THE EFFECTS OF DIFFERENT FORMS OF VITAMIN A ON INTESTINAL HEALTH: INSIGHTS FROM A GENETIC MOUSE MODEL OF VITAMIN A DEFICIENCY

by

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ABSTRACT OF DISSERTATION

The Effects Of Different Forms Of Vitamin A On Intestinal Health:

Insights From A Genetic Mouse Model Of Vitamin A Deficiency

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Vitamin A deficiency (VAD) is one of the most widespread micronutrient deficiencies affecting hundreds of millions of children and pregnant women in over half of the countries worldwide, despite the implementation of numerous intervention programs. Emerging evidence suggests that VAD can impact intestinal microbiota diversity and fitness suggesting a combined effect of dietary vitamin A and status. Not only vitamin A can be obtained from food of animal origin as preformed vitamin A, but also from vegetables and fruits containing provitamin A carotenoids, mainly as βC. In fact, βC is the most abundant dietary source of vitamin A worldwide, and often the sole source of the vitamin in certain regions of the world. Yet, its impact on intestinal functions and microbiome is unknown, especially during VAD.

The goal of this study was to understand the impact of different forms of dietary vitamin A (preformed retinoids or provitamin A carotenoids) *vs.* vitamin A status on intestinal health during VAD. To address this question, we used a genetic mouse model of VAD, the *Lrat-/-Rbp-/-* mice. Unable to store and deliver retinoids (vitamin A and its derivatives) to peripheral tissues, these mice display a tenuous vitamin A status. Fecal 16S rRNA gene analysis showed that VAD in our genetic model was concomitant with fecal microbial dysbiosis and dietary vitamin A did not impact the fecal microbial taxonomic

profile. Also, VAD resulted in impaired structure and functions of the intestinal barrier as manifested by reduced mucins and antimicrobial defense, leaky gut, increased inflammation and oxidative stress, and impaired mucosal immunocytokine profiles. We also showed that that β-carotene, as the sole source of the vitamin, not only improved the vitamin A status of the vitamin A deficient mutant mice but also improved intestinal dysfunctions and modified the taxonomic profile of the fecal microbiome of VAD.

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LIST OF ABBREVIATION

Abbreviation	Meaning
Alpi	Intestinal alkaline phosphatase
Alppl2	Alkaline phosphatase,
ApoE	Apolipoprotein E
ARAT	Acyl-CoA:retinol acyltransferase
BCO1	β-carotene-15,15'-oxygenase
BCO2	β-carotene-9',10'-oxygenase
CRBP2	cellular retinol binding protein 2
CLDN	claudin
DGAT1	diacylglycerol acyltransferase 1
DKO	double knockout
GC	gas chromatography
Gpx	glutathione Peroxidase
Hifa-1	hypoxia-inducible factor-1
H&E	Hematoxylin and eosin
HPLC	High performance liquid chromatography
HSC	Hepatic stellate cells
ILC	innate lymphoid cell
Il	interleukin
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LpL	lipoprotein lipase
LRAT	lecithin:retinol acyltransferase
Lrat$^{-/-}$	lecithin:retinol acyltransferase-deficient
MUC	Mucin
PrxIII	peroxiredoxins
P22phox	cytochrome b-245 alpha chain
RA	retinoic acid
RAR	Retinoic acid receptor
RBP4	retinol-binding protein

Rbp4$^{-/-}$	retinol-binding protein-deficient
RegIII	Regenerate isle III
REH	retinyl ester hydrolase
ROH	retinol
RXR	Retinoid X receptor
SCFA	Short chain fatty acid
Sod	superoxide dismutase
SR-B1	scavenger receptor class B
STRA6	Stimulated by retinoic acid 6
TG	Triacylglycerol
TJ	Tight junction
Tnfα	Tumor necrosis factor-alpha
VA-def	vitamin A deficient diet
VA-suf	vitamin A sufficient diet
VA	vitamin A
VAD	vitamin A deficiency
VAS	vitamin A sufficiency
WT	Wild-type
ZO	Zonnula occluden

CHAPTER 1 :

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

This Introduction will provide an overview of vitamin A absorption, metabolism and current methodology to assess vitamin A status. These topics will be followed by a review of the current knowledge on how vitamin A deficiency impairs morphological and physiological functions of the gastrointestinal tract as well as the intestinal microflora. Lastly, this introduction will address current knowledge related to the modulatory role of β-carotene as provitamin A and antioxidant in maintain intestinal health.

1.2 VITAMIN A

1.2.1 Vitamin A definition

The terms vitamin A refers to *all-trans*-retinol (Blaner et al., 2016) whereas the term retinoids refers to the totality of all the different natural metabolites of vitamin A, including retinol, retinyl ester (its storage form) and retinaldehyde and retinoic acid (its active forms), as well as the synthetic chemical species resembling the *all-trans*-retinol structure, with or without the biological activity of vitamin A (Blaner et al., 2016). The term retinoids is more commonly used in the literature when the molecular action of these compounds is considered (Blaner et al., 2016).

1.2.2 Vitamin A and dietary sources

Vitamin A is an essential fat-soluble micronutrient that cannot be synthesized in mammalian cells (Tanumihardjo, 2015). Therefore, humans and other mammals must acquire it through the diet. In humans, an average of 800 μg of vitamin A is recommended to maintain an adequate vitamin A storage in the body (Miller et al., 2002). It is worth mentioning that the safe amount of daily vitamin A intake is the amount that is needed to

maintain all the vitamin A related biological functions, as well as the minimum liver storage of 20 μg vitamin A/g of tissue (Miller et al., 2002). Vitamin A is predominantly found in animal-based foods (liver, fish, egg, milk and milk products) as preformed vitamin A, mainly retinol and retinyl esters. It can also be obtained as provitamin A from fruits and vegetables, mainly β-carotene (βC), α-carotene and β-cryptoxanthin with the highest amount in carrots, sweet potatos, pumpkin, kale, spinach, collards and squash (Harrison, 2012). It has been estimated that, in developing countries, more than 70% of the dietary retinoid comes from provitamin A carotenoids, while in industrialized countries up to 75% of the total dietary retinoid is obtained as preformed retinoids (Li et al., 2014).

1.3 INTESTINAL ABSORPTION AND TISSUE METABOLISM OF VITAMIN A

1.3.1 Intestinal vitamin A absorption and circulation

The proximal part of the small intestine is the primary site where dietary retinoids (mainly *all-trans*-retinol and its fatty acids esters) and pro-retinoid carotenoids are absorbed (Goodman and Blaner, 1984). Upon being resealed from the food matrix during digestion, retinyl esters are hydrolyzed in the intestinal lumen by the synergistic action of the pancreatic enzymes triglyceride lipase and lipase-related protein 2, as well as by intestinal phospholipase retinyl ester hydrolases (Chelstowska et al., 2016; Reboul et al., 2006). It is believed that free retinol (0.5 to 130 μM), either from the diet or from dietary retinyl esters, can be taken up by enterocytes through a simple diffusion mechanism (During and Harrison, 2007). However, other studies showed that at physiological concentration, retinol absorption occurs through saturable carrier-mediated action, while passive diffusion prevails at pharmacological doses (During and Harrison, 2007).

Upon uptake by the enterocytes, retinol binds to cellular retinol binding proteins (CRBPII or RBP2). CRBPII (~15 KDa), which belongs to the fatty acid–binding protein (FABP) family of intracellular lipid–binding proteins, is exclusively express in the small intestine, mainly jejunum, where it binds to *all-trans*-retinol as well as monoacylglycerols including 2-arachidonoylglycerol (2-AG), 2-oleoylglycerol (2-OG), 2-lineoylglycerol (2-LG), and 1-AG (Lee 2020). Within the enterocytes, retinol bound to CRBPII serves as a substrate for the esterification by lecithin:retinol acyltransferase (LRAT) enzyme to synthesize retinyl esters (Blaner et al., 2016; O'Byrne and Blaner, 2013). LRAT, a 25 KDa membrane-bound protein that belongs to the NlpC/P60 superfamily, is the key enzyme that synthesizes retinyl esters in many tissues, with relatively higher activity in liver, testis, intestine and eyes (Golczak and Palczewski, 2010). LRAT transfers long-chain fatty acyl moieties, such as palmitic, stearic, oleic, and linoleic acid, present at the sn-1 position of the membrane bilayer phosphatidylcholine, to retinol (Golczak and Palczewski, 2010; O'Byrne and Blaner, 2013). However, trace amount of retinyl esters in the eye, liver and lung of *Lrat*$^{-/-}$ mice suggest that another enzyme(s) is able to esterify retinol in these tissues (O'Byrne et al., 2005). An alternative retinol esterifying activity, utilizes acyl CoAs as donors of the fatty acyl moieties (acyl CoA:retinol acyltransferase; ARAT), although the molecular identity of this enzyme remains unclear to date (Randolph et al., 1991). The literature suggests that this activity, unlike the LRAT enzyme, can esterify free retinol (Blomhoff, 1994), and especially excess of retinol when CRBPII is saturated by large doses of vitamin A (Blomhoff, 1994). One of the potential candidate enzymes with ARAT activity is diacylglycerol acyltransferase1 (DGAT1), at least in the intestine. Indeed, at a physiological oral dose of retinol (6μg), residual retinyl esters were found in the intestinal

chylomicrons of the *Lrat*-deficient mice, but not in those of mice lacking both *Lrat* and *Dgat1* (Wongsiriroj et al., 2008a). Interestingly, when an oral pharmacological dose of retinol (1mg) was administered to mice lacking both *Lrat* and *Dgat1*, retinyl esters could still be detected in the small intestine, suggesting the existence of yet another enzyme with esterification activity, at least in this organ (Wongsiriroj et al., 2008a). In the intestine the major forms of retinyl esters is retinyl palmitate (up to 70%), but other forms of esters such as retinyl oleate, linoleate, and stearate can also be found (During and Harrison, 2007). However, the proportion of retinyl esters appears to be substrate- and enzymatic activity-dependent. For example, more retinyl palmitate and less linoleate and oleate are present in the intestine in the presence of LRAT (O'Byrne et al., 2005). However, in the absence of intestinal LRAT, retinol is the main retinoid incorporated in the chylomicrons and the remaining retinyl esters within chylomicrons are more representative of an ARAT activity, such as linoleate and oleate (O'Byrne et al., 2005).

In the postprandial period, retinyl esters along with other dietary lipids are packaged in chylomicrons to be secreted from the basolateral side of the enterocytes into the lymph (**Fig. 1.1**) (Harrison, 2012). Chylomicrons are intestinal lipoproteins produced in enterocytes from dietary lipids and mainly consisting of triacylglycerols, cholesterols and phospholipids (Hussain et al., 2005). Dietary lipids are conjugated with apolipoproteins (apoB-48) in the endoplasmic reticulum and Golgi to form mature chylomicrons (Dash et al., 2015). The assembly and secretion of chylomicrons is highly regulated by the activity of microsomal triglyceride transfer protein (MTP) that resides in the endoplasmic reticulum lumen (Quadro et al., 2019). MTP binds and chaperones lipids to the nascent apoB to avoid abnormal folding and degradation by proteasomes and help in the intracellular assembly of

apoB lipoproteins, such as chylomicrons and very low density lipoproteins (VLDL) (Hussain et al., 2011). Retinoids within the chylomicrons are secreted into the lymph and from here in the general circulation through the thoracic duct. Depending on the retinoid content of the ingested food, postprandial retinyl esters in the circulation can reach a concentration of 5-10 μM; however, in the fasting state this concentration will drop to 100-200 nM (Li et al., 2014).

In the general circulation, lipoprotein lipase (LPL) remodels chylomicrons to smaller retinyl ester-containing particles, namely chylomicron remnants. Specifically, chylomicron remnants are the result of LPL hydrolysis on triglycerols, and acquisition of apolipoprotein E (apoE) from circulating lipoproteins (Redgrave, 2004). More than 75% of the retinyl ester within chylomicron remnants is cleared by the liver, the largest storage site of vitamin A in the body, whereas the remaining is cleared by extrahepatic tissues (Li et al., 2014).

1.3.2 Hepatic vitamin A metabolism and storage

Retinyl esters in the chylomicrons are cleared out from the circulation through the cell surface LDL receptor, that has high affinity to apoE (Cooper, 1997; Redgrave, 2004). During vitamin A sufficiency, upon uptake of vitamin A by the hepatocytes, retinyl esters are rapidly hydrolyzed to retinol by retinyl ester hydrolysis (REHs) to be transferred to hepatic stellate cells (HSC or Ito cells) for storage (**Fig. 1.1**) (O'Byrne and Blaner, 2013). Currently, it is not entirely clear how vitamin A is transferred from hepatocytes to hepatic stellate cells for storage. Cellular-retinol binding protein I (CRBPI or RBP1), which is found in both hepatocytes and stellate cells, is essential for efficient retinyl ester synthesis in the liver by LRAT (Herr and Ong, 1992). Indeed, in the absence of the CRBPI in mice,

hepatic retinyl esters dropped significantly likely due to the impaired delivery of retinol to LRAT for esterification (Ghyselinck et al., 1999). However, CRBPI it is not a secreted protein, therefore cannot leave the intracellular environment to transfer retinol from hepatocytes to HSC (Shirakami et al., 2012). In contrast, being a secreted protein, it was proposed that RBP could shuttle retinol from hepatocytes to HSC (Blomhoff et al., 1988). However, later works demonstrated that this was not the case (Sauvant et al., 2001). It is also possible that this transfer may occur *via* small lipid-rich particles which carries retinoids, but this hypothesis has not been confirmed (Shirakami et al., 2012). HSC, that constitute 5-8% of the liver cells, store more than 90% of hepatic vitamin A as retinyl esters in lipid droplets (Shirakami et al., 2012) (**Fig. 1.1**). The size and number of lipid droplet correlate with the dietary retinoids intake. Indeed, during vitamin A sufficiency the number and size of lipid droplets increase compared with vitamin A insufficiency (Blaner et al., 2009). When vitamin A is needed by the extrahepatic tissues, retinyl esters within the stellate cells are hydrolyzed to retinol and transferred to hepatocytes (O'Byrne and Blaner, 2013), once again through a mechanism that has not been fully elucidated. In the hepatocytes, one molecule of retinol binds noncovalently to retinol-binding protein (RBP or RBP4) to form the holo-RBP complex to be secreted into the bloodstream for distribution to the peripheral tissues (Shirakami et al., 2012). RBP is a 21 KDa protein with a single binding site for retinol, synthesized predominantly in the liver, even though it is expressed in other tissues including adipose, intestine, retinal pigment epithelium, kidney and ovary (Shirakami et al., 2012). Retinol-RBP circulates in the bloodstream in a complex with another serum protein called transthyretin (TTR) also mainly synthesized by liver. TTR is a homo-teratmer with individual subunits of 14 kDa (Kanda et al., 1974). The

formation of this ternary complex increases the molecular weight of the complex retinol-RBP and reduces its filtration through the kidney glomeruli (Gaetani et al., 2002; Monaco et al., 1995).

