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Collaborative effects of *Spirulina platensis* supplementation and weaning time on productive performance and immune status of suckling buffalo calves

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ABSTRACT

Background: The productive performance and immune status of calves during the pre-weaning phase significantly impact their lifelong productivity. *Spirulina platensis* (SP) supplementation in suckling calf's starter enhanced immune function, health status and growth performance. The goal of this study was to investigate the productive performance and some immune parameters of suckling buffalo calves associated with *Spirulina platensis* (SP) as a feed additive in early-weaned (EW) and late-weaned (LW) calves.
Methods: Twenty-four newborn buffalo calves with an average live body weight (LBW) of 31.15±1.03 kg were randomly allocated to six groups (four calves each) in a 2× 3 factorial design for weaning time and SP treatments as follows: G1, G2 and G3 were EW and received SP at 0.0, 0.01 and 0.02% of LBW, respectively and given milk at level of 12.5% of LBW until the 5th week. Then the milk intake (MI) was reduced weekly (25, 50, 75%) to achieve early weaning at 8th week, then the SP was added to drinking water from 9th to 12th weeks in EW calves. While, G4, G5 and G6 were LW and received the same SP levels, with milk adjusted weekly at level of 12.5% of LBW until 6th week, then reduced biweekly (25, 50, 75%) to achieve late weaning at 12th week. The parameters of productive performance were evaluated in all groups. Heparinized blood samples were obtained at the 12th week of age to assess the phagocytic activity of peripheral blood mononuclear cells (PBMCs) and to determine the expression of IFN-γ and IL-2 mRNA. Serum samples were collected every four weeks to evaluate nitric oxide (NO) and lysozyme levels.

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Results: Late weaning induced significantly (P<0.05) higher LBW, total weight gain (TWG), average daily gain (ADG), MI and feed conversion ratio (FCR) compared to early weaning. However, early weaning showed significantly (P<0.05) higher calf starter (CS) and berseem hay (BH) intake and economic efficiency than those of late weaning. SP additive induced significant increases (P<0.05) in LBW, TWG, ADG, feed intake, FCR and economic efficiency. The interaction between weaning period and SP additive revealed significant (P< 0.05) differences among the different groups with the highest values of LBW, TWG, ADG, MI, gross energy (GE), dry matter (DM) and crude protein (CP) per kg weight gain (FCR) in G6 and the lowest values in G1. Average daily intake of CS and BH were highest in G3 and lowest in G4. The intake of DM, GE and CP were highest in G6 and lowest in G1 during the period 0-8th weeks, and highest in G3 and lowest in G4 during the periods of 8th to 12th and 0 to12th weeks. Moreover, the economic efficiency values was the highest in G3 and the lowest in G4. Concerning the immune parameters, both levels of SP in EW groups (G2 and G3) and LW groups (G5 and G6) improved all the measured immune parameters in a concentration-dependent way compared to the SP non-supplemented calves (G1 and G4). Whereas, the highest significant values ($p < 0.05$) were observed at the 12th week in LW calves supplemented with SP (G5 and G6). In conclusion, SP may function effectively as a feed additive at 0.01 or 0.02% of gestational weight for suckling buffalo calves during the pre-weaning stage to improve their productive performance and immune function so that early weaning can be achieved to provide milk and reduce feeding costs during suckling period.

INTRODUCTION

The neonatal phase constitutes a critical stage in a calf's life, during which they are highly susceptible to environmental stressors and pathogenic infections that can significantly influence their productive performance and immune function **(Cangiano et al. 2024)**. A potential strategy to optimize the nutrition outcome during the pre-weaning and early weaning periods, involves nutritional supplementation of appropriate natural feed additives, which can support the immune system, maximize productive performance, achieve optimal weaning weight, and minimize both the duration and cost of the suckling period **(Stefańska et al. 2022)**.

Among natural feed additives, Spirulina platensis (SP) has been recognized as a promising feed additive. Spirulina is a thread-like helical cyanobacterium, has been categorized as a blue-green microalga **(Becker, 2007)**. It is nutrient rich and it can be used as nutritional supplement for both animals and humans **(Buddhadasa and Adorno, 2004**, **Babadzhanov et al. 2004** and **Muhling et al. 2005).** It contains proteins (55%-70%), all essential amino acids, essential fatty acids (18%), carbohydrates (15%-25%), minerals, vitamins and pigments such as phycocyanin, chlorophyll, and carotenes. Spirulina is characterized by its beneficial effects against viral infections, anemia, malnutrition and tumor progress **(Sanchez et al. 2003** and **Habib et al. 2008).** The inclusion of SP (10 g/day) in cow milk has been improved the live weight and growth performance of lambs aged 15-30 days compared to the control group **(Bezerra et al. 2010)**. Spirulina has been utilized in feeding trials to enhance the productive efficiency for calves **(Glebova et al. 2018).** Furthermore, **Chesmberah et al. (2023)** found that the using of SP (3%) in the starter diet of Holstein suckling calves developed the final body weight in comparing with the control group (P<0.05). Also SP in concentration of 1, 2 and 3% in diet has significantly increased the daily weight gain of suckling calves. The same result was stated by **El-Moghazy et al. (2023)** when added SP powder at levels; 5, 10 and 15 g/head/day for suckling Egyptian buffalo calves three months suckling phase. Conversely, **Ghattas**

et al. (2019) noted that there were non-significant increases in the weight of Holstein newborn calves group which were treated with SP powder (6 gm/ calve/ day) for 45 days compared to control group.

Neonatal calves are born with an undeveloped adaptive immunity, limiting their ability to support an active immune response during the initial weeks of age **(Stelwagen et al. 2009)**. This increase their reliance on innate immunity, which functions as the first line of defense against infections and exerts a vital role in early immune protection before the activation of adaptive immunity. Strengthening innate immunity during this critical period is essential for reducing disease susceptibility and supporting overall health and optimal growth performance **(Kim et al. 2020 and Mattioli et al. 2020)**. The function of SP has been displayed in many previous studies as an immune stimulant **(Youssef et al. 2023)**, antioxidant and antibacterial **(Abdel-Daim et al. 2013 and Kavisri et al. 2023).** Spirulina has been reported to activate the innate immunity, promote macrophage activity **(Yehia et al. 2024 and Youssef et al. 2023)**, enhance cytokine and antibody production, and activate macrophages, NK cells, T cells, and B cells **(Hirahashi et al. 2002 and Hayashi et al. 1994)**. It has also been shown to raise NO synthase level in chicken macrophages **(Al-Batshan et al. 2001)** and improve lysozyme and nitric oxide secretion **(Yehia et al. 2024)**. Spirulina's high-molecular-weight polysaccharide (Immulina) has been found to increase antigen-specific production of Th1 cytokines such as IFN-γ and IL-2 **(Lobner et al. 2008).** However, limited information is available concerning the outcome of SP as a feed additive on the productive performance and immune status in newborn calves from birth to weaning. Hence, the current study was designed to investigate the impact of SP as a feed additive on the productive performance and some immune parameters in newborn buffalo calves weaned either early at 8^{th} week or late at 12^{th} week of age.

MATERIALS and METHODS

This study was performed in Animal Production Research Station at Mahalet Mosa in Kafr El Shekh Governorate, APRI and AHRI, ARC, Ministry of Agriculture, Egypt. The SP powder was obtained from National Institute of Oceanography and Fisheries (NIOF), Egypt.

