

Effect of *Bacilli* as feed additive on immune response of pregnant she-camel and its newborn calf

Mohie El-Dinn, M.M.^{1,2@}; Abdel-Aal, E.S.²; Eid, L.N.²; Farid, A.A.¹; Aly, I.R.³ and El-Deeb, S.O.¹

¹Department of Zoology. Faculty of Science. Cairo University. Egypt.

²Animal Production Research Institute. Agricultural Research Center. Egypt.

³Theodore Bilharz Research Institute. Egypt.

SUMMARY

Pregnancy in dromedary camel is associated with down-regulation of immune responses that may lead to susceptibility to viral and bacterial infections so it is expected that feeding probiotics may help in strengthening pregnant dams' immunity. This study aimed to evaluate the impact of *Bacilli* mixture on the immunity of pregnant dams and their newborn calves' peri-parturation. A total of 10 dams at late gestation to one-month post calving were divided into two equal groups: G1 (control) was fed the basal diet while G2 was fed diet supplemented with a mixture of [*Bacillus subtilis* - *Bacillus licheniformis*] (Enhancer 1.3, 10g/head/day). Dams', calves' plasma and milk were collected at certain durations. Results revealed a significant increase in dams; IgG levels of G2 compared to G1 at one month pre calving, one week and one month post calving. Milk IgG levels showed significant increase in G2 at one week post-calving as well. Calves' IgG levels showed significant increases in G2 at all durations. IgA in dams and calves didn't show significant change at all. Milk IgA levels showed increases in G2 more than G1 at calving and one week post calving. IFN- γ level in dams' plasma showed significant increase in G2 more than G1 at calving and at one week while in milk, levels showed significant increase in G2 only at one week post calving and in calves, levels were higher in G2 at one week and one month after birth. In conclusion, *Bacilli* mixture-based feed additive to the feed of pregnant dromedary she-camels improved some immunological parameters of them and their newborn calves.

Efecto de los *Bacilli* como aditivo alimenticio en la respuesta inmune de la camella y de su guelfo recién nacido

RESUMEN

La preñez en el dromedario se asocia con la regulación descendente de las respuestas inmunitarias que pueden llevar a la susceptibilidad a infecciones virales y bacterianas por lo que se espera que la alimentación a base de probióticos puede ayudar a fortalecer la inmunidad de las camellas preñadas. Este estudio tuvo como objetivo evaluar el impacto de la mezcla de bacilos en la inmunidad de las camellas preñadas y en el parto de sus guelfos recién nacidos. Un total de 10 camellas durante su gestación tardía hasta un mes después del parto se dividieron en dos grupos iguales: El G1 (control) se alimentó con la dieta basal, mientras que G2 fue alimentado con dieta suplementada con una mezcla de [*Bacillus subtilis* - *Bacillus licheniformis*] (Potenciador 1.3, 10g/cabeza/día). El plasma de las camellas, guelfos y leche se recolectaron en diferentes ensayos. Los resultados para las camellas revelaron un aumento significativo de los niveles de IgG al comparar el G2 con G1 un mes antes del parto, una semana y un mes después del parto. Los niveles de IgG en leche mostraron un aumento significativo en el G2 a la semana después del parto también. Los niveles de IgG de los guelfos mostraron aumentos significativos en G2 en todos los periodos. Las IgA en las camellas y guelfos no mostraron cambios significativos en absoluto. Los niveles de IgA de leche mostraron aumentos en G2 más que en G1 en el momento del parto y una semana después del parto. El nivel de IFN- γ en el plasma de camellas reveló el aumento significativo en el G2 más que en el G1 en el momento del parto y a la semana mientras que en leche, los niveles demostraron el aumento significativo en G2 solamente una semana post-parto y en guelfos, los niveles eran más altos en G2 a la semana y un mes después del nacimiento. En conclusión, la inclusión de un aditivo a base de una mezcla de bacilos en la alimentación de las camellas preñadas mejoró algunos de sus parámetros inmunológicos y de sus guelfos recién nacidos.

ADDITIONAL KEYWORDS

Dromedary she-camel.
Bacilli species.
IgG.
IgA.
IFN- γ .
Newborn calves.

