# Production performance of pigs reared in different systems and fed increased energy content diets with or without green alfalfa

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## Abstract

The objective of this study was to determine the effects of various feeding and housing systems on fattening performance, slaughter value and biochemical serum parameters in growing-finishing pigs. The experimental material comprised 90 growing-finishing pigs, divided into six groups of 15 animals that were diverse in terms of rearing (with or without free access to outdoor runs) and feeding systems (fed increased metabolizable energy (ME) content diets with or without green alfalfa). Different feeding regimes and rearing systems had no significant effects on most fattening results and the carcass traits of the pigs except for daily water and diet intake and feed/gain ratio. Daily water intake was lower in pigs fed complete diets plus green alfalfa forage. Low-density lipoprotein (LDL) cholesterol concentrations were statistically lower in the blood serum of Groups 3 and 6 than in Groups 2 and 4. High-density lipoprotein (HDL) cholesterol levels were significantly higher in the serum of Group 6, compared with other groups. Group 3 had significantly higher triglycerides levels compared with Groups 1, 2, 4, 5 and 6. Taking into consideration all these dependencies, it could be assumed that feeding regime and rearing with access to outdoor runs might improve not only blood lipid profile, but also the dietetic value of pork. It seems that feeding a complete diet with increased ME, plus green alfalfa forage and rearing indoors with free access to outdoor runs, gave the best results.

**Keywords:** Carcass quality, finishing system, lipids, pig nutrition, production system <sup>#</sup> Corresponding author: kozwoj@uwm.edu.pl

# Introduction

In recent years the dramatic shift in availability of feed ingredients for the pig industry has meant that a wider range of feedstuffs must be considered in formulating pig diets, including those that contain considerable amounts of fibre. Because energy is the single most expensive component of a pig diet, optimizing its utilization is critical for efficient pork production (Velayudhan *et al.*, 2015).

Numerous studies worldwide have attempted to compare the growth performances of pigs kept indoors with outdoor production systems, but their results have been inconclusive and, in many cases, contradictory (Lebret, 2008; Lebret *et al.*, 2014). In a study by Gentry *et al.* (2002), pigs reared outdoors were characterized by higher growth rates than pigs born and finished in an indoor environment. Similar trends were noted by Stern *et al.* (2003) and Millet *et al.* (2005). In another experiment (Gentry *et al.*, 2004), pigs reared outdoors were heavier and had higher gain/feed ratios. Different results were reported by Hoffman *et al.* (2003) and Enfält *et al.* (1997), who noted higher daily gains in pigs kept indoors. According to other authors (Sather *et al.*, 1997; Stern *et al.*, 2003; Lebret, 2008; Lebret *et al.*, 2014), free-range pigs needed a longer period to reach the desired market weight, and used their feed less efficiently.

These results suggest that the effects of diets that differ in energy concentration should be analysed, because pigs reared indoors and those raised outdoors (with access to open-air runs or pasture) differ in their protein and energy requirements because free-range pigs use more energy owing to more extensive physical activities and the need to maintain body temperature during cold periods. There are still divergences in the results of many studies about the influence of rearing conditions and feeding regime on carcass traits and quality of pig meat (Enfält *et al.*, 1997; Klont *et al.*, 2001; Millet *et al.*, 2005; Jordan *et al.*, 2008; Lebret, 2008; Karpiesiuk *et al.*, 2013; Lebret *et al.*, 2014).

Taking these aspects into account, it is worth remembering that there has been increasing interest in alternative pig feeding and housing systems in recent years. Farming practices of environmental enrichment

are methods of improving animal welfare and increasing disease resistance (Klont *et al.*, 2001; Gentry *et al.*, 2002; 2004) as well as supporting the production of organic, health-promoting foods with high nutritional value, in response to growing consumer demands (Kozera, 2007).

The objective of this study was to determine the effects of various feeding systems (addition of green alfalfa forage, increased metabolizable energy content) and housing (indoors or indoors with free access to outdoor runs) on fattening performance, slaughter value and biochemical serum parameters in growing-finishing pigs.