Experimental animals, diets and design:

Twenty-four newborn buffalo calves with average initial LBW of 31.15 ± 1.03 kg were used in this study. They suckled colostrum of their dams for the first three days. The calves were randomly separated to six groups (four calves in each group) in a 2 × 3 main factorial design for weaning time and SP treatments. Factor I: was weaning age, either early-weaned (EW) at the 8^{th} week of age (G1, G2 and G3) or late-weaned (LW) at the 12^{th} week (G4, G5 and G6). Factor II: was the level of SP treatments, whereas the different levels of SP were unsupplemented (0.0% of LBW) in both G1 and G4 groups, 0.01% SP of LBW in G2 and G5 and 0.02% SP of LBW in G3 and G6. According to this design, G1: EW + 0.0% SP, G2: EW + 0.01% SP, G3: EW + 0.02% SP, G4: LW + 0.0% SP, G5: LW + 0.01% SP, and finally G6: LW + 0.02% SP. All calves received fresh buffalo milk two times daily and adjusted weekly at level of 12.5% of LBW in EW calves until 5^{th} week and in LW calves until 6^{th} week of age. Then the intake of milk was reduced weekly in EW calves by 25, 50 and 75%, while in LW calves the actual milk intake was reduced biweekly by 25, 50 and 75% **(Rashid et al. 2013).** Spirulina powder was given daily; either suspended in the sulking milk or water till 12^{th} week of age in both EW and LW calves. Pens were designed to allow calves from the 2^{nd} week to have free access to their total feed solids, which contained calf starter (CS contained 19.76% CP and 70% total digestible nutrients (TDN)) and good berseem hay (BH). In addition, they were provided with clean drinking water, blocks of mineral salt and vitamins throughout the experimental periods. The chemical composition and constituents of the used rations are illustrated in Table 1.

Table 1. Chemical composition of ingredients of the experimental rations.

Item	DM %	Composition on DM basis (%)						GE Mcal/kg
		OM	CP	CF	EE	NFE	Ash	
Calf starter (CS)	89.54	92.64	19.76	4.98	2.40	65.50	7.36	4.52
Berseem hay (BH)	90.32	89. 42	11.66	30.87	2.73	44.16	10.58	4.23
Spirulina (SP)	88.45	89.90	55.80	4.90	6.20	23.00	10.10	4.88

Ingredients % of calf starter as fed basis were 44% Maize grains, 24.70% Soybean meals (44%), 15% Linseed meals, 8% Wheat bran, 4% Molasses, 2% Di-calcium phosphate, 1% CaCO3, 1% Salts and 0.3% Vitamin premix. The constituents of vitamin premix /kg: Vitamin A = 27,000,000 IU, Vitamin D3 = 5,400,000 IU, Vitamin B1=1000mg, Vitamin E= 9000 IU, Nicotinic acid =17,500 mg, Calcium Pentothenate = 12,500 mg, Vitamin K3 =2,500 mg, Folic acid = 250 mg, and Vitamin B2= 7,500 mg. Buffalo milk contained Fat 6.5%, protein 3.9%, lactose 5.2%, ash 0.72%, total solids 16.32% and gross energy 1.08 Mcal/kg.
DM: dry matter. CP: crude protein. OM: organic matter. GE: gross energy. EE: ether extract. CF: crude fiber. NFE: nitrogen free extract

Sampling:

Samples from milk were taken every two weeks and frozen at -20 ^{o}C until the chemical analysis. Milk samples were examined for fat, protein, total solid and ash **(Ling, 1963).** Solids not fat (SNF) was estimated by difference between total solid and fat. Lactose % was calculated according to **Economides (1986).** Representative samples of CS and BH were examined for DM, CP, EE, CF and ash contents following the **AOAC (1995)**. Digestible energy (DE) was measured according to **AAFCO (1997)** and the GE according to **MAFF (1975).** GE (Mcal/kg DM) = (0.226 * CP + 0.177 * CF + 0.407 * EE + 0.192 * NFE) / 4.184

Blood samples:

Heparinized blood samples were obtained at the 12th week of age to separate the peripheral blood mononuclear cells (PBMCs) which utilized in the phagocytosis assay and in the determination of IFN- γ and IL-2 mRNA expression level using quantitative real time-PCR (qRT-PCR). Blood samples were obtained from all groups at 4, 8 and 12th weeks of the experiment and centrifuged to separate serum for assessment of nitric oxide (NO) and lysozyme levels.

Evaluation of the productive performance:

During 12 weeks of the experimental duration, LBW was measured biweekly and ADG was calculated. Also, total fresh milk intake (MI), total milk dry matter intake (MDMI), total CS dry matter intake (CSDMI), berseem hay dry matter intake (BHDMI) and total dry matter intake (total DMI) were reported weekly. Total DMI is calculated as follow: MDMI + CSDMI + BHDMI. Feed conversion ratio (FCR) estimated as the quantity of DM, GE and CP per kg weight gain and was calculated as follows: FCR (Kg) = TDMI/ ADG, total GE intake/ADG and total CP intake/ADG.

The Evaluation of the immune parameters:

Phagocytic activity of peripheral blood monocyte cells (PBMCs) from calves:

The separation of peripheral blood mononuclear cells (PBMCs) from the calves was estimated according to **Boyum (1968)**. Briefly, heparinized blood samples were obtained from calves at the 12th week old, diluted 1:1 with RPMI media, layered gently on lymphocyte separation medium, and then centrifuged in a cooling centrifuge at 2400 rpm for 30 min. The layer formed between the plasma and the lymphocyte separation medium was aspirated cautiously and washed three times. The resulting cell pellet (PBMCs) was then utilized for the phagocytic activity test and the extraction of IFN-γ and IL-2 mRNA from PBMCs. The phagocytic activity of calves' monocytes was assessed using *Candida albicans* spores, in accordance with **Bos and De Souza (2000)**. By dividing the number of phagocytic macro-

phages by the total number of macrophages, the phagocytic percentage (PP) was ascertained. The phagocytic index (PI) was determined by dividing the total number of macrophages by the number of macrophages that engulfed ≥3 Candida spores.

Measuring of serum nitric oxide (NO) and lysozyme:

They were done on all the serum samples; the nitric oxide assay was performed referring to **Rajaraman et al. (1998).** The lysozyme assay was done by the agarose gel plate lyses assay by using micrococcus lysodicticus bacteria according to **Schultz (1987).**

Determination of IFN-γ and IL-2 mRNA in peripheral blood mononuclear cells (PBMCs) by quantitative real time-PCR (qRT-PCR):

The extraction and purification of RNA from calves PBMCs samples were prepared by QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). The oligonucleotide primers of IFN-γ and IL-2 were shown in Table 2 (Metabion, Germany). The qRT-PCR SYBR green procedure was estimated in a Stratagene MX3005P real time PCR machine and the analysis of the obtained data of qRT-PCR were assayed by the amplification curves and threshold cycles (CT) values (stratagene MX3005P software). The differences of fold changes in the mRNA gene expression of all samples, were detected by comparing the CT of each sample with that of the normal control group following the "$-2^{\Delta\Delta Ct}$" procedure **(Yuan et al. 2006).**

Table 2. Target genes, primers sequences and cycling conditions for quantitative real time-PCR.