PALABRAS CLAVE ADICIONALES

Camella.
Especies de *Bacilli*.
IgG.
IgA.
IFN- γ .
Guelfos recién nacidos.

INFORMATION

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mm.arc@hotmail.com

INTRODUCTION

Camelus dromedarius (One-humped camel) is one of the most important domestic animals in the arid and semi-arid regions, due to their potential to produce low cost and high quality milk and meat (Zayed 2011; Muz-

zachi et al., 2015, pp. 367-373). According to FAO Statistics (2013), the camel population in Egypt is 152,946 head. Like most of livestock animals, imbalances of nutrients can affect the camel immune function by modulating some physiological activities, especially under stressful conditions (FAO 2012). Pregnancy is known to

be one of the physiological stressors that might affect she-camel's performance (Al-Zamely 2011, pp. 46-51). During the peri-parturient period, hormone fluxes may adversely affect immune cell function. As reported by previous studies, during the transition period (from pregnant, non-lactating to non-pregnant, lactating state), a damage of biological macromolecules and disruption of normal metabolism and physiology could happen (Abdel-Aal & Eid 2014, pp. 255-270).

During peri-parturition, the most important factor causing immune-suppression might be the metabolic stress resulting from hormonal and metabolic fluctuations, a negative energy balance, and shortage of proteins, minerals and vitamins which are required to meet the demands of the fetus until lactation. Therefore, dietary supplementation with microbial feed additives has been reported to improve growth performance, nutrient digestibility, and immune status of the pigs (Meng et al. 2010, pp. 3320-3326; Lei & Kim 2014, pp. 171-176).

Camel productive efficiency is greatly influenced by the high mortality rate of camel calves in their first 3 months. The main causes for mortality are the harsh desert conditions and infectious diseases. Improving she-camel's health during the periparturient period might be important for the health status of their newborn calves. The survival of camel calves depends on their successful body defense against microbial invasions. Camel calves come to life almost deprived of serum immunoglobulin and depend on colostrum for virtually all its humoral passive immunity (Ghazi et al. 1994, pp. 337-342). The amount of colostrum immunoglobulins that can be absorbed depends on their passive transfer through intestine in the early days (Ghazi et al. 1994, pp. 337-342).

Probiotics are known as one of the feed strategies that have been widely used in various livestock species for improving health and production (Vivek et al. 2014, pp. 188-199), especially, after antibiotics banning by the European Union. Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit to the host" according to FAO (2009). Probiotics have also been reported to improve growth performance and nutrient digestibility, balance of the intestinal microflora and importantly promote immune function and benefit the intestinal morphology in which animals (Sinol et al. 2012, pp. 264-268; Zhang and Kim 2013, pp. 343-347).

Bacilli species are known as popular examples of probiotics due to their resistance against high temperature so they are easier to handle during manufacturing, pelleting, storage and transportation. Two of the 13th identified *Bacilli* species are *B. subtilis*, and *B. licheniformis*, which are safely used as a probiotic in animal feeds (EFSA FEEDAP Panel 2016, p.11). It is previously reported that *Bacillus subtilis*, *Bacillus licheniformis* enhance animal's growth (Vivek et al. 2014, pp. 188-199), improve ruminant's health through immunity (Oetzel et al., 2007). The positive effects conferred on animal's health can be attributed to either the secretion of lytic enzymes against harmful pathogens, supportive enzymes to the digestive functions or releasing antimicrobial peptides by *Bacilli* species as explained by Sorokulova, (2013). *Bacilli* strains bind to the intestinal epithelium compete against pathogenic bacteria and stimulate the

animal's immune system (Beauchemin et al. 2006, pp.251-284; Riddell et al. 2010). The aim of the study is to investigate the influence of probiotic consisting of *Bacillus subtilis* and *Bacillus licheniformis* on the immune response in pregnant dromedary camels during peri-partum period and its reflection on their newborn calves.

