gave a total basal area of about 377.6 m^2 and a total volume of about 9,132 m^3. The basal area and volume per hectare of the forest reserve were estimated as 62.9 m^2 and 1,522 m^3 respectively (Table 5). The distribution of the basal area per hectare among the diameter size classes (Table 5) showed that diameter class 9 had the highest basal area of about 17.6 m^2 per hectare followed by diameter classes 7, 8 and 6, with standing volumes of about 385.8 m^3, 286.8 m^3 and 337.2 m^3 per hectares respectively.

Table 5

Basal area and volume per hectare of the species

Class size [cm]	Basal area/4 ha	Volume/4 ha
20–29	2.5	65.0
30–39	3.0	73.9
40–49	3.3	87.1
50–59	14.5	372.7
60–69	39.5	1,014.2
70–79	58.7	1,459.6
80–89	81.4	2,023.1
90–99	69.6	1,721.2
100 cm above	105.1	2,315.3
Total	377.6	9,132.0

Discussion

The regeneration potential indices calculated for the species encountered in the study showed the various species' regeneration capabilities and conservation status in the study area. The results in table 1 and 2 showed that the regeneration potentials of the tree species in the study area was generally poor, with only 45% (18 species) of the tree species encountered having more than 100 juvenile trees per hectare and their regeneration potential indices greater than one (>1). This was not even up to half of the 40 species in table 3 that were represented by both the juvenile and adult individuals. This has a serious implication on the regeneration and conservation of the various species encountered and the renewal of the forest in general. Since regeneration is one means of forest renewal (NWABOSHI 1982). This is similar to the result obtained by NUR et al. (2016) where 36% of the tree species (17 out of 47) are regenerating in the study area, while majority of the tree species (64%) are not getting favourable con-

ditions to regenerate. ADDO-FORDJOUR et al. (2009) also reported only 29 regenerating tree species from 12 families for the Tinte Bepo Forest Reserve in Ghana. CECCON et al. (2004) and WALE et al. (2012) also noted that lack of adequate regeneration is an issue recognized by foresters and ecologist. MALIK and BHATT (2016) also observed limited regeneration and subsequently declining populations of some dominant native species. Furthermore, JAYAKUMAR and NAIR (2013) observed in their study that only 10 lspecies regenerated well with one of the dominant species having no seedlings which is an indicative of poor regeneration potential. Species such as *Gossweilerodendron balsamiferum, Funtumia elastica, Hylodendron gabunense* and others in Table 2 had high regeneration potential indices (>1), exhibited high potentialities to perpetuate their populations in the community and renew the entire forest. This implies that they have high ability to replace their population effectively and can also replace other tree species which have threatened status in the community. These species were able to regenerate successfully in the area probably because of their ability to produce large quantities of viable seeds, withstand shading, suppression and compete favourably for growth resources in the micro climate under the closed canopy.

In terms of sustainable forest management, the natural regeneration of *Funtumia elastica, Strombosia postulata, Gossweilerodendron balsamiferum, Hylodendron gabunense* and others with high regeneration potential indices, offer enough potential to support sustained yield harvest without any need for artificial intervention. Artificial regeneration is useful where natural seeding of the desired species is absent and difficult to obtain or where their performance is poor and unreliable, and there is need to correct any imbalance between regeneration and exploitation (NWOBOSHI 1982). On the other hand, *Omphalocarpum procerum, Harungana madagascariensis, Pycnathus angolensis* and other species in Table 2 with low regeneration potential indices (< 1) have threatened status. A tree species with less than 1.0 regeneration potential index is considered as a rare species (PATHASARANTHY and KARTHIKEYAN 1997, FORMECU 1999). They have very low potentialities to perpetuate their own populations in that community. Their continued existence in that community is therefore threatened because of their low natural abilities to replace their adult populations in that community. These species could not regenerate successfully in the area probably because they could not do well in the micro climate under the closed canopy.

The species that were considered most threatened in the study area were those with regeneration potential index of zero (0.00). They were represented by either very few adult or juvenile trees as shown in Table 2.

Those species with less than one (>1) adult tree per hectare without regeneration included *Pycnathus angolensis, Brenania, brieyi, Ananidium mommi, and Tabamagmontiana pachysipton*. Species with few regenerations per hectare, but no adult individuals were *Entandrophragma angolense, Khaya ivorensis, Anthenotha macrophylla* etc. The scanty representation of some trees species in this study is typical of the tropical rain forest ecosystem NWOBOSHI (1982) and ETUKUDO (2000) reported that a forest may be rich in tree species with hundreds of them sometimes found in a single hectare, but some species may be represented by only one to three individuals per hectare. According to PARTHASARATHY and KARTHIKEYAN (1997), a species with less than ten individuals per hectare is considered as rare and endangered species.

Generally, the absence of either their adults or regeneration from all the sample plots indicated their scarcity in the area, which is a sign of endangered or threatened status. The scarcity representation or low regeneration potentials and threatened status of some of the species in the area may be due to their low seed production and viability and/or high mortality rate in the area. This is because the success of the natural regeneration of any species depends on its seed availability, viability and seeding establishment as well as shade tolerance. Unfortunately, some economically desirable species in the tropics, example *Triplochiton scleroxylon* do not fruit yearly. Those that do, hardly have good seed years at regular intervals. Sometimes, the seeds may be eaten by insects, birds and rodents before or after falling from the trees (NWOBOSHI 1982). Although there was no sign of any recent exploitation of timber in the area, the scanty representation and low regeneration of some tree species especially those with non-timber values could be attributed to their over exploitation for non-timber uses. For example, the fruits and trees of *Irvingia gabonensis*, *Afzelia* spp., *Brachystegia* spp., *Allanblackia floribunda*, *Baillonella toxisperma* and *Garcinia cola* are collected for use by humans. This would render their seeds unavailable for their successful natural regeneration and thus be responsible for their scarcity in the area. The stem and branches of *Garcinia mannie* are used as chewing sticks for hygiene. Over exploitation of this species could make its regeneration scarce in the area, since the adult individuals that should produce more seeds for successful natural regeneration would be lacking. Most of the well-known and highly demanded economic species encountered in the study area, such as *Brachystegia* spp., *Afzelia, Bipindensis, Pterocarpus seyauxii, Piptadeniastrum africanum, Ballonella toxisperma, Lovoa trichilioides, Nauclea diderrichii, khaya ivorensis, Entandrophragma utile*, and *Termimalia superba* had threatened status. The management and conservation impli-

cations or species with threatened status in the area include impossibility of sustained yield harvest of such species from the forest without artificial intervention. This means that the application of appropriate silvicultural techniques would be necessary to enhance the seeding production, growth and survival of such species in the area.

With the very large number of smaller trees, the population structure in the study area would favour sustained yield harvest through regular recruitment from lower diameter-size classes into higher diameter-size classes. OGBONAYA (2002) opined that a juvenile size class population of about 35% is ideal for sustained yield harvest. An estimated juvenile tree population of about 4,105 per hectare (Table 2) which was about 99% of the total tree population per hectare, was therefore more than enough to support sustained yield harvest per hectare.

Conclusion

The regeneration potential indices of the species encountered showed that only 45% of the species had their regeneration potential indices greater than one (>1). The regeneration potentials for the various species in the reserve indicated that sustained yield production of wood is very possible. This will be achieved with the adoption of appropriate silvicultural measures that can enhance the regeneration, survival and growth of the species with threatened status. It also requires the adoption of natural forest management to ensure the conservation and sustainable utilization of all tree species in the reserve and the genetic resources. It is therefore recommended that seed trees should be left standing during harvesting to produce seeds for the next generation of tree crops.

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BLA$_{\text{CTX-M}}$ AND *BLA*$_{\text{SHV}}$ GENES ENCODING *ESCHERICHIA COLI* FROM THE ENVIRONMENT AND CLINICS OF SECONDARY AND TERTIARY LEVEL HOSPITALS

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Key words: ESBL genes, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM,}}$ waste-water, antibiotic resistance genes.

Abstract

Escherichia coli from environmental and clinical sources encoding Extended-Spectrum *β*-lactamase (ESBL) genes in Abeokuta, Nigeria were investigated. *Escherichia coli* strains from clinical sources and hospital waste water (HWW) were isolated from tertiary and secondary level hospitals in Abeokuta and swimming pool water (SPW) from hotels in Abeokuta. 103 *Escherichia coli* isolates were identified using standard methods. Antimicrobial susceptibility was by Kirby--Bauer method. ESBL-producers were confirmed by double disk test. *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ in ESBL-producing *Escherichia coli* were detected with PCR. *Escherichia coli* were 45.6%, 43.6% and 9.7% of all isolates from HWW, clinical and SPW respectively. ESBL was expressed in 41 (39.8%) isolates. Isolates from HWW were more resistant to antibiotics than others. Sixty percent of the isolates harbored the ESBL *bla*$_{\text{SHV}}$ gene, while fifty percent had *bla*$_{\text{CTX-M}}$. One isolate had both *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$. This is of grave health implications in the clinics and environment.

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Introduction

Antimicrobial resistance is an emerging problem throughout the world including Nigeria. Particularly resistance associated with Gram-negative bacteria has increased globally (FROST et al. 2019). Serious hospital acquired and community onset bacterial infections in humans including urinary tract infection are commonly caused by *E. coli* (PATERSON 2006). In addition to the prospect of untreatable infections, antimicrobial resistance results in higher economic costs due to longer stay in hospital by infected patients, the requirement for additional diagnostics and more expensive drugs. *β*-lactam antibiotics are important drugs in treating infections caused by *E. coli*, however, *β*-lactamase are the bacterial resistanct enzymes which hydrolyse and make these antibiotics inactive (LIVERMORE and WOODFORD 2006). Resistant genes can be transferred between bacteria via mobile genetic elements (DROGE et al. 1998). One of the mobile elements, integrons that mediate integration of resistance genes may also be involved (PARTRIDGE et al. 2009). Resulting in the development of multidrug-resistant bacteria. Most studies surveying antimicrobial resistance have focused on clinical isolates, while the survival and prevalence of antibiotic-resistant bacteria in the environment has not been well investigated. Furthermore, while hospitals have been regarded as a major source of dissemination of antibiotic-resistant bacteria, very little is known about the contribution of other sources in the spread of these strains. Hence, this study focused on determination of means of community spread of ESBL producing multidrug resistant *Escherichia coli* genes with reference to swimming pools, hospital settings and environments in Abeokuta, Southwest, Nigeria.

Materials and Methods

Sample Collection and Preparation

Clinical isolate of *E. coli* were collected from Sacred Heart Hospital Lantoro, Federal Medical Centre Idiaba, Abeokuta and Ijaye State Hospital. Swimming pool water was collected from 2-star, 3-star and 4-star hotels in Abeokuta in sterile containers. Samples were collected from a depth of 0.5–1 meter. While hospital waste water were collected from effluent of Federal Medical Centre Idiaba, Sacred heart hospital Lantoro and Ijaye State Hospital all in Abeokuta. The samples were transported to the laboratory in ice chests.

Isolation and Identification of *E. coli*

Isolation and identification of *E. coli* was done using bacterial culture media and different biochemical tests. Each samples were cultured on MacConkey agar and incubated at 37°C for 24 hours. Three to five colonies presumptive *E. coli* were randomly selected and identified by subculturing on Eosin Methylene Blue plate (EMB) Representative of pure colonies were picked based on morphology. The isolated colonies were identified as *E. coli* biochemically following standard protocols (OMBARAK et al. 2016).

Antimicrobial Susceptibility Profile of *Escherichia coli* Isolates Using Agar Diffusion Test

Isolates were tested to evaluate the pattern of antimicrobial susceptibilities by Kirby-Bauer disk diffusion method following CLSI guidelines. The following antimicrobial agents were used Nitrofurantoin (300 µg), Ciprofloxacin (5 µg), Ceftazidine (30 µg), Cefuroxime (5 µg), Gentamicin (10 µg), Ofloxacin (5 µg), Cefotaxime (30 µg), Cefixime (5 µg), Cefotaxime (30 µg) (Oxoid UK). After preparation of bacterial suspension, the turbidity of each of them was adjusted to 0.5 McFarland standard equivalent and then inoculated on Mueller-Hinton agar. After overnight incubation at 37°C, diameters of inhibition zones were measured and the results were interpreted as susceptible, intermediate, and resistant (CLSI, 2010).

Screening Test for ESBL Producing *E. coli*

Double Disk Synergy Test (DDST) was done by using cefotaxime (30 mg) and ceftazidime (30 mg) with and without clavulanic acid (10 mg) disks on Mueller-Hinton agar (Oxoid, UK) with 25 mm apart from each other. An increase of equal or more than 5 mm in zone diameter for either antimicrobial agent tested with clavulanic acid versus its zone when tested without clavulanic acid indicated the presence of ESBL (CLSI, 2010).

Genotypic Analysis of the Isolates for ESBL Production DNA Extraction From *E. coli* Isolates

DNA extraction from isolates was performed as described by BAZZAN et al. (2016). Where a few colonies were suspended in 300 µL sterile distilled water and heated at 95°C for 10 minutes. Afterward, they were placed on ice for 5 minutes and then centrifuged at 13 000 rpm for 10 minutes and the supernatant was used as the DNA template.

PCR of *bla*$_{TEM}$, *bla*$_{SHV}$ *and bla*$_{CTX-M}$ *genes*

E. coli isolates were screened by PCR method and using specific oligonucleotide primers to determine *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla* $_{CTX-M}$ genes (SIMA et al. 2016). Primer sequences and their size were used for the detection of *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla* $_{CTX-M}$ genes (Table 1). The PCR reactions for detection of *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla* $_{CTX-M}$ genes were done in a total volume of 25 mL by using Master Mix Red, Taq DNA polymerase with $MgCl_2$. Amplicons were separated by agarose gel electrophoresis at 80 V for 2 h. After which, fragments were stained and visualized using ultraviolet light. Polymerase chain reaction products were sequenced. Then, nucleotide sequences were compared with sequences in the GenBank and European Molecular Biology Laboratory databases by using the BLAST.

Table 1

Primer used for polymerase chain reaction

Gene	Nucleotide sequence fragment	Length (bp)	Reference
bla $_{TEM}$	5-TCGGGGAAATGTGCGCG-3 5-TGCTTAATC AGTGAGGCACC-3	972	SIMA et al. (2016)
bla $_{SHV}$	5-GGGTTATTCTTATTTGTCGC-3 5-TTAGCGTTGCCAGTGCTC-3	615	SIMA et al. (2016)
bla $_{CTX-M}$	5-ACGCTGTTGTTAGGAAGTG-3 5-TTGAGGCTGGGTGAAGT-3	450	SIMA et al. (2016)

Data Analysis

Data were analyzed using SPSS version 17 statistical package and the association between ESBL producing organism's comparison was determined using Chi square test.

Results and Discussion

Distribution of *Escherichia coli* Isolates According to Sampled Areas

In this study a total of 103 samples were randomly collected from the three sampled areas clinic, hospital waste water and swimming pool water (Table 2). The highest percentage occurrence of *Escherichia coli* were obtained from hospital waste water (HWW) (45.6%), followed by clinical (43.6%) while swimming pool water (SPW) (9.7%) had the least. This result is in agreement with the report of DIWAN et al. (2010) and KORZENIEWSKA et al.

(2013). Both reported higher occurrence of this important member of the *Enterobacteriaceae* in clinical and waste water effluent. Isolation of lower percentage of these coliforms in swimming pool water might be linked to low pH.

Table 2

Escherichia coli from clinical, hospital waste water and swimming pool water

S/N	Sample source	*E. coli* [%]
Clinical		
1	FMC	20(44.4)
2	LANT	12(26.7)
3	IJAYE	13(28.9)
Total		45(43.6)
Waste water		
4	FMC	24(50.0)
5	LANT	10(21.3)
6	IJAYE	13(27.7)
Total		47(45.6)
Swimming pool		
7	IDB	3(30)
8	MTG	5(50)
9	LYR	3(20)
Total		11(9.7)
Grand total [%]		103(100)

Explanations: FMC – Federal Medical Center Idi Aba; LANT – Lantoro Catholic Hospital; IJAYE – State Hospital Ijaye; IBD, MTG and LYR are two-star-, three-star, and four-star hotels within Abeokuta Metropolis

Also, it may be as a result of low effectiveness of the chlorine used in the swimming pool water. However the detection of *E. coli* in swimming pool water indicate fecal contamination of the water (ABD EL-SALAM 2012) According to sample type, waste water had more *E. coli* isolates than swimming pool water. This result is in line with the findings of DIWAN et al. (2010) and JORGENSEN et al. (2017). Both of them reported high prevalence of *E. coli* from waste water sources.

Based on sample source the occurrence of *E. coli* were more from waste water sample collected from FMC (44.4%) followed by those from Ijaye (28.2%) then Lantoro (26.2%) (Table 2 and Table 3). A study by ALIPOUFARD and NILII (2010) reported most of the ESBL-producing isolates were from the medical wards, followed by the out-patient's clinic. Adequate Hospital planning and surveillance can be a powerful tool to improve and decrease the burden of communicable diseases (NEIDERUD 2015).

Table 3

Escherichia coli isolates based on sample source

S/N	Sample sources	Sites of collection of *E. coli* (n = 103)		
		FMC	LANT	IJAYE
1	clinic	20	12	13
2	waste water	24	10	13
3	swimming pools	DBI	MTG	LYR
		3	5	3

Explanations: FMC – Federal Medical Center Idi Aba; LANT – Lantoro Catholic Hospital; IJAYE – State Hospital Ijaye; IDB, MTG and LYR are swimming pools located in hotels within Abeokuta; n – number of samples

Among the isolates evaluated (Table 4), ESBL was expressed phenotypically in 39.8%. The percentage of ESBL recorded in this study concurred to report by IROHA et al. (2009) and can be compared to the report of AKANBI et al. 2013. The variation in ESBLs prevalence rates reported between geographical areas and institutions may be attributed to the complex epidemiology of ESBLs, specific type of bacteria involved and methods used for ESBL detection among other factors (AL-JASSER et al. 2006, KAUR et al. 2013).

Table 4

ESBL producing *Escherichia coli* isolates from various sources

S/N	Sample sources	Sites of collection of *E. coli* (n = 41)			Total
		FMC	LANT	IJAYE	
1	clinic	2	6	7	15(36.6)
2	waste water	11	5	5	21(51.2)
3	swimming pools	DBI	MTG	LYR	
		1	2	2	5(12.2)
Grand total					41(39.8)

Explanations: FMC – Federal Medical Center, Idi Aba; LANT – Lantoro Catholic Hospital; IJAYE – State Hospital Ijaye; DBI, MTG and LYR are swimming pools located within within Abeokuta, n – number of samples

On comparing the distribution of ESBL based on sample source, isolates from Hospital waste water had the highest ESBL occurrence of 51.2%, followed by 36.6% from Clinic and then 12.2% from swimming pool water (Table 4). The findings reported in this study is in line with the report of JORGENSEN et al. (2017) who reported high occurrence of ESBL among isolates from hospital waste water, compared to clinical sample. This justify the possibility of ESBL to be transferred between humans and animals, but may also spread in aquatic environments and potentially contaminate and infect exposed individuals (CONTROL ECFDPA 2015).