Target genes	Primers sequences	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Ref.
				Secondary denaturation	Annealing (Optics on)	Extension	
ß2M	AGACACCCAC-CAGAAGATGG TCCCCATTCTTC AGCAAATC				60˚C30 sec.	72˚C 30 sec.	Harrington *et al.* 2007
IFN-γ	GCGCAAAGCCA-TAAATGAAC CTCAGAAAGCG-GAAGAGAAG	50˚C 30 min.	94˚C 5 min.	94˚C 15 sec.	53˚C30 sec.		Zaros *et al.* 2007
IL-2	TCCAA-GCAAAAACCTG AACC CAGCGTTTACTG TTGCATCATC				57˚C30 sec.		

ß2M * (ß2-Microglobulin) served as housekeeping gene (internal control)

Statistical analysis:
Statistical analysis of the productive performance results was done using the General Linear Model's procedures (**SAS program, 2002**). The model of statistical analysis was as follows: $Y_{ij} = \mu + F_{1i} + F_{2j} + (F_1 \times F_2)_{ij} + +e_{ij}$. Where Y_{ij} is dependent variable, μ is over all mean, $F_{1i}$is the fixed effect of factor I. Where i =weaning age, F_{2j} is the fixed effect of factor II, where = level of SP 0, 0%, 0.01% and 0.02% of the BW, e_{ijk} = residual error. Differences among means of all groups were subjected to Duncan's Multiple Range test at a level of P<0.05 **(Duncan, 1955).**

Results

Live body weight and weight gain:
Live body weight (LBW) of calves as impacted by weaning and SP addition is illustrated in Table (3) and Figure (1). In spite of LBW was nearly similar in the first week (0 w), LBW at 8th and 12th weeks were significantly higher (P< 0.05) with late weaning related to early weaning and significantly increased with SP addition. The interaction between weaning and SP addition revealed significant (P< 0.05) differences in LBW at 8th and 12th weeks between the different groups with the highest values in G6 and the lowest values in G1. Also, TWG and ADG at the periods of 0-8th, 8th -12th and 0 -12th weeks were higher significantly with LW than those of EW calves and increased significantly (P< 0.05) with SP addition. The interaction between weaning and SP addition induced significant (P< 0.05) differences in TWG and ADG at the periods of 0-8th, 8th -12th and 0-12th weeks among the different groups with the highest levels in G6 and the lowest in G1.

Table 3. Live body weight (LBW), total weight gain (TWG) and average daily gain (ADG) of suckling buffalo calves weaned early or late and offered SP supplement.

Item	Weaning		Treatments			Interactions (weaning x treatments)						±SE
						Early weaning			Late weaning			
	Early	Late	0.00%	0.01%	0.02 %	G 1	G 2	G 3	G 4	G 5	G 6	
LBW (kg)												
0 week	30.58	31.72	30.95	31.38	31.13	30.50	30.75	30.50	31.40	32.00	31.75	0.24
8 week	63.55b	68.97^{a}	62.48 ^{b}	67.16 ^{a}	69.14^{a}	60.52^{c}	64.35^{b}	65.78^{b}	64.44^{b}	69.97^{a}	72.50^{a}	1.03
12 week	78.62b	85.79^{a}	75.89 ^{b}	83.94 ^{a}	86.78^{a}	72.83^{c}	80.44^{b}	82.58^{b}	78.95^{b}	87.44^{a}	90.98^{a}	1.50
TWG (kg)												
0-8 week	32.97^{b}	37.25^{a}	31.53^{b}	35.78^{a}	38.02^{a}	30.02^{e}	33.60^{cd}	35.28^{c}	33.04^{d}	37.97^{b}	40.75^{a}	0.87
8-12 week	15.07^{b}	16.82^{a}	13.41^{b}	16.79^{a}	17.64^{a}	12.32^{e}	16.09^{cd}	16.80^{c}	14.51^{d}	17.48^{b}	18.48^{a}	0.50
0-12 week	48.04^{b}	54.07 ^{a}	44.94^{b}	52.57^{a}	55.66^{a}	42.34^{e}	49.69^{cd}	52.08^{c}	47.55^{d}	55.44^{b}	59.23^{a}	1.35
ADG (kg)												
0-8 week	0.59^{b}	0.67 ^{a}	0.56^{b}	0.64^{a}	0.68^{a}	0.54^{e}	0.60^{cd}	0.63^{c}	0.59^{d}	0.68^{b}	0.73^{a}	0.02
8-12 week	0.54^{b}	0.60 ^{a}	0.48^{b}	0.60 ^{a}	0.63^{a}	0.44^{e}	0.57^{cd}	0.60^{c}	0.52^{d}	0.62^{b}	0.66 ^{a}	0.02
0-12 week	0.57^{b}	0.64 ^{a}	0.54^{b}	0.63^{a}	0.66^{a}	0.50^{e}	0.59^{cd}	0.62^{c}	0.57^{d}	0.66 ^{b}	0.71^{a}	0.02

a b c d e Means within rows with different superscript are significantly different (P< 0.05). G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW. LBW: live body weight. TWG: total weight gain. ADG: average daily gain.

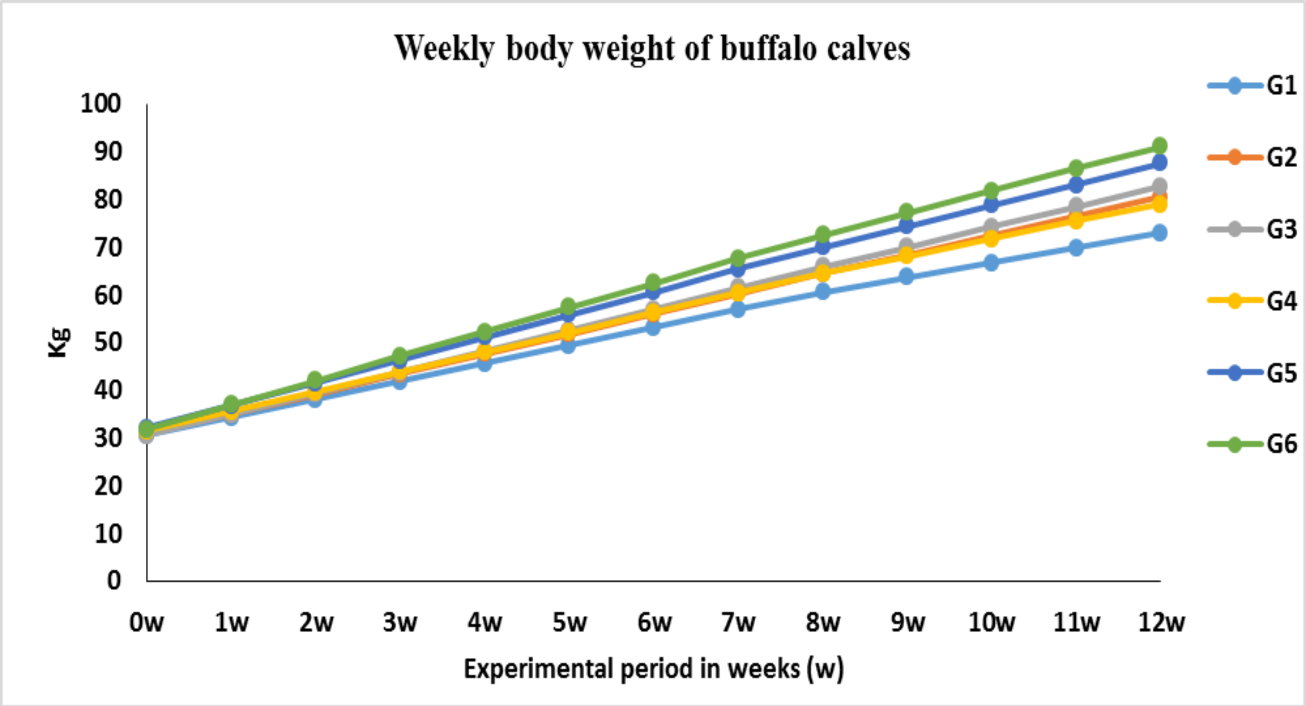

Figure 1: Weekly body weight of EW and LW buffalo calves and given Spirulina supplement. G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW.