MATERIALS AND METHODS

PROBIOTIC STRAIN

A local commercially available product; mixture of two *Bacilli* strains [*Bacillus subtilis* + *Bacillus licheniformis*] (Enhancer 1.3, International Free Trading Co. "IFT Corporation") was used

ANIMAL GROUPING

Ten healthy pregnant dromedary she-camels at their late gestation period (at 10th month of pregnancy) with starting body weights ranged between 570 and 600 kg were housed in Camels Studies and Production Development Center (CSPDC), Matrouh Governorate, northern Egypt. All dams were fed the basal diet for two months pre-calving and till one month post-calving. Dams were divided into two groups (five dams/group), as follows:

Group1 (G_1); (control group), were fed the basal diet only. The basal diet recommended by Animal Production Research Institute (APRI) - Egypt to be introduced to dams at transient period consists of [Concentrate feed mixture; CFM \approx 5-6 kg (12% protein) + Clover hay \approx 2-3 kg + Rice straw \approx 5-6 kg].

Group2 (G_2); were fed the basal diet + 10 g/head/ day of [*Bacillus subtilis* + *Bacillus licheniformis*] mixture (mixed with each individual's feed).

EXPERIMENTAL DURATIONS

The experiment began at the transition period of pregnancy (especially, from the 10th month of pregnancy) and continues until one month post-calving. Various samples (Plasma or Milk) were taken at selected times as illustrated in the following **Table I**.

BLOOD SAMPLING

Blood samples were collected from the jugular vein of dams and their calves into vacutainer tubes containing EDTA as an anti-coagulant, centrifuged at 3000 rpm/10

Table I. The experimental durations at which samples were taken (Ensayos experimentales en los que se tomaron las muestras).

Time from calving	Dams' plasma sampling	Milk sampling	Calves' plasma sampling
Two months pre-calving	√	—	—
One month pre-calving	√	—	—
At calving	√	√	√
One week post calving	√	√	√
One month post calving	√	√	√

min to obtain plasma which was kept frozen at -20° C until analysis.

MILK SAMPLING

Milk was collected in clean containers and separated into whey. About 10 ml of colostrum or milk was centrifuged at 4000 rpm at 4° C for 30 minutes to remove the floating lipid drop then warmed in a water bath at 40 to 45° C for 30 minutes, after that some drops of freshly prepared Rennet solution were added and incubated in water bath. Casein clot aggregated with the remaining fats was filtered to obtain clear whey that was stored at -20° C until analysis (Hyun et al. 2014, pp.1976-1982).

IMMUNOLOGICAL ANALYSIS

Immunoglobulins IgG, IgA and the cytokine IFN-γ were quantitatively detected by Sandwich Enzyme Linked Immunosorbent Assay (ELISA) (Engvall and Perlmann 1971, pp. 871-874). Procedures were performed according to the manufacturer’s (Camel IgG ELISA, Life Diagnostics Inc., Catalog Number: IGG-16), (Camel IgA ELISA, Mybiosource, Catalog Number: MBS073806) and (Camel IFN-γ ELISA, Wuhan Fine Biological Technology Co., Ltd., Catalog Number: ECM0009).

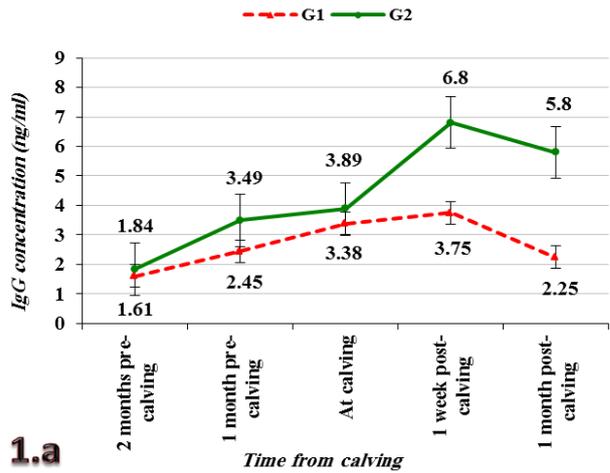
STATISTICAL ANALYSIS

Statistical analysis of the data was carried out using SAS User’s Guide: Statistics procedure of SAS version 9.1 for Windows (SAS 2004). Duncan’s multiple range and multiple F-test was used to detect significant differences between treatment means where differences were considered significant when (P<0.05) (Duncan 1955, pp. 1-42).