Resistance Profile of ESBL Producing Isolates From Clinics and Environments

Table 5 showed antibiotics resistant profile of *E. coli* based on sample collection source. Generally, isolates from hospital waste water recorded the highest resistance of 85.1% while the least resistance of 35.6% was from clinical isolates. This is in agreement with the findings of IBRAHIM et al. (2012), who reported high resistance rates of MDR *E. coli* isolates to the first-line oral antimicrobial agents. Generally, most isolates were susceptible to Cefotaxime, because of the low resistance (35.6%) recorded compared to other antibiotics. Among clinical isolates, 80% were resistant to Ceftazidime while most of them were susceptible to Cefotaxime. In HWW, most of the isolates were resistant to Ciprofloxacin (85%) and susceptible to Ofloxacin.

Table 5

Antibiotics resistance profile of ESBL producing *E. coli* based on sample source

S/N	Antibiotics	*E. coli* resistant profile		
		clinic (n = 45) [%]	hospital waste water (n = 47) [%]	swimming pool water (n = 10) [%]
1	CRX	29 (64.4)	33(70.2)	6(60.0)
2	GEN	27 (60.0)	19(40.4)	5(50.0)
3	CXM	34 (75.6)	37(78.7)	6(60.0)
4	OFL	24(53.3)	16(34.0)	8(80.0)
5	AUG	24(53.3)	35(74.5)	4(40.0)
6	NIT	26(57.8)	32(68.1)	5(50.0)
7	CPR	22(48.9)	40(85.1)	7(70.0)
8	CAZ	36(80)	37(78.7)	8(80.0)
9	CTX	16(35.6)	23(48.9)	4(40.0)

Explanations: n – number of isolates CRX (cefuroxime); GEN – gentamycin; CXM – cefixime; OFL – ofloxacin; AUG – augmentin; NIT – nitroflaxacin; CPR – ciprofloxacin; CAZ – Ceftazidine; CTX – cefotaxime

Escherichia coli isolates from SPW showed susceptibility to Augmentin and Cefotaxime. This could be attributed to insufficient decontamination in waste water treatment plants, which induces the risk of artificial selection of extended-spectrum β-lactamase production among *Escherichia coli* isolates (GUNDOGDU et al. 2017). High level resistance to these drugs has also been reported by UGWU et al. (2017). This might be linked to recent misuse of antibiotics in hospital settings and low dose of first-line therapeutic drugs (SAHUQUILLO-ARCE et al. 2011). In the clinic this study observed that the best antibiotics which could be recommended for the treatment of *E. coli*

associated infection is Cefotaxime followed by Ciprofloxacin. In Sewage Ofloxacin, Gentamicin and Amoxicillin-Clavulanic could be recommended, while in swimming pool Amoxicillin-Clavulanic could be the drug of choice. The selective efficacy of these drugs could be associated to lower selective pressure due to their restricted use (SAHUQUILLO-ARCE et al. 2011). Gentamycin, and Ceftazidine were observed to be more resistant in isolates from clinic followed by those from the swimming pool and waste water while to Ciprofloxacin and Cefotaxime isolates from the waste were more resistant than those from the Swimming pool and Clinic, respectively.

PCR Detection of the Presence of *bla*$_{TEM}$ Genes in ESBL Producing *E. coli* strains From Swimming Pools, Hospital Waste Water and Clinics

The result showed that none of the *E. coli* isolates encoded the *bla*$_{TEM}$ genes (Fig 1).

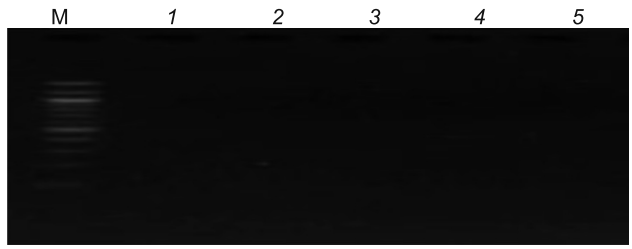

Fig 1. Gel electrophoresis of PCR assay for the detection of *bla*$_{TEM}$ gene from ESBL producing *E. coli* from clinics, hospital waste water and swimming pool water

PCR detection of *bla*$_{CTX\text{-}M}$ genes among ESBL producing *E. coli* strains isolated from clinical samples, hospital waste water and swimming pool water.

The results from Figure 2 showed amplifications which indicate the presence of the *bla*$_{CTX\text{-}M}$ genes.

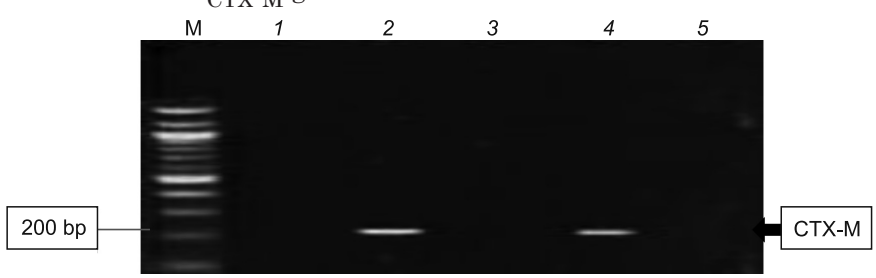

Fig 2. Gel electrophoresis of PCR assay for the detection of *bla*$_{CTX\text{-}M}$ genes from ESBL producing *E. coli* strains from clinics, hospital waste water and swimming pool water. Explanations: lane M – Ladder; *2* – *E. coli* (Clinic FMC); *4* – *E. coli* (Clinic Ijaye)

PCR detection of *bla*$_{\text{SHV}}$ genes among *E. coli* isolated from hospital waste water, clinics and swimming pool water.

Previous studies have noted that most prevalent types of ESBLs are *bla*$_{\text{SHV,}}$ *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M}}$ which can arise because of mutation in the *β*-lactam genes. *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$ were the *β*-lactams genes observed among the *E. coli* isolates (Fig. 3). Even though *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ are the most common type of the ESBLs genes in the past decade, recently *bla*$_{\text{CTX-M}}$ have been found more prevalent than *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ genotypes

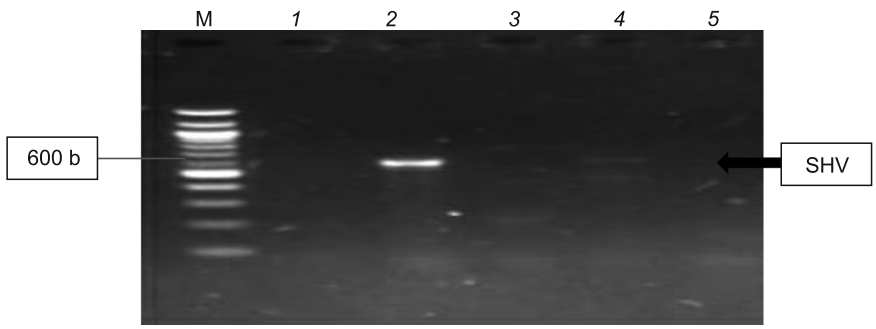

Fig. 3. Gel electrophoresis of PCR assay for detection of ESBL genes for *bla*$_{\text{SHV}}$ among *E. coli* from hospital waste samples, clinics and swimming pool water.
Explanations: lane M – Ladder; *1 – E. coli* (Clinic FMC); *2 – E. coli* (Clinic FMC); *3 – E. coli* (Clinic Lantoro); *4 – E. coli* (Clinic, Ijaye); *5 – E. coli* (Clinic, Lantoro)

(BARGUIGUA et al. 2011, GAUTAM et al. 2019). In our study *bla*$_{\text{CTX-M}}$ was more prevalent (40%) followed by *bla*$_{\text{SHV}}$ (20%) and none of the isolate encode the genes for *bla*$_{\text{TEM.}}$ The high prevalence in our study concurred with earlier report by SEPUTEINCE et al. (2009). This is in line with the report of HASSAN et al. (2014) in Saudi Arabia and KIRATISIN et al. (2014) in Thailand. These workers recorded low prevalence of *bla*$_{\text{SHV}}$ compared to *bla*$_{\text{CTX}}$ in their countries, respectively.

Conclusion

This study showed the presence of MDR ESBL producing *Escherichia coli* encoding *β*-lactamase genes *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$ from waste water and clinical samples. ESBL were more predominant in waste water sample followed by those from the clinic. This report is of public health importance because if these isolates find their way to surface or groundwater. It can transfer the resistant genes to other strains and other pathogenic microorganisms. Thereby acting as reservoir for further dissemination of the *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX}}$ ESBL resistant genes.

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BIOSTIMULATION POTENTIALS OF CHICKEN DROPPING ON SPENT OIL CONTAMINATED SOIL FROM AUTOMOBILE WORKSHORPS IN KEFFI, NASARAWA STATE NIGERIA

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Key words: biostimulation, chicken dropping, automobile workshops, hydrocarbon utilizing bacteria.

Abstract

Research into the use of organic waste for the biostimulation and bioremediation of hydrocarbon contaminated soils has expanded rapidly over the years. In this study, the possibility of chicken dropping to stimulate the growth of hydrocarbon utilizing bacteria and optimize total hydrocarbon biodegradation of oil contaminated soil from mechanic workshop was investigated. Soil samples from five (5) different automobile workshops within Keffi, Nasarawa State Nigeria were mixed with poultry droppings at concentrations of 0% (control), 10% and 30%. The set up was then left for a period of 4 weeks. The chicken droppings had total bacteria and fungi counts of $2.51 \cdot 10^7$ cfu g^{-1} and $1.98 \cdot 10^6$ cfu g^{-1} respectively, while soil samples had the bacterial count in the range of $3.92 \cdot 10^6$–$4.87 \cdot 10^6$ cfu g^{-1}. Chicken dropping had mean pH, nitrogen, phosphorus, carbon and moisture contents of 7.06±0.78, 0.88±0.07%, 36.90±3.11 mg kg^{-1}, 3.08±0.11% and 44.78±1.90% respectively. The nitrogen and carbon content in all experimental soil decreases with increase in bioremediation time. All the bio-remediated soil had significantly ($p < 0.05$) higher

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hydrocarbon utilizing bacteria (HUB) than the control (un-remediated). In all the soil samples remediated, 30% remediation had significantly ($p < 0.05$) highest HUB in the range of $16.87 \cdot 10^6$–$19.87 \cdot 10^6$ when compared with those remediated with 10% chicken droppings ($14.78 \cdot 10^6$–$16.89 \cdot 10^6$). This study demonstrated that chicken droppings are good organic substrate containing nitrogen, phosphorus, and carbon, with great potentials for biostimulation of hydrocarbon utilizing bacteria in oil contaminated soil.

Introduction

Petroleum-based products are the major source of energy for industry and daily life. The amount of natural crude oil seepage was estimated to be 600,000 metric tons with a range of uncertainty of 200,000 metric tons per year (AL-MUTAIRI et al. 2008). In Nigeria, oil pollution problems have been prevalent since the commencement of oil exploration and development of the petroleum industry (OKOLO et al. 2005). During the exploration and production of crude oil, numerous oil fields, tank farms, flow stations, pipelines, tankers and loading jetties constantly provide potential sources of oil pollution (IJAH and ANTAI 2003). However, common forms of pollution come from household wastes, agricultural wastes, gas flaring, oil spills and spent lubricating oil (OFOEGBU et al. 2014).

Spent engine oil, usually obtained after servicing and subsequently draining used oil from automobiles and generator engines, is indiscriminately disposed into gutters, water drains, open vacant plots and farms in Nigeria by auto mechanics and allied artisans with workshops (NWANKWEGU et al. 2016). Soils polluted with petroleum hydrocarbons (PHCs) or spent engine oil differ from unpolluted soils and are not able to support adequate crop growth and development (AL-MUTAIRI et al. 2018). There is need to treat these soils so as to satisfy the food requirement of the ever-increasing world population.

Bioremediation is a cost-effective method of soil remediation which uses organisms for the treatment of polluted soils. It has been used across the globe for the treatment of a wide range of organic soil pollutants (CAI et al. 2010). However, nutrient limitation in hydrocarbon-contaminated soils presents a challenge to bioremediation; nonetheless, addition of nutrients generally benefits soil hydrocarbon utilizing bacteria via biostimultion resulting in enhanced bioremediation of hydrocarbon polluted environment (LIU et al. 2014).

Poultry droppings is a mixture of poultry excreta, spilled feed, feathers, and material used as bedding in poultry operations (OFOEFULE and UZODINMA 2006). Poultry manure is rich in organic manure since solid and liquid excreta are excreted together resulting in no urine loss. Poultry manure is used as a source of N, P and K and some micronutrients

(MULLINS et al. 2002). These nutrients could be very useful in providing adequate environmental medium for efficient growth and reproduction of the hydrocarbon utilizing bacteria. The present study aimed at evaluating the biostimulation effect of chicken dropping on hydrocarbon utilizing bacteria in soil sample collected from mechanic workshop.

Materials and Method

Description of Study Area and Sample Collection

The soil sample used in this study was collected from Keffi, Nasarawa State, Nigeria. The main occupations of the people are land farming and subsistent agriculture, of which there is no history of crude oil pollution in this environment. Oil contaminated soil was collected from five different automechanic workshops at Keffi garage, Opposite New Keffi hotel, Angwan kwara, High court, and Angwan Tanko, all in Keffi, Nasarawa State Nigeria. At each sampling point, two samples were collected at a depth of 10 cm and bulk samples were collected in polythene bags and immediately transported to the laboratory for analysis. The soil amendment material (chicken droppings) was collected from Lawal Poultry Farms, Pyanku, Nasarawa State, Nigeria.

Isolation and Identification of Isolates From the Spent Oil Contaminated Soil

Total microbial analysis was carried out on the soil by weighing 10 g of soil sample, serially diluted and inoculated unto nutrient agar and potato dextrose agar to culture bacteria and fungi. The culture plates were incubated for 24 and 78 hours respectively. On completion of the culture, microbial strains were identified. Each isolate was examined for its size, shape, margin, consistency, elevation, pigmentation, Gram reaction and cell morphology. The isolates were characterized as described by HOLT et al. (1999). Biochemical tests which were carried out included production of catalase, indole and oxidase enzymes. Spore production and oxidation/fermentation of sugars were also examined.

Isolation and Identification of Hydrocarbon Utilizing Bacteria (HUB)

HUB counts in the soil was determined by plating an aliquot of 0.1 ml of the serially diluted 1 g of the soil on oil agar (OA) [1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.1g

NaCl, 20 g agar, 1% used engine oil in 1000 ml distilled water, pH 7.4], and incubated at 30ºC for 72 hours. Discrete colonies that developed were counted and expressed in cfu g^{-1} (ADAM et al. 2014).

Physicochemical Analyses of Soil Sample and Amendment Material

Physicochemical parameters including moisture, pH, total nitrogen, phosphorus and carbon contents were carried out on both the chicken droppings before amendment and on the soil sample after amendment with chicken droppings. The analysis was carried out on a weekly basis throughout the study period. The pH was determined by the according to the modified method of McLean (1982), the total organic carbon was determined by the modified (NELSON and SOMMERS 1982) wet combustion method (WALKEY and BLACK 1934) available nitrogen was ascertained using semi-micro Kjeldhal method (BREMNER and MULVANEY 1982), the available phosphorus by Brays No.1 method (OLSEN and SOMMERS 1982).

Bioremediation Study

This was carried out ex situ in the Microbiology laboratory at Nasarawa State University Keffi, Nasarawa State. Eighty grams (80 g) of the soil samples was mixed with chicken droppings at various concentrations of 10% and 30% (Table 1). The control was left without amendment. This remediation exercise was carried out in a well perforated 1.5 litres plastic container with an estimated depth of 13 cm. This research work was conducted for four weeks, during which samples from each group were taken to the laboratory for analysis of total hydrocarbon content, once in every 7 days. Hydrocarbon-Utilizing Bacterial (HUB) content was analysed using standard methods (APHA 1992).

Table 1

Experimental design for bioremediation

Experimental groups		Description
T1	chicken dropping added at 10%	80 g of polluted soil + 8 g of chicken droppings
T2	chicken dropping added at 30%	80 g of polluted soil + 24 g of chicken droppings
T3	control	80 g of polluted soil only (control)

Results

Bacteria and Fungi Species Isolated from Auto-Mobile Polluted Soil

The morphological and biochemical characteristic of bacteria species isolated from the contaminated soil are shown in Table 2. Data confirmed the identity of the organism to be *Bacillus subtilis*, *Micrococcus luteus* and *Pseudomonas aeruginosa* strain. Fungal species including *Aspergillus niger, Aspergillus fumigatus* and *Mucor* species were isolated (Table 3).

Table 2

Morphological biochemical characteristic of bacterial isolate

Test	*Micrococcus species*	*Bacillus species*	*Pseudomonas species*
Shape	Cocci	Rod	Rod
Gram Stain	*G*+	*G*-	*G*-
Catalase	+	+	+
Methyl Red	+	-	-
Oxidase	+		
Indole	-	+	+
Glucose	AG	AG	AG
Fructose	*A*	*G*	AG
sucrose	AG	AG	AG
Lactose	AG	AG	AG

Key: (+) – positive; (-) – negative; *G*+ – gram positive; *G*- – gram negative; *A* – acid; AG acid and gas production

Table 3

Morphological characteristics of fungal isolated

Test	*Aspergillus niger*	*Aspergillus fumigatus*	*Mucor* species
Macroscopy	black and powdery like	gray – green fluggy colonies	whitish/light cotton like
Microscopy	conidiophores smooth walled and non-septate	long erect non-septate conidiophores	round, conidia non-septate

Total Bacteria and Fungi Count

The total bacteria and fungi counts in the soil contaminated samples and the chicken droppings used for bioremediation are shown in Table 4. The chicken droppings had total bacteria and fungi counts of $2.51 \cdot 10^7$ cfu g^{-1} and $1.98 \cdot 10^6$ cfu g^{-1} respectively. The soil samples had the bacterial count in the range of $3.78 \cdot 10^6$ –$4.87 \cdot 10^6$ cfu g^{-1} and fungal count in the range $2.89 \cdot 10^5$–$3.11 \cdot 10^5$ cfu g^{-1}.

Table 4

Bacteria and fungi count in the soil contaminated samples and the chicken droppins

	Bacteria count	Fungi count
Keffi garage	$4.87 \cdot 10^6$	$3.06 \cdot 10^5$
Opposite New Keffi hotel	$3.78 \cdot 10^6$	$2.89 \cdot 10^5$
Angwan kwara	$4.56 \cdot 10^6$	$2.91 \cdot 10^5$
High court	$4.08 \cdot 10^6$	$3.11 \cdot 10^5$
Anguwan Tanko	$3.92 \cdot 10^6$	$2.81 \cdot 10^5$
Chicken droppings	$2.51 \cdot 10^7$	$1.98 \cdot 10^6$

Physicochemical Properties of Chicken Droppings

Physicochemical properties of chicken droppings used for bioremediation are presented in Table 5. Chicken dropping had mean pH of 7.06±0.78, nitrogen content of 0.88±0.07%, phosphorus contents of 36.90±3.11 mg kg^{-1} while the organic carbon and moisture content were 3.08±0.11% and 44.78±1.90 % respectively.