Feed intake:

The main impacts of weaning age and SP levels on the intake of feed by calves are presented in Table (4) and Figure (2). Average daily MI was increased significantly (P< 0.05) with late weaning than those of early weaning and also it was increased significantly with SP additive than non-additive. The interaction between weaning and SP addition revealed significant (P< 0.05) differences in MI between the different groups with the highest values in G6 and the lowest values in G1 (Figure 2). While, the intakes of CS and BH were higher significantly (P< 0.05) with early weaning than those of late weaning and insignificantly increased with SP addition. The interaction between weaning and SP addition revealed significant (P< 0.05) differences in CS and BH intakes among the different groups with the highest values in G3 and the least values in G4. Meantime, the intake of DM, GE and CP showed inconstant significant differences (P< 0.05) between early and late weaning and increased significantly (P< 0.05) with SP additive. The interaction between weaning and SP addition revealed significant differences in the intake of DM, GE and CP between the groups with the highest values in G6 and the lowest values in G1 during the period 0-8th weeks and the highest values in G3 and the least values in G4 during the periods of 8th -12th and 0-12th weeks. The results of milk on a dry matter basis go in the same direction as the results of fresh milk.

Table 4. Average daily feed intake (kg/head/day) of suckling buffalo calves fed different treatments.

Item	Weaning		Treatments			Interactions (weaning x treatments)						±SE
						Early weaning			Late weaning			
	Early	Late	0.00	0.01	0.02	G1	G2	G3	G4	G5	G6	
Milk												
0-8 week	3.32^{b}	4.29^{a}	3.67^{b}	3.84^{a}	3.89^{a}	3.23^{c}	3.34^{c}	3.36^{c}	4.10^{b}	4.34^{a}	4.41^{a}	0.13
8-12 week	0.00^{b}	2.04^{a}	0.96^{b}	1.04^{a}	1.07^{a}	0.00	0.00	0.00	1.93^{b}	2.07^{a}	2.13^{a}	0.26
0-12 week	2.21^{b}	3.54^{a}	2.77^{b}	2.90^{a}	2.95^{a}	2.16^{c}	2.23^{c}	2.24^{c}	3.38^{b}	3.58^{a}	3.65^{a}	0.17
Calf starter (CS)												
0-8 week	0.70^{a}	0.66^{b}	0.65	0.69	0.70	0.67^{a}	0.70^{a}	0.71^{a}	0.63^{b}	0.67^{a}	0.69^{a}	0.01
8-12 week	1.95^{a}	1.45^{b}	1.63	1.72	1.74	1.88^{b}	1.96^{ab}	1.99^{a}	1.38^{d}	1.47^{cd}	1.50^{c}	0.06
0-12 week	1.11^{a}	0.92^{b}	0.98	1.03	1.05	1.07^{b}	1.12^{ab}	1.14^{a}	0.88^{d}	0.94^{c}	0.96^{c}	0.03
Berseem hay (BH)												
0-8 week	0.42^{a}	0.32^{b}	0.35	0.37	0.38	0.40^{b}	0.42^{a}	0.42^{a}	0.30^{d}	0.32^{cd}	0.033^{c}	0.01
8-12 week	1.35^{a}	0.96^{b}	1.11	1.17	1.19	1.30^{b}	1.36^{ab}	1.38^{a}	0.91^{d}	0.97^{cd}	0.99^{c}	0.05
0-12 week	0.73^{a}	0.53^{b}	0.60	0.64	0.65	0.70^{b}	0.73^{ab}	0.74^{a}	0.51^{d}	0.54^{cd}	0.55^{c}	0.03
Total DM												
0-8 week	1.54	1.58	1.50^{b}	1.57^{a}	1.60^{a}	1.49^{c}	1.55^{b}	1.57^{b}	1.51^{c}	1.60^{a}	1.63^{a}	0.02
8-12 week	2.97^{a}	2.50^{b}	2.62^{b}	2.76^{a}	2.81^{a}	2.86^{b}	2.99^{ab}	3.03^{a}	2.37^{d}	2.53^{c}	2.59^{c}	0.06
0-12 week	2.02^{a}	1.89^{b}	1.87^{b}	1.97^{a}	2.00^{a}	1.95^{ab}	2.03^{a}	2.05^{a}	1.80^{c}	1.91^{b}	1.95^{ab}	0.03
Gross energy (GE)												
0-8 week	8.49^{b}	8.97^{a}	8.39^{b}	8.81^{a}	8.94^{a}	8.21^{d}	8.54^{c}	8.62^{bc}	8.57^{c}	9.08^{ab}	9.26^{a}	0.10
8-12 week	14.55^{a}	12.82^{b}	13.09^{b}	13.82^{a}	14.05^{a}	14.01^{b}	14.64^{ab}	14.82^{a}	12.17^{d}	12.99^{c}	13.28^{c}	0.24
0-12 week	10.51	10.25	9.96b	10.48^{a}	10.65^{a}	10.15^{ab}	10.57^{a}	10.69^{a}	9.77^{b}	10.39^{a}	10.60^{a}	0.10
Crude protein (CP)												
0-8 week	315^{b}	335^{a}	313^{b}	328^{a}	333^{a}	305^{b}	317^{b}	320^{b}	320^{b}	339^{a}	346^{a}	3.88
8-12 week	544^{a}	478^{b}	489^{b}	516^{a}	524^{a}	524^{b}	547^{ab}	554^{a}	454^{d}	485^{c}	495^{c}	9.08
0-12 week	392	383	371^{b}	391^{a}	397^{a}	378^{ab}	394^{a}	398^{a}	365^{b}	388^{a}	396^{a}	3.66

a b c d e Means within rows with different superscript are significantly different (P< 0.05). G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW.