## **Materials and Methods**

The experimental material comprised 90 growing-finishing pigs produced by mating (Polish Large White x Polish Landrace) sows with (Duroc x Pietrain) boars. The pigs were randomly allocated to six experimental groups of 15 animals (5 pigs/pen) based on their initial bodyweights, sex (7 barrows and 8 gilts/group) and origin of litter (piglets from 24 litters): Group 1 was fed a complete diet and kept indoors with free access to outdoor runs; Group 2 was fed a complete diet and was kept indoors; Group 3 was fed a complete diet with an increased ME content and was kept indoors with free access to outdoor runs; Group 4 was fed a complete diet plus green alfalfa forage, and was kept indoors with free access to outdoor runs; Group 5 was fed a complete diet plus green alfalfa forage, and was kept indoors and Group 6 was fed a complete diet with increased ME content, plus green alfalfa forage, and was kept indoors with free access to outdoor runs; Group 5 was fed a complete diet plus green alfalfa forage, and was kept indoors and Group 6 was fed a complete diet with increased ME content, plus green alfalfa forage, and was kept indoors with free access to outdoor runs. Pigs of all experimental groups were located in the same location and were kept in pens (3 m x 3 m) without litter. The experiment was conducted from May to August, that is in summer.

Animals of Groups 1, 2, 4 and 5 received complete control cereal-soybean diets (Table 1), formulated in accordance with Pig Nutrient requirements (1993) for one-phase feeding (from 30 kg to 110 kg Rbodyweight), offered ad libitum from automatic feeders. Pigs of Groups 3 and 6 were fed the complete experimental diets in which the concentration of ME was increased by 10% (by adding rapeseed oil) relative to the control diet used in pigs feeding in other groups (i.e. Groups 1, 2, 4 and 5). Pigs in Groups 4, 5 and 6 were fed additional green alfalfa forage (3 kg per pen), offered once a day at 08:00 in stoneware troughs.

The experiment started when the pigs reached an average initial bodyweight of 28.5 kg, and was carried out for 122 days to the average final bodyweight of 118.5 kg. Feed intake, green alfalfa forage consumption and water intake from nipple drinkers were monitored throughout the experiment. The chemical composition of experimental diets and green alfalfa forage was determined by AOAC (2006) methods. In order to determine the effects of feeding regimes and housing systems on the lipid profile, blood was sampled from all the pigs (from *vena cava cranialis*) a week before expected slaughter. The serum levels of total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides and cortisol were determined. All analyses were performed with the Cobas Integra 800 analyzer (protein was determined by the biuret assay, urea by the kinetic test with urease and glutamate dehydrogenase, total cholesterol and triglycerides by enzymatic-colorimetric methods, HDL cholesterol by an enzymatic colorimetric test. LDL cholesterol levels were calculated based on Friedewald formula (Friedewald *et al.* 1972).

Specification	Complete diets							
Specification	Control (Groups 1, 2, 4 and 5)	Experimental (with increased ME level) (Groups 3 and 6)						
Ground wheat	40.00	40.00						
Ground barley	44.88	39.88						
Soybean meal	12.00	13.00						
Rapeseed oil	-	4.00						
Premix*	3.00	3.00						
L-lysine	0.12	0.12						

Table 1 Composition of experimental diets

\*Composition of premix: 341600 j.m.vitamin A; 54 165 j.m.vitamin D<sub>3</sub>; 2166 mg vitamin E; 75 mg vitamin K; 37.5 mg vitamin B<sub>1</sub>; 116.6 mg vitamin B<sub>2</sub>; 66.6 mg vitamin B<sub>6</sub>; 0.58 mg vitamin B<sub>12</sub>: 600 mg vitamin B<sub>3</sub>; 275.8 mg pantothenic acid; 10 mg folic acid; 10 000 mg choline chloride; 1.33 mg vitamin H; 3000 mg Fe; 700 mg Cu; 3666 mg Zn; 2000 mg Mn; 5.33 mg Se; 13.3 mg I; 8.33 mg Co; 5 mg Mg; 33.3 g Na; 40 g P; 226 g Ca; 61.66 g lysine; 8.33 g methionine; 9.16 g threonine. ME: metabolizable energy.

Slaughter, post-slaughter processing and carcass evaluation were carried out in accordance with meat industry regulations. Lean meat content was determined on suspended hot right half-carcasses, using the CGM (Capteur Gras/Maigre) apparatus by Sydel, operated by authorized and trained personnel. CGM is a hand-held device equipped with an optical probe that determines the thickness of the loin muscle and the fat layer by measuring the light reflected in the probe. The device determines the lean meat content of the carcass, that is, the ratio of the total mass of the striated muscles to the mass of the carcass:

 $LMC_{CGM} = 59.42 + 0.1322M_2 - 0.6275T_2$ 

 $T_2$  is the thickness of the backfat between the 3rd and 4th ribs, 6 cm from the line of carcass partition;  $M_2$  is the thickness of the *longissimus dorsi* muscle, 6 cm from the line of carcass partition. It is measured no longer than 45 minutes after the animal was stunned.