Table 5

Physicochemical properties of chicken droppings used for bioremediation

Parameter	Chicken droppings
pH	7.06±0.78
Nitrogen [%]	0.88±0.07
Phosphorus [mg kg^{-1}]	36.90±3.11
Organic C [%]	3.08±0.11
Moisture [%]	44.78±1.90

Values are mean ± standard error of mean (SEM) of 3 determinations

Nutrients Compositions of Oil Contaminated Soil Sample for Remediation

The physicochemical properties of engine oil contaminated soil are shown in Table 6. There were no significant differences ($p > 0.05$) in the pH, nitrogen, organic carbon and moisture contents of the soil samples from the five (5) locations. However, the phosphorus content was significantly higher ($p < 0.05$) in soil sample collected from "anguwan kwara workshop' when compared with soil collected from other sample location. pH of the soil ranged between 6.22±0.29 and 7.90±1.02, nitrogen contents ranged between 11.74±0.43% and 1.85±0.12%, phosphorus ranged between 15.07±0.95 mg kg^{-1} and 19.78±0.45 mg kg^{-1}, organic carbon ranged between 2.30±0.11% and 2.94±0.09% while moisture contents ranged between 8.67±0.33% and 9.56±0.56%.

Table 6

Physicochemical properties of engine oil contaminated soil

	Soil sample location				
Parameters	Keffi garage	Opposite New Keffi hotel	Angwan kwara	High court	Angwan Tanko
pH	$6.23±0.06^a$	$6.55±0.10^a$	$7.90±1.02^b$	$6.22±0.29^a$	$6.41±0.31^a$
Nitrogen [%]	$1.78±0.10^a$	$1.81±0.11^a$	$1.74±0.43^a$	$1.80±0.09^a$	$1.85±0.12^a$
Phosphorus [mg kg^{-1}]	$15.98±0.67^a$	$16.89±1.32^a$	$19.78±0.45^b$	$16.90±1.89^a$	$15.07±0.95^a$
Organic C [%]	$2.56±0.12^a$	$2.89±0.15^a$	$2.94±0.09^a$	$2.30±0.11^a$	$2.42±0.54^a$
Moisture [%]	$9.56±0.45^a$	$8.74±0.28^a$	$8.67±0.33^a$	$8.75±0.45^a$	$9.56±0.56^a$

Values are mean ± standard error of mean (SEM) of 3 determinations. Values along the same column with different superscripts are significantly different ($p < 0.05$)

Effect of Remediation on Nutrient Composition of the Contaminated Soil Sample

Nitrogen Content

The nitrogen content in all experimental soil decreases with increase in bioremediation time (Fig. 1). However, the un-remediated control soil had the highest nitrogen content. The decrease in nitrogen content was dependent on concentration of chicken droppings except for sample collected from Anguwan tanko and high court automobile workshop.

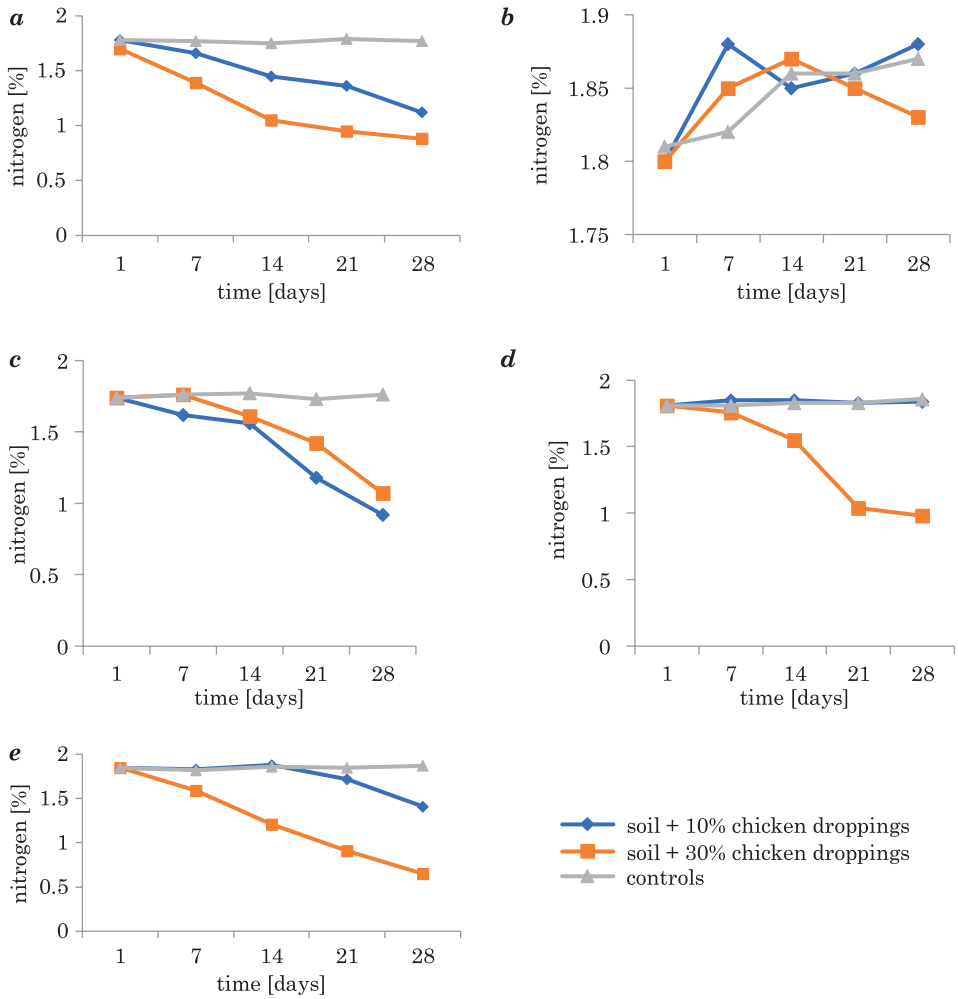

Fig. 1. Effect of remediation time on nitrogen contents of contaminated soil sample: *a* – Keffi garage automobile workshop; *b* – high court automobile workshop; *c* – Anguwan kwara automobile workshop; *d* – New Keffi hotel automobile workshop; *e* – Anguwan tanko automobile workshop

Carbon Content

The carbon content in all experimental soil decreases with increase in bioremediation time (Fig. 2). However, the un-remediated soil had the highest carbon content. The decrease in carbon content was dependent on concentration of chicken droppings for all the soil sample except for high court automobile contaminated soil.

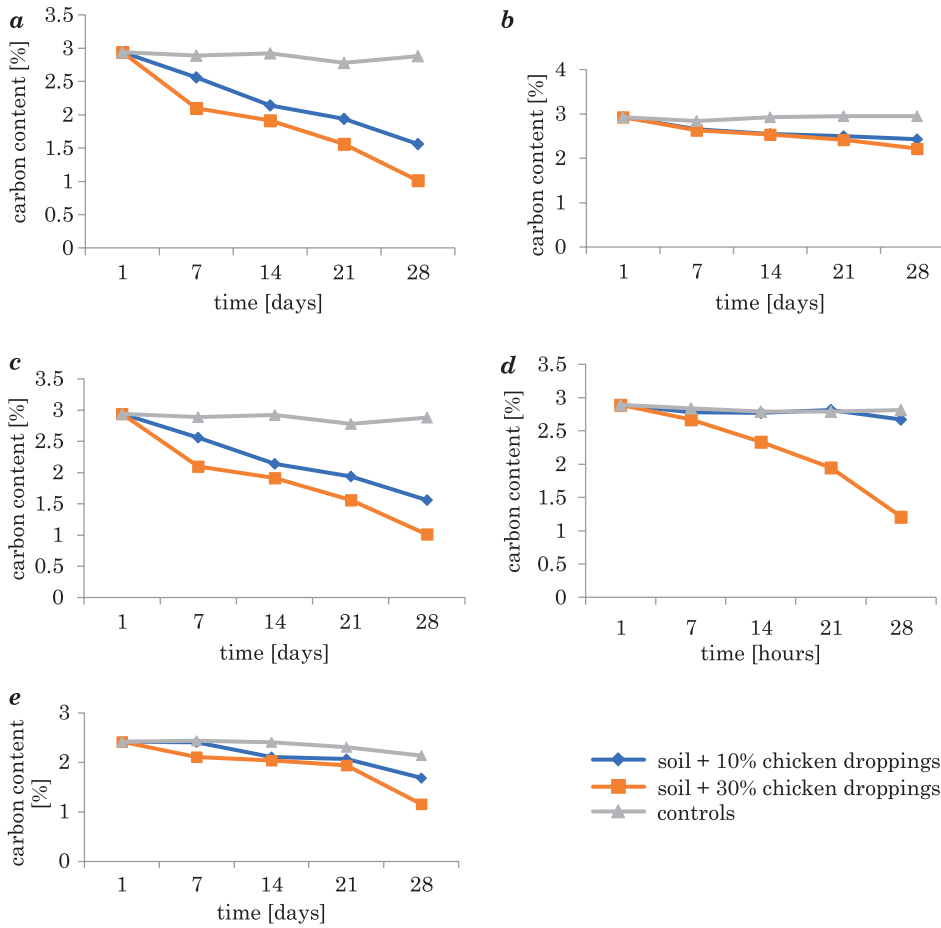

Fig. 2. Effect of remediation time on carbon contents of contaminated soil sample: *a* – Keffi garage automobile workshop; *b* – high court automobile workshop; *c* – Anguwan kwara automobile workshop; *d* – New Keffi hotel automobile workshop; *e* – Anguwan tanko automobile workshop

Hydrocarbon-utilizing bacterial (HUB)

The hydrocarbon-utilizing bacterial (HUB) count in all experimental soil after four (4) weeks of bioremediation with chicken droppings are presented in Figure 3. All the bio-remediated soil had significantly ($p < 0.05$) higher HUB than the control (un-remediated) soil. In all the soil samples remediated, 30% remediation had significantly ($p < 0.05$) the highest HUB in the range of $16.87 \cdot 10^6$ –$19.87 \cdot 10^6$ cfu g^{-1} when compared with those remediated with 10% chicken droppings ($14.78 \cdot 10^6$– $16.89 \cdot 10^6$ cfu g^{-1}). The non-remediated control had the least HUB count range of $4.87 \cdot 10^6$–$7.32 \cdot 10^6$ cfu g^{-1}.

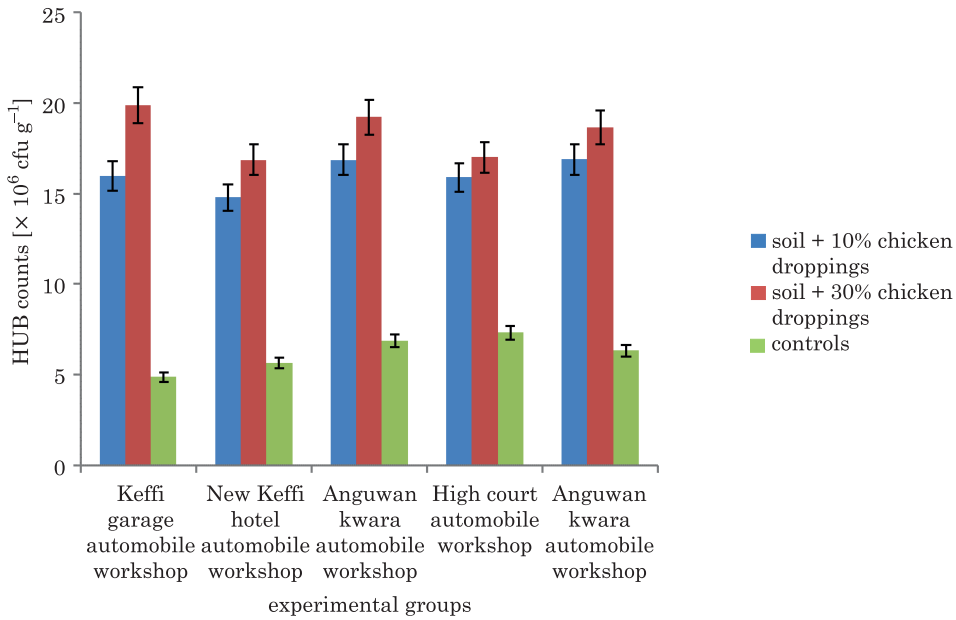

Fig. 3. Counts of Hydrocarbon-Utilizing Bacterial (HUB) population in polluted soil after 4 weeks of bioremediation

Discussion

Biodegradation is increasingly being considered as a less expensive alternative to physical, mechanical and chemical means of disposing hydrocarbon pollutants (UMAR et al. 2013). Studies have reported that microbial growth in soil is controlled not by the total amount of resource available but by the scarcest resources (limiting factor), which are, in this case, N, P and C (GORBAN et al. 2011).

Morphological and biochemical identification of organism in the contaminated soil confirmed the identity of *Bacillus subtilis*, *Micrococcus luteus* and *Pseudomonas aeruginosa* strain *Aspergillus niger, Aspergillus fumigatus* and *Mucor* species in the soil sample. Previous studies have implicated *Acinetobacter, Micrococcus, Pseudomonas, Nocardia,* and *Bacillus* as the major hydrocarbon utilizing bacteria in oil contaminated soil (DAS and MUKHERJEE 2007). This is also in consistence with the findings of OKAFOR et al. (2016) who identified *Bacillus* spp., *Pseudomonas* spp., *Flavobacterium* spp., *Fusarium* spp. and *Aspergillus* spp. in crude oil contaminated soil from Anambara State. The ability to isolate high numbers of certain oil degrading microorganisms from an environment is commonly taken as evidence that those organisms are the active degraders of the constituents

of that environment. Therefore, the presence of these organisms in crude oil contaminated soil is an indication that these organisms are indigenous hydrocarbon utilizing organism for carbon and energy source (OKOLO et al. 2005). It has been reported that some organism cannot survive high diesel contamination level of up to 11.11% (STEPHEN et al. 2013). However, the hydrocarbon (HC) degrading activities of the surviving microbes could be enhanced by the addition of nutrients required for optimum activities and reproduction of the organism. Although microorganisms are present in contaminated soil, their numbers might not be sufficient to initiate remediation of contaminated sites. The microbial (Fungi and bacteria) population of chicken droppings in this study are higher than $3.4 \cdot 10^4$ cfu g^{-1} and $1.77 \cdot 10^4$ cfu g^{-1} for bacteria and fungi count reported for chicken droppings use for bioremediation in Anambara State (OKAFOR et al. 2016). These discrepancies could be attributed to the differences in the nutritional quality of feed been fed to the chicken which results to differences in nutrient contents of the droppings and consequently effect bacterial growth. Similarly, differences in environmental factors such as temperature, pH, humidity could affect the optimum activities and reproduction of the organism. The high microbial count reported in this study would therefore favour hydrocarbon degradation when used in bioremediation.

The contaminated soil sample from the automobile workshop in this study had the bacterial count in the range of $3.92 \cdot 10^6$–$4.87 \cdot 10^6$ cfu g^{-1} and fungal count in the range 2.91 · 105–$3.11 \cdot 10^5$ cfu g^{-1}. In agreement with this study, DANIEL et al. (2017), reported bacteria count in the range of $2.94 \cdot 10^6$ cfu g^{-1} and $2.39 \cdot 10^6$ CFU g^{-1} from automobile workshops in Benue State. However, STEPHEN et al. (2015), reported a low bacteria count in range of $8.0 \cdot 10^3$ to $9.8 \cdot 10^4$ cfu g^{-1} in mechanic workshop soil from Kogi State.

During the bioremediation, it was observed that C and N decrease as the remediation time increases. This observation is in agreement with previous studies that reported enhanced degradation of crude oil using other animal manures i.e goat manure (NWOGU et al. 2015), chicken-drop (Ijah and ANTAI 2003), poultry manure (ADESODUN and MBAGWU, 2008). The decrease in total N and C is due to their utilization for microbial growth. The counts of HUB in all the soil remediated with chicken droppings were higher compared to that of non-remediated control soil; these counts are comparable to those of Ibiene et al. (2011) who reported that the total culturable hydrocarbon utilizing bacterial count in crude oil contaminated soil ranged between · 10^3 cfu g^{-1} to · 10^6 cfu g^{-1} , also IJAH and ANTAI (2003), who observed counts of hydrocarbon degraders in oil polluted soil to be · 10^6 cfu g^{-1} but lower than those obtained by ANTAI and MGBOMO (1989) whose counts of HUB in hydro-

carbon-contaminated soil was · 10^8 CFU g^{-1} ; this may be due to differences in microbial ecology of the soil or characteristics of the experimental soils. The reason for higher counts of bacteria in bio-remediated soil may be the result of the presence of appreciable quantities of nitrogen and phosphorus in chicken droppings, which are necessary nutrients for bacterial biodegradative activities (ADESODUN and MBAGWU 2008).

Hydrocarbon-polluted soil is toxic for plants, possibly due to direct inhibiting effects of hydrocarbons or their metabolites as well as to changed soil conditions (MORELLI et al. 2005). Apart from this, oil pollution may lead to changes of the microbial community structure, favoring the dominance of phytotoxin-producing species (LABUD et al. 2007, STELIGA et al. 2012). Fortunately, the decreases in nutrient composition of the chicken dropping amended soil lead to corresponding increase in Hydrocarbon utilizing bacteria, this will consequently lead to increased oil biodegradation in the soil. By implications, the biostimulation technique proposed here for soils polluted with crude oil could be suitable in field, because of its low costs and its low environmental risk associated with volatile hydrocarbon losses.

Conclusion

This study demonstrated that chicken droppings are good organic substrate containing nitrogen, phosphorus, and carbon, which have great potentials for enhanced bioremediation of diesel contaminated soil. The wide availability of chicken droppings in commercial quantity in Nigeria makes these waste products a potential and viable biostimulant for enhanced bioremediation of crude oil polluted environments.

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Conflict of Interest

The authors declared no conflict of interest exist.

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EFFECT OF LIGHT SPECTRUM ON THE PHYCOCYANIN PRODUCTION BY *ARTHROSPIRA PLATENSIS*

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Key words: *Arthrospira platensis*, phycocyanin, light, cyanobacteria, biomass.

Abstract

The global market shows high demand for products of natural origin to reduce the use of synthetic substances in the industries. One of the opportunities for acquiring natural compounds of industrial value is the use of cyanobacteria biomass. In terms of biomass composition, cyanobacteria of the species *Arthrospira platensis*, which contains phycocyanin, deserve special attention. Phycocyanin is a pigment-protein complex widely used in the food and cosmetics industries, histochemistry, fluorescence microscopy and flow cytometry. The high demand for this pigment determines the search for methods to intensify phycocyanin production by *A. platensis*. The aim of the study was to determine the effect of light of different wavelengths on phycocyanin productivity by cyanobacteria of the species *Arthrospira platensis*. The highest biomass concentration and biomass production efficiency were obtained in a culture using Blue-Red LED lighting and they amounted to 5.619 ± 0.053 g TS dm^{-3} and 656 ± 7 mg dm^{-3} d^{-1}. The highest phycocyanin concentration and purity were observed in a culture using Red LED lighting and they amounted to 17.61 ± 0.51% TS and 0.710 ± 0.01.

Introduction

The global market shows high demand for products of natural origin to reduce the use of synthetic substances in the food, pharmaceutical and cosmetics industries. This has led to a growing interest in biotechnological research focused on increasing the rate and efficiency of acquiring natural products through the elimination of the main limiting factors, such as e.g. crop seasonality. One of the opportunities for acquiring natural compo-

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unds of industrial value is the use of cyanobacteria biomass that exhibit high biomass productivity and can be cultured in photobioreactors, which restricts the impact of external conditions on the culture and hinders the access of both parasites and competing microorganism species (KIM et al. 2013). In terms of biomass composition, cyanobacteria of the species *Arthrospira platensis* deserve particular attention. They are characterised by high contents of protein, γ-linolenic acid (COHEN 1997), polysaccharides (De PHILIPPIS and VINCENZINI 1988), β-carotene (GIREESH et al. 2001), chlorophyll and phycocyanin (RANGEL-YAGUI et al. 2004). The species *Arthrospira platensis* is cultured on a commercial scale for the extraction of phycocyanin, a compound with a high added value which is used in a variety of industries.