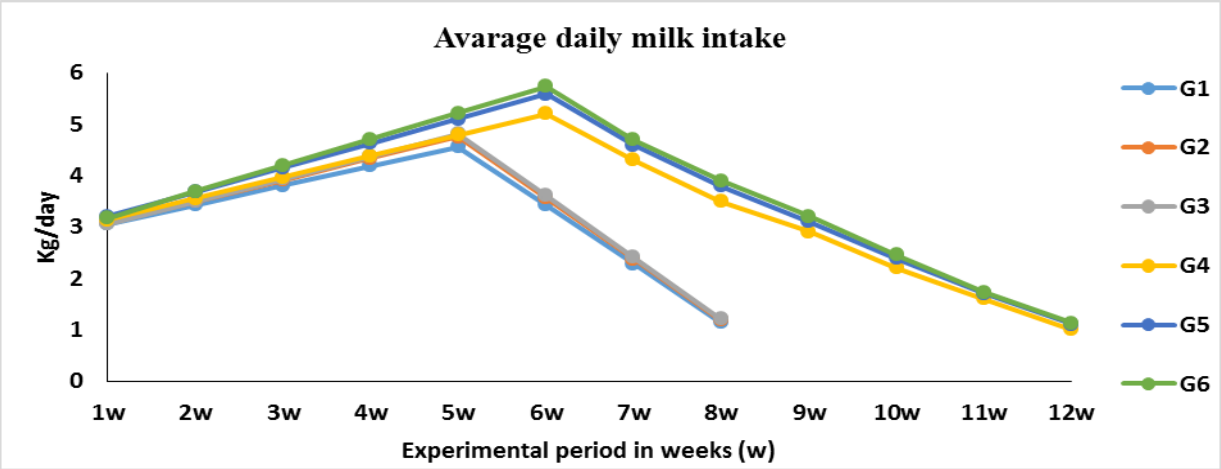

Figure 2: Average daily milk intake of EW and LW buffalo calves and given Spirulina supplement. G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW.

Feed conversion ratio (FCR):

Feed conversion ratio (Table 5) was calculated as the quantities of DM, GE and CP per kg weight gain. The amounts of DM, GE and CP per kg weight gain were significantly (P< 0.05) better in late weaning compared to early weaning as well as with SP additive than non-additive. The interaction between weaning and SP additive revealed significant (P< 0.05) better differences in the quantities of DM, GE and CP per kg weight gain among the different groups than G1, with the best one in G6.

Table 5. Feed conversion ratio (FCR) of suckling buffalo calves fed different treatments.

Item	Weaning		Treatments			Interactions (weaning x treatments)						±SE
						Early weaning			Late weaning			
	Early	Late	0.00	0.01	0.02	G1	G2	G3	G4	G5	G6	
DM												
0-8 week	2.59^{a}	2.39^{b}	2.67^{a}	2.47^{b}	2.36^{b}	2.78^{a}	2.58^{b}	2.48^{bc}	2.56^{b}	2.36^{cd}	2.24d	0.04
8-12 week	5.47^{a}	4.19^{b}	5.54^{a}	4.63^{b}	4.48^{b}	6.51^{a}	5.20^{b}	5.04^{b}	4.58^{c}	4.06^{d}	3.92d	0.21
0-12 week	3.49^{a}	2.95^{b}	3.52^{a}	3.16^{b}	3.04^{b}	3.86^{a}	3.43^{b}	3.31^{bc}	3.17^{c}	2.90^{d}	2.77d	0.09
GE												
0-8 week	14.30^{a}	13.55^{b}	14.92^{a}	13.81^{b}	13.21^{b}	15.32^{a}	14.23^{bc}	13.69^{cd}	14.52^{a} b	13.40^{d} e	12.73^{e}	0.22
8-12 week	26.78^{a}	21.48^{b}	27.68^{a}	23.15^{b}	22.41^{b}	31.86^{a}	25.47^{b}	24.71^{bc}	23.49^{c}	20.82^{d}	20.12^{d}	0.89
0-12 week	18.20^{a}	16.01^{b}	18.70^{a}	16.80^{b}	16.14^{b}	20.14^{a}	17.87^{b}	17.24^{b}	17.26^{b}	15.74^{c}	15.03^{c}	0.39
CP												
0-8 week	531	506	556^{a}	515^{b}	492^{b}	569^{a}	529^{bc}	509^{c}	542^{ab}	501^{cd}	476^{d}	7.88
8-12 week	1001^{a}	801^{b}	1033^{a}	864^{b}	837^{b}	1190^{a}	952^{b}	923^{bc}	876^{c}	776^{d}	750^{d}	33.32
0-12 week	678^{a}	598^{b}	697^{a}	627^{b}	602^{b}	750^{a}	666^{b}	642^{b}	644^{b}	587^{c}	561^{c}	14.51

a b c d e Means within rows with different superscript are significantly different (P< 0.05). G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW.

Economic efficiency:

Economic efficiency results (Table 6), stated that average daily feed cost was higher significantly (P< 0.05) for late weaning compared to early weaning, while it was increased insignificantly with SP addition. The interaction between weaning and SP additive revealed that average daily feed cost was higher significantly (P< 0.05) in G5 and G6 followed by G4, but G1, G2 and G3 had the lower values. The output of ADG was higher significantly (P< 0.05) for late weaning compared to early weaning and was increased significantly (P< 0.05) with SP additive. The interaction between weaning and SP additive revealed significant differences (P<0.05) in output of ADG among the different groups with the highest values in G6 and the lowest values in G1. However, economic efficiency was higher significantly (P< 0.05) for early weaning than those of late weaning and increased significantly with SP additive. The interaction between weaning and SP additive revealed significant differences in economic efficiency among the different groups with the highest values in G3 and the lowest values in G4. These results might be to the reduction of high-cost milk with early weaning

Table 6. Economic efficiency of suckling buffalo calves fed different treatments.

Item	Weaning		Treatments			Interactions (weaning x treatments)						±SE
						Early weaning			Late weaning			
	Early	Late	0.00	0.01	0.02	G1	G2	G3	G4	G5	G6	
Feed cost												
0-8 week	77.75^{b}	96.28^{a}	83.82	87.78	88.93	75.49^{c}	78.07^{c}	78.64^{c}	92.13^{b}	97.49^{a}	99.22^{a}	2.43
8-12 week	31.03^{b}	64.10^{a}	45.13	48.04	49.08	29.67^{c}	31.06^{c}	31.46^{c}	60.60^{b}	65.02^{a}	66.69^{a}	4.17
0-12 week	62.18^{b}	85.56^{a}	70.92	74.53	75.65	60.22^{c}	62.40^{c}	62.91^{c}	81.62^{b}	86.67^{a}	88.38^{a}	2.99
Gain output												
0-8 week	89.29^{b}	99.79^{a}	84.45^{b}	95.85^{a}	101.83^{a}	80.40^{e}	90.00^{cd}	94.50^{c}	88.50^{d}	101.70^{b}	109.16^{a}	2.33
8-12 week	82.58^{b}	90.12^{a}	71.85^{b}	89.92^{a}	94.50^{a}	65.97^{e}	86.22^{c}	90.00^{bc}	77.73^{d}	93.63^{b}	99.00^{a}	2.69
0-12 week	87.05^{b}	96.56^{a}	80.25^{b}	93.87^{a}	99.39^{a}	75.59^{e}	88.74^{cd}	93.00^{c}	84.91^{d}	99.01^{b}	105.77^{a}	2.42
Economic efficiency												
0-8 week	114.76^{a}	103.46^{b}	101.27^{b}	109.80^{a}	115.09^{a}	106.49^{c}	115.29^{b}	120.17^{a}	96.05^{e}	104.31^{c}	110.01^{c}	2.01
8-12 week	265.95^{a}	140.26^{b}	175.42^{b}	210.95^{a}	217.41^{a}	222.54^{b}	277.87^{a}	286.36^{a}	128.30^{d}	144.03^{cd}	148.46^{c}	16.58
0-12 week	139.88^{a}	112.65^{b}	114.78^{b}	128.23^{a}	133.75^{a}	125.53^{c}	142.22^{b}	147.83^{a}	104.02^{f}	114.24^{e}	119.68^{d}	3.95

a b c d e f: Means within rows with different superscript are significantly different (P< 0.05). G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW. Prices of kg were 20 LE for milk, 13 LE for calf starter, 3 LE for berseem hay, 200 LE for SP and 150 LE for weight gain according to 2022-2023 prices