Whereas after a vitamin A-rich meal retinyl esters in chylomicrons can account for the majority of circulating vitamin A, in the fasting state, retinol-RBP constitutes the major form of vitamin A in the bloodstream (Blaner, 2007). The plasma concentration of retinol (bound to RBP) is tightly regulated and maintained within a specific range, i.e. 2–3 μM in humans and about 1-1.5 μM in mice, regardless of normal variations in daily dietary retinoid intake (O'Byrne and Blaner, 2013; Quadro et al., 2000).

Deviations from these ranges, in humans, only occur under pathological conditions, including blindness, anemia, infectious diseases, severe diarrhea (Wiseman et al., 2017) as well as metabolic disorders (Thomas-Valdés et al., 2017).

The mechanism(s) whereby retinol bound to RBP is taken up by extrahepatic tissues is another controversial aspect of this field of research, devided between those who believed that this uptake could occurs *via* passive diffusion and those who claimed the existence of a specific RBP receptor (Blaner, 2007). It was only in 2007 that this cell surface receptor was identified by Sun and colleagues (Kawaguchi et al., 2007) as the Stimulated by Retinoic Acid 6 (STRA6) protein, at least in certain tissues such as eye, female reproductive tissues and placenta where retinoids uptake is significantly high (Kawaguchi et al., 2015, 2007). STRA6 is a 74 kDa multi-transmembrane transporter that functions as a specific receptor for RBP mediating retinol uptake (Chen et al., 2016; Kawaguchi et al., 2007). It has been proposed that the coupling of STRA6 with LRAT enzyme is essential to increase the retinol uptake (Amengual et al., 2012) and that STRA6

can also facilitate the efflux of retinol from cells by catalyzing the release of retinol from RBP (Isken et al., 2008; Kawaguchi et al., 2012). Additionally, it has been proposed that STRA6 also facilitates retinol exchange between intercellular CRBPI and extracellular RBP (Kawaguchi et al., 2012). Overall, the molecular details of the transfer of retinol across the plasma membrane still need to be fully elucidated.

1.4 RETINOIC ACID SYNTHESIS AND ITS ACTIVITY AS A TRANSCRIPTIONAL REGULATOR

After plasma retinol is taken by target cells, retinol is converted to retinoic acids, the biologically active form of vitamin A, in two oxidative steps. The first one is the reversible oxidation of retinol into retinaldehyde. This reaction can be catalyzed by two different family of enzymes. The cytosolic alcohol dehydrogenases (ADHs) belong to the medium-chain dehydrogenase/reductase (MDR) superfamily of proteins. The ADHs familiy of proteins comprises six classes of ADH enzymes that are NAD^+-dependent (Belyaeva et al., 2019; Kedishvili, 2016). It is likely that the ADH enzymes contribute to the oxidation of retinol postnatally in specific tissues during vitamin A excess (ADH1) or deficiency (ADH4) but that they are not essential for atRA biosynthesis from a physiologically relevant supply of vitamin A during embryogenesis or adulthood (Kedishvili, 2013). Therefore, under extreme dietary conditions, ADHs appear to play a role as backup enzymes. Independent from the cytosolic ADH enzymes, the microsomal dehydrogenases (mRDH) of the short-chain dehydrogenase/reductase (SDR) superfamily of proteins can also catalyze the conversion of retinol to retinaldehyde as well as the reverse reaction from retinaldehyde to retinol (Kedishvili, 2016). This oxidoreductase activity in the cells appears to be the rate limiting step in the retinoic acid biosynthesis by maintaining

steady-state levels of retinaldehyde (Kedishvili, 2016). Since high levels of retinaldehyde as well as retinoic acid can be toxic, it is not surprising that the production of retinaldehyde is tightly controlled. Members of two families of SDRs are involved in the regulation of retinoic acid homeostasis, SDR16C and SDR7C (Kedishvili, 2013). Interestingly, the two groups show distinct differences in their cofactor preferences: most members of the SDR16C family (except for DHRS3 mentioned below) have higher binding affinities for NAD(H) as cofactor, while SDR7C family members prefer NADP(H) cofactor. If retinol and retinaldehyde are equally available, the cofactor preference largely determines the direction of the reaction *in vivo*. In general, the concentration of the oxidized NAD^+ greatly exceeds that of NADH (1000:1), while the concentration of NADPH exceeds that of $NADP^+$ (1:100) (Liu et al., 2007). Thus, the NAD(H)-dependent oxidoreductases usually function in the oxidative direction, whereas the NADP(H)-dependent enzymes function in the reductive direction. Belyaeva and colleagues showed that RDH10 and DHRS3 (two members of the SDR16C family), despite existing in the cells as individual proteins, can form a complex by protein-protein interaction that can activate and stabilize the function of each of the individual proteins to maintain homeostatic concentrations of retinaldehyde and ultimately retinoic acid (Belyaeva et al., 2017). When bound to RDH10, DHRS3 recycles retinaldehyde produced by RDH10 back to retinol attenuating the output of retinaldehyde and ensuring the robustness of retinoic acid homeostasis (Belyaeva et al., 2017). Interestingly, the whole-body knockouts of *Rdh10* and *Dhrs3* genes are not viable, in contrast to mice heterozygous for the mutation (Billings et al., 2013; Rhinn et al., 2011). However, Rdh10+/- mice display partial retinoic acid deficiency (Yang et al., 2018), whereas in Dhrs3+/- mice a relative retinoic acid overproduction has been reported

(Billings et al., 2013). Thus, it has been proposed that a stable oxidoreductase complex is required to maintain an appropriate amount of cellular retinoic acid in adult tissues.

Retinaldehyde bound to CRBPI is irreversibly further oxidized to retinoic acid by cytosolic aldehyde dehydrogenase (ALDHs) enzymes such as ALDH1A1 (RALDH1), ALDH1A2 (RALDH2) and ALDH1A3 (RALDH3) (Adams et al., 2014; Belyaeva et al., 2019; Isoherranen and Zhong, 2019; Kedishvili, 2016). ALDH1A3 is the most catalytically efficient enzyme, but it has a relatively high K_m value for all-*trans*-retinaldehyde (Sima et al., 2009). ALDH1A1 and ALDH1A2 have similar catalytic efficiency, but the K_m value of ALDH1A2 is much lower than that of ALDH1A1 (Gagnon et al., 2003, 2002). Thus, ALDH1A1 is the least potent retinaldehyde dehydrogenase of the three enzymes. ALDH1A2 is the primary enzyme responsible for retinoic acid biosynthesis at most sites during embryogenesis. Indeed, lack of *Aldh1A2* results in embryonic lethality due to the heart defects (Niederreither et al., 2001). Mice lacking *Aldh1A3* will died shortly after birth due to nasal premature development (Dupé et al., 2003). Lastly, *Aldh1A1-/-* mice are viable but exhibit defects in the development of the dorsal retina and in retinoic acids biosynthesis in the liver, overall indicating a more important role of this enzyme during adulthood (Fan et al., 2003; Molotkov and Duester, 2003).

Once retinoic acid is synthesized in tissues, it can bind to cellular retinoic acid binding proteins (CRABPs) and be transferred to the nucleus to bind to retinoic acid receptors (RARs) and/or retinoic acid X receptors (RXRs) to regulate the transcription of hundreds of genes, and hence modulate diverse biological processes (Rhinn et al., 2011). Retinoic acid itself activates three members of the nuclear retinoic acid receptors family of proteins, RAR-α, -β and -γ. These receptors function as ligand-dependent transcriptional

regulators by binding as homo-dimers or hetero-dimers to retinoid X receptors (RXR-α, -β and -γ) (Rhinn et al., 2011). RARs are activated mainly by *all-trans*-retinoic acid and its isomer *9-cis*, whereas RXRs are only activated by *9-cis*-retinoic acid (Grenier et al., 2007). Three receptors (RAR-α, RXR-α and RXRβ) have widespread expression patterns, whereas the others (RAR-β, -γ and RXR-γ) show more complex tissue-specific expression (Rhinn et al., 2011). Thus, most tissues are potential targets of retinoic acid actions. In the absence of retinoic acid, in the nucleus, the receptors form a homo- or hetero-dimer that can bind to a DNA motif known as retinoic acid response element (RARE) sequences, located in the promoter of target genes. These RAREs can function as a ligand-inducible transcriptional enhancers or repressors for a given target gene (Bastien and Rochette-Egly, 2004). Through this transcriptional regulatory mechanism, retinoic acid regulates more than 500 target genes (**Fig. 1.1**) (Balmer and Blomhoff, 2002; Grenier et al., 2007). It should be mentioned that retinoic acid can modulate gene expression through binding to peroxisome proliferators-activated receptor PPARβ/δ which then hetrodimerizes with RXR, similar to RAR (Manicassamy and Pulendran, 2009; Rhinn et al., 2011; Schug et al., 2007).

In the cytoplasm, the cellular retinoic acids binding protein II (CRABPII) can channel retinoic acid to the cytochrome P450 (CYP26s) family of enzymes for degradation (Cunningham and Duester, 2015; Isoherranen and Zhong, 2019; Zhang et al., 2015). The CYP26s enzymes hydroxylate retinoic acid on the C4 position on the beta-ionone ring to more polar and inactive forms, such as 4-hydroxy- and 4-oxo-retinoic acid, as well as 18-hydroxy-retinoic acid and 5,6-epoxy-retinoic acid (Isoherranen and Zhong, 2019; Ross and Zolfaghari, 2011; Zhang et al., 2015). The CYP26 familiy of enzymes comprises three

isoforms, CYP26A1, CYP26B1, and CYP26C1. CYP26A1 is highly expressed in the liver, duodenum, colon, and placenta. Its promoter contains a RARE *cis*-element, thus the catabolism of retinoic acid by Cyp26A1 is enhanced by its own excessive levels. Cyp26B1 catabolizes retinoic acid mostly in placenta, ovary, testes, and intestine. Being the family member with high affinity and low capacity for its substrate, Cyp26B1 is proposed to be the main enzyme regulating endogenous *all-trans* retinoic acid concentrations, especially in extrahepatic tissues. Importantly, *Cyp26B1* expression is upregulated by retinoic acid, even though a RARE *cis*-element(s) has not been identified in this gene. Thus, its expression levels (and activity) normally correlate with tissue retinoic acid concentrations. (Topletz et al., 2012). Lastly, CYP26C1 which is still inducible by retinoic acid, is expressed in a fewer tissues, such as brain and liver (Isoherranen and Zhong, 2019).

Tissue retinoic acid concentrations can also be controlled through posttranslational regulation of its receptors RAR and RXR *via* proteasomal degradation and phosphorylation (Adams et al., 2014; Grenier et al., 2007).

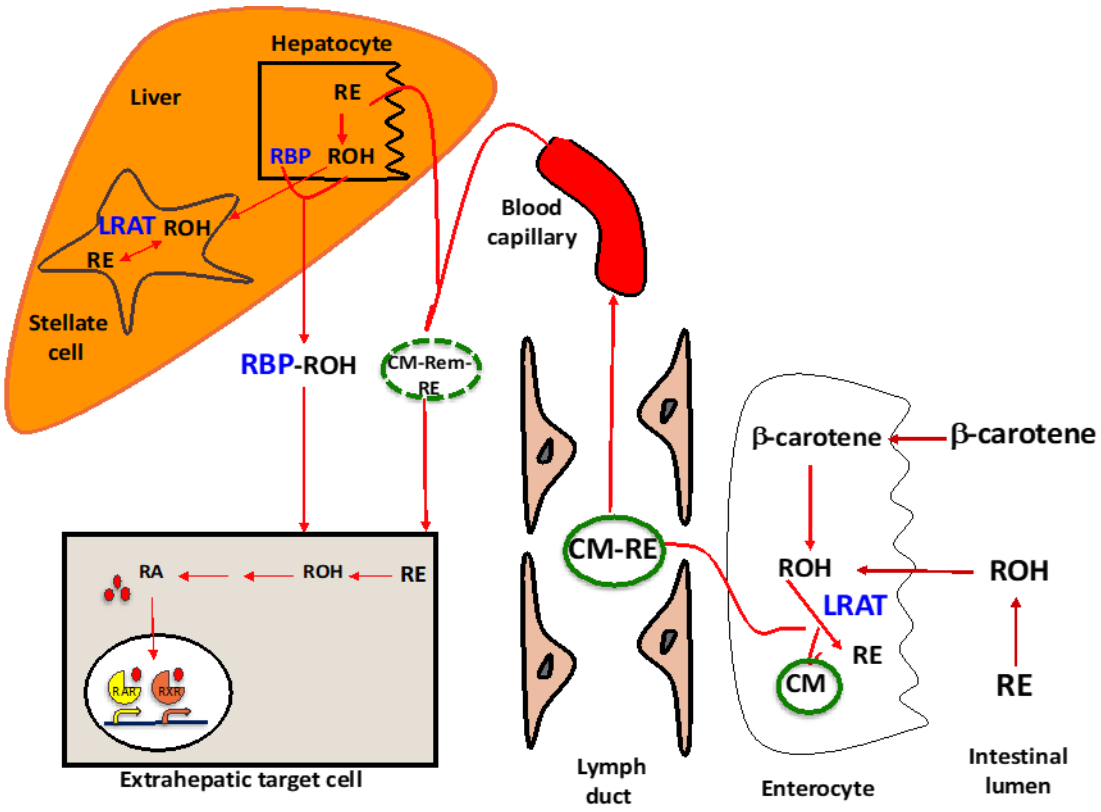

Figure 1.1: Schematic representation of vitamin A absorption, transport and metabolism in liver and extra hepatic tissues.