In the *A. platensis* cells, this compound is used as the main photoreceptor in the photosynthesis process and is found, along with other phycobiliproteins, in complexes referred to as phycobilisomes (GANTT 1981, MIMURO and KIKUCHI 2003). It is estimated that the phycocyanin content of the *Arthrospira platensis* cells may be as high as 15% of the dry matter (WAN et al. 2016).

Phycocyanin is a pigment-protein complex used in food products to increase nutritional value. It is used as food colourants, antioxidants and emulsifiers which can replace or reduce the use of synthetic additives. The substance is also widely used as a pigment in the cosmetics industry and as a fluorescent biomarker in laboratory testing (ZHAO et al. 2014). The pigment is used as fluorescent probes in histochemistry, fluorescence microscopy, flow cytometry and fluorescence immunoassay (SEKAR and CHANDRAMOHAN 2008).

Photosynthesising organisms such as *Arthrospira platensis* can be cultivated using advanced technologies enabling thorough monitoring and controlling the conditions as well as in open ponds (DĘBOWSKI et al. 2012). In closed systems, photobioreactors are used, which, unlike open cultures, enable continuous monitoring of the temperature and the pH of the culture medium and the control of the method, intensity and duration of lighting (KAEWPINTONG et al. 2007, PULZ 2001). The provision of adequate lighting is one of the most important factors affecting the biomass productivity and the content of assimilation pigments. Scientific research has shown that the type of light source and the wavelength not only affect biomass productivity but also the chemical composition of cyanobacteria (HO et al. 2014a, HO et al. 2014b). Compared to traditional fluorescent lamps, light-emitting diodes (LED) characterised by narrow-band wavelength, low energy consumption and high reliability are regarded as the optimal light source for the cultivation of photosynthesising organisms (SCHULZE et al. 2014).

The aim of the study was to determine the effect of light of different wavelengths on phycocyanin productivity by cyanobacteria of the species *Arthrospira platensis*.

Materials and Methods

Microorganism Strain and Culture Medium

The biomass of cyanobacteria *Arthrospira platensis* used in the experiment originates from a culture carried out under controlled conditions, initiated from a culture acquired from the Experimental Phycology and Culture Collection of Algae Centre in Göttingen (Fig. 1).

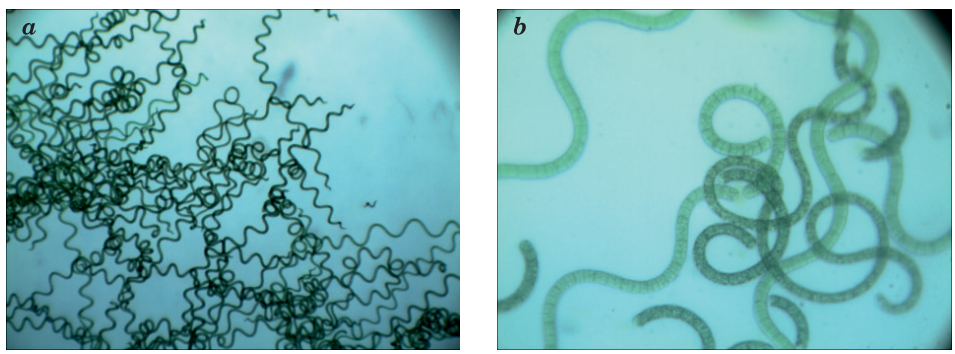

Fig. 1. *Arthrospira platensis* seen in microscope, magnified: *a* – 100x; *b* – 40x

In the experimental culture, a modified medium AIBA and OGAWA was used (Table 1) (AIBA and OGAWA 1977).

Table 1

The composition of the culture medium used in the experiment

Component	Unit	Amount
$NaHCO_3$	g dm^{-3}	13.61
Na_2CO_3	g dm^{-3}	4.03
K_2HPO_4	g dm^{-3}	0.50
$NaNO_3$	g dm^{-3}	2.50
K_2SO_4	g dm^{-3}	1.00
NaCl	g dm^{-3}	1.00
$MgSO_4 \cdot 7\ H_2O$	g dm^{-3}	0.20
$CaCl_2 \cdot 2\ H_2O$	g dm^{-3}	0.04
$FeSO_4 \cdot 7\ H_2O$	g dm^{-3}	0.014

EDTA (Titriplex III, MERC)	g dm^{-3}	0.084
$ZnSO_4 \cdot 7\ H_2O$	µg dm^{-3}	5
$MnSO_4 \cdot 4\ H_2O$	µg dm^{-3}	10
H_3BO_3	µg dm^{-3}	50
$Co(NO_3)2 \cdot 6\ H_2O$	µg dm^{-3}	5
$Na_2MoO_4 \cdot 2\ H_2O$	µg dm^{-3}	5
$CuSO_4 \cdot 5\ H_2O$	µg dm^{-3}	0.005

Culture Condition

Arthrospira platensis were cultivated in pipe photobioreactors with a vertical orientation and an active volume of 2 dm^3. The temperature of the culture was 30 ± 1°C. The test stand was equipped with an aeration pump connected to the reactors from below. This solution supplied carbon dioxide from atmospheric air to the system and mixed the cyanobacteria culture. The volumetric aeration ratio was 0.6 v/v. The cultivation time was 8 days, then physicochemical analyzes were performed.

Light Sources

The study was divided into four experimental series with the light source as the division criterion:

– red-red LED (660 and 630 nm),
– blue-blue LED (470 and 430 nm),
– blue-red-blue-red LED (660, 630, 470 and 430 nm),
– white-white fluorescence lamp (5600 K).

In the experimental series using LED lighting, modules comprised of 1 Watt Helixeon diodes were used (Helio Opto, Taiwan). Each module comprised 28 diodes and a 36 W power supply unit (Philips, the Netherlands). In the control series using a fluorescent lamp, a 28 W lamp with a colour temperature of 5600 K was used (Osram, Germany). The lighting intensity in all series was about 7500 lux.

Measurement of Biomass Concentration

Dry matter content, or total solids (TS) was determined by filtering 50 ml of culture samples through a 110 mm diameter hard cellulose filter. Following the filtering process, the filter was dried in a laboratory oven (Binder, Germany) until a constant weight was obtained. In order to determine dry matter content, the difference between the weight of a dried empty filter and the weight of a dried filter following filtration was determined.

Determination of Growth Parameters and Phycocyanin Productivity

The biomass productivity (P_b, g TS dm^{-3} d^{-1}) was calculated based on the equation:

$$P_b = \frac{\Delta X}{\Delta t}$$

where:

ΔX – the difference in biomass concentration [g TS dm^{-3}] over a cultivation time of Δt (d).

Moreover, according to the mass balance of microalgae, the phycocyanin productivity ($P_{phy,}$ mg dm^{-3} d^{-1}) was calculated from the relationship between the phycocyanin content and volumetric growth rate of the microalgal cell, as indicated in the equation:

$$P_{\text{phy}} = P \cdot \text{EY}$$

where:

P – the biomass productivity [g TS dm^{-3} d^{-1}]

EY – the phycocyanin extraction yield [mg g^{-1} TS].

Phycocyanin extraction

To extract phycocyanin from the *Arthrospira platensis* cells, 0.5 g dry biomass was added to 50 cm^3 phosphate buffer (pH of 7.0). The prepared solution was subjected to ultrasonic disintegration. The ultrasonic treatment process was carried out using a 400 W UP400S ultrasonic processor with a frequency of 24 kHz (Hielscher, Germany). The ultrasound amplitude was 70%, and the disintegration time was 30 s. Following the ultrasonic disintegration process, the samples were shaken in the dark for 4 h. The samples were then centrifuged at 9,000 xg for 15 minutes and the supernatant was subjected to a spectrophotometric analysis.

Determination of Phycocyanin Concentration

After centrifugation, the supernatant's optical density was measured using a DR5000 spectrophotometer (Hach, USA). Phycocyanin content (PC, mg cm^{-3}) was calculated according to the following equation (BENNETT and BOGORAD 1973):

$$\text{PC} = \frac{\text{OD}_{615} - 0.474(\text{OD}_{652})}{5.34}$$

where:

OD_{615} – the optical density of the sample at 615 nm

OD_{652} – the optical density of the sample at 652 nm.

The purity index of phycocyanin is determined by dividing the phycocyanin maximum absorbance wavelength to a specific absorbance wavelength of total protein (OD_{615}/OD_{280}). The purity of phycocyanin extract (EP) was calculated according to the following equation (ABALDE et al. 1998):

$$EP = \frac{OD_{615}}{OD_{280}}$$

where:
OD_{615} – the optical density of the sample at 615 nm
OD_{280} – the optical density of the sample at 280 nm
indicating the total concentration of proteins in the solution.

The extraction yield (EY, mg g^{-1} TS^{-1}) was calculated as:

$$EY = \frac{PC \cdot V}{d_w}$$

where:
PC – the phycocyanin content [mg cm^{-3}]
V – the volume of solvent [cm^3]
d_w – the dried biomass [g TS].

Results and Discussion

Effects of Light Wavelength on Cell Growth and Biomass Productivity

In the experiment, cyanobacteria *Arthrospira platensis* were cultured using various light sources i.e. a fluorescent lamp and red, blue, and red-blue electroluminescent diodes, and the illuminance of light emitted on the photobioreactor surface was 7500 lux. The initial biomass concentration in all experimental series was 0.369 ± 0.014 g TS dm^{-3}. As shown in Figure 2, the highest biomass concentration was obtained in the culture with blue-red LED lighting and it amounted to 5.619 ± 0.053 g TS dm^{-3}. In terms of biomass concentration, the culture using red LED lighting appeared to be the second highest with a value of 3.915 ± 0.083 g TS dm^{-3}. The lowest biomass concentration of 2.789 ± 0.032 g TS dm^{-3} was noted in the culture lit by the fluorescent lamp (Fig. 2). The experiment results are consistent with a study by LEE et al. (2016). The *Arthrospira platensis* were cultured using three light sources: red LED (660 nm), red-blue LED (660 and 450 nm), and blue LED (450 nm). The obtained results show that the highest biomass concentration of 3.2 g TS dm^{-3} was obtained in the culture using red-blue LED lighting. WANG et al. (2007) and CHEN et al.

(2010) investigated the growth of *Arthrospira platensis* using red, yellow, blue, white and green LED diodes. They observed that the highest biomass concentration is obtained when using a red LED diode. However, they carried out no testing with the simultaneous use of blue and red culture lighting.

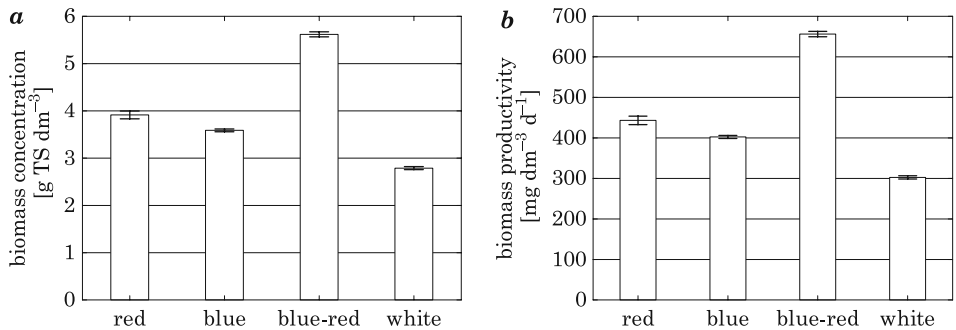

Fig. 2. Effects of light wavelength on: *a* – biomass concentration; *b* – biomass productivity

The values of biomass productivity in particular series were proportional to biomass concentrations. For the culture based on blue-red LED lighting, the biomass productivity was 656.250 ± 6.592 mg TS dm^{-3} d^{-1}, and for a fluorescent lamp it was 302.500 ± 4.005 mg TS dm^{-3} d^{-1} (Fig. 2). A study by Lima et al. (2018) also indicated that cultures using mixed red and blue light exhibit a higher biomass concentration than cultures with a separate red or blue lighting.

Effects of Light Wavelength on Phycocyanin Content and Phycocyanin Productivity of Arthrospira platensis

Figure 3 shows the effect of light of different wavelengths on phycocyanin content and phycocyanin productivity of *Arthrospira platensis*. The highest phycocyanin content was noted for biomass cultured using red LED lighting and it amounted to 17.61 ± 0.51% TS. The culture with blue LED lighting was characterised by the lowest phycocyanin content in the cells, which was 2.47 ± 0.03% TS (Fig. 3). LIMA et al. (2018), when researching the effect of the spectral quality of light on the accumulation of pigments in *Arthrospira platensis* biomass, also observed that the highest phycocyanin concentration (16.71% TS) was obtained with the use of a red LED (660 nm). On the other hand, when a blue light (450 nm) was used, no presence of phycocyanin was demonstrated in *Arthrospira platensis* biomass. CHEN et al. (2010), when researching the effect of red, white, blue, yellow and green LEDs, also observed the highest phycocyanin content of 15.2% TS in the culture using red light.

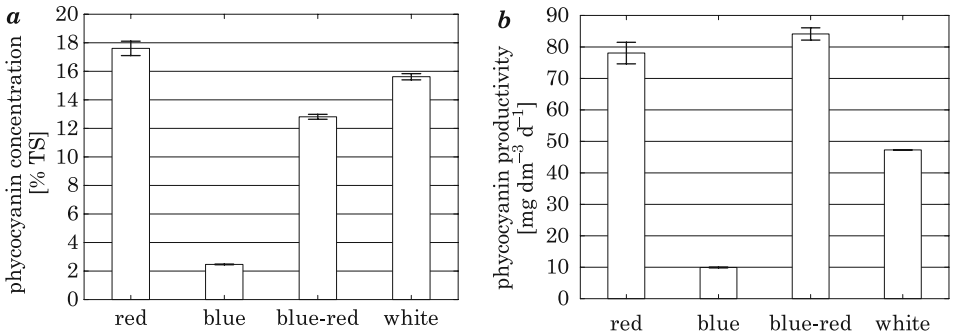

Fig. 3. Effects of light wavelength on: *a* – phycocyanin concentration; *b* – phycocyanin productivity

Despite the highest phycocyanin content in the culture with Red LED lighting, the average phycocyanin productivity was lower by 7.76% compared to the culture using blue-red diodes, which was associated with higher biomass productivity. The highest phycocyanin productivity was 84.12 ± 1.95 mg dm^{-3} d^{-1}. The lowest phycocyanin productivity was recorded in culture using blue LED lighting, which was 9.93 ± 0.14 mg dm^{-3} d^{-1} (Fig. 3).

Effects of Light Wavelength on Purity Grade of Phycocyanin

Phycocyanin purity is determined through the absorbance ratio OD_{615}/OD_{280}, with a purity of ≥ 0.7 regarded as food grade, ≥ 3.9 as reactive grade, and ≥ 4.0 as analytical grade (PATIL et al. 2006). As shown in Table 2, the purity of phycocyanin obtained in the experiment only in the culture using red LED lighting falls in the lowest category with a value of 0.710 ± 0.01. Prior to further uses, phycocyanin from other cultures would have to be purified to the required ranges. WALTER et al. (2011) analysed the production of phycocyanin using various light spectra and also observed that the extract from the culture using a red LED was characterised by the highest purity.

Table 2

The purity grade of phycocyanin in experimental culture

Series	Phycocyanin extraction yield [mg g^{-1} TS]	Phycocyanin purity
Red	176.08 ± 5.08	0.710 ± 0.010
Blue	24.67 ± 0.22	0.251 ± 0.005
Blue-red	128.17 ± 1.75	0.537 ± 0.001
White	156.29 ± 2.18	0.485 ± 0.005

Conclusions

This study concerning the effect of lighting on the intensification of phycocyanin production in a culture of *Arthrospira platensis* demonstrated that the light spectrum affected both an increase in the biomass of the tested cyanobacteria, phycocyanin content of the biomass and the purity of the obtained phycocyanin.

The highest biomass concentration and biomass production efficiency were obtained in a culture using Blue-Red LED lighting and they amounted to 5.619 ± 0.053 g TS dm^{-3} and 656 ± 7 mg dm^{-3} d^{-1}.

The highest phycocyanin concentration and purity were observed in a culture using Red LED lighting and they amounted to 17.61 ± 0.51% TS and 0.710 ± 0.01.

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COMPOSITION OF ROSEMARY'S ESSENTIAL OIL AND ITS ANTIOXIDANT ACTIVITY IN THE REGION OF TALSINT (MOROCCO) WITH FOCUS ON THE ALTITUDE FACTOR

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Key words: Rosmarinus officinalis, essential oil, antioxidant, Talsint.

Abstract

The *Rosmarinus officinalis* L. (rosemary) is an evergreen shrub used worldwide for its aromatic and medicinal virtues. It plays an important role in the local economy of Talsint (Eastern Morocco). In this study, eight samples of wild population of rosemary were collected from different altitudes in the region of Talsint, in order to characterize the chemical composition and antioxidant activity of their essential oils and investigate the altitude effect on their variability. The volatile profiles were determined by Gas chromatography-mass spectrometry (GC-MS). Thus, the major constituents were: 1.8-cineole (50.60–64.27%), camphor (1.77–14.12%), α-pinene (6.61–9.02%), and borneol (1.98–6.20%). The antioxidant activity to scavenge free radicals was determined by the 1,1-diphenyl-1-picrylhydrazyl (DPPH) assay. All the eight tested oils showed considerable antioxidant activity, although it was lower than that of ascorbic acid. Based on the linear regression between the altitude and the concentration of the main compounds, we conclude that altitude doesn't have an effect on the chemical composition of rosemary's essential oil.

Introduction

Rosmarinus officinalis L., is an aromatic plant of Lamiaceae family. It's an evergreen shrub reaching a height of up to 1.5 m. It grows spontaneously in many Mediterranean countries (PORTE at al. 2000).

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Rosemary is used widely since antiquity in culinary, cosmetics, and medicinal products (CELIKTAS et al. 2007, JEMIA et al. 2013). Many studies have demonstrated that it is a very efficient plant as natural antioxidant (RASKOVIC et al. 2014, ALI et al. 2015). In addition, it presents strong anti--inflammatory (JUHÁS et al. 2009), and antimicrobial properties against yeast (KIVRAK et al. 2009), mold (CHIFIRIUC et al. 2012) and gram-positive bacteria (JIANG et al. 2011).

Numerous researches have focused on the chemical composition of *Rosmarinus officinalis* essential oil. The main compounds detected were 1.8-cineole, camphor, *α*-pinene, borneol, verbenone, and camphene (NAPOLI et al. 2010, SEDIGHI et al. 2015, MALDINI et al. 2016, Miraj 2016).

However, several researches have revealed the variability of the composition and the yield of the rosemary essential oil which depends on different intrinsic factors as genetic background and plant age (HIDALGO et al. 1998) or extrinsic factors such as soil (SINGH and GULERIA 2013, TAWFEEQ et al. 2016), seasons (LEMOS et al. 2015), climate conditions (LAKUŠIĆ et al. 2012, JORDÁN et al. 2013), or extraction methods (SZUMNY et al. 2010, FADEL et al. 2011) as well as a combination of convective pre--drying and VM finish-drying (CPD-VMFD).