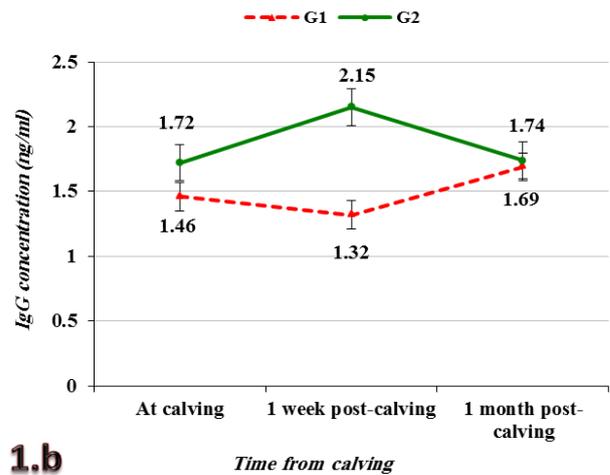
RESULTS

PROFILE OF IGG LEVELS (NG/ML) IN SHE-CAMELS’ PLASMA, THEIR MILK WHEY AND NEWBORNS’ PLASMA

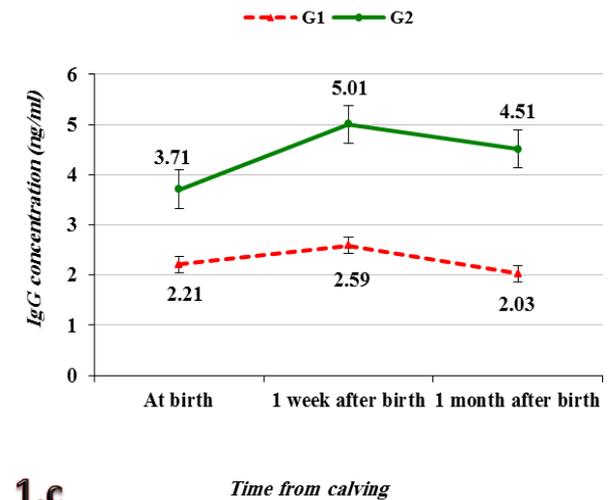
IgG profile in she-camels’ plasma at different durations pre- and post-calving presented in Fig (1,a) showed that there was no significant difference in IgG concentration neither at two month pre-calving nor at calving in G1 and G2 (1.61±0.18 and 1.84±0.33 ng/ml, respectively). At one month after oral administration (one month pre-calving) of *Bacilli* mixture, G2 recorded a significant (P<0.05) higher concentration of IgG (3.49±0.64) compared to G1 (2.45±0.23). The same trend was noticed for IgG at one week post-calving where G2 was higher than G1 (6.8±0.18 vs. 3.75±0.41) ng/ml and till one month post-calving (5.8±0.44 vs. 2.25±0.36), respectively. There was no significant difference in IgG concentration at the time of calving (Figure 1A). In the current study, milk IgG levels were shown to have significant differences (P<0.05) at calving (1.46 ± 0.03 for G1) vs. (1.72 ± 0.13 for G2), and one week post calving (1.32 ± 0.14 for G1) vs. (2.15 ± 0.41 for G2), but no differences were detected at one month after delivery as shown in Figure 1B. As shown in Figure 1C, Igs in plasma of newborn camel calves belonging to G1 and G2 groups, recorded significant differences during the studied durations (P<0.05). Calves to dams administered *Bacilli* mixture (G2) had elevated levels of IgG compared to the untreated group (G1) at calving (3.71±0.50 vs. 2.21±0.91), at one week (5.01±0.90 vs. 2.59±0.70) and one month post calving (4.51±0.59 vs. 2.03±0.48) Figure 1C.



1.a



1.b



1.c

Figure 1. Profile of IgG levels (ng/ml) in She-camels' plasma (A), their milk whey (B) and newborns' plasma (C) (Perfil de los niveles de IgG (ng/ml) en plasma de camellas (A), suero de leche (B) y plasma de los gélfos recién nacidos (C)).