Carotenes (from plants), as well as retinyl esters (RE) and retinol (ROH) from animal sources are the main dietary sources of vitamin A. They are absorbed in the proximal small intestine by the enterocytes and from here transported as retinyl esters in chylomicrons (CM) in the circulation where they are further remodeled into smaller retinyl ester-containing particles, called chylomicron remnants (CH-rem). These lipoprotein particles are taken up by the hepatocytes where retinyl esters are hydrolyzed to form retinol. From the hepatocytes, retinol is then either transported to hepatic stellate cells (or Ito cells) for storage as retinyl esters or it can be secreted into the bloodstream bound to retinol-binding protein (RBP or RBP4) to be transported to the extrahepatic tissues. Here, upon uptake, retinol can be converted to either retinyl esters for storage or retinoic acids (RA) that regulate transcription via retinoic acid receptor (RAR) and/or retinoid X receptor (RXR). Hepatocytes synthesize retinol-binding protein 4 (RBP4) and ROH binding to RBP4 will be secreted into the circulation.

1.5 THE VITAMIN A PRECURSOR B-CAROTENE AND ITS METABOLISM IN MAMMALIAN TISSUES

Carotenoids are pigments synthesized mostly by photosynthetic organisms to function as light-harvesting scavengers during photosynthesis (Coronel et al., 2019). There are >700 carotenoids in nature; 50 of which are abundant in the human diet. Among the latter group, only 20 are present in human plasma in significant concentrations (Moran et al., 2018). Fruits and vegetables are the primary sources of dietary carotenoids, but they can also be obtained from meat and other products (eggs, milk, etc.) of animals that have ingested these pigments (Coronel et al., 2019). Among the carotenoids, only a handful of them display provitamin A activity, including β-Carotene (βC), α-carotene and β-crypthoxanthin with the highest in βC. The presence of at least one unsubstituted β-ionone ring confers provitamin A capacity to carotenoids (Green and Fascetti, 2016). βC is a 40 carbon-hydrogen molecule, abundant in yellow-orange fruits and dark green leafy vegetables such as pepper (42,891 μg βC /g), carrot (33,954 μg βC /g) and paprika (26,162 μg βC /g food) (Coronel et al., 2019). βC has the highest provitamin A activity due to two β-ionone rings which lead to two molecules of vitamin A (von Lintig et al., 2019).

1.5.1 βC metabolism

βC is the most common source of vitamin A among both developed and developing countries (Grune et al., 2010). In the human intestine, up to 40% of the ingested βC is packaged within lipoproteins in its intact form, for distribution to various organs and tissues to be stored or metabolized into retinoids, as needed (Parker, 1996). Upon βC absorption by enterocytes and other cells types, βC is cleaved symmetrically by the action of the cytoplasmic enzyme called β-carotene-15-15'-dio-oxygenase (BCO1) to yield two

molecules of retinaldehyde (von Lintig et al., 2019). Retinaldehyde can be either oxidized to retinoic acid or reduced to retinol and then esterified to retinyl esters by LRAT enzyme (von Lintig et al., 2019). βC can also be cleaved asymmetrically by the action of another enzyme, localized in mitochondria, β-carotene-9',10'-dio-oxygenase (BCO2), generating one β-ionone ring and β−apo10'-carotenal (Amengual et al., 2013a). β−apo10'-carotenal can be further metabolized by BCO1 to yield one molecule of retinaldehyde (Amengual et al., 2011). Interestingly, β-apocarotenoids of various chain length could also function as transcriptional regulators, specifically as nuclear receptor antagonists, exerting anti-retinoic acid activities (Harrison and Quadro, 2018). It has been showed that β-apocarotenoids can directly compete with retinoic acid for the binding to all three isoforms of RAR and can also modulate the RXR oligomeric state (Eroglu et al., 2012; Sun et al., 2014). Aside from differences in the localization of BCO1 and BCO2 (cytoplasm vs. mitochondria, respectively), it has been demonstrated that these two enzymes have different substrate preference both *in vitro* (Hu et al., 2006) and *in vivo* (Kelly et al., 2018). *In vivo*, a 1000 folds accumulation of βC over β-apo-10'-carotenal (Apo10al) in *Bco1*$^{-/-}$ mice supplemented with βC suggest that βC is not favorable substrate for the BCO2 enzyme. Instead, other carotenoids such as β-cryptoxanthin with a 3-OH-β-ionone ring is a better substrate for BCO2. Indeed, when *Bco2*$^{-/-}$ mice were supplemented with β-cryptoxanthin, this compound accumulated in the tissues of the mutant mice (Amengual et al., 2013a). These data also indicate that BCO1 is the main enzyme that contributes to formation of vitamin A from βC. In contrast, BCO2 seem to play a major role in cleaving carotenoids, including βC, to prevent their toxic accumulation in the mitochondria (Amengual et al., 2011, 2013a; Palczewski et al., 2014).

1.5.2 βC as a "safe" source of vitamin A

The intestine is the first site within the body where regulation of vitamin A metabolism occurs. The dietary intake of βC is variable among populations, ranging from 2 - 10 mg/day (Grune et al., 2010). Bioaccessibility and bioavailability of βC is also variable from 10 to 90% (Grune et al., 2010) depending on many factors such as food matrix, dietary fat, dietary fiber and vitamin A status of the body (Grune et al., 2010; Moran et al., 2018). Both intestinal βC absorption (mediated by the scavenger receptor SR-BI) and conversion into retinoids (mediated by BCO1) are repressed by elevated intake or tissue levels of vitamin A (Lobo et al., 2010a). This negative feedback regulatory mechanism is mediated by ISX, an intestine-specific homeobox transcription factor, which is upregulated by retinoic acid and functions as a transcriptional repressor of *Bco1* and *Srb1* (Lobo et al., 2010b). Genetic and dietary manipulations confirmed that intestinal vitamin A uptake and production from βC increases when dietary vitamin A is limited (Lobo et al., 2010b). Conversely, if sufficient dietary vitamin A or provitamin A is converted to retinoic acid, the uptake and cleavage of βC are reduced, preventing accumulation of toxic levels of vitamin A (Lobo et al., 2010b). This regulatory mechanism explains why βC supplementation does not likely cause the toxic state of hypervitaminosis A and is considered a preferred source of dietary vitamin A (Grune et al., 2010); whereas preformed vitamin A is absorbed in a non-regulated manner and can be toxic (Allen and Haskell, 2002). However, complete knowledge of the factors affecting βC absorptions and transport, tissue storage or enzymatic accessibility, specifically in the intestine, the first site to receive the dietary βC, is still needed.

1.5.3 Other activities of βC

βC can also serve as an antioxidant. Being highly lipophilic, βC localizes in the interior side of the cell membrane and quenches radicals in the hydrophobic part or nonpolar fatty acid tail of the membrane. (Mueller and Boehm, 2011; Tapiero et al., 2004). βC has an electron-rich C_{40} polyene backbone with a series of conjugated C=C bonds which are primarily responsible for their pigmentation properties and antioxidant activities (Green and Fascetti, 2016; Young and Lowe, 2018). There are at least three possible mechanisms for the reaction of carotenoids with radical species. They include (1) radical addition, which is the binding of the radical to βC through an organic addition reaction to form an adduct; (2) electron transfer to the radical from βC that results in the formation of the βC cation radical; and (3) allylic hydrogen abstraction from the 4- and 4' carbon position by the radical (Fiedor and Burda, 2014; Mueller and Boehm, 2011). Overall, the antioxidant activity of carotenoids prevents the formation of peroxides (H2O2) and other extreme ROS such as the hydroxyl radical (Fiedor and Burda, 2014; Skibsted, 2012). The consumption of food-based antioxidants like βC seems to have protective effects on macular degeneration, cataracts, liver carcinogenesis, lung disease, oesophageal adenocarcinoma and Barrett's oesophagus (Kubo et al., 2010). Hence, antioxidants from the diet, such as βC, are essential for the neutralization of oxidative stress (Z. Liu et al., 2018). Human and animal βC supplementation studies have reported improvements in efficient quenching of singlet oxygen. In human, oral βC supplementation reduced production of blood oxidative stress and increased antibody response to vaccination (Otomaru et al., 2018). Interestingly, accumulation of ROS in the intestinal epithelial cells could result in apoptosis and damage of the tight junction proteins (Kajino-Sakamoto et al., 2010), in turn leading to leakage of

ROS into the lumen from the damaged epithelial cells (Kuhn et al., 2018). Elevation of ROS in the intestine could also shift the microbial composition and exacerbate the microbiota-linked intestinal dysregulation. For example, during inflammation *H. pylori* has been associated with induction of the neutrophils to produce ROS in addition to its capability to produce ROS by itself (Handa et al., 2010; Tian et al., 2017). Conversely, other bacteria (i.e. *Bifidobacterium* and *Lactobacillus)* are known to relieve the oxidative stress and ROS accumulation by producing and secreting antioxidant enzymes, including bacterial SOD (Tian et al., 2017). Interestingly, one dose of βC administration in mice can reduce accumulation of intestinal ROS induced by a high fat diet regimen (Kuhn et al., 2018). Overall, these findings indicate that intestinal microbiota can exacerbate oxidative stress; however, the impact of intestinal ROS on the microbiota *per se* is still not fully understood. Given that VAD *per se* is associated with high oxidative stress (Arruda et al., 2009; Spiegler et al., 2018), the impact of dietary βC in attenuating the excess of intestinal ROS caused by VAD should not be discounted.

Despite its potential antioxidant activity, in some cases, βC can function as a prooxidant (Toti et al., 2018). Interestingly, adverse effects of βC were seen in the β-Carotene and Retinol Efficacy Trial (CARET) and in the Alpha-Tocopherol, β-Carotene Cancer Prevention (ATBC) Studies. In the CARET study, the daily βC (30 mg) and retinyl palmitate (25,000 IU) supplementation was examined in regard to the incidence of lung cancer. However, this trial was interrupted as it increased morality by 46% among participants with history of smoking or asbestos exposure (Redlich et al., 1998). In the ATBC trial, the effects of alpha-tocopherol (50 mg) and βC (20 mg) supplementation was also tested to prevent lung cancer. However, the results also did not show any preventive

effect of the supplementation on lung cancer, particularly among smokers and asbestos workers. Although it is not clear, it seems like pharmacological βC supplementation, heavy smoking and higher alcohol intake could indeed increase lung cancer (Albanes et al., 1996). Whereas this detrimental effect was initially ascribed to the pro-oxidant activity of βC, It was later suggested that short-chain and long-chain cleavage products of βC could have played a role in modifying the respiratory burst, increase apoptosis of neutrophils and ultimately increase the oxidative stress in the lung through their transcriptional regulatory function (Haider et al., 2017; Willey et al., 1987).

1.6 VITAMIN A STATUS

1.6.1 Definition of vitamin A status

The vitamin A status is defined by the total body reservoir of vitamin A that reflects the hepatic concentration of the vitamin, given that the liver is its main body storage site (Blaner et al., 2016). In turn, the concentration of vitamin A in the liver is a measure of the dietary intake of the vitamin and of the efficiency of its intestinal absorption and metabolism. Therefore, a variety of factors that can influence these latter processes can ultimately affect vitamin A status (Green and Fascetti, 2016). For example, factors such as socio-demographic and socio-economic status, dietary habits, gender, age, chronic infections and pathological conditions can contribute to the overall vitamin A status (Faure et al., 2006; West and Mehra, 2010).

1.6.2 How to assess vitamin A status

In humans, vitamin A status is commonly defined based on the concentration of serum retinol. Current cut-offs define vitamin A sufficiency when 0.7 μmol/L < retinol <

2.07 μmol/L (Ross and Harrison, 2013; Zeng et al., 2017). When circulating retinol levels drops below 0.7 μmol/L, vitamin A deficiency is diagnosed. Moreover, 0.35 μmol/L < serum retinol < 0.7 μmol/L defines marginal or subclinical vitamin A deficiency, whereas serum retinol < 0.35 μmol/L defines a status of severe vitamin A deficiency (Ross CA, 2005). However, circulating retinol levels do not necessarily reflect accurately the concentration of hepatic retinoids and thus the overall body vitamin A content (Tanumihardjo, 2011). Circulating levels of retinol bound to RBP are maintained very constant within a rather large range of concentration that average around 1 μmol/L (Quadro et al., 2000). Thus, serum retinol levels do not decline until liver vitamin A content is dangerously low (Furr et al., 1989; Tanumihardjo, 2015). Under conditions of vitamin A sufficiency, the ratio of serum retinol:RBP is 1:1 (Gamble et al., 2001). However, in vitamin A deficiency RBP secretion form the liver is attenuated and the protein accumulates in the endoplasmic reticulum of the hepatocytes (Dixon and Goodman, 1987). Overall, the molecular mechanisms that regulate hepatic secretion of the complex RBP-retinol are still not fully understood. The ratio retinol:RBP in the circulation could also be affected by pathological conditions including obesity and insulin resistance, that could negatively impact the accuracy in assessing the vitamin A status (Rubin et al., 2017). Furthermore, under acute hepatic inflammation, serum retinol levels might also be underestimated due to lower production of RBP from the liver (Rubin et al., 2017). Indeed, the release of inflammatory cytokines, such as interleukin-6 and -1, during systemic or local hepatic inflammation, activate the systemic acute phase response (APR). The liver in response to APR, reduce the production of negative acute-phase proteins such as RBP by the hepatocyte and thus indirectly reduces circulating retinol levels (Stephensen, 2001).