In Morocco, rosemary, locally known "Azir", grows spontaneously in various regions, especially in the eastern parts of the country where it covers approximately 500 000 ha in the three provinces of Taourirt, Jerada and Figuig. Just in Talsint, which is a remote region in the province of Figuig, rosemary's shrub is covering more than 200 000 ha (NAGGAR and IHARCHINE 2015).

Talsint is located in the southeast of the High Atlas Mountains series. It is composed half of mountains and half of plains. It's characterized by an arid climate with a weak annual rainfall of 140 mm (DAOUDI et al. 2017).

Rosemary commerce and harvesting by the population and cooperatives play a huge role in the local economy of Talsint. Thus, this plant is exposed to immoderate utilization by local residents. Besides, the means of essential oil production and marketing are still traditional due to a lack of scientific studies concerning the characteristics of the local rosemary leaves and its essential oil.

Therefore, given the lack of chemical information concerning Talsint's rosemary essential oil, and the importance that plays this plant on the local economy, this work aims to investigate the essential oil chemical composition and antioxidant activity of *Rosemarinis offinalis* in the region of Talsint. This information will represent an added value to the rosemary essential oil of the region as a commercial product.

Material and Methods

Plant Material

Eight samples of *Rosmarinus officinalis* L. leaves were collected from Talsint vicinity (Province of Figuig, Morocco), within an area of 150 km^2 (Fig. 1)

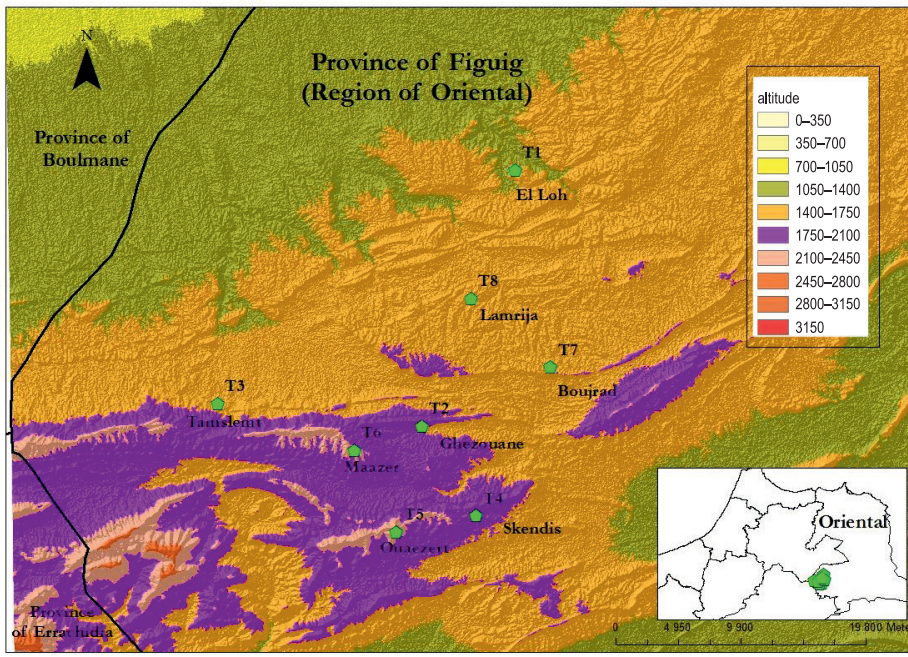

Fig. 1. Map of samples' location in Talsint vicinity (Provinces of Figuig, Region of Oriental Morocco)

Table 1

Lambert coordinates altitude, and location name of samples of wild rosemary populations collected in 2017 from the forest of Ait Seghrouchen, Talsint (Province of Figuig, Morocco)

Sample	Location	Lambert coordinats		Altitude
		X	*Y*	
T01	El Loh	672 760	255 492	1447
T02	Ghezouane	663 922	225 465	1760
T03	Tamslemt	647 714	227 243	1635
T04	Skendis	668 223	218 339	1914
T05	Ouaezart	661 912	217 043	2143
T06	Maazer	658 584	223 526	2153
T07	Boujrad	674 144	230 096	1739
T08	Lamrija	667 834	235 577	1581

and from different altitudes of the North Slope of High Atlas Mountain on May 2017. Thus, the T01, T03 and T08 samples are collected below the 1580 m altitude threshold, T02 and T07 within 1739 and 1760 m, whereas T04, T05 and T06 are above 1913 m (Tab. 1). We mention that samples were collected at bloom stage from new shoots of individual plants in Ait Seghrouchen Forest. Afterwards, the plant material was dried at room condition (25°C) for 7 days.

Essential Oil Extraction

The leaves of *Rosmarinus officinalis* samples underwent hydrodistillation for 3 h with a Clevenger-type apparatus according to the European Pharmacopoeia (1996). The essential oil extracted was isolated from water and dried over anhydrous sodium sulphate and kept in amber vials at 4 C. The yield of each sample was calculated per 100 g of plant dry matter.

Chromatography – Mass Spectrometry Analysis

The essential oil extracted was analyzed by gas chromatography--mass spectrometry (GC-MS) using a Hewlett Packard 6890 mass selective detector coupled with a Hewlett Packard 6890 gas chromatograph with a 30 m × 0.25 mm HP-5 (cross-linked Phynel-methyl Siloxane) column with 0.25 µm film thickness (Agilent). The carrier gas is Helium and the flow rate along the column was 1.4 mL min-1. The temperature of the column was of 10°C min^{-1} and increased finally, at rate of 30°C min^{-1}, from 230 to 280. The parameters of the mass-spectrometry used in this study were as follows: ionization energy of 70 eV and ionization current was 2 A. The ion source temperature was 200°C and resolution was 1000. Mass unit were set from 30 to 450 m/z. The components of essential oil were identified based on relatives retention indices, Kovats index, WILEY 275 Library and by comparison to the literature data (VIUDA-MARTOS et al. 2007).

DPPH Radical-Scavenging Activity

The scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was carried out by the method reported by TAHRI et al. (2015) with minor modifications. 0.6 Ml of essential oil solution with various concentrations (50 µl ml^{-1}, 100 µl ml^{-1}, 150 µl ml^{-1} and 20 µl ml^{-1}) were mixed with 2.4 mL of DPPH solution (diluted in methanol at 0.004%) and incubated afterwards for 30 minutes in dark at room temperature. Then, the absorbance is measured at 517 nm. Methanol is used as negative control meanwhile ascorbic acid (vitamin C) as a standard antioxidant. The percentage of inhibition activity of DPPH radicals was calculated as:

$$\text{Inhibition Activity [\%]} = \left[\frac{(A0 - A1)}{A0}\right] \cdot 100$$

where:
A0 – the absorbance of the negative control
A1 – the absorbance of the essential oil solution.

The minimal concentration to reduce 50% of DPPH radicals (IC50) was worked out from linear regression analysis.

Statistical Analyses

To assess the affinity between the essential oil belonging to different localities, a hierarchical cluster analysis (HCA) was conducted with the Euclidean distance as a measure of dissimilarity based on the main five components. All the analyses were conducted using SPSS software, version 19 (IBM SPSS, Chicago, IL, USA).

In order to study the relationship between the altitude and the other variables such as the yield and chemical composition, we used the linear regression and the Pearson coefficient (p).

Results and Discussion

Yield of the Essential Oils

Table 2 reports the yield of the extracted essential oils. The yield ranges from 0.58 to 1.67%. We notice that the lowest value belong to Boujrad and Mrija localities that have relatively medium altitude (1739 and 1581 respectively); meanwhile, the highest yields were found in the samples of Skendis, Ouaezart, and Maazer, characterized by high altitudes (more than 1900 m). However, we can't attribute the yield variability to the altitude since Tamslemt and Ghezouane, which are samples with the same range of altitude as Boujrad and Mrija; have higher yields (1.32% and 1.09% respectively). Those observations are confirmed by the regression line (Fig. 2) set between the essential oil yield and the altitude that showed a non-significant correlation ($R^2 = 0.37$; $p > 0.1$). As a result, we can assume that the yield is not influenced by the altitude effect.

Many studies that investigate rosemary's essential oil yield in different regions. Thus, the rosemary studied in the region of Boulmane (Morocco) yielded 0.54% (DERWICH et al. 2011). Meanwhile, the *Rosmarinus officinalis* of Taourirt (Morocco) yielded 1.8% (AIT-OUAZZOU et al. 2011).

Table 2

Chemical compositions of the essential oil of rosemary leaves of Talsint (Figuig, Morocco)

Locations	El Loh	Ghezouane	Tamslemt	Skendis	Ouaezart	Mâazer	Boujrad	Mrija	RT*	IK**
Yield (g/100 mg)	**1.33**	**1.32**	**1.09**	**1.64**	**1.67**	**1.52**	**0.58**	**0.60**	–	–
Compounds									–	–
Monoterpenes Hydracarbons	**20.7**	**19.28**	**24.77**	**21.13**	**21.65**	**21.11**	**18.87**	**19.11**	–	–
α-pinene	8.58	6.61	8.29	8.71	8.64	8.15	6.03	9.02	4.63	137
Camphene	3.61	3.93	3.78	2.93	3.04	2.8	1.64	3.04	5.06	189
β-pinene	8.51	5.96	8	5.3	5.97	5.96	5.89	4.66	5.613	254
β-myrcene	–	0.75	0.82	1.04	0.95	1.01	1.12	0.63	5.695	265
α-terpinene	–	–	–	–	–	–	0.54	–	6.39	334
D-limonene	–	1.3	2.99	1.39	1.59	1.78	1.91	1.28	6.49	360
β-terpinene	–	–	–	–	–	–	0.48	–	6.64	378
β-cymene	–	–	–	0.8	0.79	0.69	–	–	6.74	391
δ-terpinene	–	0.73	0.89	0.96	0.67	0.72	1.26	0.48	7.10	433
Monoterpenes Oxigenated	**79.3**	**79.53**	**74.47**	**77.85**	**77.13**	**76.43**	**81.13**	**79.42**		
(+)-2-Carene	0	0.37	–	–	–	–	0.56	–	6.27	492
1.8-cineol	64.27	58.81	58.24	53.16	53.35	50.6	57.24	61.73	6.81	399
Linalool	–	0.66	–	1.2	1	1.15	1.15	–	7.99	539
Camphor	6.39	9.36	1.77	12.82	12.96	13.33	14.12	11.47	9.62	736
Borneol	5.73	4.63	6.2	5.07	4.85	5.26	5.51	1.98	9.66	741
α-terpineol	–	3.71	4.65	4.39	3.87	4.97	1.08	3.11	10.00	781
Bornyl acetate	2.91	1.99	3.61	1.21	1.1	1.12	1.47	1.13	11.18	924
Sesquiterpenes	**–**	**1.19**	**0.76**	**1.02**	**1.22**	**1.43**	**–**	**0.98**		
Careophyllene	–	1.19	0.76	1.02	1.22	1.43	–	0.98	12.771	1113
Others	–	–	–	–	–	1.03	–	–		
Diethyl Phthalate	–	–	–	–	–	1.03	–	–	16.6	1504
Total identified	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**99.51**	**–**	**–**

Explanations: * RT – retention index; ** IK – Kovats index

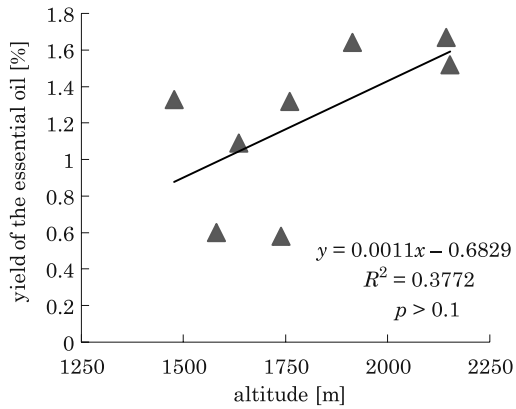

Fig. 2. Linear regression of the yield of the essential oil of the studied samples of rosemary along altitude

Those values, that were registered in Morocco, are less than those found in Iran by JALALI-HERAVI, SADAT and SERESHTI (2011), who reported a yield of 2.05%. They are also less than those found in Brazil where it yielded 2.5% (PORTE et al. 2000). Nevertheless, according to ANGIONI et al. (2004), who studied Sicilian rosemary, and to JORDÁN et al. (2013), who studied Spanish rosemary, didn't attribute the yield variability to the bioclimatic factor.

The Chemical Composition of The Essential Oil

The GC-MS analysis revealed 18 components, which represent from 99.5 to 100% of the total of the identified compounds in the extracted essential oil (Table 2). We report that the major compounds were 1.8-cineole, camphor, and *α*-pinene are present in all samples with variable concentrations depending to the location (Fig. 3): 1.8-cineole is ranging from 50.60%

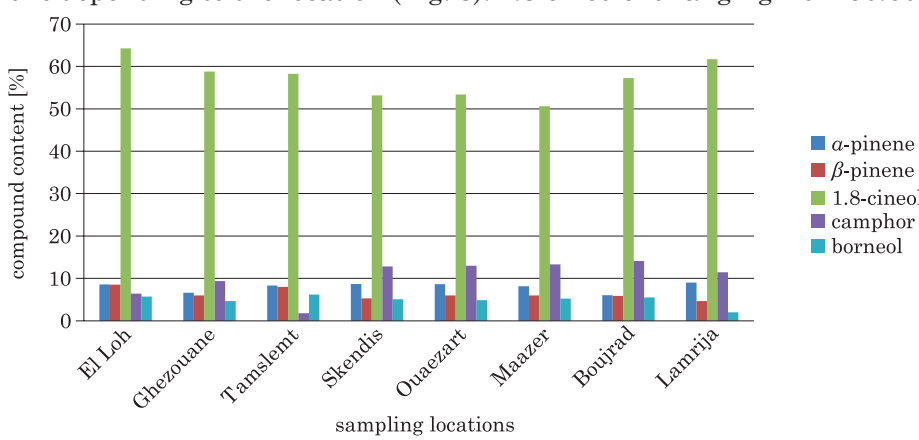

Fig. 3. Volatile profile of the main compounds in 8 populations of rosemary collected in the region of Talsint

(Maazer) to 64.27% (El Loh); camphor change largely from 1.77% (Tamslemt) to 14.12% (Boujrad); meanwhile the concentration of *α*-pinene goes from 6.61% to 9.02%. Other compounds as *β*-pinene (4.66–8,51%), borneol (1.98–6.20) and camphene (1.64–3–93%) are also present, with less importance, in all the analyzed samples.

Many studies conducted across the world concerning *Rosmarinus officinalis* essential oil reported a wild range of volatile profiles. Thus, the main constituent in Montenegrin and Albanian rosemary essential oil is camphor with a relative concentration of 24.4 and 17.3% respectively (LAKUŠIĆ et al. 2012). Whereas, Mexican rosemary essential oil is rich with *α*-pinene (31.07%) and Verbenone (15.26%). The literature related to Moroccan *Rosmarinus Officinalis* essential oil pointed out volatile profiles that are close or similar to our finding with 1.8-cineol as a major constituent with more than 40%.

The regression drawn between the four major components (*α*-pinene, *β*-pinene, 1.8-cineol and camphor) and the altitude in the studied rosemary essential oil is illustrated in Figure 4 in order the emphasis the relationship between the altitude and the volatile composition. Excepted for 1.8-cineole content which showed a significant relationship (R^2 = 0.88, $p < 0.0005$) with a declining trend across the increasing altitude, the tree other compounds show a very week correlation with non-significant correlation.

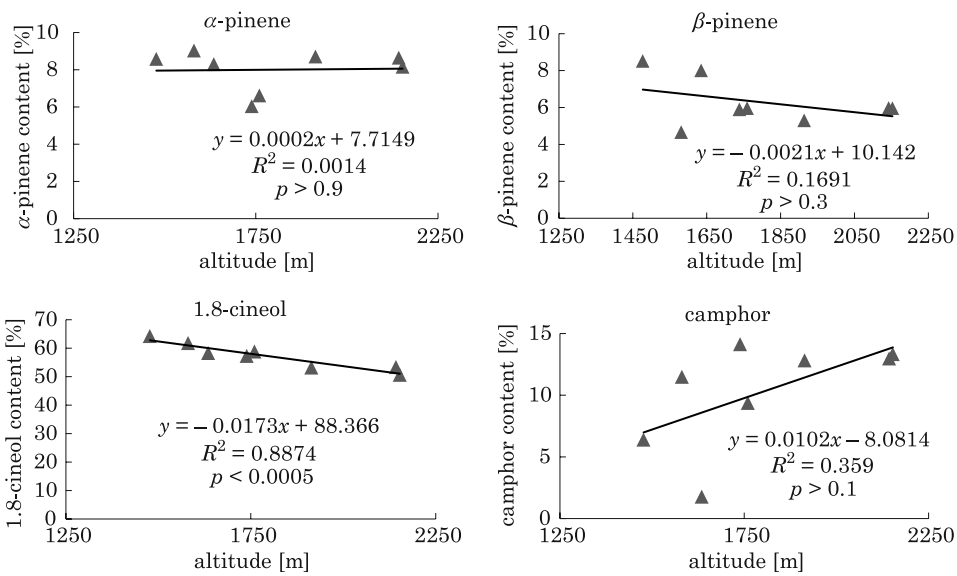

Fig. 4. The regression analysis of the *α*-pinene, *β*-pinene, 1.8-cineol, and camphor content along the altitude

Explanations: p is the coefficient of Pearson and R^2 is coefficient of determination

Thus, we can say that the composition of the essential oil isn't depending on the altitude of the rosemary shrub. Our results are congruent with the reports made by Li et al. (2016) who confirmed that volatile variability of *Rosmarinus officinalis* essential oil from Italy, Corsica, isn't influenced by altitude and assumed that this variability is due to genetic backgrounds rather than environmental conditions.

As regard to molecule groups, Table 3 showed that the oxygenated monoterpenes are the dominant constituents of the essential oil of Talsint's rosemary. They range from 74.47 to 81.13% of the total of the constituents; meanwhile the monotepenes hydrocarbons represent from 18.87% to 24.77%. In contrast, the sesquiterpenes don't exceed 1.43%. Our finding are in agreement with the results of TAHRI et al. (2015) and LIU et al. (2011).

