CHAPTER 5

DISCUSSION

5.1 Nutritional analysis of date palm and goat milk

Consumption of date palm and goat milk is highly beneficial for improving general health and wellbeing. Nutrients present in date palm and goat milk like carbohydrate, iron, manganese, calcium, magnesium, zinc, and ascorbic acid all contribute to the health and wellbeing of the body. The predominant sugars found in Ajwa date palms are reducing sugars particularly glucose and fructose with concentrations of 31.60g/100g and 29.50g/100g of fresh weight, respectively. The sugar content was higher than those found in the previous study reported by Hamad *et al.*, (2015) with glucose and fructose were 35.4mg/100g and 39.4mg/100g, respectively. The study also detected 13.45mg/100g sucrose which was not detected in this study. The result from this study showed that all sucrose was converted into simple reducing sugar, glucose and fructose during the ripening process which make it taste very sweet. Every 1 kg of fresh Ajwa date palm contains 536mg calcium, 418mg magnesium and 10.70mg iron. The Ajwa flesh was also found to contain trace amount of manganese and zinc of 5.23mg/kg and 2.39mg/kg fresh weight, respectively. In this study, fresh Ajwa date palm was found to contain moderate amount of ascorbic acid of 9.80mg/100g. Previous studies demonstrated that Tunisian date variety, contains ascorbic acid ranging between 17.7mg/100g to 31.86 mg/100g while Bahraini varieties ranging from 2.0 mg/100g to 6.7mg/100 (Allaith, 2008; Chaira *et al.*, 2009). It had been documented that the variation in nutritional composition in Ajwa date palm was a result of genetic variation, soil mineral content and fertilizers used. The maturity stages from Kimri to the Tamer stage notably affects the mineral composition in date palm, in which the sugar content had increased and other constituents like moisture, crude protein,

crude fat, crude fibre decreased (Iqbal *et al.*, 2011). Such variations reflect the diversity of date palm cultivars. More research on the nutritional and biochemical features of the date palm is required.

In this study, the amount of lactose contained in goat milk was 3.38g/100mℓ fresh weight. Glucose, sucrose, galactose, fructose and maltose were not detected. Fresh goat milk contains trace amount of iron and zinc of 0.90mg and 0.99mg/L, respectively. Every 1L of fresh goat milk contains 722mg calcium and 100mg magnesium. These results obtained are lower than those previously reported with 1550mg/L calcium and 142mg/L magnesium were detected in fresh goat milk (Moreno-Rojas et al., 1994). No ascorbic acid was detected in fresh goat milk. Genetic variation, environmental condition and goat farming practice such as duration of lactation, milking type, frequency and duration of milking effects the biochemical composition and quality of milk (Yangilar, 2013). These results suggested that date palm and goat milks are nutritious and beneficial for overall well-being.

5.2 Effects of date palm and goat milk on body weight changes in IDA-induced rat

The decline in rat's body weight showed that IDA adversely affects growth and development. In comparison to the normal control group, the body weights of rats in the negative group decreased significantly after 4 weeks of feeding with the low iron, which is consistent with prior study by He *et al.*, (2019). The final body weight after supplementation of date palm and goat milk was significantly increased, as compared to the negative control, and showed no difference with a positive control group given ferrous fumarate, indicating that date palm and goat milk have positive impacts on improving the growth of IDA rats.

5.3 Effects of date palm and goat milk on the iron bioavailability in IDA-induced rat

HRE is used to estimate the percentage of ingested iron that is absorbed and converted into haemoglobin. Recycled iron from macrophage and senescent erythrocytes are typically used for haemoglobin production. However, if the body iron is inadequate to meet the physiological demands, intestinal iron absorption will be stimulated. During an iron deficiency state, more iron is being absorbed from the small intestines into the circulation. As iron store in the body increases, the iron absorption in the small intestine will decrease. A higher HRE value indicates increase dietary iron utilization (Xiao *et al.,* 2016). In this study, HRE percentage was higher in negative control with low Hb level. A study in the human population also showed that iron absorption was low in iron-repleted subjects but was upregulated considerably in IDA subjects (DellaValle *et al.,* 2015; Thankachan *et al.*, 2012). The efficiency of haemoglobin recovery in anaemic subjects reflects the dietary iron incorporation into haemoglobin throughout the intervention period. The absorption of iron depends on several factors such as the chemical form of dietary iron, organic acids, gastric acid secretion and the presence or absence of enhancers and inhibitors in the food (Mackenzie & Garrick, 2005). Iron must be reduced to the ferrous ion (Fe^{2+}) or bound to a protein such as heme in order to be effectively absorbed. The low pH of gastric acid in the proximal duodenum enables ferric reductase enzyme (Dcytb) to convert insoluble dietary ferric (Fe^{3+}) ions to absorbable ferrous (Fe^{2+}) ions, before being transported into the cell via iron transporter DMT1. This process of iron absorption can either be enhanced or inhibited by certain dietary compounds. Phytate, polyphenol, calcium, animal protein and oxalic acid have been reported to impede dietary iron absorption (Milman, 2020).