PROFILE OF IGA LEVELS (NG/ML) IN SHE-CAMELS’ PLASMA, THEIR MILK WHEY AND NEWBORNS’ PLASMA.

Levels of IgA in the experimental animals administered *Bacilli* mixture (G2) were numerically higher at

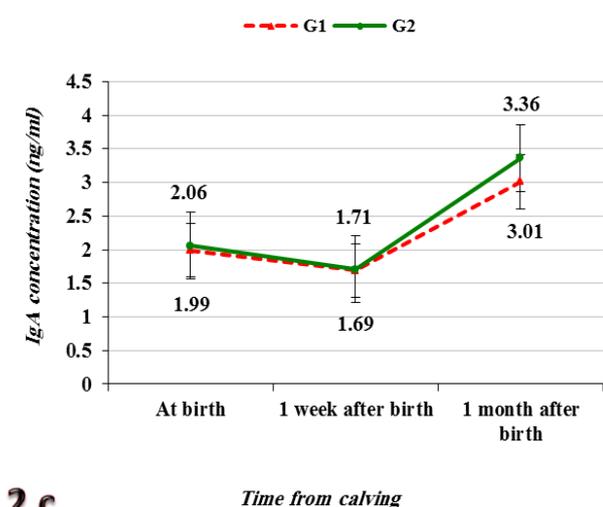
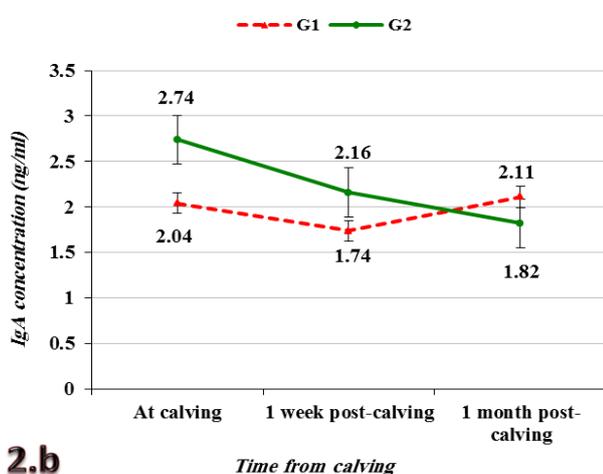
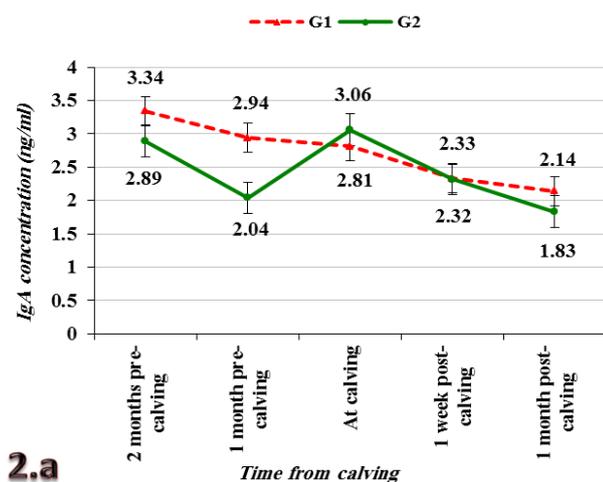


Figure 2. Profile of IgA levels (ng/ml) in She-camels' plasma (A), their milk whey (B) and newborns' plasma (C) (Perfil de los niveles de IgA (ng/ml) en plasma de camellas (A), suero de leche (B) y plasma de los gélfos recién nacidos (C)).

calving (3.06 ± 0.45 vs. 2.81 ± 0.11) when compared to the untreated counterpart. The difference between groups disappeared at one week and one month post calving as shown in **Figure 2A**. IgA concentrations transferred into milk of she-camels showed significant differences

($P < 0.05$) between G1 and G2 at calving (2.74 ± 0.49 vs. 2.04 ± 0.51) and at one week (2.16 ± 0.54 vs. 1.74 ± 0.15), but not at one month post calving as shown in **Figure 2B**, in response to administration of *Bacilli* mixture. An opposite trend was observed for IgA levels in newborn calves' plasma in G1 and G2, since IgA concentrations didn't show differences at any of the experimental periods (calving, one week and one month after delivery (**Figure 2C**)).