Table 3

Free radical-scavenging activities [%] of rosemary essential oil of the Talsint region in Morocco and of ascorbic acid, and the total of monoterpenes hydrocarbons and oxygenated monoterpenes

Sample	Location	Concentration of essential oil [µl ml^{-1}]				R^{2a}	DPPH^b IC50 [µl ml^{-1}]	Total MH	Total MO
		20	**40**	**60**	**80**				
T01	El Loh	41.98	50.09	64.98	78.73	0.986	32.81	20.7	79.3
T02	Ghezouane	43.73	54.02	60.14	72.64	0.984	33.57	19.28	79.53
T03	Tamslemt	42.26	57.86	64.96	77.44	0.981	31.13	24.77	74.47
T04	Skendis	43.16	54.37	60.83	72.85	0.988	33.7	21.13	77.85
T05	Ouaezart	43.85	52.69	65.26	74.65	0.986	32.23	21.65	77.13
T06	Maazer	43.89	53.09	64.08	78.64	0.989	32.79	21.11	76.43
T07	Boujrad	43.08	55.16	61.07	73.12	0.983	33.26	18.87	81.13
T08	Lamrija	43.3	55.04	60.94	72.85	0.984	33.07	19.11	79.42
Ascorbic acid^c	-	**46.61**	**63.34**	**71.16**	**82.77**	**0.97**	**22.65**	–	–

Explanations: ^{a}, R^2 – the regression coefficient used to measure the linear relationship between the essential oil concentration and the percentage of radical scavenging; ^{b} – essential oil concentration required to scavenge 50% of DPPH solution; ^{c} – ascorbic acid used as a reference antioxidant

The dendrogram of the cluster analysis of the essential oil volatile profile is carried out in Figure 5. Therefore, we can split the studied samples to two main groups: *A* and *B*. The group *A* can be divided to two subgroups (A1 and A2). Group *A* is characterized by a relatively high content of camphor (9.36–14.12%). Actually, 1.8-cineol content is the one what makes the difference between Sub groups since in Sub Group A1 (Skendis, Ouaezrt and Maazer), it is ranging from 50.6 to 53.35% which is lower than the values registered in Sub Group A2 (Ghezouane, Boujrad and Lamrija) where 1.8-cineole percentage attains 57.24 to 61.73%. By contrast, the

Group B (El Louh and Tamslemt) is characterized by a relatively weak content of camphor (1.77–6.39%) and high content of 1.8-cineole (58.28–64.27%). Based on this classification, we can say that samples of moderate altitude are richer of 1.8-cineol. However, there is a quite difference between SubGroub A1 and Group *B* concerning the content of camphor, even if they belonged to the same range of altitude, since we find a low content in Group *B*. As a consequence, we can report that the effect of altitude remains unclear. This variability could be explained through further studies which target the interaction of the different factors influencing the chemical composition of the essential oil like the genetics, age and environmental conditions.

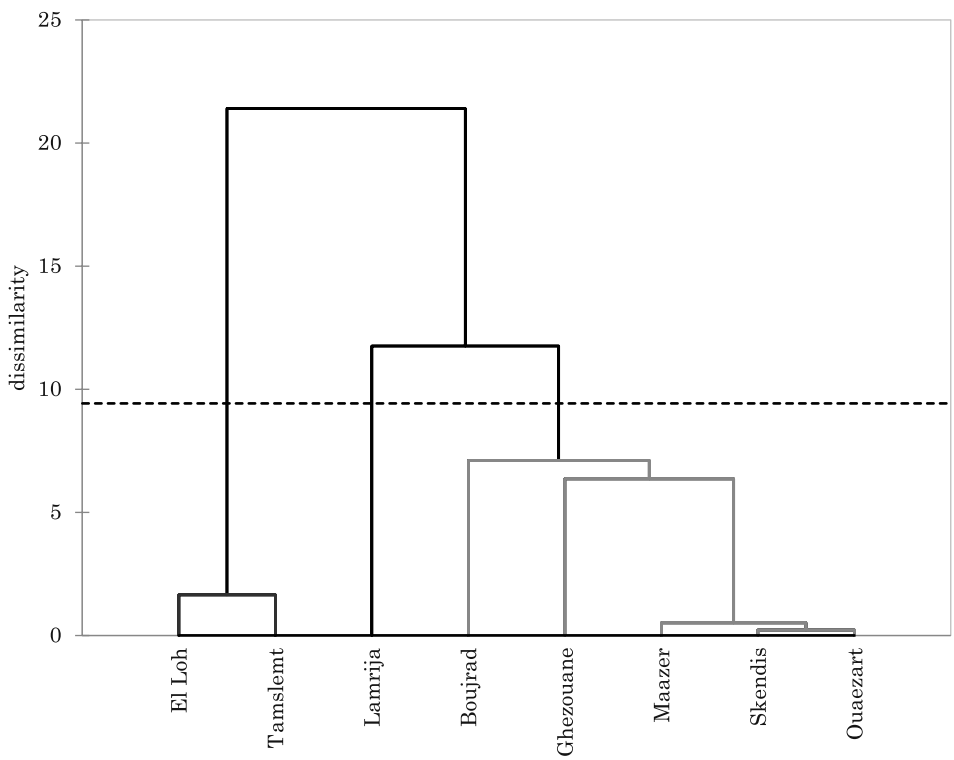

Fig. 5. Cluster dendrogram using the main five constituents of the eight populations of rosemary of Talsint (Province of Figuig)

Anti-oxidant activity

The eight samples of the essential oil extracted were submitted to the test of radical scavenging capacity through the original DPPH assay (Tahri et al. 2015). The results of the DPPH test are summarized in Table 3.

The Radical scavenging capacity of the analyzed samples showed an increase in line with the oil concentration: the most potent essential oil was the one of Skendis (IC50 = 31.13%). However, the values are judged very close to each other's (31.13–33.70%).

In addition, our samples show less antioxidant capacities compared to ascorbic acid (Table 3), which was used as standard antioxidant (IC50 = 22.65 ± 0.61 µl ml^{-1} with r^2 = 0.97). Anyhow, according to those results, the essential oil of the rosemary of Talsint is judged as a very potent antioxidant to neutralize the free radicals. Our findings are in congruent with the results of BERETTA et al. (2011), and TAHRI et al. (2015).

In order to investigate the relation between the content of molecules and the bio activity, we calculated the correlation of the IC50 and the percentage of hydrocarbons. Thus, we conclude that the antioxidant activity is negatively proportional to the percentage of monoterpenes hydracarbons (r^2 = 71%). Many studies pointed out that the capacity of an antioxidant component to scavenge DPPH is mainly depending to its ability to free hydrogen molecules, and this is too related the abundance of functional groups of monoterpenes hydrocarbons (TAHRI et al. 2015).

Conclusion

As regard to chemical composition, 1.8-cineol is the major component of *Rosmarinus officinalis* L. essential oil in the region of Talsint. In addition, camphor, as the second major component, is present with relatively less concentration than other regions in Morocco. Nonetheless, there is certain variability concerning the percentage of the chemical components among the studied rosemary essential oil that couldn't be explained by the altitude difference of the rosemary shrubs Furthermore, Talsint's rosemary essential oil showed an important anti-oxidant activity that could be used in food industry as non-synthetic anti-oxidant.

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OCCURRENCE AND CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM COWS WITH MASTITIS

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Key words: *Staphylococcus aureus*, mastitis, phenotyping, genotyping, biotyping.

Abstract

Mastitis is the most common disease of dairy cows, which causes significant economic losses in dairy farming. Mastitis caused by *Staphylococcus aureus* is particularly difficult to treat and eradicate, which is why rapid identification and evaluation of the pathogen and strain is important. The study presents occurrence and characterisation of *S. aureus* in the North – East region of Poland, and especially in the Podlaskie Voivodeship. Of the 9,617 samples of milk from cows suspected of mastitis, *S. aureus* was isolated from 1,330 samples, representing 19.4%. 225 strains of *S. aureus* were biotyped according to Umeki and classified into three biotypes: I – 19 (8.45%), III – 115 (51.11%), IV – 91 (40.44%). Biotype IV strains showed the most pathogenic characteristics. Randomly selected *S. aureus* strains were genotyped using ADSRRS-fingerprinting, this method was a better tool for differentiating strains compared to biotyping.

Introduction

Mastitis is the most common dairy cows' disease and contributes to significant economic losses in dairy farming. A particular problem is the inflammation caused by staphylococci, especially *Staphylococcus aureus*. This is due to the specific properties of *S. aureus*, among others: the ability to adhere to mammary gland cells, which facilitates tissue colonization (FROST 1975, AGUILAR et al. 2001), the ability to produce enzymes and toxins, which in turn enables the spread of bacteria in the mammary gland, while the polysaccharide capsular and coagulase impair immune

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defense (SORDELLI et al. 2000, SZYMAŃSKA and BUCZEK 1999). Slime and biofilm production enables staphylococci to survive in the mammary gland (VASUNDEVAN et. al. 2003, FOX et al. 2005, MELHIOR et al. 2006). In addition, the production of β-lactamase and the presence of the PBP2a protein reduces the sensitivity of these bacteria to β-lactam antibiotics, including methicillin (ŁOPACIUK and DZIERŻANOWSKA 2002). This results in mastitis which is difficult to treat and combat. In preventing the occurrence of new infections, it is important to quickly recognize and thoroughly understand the etiological factor. Effective typing of bacteria, especially staphylococci shows the pattern of transmission of infection, enables the identification of a reservoir of microorganisms and helps to eliminate existing infections and prevents the formation of new ones (MIĘDZYBRODZKI et al. 2008). Many phenotypic and genotypic methods are used to differentiate *S. aureus* strains isolated from cow's mammary gland. Phenotyping is the classification of strains based on the phenotypic characteristics of microorganisms. The phenotyping of *S. aureus* strains isolated from cow's milk uses: biotyping, i.e. the classification of bacteria based on specific biochemical characteristics (HAJEK and MARSALEK 1961, DEVRIESE et al. 1984, HAKIMI et al. 2016), serotyping (TOLLERSRUD et al. 2000, GUIDRY et al. 1998, SUTRA 1990, POUTREL 1988, BARDIAU et al. 2014, AMBROGGIO et al. 2018), phagotyping (MACKIE et al. 1987, AARESTRUP et al. 1997, FOX et al. 1991, LARSEN et al. 2000, VINTOV et al. 2003). Methods based on electrophoretic techniques are also used to analyze cellular protein profiles such as immunoblotting (LEITNER et al. 2003, YOUNIS et al. 2002) and MLEE (Multilocus Enzyme Electrophoresis) (FITZGERALD et al. 1997, TOLLERSRUD et al. 2000). One of the phenotypic methods for typing staphylococci is testing the antibiotic resistance profile (AARESTRUP et al. 1995, RAIMUNDO at al. 1999, LARSEN et al. 2000, VINTOV et al., 2003, ANDERSON et al. 2006, JAGIELSKI et al. 2014). It is a method commonly employed because of routine use of antibiograms, simplicity and low costs of testing. Commercial tests such as API 20 Staph system (bioMerieux), Api ID 32 Staph (bioMerieux), Staph-Zym (Rosco), the VITEK system (bioMerieux), Microgen Staph ID (Microgen Bioproducts) and STAPHYtest (PLIVA-Lachema) are commonly used to identify and type *S. aureus* strains (LISOWSKA-ŁYSIAK et al. 2018). Currently, genotypic methods are used for detection, identification, typing (differentiation) as well as in taxonomic studies or phylogenesis of *S. aureus*. In the study of genetic variation, molecular biology techniques are used, including chromosome size analysis, restricted digestion of the genome or plasmids and electrophoretic separation of its products. Analysis of plasmid profiles of *S. aureus* strains was one of the first methods used in research, but due to high variability and low

differentiating potential it is rarely used nowadays in epidemiological studies (AARESRUP et al. 1995, MATTHEWS et al. 1993, NASCIMENTO et al. 2005). REA-PFGE (Restriction Enzyme Analysis with Pulsed Field Gel Electrophoresis) is the "gold standard" in staphylococcus typing and is useful in epidemiological studies, detection of infection sources and in the case of infections caused by one strain (SABAT et al. 2013, LUNDBERG et al. 2016, JAGIELSKI et al. 2014, HATA et al. 2016, KOT et al. 2012, CASTELANI et al. 2013). The PCR (Polymerase Chain Reaction) technique that mimics the phenomenon of DNA replication is one of the most popular research techniques used to detect, identify, and determine phylogenetic relationships. Due to the advantage of sensitivity and speed of analysis, it is used in laboratories in various forms. For typing of *S. aureus* strains isolated from cow mastitis amplification of known genome regions combined with PCR/RFLP restriction analysis (LANGE et al. 1999) is used where strains are most often differentiated based on the amplification for example: coagulase (*coa*) gene fragments (AARESTRUP et al. 1995, DE SILVA and DE SILVA 2005, KARAKULSKA et al. 2011) and protein A (*spa*) (ANNEMÜLLER et al. 1999, LANGE et al. 1999, KUŹMA et al. 2005, HATA et al. 2016), genes encoding biofilm formation protein e.g. *ica* gene or *bap* gene (VASUNDEVAN et. al. 2003, SZWEDA et al. 2012, SALIMENA et al. 2016), genes encoding the ability to produce toxins (HAYAKAWA et al. 2001, LARSEN et al. 2002) or antibiotic resistance genes including methicillin resistance – the gene *mec*.

Next typing method is Random Amplified Polymorphic DNA amplifications (RAPD) – PCRs used to quickly differentiate *S. aureus* strains (LAM et al. 1996, SACHANOWICZ et al. 2007, NAWROTEK et al. 2009). Another variation of the PCR technique is the amplification of two or more different DNA fragments, i.e. Triplex PCR (SABAT et al. 2006, JAGIELSKI et al. 2014), Multiplex PCR (PUACZ et al. 2015). Ribotyping is based on differentiation of genes encoding the chromosomal RNA (rRNA) of the small and large ribosome subunits. The PCR (AARESTRUP et al. 1995) or hybridization technique (LARSEN et al. 2000) is used. Microarrays (CHIP-DNA) are recently used to type *S. aureus* strains through hybridization (LISOWSKA-ŁYSIAK et al. 2019). Another method is fingerprinting based techniques – which involves amplifying a polymorphic DNA fragment in a PCR reaction using appropriately designed primers. In the *S. aureus* typing, the presence in the genome of tandem repetitive substances (VNTR, Variable – Number Tandem Repeat) (SABAT et al. 2006, PICHETTE-JOLETTE at al. 2019) can be used. Among them *ssp, coa, spa, sdr* regions or the AFLP (Amplified Fragment Length Polymorphism) method (SAKWIŃSKA et al. 2011, VAN LEEUWEN et al. 2005) and ADSRRS fingerprinting (Amplification of DNA fragments Surrounding Rare Restriction Sites) (KRAWCZYK et al. 2007).

Recently the methods utilizing Multi-Locus Sequence Typing (MLST) (SABAT et al. 2013, JAGIELSKI at al. 2014, RABELLO et al. 2015) are gaining significance. It is low cost, easy and fast as well as a highly repeatable method. A complementary method to MLST typing is *spa typing*, which is increasingly used in typing staphylococcal strains isolated from cows with mastitis (LISOWSKA-ŁYSIAK et al. 2019). Due to the increasingly common methicillin resistance and the associated presence of the *mec* gene, it has become necessary to subtype the variable elements responsible for methicillin resistance called the chromosomal staphylococcal mec cassette (SCCmec), however, for the correct classification of the clone MLST and *spa typing* as well as SCC-mec PCR based methods should be performed. Many phenotypic and genotypic methods are used simultaneously for the proper assessment and typing of staphylococcal strains, including those isolated from cows' mastitis.

The aim of the study was to assess the incidence of bovine mastitis caused by *S. aureus* in Poland, and to evaluate strain diversity based on biotyping, according to Umeki and ADSRRS-fingerprinting.

Materials and Methods

Isolation and Identification of *S. aureus* Strains

The research material consisted of 9,617 quarters milk samples from cows with suspected mastitis among which 1,330 strains of *S. aureus* were isolated, which were subjected to further testing. Isolation and identification of strains was carried out in accordance with the recommendations of MALINOWSKI and KŁOSOWSKA (2002). The study was performed in the Bacteriology Laboratory of the Veterinary Hygiene Institute in Łomża (Poland). The material was plated on blood agar medium and Chapman medium (BIOMED), and then incubated 24 h at 37°C under aerobic conditions. Gram staining preparations were made from a single colony after incubation. After finding Gram-positive spherical bacteria, an additional catalase test was performed using 3% hydrogen peroxide (Catalase Test Difco). Additional tests were used to identify isolated Gram-positive bacteria: a coagulase test using a classic tube test using freeze-dried plasma (Biomed S.A. Kraków), detection of the clumping factor according to KĘDZIA (1997), production of hemolysins on agar medium with the addition of 5% defibrinated sheep blood. In order to identify microorganisms more accurately, API-Staph (Bio Merieux) biochemical tests were performed.

The Ability to Produce β-lactamase

The ability to produce penicillinase by the tested strains of *S. aureus* was determined using BR66A β-lactamase identification sticks (OXOID) – Identification Sticks β-Lactamase (Nitrocefin).

Lipase Production

The lipase production capacity was tested on Baird-Parker medium (BBL) with the addition of 5% egg yolk. Tested staphylococcal liquid cultures were inoculated on Baird-Parker medium. Plates were incubated for 48 hours at 37°C. Turbidity around the colony was considered positive.

Biotyping According to Umeki Method

The biotyping according to Umeki involves classifying strains into one of the four biotypes based on the disintegration of three sugars. Biotype I decomposes- mannitol, biotype II decomposes mannose, biotype III decomposes mannitol and mannose, biotype IV decomposes mannitol, mannose, and ribose (Table 1). The decomposition of sugars was made on the liquid medium according to Brailey'a and Scott'a (Kędzia 1997). The basic medium was heated to boiling, next the medium was filtered through tissue paper, brought to pH 7.7–7.8 and in the end, it was sterilized in an autoclave at 120ºC for 20 minutes. According was made 100 ml 5% sugar solution.

Table 1

Biotype *Staphylococcus aureus* strains by Umeki et al. (1992)

Sugar	Biotype			
	I	II	III	IV
Mannitol	+	-	+	+
Mannose	-	+	+	+
Ribose	-	-	-	+

Explanations: + ability to decompose the tested sugar; - no ability to decompose the tested sugar

The basic medium and the sugar solution were mixed, afterwards 10 ml Andreadea's reagent was added, which earlier was sterilized by filtration methods. The Andreadea's reagent is obtained from 0.2% aqueous solution of acid fuchsin, it was neutralized with 1 M NaOH at pH 7.2. The obtained substrate was diffused into tubes. The single colony of *S. aureus* was cultured into a medium and it was incubated at 37ºC for 24 h. The discoloration from pink to yellow indicated the ability of *S. aureus* to decompose of sugar.

Genotyping by ADSRRS – Fingerprinting

From the study population of 225 strains, 45 randomly selected *S. aureus* strains were subjected to genotypic evaluation by ADSRRS – fingerprinting. The method was described in the publication of DZIEKIEWICZ-MRUGASIEWICZ et al. (2008). The original ADSRRS – fingerprinting method was developed by MASNY and PLUCIENNICZAK (2001) and its application to bacterial DNA-fingerprinting was shown by KRAWCZYK et al. (2003, 2007)

Statistical Analysis

Statistical package SPSS 12.0 was used to perform the statistical analyses and non-parametric compatibility tests Chi2 Pearson and UNINOVA.

Results

In study 9,617 samples of quarter milk, pathogenic microorganisms were found in 6,947 samples, which constituted 72.24%. *S. aureus* was detected in 1,330 milk samples, accounting for 19.14% of all isolated microorganisms. All strains tested produced coagulase, clumping factor, degraded mannitol and caused beta hemolysis. In this study 225 *S. aureus* strains were biotyped according to Umeki, 115 (51.11%) of *S. aureus* strains were classified into biotype III and 91 strains (40.44%) were included in biotype IV, nine strains (8.45%) belonged to biotype I, while no strain was classified as biotype II. The affiliation of the tested *S. aureus* strains to a specific biotype according to Umeki is shown in Table 2.