Date palm has a significant amount of iron, which, when combined with ascorbic acid, increases iron bioavailability. Ascorbic acid is vital in iron metabolism as it enhances iron uptake into the enterocytes by reducing Fe^{3+} to Fe^{2+} during iron acquisition in the small intestine. Ascorbic acids were also reported to chelate iron and stimulate the synthesis of iron store ferritin (Lane & Richardson, 2014). Numerous prior studies have shown that goat milk provides a better use of iron, which minimizes possible interaction between iron and other minerals and hence, improve the bioavailability of copper, zinc, magnesium, phosphorus and selenium in animal models (Barrionuevo *et al*., 2002, 2003; Campos *et al.*, 2003). A study by López-Aliaga *et al.*, (2009) revealed that, despite the high calcium content in calcium-supplemented goat milk, it consumption had no deleterious impacts on iron absorption in IDA rat. Similar finding was reported by Díaz-Castro *et al*., (2011), in which they discovered that not only does calcium-fortified goat milk favour iron deficiency anaemia recovery, but it also increases the bioavailability of copper, a mineral essential for erythropoiesis.

5.4 Effects of date palm and goat milk supplementation on the RBC parameter and iron profile in IDA-induced rat

In this study, the haematological parameters of iron-deficient state were drastically different from those of a normal condition with a significant reduction in RBC, Hb concentration, PCV, MCV and MCH level. After 2 weeks of anaemic induction there was no significant difference was observed between negative control and normal control in regards to MCV, MCH and MCHC level. MCV, MCH and MCHC may remain relatively constant in IDA due to compensatory mechanisms that occur in response to the anaemia. In IDA, there is a deficiency of iron, which leads to a decrease in haemoglobin production and a decrease in the amount of oxygen-carrying capacity of the blood. To compensate for this, the body may produce smaller red blood

cells which can help maintain the total amount of haemoglobin in the blood by increasing the number of cells. As a result, the values of MCV, MCH, and MCHC may remain relatively constant in IDA, despite a decrease in haemoglobin levels. MCV is a measure of the average volume of red blood cells, while MCH is a measure of the average amount of haemoglobin in each red blood cell, and MCHC is a measure of the concentration of haemoglobin in the red blood cells. In IDA, the compensatory mechanism of producing smaller red blood cells can offset the decrease in haemoglobin content per cell, leading to relatively constant values for these indices.

The iron profile showed reduced serum iron and transferrin saturation as iron available for erythropoiesis is restricted. Serum iron showed a remarkable reduction following iron depletion with a final decrease of 3.3µmol/L as compared to normal control of 39.90µmol/L. In normal condition, transferrin is usually 30% saturated with iron and low transferrin saturation of less than 16% indicates iron deficiency (Thurnham & Northrop-Clewes, 2013). In this study iron depletion decreased transferrin saturation to 6.75%, indicating a serious iron-deficient condition. Previous studies demonstrated similar findings with iron-depleted rats that had decreased Hb concentration and serum iron (Chaeychomsri, 2015; Haro-Vicente *et al*.,2009).

In this study, supplementation of date palm and goat milk in IDA rats had significantly promoted RBC production showing recovery from the iron-deficient state. Improvement in Hb concentration, PCV level, serum iron and transferrin saturation were also noted. Prior works have documented the effectiveness of date palm in improving IDA in both animals and humans. Onuh *et al.*, (2012) reported an improvement in haemopoietic activity in which crude methanolic and crude aqueous extract of date palm fruit significantly increased RBC, Hb and PCV levels in anaemic rats. Zen *et al.*, (2013) reported that date palm juice significantly increased the Hb level

in male rats fed on a low iron diet while Abdelsalam *et al.*, (2014) demonstrated a significant increase in Hb concentration in anaemic late pregnant Najdi ewes when treated with sukkary date palm extract. Ammar *et al*., (2020) showed that Djamaa date syrup administered to wistar anaemic rats increased Hb, RBC, haematocrit and serum iron level. In human study, Irandegani *et al.*, (2019) revealed that consumption of date palm as part of nutritional program increased Hb, haematocrit and serum ferritin level in primary school girls with IDA, while according to Widowati *et al*., (2019), the consumption of date extract significantly increase Hb content of pregnant woman with anaemia. Indrayani *et al*., (2018) showed that in addition to higher Hb level, date palm intervention also gives positive effects in bowel movement in anaemic subjects.