The pH was measured in the *longissimus lumborum* (LL) 45 min after bleeding (pH<sub>45</sub>) and after 24 hours of carcass chilling (pH<sub>24</sub>). The parameters, pH<sub>45</sub> and pH24, were determined with the WTW 3310 pH meter and combination electrode (WTW-Wissenschaftlich-Technische Werkstaetten GmbH, Weilheim, Germany) and calibrated with the same standard solutions of pH 4.01 and 7.00 at 20 °C. Additionally, their accordance was tested with meat samples at the beginning of and regularly during the measuring period.

On cold carcasses, backfat thickness (BFT) (means of five measurements) was measured at five points with callipers: At the thickest point over the shoulder, on the back behind the last rib, over the cranial border of *m. gluteus medius* (loin I), over the centre of *m. gluteus medius* (loin II), over the caudal border of *m. gluteus medius* (loin II).

All parameters were processed statistically, and the significance of differences between means in groups was determined by two-way analysis of variance (ANOVA) and the Duncan's test. A two-way analysis was carried out in order to assess the significance of different feeding and rearing systems and was performed using the general lineal model (GLM) procedure of StatSoft software (version Statistica PL 12.5). The model of analysis was:

$$Y_{ijk} = \mu + FT_i + RS_j + (FT \times RS)_{ij} + \varepsilon_{ijk}$$

where FT<sub>i</sub> is the feeding type (I = 1,2,3), RS<sub>j</sub> the rearing systems (j = 1,2), (FT x RS)<sub>ij</sub> the interaction between the feeding and rearing effects and  $\varepsilon_{ijk}$  is the residual error.

The experiment was approved by the local Ethics Committee for Animal Experimentation at Olsztyn.

### **Results and Discussion**

Table 2 presents the chemical composition of experimental diets. The total protein content of the control and experimental diets was 165.6 and 163.0 g/kg, respectively, which meets the protein level specified for a one-phase feeding of growing-finishing pigs in the Pig Nutrient Requirements (1993). The ME content of diets, calculated from tabular data, was consistent with the methodological assumptions.

 Table 2 Chemical composition of experimental diets and green alfalfa forage

	Comple	Green alfalfa forag			
Specification	control (Groups 1, 2, 4, 5)	experimental (Groups 3, 6)	(Groups 4, 5, 6)		
Dry matter	88.83	89.26	18.40		
Crude protein	16.56	16.30	3.55		
Crude fat	1.65	5.27	0.39		
Crude fibre	4.41	4.08	5.52		
Crude ash	4.11	3.77	1.90		
ME <sup>1</sup> MJ/kg (calculated)	12.72	13.84	8.80		
N-free extractives	62.10	59.84	7.04		
Organic matter	84.72	85.49	16.50		

<sup>1</sup> ME: metabolizable energy, calculated values (Pig Nutrient Requirements, 1993).

Daily feed intake per pig was different between groups, ranging from 2.15 kg in Group 2 to 2.40 kg in Group 4. Significant differences were observed between all groups (Table 3). Average feed/gain ratio (i.e. kg of complete diet/kg gain) was highly satisfactory, at 2.98, 2.93, 3.25, 3.19, 3.04 and 2.96 kg in groups 1 - 6, respectively, whereas green alfalfa forage intake per kg bodyweight gain was 1.59 kg in Group 4, 1.68 kg in Group 5 and 1.67 kg in Group 6. Daily water intake was lower in pigs fed complete diets with an addition of green alfalfa forage, compared with animals fed complete diets (Table 3). Higher water intake was noted in Groups 1, 2 and 3, at 5.14, 5.48 and 4.95 L/head/day, respectively. In Groups 4, 5 and 6, fed green alfalfa forage as additional feed, water intake was 4.76, 4.43 and 4.34 L/head/day, respectively. Differences between groups were statistically different ( $P \le 0.05$ , 0.01).