The results of the immune parameters:

The result of the phagocytic activity of peripheral blood monocyte cells (PBMCs):

After 12th week of the experiment, the mean values of phagocytic activity (PP and PI) (Figure 3 & 4) were markedly increased in all groups supplemented with SP, in a concentration-dependent manner, whether EW at 8th week (G2 and G3) or LW at 12th week (G5 and G6), compared to those did not receive SP (G1 and G4). While, G6 (LW + 0.02% SP) showed the highest significant (P< 0.05) values of PP and PI (59.67 ± 1.2 and 0.48 ± 0.02, respectively) compared to other groups. Moreover, the results obtained from the EW groups and receiving spirulina at either low or high levels (G2 and G3) demonstrated a numerical increase in PP and PI values in a concentration dependent way compared to G4 (LW + 0.0% SP) and approached the value of G5 (LW + 0.01% SP). Furthermore, LW groups with or without SP revealed higher values than those of the comparable EW groups.

The results of the serum NO and lysozyme assays:

The results in Figures 5 & 6 demonstrated that the SP supplementation to the suckling calves at 4th and 8th weeks of the experiment resulted in numerical increases in a concentration and time-dependent way in the levels of NO and lysozyme in G2, G3, G5 and G6, as compared to the non-supplemented spirulina groups (G1 and G4). At week 12 of the experiment, SP supplementation induced obvious higher values of NO and lysozyme in both EW (G2 and G3) and LW (G5 and G6) groups compared to the non-supplemented groups (G1 and G4). Whereas NO levels were significantly (P< 0.05) higher in G6 (9.67±0.16) than in G1 (7.09 ±0.19), G2 (8.32±0.17), G3 (8.64±0.29) and G4 (8.11±0.46). Also, the NO was significantly higher in G5 (9.12±0.47) than in G1. The lysozyme level was significantly increased in G6 (174.5±14.7) than in G1 (108.3±20.2) and G4 (124±5.5). Moreover, the values in LW groups with or without SP revealed higher values than those of the comparable EW groups all over the experiment.

The result of IFN-γ and IL-2 mRNA gene expression in PBMCs cells by quantitative real time-PCR (qRT-PCR):

At week 12 of the experiment, the fold changes of mRNA for IFN-γ and IL-2 gene expression in PBMCs were upregulated in all groups compared to G1 (Figure 7). Whereas, calves in G5 (LW + 0.01% SP) and in G6 (LW + 0.02% SP) exhibited a significantly (P<0.05) higher value of IFN-γ (3.17 ± 0.25 and 3.39 ± 0.15 respectively) and IL-2 (3.62 ± 0.14 and 3.95 ± 0.22 respectively) compared with other groups. Moreover, G2 (EW + 0.01% SP) and G3 (EW + 0.02% SP) showed significantly higher values of IFN-γ and IL-2 than in G1 (EW + 0.0% SP). Also it was noticed that G2 and G3 showed a dose-dependent numerically increases in IFN-γ and IL-2 than in G4 (LW + 0.0% SP).

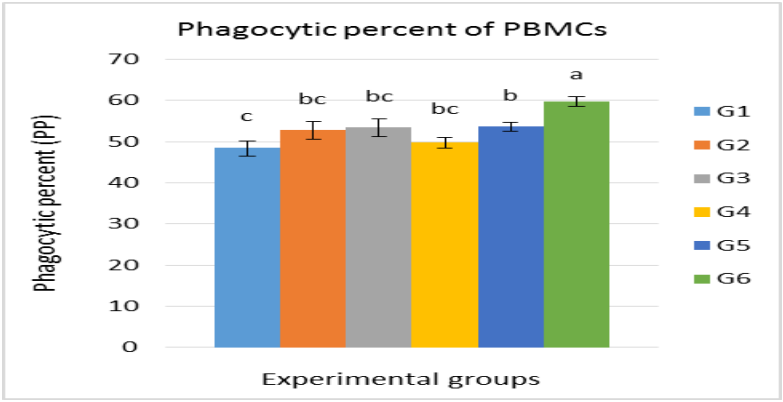

Figure 3. The results of phagocytic percent (PP) of peripheral blood monocyte cells (PBMCs) were evaluated at 12th w of age. The data are displayed as the mean values ± SE bar. Columns with same small letter indicate no significant differences between groups at $p < 0.05$. G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW.

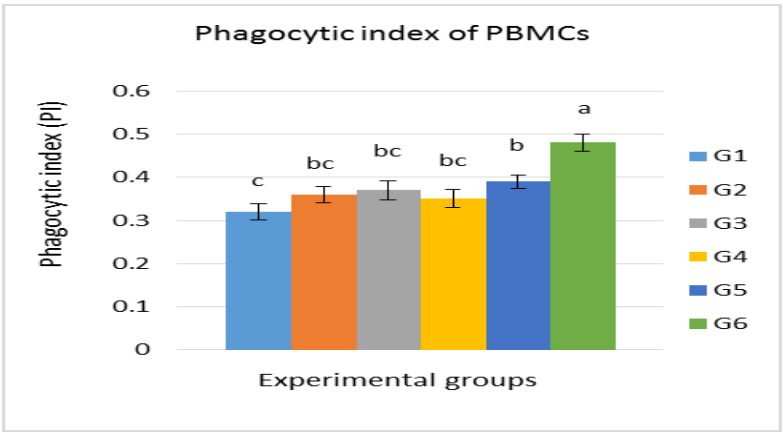

Figure 4. The results of phagocytic index (PI) of peripheral blood monocyte cells (PBMCs) were evaluated at 12th w of age. The data are displayed as the mean values ± SE bar. Columns with same small letter indicate no significant differences between groups at $p < 0.05$. G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW.

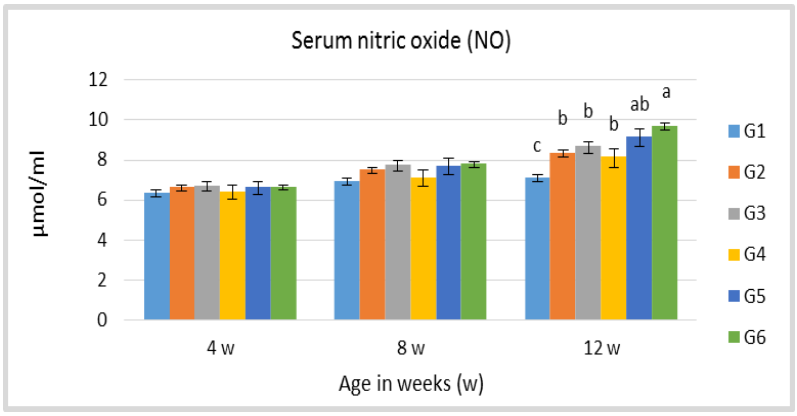

Figure 5. The results of serum NO assays. The data are displayed as the mean values ± SE bar. Columns with same superscripted small letter indicate no significant differences at the same time between groups at $p < 0.05$. G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW.