5.5 Effects of date palm and goat milk on the expression of iron metabolism related genes in IDA-induced rat

The body iron status affects the expression of iron metabolism-related genes in both the small intestine and liver. Both organs play a crucial role in iron homeostasis in which the small intestine acts as the main site of dietary iron absorption while the liver regulates iron distribution and storage. Our data documented small intestine and liver expression of DMT1, Dcytb, ferroportin, transferrin, transferrin receptor (TfR), ferritin and hepcidin and that iron level affects the expression of these genes.

In this present study, the qPCR results demonstrated that the expression of DMT1 in the small intestine was indifferent in all groups regardless of the iron status. This result is in contrast with previous findings in which an increased level of DMT1 mRNA was detected in subjects with iron deficiency anaemia (Leong *et al.,* 2003; Martini, *et al.,* 2002; Shawki *et al.,* 2015; Surekha *et al.*, 2020; Trinder *et al*., 2000; Zoller *et al*., 2001). A similar result was seen in the liver, where the DMT1 mRNA expression remains unchanged despite differences in iron status. DMT1 is a

transmembrane protein that involved in iron transport, responsible in transporting ferrous iron into the cell. Urrutia *et al.*, (2013) demonstrated that intestinal inflammation alters the expression of DMT1 and thus interferes with iron metabolism. The abnormality of iron metabolism thus subsequently influences the level of erythropoietic activity.

A possible explanation for those disparate observations can be that there is more than one metal-ion transporter exists for iron metabolism. ZRT/IRT-like protein 14 (ZIP14) is a transmembrane metal ion transporter that is abundantly expressed on the surface of the liver, heart and pancreas. Primarily responsible in transporting zinc into the cytosol and intracellular organelles, Liuzzi *et al.*, (2006) demonstrated that ZIP14 is actively involved in non-transferrin bound iron (NTBI) uptake. Zhao *et al.*, (2010) also reported that ZIP14 mediates the transport of iron-bound transferrin *in-vitro*, exploring a potential role of ZIP14 in iron metabolism. Geiser *et al.*, (2012) documented that ZIP14 is expressed along the intestine and is critical for zinc absorption and normal intestinal epithelium function. DMT1 is also known to be involved in transporting wide range of other divalent metal ions such as copper (Cu^{2+}), zinc (Zn^{2+}), manganese (Mn^{2+}), and cadmium (Cd^{2+}) suggesting these metals share a common uptake pathway albeit with different affinities (Ludwiczek *et al.*, 2007). Further studies, however, are required to specifically define the role of ZIP14 in iron metabolism as currently, DMT1 was the only transmembrane transport protein known to participate in iron transport. Second explanation would be that DMT1 is integral to both dietary iron uptake and macrophage iron recycling, thus it is ubiquitously expressed to maintain normal body iron level. Thus, constant DMT1 expression in small intestine may indicate that iron is constantly being absorbed from dietary site of absorption to compensate for iron loss during daily activity. DMT1 are also crucial for the efficient turnover of iron acquired from

senescent erythrocytes. According to Soe-Lin *et al*., (2010), the loss of DMT1 diminished the ability of macrophages to recycle haemoglobin derived iron; hence, constant DMT1 expression in liver is expected for continual iron recycling.