Table 2

Affiliation of *Staphylococcus aureus* strains isolated from cow's milk samples to the biotype by UMEKI et al. (1992)

	Biotypes according to UMEKA (1992)			
	I	II	III	IV
S. aureus strains $N = 225$	19 (8.45%)	0 (0%)	115 (51.11%)	91 (40.44%)

In the next stage of the study, the strain belonging to a particular biotype and selected pathogenic features were compared. Biotype I included strains with the least pathogenicity features, in this group only 37% of the strains produced lipase, 12.5% produced β-lactamase. The biotype III strains produced 37.04% of lipase, 44% produced β-lactamase, biotype IV

staphylococci showed the most pathogenicity, 62.96% of them produced lipase, 66.67% had the ability to produce β-lactamase.

Genotyping examination by the ADSRRS method – fingerprinting showed that biotype III was the most diverse, it included seven different genotypes: A, B, C, D, E, F, H, of which 17 (38.6%) belonged to genotype D, five to A, two – C, three – E and one for B, I and F. Three different genotypes D, E and I belonged to biotype IV, and 12 strains were classified as genotype D, and one for E and I. Other genotypes A, B, C, E, F, H belonged mainly to biotype III. 17 (58.6%) strains belonging to genotype D were mainly classified to biotype IV 17 (58.6%), 12 (41.4%) to biotype III, no strain from this group was classified to biotype I (Table 3).

Table 3

Differentiation of *S. aureus* strains based on their classification to biotypes and genotypes

Genotype	Number of strains n = 45	Biotype		
		I n [%]	III n [%]	IV n [%]
A	5	0 0	5 100%	0 0
B	1	0 0	1 100%	0 0
C	2	0 0	2 100%	0 0
D	29	0 0	12 41.38%	17 58.62%
E	4	0 0	3 75%	1 25 %
F	1	0 0	1 100%	0 0
G	1	1 100%	0 0	0 0
H	1	0 0	1 100%	0 0
I	1	0 0	0 0	1 100 %
Total	45	1 2.22%	25 56.56%	19 42.22%

As in the case of Umeki biotyping, the majority of genotype D strains (41.4%) showed the ability to produce β-lactamase, among the remaining genotypes β-lactamase was produced only by 18.8% of the strains.

Discussion

In Poland and in the world, the occurrence of mastitis caused by *S. aureus* varies and depends on: the region, the breed of cows, environmental conditions and *mastitis* control programs. At the beginning of the 90s, infections of *S. aureus* in the Podlasie region constituted only 5.7% (JAKUBCZAK et al. 1998), since 1997 *S. aureus* already constituted 18.7% (JAKUBCZAK et al. 2001), in later studies in this region, the percentage of isolation of *S. aureus* averaged 19.14%, of which 28.15% of isolated strains in the last year of the study. In subsequent years, the percentage of mammary gland infections with this microorganism in the studied region remained at a similar level (19.8%) (NIEDZIELA et al. 2008). During the analyzed period, *S. aureus* was most often isolated in northern Poland, namely 34.8–50.7% of samples delivered to the laboratory in Malbork (CZUPA and CZUPA 2001). In the Bydgoszcz region, MALINOWSKI et al. (2003) classified 34.5% of the strains as *S. aureus*, while in subsequent analyzes only 8.6% (MALINOWSKI et al. 2006), while in the Lublin region 10.4% (KRUKOWSKI et al. 2000). Recent studies have shown that the percentage of isolation of *S. aureus* in the country is at a similar level, namely 13% (SMULSKI et al. 2011) and 12.1% (SZTACHAŃSKA et al. 2016) in the region of north-eastern Poland and 20.8% (BURMAŃCZUK et al. 2016) in the West Pomeranian Voivodeship. In the research of Rola et al. (2015) involving small farms, as much as 50% of milk samples were infected with *S. aureus*. It should be noted that the latest data related to research carried out at specific farms, while the previous data concerned mainly analyzes of the number (percentage) of inflammations occurring during the year generally (SMULSKI et al. 2011). The author also noted that larger farms had better health status. In the world literature, the occurrence of mammary gland infections caused by *S. aureus* is diverse. Currently, following the introduction of *mastitis* eradication plans, the percentage of isolation of *S. aureus* from the mammary gland of cows is lower.

Understanding the etiology of mammary gland inflammation in the studied region allows establishing a strategy for controlling and preventing mastitis, which is important in the case of *S. aureus*, which often causes subclinical infections. In addition, bacterial differentiation is an essential tool to control the spread, carrier of *S. aureus* strains and in epidemiological research. A properly selected typing method allows you to show the direction of infection spread and indicate sources of origin of a given strain (MIĘDZYBRODZKI et al. 2008). In the past, phenotypic methods dominated in strain typing, currently genotypic methods are commonly used to differentiate microorganisms. Phenotyping of *S. aureus*

strains and especially biotyping (HAJEK and MARSALEK 1971, DEVRIESE et al. 1984) is mainly a historical method due to high variability, low accuracy, poor repeatability of results and low discriminatory power (TENOVER et al. 1994) as well as occurrence of *S. aureus* strains with atypical biochemical features (MIĘDZYBRODZKI et al. 2008). Despite this, research continues to use these methods as a complement to genotyping (MYLLES et al. 1997) and more recently Hakimi et al. (2016). The authors compared Devriese biotyping methods of *S. aureus* strains to genotypic methods such as RAPD PCR (MYLLES et al. 1997), PCR (HAKIMI et al. 2016). Both noticed the relationship between given phenotypic traits and the ability to spread and transmit strains. In microbiological studies, the occurrence of specific phenotypic traits is often used, e.g. biofilm production capacity (FOX et al. 2005), toxin production capacity (KOT et al. 2011) or antibiotic sensitivity (JAGIELSKI et al. 2014) compared to studies with application of genetic methods. The literature often compares the occurrence of given phenotypic traits and genotypic methods.

In our own research, two little-known typing methods were used: Umeki biotyping involving the breakdown of 3 sugars and ADSRRS fingerprinting genotyping. As a result of biotyping according to Umeki, 8.45% of the tested strains were included in the biotype I, they showed the least pathogenic characteristics, no strain was classified to biotype II, 51.11% was included in III and 40.44% of the tested strains in IV. The obtained results show similarity to the research of Umeki et al. (1992), in which biotype III was also the dominant biotype, strains of this biotype were found on all farms. Rarely, strains belonging to biotype II (4.6%) were isolated, and biotype I and IV represented 16.9% of the strains. Other results were obtained by SACHANOWICZ and JAKUBCZAK (2004), who most often detected strains of biotype IV – 55.8%, III – 20.3%, II – 1.3% and I – 17%, respectively. In addition, UMEKI et al. (1993) studied the relationship between belonging to a given biotype and the occurrence of selected pathogenicity features. They noticed that the ability to produce coagulase, lipase and fibrinolysin in staphylococci increased with belonging to a higher biotype, reaching the highest values among strains belonging to biotype IV. This thesis is confirmed by our own research, which showed that as much as 62.96 % of *S. aureus* strains of this biotype produced lipase, 66.67 % had the ability to produce beta-lactamase. Among strains belonging to biotype I, only one strain (12.5%) produced β-lactamase by analogy, 37% produced lipase, slime, and biofilm also only 1 strain, which accounted for 12.5%. The results obtained coincide with those of SACHANOWICZ et al. (2003) and UMEKI et al. (1992) which confirm the thesis on the relationship between the virulence of the *S. aureus* strain and belonging to a specific biotype.

In own research, strains belonging to individual biotypes were subjected to ADSRRS fingerprinting genotyping. This technique was developed by Polish scientists MASNY and PŁÓCIENNICZAK (2001), modified by KRAWCZYK et al. (2003). The ADSRRS fingerprinting was used in human medicine to differentiate clinical strains of *E. coli, Klebsiella oxytoca, Klebsiella pneumoniae* (MASNY and PŁÓCIENNICZAK 2001, KRAWCZYK et al. 2005), *Serratia marcescens, Enterococcus faecium* (KRAWCZYK et al. 2003) as well as *S. aureus* isolated from skin furuncles (KRAWCZYK et al. 2007). The method has also been used in veterinary medicine to differentiate *Corynebacterium pseudotuberculosis* strains isolated from goats (STEFAŃSKA et al. 2007). Whereas NOWAKIEWICZ et al. (2017) used this method for typing *Enterococcus* strains isolated from pigs and in the assessment of *Staphylococci* isolated from wild animals (2016). This method was first used for typing *S. aureus* strains isolated from milk of cows with mastitis by DZIEKIEWICZ-MRUGASIEWICZ et al. (2008), where studies have shown the occurrence of nine different genotypes marked from A to G. The dominant was the D genotype, which was characterized by high pathogenicity and spreading ability, namely in the first year of the study the D genotype isolates constituted 14.29%, and in the second year of the study 73.64% of all isolates. Moreover *S. aureus* strains belonging to D genotype had higher ability to adhere to the mammary epithelial cells and slime and biofilm production. The next study compared the belonging of *S. aureus* strains of individual genotypes to biotypes according to Umeki. The results showed that all genotype D strains belonged to pathogenic biotypes III and IV, most of which belonged to biotype IV. This is confirmed by the relationship between phenotypic and genotypic traits, which affects the ability of the strains to spread.

Own and other authors' research showed the usefulness of the ADSRRS fingerprinting method in strain typing and epidemiological analyzes. NOWAKIEWICZ (2016) additionally showed a strong correlation between genotype and phenotype profile together with antibiotic resistance. In contrast, STEFAŃSKA (2007) showed that ADSRRS fingerprinting has a higher discriminatory power, better repeatability than RAPD-PCR and Box PCR methods. KRAWCZYK et al. (2007) showed that ADSRRS fingerprinting has similar discriminatory power as PFGF considered as the gold standard in genetic testing. Currently, molecular based methods such as MLST or *spa* typing predominate in molecular research, they are more accurate and have a high discriminatory power, unfortunately there are no studies comparing both techniques.

Conclusion

Mastitis caused by *S. aureus* is the most common problem in dairy farming in Poland and in other countries. Umeki biotyping and ADSRRS--fingerprinting genotyping can be useful method to typing *S. aureus* strains. In addition, the ADSRRS-fingerprinting can be used in the study of etiological factors. The advantage of both methods is the ease of implementation, low costs, and no need to have specialized equipment.

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ANIMAL DEFECTS IN THE ARMENIAN LAW IN THE CROWN OF THE KINGDOM OF POLAND

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Key words: veterinary medicine, warranty, guarantee, redhibitory defects, legal history, sale of animals.

Abstract

The aim of the study is to research and analyze juridical and veterinary aspects of the legal norms on animal defects in the *Statute of Polish Armenians*, 1519. The seller's liability for defects of horses, oxen, cows and bees is described along with general clauses, and subject to historical-legal, linguistic, logical, teleological methods of analyzis. The work reveals warranty, stipulatory, and guarantee norms, derived by Armenian legislators from many sources, and innovatively processed. Among these ideas, many original legal concepts can be found; new and independent way of Roman law reception is revealed. Both the discussed Armenian Statute and its main basis, the Datastanagirk' of Mkhitar Gosh, mix Roman concepts of redhibitory action with the theory of major and minor defects. Although this Statute is an opus of medieval Middle--East immigrants, it presents the same high level of legal development as the modern-era Western-European legal acts on animal defects.

Introduction

The sale of animals, due to the unique nature of its subject, which is the living and painful organism, as well as the economic importance of animal husbandry, is regulated by specific norms in various legal systems. Animals may be burdened with various defects, usually described in modern legal doctrine as legal and physical defects.

The subject of the work is a normative analyzis of the provisions of the Armenian Statute, regarding the defective nature of animals, including: the premises of the seller's liability for latent defects in the subject of the

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contract of sale, and the buyer's claims derived from the burdening of an animal with a defect. This database was used to analyze legal and veterinary connections in the Armenian Statute and to deepen knowledge about the defects of animals being subject to commercial transactions of the Polish Armenians. The legitimacy of such a research scope is indicated by the fact that there were no previous studies on the level of veterinary knowledge and its impact on the legal norms of Datastanagirk' and other normative acts based on it.

Material and Methods

Normative analyzis of the discussed *Statuta iuris Armenici* (BALZER 1906) was made, using the following methods of interpretation: historical--legal, comparative, linguistic, logical, teleological.

The Latin normative text accepted by Sigismund I of Poland, preserved in the *Metrica Regni Poloniae* and the diploma issued for the Lwów Armenians (BALZER 1910), will be analyzed. The pre-original text (in Armenian language) has not survived. Apart from slightly different Latin variants there are also translations available: compatible Polish official translations of 1528, 1595, and 1601, 19th-century Polish unofficial, as well as an Armenian-Kipchak translation (BALZER 1910).

The regulation of liability for animal defects in the contracts of sale is contained in chapters (capitula) forty-eight to fifty-one and in chapter eighty-two of the Armenian Statute (DAT. II.55–58, II.100, II.110 = DAT. 94–97, 185, 221; Statut warcki No. XII; Sachsenspiegel No. I.9, III.4.83; Ius Municipale No. 21.30.96.

The legal text is as follows:

"Capitulum quadragesimum octavum. De emptione equi. Vendens equum alter ab altero, forum emptionis equi debet fieri in praesentia duorum vel trium testium, propterea ne equus esset furtivus, et ne antiquam claudicaturam habeat, et quod non esset ptisicus alias dychawiczny, aut nosaty. Si vero cognitum fuerit ad septimum diem aliquod vitium ex praedictis in equo empto, tunc emptor talem equum vitiosum restituere venditori poterit. Si vero equus ad septimum diem praedicta vitia aut unum eorum in se repertum non habuerit, tunc forum venditionis equi suum effectum sortiri debet. Si vero equus ille furtivus fuerit, tunc intercessor tenebitur emptorem pro praefato equo suo grosso et impensa eliberare, intercedere et indemnem reddere.

Capitulum quadragesimum nonum. De vendito bove. Vendens bovem alter alteri coram tribus testibus tale forum debet facere, et vendens bovem huiusmodi de iure tenebitur talem bovem dare illi ementi ad aratum sive currum ad tentandum, quod talis bos non esset nocivae consuetudinis, nec furatus. Si vero bos fuerit nocivae et ferae consuetudinis, ad septimum diem potest illi venditori restitui. Si fuerit furtivus et aliquis alloqueretur se ad illum, tunc emptor debet se trahere ad principalem intercessorem, qui intercessor debet eum suo grosso ubilibet intercedere et indemnem reddere iure ita dictante.

Capitulum quinquagesimum. De vendita vacca. Vendens alicui vaccam debet emptori cavere, quod talis vacca quolibet anno consuevit inpraegnari; sin aliter compertum fuerit in praedicta vacca, quam ipse venditor spopondit, tunc in uno integro anno eandem vaccam emptor venditori restituere potest. Si vero ipsa vacca fuerit prolificans bene, tunc forum debet suum effectum sortiri.

Capitulum quinquagesimum primum. De apibus. Vendens alicui apes in autumn in alveario cum melle, et venditor spopondit emptori, quod in huiusmodi alveario est tantum mellis, et nominat certam mensuram et expressam quantitatem, si ille emptor credere venditori noluerit, tunc poterit alvearium aperire et mel mensurare; et quicquid mellis ad illam quantitatem et mensuram defecerit, hoc ille venditor aut melle apponat, aut pecuniis solvet defectum mellis. Si mel deficiens apponere noluerit, tunc forum huiusmodi ad nihilum redigitur. Si vero illud mel excesserit quantitatem et mensuram condictatam in alveario, tunc emptor restituere venditori huiusmodi excrescentiam mellis non tenebitur, quia spe lucre, non damni emit. Si aliquis vendiderit vernali tempore propter examen apum, talis recipiendo huiusmodi apes debebit ponere in suo mellificii loco, ad decimam vel vigesimam diem eas Servando propter inquirendum, si illae apes emittent examen vel non. Et forum pro huiumodi apibus debet fieri coram duobus vel tribus testibus. Et introitus ac exitus apum ita fiat et inveniatur, sicut in foro condictatum est. Si exitus et introitus apum ita invenietur, sicut contractus fori conclusus est inter venditorem et emptorem, tunc huiusmodi forum debet suum effectum sortiri. Si vero inter huiusmodi apes aliquid nocivi fuerit, aut mater earundem moreretur inter spatiumdecem aut viginti dierum, poterit emptor venditori tales apes vice--versa restituere. Post decursum vero viginti dierum, si aliquid nocivi praedictis apibus evenerit, tunc emptoris, non autem venditoris debet esse damnum".

Legislators – beginning with Mkhitar Gosh himself – focused only on three animal species: bees, horses and cattle, establishing a separate legal regime for oxen (males) and cows (females). This indicates the significant

economic position of these animals in the former society and the commercial, economic and legal importance of the contracts of sale concluded. Their health status was crucial for the parties to the contract and its valuation was dependent on the current veterinary knowledge.

All the aforementioned provisions of chapters forty-eight to fifty-one of the Armenian Statute should be analyzed in terms of: firstly, statutory requirements necessary to conclude a contract of sale; secondly, conditions under which the legal act concluded will exert legal effects; thirdly, liability for defects of the item sold. In individual cases, also the seller's oath (sponsio) is included; in the case of the autumnal sale of bees (in a beehive) – the issue of dispute between parties regarding the amount of honey declared by the seller. Apart from that, other appropriate norms of Datastanagirk' and Statute shall be researched and critically analyzed.

It should be noted that the subtle civilian considerations were not shared by the authors of the Statute, due to, among others, poorly-developed animal production and modest market trading of animals in the existing economic and social conditions.

As to the legal nature of the contracts, it is indispensable to stress that their classification into the contemporary conceptual grid of civil law, shaped by the case-law and doctrine, will never be fully accurate, faithful or satisfactory.

Results and Discussion

Genesis of the Legal Act

When trying to properly read and interpret the statutory norms, one should refer to its history, as well as the goals set by the authors. This especially applies to Mkhitar Gosh (Mxit'ar Goš) – the creator of the Datastanagirk' (referred as: DAT., T'OROSYAN 1975, BASTAMEANC' 1880). It is the oldest Armenian private legal code – or rather a legal treaty, or a sui generis commentary (BALZER 1910, THOMSON 2000). This work was created ca. 1184 A.D. The Mkhitar's code is based on biblical law, combined with the legacy of various cultural origin, including canonical regulations, traditional Armenian law, Roman and Muslim law, among others (THOMSON 2000).

The content of the HOLY SCRIPTURE constituted the basis for Mkhitar Gosh and his unknown successors, but it has allways been subject to their creative interpretation. Their modern viewpoint allowed them not only to describe and duplicate the divine law, but also to create new norms, called

praescriptio (THOMSON 2000). The thought accompanying Gosh during his works was to indicate the proper way and improve the human world by eradicating evil and sin. It is definitely not a strice legislative act, but rather a beacon, which had a significant impact on the sanctions present in his code (DZIKOWSKI 2016). The aim of the code was also to popularize the legal knowledge, which – according to the author – contains within one system both God's law and secular – civil and penal – law. Gosh's work was not intended as a universally binding law at first, but over time – after shortening and simplifying the arguments – it became firstly an unofficial source of law, and later on, at the turn of 13th and 14th century, the basis of the official Smpat's Code of the Lesser Armenia (KARST 1905). It was also used as the diaspora law.

Over the centuries, there has been a gradual, multifaceted and diverse – quantitatively, qualitatively, and territorially – evolution of the initial text. Among the many textual variants, the most independent and innovative path of legal thought-development seems to be the so-called the Lwów matrix (KUTRZEBA 1909, BALZER 1906). Although its text is not preserved, it is possible to interpret it from the Statute under discussion. It did not strictly correspond to any of the other known variations of the text. Within the matrix, the DATASTANAGIRK' and SYRO-ROMAN LAW (BRUNS and SACHAU 1880, BALZER 1906) were merged.