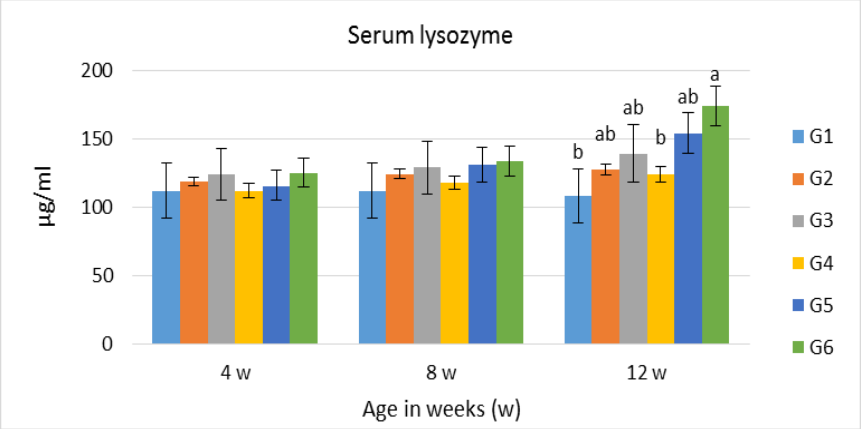

Figure 6. The results of serum lysozyme assays. The data are displayed as the mean values ± SE bar. Columns with same superscripted small letter indicate no significant differences at the same time between groups at $p < 0.05$. G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW.

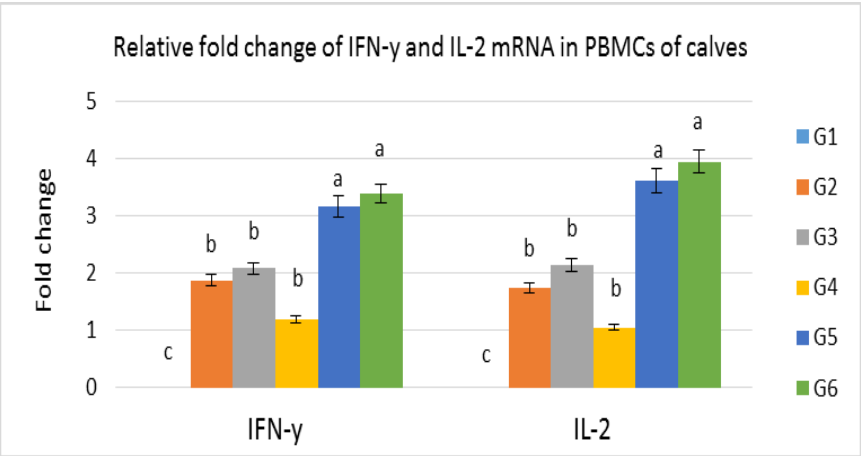

Figure 7. The results of quantitative real time-PCR (qRT-PCR) of IFN-γ and IL-2 mRNA in PBMCs cells were evaluated at 12th w of age. The relative fold change of IFN-γ and IL-2 mRNA is calculate by $2^{-\Delta\Delta Ct}$ method. The CT of each sample was compared with that of G1. Data are displayed as mean ± SE bar. Means with same small letter indicate no significant differences ($p < 0.05$) between groups in the same examined cytokine. G1: EW + 0.0% SP. G2: EW + SP 0.01% of BW. G3: EW + SP 0.02% of BW. G4: LW + 0.0% SP. G5: LW + SP 0.01% of BW. G6: LW + SP 0.02% of BW.

DISCUSSION

In the current study, the impacts of SP supplementation at different levels, in combination with early or late weaning, were evaluated in newborn calves with respect to growth performance, innate immune parameters, and immune-related cytokine responses. The findings demonstrated that SP supplementation induced a positive impact on calf performance. This improvement appeared to be achieved by numerous factors, including the quantity of milk intake associated with the length of suckling period, in addition to the positive impacts of SP supplementation which in turn led to an increase in the intake of feed, LBW, ADG, and improve the FCR of the tested calves. When comparing the main effects of the late weaning to the early weaning (Factor 1), it was found that all LW calves sulking more milk (MI) than EW (Table 3). These findings were in agreement with those recorded by **Rashid et al. (2013)** and **Abbas et al. (2017)**. Moreover, the improvement in LBW, TWG, ADG and FCR in the current study was higher during the first

eight weeks than in the last four weeks. This was due to the high MI during the first eight weeks whereas the EW calves stopped suckling milk from the 8th weeks, and hence they ingested more starter ration, which has less FCR than milk (Table 5). Calf growth is most rapid during the first eight weeks of suckling, with average daily gains often exceeding 1.2 kg, as calves rely heavily on milk for nutrition. In the final four weeks of suckling, which typically involves weaning at around 6-8 weeks in dairy systems, growth rates may slow as calves rely more on solid feed and may experience a slight growth check around the weaning period **(Hepola et al. 2007)**.

Regarding the impacts of the interaction of weaning time and spirulina levels (F1× F2) on the intake of milk, starter, BH, total DM and calves performance. When comparing the early weaning and the late weaning, it was found that in the absence of SP, the EW calves (G1) consumed less ($p< 0.05$) milk than those of the LW (G4) during 12 w (Table 4), and as the result they had ($p <0.05$) lower BW. These findings agree with those reported by **Abbas et al. (2017)** who recorded that during first twelve weeks, the EW calves consumed less milk (175.6 L vs. 318 L) and showed less BW (62.7kg vs.77.7kg) and the maximum MDMI / BW% was nearly 1.5% and 1.6% at the 5th and 6th w respectively at offered milk 10% of BW. However, in the existing study, using SP supplementation, there was a favorable effect ($p< 0.05$) on milk consumption, which was initially low and then gradually increased during early or late weaning.

With regard to the milk offered and the actual milk suckling / LBW, the obtained results showed that, in general, the milk suckling was lower than the actual allowance by (12.5%), especially at an earlier age. Whereas, the milk as dry matter intake (MDMI) as a percent of LBW ranged between (5.5%) and (6.29%) during the first two weeks of age. These results may be attributed to the lack of turnout in suckling milk through buckets **(Abbas et al. 2017).** Conversely, the solid intake showed an opposite trend compared to milk suckling during the trial (Table 4). Especially, in calves that are fed without additives at either early or late weaning (G1) or (G4). After that, as a result of early weaning or decreased milk suckling, the MI dramatically increased in G1 and G4 till the end of the experiment. Regarding the influence of SP supplementation on solids intake, the current study showed that the supplementation had a greater positive impact at early weaning compared to late weaning (Table 4). This may be attributed to the impact of SP supplementation in reducing the opposing impacts of the weaning stress (shock) on feed intake and hence on the LBW, especially in the early weaning. These findings stated that the substandard effects of the early weaning accompanied by the absence of SP induced a significant decrease in solid intake, LBW and TWG which may be due to one of the opposing effects of early weaning stress on feed intake and body weight **(Neamt et al. 2019)**.