Therefore, serum retinol level alone does not represent a fully reliable estimate of the vitamin A status in both human and animals.

Since the bulk of the body vitamin A is stored in the liver, direct measurements of vitamin A levels in hepatic biopsy could be the ideal approach to correctly estimate vitamin A status in humans (Lietz et al., 2016). However, this is obviously not feasible. Based on the hepatic concentrations of vitamin A the current cut off to define vitamin A deficiency in human is <0.1 μmol/g, whereas under vitamin A sufficiency hepatic vitamin A is estimated to be 0.1-0.7 μmol /g (Furr et al., 2005, 1989).

The relative dose response (RDR) test (Loerch et al., 1979; Tanumihardjo et al., 1994) and the modified relative dose response (MRDR) test (more refined form of RDR) (Tanumihardjo et al., 1990), are among the methods that can estimate the concentration of vitamin A in the hepatic stores. The general concept behind these methods is that RBP that accumulates in the liver under vitamin A deficiency is resealed quickly bound to retinol upon administration of a single oral dose of retinyl esters. The concentration of serum retinol before and after the bolus dose can be measured by HPLC (Tanumihardjo, 2015). Therefore, assessments of vitamin A status based on both RDR and MRDR are more accurate when hepatic concentrations of vitamin A are very low, i.e., under vitamin A deficiency. These methods are less reliable in instances of high or toxic concentrations of hepatic vitamin A (Tanumihardjo et al., 2016b).

The retinol isotope dilution (RID) method is one of the most sensitive methods to estimate the total body vitamin A status in humans with relatively noninvasive procedure (Furr et al., 2005, 1989). This method is based on the assumptions that 80% to 90% of the total body vitamin A is stored in the liver of well-nourished individuals; and the weight of

the liver has a fixed relationship with body weight at different ages (Tanumihardjo et al., 2016b). Briefly, this method requires one baseline measurement of serum retinol, followed by administration of a stable retinoid isotope tracer. After allowing the tracer to mix fully with the body vitamin A stores over a certain number of days (about 14-21 days), blood samples are taken and vitamin A is measured. To calculate the body vitamin A from RID technique, modified version of the "Olson equation" (TBS = F X dose X S X H:D) is currently used. TBS is total body vitamin A store; F is a factor for efficiency of absorption and storage of the test dose in the liver (0.5); "dose" is the oral dose in moles; S is the ratio of specific activities of retinol in serum compared to liver (0.65); TTR is the stable isotope analog to specific activity, which is commonly used for radioisotopes, so S can be defined as the ratio of TTR in serum to TTR of stores; and H:D is the ratio of hydrogen : deuterium retinol (equal to 1/TTR) in plasma after an equilibration period (Gannon et al., 2018; Green et al., 2016).

Comparison of RID methods with liver biopsy, showed a good agreement between the two methods (Tanumihardjo et al., 2016b). However, some circumstances such as intestinal infection and inflammation, iron deficiency or high hepatic vitamin A status may impair the accuracy of estimating the total body vitamin A stores using RID method (Lietz et al., 2016).

Overall, it seems that a more user-friendly biomarker is needed to precisely evaluate the vitamin A status. Not only are biomarkers important to measure vitamin A status in populations with vitamin A deficiency, but sensitive biomarkers are also needed to measure the effectiveness of intervention programs that are applied in regions with vitamin A deficiency (Tanumihardjo et al., 2016a).

1.7 VITAMIN A DEFICIENCY

1.7.1 Severe vitamin A deficiency

Poverty and food insecurity in developed and developing countries are associated with inadequate nutrients intake, including vitamin A (Tanumihardjo et al., 2007). Vitamin A deficiency (VAD) is one of the most widespread micronutrient deficiencies, with more obvious health consequences among populations with higher nutritional demand such as children and pregnant women among whom vitamin A deficiency and general malnutrition are often linked (Bailey et al., 2015). Using the current cut off value (serum retinol < 0.7 μM) to define vitamin A deficiency, about 200-250 million preschool children are blind due to the lack of vitamin and about 50% of them die within a year of losing sight, primarily from measles and diarrhea (Bailey et al., 2015; Dubock, 2017; Stevens et al., 2015). Additionally, 10-20% pregnant women in low-income families in the developing countries develop vitamin A deficiency, which is associated with impaired maternal immunity and fetal development (Bailey et al., 2015).

Vitamin A deficiency results in xerophthalmia, metaplasia, anorexia, and more recently it has been associated also with cognitive impairment, such as Alzheimer's disease (Sommer, 2008; Underwood, 2004; Zeng et al., 2017). Additionally, vitamin A deficiency compromises the function of both innate and adaptive immunity and increases the susceptibility to infectious diseases, such as measles, malaria and HIV, as well as respiratory diseases (emphysema, chronic obstructive pulmonary disease - COPD, pulmonary fibrosis) (Stephensen, 2001; Timoneda et al., 2018). Vitamin A deficiency is also associated with increased oxidative stress that can exacerbate diseases such as diabetes, cancer, intestinal bowel disease (Arruda et al., 2009; Bhattacharyya et al., 2014).

Oxidative stress that is due to the accumulation of reactive oxygen species (ROS) is the result of imbalance between the rate of production of ROS and elimination of it (Arruda et al., 2009). Furthermore, vitamin A deficiency causes a rapid increase in mitochondrial-derived ROS production which leads to mitochondrial dysfunction and ultimately cell death (Kim and Hammerling, 2020).

1.7.2 Marginal vitamin A deficiency

While severe or clinical vitamin A deficiency is less common in industrialized countries, emerging data suggest that marginal or subclinical vitamin A deficiency (0.35 μM< serum retinol < 0.7 μM), is more widespread than expected also among western populations from low socioeconomic background that consume a poor diet, particularly low in fruits and vegetables, but not necessarily low in calories (Bird et al., 2017; Garretto et al., 2019; Hanson et al., 2018; Stephens et al., 1996). Additionally, it has been shown that marginal vitamin A deficiency is also prevalent among the elderly in industrialized countries (Lahiri et al., 2007). Recent animal studies also provided support that marginal vitamin A deficiency during the early stage of life could be associated with cognitive impairment (Zhang et al., 2017) as well as Alzheimer's later in life (Zeng et al., 2017), at least in animal models. Due to the lack of clear clinical symptoms and awareness of risk factors, marginal vitamin A deficiency has been widely neglected, even though it still represents a public health threat.

1.7.3 Intervention programs to overcome vitamin A deficiency

A number of strategies have been implemented for more than 20 years to overcome vitamin A deficiency, including vitamin A supplementation, food fortification,

biofortification and dietary diversifications (Dubock, 2017). Currently ~82 countries have implemented different types of intervention programs, sometimes simultaneously (Wirth et al., 2017).

The WHO recommends, a high dose of vitamin A supplementation (60 mg retinol equivalent) every 4-6 months for children between the ages of 6-59 months (WHO, 2009). The supplementation program for children, often coupled with immunization programs, reduced mortality associated with measles, diarrhea, and other illnesses up to 24% globally (Mostafa et al., 2019; Ramakrishnan and Darnton-Hill, 2002). Note that the vitamin A supplementation coverage in Bangladesh improved vitamin A deficiency in up to 90% of the children based on serum retinol levels (Mostafa et al., 2019). However, in this country – as well as in others – the program coverage was not homogenous. For example, in 2014, in Bangladesh only 62% of children with vitamin A deficiency received the next vitamin A supplement after 6 months from the first dose (Mostafa et al., 2019). Another key factor for the success of this strategy has been the parental education. Parents lacking knowledge about the health consequences of vitamin A deficiency were more reluctant to participate in the program mostly in Africa (Guinea), India and Cambodia (Agrawal and Agrawal, 2013; Grover et al., 2008). Vitamin A supplementation is considered to be a cost ineffective intervention method to combat VAD. Although individual vitamin A capsule costs about $1 to be produced and delivered for every child twice a year, the annual overall cost of the supplementation is one billion of dollars due to the high number of people with VAD (Dubock, 2017). Thus, vitamin A supplementation cannot successfully address VAD in regions with chronic VAD and insufficient vitamin A intake (Dubock, 2017; Klemm et al., 2016).

Many countries have implemented the fortification strategy whereby preformed vitamin A is added to staple foods, such as oil (18 mg/kg), sugar (5-9 mg/kg) or flour (2.8 mg/kg) (Dary et al., 2002; Wirth et al., 2017), with sugar being the most common and successful of these foods (Klemm et al., 2016). This strategy can be cost-effective compared to the vitamin A supplementation when there is a suitable food vehicle, and the national food industry is sufficiently developed to implement this technology (Bouis et al., 2011; Tanumihardjo et al., 2016a). An example of the successful fortification strategy to combat VAD is the use of fortified sugar in Guatemala between 1981-1988. In this country, based on the increase in serum retinol levels particularly among children, VAD is no longer a public health problem (PMID: 7446457). However, evaluation of sugar fortification programs in Nicaragua by isotope dilution testing showed that 9 out of 21 children had toxic levels of vitamin A in their liver, one year after the beginning of the program (Ribaya-Mercado et al., 2004). Although food fortification with vitamin A can have positive heath impact, factors such as the degree of fortification, the stability of fortified food and the high consumption of some of these fortified foods in some regions raised concerns of toxicity of the vitamin (Tanumihardjo, 2011). In some countries, such as Zambia, fortification strategy has been implemented together with other strategies, such as high doses of vitamin A supplementation (200,000 IU of vitamin A) (Kafwembe et al., 2009). While the high dose vitamin A supplementation and low dose of fortification (i.e. 10 mg retinyl ester/kg of sugar) was successful in most cases, there were instances where vitamin A levels exceeded the upper limits of dietary vitamin A (Tanumihardjo et al., 2015). Similarly, excessive vitamin A intake has been reported in countries, such as Uganda, where multiple foods were fortified at the same time (Kyamuhangire et al., 2013).

Biofortification of agriculture products with provitamin A carotenoids aims at increasing the concentration of provitamin A carotenoid through traditional breeding or genetic engineering (Garg et al., 2018). For instance, orange maize and yellow cassava produce about 17-24 μg/g, (Gannon et al., 2014; Taleon et al., 2017) and 13.5 μg/g (De Moura et al., 2015) of provitamin A, respectively. These crops were generated either through plant breeding strategy from common yellow maize (Gannon et al., 2014) or through transgenic and breeding approaches of wild type white cassava (Carvalho et al., 2016; Welsch et al., 2010). Similarly, the transgenic "Golden Rice" was genetically engineered to synthesize β-carotene up to 3.57 mg of β-carotene per kilogram of rice (Swamy et al., 2019). The golden rice, on the other hand, has been widely criticized due to the controversy around the genetically modified organism (GMO) products (Kettenburg et al., 2018). In contrast to the vitamin A supplementation and fortification strategies, the biofortification offers the advantage of reducing the risk of excessive vitamin A intakes (Gannon et al., 2014). Indeed, negative feedback regulation of β-carotene absorption and bioconversion into vitamin A limits the risk of hypervitaminosis. Thus, biofortification is considered a cost-effective and long-term approach to combat VAD (Bouis et al., 2011).

Dietary diversification increases availability, access, and consumption of foods with a high content and bioavailability of micronutrients, particularly enriched with β-carotene. When combined with nutritional education, dietary diversification is another long-term and sustainable food-based strategy to eradicate VAD in both developed and developing countries (Gibson and Hotz, 2001).

Although intervention programs, in combination or separately, have shown improvement in vitamin A status among population with the risk of clinical VAD, this is

still one of the most prevalent micronutrient deficiencies in the world (Bailey et al., 2015; Tanumihardjo et al., 2015; Wirth et al., 2017). It is important to mention that application of the "right" strategy(s) to the "right" group of vitamin A deficient people is a key factor to eradicate this disease. Clearly, integrating at least two different strategies in regions with clinical VAD could be the best approach. Children in developing countries with severe VAD, such as Ghana, Uganda, Bolivia and Cameroon are born with extremely low vitamin A stores in the liver because of the maternal poor vitamin A status and insufficient dietary intake of the vitamin (Tanumihardjo et al., 2016a; Wirth et al., 2017). To maintain the minimum requirement of vitamin A in the liver (20 μg/g of liver), these children would need to eat 10 times more fruits and vegetables than normal. Thus, in these cases, integrating multiple strategies can be more effective (Ramakrishnan and Darnton-Hill, 2002).

An important factor that could jeopardize the efficacy of the intervention programs to eradicate VAD, may be linked to the detrimental effects of vitamin A deficiency on intestinal morphology and functions that could limit first of all intestinal vitamin A absorption and processing.