 Table 3 Average daily feed and water intake and feed/gain ratio of experimental pigs

Specification	Group (feeding/rearing)						0 EM	Significance level	
Specification	1 2 3 4 5 6		6	SEM	Rearing	Feeding			
Daily intake of diets (kg of mixture/pig)	2.21 <sup>B</sup>	2.15 <sup>BD</sup>	2.31 <sup>Cb</sup>	2.40 <sup>Aa</sup>	2.30 <sup>B</sup>	2.27 <sup>B</sup>	0.021	NS	*
Daily intake of alfalfa (kg/pig)	-	-	-	0.47	0.43	0.46	0.010	NS	NS
Daily water intake (L/pig)	5.14 <sup>BCa</sup>	5.48 <sup>A</sup>	$4.95^{\text{BbE}}$	4.76 <sup>BDF</sup>	$4.43^{\text{BDF}}$	$4.34^{\text{BDF}}$	0.097	NS	**
Feed/grain ratio (kg of mixture /kg gain)	2.98 <sup>B</sup>	2.93 <sup>Bb</sup>	3.25 <sup>A</sup>	3.19 <sup>A</sup>	3.04 <sup>Ba</sup>	2.96 <sup>B</sup>	0.031	NS	*
Feed/gain ratio (kg alfalfa/kg gain)	-	-	-	1.59	1.68	1.67	0.021	NS	NS

Means within rows followed by different letters or \* are different in the Duncan test: \* a b, c d:  $P \le 0.05$ ; \*\* A B, C D, E F:  $P \le 0.01$ ; NS: not significant.

Water intake was lower in pigs fed alfalfa (Groups 4, 5 and 6) as green forage provides some of the daily water requirements. These data corroborate the findings of Karpiesiuk & Falkowski (2008), who noted water intakes of 4.82 L to 6.36 L in groups that had no access to green forage and 3.39 L to 4.48 L in groups fed green alfalfa as an additional feed. Gadd (2005) reported that water requirements of pigs with different body weights (BW) were 15 - 40 kg BW: 2.25 L/day/head; 40 - 60 kg BW: 5.00 L/day/head; 60 kg BW and above: 6.00 L/day/head. According to the guidelines of the nutrient requirements of swine (NRC, 2012), the water requirements of growing pigs fed ad libitum are *ca.* 2 L per kg feed.

The average values of pig serum biochemical parameters of pigs are shown in Table 4. No significant differences in total cholesterol concentrations were found between groups, and the average level of this compound ranged from 2.24 mmol/L (Group 6) to 2.04 mmol/L (Group 4).

The feeding regimes and housing systems contributed to highly significant and significant differences in HDL cholesterol, LDL cholesterol and triglycerides concentrations among groups. The level of HDL cholesterol was highest in pigs reared outdoors and fed the complete experimental diet (with increased ME level) plus green alfalfa forage (1.25 mmol/L). It was confirmed statistically on a high significant level ( $P \le 0.01$ ) compared with Groups 1, 2, 4 and 5 and on a significant level ( $P \le 0.05$ ) in Group 3 (1.11 mmol/L). At the same time, the lowest level of LDL cholesterol was noted in Group 3 (0.77 mmol/L) and this value was statistically differentiated from Groups 2 ( $P \le 0.01$ ) and 4 ( $P \le 0.05$ ). Group 3 had significantly higher triglycerides levels (0.65 mmL/L), compared with Groups 1, 2, 4, 5 and 6, where these values were noted, namely 0.35, 0.33, 0.26, 0.33 and 0.43 mmL/L, respectively. Significant differences were observed between Groups 6 (0.43 mmL/L) and 4 (0.26 mmL/L). There were no statistically confirmed differences in cortisol level between animals of experimental groups.

Blood cholesterol levels are determined by genetic factors and environmental conditions, including nutrition (Hanczakowski *et al.*, 2009). In the present study, total cholesterol concentrations were slightly above the reference range (Winnicka, 2011) in Groups 2, 3 and 6 (2.19 mmol/L, 2.14 mmol/L and 2.24 mmol/L, respectively). Migdał *et al.* (1999) reported a rising tendency in concentrations of lipids and cholesterol in the serum (including LDL) of meat-type crossbred pigs. Differences between groups, confirmed by statistical analysis, were noted for HDL and LDL cholesterol concentrations. According to Winnicka (2011), levels of HDL cholesterol in pig serum should account for at least 40% of total cholesterol concentrations, since a decrease in the HDL fraction below this value is undesirable. In the current study, the