Iron is largely adopting the oxidised state and it must be reduced before being transported into the cell. Dcytb reduces extracellular ferric iron and other physiological substrates by utilising intracellular ascorbate as an electron donor. Dcytb mRNA has been demonstrated in several previous studies to be highly regulated in iron metabolism pathway (Dupic *et al*., 2002; Frazer *et al*., 2003; Latunde-Dada *et al*., 2004; Muckenthaler *et al*., 2003). In this present study, increased expression of Dcytb mRNA was noted in the small intestine of the negative control group. Increased Dcytb mRNA expression reflects the demand for iron during iron depletion, promoting the availability of reduced iron (Fe^{2+}) for the iron transporter, that subsequently results in enhanced iron uptake during dietary iron absorption. This result corresponds to a report by Dupic *et al*., (2002), in which Dcytb mRNA was globally upregulated in the small intestine of mice fed with no iron diet for 14 days. Moreover, a study by Latunde-Dada *et al.*, (2008) also showed that iron uptake was significantly greater in Dcytb-transfected Caco-2 cells than in cells with control empty vector, further confirming Dcytb as a ferric reductase that can stimulate iron uptake *in-vitro*. The intervention of date palm and goat milk significantly normalized Dcytb mRNA expression, which may indicate improvement in iron absorption. In the liver, the expression of Dcytb mRNA was indifferent regardless of iron status corresponding with previous finding by Latunde-Dada *et al.*, (2002) in which they found that iron status does not appear to modulate Dcytb expression in the liver and also in the spleen.

Iron, once inside the enterocytes, is either stored within intracellular ferritin or be transported across the basolateral membrane through ferroportin to meet the systemic

requirement. As the only identified mammalian iron export proteins, ferroportin is essential in iron homeostasis and intestine-specific ferroportin-knockout mice developed severe systemic iron deficiency and shows iron accumulation in enterocytes (Donovan *et al.*, 2005). Ferroportin is a transmembrane iron transporter and has been described to be expressed abundantly in tissues that transport large amounts of iron such as in i) duodenal enterocytes that absorb dietary iron, ii) Splenic and liver macrophages that recycle iron from senescent erythrocytes, iii) hepatocytes which store and release iron according to body requirement and iv) placental trophoblast which transport iron from maternal to fetal circulation (Nemeth, 2010). In this study, the mRNA ferroportin expression in the small intestine of negative control was significantly increased, corresponding with earlier finding by Li *et al.*, (2013). Increased in ferroportin expression during an iron-deficient state allows more iron to be transported out from enterocytes into the bloodstream where demand for iron is high for active erythropoiesis. The ferroportin mRNA expression was significantly decreased post-intervention with date palm and goat milk, meaning that less dietary iron is being transported out into the circulation. The expression of ferroportin mRNA in the liver, however, was unaffected regardless of iron status. This result is correspondence to the study by Chiabrando *et al*., (2013) that showed experimentally induced acute anaemia in mice caused ferroportin mRNA level in small intestine and spleen macrophages to be up-regulated, whereas it remains unchanged in the liver and down-regulated in erythroid precursor, showing the specificity of ferroportin regulation. The previous study by Li *et al.*, (2013) also showed a similar result in which ferroportin mRNA expression in the liver remain unchanged following iron deficiency anaemia for four weeks. This result may suggest that hepatocyte as major iron storage is still required for active erythropoiesis, thus iron efflux from this organ is vital. This result can be also

reflected in ferritin expression in the liver, in which ferritin expression was significantly low as compared to normal control. The production of ferritin is majorly triggered by the presence of iron; thus, this result may suggest that a longer recovery time was needed to normalize body iron level following iron-deficient condition.

Hepcidin-ferroportin interaction play a role as a control of systemic iron homeostasis. Hepcidin acts through proteolytic degradation of ferroportin, thus controlling iron efflux into the bloodstream circulation. In the absence of hepcidin, erythropoietic activity enhances dietary iron absorption in the small intestine as well as iron release from recycling macrophages and iron storage in hepatocytes. In this study, downregulation of hepcidin mRNA was noted in the small intestine and liver of negative control group correspondence with previous findings (Khaled *et al*., 2021; Pagani *et al.*, 2019; Mahajan *et al.*, 2017: Diaz-Castro *et al.*, 2014; Frazer *et al.*, 2002). In an iron-deficient state, hepcidin production is reduced to encourage dietary iron absorption and to allow the release of stored iron from macrophages and hepatocytes. This adaptation mechanism subsequently provide a greater iron supply for haemoglobin synthesis (Camaschella, 2018). In this study, the hepcidin-ferroportin relationship was demonstrated in iron-deficient state, in which hepcidin suppression in the liver boosted ferroportin expression in the small intestine, increasing iron efflux into the blood circulation, allowing for compensatory increases in iron acquisition. Post-intervention iron repletion with date palm and goat milk significantly up-regulates hepcidin mRNA in the liver which in turn suppressed ferroportin expression in the small intestine. Albeit there was no significant difference was noted between intervention groups, the positive control given iron tablet showed the highest hepcidin expression of nearly three-fold when compared to other groups post-intervention, suggesting that iron tablet is indeed more effective and faster in restoring iron deficiency. Hepcidin is primarily produced

by the liver and a study by Li *et al*., (2013) showed that the expression of hepcidin mRNA is nearly 32-fold greater in the liver than that of the small intestine. The basis for the hepcidin expression in the small intestine requires further investigation.