PROFILE OF IFN- γ LEVELS (NG/ML) IN SHE-CAMELS' PLASMA, THEIR MILK WHEY AND NEWBORNS' PLASMA

IFN- γ levels in the experimental animals began to show significant elevation at calving in G2 (0.05 ± 0.004) compared to G1 (0.03 ± 0.001), and this trend continued only till one week post calving (0.08 ± 0.005 vs. 0.04 ± 0.001). The level of IFN- γ at one month post calving showed no significant differences between the experimental groups (**Figure 3A**). Similar trend for IFN- γ levels in milk had been observed, showing significant higher results ($P < 0.05$) in G2, at one week (0.04 ± 0.018) compared to G1 (0.02 ± 0.003), while no effect detected at calving or at one month post calving due to administration of *Bacilli* mixture, as shown in **Figure 3B**. Levels of IFN- γ showed numerical differences in calves to dams administered *Bacilli* mixture when compared to their untreated counterparts, as shown in **Figure 3C**.

DISCUSSION

Stimulation and enhancing the immune response of dams to overcome gestation stress during the last trimester of pregnancy and at early lactation is reviewed by Modrak and Stewart (2015, pp. 57-84) and Broadway et al. (2015, pp. 417-427). The participation of all immune cells associated with the gut (macrophages, B and T lymphocytes) is necessary for effective immune response (Mestecky & Mc Ghee 1992, pp. 75-88; Czerkinsky et al. 1993, pp. 106-116).

It is known that she-camel is unlike other female ruminant, where it has what is called epitheliochorial placenta (Fowler & Bravo 1998, pp. 381-429; Dantzer 1999, pp. 325-368). The epitheliochorial placenta is impermeable and prevents intra-uterine passage of antibodies and other immunological factors from mother to fetus during pregnancy (Moffett & Loke 2006, pp. 584-594). The reason of that impermeability is related to its unique structure of six cell layers between the maternal and fetal circulatory (maternal capillary endothelium, uterine connective tissue, uterine epithelium, chorionic epithelium, fetal connective tissue and fetal capillary endothelium) (Koterba et al. 1990) so calf is born with hypogammaglobulinemia and immature immunity at birth (Enders & Carter 2004, pp. 53-59) and hence the maternal protection to fetus during pregnancy postpones the calf specific immunity, (Silva et al. 2013, pp. 68-74).

The innate immune system of fetus or newborn calves is immunosuppressed because of maternal hormones released during late pregnancy (Benesi et al. 2012, 352-356). Therefore colostrum is the only source for immune protection which ensures the survival of