Specification	Group (feeding/rearing)						054	Significance level	
	1	2	3	4	5	6	SEM	Rearing	Feeding
Total cholesterol (mmL/L)	2.05	2.19	2.14	2.04	2.07	2.24	0.026	NS	*
HDL cholesterol (mmL/L)	1.01 <sup>B</sup>	1.05 <sup>B</sup>	1.11 <sup>b</sup>	1.00 <sup>B</sup>	1.01 <sup>B</sup>	1.25 <sup>Aa</sup>	0.020	NS	**
LDL cholesterol (mmL/L)	0.89	1.01 <sup>Aa</sup>	$0.77^{Bd}$	0.94 <sup>c</sup>	0.93	0.81 <sup>b</sup>	0.023	*	*
Triglycerides (mmL/L)	0.35 <sup>B</sup>	0.33 <sup>B</sup>	0.65 <sup>A</sup>	0.26 <sup>Bb</sup>	0.33 <sup>B</sup>	0.43 <sup>Ba</sup>	0.024	*	**
Cortisol (µg/dL)	5.08	5.64	4.31	4.04	6.03	5.77	0.330	NS	NS

**Table 4** Serum biochemical parameters of experimental pigs

Means within rows followed by different letters or \* are different by the Duncan test: \* a b, c d:  $P \leq 0.05$ .

\*\* A B:  $P \leq 0.01$ ; NS: not significant.

HDL fraction had a 47% to 55% share of total cholesterol.

In the blood serum of animals fed a complete diet supplemented with rapeseed oil (Groups 3 and 6), which contains a relatively high level of unsaturated fatty acids (PUFA/MUFA), a significant increase in the levels of HDL cholesterol and a decrease in the levels of LDL cholesterol were confirmed, compared with other the groups. The differences between the groups were statistically significant ( $P \le 0.05$ ) or highly significant ( $P \le 0.01$ ). Obtained dependencies confirmed the theory that PUFA/MUFA in animal food has an influence on the lipid profile of animals' blood serum (i.e. an increase in HDL and decrease in LDL cholesterol levels precisely) (Froyland et al., 1996; Barowicz & Pietras, 1998). In addition, in the blood serum of the animals in Group 6, a statistically significant and higher level of HDL cholesterol was recorded compared with pigs of Group 3. This could be a positive result of the addition of green alfalfa as a forage feed in Group 3. It could be explained that a higher crude fibre level in the diet influences a growing level of HDL cholesterol. At the same time it induces a reduction in the LDL cholesterol level in blood serum. Such a dependence was noted in earlier studies (Kishimoto et al., 1995; Kozera et al., 2006; Hanczakowski et al., 2009). Taking into consideration all these dependencies (i.e. HDL and LDL levels), it could be assumed that feeding regime (diet with increased ME content from rapeseed oil supplementation and green alfalfa forage addition) and rearing conditions (with access to outdoor runs) might improve the dietetic value of meat. Demigne et al. (1995) stated that there is dependence between a cholesterol production in animals` livers and the dietetic value of pork.

With regard to triglycerides, Group 3 had significantly higher triglyceride levels compared with all other groups. In addition, significant differences were observed between Groups 6 and 4 for this parameter. It could be concluded that feeding growing-finishing pigs a diet with increased ME content had a significant influence on the serum triglycerides level. However, animals fed in this way, but with green alfalfa as additional forage (Group 6), had a significantly lower level of serum triglycerides compared with Group 4. This is in agreement with earlier investigations (Kozera *et al.*, 2006), which proved that increased crude fibre levels in experimental diets resulted in an increase in triglycerides.

Serum cortisol is widely used as an indicator of stress (Choe *et al.*, 2014). In the present study, the levels of this hormone were similar and not differentiated among groups. Nevertheless, there was a tendency to lower the level of cortisol serum in pigs reared with free access to outdoor runs compared with those reared indoors. It could be understood as a determinant of better welfare.