Iron storage is a critical component of cellular iron homeostasis since it permits iron to be sequestered in a nontoxic form while simultaneously serve as a reservoir from which iron can be utilised for future metabolic demands. Ferritin is the major intracellular iron storage protein, with the greatest concentrations of ferritin are typically stored in the liver. Ferritin expression is regulated post-transcriptionally (Torti & Torti, 2002) and once required, iron will be released by ferritin degradation. The expression of ferritin mRNA was significantly reduced in the iron-deficient state in both small intestine and liver corresponding with a previous study (Li, *et al*., 2013). In iron-deficient state, the ferritin available in the small intestine and liver were significantly reduced as iron was continuously released into the bloodstream for normal body function. In this study, results showed that iron repletion for 4 weeks with iron tablets, date palm and goat milk was unable to normalise the ferritin level in both small intestine and liver. The total body iron store is reflected in the enterocytes' iron storage. Iron entering the enterocytes become intracellular labile iron temporarily, with extra iron is synthesised into ferritin. When iron demand is low, iron is lost via subsequent exfoliation of intestinal epithelial cells. As only 1-2mg of dietary iron is absorbed daily, the primary sources of plasma iron are senescent red blood cells phagocytosed by the macrophages in the spleen, liver, and bone marrow (Gkouvatsos *et al.*, 2012). Liver houses the largest population of reticuloendothelial macrophages in the form of Kupffer cells, thus during recovery from the iron-deficient state, iron is also being released from the liver to support erythropoiesis. The result showed that additional time may be required to improve total iron body store following iron deficiency. Depending on the

severity of IDA and underlying cause, it may take up to several months to build up iron reserves following iron replacement therapy (Jimenez *et al.*, 2015).

Transferrin is a blood-plasma glycoprotein, that binds to circulating Fe^{3+} preventing it from travelling in its toxic form. Transferrin transports iron through the blood to bone marrow for the production of haemoglobin and erythrocytes and also to various tissue such as the liver and spleen. High transferrin level signifies low iron state, as more transferrin is being produced by liver to use all the iron available in the body (Miller, 2013). In this study, the level of transferrin mRNA was significantly increased in both small intestine and liver of negative control group. This result indicated that cellular iron scarcity increases the expression of transferrin mRNA, allowing more iron to be transported in the circulatory system to supply for erythropoiesis. Post iron repletion with date palm and goat milk significantly down-regulated transferrin mRNA expression in the small intestine, showing no significant difference with normal control group. In the liver, transferrin mRNA expression, however, remains high and unchanged 4 weeks post-intervention. Liver is the primary source of transferrin synthesis with low level of transferrin expression has been documented exists in other organs such as in the brain, spleen, and kidney. The transferrin production is influenced by several factors such as the amount of iron absorbed from the diet, the amount of iron recycled and released by macrophages, and the amount of iron utilized by the bone marrow and other tissue (Gkouvatsos *et al.*, 2012). Developing erythroid cells also heavily depends on transferrin bound iron for Hb synthesis (Muckenthaler *et al.*, 2017). Post intervention with date palm and goat milk decrease transferrin mRNA expression in small intestine indicating normal iron absorption. Meanwhile, increased transferrin mRNA expression in the liver post intervention may indicate on-going demand for transferrin following IDA.