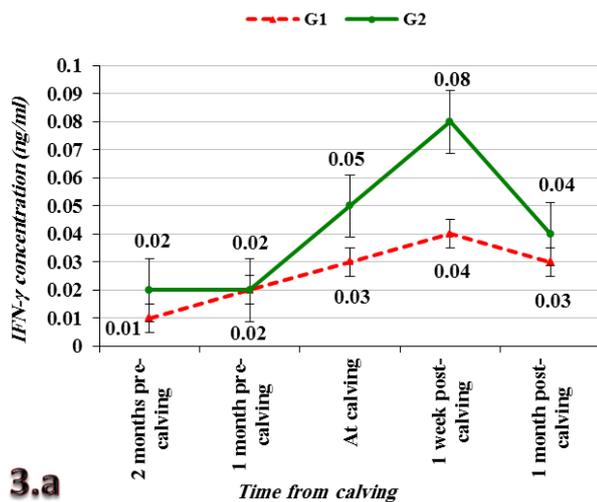
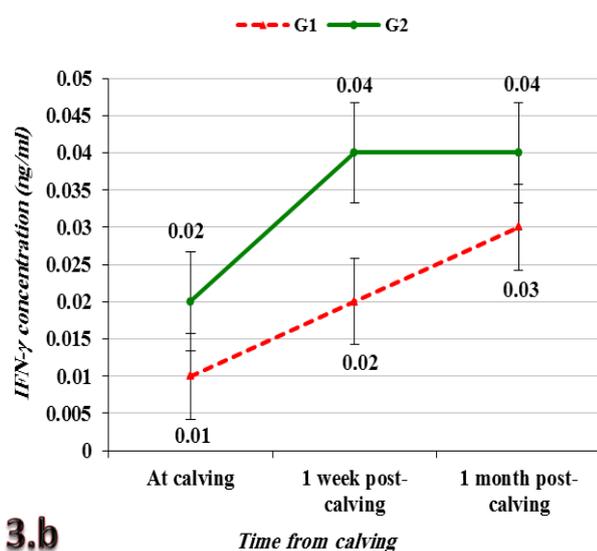
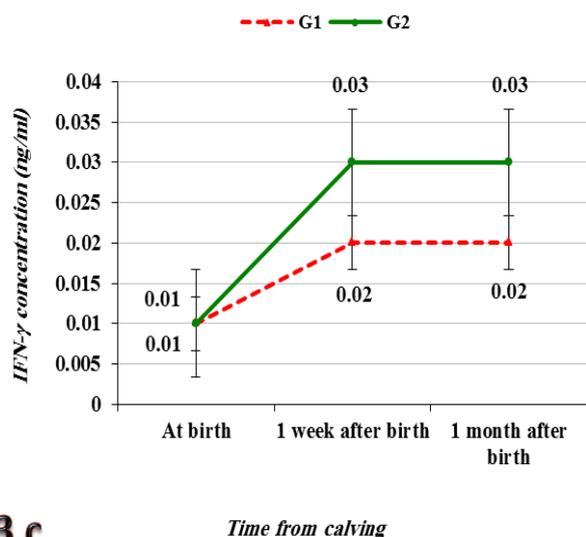

3.a

3.b

3.c

Figure 3. Profile of IFN- γ levels (ng/ml) in She-camels' plasma (A), their milk whey (B) and newborns' plasma (C) (Perfil de los niveles de IFN- γ (ng/ml) en plasma de camellas (A), suero de leche (B) y plasma de los gélfos recién nacidos (C)).

calves in their first months of life by passive transfer of immune factors such as Igs, and cytokines (Silva et al. 2013, pp. 68-74).

Milk is enriched with Igs that come from blood of dam where their levels in milk gradually decrease over time until become very low at 24 hours after birth (Tizzard, 2002). During early lactation Igs in colostrum are absorbed via unselective permeability of epithelial cells in calf's small intestine then are transported through intestinal lymphatic tissue to reach its circulatory system (Riddle 2003, pp. 1-5). This type of natural immunization is called, passive immunity that provides protection for newborn calves after birth (Salmon 1999, pp. 143-155). The majority of Igs in colostrum originate from mother's blood rather than the mammary glands (Portter 1979, pp. 197-205).

Proinflammatory cytokines such as IFN- γ are present in maternal colostrum, is possible to be absorbed by the intestinal mucosa of calves, reaching highest concentrations in newborns bloodstream in the first 72 hours of its life (Madureira 2011), but a calf may have IFN- γ levels prior to colostrum intake, probably due to antigen contact during pregnancy where some microorganisms can cross the placenta and the innate immune system of calves is then the main defense during pregnancy (Gomes et al. 2014, pp. 77-83). IFN- γ is secreted primarily by T helper cells and Natural Killer (NK) cells. IFN- γ decreases with the progression of lactation, being undetectable on the seventh day postpartum (Hagiwara et al. 2001, pp. 59-69).

The aim of our work is to enhance the immunity of pregnant she-camels and hence this reflects on the improvement of their health and their milk immunological value that passively transfers to their newborn calves.

Previous studies on different animal species have revealed strong evidence that oral administration of bacterial spores stimulates the immune system (Anu et al. 2014). *Bacilli* spores are recommended as feed additives because they are strong, stable and resistant to heat so that they could be mixed with animal feed when manufactured. Besides to that, their fate after ingestion is not affected by the acidic condition in stomach or pH extremes of enzymes in the small intestine (Nicholson et al. 2000, pp. 548-572).