1.8 THE INTESTINE: MORPHOLOGY AND FUNCTIONS

The intestine covers about 400 m^2 of the surface of the body with a complex multilayer of cells that constitute the intestinal barrier. This latter separates the gut lumen from the inner parts of host's body and includes mechanical (mucus, epithelial layer), hormonal (defensins, IgA), immunological (lymphocytes, innate immune cells), muscular and neurological components. All together, these components protect the body against microbes and antigens present in food (Bischoff et al., 2014).

The gastrointestinal (GI) tract is an organ shaped as a long tube that is divided into different segments with specialized functions, such as nutrient absorption, permeability and barriers (Turner, 2009). The more proximal region of the intestine is called small intestine (further divided in duodenum, jejunum and ileum, from proximal to distal) and the more distal segment, called large intestine, comprises cecum and colon. The GI tract, regardless of the region, contains several layers of different tissues including mucosa, submucosa, muscularis, and serosa (**Fig. 1.2).**

1.8.1 Intestinal mucosa

In the small intestine, where most of the nutrients' absorption takes place, the mucosa forms small and long finger-like folds called villi, lined with a specialized type of epithelial cells, called enterocytes (Mowat and Agace, 2014; Pelaseyed et al., 2014). Conversely, the mucosal layer in the cecum and colon is flat with no villi, indicating that, this mucosa is not specialized in the absorption of nutrients but rather in the reabsorption of water and electrolytes. The mucosa of the large intestine also harbors 10^{13}-10^{14} commensal microorganisms that are essential for life (Johansson et al., 2013; Mowat and Agace, 2014). The enterocytes are the main cell type in the intestine. They have a columnar shape and specialized membrane domains. The apical side of the enterocyte's plasma membrane allows the enterocytes to absorb nutrient and ions through specific transporters from the intestinal lumen into the cytoplasm. To optimize this process the apical side of the enterocytes is expanded to form the microvilli (Pelaseyed et al., 2014). Specific receptors on the apical side of the plasma membrane, such as the Toll-Like receptors (TLR), also enable the enterocytes to "sense" changes in the microbial community residing in the intestinal mucosa (Pelaseyed et al., 2014). Enterocytes can also produce mucins and

antimicrobial proteins to inhibit and control the overgrowth of the intestinal bacterial (Okumura and Takeda, 2016).

Goblet cells constitute about 10% and 25% of the epithelial cells in the small intestine and colon, respectively, and are the major contributor to the formation of the mucus layer (Birchenough et al., 2015; Mowat and Agace, 2014). The mucus layer, mainly composed of mucin proteins, water, ions, antimicrobial peptide and IgA, it is juxtaposed to the surface of the intestinal epithelial cells that physically separate the internal milieu from luminal material, especially bacteria (**Fig. 1.3**) (Bischoff et al., 2014; Kim and Khan, 2013; Okumura and Takeda, 2016). The carbohydrate-rich polymeric mucin is divided in acidic and neutral types, and subdivided into sulfated and non-sulfated. Both acidic and neutral mucins are expressed throughout the intestine, but the acidic type is more abundant in the colon which is more resistant to bacteria degradation (Deplancke and Gaskins, 2001). In the small intestine there is a loose layer of mucus. In the colon two mucus layers can be identified: the inner mucus layer is firmly attached to the cells of the mucosa and normally is free from bacteria, whereas the outer layer is looser and is the habitat for bacteria residing in the intestine (Johansson et al., 2013). The number of the goblet cells increases from the proximal to the distal region of the GI tract and it is correlated with the abundance of the bacterial load in the intestine (Mowat and Agace, 2014). The function of the goblet cells can be regulated by a number of bioactive factors that are generated by the intestinal microbiota such as LPS and SCFA, as well as the host immune system, such as interferon-γ, IL-9 and IL-13 (Deplancke and Gaskins, 2001; Mowat and Agace, 2014). In addition to the well-known role of goblet cell as secretory cells, recent studies have shown that goblet

cells can have endocytosis activity and take up the luminal material and transfer them dendritic cells located in laminal propria (Pelaseyed et al., 2014).

Paneth cells constitute another group of epithelial secretory cells located in the small intestine, particularly concentrated in the ileum (Mowat and Agace, 2014). These cells contribute to the intestinal barrier by producing antimicrobial peptides such as RegIIIβ, RegIIIγ, defensins (such as α and β defensins) and lysozyme (**Fig. 1.3**) (Mowat and Agace, 2014). In the absence of the Paneth cells in the colon, other cell types, such as enterocytes, produce antimicrobial peptides (Pelaseyed et al., 2014). Generally, antimicrobial peptides bind to the peptidoglycan of the bacteria and damage the bacterial cell walls (Natividad et al., 2013). The fact that in the germ free mice, or mice with low intestinal diversity of bacteria, the level of antimicrobial is low, suggest that the complexity of the bacterial community can be one key factor regulating the production of antimicrobial peptides (Natividad et al., 2013). Studies have revealed that some bacterial species, such as *Bifidobacteria sp.*, can induce the production of RegIII through the MyD88-mediated TLR signaling pathway (Natividad et al., 2013). The synthesis of antimicrobial peptides, specifically RegIII, by epithelial cells can also be regulated by interleukin-22 (IL-22) produced by different immune cells such as innate lymphoid cells (ILCs), T helper (Th) cells, and dendritic cells (DCs) (Parks et al., 2015; Zindl et al., 2013).

Tuft cells are epithelial cell type that can also be found in different regions of the intestine. They have unique morphology, with a cylindrical cell body that narrows at the apical and basal ends and thick microvilli projecting into the lumen (Banerjee et al., 2018). They are known to express chemosensory receptors such as such as TRPM5 and α-gustducin and it has been suggested that tuft cells are innervated by neighboring neurons.

Recent studies have linked the functions of the tuft cells to the recovery of the intestinal epithelium from damage, and the induction of a type 2 immune response (mediated by ILC2) against parasitic worms, or helminths; however, the clear function of the tuft cell is still not fully understood (Gerbe et al., 2016). In mice, tuft cells continuously express IL-25 to maintain ILC2 homeostasis in the lamina propria. Upon helminth infection, IL-25 further activates ILC2s to secrete IL-13, which in turn regulates epithelial crypt progenitors to induce the differentiation of both tuft and goblet cells. Therefore, tuft cells, ILC2s and epithelial progenitors are interconnected and regulate each other in response to intestinal infections (von Moltke et al., 2016). Thus, tuft cells are considered as mediators between the microbiome and the host immune system (Banerjee et al., 2018).

All mucosal epithelial cells are constantly renewed from stem cells located at the crypt of the villi to form enterocytes, Paneth, goblet and endocrine cells (Mowat and Agace, 2014). The intestinal mucosa epithelium is formed by a single and continuous layer of epithelial cells which are perfectly aligned and held together by tight junctions, adherence junction and desmosomes (Garcia et al., 2018). Among these proteins, tight junctions, such as claudins and occludin, which are localized to the most apical site of the cells are the primary regulators of the paracellular permeability by either tightening or loosening this structure (Yano et al., 2017). Adherence junctions and desmosomes also maintain cell-cell adhesion, development and integrity of the intestine (Yano et al., 2017). High permeability of the intestine, specifically in the colon where high abundance of bacteria exist, could lead to the translocation of unwanted compounds, for example, bacterial lipopolysaccharide (LPS), from the intestinal lumen to the bloodstream causing systemic inflammation (Bischoff et al., 2014).

1.8.2 Other intestinal layers

Located underneath the mucosa, the lamina propria is made of loose connective tissue that provides blood, lymph drainage and nervous termini for the mucosa. Additionally, this layer harbors both innate and adaptive immune cell types. The submucosa is a thick muscle layer that provides support for the mucosa and is essential for its plexus parasympathetic nerves. Underneath the submucosa, the muscularis layer, another muscle layer, provides further supports to move the food through the intestine. Lastly, a tick fibrous layer, called serosa, separates the intestine from the surrounding peritoneal cavity **(Fig. 1.2)** (Mowat and Agace, 2014).

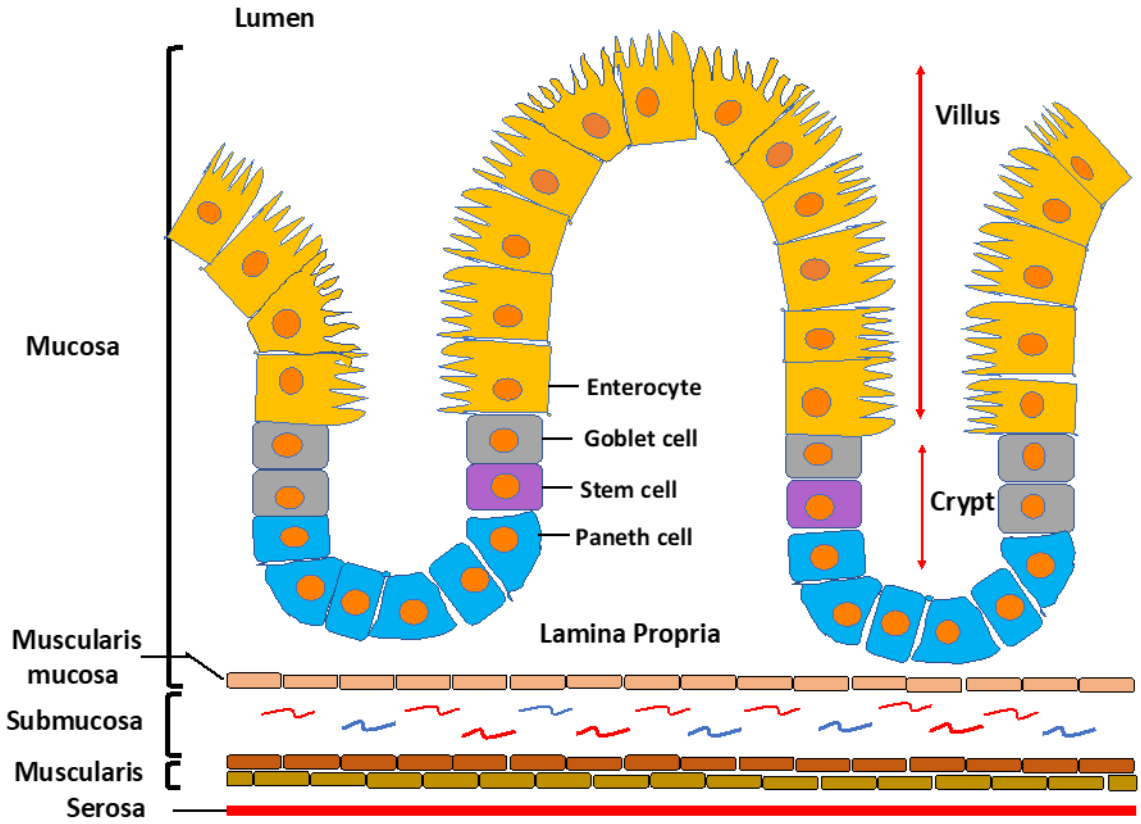

Figure 1.2: Schematic representation of intestinal layers.

Overall organization of the physiological layers in the Intestine. Mucosa layer composed of three sub-layers of muscularis mucosa, lamina propria and epithelial layer (villus and crypt). The submucosa consists of a layer of fibroelastic connective tissue containing blood vessels and nerves. The muscularis layer consists of two muscle layers. The serosa, a single layer of flatt mesoepithelial cells, is the outermost layer of the intestine.[1]

[1] *Figure adapted and modified from Mowat and Agace 2014.*

1.8.3 Vitamin A regulates non-immune intestinal cellular functions

One of the key characteristics of the intestine is the fast (turnover of 2-5 days), continuous proliferation and migration of the epithelial cells within the crypt (Barker, 2014; Cheng and Leblond, 1974) This makes this organ highly susceptible to fluctuation in vitamin A availability, as retinoids are known modulators of cell proliferation and differentiation, mainly owing to the transcriptional regulatory action of retinoic acid (Amit-Romach et al., 2009; Cha et al., 2010; Rubin et al., 2017; Sirisinha, 2015). For example, *in vitro* studies have shown that retinoic acid, perhaps through RAR/RXR promotes intestinal tight junction and decreases permeability by regulating the expression of the key junction proteins, such as Zonula Occludens 1 (ZO-1), occludin and claudin-1 (He et al., 2019; Kubota et al., 2001), claudin-6, -7 (Kubota et al., 2001), whereas indirectly regulate the expression of ZO-2 through TLR4 (Li et al., 2017). Indeed, several studies in developing countries showed that vitamin A supplementation prevents diarrhea and gut barrier dysfunction (Lancet, 1993). Vitamin A deficiency is also associated with shorter villi in the small intestine of animal model (Cha et al., 2010). Similarly, vitamin A deficiency is associated with dysregulation of the mucin expression, which plays a fundamental role in the intestinal barrier. In the small intestine, vitamin A deficiency is associated with higher expression of the *Muc2* (Cha et al., 2010), whereas in the colon it has been associated with the reduction of *Muc2* (Amit-Romach et al., 2009). Aberrant intestinal mucin production due to vitamin A deficiency, could be the result of dysregulation of epithelial cell proliferation (Wang et al., 1997) and/or mucin expression by retinoic acid (Choi et al., 2003; Koo et al., 1999). Altogether these studies provide evidence that vitamin A regulates intestinal morphology and functions.