Transferrin receptor (TfR) is a carrier protein for transferrin, ubiquitously expressed at low levels on the surface of virtually all cells and is essential for haematopoiesis and erythropoiesis through the internalization of transferrin-bound iron by endocytosis (Wang *et al.*, 2020). Its expression is however, elevated, in rapidly dividing cells including variety of cancers or during inflammation. In small intestine, TfR also supplies iron to the developing epithelial cells in the intestinal crypts, thus essential in maintaining epithelial homeostasis independently of its function of an iron importer. Intestinal epithelium cells have an extremely short lifetime of 3 to 5 days and have the fastest turnover rate of any fixed-cell population in the body, with estimated $2x10^8$ cell are shed from the small intestine daily (Williams *et al.*, 2015). The new intestinal epithelial cells are generated in the crypts region and will migrate and differentiate as they move apically toward the villus where they are shed at the extrusion zone at the villus tip. In this study, the expression of the TfR gene during iron deficiency was significantly upregulated in both small intestine and liver. This finding is corresponding with previous studies (Li *et al.*, 2013; Dupic *et al*., 2002; Mckie *et al.*, 1996; Lu *et al.*, 1989) in which they found that TfR mRNA expression increased in iron-deficient conditions. Increased TfR mRNA expression in small intestine encourages more iron to be taken up from dietary absorption site to compensate for erythropoiesis activity and also to maintain intestinal integrity. The level of iron in the labile iron pool (LIP) is noted to strongly regulate the expression of TfR Low iron levels in LIP cause increased expression of TfR and vice versa (Jamnongkan *et al.*, 2017). In an iron-deficient state, a low LIP level up-regulates TfR expression, which then will promote transferrin-mediated iron uptake. TfR mRNA expression was significantly reduced in both the small intestine and the liver after iron repletion with date palm and goat milk.

5.6 Effects of date palm and goat milk on the expression of the iron metabolism-related protein on the small intestine and liver of IDA-induced rat

In small intestine, DMT1 protein showed prominent immunoreactivity on the apical membrane of enterocytes with a striking abundance at the brush border membrane. DMT1 staining was specific for enterocytes, as goblet cells did not stain for the protein. DMT1 expression is strongest at the brush border of the apical pole in correspondence to the primary function of DMT1 as the iron importer that is responsible for the unidirectional uptake of ferrous iron released by dietary digestion with the help of ferrireductase present within the brush border. This finding is consistent with previous findings (Canonne-Hergaux *et al*., 1999; Griffiths *et al*., 2000; Zoller *et al*., 2001). In normal liver, DMT1 protein was expressed on the hepatocyte's plasma membrane with an even cytoplasmic expression. No staining was observed in Kupffer cells corresponding with previous findings (Trinder *et al*., 2000). DMT1 protein expression in the small intestine and liver of all treatment groups showed a similar pattern of localisation and staining intensity with that of the normal control. These findings were consistent with the observed mRNA level, in which the DMT1 expression remains unchanged in all groups irregardless of iron level. As previously mentioned, this may due to either the existence of other metal-ion transporter for instances, ZIP4 that transport iron into the cell or that DMT1 is being ubiquitously expressed in both small intestine and liver to maintain body iron level, following iron deficient state.

In normal small intestine, Dcytb protein was observed within the enterocytes, along the villi axis consistent with that previously reported (McKie, 2001). Increased expression of Dcytb was noted in the negative control although not significant, suggesting that Dcytb was indeed necessary for iron absorption corresponding with previous literatures (Choi *et al.*, 2012; McKie, 2008). Staining for reductase activity

was lower in the small intestine of the supplemented group, associated with reduced duodenal ferric reductase activity. In liver tissue of normal control, Dcytb expression is mainly cytoplasmic, with Kupffer cell stained negative. Meanwhile, in negative control group, increased staining intensity was observed with both nucleus and Kupffer cell stained positive for Dcytb protein. A similar expression was seen in the date palm treated group with milder immunoreactivity. Meanwhile, the Dcytb protein expression in positive control group, goat milk treated group and date palm and goat milk treated group showed similar expression with that of normal control with an even cytoplasmic distribution and negative reactivity in the nucleus and Kupffer cells. Discovered in 2001 by McKie *et al*., (2001) , there are limited literatures on the Dcytb expression, with most studies have relied on the expression of Dcytb in the small intestine. Dcytb has been documented to be found in lung epithelial cells, in plasma membrane of mature RBC and in both epithelial and myoepithelial cell of breast tissue. To our knowledge, no prior studies have examined the localisation of Dcytb in the liver, thus further investigation is required.