Apart from the Syro-Roman law, the influence of: Sachsenspiegel (ECKHARD 1955), German IUS MUNICIPALE, and local Polish law is also visible (BALZER 1906). This act is probably a multi-author work, formed from ca. 1280 A.D. until the 1460s, with slight additions in 1518–1519 (BALZER 1906, BALZER 1910).

The discussed Statute is an official, public, formal, Latin translation of the earlier Armenian-Tatar text (BALZER 1910). The reason for its creation was the controversy arising between the Armenian autonomous government and the Lwów authorities in 1518: judging in a mixed court was not possible due to the lack of knowledge of the Armenian-Tatar language, and therefore also legal norms, by the city authorities.

The partition of Armenia in 1080 A.D., and Mongol invasions in the 13th century resulted in the mass emigration of the Armenians from their homeland. Armenians arrived in Lwów as early as the beginning of the 13th century. They settled also in other towns, including: Bar, Brzeżany, Horodenka, Jazłowiec, Kamieniec Podolski, Kazimierz/Vistula, Kuty, Łysiec, Mohylów Podolski, Śniatyń, Stanisławów, Tyśmienica, and Zamość. The privileges of King Casimir the Great and his successors formed the legal, political, and religious basis for the existence of the Armenians in Poland. For centuries they created and still create an interesting element

of the multinational and multicultural makeup of Poland. This statute is the law of immigrants. They brought with them their own laws, customs, faith and language – and after settling in a new place, they were able to interact with other residents and creatively transform and develop their legal system.

The discussed act was in force not only in Lwów, but gradually also adopted in Kamieniec Podolski, Zamość and in other Armenian settlements in the Crown of the Kingdom of Poland. It was in fact a common and universal act of all the citizens of Armenian nationality living in the Polish-Lithuanian Commonwealth, which was identical with members of the Armenian Church, firstly independent from the papacy, and since the 17th century – the Armenian Catholic Church.

Elements of the Contract, Exertion of Legal Effects

To the essentialia negotii of the contract of sale, meaning the subject--essential provisions of the legal action, allowing to classify a contract to a given type and conditioning the nature of the legal relationship created, the parties define the subject of the benefits of the parties, hence: price and subject (which can be marked in relation to the identity of an animal, e.g. horse, ox, cow, or a swarm of bees, or a beehive containing a bee family and a precisely marked amount of honey).

It should be assumed that in relation to the analyzed statutory act, a principle is applicable, stating that legal actions evoke not only these legal effects explicite expressed by the parties, but also those resulting from the norms of universally-binding and customary law.

The analyzed norms refer to the cash sale of animals, do not set minimum or maximum prices, nor do they comment on the payment date, leaving a large margin of contractual freedom to the parties. The regulations do not expressly refer to any prohibition on introducing provisions in the form of accidentalia negotii by the parties to the contract of sale. A large margin of contractual freedom can be observed.

A special requirement was revealed regarding the form of legal action – the presence of witnesses (except for sales of cows).

The autumnal purchase of bees is a classic contract of sale. The purchase of an ox (chapter forty-nine) is an example of buying for a trial period for a plough or a cart – ad aratrum sive currum. A specific deadline for detecting defects – bad habits and being stolen, was established – quod talis bos non esset nocivae consuetudinis, nec furatus. The same can be found in DAT. ninety-five. Similarly, the existence of a trial period can be observed in the spring sale of bees (chapter fifty-one), and in DAT. ninety--six also for cows.

In other cases, the sale may be understood as an agreement of suspended effectiveness, or a conditional contract, or may appear as the conclusion of the contract only as a result of the lapse of the trial days, or as the conclusion of a rebuttable contract. As for horses (chapter forty-eight), bees in spring (chapter fifty-one) and cows (chapter fifty, differently from the parental DAT. ninety-six) the legal nature of the contract can be best reflected as defining it as a sale with a suspended effect.

The purchase of a cow is an example of conditional sale. The seller had to solemnly promise the buyer that the cow would be with calf every single year. The legal action has its effect only one year following the conclusion of the contract, provided that the cow at that time was pregnant. With regard to the oath, a term was used, derived from Roman law: sponsion (ZIMMERMANN 1996).

In case of the autumnal sale of a beehive with honey, the defect was detected at a stage preceding the conclusion of the contract – and, as in the case with cows, it was based on the seller's responsibility for his stipulation. The difference was that if the seller did not comply with the statutory obligations, the contract of the sale of bees has not been concluded at all.

The examined contracts characterize the suspension of their effects until the time of ineffective (no claims from the buyer or third parties) expiration of time limits for exercising the warranty rights (until the defect--detection deadline has elapsed). This was indicated by the repeated phrase: forum venditionis suum effectum sortiri debet – the contract of sale shall exert its effect.

Only the term contractus fori conclusus est – the contract is concluded, used by the authors of the statutory act in relation to the spring sale of bees (chapter fifty-one) clearly indicates that the contract was concluded definitively only after the expiry of the warranty claims' deadline – and this, however, can be equated with the end of the trial period described in chapter forty-nine.

Moreover, there is no concept of a contingent contract – called also pactum de contrahendo, pactum praeparatorium, or l'avant-contrat – observed, because there is no noticeable idea of a contract duality in which the parties to the preliminary contract undertake to conclude a final agreement and a final contract.

The basic requirements necessary for the validity of the contract of sale were: consistent declarations of the will of the parties to the contract, in relation to the subject of the contract, a certain price, the obligation to conclude the contract (called forum, forum emptionis), and the transfer of the possession in the presence of witnesses.

Witnesses were required by the norms of both the Statute and the Datastanagirk' as assistants in concluding and completing sales contracts. In the case of oxen – three witnesses of Armenian nationality (chapter forty-nine, and DAT. ninety-five), in the case of horses – two or three witnesses (chapter forty-eight, but in DAT. ninety-four – three witnesses). In the case of the spring sale of bees – two or three, present both at the conclusion of the contract and when transferring possession – in- and outgoings of bees. The requirement for the presence of witnesses in this case was decisive for the legal effectiveness of the contract previously concluded in front of them. Si exitus et introitus apum ita invenietur, sicut contractus fori conclusus est inter venditorem et emptorem, tunc huiusmodi forum debet suum effectum sortiri – the contract will exert its effect only if the in- and outgoings of bees take place in the same way as the conclusion of the contract. In the case of cows, however, witnesses were not required, according to the Lwów standards – differently in DAT. 96.

Their role, apart from assuring the validity of a legal action as they were necessary to conclude a contract and exert its effects, was to declare the absence of defects in the sold animals. It should be concluded that this was to take place after a simplified clinical veterinary examination, at least after seeing the animal in question. Another function they had was – although it is not explicitly regulated in the Statute – to play an evidential role in a possible future lawsuit (DZIKOWSKI 2016).

Defects

Among the defects (vitia) of animals sold, which give rise to liability on the part of the seller, two groups can be distinguished: physical and legal defects. Their appearance or detection in the time prescribed by law, gives rise to liability under the warranty. It is the seller's obligatory responsibility towards the buyer, depending only on the existence of statutory prerequisites, and not on the parties' will. The Statute recognizes also guarantee and stipulatory liability for sponsiones and a general clause of chapter eighty-two.

The physical defects established by the legislation of the Armenian Statute include, in the case of the sale of horses (chapter forty-eight), strictly defined diseases: inveterately lameness, long-term cough (equine asthma, recurrent laryngeal nerve paralysis/vocal fold paresis, recurrent airway obstruction/chronic obstructive pulmonary disease) and glanders (Burkholderia mallei (former Pseudomonas mallei) infection). It is worth noting that knowledge of these health disorders testifies to the development of medieval veterinary medicine. The detection of these enumerative diseases within seven days resulted in redhibition – return of the horse.

Chapter forty-eight de emptione equi (on the sale of a horse) has its source in the provisions of DAT. ninety-four, which states the defects of quadrupeds and not just horses, but in contrast to the original: it narrows the scope of normalization only to horses, so the regulation has been changed here from an abstract to a casuistic one.

While the catalogue of major (qualified) defects has been taken over with changes (apart from nightly blindness, vicious kicking, fear of and refusal to cross a bridge, so therefore not only stricte physical defects, but also mental ones), the minor defects (all others) are not distinguished in the Statute at all. Ravages associated by DAT. ninety-four with the occurrence of minor defects (redhibition in the seven-day period) was assigned in chapter forty-eight to the qualified defects. The annual deadline for the detection of a qualified defect was waived, and the buyer's right was reduced only to the possibility of returning the purchased animal, omitting the right to demand a price reduction (actio quanti minoris).

Responsibility for a legal defect (of a stolen quadruped) in DAT. ninety--four was based on the principle of redhibition: the rightful owner may take his animal, and the buyer should demand reimbursement from the seller. It is debatable whether the seller responded for his false statement. In chapter forty-eight of the discussed Statute, the institution of an eviction was introduced, meaning the defendant sued for the return of a thing, referred to someone else, from whom he bought the item. The Latin term "intercessio" and Polish "zachodztwo" were also in use for eviction (Bardach 1964). This was modelled on Western-European laws (Sachsenspiegel No. I.9, III.4.83, Ius Municipale No. 21.30.96, Statut warcki No. XII). The deadline for detecting the legal defect (unlimited term) remained unchanged.

It should be noted that in Polish law, the division of defects into major (qualified – wady glowne) and the others was present until 2014, while the main defects included, i.a. equine asthma, with a fifteen-day deadline to detect it (Rozporządzenie Ministra… DZ.U. 1966, nr 43, poz. 257, Ustawa z 30 maja 2014… DZ.U. 2020, poz. 287).

In the case of oxen sales (chapter forty-nine, buying for a trial) physical defects were: inveterate, existing and lasting for years, defects or harmful habits such as restiveness or mental defects, preventing the animal from working in a plough or a cart, detected in a seven-day period (the trial period). Chapter de vendito bove, is derived from DAT. ninety-five, which imposed on the seller the obligation to make a statement in the presence of three witnesses, that there were no specific qualified defects and that the animal had specific characteristics (to be tested). In addition, Gosh added that in the case of young males, when it was unknown what

their experience in working on a farm, at ploughing, at treshing floor, or as beasts of burden, pulling a cart is, they could be examined.

The paragraph discussed underwent a similar evolution as described in the case of horses: it does not differentiate defects into minor and major ones, and the term for redhibition was unified. In DAT. 95 inveterate lameness, long-standing cough, kicking, and nocturnal blindness were classified as major defects.

In the case of the theft of an oxen, the institution of evictio was in force at any time (in spite of redhibition in an unrestricted period of time, according to DAT. ninety-five).

Chapter fifty on the sale of cows has its genesis in DAT. ninety-six. In the Polish-Armenian text, contrary to Mkhitar's version, there is neither a requirement of witnesses, nor the possibility of demanding a reduction in the price of a purchased cow (actio quanti minoris), nor any liability for minor (other than lack of pregnancy) defects, within seven days.

In the case of cows, a defect was a lack of calving within one year (uno integro anno) from the conclusion of the contract, if the seller swore (sponsio) that the cow had been, and would be, with calf annually. In the latter case, the term sponsio, deriving from Roman law and meaning solemn oath was used (ZIMMERMANN 1996). The basis of the seller's liability in this case was not the cow's fault itself, manifested in infertility of various types, but the oath of assurance. It is therefore not a classic warranty responsibility, but one based on the principle of actio ex stipulatu.

Chapter fifty-one treats bees and is in compliance with the provisions of DAT. ninety-seven, developing its norms, with the exception of the buyer's loss foreseen by Gosh. This chapter contains essentially two legal norms that require separate discussion.

The autumnal sale of bees in a beehive with honey should take place according to the weight of the honey indicated by the seller's sponsio: certa mensura et expressa quantitas – specified measure and expressed quantity. A buyer who disagreed with the aforementioned assurances could have, in the framework of the preceding and preparatory steps to conclude the contract, demanded opening the hive and weighing its contents – alvearium aperire et mel mensurare. If it was revealed that is not enough honey – less than it was stipulated, the seller should supplement this difference with honey or cash to the previously determined weight (he has ex lege debt towards the buyer). Failure to fulfil this provision resulted in the failure to conclude the contract. If, on the other hand, there was more honey than the amount stipulated, the buyer acquired the surplus free of charge, according to the principle that he bought in the hope of profit, not loss (spe lucri, non damni). The discussed regulation is a development of Gosh's rules and continues his legal thought.

In the case of the sale of bees in the spring, the disadvantages resulting in the redhibition were: rejuvenation of bees, death of the queen bee, as well as other cases of harmful disorders (aliquid nocivi – anything harmful, so an open catalogue), within ten or twenty days of testing (unspecified term) from the date of purchase. DAT. ninety-seven on the other hand, treats the mere assumption of the queen bee's death or leprosy as defects. One must however take into account the low level of development of contemporary veterinary science and the fact that it was difficult to be ascertained.

Both DAT. ninety-seven and chapter fifty-one of the Statute clearly underline that after a period of ten or twenty days the responsibility is transferred to the buyer. It should be noted that, despite the lack of specified deadline, the part referring to bees contains the clearest civilian regulations. The legislator clearly states that until the deadline the liability for the latent defects is borne by the seller and the buyer can return the property, but after the deadline the liability is transferred to the buyer.

Once again, it should be emphasized that the presence of two or three witnesses was in this case a condicio sine qua non of the conclusion of the contract and transfer of ownership and possession. In DAT. ninety-seven the number of witnesses was not specified.

Apart from these casuistic rules, both DAT. one hundred eighty-five, and chapter eighty-two, bring the general rule of seller's liability for false, fraudulent and malicious statements, aimed at misleading the customer to buy a defective item.

Chapter eighty-two provides for miscellaneous things, but especially for animals like cattle, horses, or gregarious animals, a general and abstract liability for all fraudulent seller's statements. If the vendor knew of any defect and, despite his knowledge, mala fide claimed that his items were good, and he boasted of or praised false quality, he was fully liable for his statement. Such an animal should be returned to him and he would be criminally punished by the jury. This is a specific type of responsibility balancing between ancient Roman liability for Aedilician stipulations and for dicta promissave. From the Aedilician law: an evident responsibility for dolus in contrahendo, obligatory character and easy execution were adopted. Extension of liability to all cases of latent defects were modelled on the responsibility for dicta et promissa (NICHOLAS 1959, ZIMMERMANN 1996, MONIER 1930, MANNA 1994, IMPALLOMENI 1955). What distinguishes the STATUTE from the Roman dicta et promissa is: narrowing the liability to only the malicious intent of the seller and his knowledge in the Lwów text.

The original norm is more compound than the analyzed one. Such behaviour was punished with an anathema, as stated in DAT. one hundred

eighty-five (THOMSON 2000). Exculpation from this canonical penalty involved declaring all defects while selling an animal. The defective item (e.g. a bull butting or having a blemish in its body or behaviour, a stubborn and skittish donkey, in situations such as defect detection by the buyer, the seller's boasting, or witnesses to his fraudulent sale) should be returned – sold back to the seller, unless the purchaser finds it accurate – then there is a place for actio quanti minoris. If the purchaser has already sold it bona fide, there should be a penance to him.

Humanitarian views of Mkhitar Gosh are also present in DAT. two hundred twenty-one in fine, according to which stubborn, kicking, goring or biting animals, which had killed a man, were to be sold only to people who knew about these incidents and could tame dangerous animal behaviour.

Conclusion

It should be noted that all the mentioned physical defects, such as infertility (lack of a bee queen, lack of pregnancy in cow), physical diseases, bee rejuvenation or mental incapacity to work, caused the inability to achieve the intended purpose of the contract, i.e., the proper economic use of animals. Distinction of these diseases is also a testimony to the veterinary achievements of the day.

The Statute consists a separate, innovative, and previously undescribed way of the Roman law reception in Europe. The primary type of seller's liability in the Statute is the warranty in form of the actio redhibitoria, derived from the Roman law (*Digesta seu Pandectae*…, 1872, 21.1, MANNA 1994): the right of the buyer to return the goods to the seller in the above-mentioned statutory terms. Actio ex stipulatu (responsibility for solemn oaths) and for a guarantee is also present in the legal text under consideration. The general clause of chapter eighty-two is also highly innovative, applicable to all kinds of items sold, enforcing the liability for false, fraudulent vendors' statements.

The norms of the Armenian Statute establish only one legal defect: the sale of goods by a non-owner – the sale of stolen animals. Although this is not explicitly expressed in the legal text under consideration, it should be interpreted that the concept of entering into the possession of any thing (including animals) contrary to legal norms (by theft), did not result in the purchase of ownership of this thing, was present in the law of the Polish Armenians. This applied to proprietors both in bad and good faith. Ownership remained with the original legal owner. According to the paremy nemo plus iuris in alium transferre potest, quam ipse habet (one cannot

transfer more rights than he has), the proprietor aiming to conclude a contract of sale was able to transfer only possession, and never the ownership of the animal. Norms of the DATASTANAGIRK' allowed for the return of the stolen things without being enslaved by specific dates.

The elements of civil and criminal material law, as well as procedural law, are mixed in the discussed legal code. Bearing in mind Roman legal tradition, Lwów Armenian Statute is a very modern and innovative legal act of its time. During the Middle Ages, Roman Aedilician responsibility for animal defects (*Digesta seu Pandectae*... 1872, 21.1) lost its popularity and nearly went out of use in Western Europe. Instead, the significance of the buyer's attention was emphasized. One should be cautious, attentive, wachtful, and heedful – as in the proverb: Augen auf, Kauf ist Kauf (be careful and keep your eyes wide open as you buy anything), or according to the English idea of caveat emptor (BURKE 1967). Apart from that, administrative control of trade was enforced by guilds, the Hanseatic League, and town authorities (ADAMCZUK 2008). The concept of major and minor defects, present in the discussed Statute has also been present in European legal systems, e.g. in Germany.

This seemed to change only after the German process of reception of the Roman law of warranty in the following centuries, but the adopted law was in force for all things – apart from animals, to which it was originally created by the curule Aediles in Rome. These creatures were subject to leges speciales (casuistic, dividing defects into categories, shaped according to the German legal model) till the modern era. Examples of such special regulations of animal minor and major defects were the original texts of the Polish and German civilian regulations (Bürgerliches… RGBL. 1896, No. 21, S. 195, §§ 459 sqq. BGB, Kaiserliches Verordnung … RGBL. 1899, No. 13, S. 219–220, DZ.U. 2019, poz. 1145, 1495, Rozporządzenie Ministra… DZ.U. 1966, nr 43, poz. 257).

It should be concluded that, after some editorial changes, the discussed norms could be present even in modern European codifications. The Armenian Statute presents the same level of legal development as statutes formed after hundreds and hundreds of years of Western juridical thought. Both the STATUTE and DATASTANAGIRK' mix in a clear and coherent way the Roman Aedilician actions and theory of minor and major defects of animal health, but the STATUTE's novelty can be seen in a gradual departure from the differentiation of defects.

The lawmakers of the Lwów Armenian diaspora shortened and simplified the writing of Mkhitar Gosh. In some cases, radically different legal solutions have been adopted – combining many legal concepts in an original way, derived from many sources, and creating their own, innovative

normative ideas. They also used their veterinary knowledge for the practical purposes of the market trade.

They were immigrants who, preserving their own traditions, customs and laws, appreciated and incorporated into their legal order both the reciped Roman law, and the achievements of contemporary European legal culture.

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