1.8.4 Intestinal immune cells

The immune response comprises innate and adaptive immune response. The innate immunity is the first response of the body to eliminate pathogens, without development of the memory of previous exposure to the pathogen/antigen. If the innate immune system cannot control the infection, then the adaptive immunity is engaged through both hormonal and cellular components (Iwasaki and Medzhitov, 2015). Immune cells in the GI tract comprise both innate and adaptive cells, such as innate lymphoid cells (ILCs), T and B lymphocytes, macrophages and dendritic cells (DC), that all together control the bacterial load in the intestine and constitute an active defense again pathogens (Mowat and Agace, 2014). ILCs are key mediators of the innate immunity that share a lot of developmental and functional similarities to T cells (Sonnenberg and Artis, 2012). Based on the function and the type of the cytokines they express, ILCs are subdivided in ILC1 and ILC2, predominantly activated during the immune response to intracellular bacteria and helminths, respectively, and ILC3 that protect the intestine against extracellular bacterial infection **(Fig. 1.3)** (Parks et al., 2015; Spits et al., 2013). Lymphocyte T and B cells are key players of the adaptive immune response. B cells hormonal response to antigen or bacteria is mediated by antibody, including IgA (Hoffman et al., 2016). T cells express cell surface receptors that upon interaction with a specific antigen trigger the differentiation into T helper, regulatory T-cells (T_{REG}) or cytotoxic T cells subsets, responsible for the defense against infections, and for inducing inflammation or tolerance to commensal bacteria and food antigen (Ma et al., 2019). Macrophages, the most abundant leukocytes in the lamina propria of the intestine, serve both the innate and adaptive immune system playing a key role during inflammation and bacterial infections. Macrophages can produce

a wide range of cytokines, such as IL10 which can prevent the inflammation, or TNFα and IL-1β that produced in response to microbial change maintain Th17 cells activity (Mowat and Agace, 2014) Dendritic cells (DC) are the mediators of the cross-talk between the innate and adaptive immune system as they "sense" antigens in the peripheral tissues and enable the activation of more specialized immune cells, such as the T helper (Th) cells by the action of specific cytokines **(Fig. 1.3)** (Czarnewski et al., 2017). Interestingly, the functions of the DC cells are tightly regulated by their ability to sense and/or metabolize retinol to retinoic acids *via* retinaldehyde dehydrogenases (ALDH) (Czarnewski et al., 2017; Jiang et al., 2018). It is generally believed that retinoic acid synthesized by DC, although not exclusively by these cells (Manicassamy and Pulendran, 2009), promote an anti-inflammatory response (Czarnewski et al., 2017; Zeng et al., 2016). Thus, DC in the presence of endogenous retinoic acid, enhance the differentiation of T cells towards immunosuppressive Foxp3^{+} regulatory T cells (T_{REG}) while inhibiting the differentiation of proinflammatory T cells, such as the IL-17- and IL-22-expressing Th17 cells **(Fig. 1.3)** (Czarnewski et al., 2017; Raverdeau and Mills, 2014; Tai et al., 2018). However, it is important to mention that the effect of retinoic acid on the function of the DC could be dependent on the microenvironment and type of cytokines that the DC cells are exposed to (Czarnewski et al., 2017). For example, in presence of both retinoic acid and IL-15, the DC can promote the secretion of proinflammatory cytokines such as IL-23 and IL-12 (DePaolo et al., 2011a).

Retinoic acid also stimulates the production of the IgA from B cells by regulating cell proliferation and differentiation (Gutzeit et al., 2014; Hui et al., 2020; Mora and von Andrian, 2009; Pantazi et al., 2015); indeed, vitamin A deficiency is usually associated

with reduction of IgA synthesis and mucosal secretion (Czarnewski et al., 2017). Similar to the T cells, the development and function of the innate lymphoid cells (ILC) is also regulated by the retinoic acids (Parks et al., 2015; Spits et al., 2013). Studies showed that the concentrations of retinoic acid gradually decrease from the proximal (duo denum) to the distal segment of the intestine (colon) (McDonald et al., 2012; Villablanca et al., 2011). Interestingly, along the gastrointestinal tract, the ILC subsets are also differentially distributed. For instance, ILC3 and ILC1 are predominant in the small intestine; while ILC2 cells are mainly in the colon (Czarnewski et al., 2017). Thus, it is tempting to speculate that retinoic acids might be one of the players involved in the complex functional regionalization of ILCs in the intestine, likely by controlling their migration, differentiation and/or function (Czarnewski et al., 2017). Interestingly, whereas vitamin A deficiency is associated with severe reduction of the number of ILC3, which protect against bacteria, it increases the number of the ILC2, especially active in the protection against nematode infections (Spencer et al., 2014). These findings suggest that even during vitamin A deficiency, which has a profound impact on adaptive immune system dysregulation, the intestine is still capable of providing protection against a specific group of infectious worms (i.e. helminth) (Spencer et al., 2014).

Altogether, these studies provide important insights into the role of vitamin A as an essential regulator of intestinal barrier functions by maintaining both proper immune response and the integrity of the intestinal epithelium (de Medeiros et al., 2018). However, many gaps still remain in our understanding of vitamin A interactions with the intestinal mucosal immune system, epithelial junctional proteins and intestinal microbiota (de Medeiros et al., 2018). The majority of the studies focused the role of vitamin A in the

small intestine where the majority of vitamin A is absorbed. However, much less is known on its role in regulating barrier function in the colon, where trillions of bacteria are also harbored.

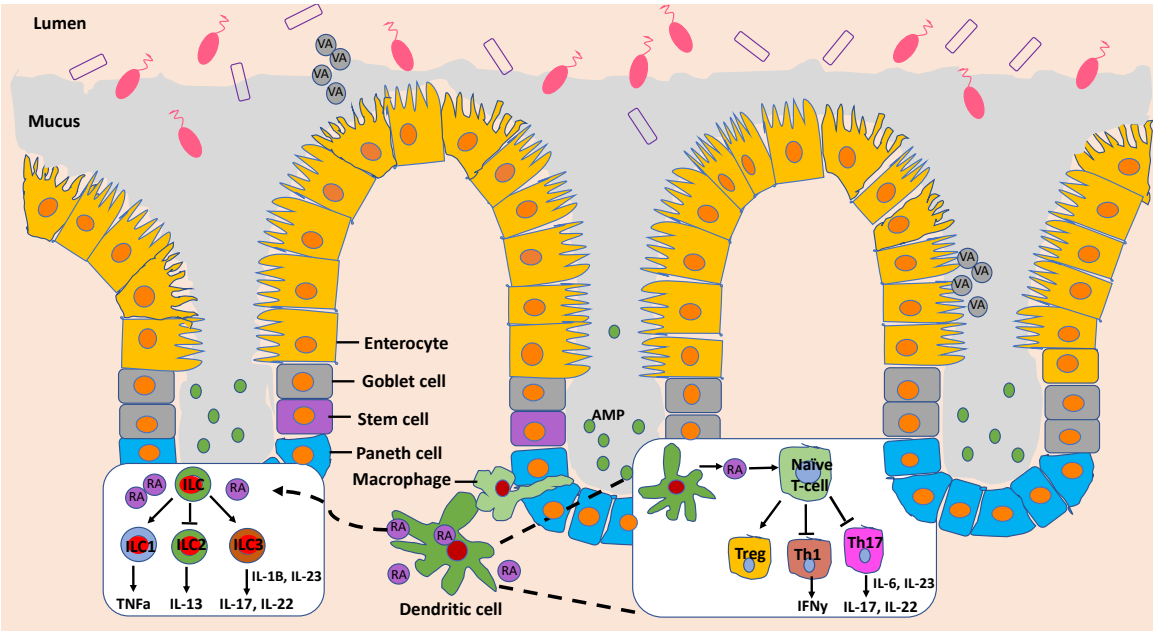

Figure 1.3: Schematic representation of intestinal non-immune and immune compartments.

Intestinal epithelial cells are produced from stem cells near the bottom of the crypts. Enterocytes are responsible for absorption of nutrients. Goblet cells are responsible for the production of the mucus, whereas Paneth cells, that migrate downwards to the bottom of the crypt after being generated from the stem cells, produce antimicrobial peptides (AMPs). Retinoic acids (RA) produced by dendritic cells (DCs) and macrophages helps to maintain immune homeostasis and mucosal tolerance by promoting the expansion of Tregs from naive T cells, while inhibiting Th1 and Th17 cells. Innate lymphoid cells (ILC) can be classified into three groups (ILC1, ILC2 and ILC3). RA produced by the DCs can also support the differentiation of ILC1/3 while inhibiting the expansion or function of ILC2. [2]

[2] *Figure adapted and modified from Mowat and Agace 2014; Zeng et al, 2019; Raverdeau and Mills 2014.*

1.9 VITAMIN A AND INTESTINAL MICROBIOME

1.9.1 Intestinal microbiome

The intestinal microbiome is a complex ecosystem of microbial species living in the lumen and in the mucosa of the GI tract. The intestine harbors tens of trillions of microorganisms, including at least 1,000 different species of known bacteria with more than 3 million different genes (Hooper et al., 2002) The intestinal ecosystem is shaped and maintained by a dynamic interaction between host and microbes (Hooper et al., 2002). Moreover, environmental factors, host metabolism and immunity, can commonly lead to changes in microbial diversity and richness. Shifts in microbial community composition, in turn, can alter the downstream microbial metabolites, predominantly short chain fatty acids (SCFA) and secondary bile acids, that play critical roles in maintaining intestinal immunity and barrier (H. Zeng et al., 2019) and act also on distant organs within the body of the host (Braniste et al., 2014; Caesar et al., 2016). Microbial dysbiosis, a disruption of intestinal microbiome homeostasis, has been associated with a wide range of diseases including inflammatory bowel disease (IBD) (Hold et al., 2014), irritable bowel syndrome (IBS) (Kassinen et al., 2007), celiac disease (Nadal et al., 2007), food allergies (Kuvaeva et al., 1984) diabetes (Forslund et al., 2015) obesity (W.-C. Liu et al., 2018), and cardiovascular diseases (Tang et al., 2019), among many others. Notably, microbial dysbiosis can further dysregulate the immune mucosal response thus contributing to chronic inflammation which could further compromise the intestinal barrier (Kim et al., 2017). There is a dynamic interaction between the microbial community and its host. Alterations in one cause changes in the other, and *vice versa*. For example, while high fat diet increases intestinal permeability by reducing the expression of the tight-junction

proteins resulting in increased metabolic endotoxemia, antibiotic treatment changes the bacterial community leading to reduced metabolic endotoxemia in mice (Cani et al., 2008).

1.9.2 The microbiome of vitamin A deficiency

A specific taxonomic profile of the microbiome of vitamin A deficiency has begun to emerge linking VAD to fewer butyrate-producing *Clostridia spp*. in the feces of both children (Lv et al., 2016) and mice (Tian et al., 2018), for example 16S rRNA gene analysis of fecal samples from children with VAD and persistent diarrhea showed that VAD is associated with lower diversity of intestinal microbiota, reduced abundance of *Clostridium* and higher abundance of the *Enterococcus* compared to children with normal levels of vitamin A status (Lv et al., 2016). Perhaps reduction of the *Clostridium*, a key butyrate producing genera, could result in reduction of the butyrate short chain fatty acids (SCFA) that can support the growth of pathogenic bacteria (Lv et al., 2016). Butyrate is an energy source for the enterocytes (Bui et al., 2015). Thus, limited availability of butyrate could result in energy deprivation, reduced expression of enzymes for the metabolism and TCA cycle and ultimately disrupt the proper intestinal cell function (Donohoe et al., 2011). Another study in mice pointed to similar effects of VAD on butyrate-producing bacteria. Tian and colleagues (Tian et al., 2018) performed 16S rRNA gene analysis of the cecal microbiome of mice on vitamin A- diet from the gestation until 7 weeks of age. VAD was associated with an increased ratio of *Firmicutes/Bacteroidetes* phyla and reduction of the *Clostridium*, *Roseburia* and increased *Bacteroides* genera. In the same study it was also observed that VAD resulted in liver dysfunction, altering the concentrations of metabolites such as lactate, glucose and branch-chain amino acids which correlated with the shifts in the microbiota population. These results support the notion that there is interaction between

the gut microbiome and distant organs, such as liver

Zhou and colleagues (Zhou et al., 2020) showed that dietary vitamin A deprivation for 8 weeks in mice is associated with impaired glucose tolerance and islet structure as well as β cell mass, proposing that these phenotypes were caused by the intestinal bacterial dysbiosis. Interestingly, by surveying the 16S rRNA of cecal samples, the authors showed that vitamin A supplementation for 8 weeks could revert the microbial dysbiosis induced in mice by the dietary restriction.

VAD in rats was associated with impairment of the gut barrier and reduced diversity of the mucosa-associated bacteria, particularly reduction of the intestinal *Lactobacillus* and *Bifidobacteria and* increased abundance of *E. coli*, compared to vitamin A sufficient animals (Amit-Romach et al., 2009).

By using a gnotobiotic mouse model, Hibberd and colleagues investigate the impact of micronutrients' deficiency on human gut microbiome development. Specifically, they demonstrated that vitamin A deprivation impacts on the taxonomy of the cultured gut bacterial species of human. However, in this study the vitamin A status of the mice, especially in the intestine, was not properly estimated. To address the direct effect of dietary vitamin A on the microbiota, Hibberd and colleagues also evaluated the vitamin A-dependence of 92 culturable bacteria of age- and growth-discriminatory taxa from the human gut microbiota *in vitro*. Among the bacteria tested, *Bacteroides vulgatus* was highly abundant in gnotobiotic mice deprived of dietary vitamin A (Hibberd et al., 2017). They showed that retinol can directly inhibit the growth of *B. vulgatus*. Further, they showed that vitamin A deprivation was associated with altered bile acid metabolism *in vivo*, raising the possibility that retinol, bile acid metabolites, and the AcrAB-TolC (broad-specificity

multidrug efflux pump) might interact to influence the fitness of *B. vulgatus* (Hibberd et al., 2017).