In this study, ferroportin protein expression was localised predominantly within enterocytes cytoplasm, concentrated to the basal and apical membranes. Thomas & Oates (2004) demonstrated that ferroportin was found throughout the enterocyte with strong expression along the basal membrane. Some expression was seen above the nucleus at the apical region with weak expression was also found at the lateral membrane. D´Anna *et al.*, (2011) demonstrated that the small intestine of healthy mice, in conjugation with ferroportin apical expression, also expressed slight basolateral ferroportin expression. Small intestine is one of the major sites for ferroportin expression and the localization of ferroportin along the basal region of enterocytes is expected for the export of dietary iron to satisfy the erythropoietic demands. The

expression of ferroportin on the apical region indicate that ferroportin may also plays an additional role other than iron exporter in the iron metabolism. Another study by Oates & Thomas, (2005) found that ferroportin mRNA was localised only to the enterocytes villus and that some goblet cells also expressed ferroportin which was not found in this study. They speculate that ferroportin may acts as a modulator of iron uptake on the apical membrane by modulating the expression and/or activity of DMT1. Meanwhile, study by Yang *et al.*, (2005) revealed that ferroportin is located on the apical membrane and not the basolateral membrane of airway epithelial cells of humans and rodents, indicating that ferroportin may potentially play a significant role in iron detoxification. In negative control, enhanced ferroportin expression was observed on the apical and basal membrane of enterocytes with weak expression was also detected on the lateral membrane. In addition to membrane expression, strong intracellular expression was also seen immediately above the nucleus and on the basal cytoplasm. This finding was consistent with prior studies that demonstrated iron deficiency enhanced ferroportin expression (D´Anna *et al*., 2011; D'Anna *et al*., 2009; Donovan *et al*., 2005; Oates & Thomas, 2005; Thomas & Oates, 2004b; Abboud & Haile, 2000). This expression was markedly reduced during iron repletion where the rats were supplemented with date palm and goat milk. Oates & Thomas (2005) found that ferroportin was also seen on the microvilli under high power imaging where the expression was diminished in iron loaded tissue. Thus, the expression of ferroportin in enterocytes was inversely related to iron loading. In liver, the distribution of ferroportin was limited to hepatocytes with an even cytoplasmic expression, with Kupffer cells remains negative. This result is inconsistent with the previous finding documented by D´Anna *et al.*, (2011) where in healthy mice, positive immunoreactivity was found on the Kupffer cell with mainly cytoplasmic expression and slight expression at the plasma

membrane. No staining was found in hepatocytes and sinusoidal endothelial cells suggesting macrophage is the prominent ferroportin positive cell in the liver. Naz *et al.*, (2012) showed that ferroportin immunoreactivity was found mainly in the nuclei of hepatocytes, while Yang *et al.*, (2002) demonstrated that ferroportin was apparent on the surface of hepatocytes and Kupffer cells.

In the normal small intestine, TfR protein was detected on the crypts and villi of the epithelial cell of enterocytes with intensity decreased as epithelial cells migrated apically toward the villus tips. TfR was shown to be prominent on the basal and lateral membrane of intestinal epithelial cells, with diffuse intracellular cytoplasmic expression, corresponding with prior studies by Deaglio *et al.*, (2002) and Kolachala *et al.*, (2007). In the negative control, a significant increase in staining intensity was observed on the apical villi, lateral and basolateral membrane of the epithelial lining with goblet cell remained negative. The TfR density is inversely proportional to iron nutritional status, as more iron is needed to be absorbed from the dietary absorption site to compensate for the lack of iron in an iron-deficient state. As iron become abundant, TfR accumulation in the apical area of enterocyte villi decreased. In positive control, a similar pattern of TfR expression with normal control was noted, with TfR protein expression concentrated on the basolateral membrane of epithelial cells. These results suggest that iron repletion with date palm and goat milk reduces the expression of TfR protein in the small intestine. The immunohistochemistry result was in line with the gene expression result, which showed that TfR expression in the small intestine increases during the iron deficiency and that supplementing with date palm and goat milk normalises TfR expression to that of normal control. In normal liver, the expression of TfR protein was limited within hepatocytes with an even cytoplasmic localization. No stain was detected in endothelial and bile duct cells. In negative control

rats, the TfR expression is retained within the hepatocytes with an increased TfR expression on the plasma membrane of hepatocytes. The positive and intervention groups showed similar TfR expression to that of the normal control.