Feed conversion ratio (FCR) expressed as the amounts of DM, GE and CP per kg weight gain in Table (5) showed a significantly ($P<0.05$) better in late weaning compared to early weaning by 9% (0-8th w), 31 % (8th -12th w) and 18% (0-12th w). Concerning the influence of SP addition on calves' performance, the results revealed a significant improvement in FCR by 7.5% , 11.6% (0-8th w), 16%, 19% (8th -12th w) and 10%, 13.6% (0-12th w) with 0.01% and 0.02% SP supplementation, respectively compared to non- supplemented (Tables 5). Moreover, there were obvious enhancements in the final LBW due to SP supplementation throughout the experiment. These results appeared to be greater than those recorded in previous researches by **Glebova et al. (2018)**. The improved productive performance observed in the this study comes in line with previous studies indicating that feeding lambs with cow milk enriched with SP (10 g/day) induced higher live BW and growth rates between 15 and 30 days' old compared to the control lambs **(Bezerra et al. 2010)**. Also, feeding with SP improved feed intake, BW, ADG and FCR in lambs **(El-Sabagh et al. 2014),** and increased ($P<0.05$) the growth efficiency in growing pigs **(Nedeva et al. 2014)**. These favorable impacts of SP may be due to its numerous influences on animal performance, among them its stimulating effects on metabolism, improvement of digestibility, in-

creased intestinal villi length and epithelial cell numbers, regulation of calcium and phosphorus balance, and improved utilization of mineral salts. As well as its immune stimulant, antioxidant, antibacterial antiviral effects, which support the immunity and thereby positively impact overall growth and productivity in calves **(Abdel-Daim et al. 2013, Glebova et al. 2018, Khan et al. 2020,** and **Kavisri et al. 2023)**.

Economic efficiency (Table 6) was lower during the first 8th weeks compared to the last 4th weeks, which might be to reduce the amount of high-cost milk intake (Table 4). Economic efficiency increased after 8th weeks of suckling period due to the decrease the amounts of high costing milk intake and increase the amounts of low costing starter and hay **(Mohsen et al. 2017)**. Also, economic efficiency improved with SP additive for EW and LW. Spirulina supplementation improves the economic efficiency of growing calves by reducing feed costs per unit of weight gain, increasing total revenue from body weight gain, and resulting in higher net revenues and economic efficiency compared to control groups **(Riad et al. 2019).** All treated groups with SP had higher ($P<0.05$) net profit and net revenue than the control group. Conclusively, it can be concluded that 200 mg/kg body weight of SP (G3) can increase productive performance and feed utilization while achieving good relative economic efficiency **(Heidarpour et al. 2011)**.

Concerning the impact of SP supplementation on the immune parameters, the current study declared that the use of SP as a natural immune-stimulant, evokes a central role in enhancing the innate immunity of newborn calves. Innate immunity is essential in protecting calves against infections during early life before full maturation of the adaptive immune response. One important aspect of innate immunity is the phagocytosis process by monocytes and macrophages cells **(Chi et al. 2014)**. These cells identify pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors, leading to pathogen engulfment, activation of intracellular signaling, and the production of immunomodulatory molecules such as nitric oxide, lysozyme, and inflammatory cytokines **(Martinez and Gordon, 2014).** The obtained results (Figure 3 and 4), showed that SP in both concentrations (0.01% or 0.02% of BW) boosted the PBMCs phagocytic activity (PP & PI) in EW and LW groups in a concentration-dependent way compared with the non-supplemented groups. And the highest significant values were due to administration of high level of SP (0.02%) parallel with late-weaning at 12th week of age in G6, which confirmed the advantage of SP supplementation besides suckling of milk for long time. The results also declared the important roles of SP for the EW calves (G2 and G3) in activating the phagocytic cells to exceed the result of LW without SP in G4 (LW + no SP) and also to approach the results of LW calves with a low concentration of SP in G5 (LW + 0.01% SP). These finding highlight the advantageous role of SP in improving non-specific immune defense and macrophage activity in both EW and LW calves. Which are compatible with previous studies, which reported that dietary spirulina enhances functionality of mononuclear phagocyte system **(Yehia et al. 2024 and Youssef et al. 2023)**.

In the same regard, the addition of SP induced obvious upregulation of the NO and lysozyme levels in a concentration and time-dependent way all over the experiment, and there were significant increases in their levels at the LW groups (G5 and G6). It was also noticeable that SP had superior NO and lysozyme in EW calves (G2 and G3) than the LW calves without spirulina (G4) (Figure 5 and 6) which may also explain the important role of SP in enhancing the non-specific immunity in EW calves. This finding is matched with **Abdellatief et al. (2018)** and **Youssef et al. (2023)**, who described that SP significantly enhanced the serum NO and lysozyme activities. The roles of these two innate immune parameters, NO and lysozyme, in host defense mechanisms are essential, while NO is generated by activated macrophages and neutrophils through inducible nitric oxide synthases (iNOS), functions as a pro-inflammatory mediator, and controls immunological reactions and defense against different infectious pathogens **(Roberts et al. 2024)**. Moreover, lysozyme has immunomodu-

latory, antibacterial and antiviral properties **(Ragland and Criss, 2017)**.

Regarding the role of SP on mRNA levels of the immune-related cytokines (IFN-γ and IL -2) in calves PBMCs (Figure 7), all SP groups showed noticeable upregulation in both cytokines gene expression compared to the non-supplemented groups. And the significant higher values were due to low and high concentration of SP parallel with late-weaning. Moreover, spirulina supplementation to the EW groups (G2 and G3) performed better cytokines expression than the LW without receiving SP (G4). This result is well matched with those of previous studies demonstrated that SP or its high-molecular-weight polysaccharide (immulina) augmented IFN-γ level in chickens **(Mobarez et al. 2018)** and the Th1 cytokines such as IFN-γ, and IL-2 in human PBMCs **(Lobner et al. 2008)**. Gamma interferon (IFN-γ) and interleuckine-2 (IL-2), play vital roles in orchestrating the immune response against infectious disease **(Fresno et al. 1997)**. Whereas, type II IFN functions as a link between non-specific and specific immunity, regulates numerous immune cells and initiates T helper 1-type immunity **(Fensterl and Sen, 2009)**. On the other hand, Interleukin-2, stimulates T and natural killer cells and boosts the proliferation of B cells and the secretion of IgM, IgG and IgA. **(Fluckiger et al. 1993)**. The elevated immune parameters in SP groups may be due to the presence of unique high-poly-nutrient and immune-stimulant substances in SP, including Braun-type lipoproteins and immulina **(Pugh et al. 2015 and Balachandran et al. 2006)**. These substances initiate the toll-like receptor 2-dependent signalling pathway as well as the transcription of Nuclear Factor-κB (NF-κB) **(Balachandran et al. 2006)** and consequently trigger the immune functions and phagocytosis by macrophages **(Parihar et al. 2010)**. Furthermore, it was observed that the best results were in LW in comparing with the EW groups (G4 compared to G1; G5 compared to G2 and G6 compared to G3), which indicates the importance of milk suckling for sufficient duration during the neonatal period. Whereas, milk is considered a primary and highly rich source of important nutrients as well as multiple immunological compounds **(Blum, 2006 and Playford et al. 2000)**. But according to this study, giving SP to EW calves, improved their immunological and performance parameters and enabled them to get closer to the LW calves at 12th week of age. Which indicated the advantage of the SP supplementation to the early-weaned calves.

CONCLUSION

The use of SP as a natural feed additive positively improved the growth performance, innate immune parameters and immune-related cytokines of newborn calves in a dose-dependent manner. Therefore, SP can be effectively used as a feed additive at 0.01 or 0.02% of gestational weight for suckling buffalo calves during the pre-weaning stage to improve their productive performance and immune function so that early weaning can be achieved to provide milk and reduce feeding costs during suckling period.

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