In our presented results, at late gestation, *Bacilli* mixture affected IgG level in dams' plasma of the treated group, exactly after one month of oral administration and till one month post-calving when compared to the control group. The peak concentration of IgG level was observed at one week post-calving.

IgG levels either transferred into dams' milk or passively transferred to their newborn calves' blood, were also higher in the group treated with *Bacilli* mixture more than untreated group. Also it was observed that oral administration of *Bacilli* spores enhanced IgA levels in milk more than untreated animals' milk specially at calving and till one week post-calving.

These results are in agreement with Barnes et al. (2007, pp. 1538-1547) who found that oral administration of *B. subtilis* had induced systemic antibody

response to some diseases in mice. Killed *B. subtilis* spores were found to enhance systemic IgG levels and mucosal IgA specific to the influenza in mice (Song et al. 2012, pp. 3266-3277). Rajput et al. (2013, pp. 69-72) proved that IgA level was also found to increase after *B. subtilis* addition to feed. Another early study by Fiorini et al. (1985, pp. 310-312) and Rhee et al. (2004, pp. 1118-1124) had shown a significant increase in B lymphocytes bearing membrane IgA. Kritas et al. (2006, pp. 170-173) reported that *B. subtilis* supplementation in ewe's feed increased average total proteins content in milk. Huang et al. (2008, pp. 195-203 & 2013, pp. 247-252) found that oral administration in mice including *B. subtilis* stimulated cytokine production *in vitro*.

Also *Bacilli* mixture used in our experiment, enhanced IFN- γ levels in blood of pregnant she-camels at calving and reached the peak at one week post-calving more than untreated animals, and levels of IFN- γ transferred through milk of dams was higher in treated groups more than untreated, and so for their newborn calves' blood. *Bacilli* had enhanced IFN- γ levels in dams at calving and till one week after calving.

Our results are in agreement with those results recorded by Huang et al. (2013, pp. 247-252) who had found that *B. subtilis* had significantly increased the levels of proinflammatory cytokines such as IFN- γ by macrophages. Also it was demonstrated that administration of a probiotic mixture of 4 *Bacilli* strains by mice increased IFN production (Maria et al. 2004, pp. S86-90). It was observed *an In vivo* synthesis of IFN in the lymphoid organs when spores of *B. subtilis* were administered to mice (Duc et al. 2003, pp. 4215-4224 & 2004, pp. 2161-2171).

Bacilli spores have shown the ability to interact with the lymphoid tissues of the Gut Associated Lymphatic Tissue (GALT) such as Peyer's Patches (PP), mesenteric lymph nodes (Duc et al. 2003, pp. 4215-4224). PP found in intestinal submucosa and extending to mucosa contain are enriched by lymphocytes, macrophages and some plasma cells that are covered by a unique thin epithelium that contains specialized cells termed Mast cells (M-cells) which are responsible for the uptake and transport of luminal antigens (Rhee et al. 2004, pp. 1118-1124).

Bacilli spores are taken up by M-cells, then carried to PP where they can interact with macrophages and dendritic cells, B and T cells so trigger humoral and cellular immune responses and instruct the host innate and adaptive immunity. This is in agreement with Duc et al. (2004, pp. 1873-1885) and Huang et al. (2012). According to Cebra et al. (1991, pp. 222-226) who termed the phenomenon "Common Mucosal System", the antigen induced B and T cells are able to migrate via lymphatics, reach the systemic circulation and could repopulate the intestine and other distant mucosal sites such as mammary glands and hence the assumption of an increased IgG, IgA and IFN- γ production in milk dams. It had been shown that Igs concentrations in the colostrum, particularly that of IgG, greatly decrease over time. Therefore, the offspring must ingest colostrum during the first six hours of life after birth

so that Igs could be detectable in the newborn's serum, (Koterba et al., 1990).

CONCLUSION

This study indicates that addition of *Bacilli* mixture-based probiotic to the basal diet of pregnant dromedary she-camels is effective in improving some of the treated dams and their newborn. Therefore, we emphasize the inclusion of probiotics in the regular diet of pregnant she-camel in order to enhance its immunity and health, which positively affects their newborn and increases its chance of survival. This systemic chain will improve the economic value of breeding this great animal.

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