On the other hand, it is emerging that bacteria can also modulate host vitamin A homeostasis. It was recently reported that *Clostridia* inhibit retinoic acid metabolism by reducing retinol dehydrogenase 7 (Rdh7) in intestinal epithelial cells and controlling IL-22 activity to prevent microbial dysbiosis (Grizotte-Lake et al., 2018). This finding reveals another mechanism whereby intestinal bacteria can modulate the host immune system by regulating vitamin A metabolism in the intestinal cells.

A recent study indicated that vitamin A can modulate intestinal microbiota through regulating of the toll like receptors-4 (TLR-4), receptors that are bound on the membrane of the immune cells and enterocytes. The comparison of the 16S rRNA gene analysis of mucosa-associated microbiota of both WT and TLR4-/- mice on vitamin A-sufficient and -deficient diet showed that the effect of the vitamin A on bacterial community is mediated by TLR4. These results suggest that TLR4 may play a pivotal role in the regulation of the intestinal mucosal bacteria and maintenance of the intestinal homeostasis mediated by vitamin A (Xiao et al., 2019).

All together, these studies suggest the existence of a bidirectional interaction between intestinal microbiota and vitamin A status. While bacteria can modulate the vitamin A metabolism of the host, host vitamin A status can also impact the fitness of the intestinal microbiota. Additionally, vitamin A insufficiency seems to favor the growth of bacteria such as *Bacteroides*, while inhibiting others such as *Clostridium* genera. However, whether this is a direct effect of the dietary vitamin A on the microbes or it is indirectly due to the changes in host physiology under VAD, remains to be clarified. Therefore, the

complex relationship between dietary vitamin A content, vitamin A status of the host and gut microbiome fitness continues to be a matter of intense studies.

1.10 KNOWLEDGE GAPS IN THE MODULATORY ROLE OF BC ON INTESTINAL HEALTH AND MICROBIOME

Growing evidence indicates that βC may play a role in modulation of intestinal inflammation and immune system. Both *in vitro* study showed βC function in reducing *TNFα, Cox2* and *Nos2* expression of mRNA on murine macrophage-like cell lines (Kawata et al., 2018). Additionally, dietary βC supplementation (5, 10 and 20 mg/kg of body weight) for 28 days ameliorated the inflammation associated with colitis by reducing IL-6 (Toti et al., 2018; Trivedi and Jena, 2015), IL-17 and TNFα protein levels in mice (Trivedi and Jena, 2015). Furthermore, higher IgA production was also observed in the intestine of mice upon consumption of 50 mg of βC/kg of diet for 14 or 21 days (Nishida et al., 2014). However, it is not clear whether this potential anti-inflammatory role of βC was associated with the retinoic acid-mediated immune response (Nishida et al., 2014), or with the intracellular reduction of ROS by its antioxidant activity (Kawata et al., 2018), or by a combination of both factors. Overall, the immunomodulatory role of βC needs to be further clarified.

Interestingly, the bioavailability of βC can be influenced by the intestinal microbiota (Grolier et al., 1998). Earlier studies by Grolier and colleagues showed that, regardless of the type of bacteria, the gut microbiota reduced the bioavailability of βC. A comparison among germ free, antibiotic-treated and conventionally-raised rats clearly showed that both germ-free and antibiotic-treated rats had the highest bioavailability of βC, suggesting that the presence of bacteria decrease the bioavailability of the provitamin A

carotenoid, even though not directly (Grolier et al., 1998). Recent observations suggest that dietary carotenoids such as astaxanthin can alter the composition of cecal microbiota, perhaps mediated by immune modulatory role of carotenoids (Lyu et al., 2018; J. Wang et al., 2019). Addition of astaxanthin to the high-fat diet as a supplement prevented weight gain, reduced plasma and liver triacylglycerol, and decreased plasma and liver total cholesterol and regulated the gut microbiota of the mice, which optimized the ratio of *Bacteroidetes* to *Firmicutes* (J. Wang et al., 2019). Similarly βC supplementation of chickens can reduce the abundance of the *Escherichia coli* while increase the abundance of *Lactobacillus* bacteria (Hui et al., 2020).

Given that the majority of the ingested βC is not absorbed in the small intestine, but it travels in its intact form towards the colon, where most of the bacteria reside, it is important to understand the relationship between intact βC in the lumen of the colon and the intestinal microbiota.

1.11 SUMMARY

VAD is one of the most widespread micronutrient deficiencies affecting hundreds of millions of children and pregnant women in over half of the countries worldwide, despite the implementation of numerous intervention programs. Emerging evidence suggests that VAD can impact intestinal microbiota diversity and fitness. However, given that vitamin A regulates intestinal epithelial cells integrity, barrier and immunity the true relationship between dietary vitamin A content, vitamin A status of the host and gut microbiome fitness still remains to be elucidated. Not only vitamin A can be obtained from food of animal origin as preformed vitamin A, but also from vegetables and fruits containing provitamin A carotenoids, mainly as βC. In fact, βC is the most abundant dietary source of vitamin A

worldwide, and often the sole source of the vitamin in certain regions of the world. Yet, its impact on intestinal microbial populations and functions is unknown, especially during VAD.

The overarching objective of this study is to understand the impact of different forms of dietary vitamin A (preformed retinoids or provitamin A carotenoids) *vs.* vitamin A status on intestinal health during VAD. To address this question, we used a genetic mouse model of VAD, the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice. Due to the absence of lecithin:retinol acyltransferase (LRAT) and retinol-binding protein (RBP), these mice cannot store vitamin A in the liver and mobilize it towards the periphery of the body. Thus, they rely exclusively on dietary vitamin A to support vitamin A-dependent functions. Therefore, unlike WT mice that are resilient to develop vitamin A deficiency, *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice are susceptible to develop severe vitamin A deficiency by dietary vitamin A manipulation. This mouse model enables us to investigate the effect of dietary vitamin A and status on intestinal health.
We addressed the above-mentioned question through the following

1.11.1 Specific AIMS

Specific Aim 1. To understand the contribution of dietary preformed vitamin A and host vitamin A status to the microbial dysbiosis and intestinal functional dysregulation of VAD.

As the majority of the dietary vitamin A is absorbed in the small intestine, the gut microbiome, mainly residing in distal segment of the intestine, is not exposed to the fluctuations of the dietary vitamin A intake. We therefore *hypothesized* that the fitness of the intestinal microbial populations should not be impacted by the dietary vitamin A *per se*, but rather by the vitamin A status of the host. To address this question, both *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice were maintained on either vitamin A-sufficient or -deficient diet for 4

weeks and then returned to the initial regular chow diet regimen for 1 week. Intestine, liver, adipose tissue and serum as well as feces were collected from the mice at different time points throughout the experiment. The taxonomic profile of the fecal microbiome was analyzed by 16S rRNA sequencing, whereas insights into the bacteria functionality were obtained through fecal short chain fatty acid measurements and a computational approach. The intestinal vitamin A status was assessed by direct measurements of retinoid concentrations by HPLC and LC-MS analysis. Intestinal structure and functions were assessed by histology, immunohistochemistry, qPCR analysis, serum LPS and *in vivo* luminal gut ROS levels measurements.

Specific Aim 2. To determine the impact of dietary β-carotene on fecal microbial fitness and intestinal functions during VAD.

βC from fruits and vegetables generates retinoids in tissues upon its enzymatic conversion mediated predominantly by BCO1. βC is often supplemented in intervention programs to overcome VAD. However, the efficiency of βC in improving the dysfunctions of the vitamin A deficient intestine has not known been fully clarified. Moreover, the majority of ingested βC is not absorbed in the small intestine, but it travels in its intact form towards the colon to be ultimately excreted with the feces. Recently, it has been shown that a single oral dose of βC can quench reactive oxygen species (ROS) in the intestinal lumen, likely due to its antioxidant capacity. We, therefore, *hypothesized* that βC may improve intestinal health and microbiota fitness during VAD acting through its retinoid-generating capacity in the intestine and antioxidant activity. We used the same experimental scheme described in Aim 1 to generate severe VAD in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice. Two

weeks after the dietary vitamin A deprivation, WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice were supplemented with βC (1mg/mouse by oral gavage every other day) for two weeks. At the end of the fourth week, tissues, serum and feces from these mice were collected and analyzed as described in Aim 1.

CHAPTER 2 :

UNDERSTANDING THE CONTRIBUTION OF DIETARY PREFORMED VITAMIN A AND HOST VITAMIN A STATUS TO INTESTINAL FUNCTIONAL DYSREGULATION AND MICROBIAL DYSBIOSIS

2.1 ABSTRACT

Emerging evidences have revealed that vitamin A deficiency (VAD) is associated with intestinal microbiome dysbiosis. Here we addressed the impact of dietary vitamin A intake and status on fecal microbiome as well as intestinal retinoid homeostasis and barrier functions using a mouse model lacking both lecithin:retinol acyltransferase (LRAT) and retinol-binding protein (RBP or RBP4). Unable to store and deliver retinoids (vitamin A and its derivatives) to peripheral tissues, these mice display a tenuous vitamin A status. At 6 weeks of age, *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice were placed on either vitamin A-sufficient or -deficient diet for 4 weeks, and then returned to the initial regular chow regimen for 1 week. Based on serum retinol levels, *Lrat*$^{-/-}$*Rbp*$^{-/-}$ manifested VAD on a vitamin A-containing diet. However, dietary vitamin A restriction further reduced serum retinol levels and their already limited vitamin A stores. Fecal 16S rRNA gene analysis showed that VAD in our genetic model was concomitant with fecal microbial dysbiosis. Vitamin A of dietary origin did not impact the fecal microbial taxonomic profile. Also, VAD resulted in impaired structure and functions of the intestinal barrier as manifested by reduced mucins and antimicrobial defense, leaky gut, increased inflammation and oxidative stress, and impaired mucosal immunocytokine profiles. Overall, our findings indicate that the vitamin A status is a critical driver intestinal functional integrity.

2.2 INTRODUCTION

Vitamin A deficiency (VAD) is amongst the most widespread micronutrient deficiencies, affecting hundreds of millions of children and pregnant women mainly in developing countries where VAD and general malnutrition are often linked (Bailey et al., 2015; Wiseman et al., 2017). In children, severe VAD causes blindness and increased the

risk of infectious diseases, stunted growth, and death (Wiseman et al., 2017). Even though vitamin A interventions are effective in certain areas of the world, VAD continues to be a problem (Song et al., 2017; Vijayaraghavan, 2018).

Mammals obtain vitamin A exclusively from food upon absorption in the small intestine (Blaner et al., 2016). Within the enterocytes the majority of ingested vitamin A, regardless of its dietary origin, is converted into retinyl esters, predominantly by lecithin:retinol acyltransferase (LRAT), to be packaged in chylomicrons along with other dietary lipids. Once in the general circulation, remodeling of chylomicrons by lipoprotein lipase results in smaller retinyl ester-containing lipoprotein particles called chylomicron remnants, which are mainly transported to the liver, the largest storage site of vitamin A in the body. Upon uptake by the hepatocytes, retinyl esters are hydrolyzed to retinol to be either transferred to stellate cells for storage or secreted into the bloodstream bound to retinol-binding protein (RBP or RBP4) for distribution to the peripheral tissues (Blaner et al., 2016). Upon uptake by the target cells, retinol can be re-esterified for storage or oxidized to its active form, retinoic acid (Kedishvili, 2016). Vitamin A owes the majority of its functions to the transcriptional regulatory activity of retinoic acid. By binding to retinoic acid receptors (RARs) and retinoid X receptors (RXRs), retinoic acid controls the expression of hundreds of genes generally involved in cellular differentiation and proliferation and is thus critical to a number of vital biological processes (Al Tanoury et al., 2013; Das et al., 2014). Hence, alterations of vitamin A intake and/or tissue homeostasis have been linked to a number of chronic diseases in humans, including metabolic disorders (Blaner, 2019), respiratory (Timoneda et al., 2018) and intestinal pathological conditions (Barbalho et al., 2019a).

Vitamin A modulates intestinal morphology and functions by maintaining epithelial integrity and barrier and by regulating gut immunity and inflammation (Cha et al., 2010; He et al., 2019; Li et al., 2017; Sirisinha, 2015). The vitamin A status has a profound impact on intestinal health. Indeed, VAD is often associated with pathological conditions of the gut (Barbalho et al., 2019a; de Medeiros et al., 2018; Sirisinha, 2015). A "healthy" intestine is also critical to support a "healthy" microbiome, a complex and large microbial community residing in the gastrointestinal tract and comprised for the most part of non-pathogenic, symbiotic commensal bacteria that help maintain proper intestinal functions in a feedback loop, positively influencing the overall health status (Byndloss et al., 2018). Interestingly, a specific taxonomic profile of the microbiome of VAD has begun to emerge linking VAD to fewer butyrate-producing *Clostridia sp*. in the feces of both children (Lv et al., 2016) and mice (Tian et al., 2018). Furthermore, a few bacterial species sensitive to the concentration of vitamin A (retinol) in the diet have been reported in a gnotobiotic mouse model (Hibberd et al., 2017). More recently, specific gut bacteria within the class *Clostridia* have been shown to prevent microbial dysbiosis by regulating retinoic acid metabolism in intestinal epithelial cells (Grizotte-Lake et al., 2018). Clearly, the relationship between dietary vitamin A content, vitamin A status of the host, and gut microbiome fitness is complex and remains to be fully elucidated.

Here, we address this issue by exploiting a genetic mouse model of VAD, the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice (Kim et al., 2008), to discriminate between the effects of the dietary vitamin A and host vitamin A status on intestinal retinoid homeostasis, fecal dysbiosis and gut barrier functions. Our findings indicate the vitamin A status, rather than the amount of dietary

vitamin A *per se*, as a key driver of intestinal mucosal integrity, functions, and microbial fitness.