5.7 Benefits of date palm and goat milk supplementation on IDA

The increased iron utilisation with the date palm and goat milk might be attributed to a variety of beneficial nutritional factors. Date palm is high in carbohydrates, dietary fibre, vitamins, and minerals and is widely recognised as an excellent food that provides a variety of essential components with numerous health benefits. Date palm contains a high percentage of carbohydrates with each date providing about 280 kcal/100g in the form of reducing sugars, such as fructose and glucose that are readily absorbed during digestion, providing instant energy (Younas *et al*., 2020). Date palm is rich in ascorbic acids, with every 100g of date palm containing 9.80mg of ascorbic acid. Ascorbic acid is vital in iron metabolism as it enhances iron uptake into the enterocytes by reducing Fe^{3+} to Fe^{2+} during iron acquisition in the small intestine. Ascorbic acids were also reported to chelate iron and stimulate the synthesis of iron store ferritin (Lane & Richardson, 2014). The combination of high carbohydrates and iron found in date palm can provide a beneficial energy boost, which is beneficial for IDA population which are often experience fatigue. Additionally, date palm also contains minerals such as zinc, magnesium, iron, and calcium that are essential for optimum growth and maintenance of the human body. Date palm particularly contains a high concentration of calcium and magnesium which is essential for bone mineralisation, muscular relaxation, immunological function, nerve transmission and several other cellular functions (Al Alawi *et al*., 2018; Beto, 2015). Date palm are excellent sources of dietary fibre with date palm flesh reported total dietary fibre

ranging between 6.2 to 8.9% (Khalid *et al*., 2017). Dietary fibres induce satiety and have a laxative effect thus can prevent a spectrum of gastrointestinal disorders (Mrabet *et al.,* 2019). Besides, date palm fruit is also rich in polyphenols such as carotenoids, phytosterols, phenolic acids, flavonoids, tannins and sterols which give health benefits when taken as a medicine drug or as a part of the daily diet (Al-Alawi *et al.,* 2017). Polyphenol acts as a potent natural antioxidant with preventive capability for many chronic diseases. A meta-analysis by AlFaris *et al*., (2021) demonstrated that the total phenolic content of 22 selected studies on date palm was high, ranging from 4.36 to 753.30 mg GAE/100g DW with high concentration was found in ripe date palm produced in Saudi Arabia compared to those produced in Algeria, Tunisia and Iran. Pharmacological studies both *in-vitro* and *in-vivo*, showed therapeutic effects of date palm on health as their consumption has been linked to anti-inflammatory activity (Zhang *et al.,* 2013), anti-cancer activity (Siddiqui *et al.,* 2019), antifungal activity (Belmir *et al.,* 2016) anti-bacterial activity (Samad *et al.,* 2016), antihyperlipidemic activity (Alqarni *et al*., 2019) and protective activity against toxicity (Bashandy *et al.,* 2016).

Meanwhile, goat milk contains soluble proteins such as β-lactoglobulin, α-lactoalbumin and serum albumin that favour iron absorption in small intestines (López-Aliaga *et al*., 2010). It also contains a high number of medium-chain triglycerides (MCT), a form of saturated fatty acid with 6-10 carbon that is readily absorbed within the intestinal cells, contributing to easier and more efficient digestion. MCT is rapidly hydrolysed and metabolised for energy discharge with a lower deposit of fat. It escalates the synthesis of protein carriers that lead to increased nutrient absorption (Díaz-Castro *et al.,* 2015). A higher level of iron digestibility was reported in rats with distal small intestines resection when supplemented with goat milk. Greater iron content was

observed in the liver, spleen and sternum, suggesting that short-chain fatty acids favour intestinal adsorption after resection (Barrionuevo *et al.*, 2002). López-Aliaga *et al.*, (2009) revealed that despite its high calcium content, goat milk minimises calcium-iron interaction and so has no negative effect on iron absorption. Similar finding was reported by Díaz-Castro *et al*., (2011), in which they discovered that not only does calcium-fortified goat milk favour iron deficiency anaemia recovery, but it also increases the bioavailability of copper, a mineral essential for erythropoiesis. Numerous prior studies have shown that goat milk provides a better use of iron, which minimizes possible interaction between iron and other minerals and hence, improve the digestion and utilization of copper, zinc, magnesium, phosphorus and selenium in animal models (Barrionuevo *et al*., 2002, 2003; Campos *et al*., 2003). In addition, various dietary components in goat milk play vital roles in iron utilisation. The high content of Vitamin D in goat milk promotes erythropoiesis by increasing erythroid progenitor proliferation (Smith & Tangpricha, 2015) while vitamin A influences iron metabolism by mobilizing available iron from the ferritin store to form haemoglobin (Michelazzo *et al.*, 2013*)*. A systematic review also reported the beneficial effect of goat milk in iron deficiency anaemia (Zahir *et al*., 2017). Numerous studies showed the promising effects of date palm and goat milk on hemopoiesis in anaemic subjects. It is worth mentioning that consumption of date palm and goat milk singly or in combination have good iron bioavailability and are excellent natural sources for iron deficiency.