Animals of all experimental groups were characterized by similar initial bodyweights, which ranged from 28.1 kg in Group 6 to 28.9 kg in Group 3 (Table 5). At slaughter, pigs from Group 6 were heaviest (121.8 kg), while the Group 3 animals were lightest (115.4 kg). Higher daily gains were noted in Groups 4 (751 g) and 6 (769 g), and the lowest in Group 3 (709 g). Carcass quality parameters are presented in Table 5. Carcass dressing percentage was similar in all the groups, and all carcasses had a high average lean meat content, which was the highest in pigs reared indoors and fed complete diets plus green alfalfa forage, and lowest in pigs kept indoors and fed complete diets without green alfalfa forage as additional feed, but these differences were not confirmed statistically. No significant differences in loin eye area of LM, backfat thickness (BFT), the levels of  $pH_{45}$  and  $pH_{24}$  were found between the groups.

The growth rates of experimental pigs were similar to those reported by other authors. In a study by Høøk-Presto *et al.* (2009), the average daily gain of growing-finishing pigs was 740 g. Karpiesiuk & Falkowski (2008) obtained daily gains of growing-finishing pigs on a similar level to the present study, that is, on average 727 g.

Regarding slaughter traits, it could be concluded that types of feeding and rearing of growing-finishing pigs did not affect values of carcass traits between groups significantly. Strudsholm & Hermansen (2005), working with crossbred animals [(Large White x Landrace) x Duroc], reported that pigs kept in confined system had a lower content of lean meat (on average 2.3%) and higher backfat thickness (on average 1.1 mm), compared with growing-finishing pigs reared in an outdoor system. In the results of studies by Parunović *et al.* (2012), the carcass weight and cold carcass yield percentage were lower in the free-range reared pigs than in those fed and reared conventionally. According to the authors, this could be explained by a type of feeding regime. That is, intake of grass fibre led to better development of the digestive system (mainly the large intestine). Finally, it must be stressed that according to meat classification based on pH<sub>24</sub> (5.44 - 5.52), meat from the experimental pigs in the current study could be considered normal and was characterized as good quality.

Specification	Group (feeding/rearing)					огм	Significance level		
	1	2	3	4	5	6	SEM	Rearing	Feeding
Initial bodyweight (kg)	28.6	28.4	28.9	28.5	28.5	28.1	0.293	NS	NS
Final bodyweight (kg)	118.9	118.1	115.4	120.2	116.7	121.8	1.166	NS	NS
Average daily gain (g)	741	734	709	751	725	769	9.276	NS	NS
Carcass weight (kg)	97.8	95.2	89.7	96.4	95.5	98.8	1.280	NS	NS
Dressing percentage (%)	78.7	79.2	78.5	79.9	79.1	79.8	0.285	NS	NS
Lean meat content (%)	55.6	54.2	55.1	55.6	56.0	54.5	0.379	NS	NS
Loin eye area cm <sup>2</sup> )	62.4	58.9	56.1	63.7	59.7	65.3	1.058	NS	NS
Backfat thickness (mm)	24.1	24.1	23.2	24.6	22.1	25.5	0.532	NS	NS
pH <sub>45</sub>	6.48	6.62	6.54	6.57	6.52	6.56	0.029	NS	NS
pH <sub>24</sub>	5.49	5.49	5.44	5.50	5.49	5.52	0.021	NS	NS

Table 5 Fattening performance and slaughter value of experimental pigs

NS: not significant.

### Conclusion

Different feeding regimes and housing systems had no significant effect on the growth rate of pigs. The production results were highly satisfactory in all experimental groups. The addition of alfalfa green forage and/or supplementing the experimental diets with rapeseed oil resulted in increasing of the HDL cholesterol levels in the blood serum and decrease in the levels of LDL cholesterol in the blood, but had no significant influence on the blood level of total cholesterol. Cortisol levels in the serum were comparable in all groups (however, a little lower in pigs reared with access to outdoor runs), which indicates that pigs were provided with adequate nutrition and housing in all analysed systems. At the same time, this is a sign of good welfare status of growing-finishing pigs of all investigated groups. It seems that feeding a complete diet with increased ME content plus green alfalfa forage and being reared indoors with free access to outdoor runs gave the best results.

#### Authors' contributions

WJK and JF coordinated the project design and implementation. KK was in charge of sample collecting and with DB and WM was responsible for laboratory tests. All co-authors participated in results, statistics and interpretation. WJK was in charge of writing the manuscript.

#### **Conflict of interest declaration**

We wish to confirm that there are no known conflicts of interest associated with the publication of this manuscript and there has been no significant financial support for this work that could have influenced its outcome. We also confirm that this manuscript has been read and approved by all authors and that the order of authors listed in the manuscript has been approved by all of us.

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