2.3 MATERIALS AND METHODS

2.3.1 Mice and nutritional manipulation

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Rutgers University Institutional Committee on Animal Care. Wild type (WT) and *Lrat*$^{-/-}$ *Rbp*$^{-/-}$ mice on a mixed genetic background (C57Bl/6J × Sv/129; (Kim et al., 2008) were maintained on a standard vitamin A-sufficient chow diet containing 18 IU vitamin A/g diet (Prolab Isopro RMH3000 5p75). At six-weeks of age (T0; **Fig. 2.1**), *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT female mice, raised on the above-described regular chow diet, were placed either on a purified vitamin A-sufficient (Research Diets, VA-suf; 20 IU vitamin A/g diet) or -deficient diet (Research Diets, VA-def: < 0.02 IU vitamin A/g diet) for four weeks (T1; **Fig. 2.1**), and then returned to the regular chow diet for one week (T2; **Fig. 2.1**). The macronutrient composition of all these diets was similar (protein 26%, carbohydrate 60%, fat 14% Kcal). The regular chow contained cellulose, hemi-cellulose and lignin (4% total fiber) whereas the purified diets contained only cellulose (3% total fiber). At each time point (T0 to T2), after collection of fresh feces, mice (n = 3-6/group) were sacrificed by CO_2 inhalation between 9:30 and 11:30 AM to collect serum, liver, adipose tissue (perigonadal), small intestine (mucosa) and colon, immediately snap frozen on dry ice and stored at −80 °C for microbiome DNA analysis and HPLC analysis. At dissection, duodenum and colon segments were opened longitudinally, and feces and food contents

were pushed out with a blunt forceps. As colon and small intestine from each mouse could be used at most for two types of analysis, additional mice at T1 were sacrificed to perform the extended analysis as follows: n = 6/group for mRNA analysis; n = 3/group for immunohistochemistry (IHC) and Periodic acid–Schiff (PAS) staining; n = 4-5/group for *in vivo* reactive oxygen species (ROS) measurement; n = 4-6 for LC-MS/MS measurements of retinoic acid. Food intake, measured weekly from T0 to T2, was similar among the groups at each time point from T0 to T1 (**Fig. 2.2A**). At T2, food intake was significantly higher compared to all other time points, regardless of the genotype (**Fig. 2.2A**). Within each genotype, body weight was similar at each time point, regardless of the diet (**Fig. 2.2B**). However, *Lrat*$^{-/-}$*Rbp*$^{-/-}$ showed a slightly but significantly lower body weight compared to WT VA-suf throughout the experiment, regardless of the dietary regimen (**Fig. 2.2B**), as already reported (Marwarha et al., 2014).

Throughout the experiment, mice had constant access to diet and water *ad libitum* and were housed in a room with a temperature of 24 ± 1°C and a 12:12-h light:dark cycle (7:00 AM - 7:00 PM).

2.3.2 HPLC and LC-MS/MS analysis of retinoids

Reversed-phase HPLC analysis was performed as previously described (Kim et al., 2008; Kim and Quadro, 2010) to measure retinol and retinyl ester concentrations in serum and tissues, including 100-150 mg small intestine and colon.

To measure retinoic acid (RA) in serum and tissues, samples were prepared as previously described (Zhong et al., 2019a). In brief, tissues (100-120 mg liver or 120-150 mg small intestine or colon) were homogenized with 0.9% saline and 100µL serum was diluted with saline, both in a 5:1 ratio of saline to tissue homogenate or serum. After the

addition of internal standard (5 μL of 2 μM all-*trans*-retinoic acid-d_5; Toronto Research Chemicals (North York, Ontario, Canada), 2 mL of acetonitrile with 1% formic acid was added to tissues homogenates and diluted serum followed by the addition of 10 mL of hexanes to extract different retinoic acid isomers (all-*trans*-, 13-*cis*- and 9-*cis*-retinoic acid). After the organic layer was transferred to a new glass tube and evaporated under N_2 flow, samples were reconstituted in 60% acetonitrile for LC-MS/MS analysis. Samples were analyzed using the AB Sciex 5500 QTRAP Mass Spectrometer coupled with an Agilent 1290 UHPLC (Agilent Technologies; Santa Clara, CA). The LC-MS/MS method used for the analysis of RA including LC conditions and MS parameter settings, was as previously reported (Zhong et al., 2019b).

2.3.3 RNA extraction and Real-Time PCR

RNA extraction and Real-Time PCR were performed as described previously (Spiegler et al., 2018). Briefly, total mRNA was extracted from mouse colon (groups at T1) using RNABee according to the manufacturer's instructions (Tel-test Inc. Friendswood, TX). The concentration and purity of RNA were determined by Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA). One microgram of RNA was reverse transcribed to complementary DNA (cDNA) using Verso cDNA synthesis kit according to the manufacturer's instructions (ThermoFisher Scientific, Dallas, TX). To quantify mRNA, real time PCRs were performed using an Applied Biosystems QuantStudio 3 Applied Biosystem instrument (ThermoFisher Scientific, Dallas, TX). The primers listed in **Table 2.1** were validated for PCR efficiency and a unique melt curve with SYBR Green chemistry. For the RT-PCR experiments, 300 nM of each specific primer (final concentration) were mixed with 25-50 ng of cDNA equivalent of the total RNA, and

7.5 µL of SYBR Green Master Mix (ThermoFisher Scientific, Dallas, TX) in a total volume of 15 µL. Each sample was run in duplicates or triplicates. Relative quantification of mRNA expression was calculated using $2(-\Delta\Delta Ct)$ method (Schmittgen and Livak, 2008), normalized to the TATA-binding protein (TBP) gene. Gene expression changes were expressed as mRNA fold change from the control group (WT on VA-suf).

2.3.4 Histochemical and Immunohistochemical (IHC) analysis

The entire colon segment of the gastrointestinal (GI) tract was dissected and fixed overnight at room temperature in 10% formalin. For each mouse, six 2.5 mm segments were collected every 10 mm along the length of the colon and randomly embedded in the same paraffin block. For each paraffin block, 5 µm sections were cut at three different depths (levels), at an average of 400 µm apart. At each level, three consecutive sections were collected. One section was stained with Hematoxylin and Eosin (H&E); one was stained with Periodic Acid-Schiff (PAS)/Alcian blue, which stains acidic and neutral mucins, respectively, for goblet cells visualization and quantification (Adams and Dilly, 1989); the third one was used for IHC analysis. For IHC studies, the sections were deparaffinized, rehydrated, and blocked with 100% normal goat serum at room temperature for 2 hr. These sections were then incubated overnight at 4°C with a primary rabbit polyclonal Claudin 2 (CLDN-2) antibody (1:200, Invitrogen, Rockford, IL), or Claudin 5 (CLDN-5) antibody (1:200, Invitrogen, Rockford, IL), followed by a 30 min incubation with a biotinylated goat anti-rabbit secondary antibody (1:10000, Vector Labs, Burlingame, CA). Antibody binding was visualized using a DAB Peroxidase Substrate Kit (Vector Labs, Burlingame, CA).

All tissue sections were scanned using a Revolve microscope (ECHO Laboratories, San Diego, CA).

2.3.4 Plasma endotoxin analysis

Plasma endotoxin levels were assessed using a PierceTM LAL Chromogenic Endotoxin Quantitation Kit (Cat no. 88282, ThermoFisher Scientific, Dallas, TX), according to the manufacturer's instructions. Samples were run in triplicate and averaged to provide a plasma endotoxin value for each mouse.

2.3.5 Preparation of hydro-indocyanine green (H-ICG) and in vivo ROS imaging and analysis

H-ICG was prepared from the cyanine dye, indocyanine green, by reduction with $NaBH_4$ as previously described (Kuhn et al., 2018). WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice at T1 VA-def, were gavaged with H-ICG reconstituted in water (0.5 mg/mL) at a dose of 2 mg/kg. *In-Vivo* ROS imaging and analysis was performed by using a Bruker In-Vivo Multispectral (MS) FX PRO imaging system (Bruker, Ettlingen, German) (Kuhn et al., 2018). Briefly, after mice were anesthetized with 2% isoflurane, they were laid down in the abdomen-down position directed towards the camera into the MARS system. Following excitation illumination at 760 nm, emission at 830 nm was recorded using a filter equipped high sensitivity cooled charged coupled device camera. Acquisition time was 30s for near infrared fluorescent (NIRF) images, followed by a bright-field light photograph (0.5 s exposure). Both NIRF and bright-field images were optically superimposed to visualize anatomical information. Fluorescence was quantified as photons/s/mm^2 using Carestream MI software v5/0.529 (Carestream Health Inc., Rochester, NY). The background intensity

of each image was set to zero and identical elliptical regions of interest were drawn on each image (91x103 pixels; interior area = 7373). The mean fluorescence intensity within the ellipse was recorded for each animal.

2.3.6 Short chain fatty acids (SCFA) by GC-MS analysis

Freshly collected fecal samples were weighed and re-suspended in 1mL of 0.5% phosphoric acid per 0.1 g of sample and frozen at −20°C immediately after collection. Once thawed, the fecal suspensions were homogenized for about 2 min and centrifuged for 10 min at 17949 × g. The aqueous fecal suspensions were extracted with diethyl ether (1:1, vol:vol) by vortexing for 30sec followed by centrifugation for 10min at 17949 × g. Organic extracts were stored at −20°C. Prior to analysis, a 600 µL volume of the organic phase was transferred into a tube and 100 µL of 1mM 2-methyl hexanoic acid (ThermoFisher Scientific, Dallas, TX) added as internal standard. The internal standard was used to correct for injection variability between samples and minor changes in the instrument response. Three independent replicate extractions were performed per sample.

The GC-MS analysis was performed as described (García-Villalba et al., 2012). Briefly, the GC-MS system consisted of an Agilent 7890A (Agilent Technologies, Palo Alto, CA), equipped with an automatic liquid sampler (MPS2) (Gerstel, Mulheim, Germany) and coupled to an Agilent 5975C mass selective detector. The GC was fitted with a high polarity, polyethylene glycol (PEG), fused silica capillary column DB-WAXetr (30 m, 0.25 mm id, 0.25 µm film thickness) and helium was used as the carrier gas at 1 mL/min. Injection was made in splitless mode with an injection volume of 1 µL and an injector temperature of 250°C. Data acquisition was performed using the Chemstation software (Hewlett-Packard, Palo Alto, CA). Identification of the SCFAs was based on the

retention time of standard compounds and with the assistance of the NIST 08 libraries. SCFA absolute concentrations were determined based upon standard curves generated for each individual fatty acid.

2.3.7 Fecal sample collection and DNA extraction

Each mouse was placed in an empty cage without bedding for 10–15 min to allow collection of fresh stool samples that were snap frozen in dry ice and kept at -80°C until further processing. Fecal genomic DNA was extracted at the University of Missouri DNA Core facility using PowerFecal kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with the exception that the samples were homogenized in the provided bead tubes using a TissueLyser II (Qiagen, Venlo, Netherlands) for three minutes at 30/sec. DNA yields were quantified via fluorometry (Qubit 2.0, Invitrogen, Carlsbad, CA) using quant-iT BR dsDNA reagent kits (Invitrogen, Carlbad, CA).

2.3.8 16S rRNA library construction and sequencing

Library construction and sequencing were performed at the University of Missouri DNA Core facility (Ericsson et al., 2015). Bacterial 16S rRNA amplicons were generated via amplification of the V4 hypervariable region of the 16S rRNA gene using dual-indexed universal primers (U515F/806R) flanked by Illumina standard adapter sequences and the following parameters: 98°C(3:00)+[98°C(0:15)+50°C(0:30)+72°C(0:30)] × 25 cycles +72°C(7:00). PCR was performed in 50 µL reactions containing 100 ng DNA, primers (0.2 µM each), dNTPs (200 µM each), and Phusion high-fidelity DNA polymerase (1U; ThermoFisher Scientific, Waltham, MA). Amplicon pools (5 µL/reaction) were combined, thoroughly mixed, and then purified by addition of Axygen Axyprep MagPCR clean-up

beads (ThermoFisher Scientific, Waltham, MA) to an equal volume of 50 µL of amplicons and incubated for 15 minutes at room temperature. Products were then washed multiple times with 80% ethanol and the dried pellet was re-suspended in 32.5 µL elution buffer, incubated for 2 minutes at room temperature, and then placed on the magnetic stand for 5 minutes. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system (Agilent, Santa Carla, CA), quantified using quant-iT HS dsDNA reagent kits (Invitrogen, Carlsbad, CA), and diluted according to Illumina's standard protocol for sequencing on the MiSeq instrument (Illumina, San Diego, CA), using the V2 chemistry to generate 2×250 bp paired-end reads.

2.3.9 Informatics Analysis

Read merging, clustering, and annotation of DNA sequences was performed at the University of Missouri Informatics Research Core Facility. Paired DNA sequences were merged using FLASH software and removed if found to be far from the expected length of 292 bases after trimming for base quality of 31. Cutadapt (Martin, 2011) (https://github.com/marcelm/cutadapt) was used to remove the primers at both ends of the contig and cull contigs that did not contain both primers. The u-search (Edgar, 2010) fastq_filter command (http://drive5.com/usearch/manual/cmd_fastq_filter.html) was used for quality trimming of contigs, rejecting those for which the expected number of errors was greater than 0.5. All contigs were trimmed to 248 bases and shorter contigs were removed. The Qiime (Kuczynski et al., 2011) 1.9 command split_libraries_fastq.py was used to demultiplex the samples and the command beta_diversity_through_plots.py was used to subsample data to a uniform read count. The outputs for all samples were combined into a single file for clustering. The uparse (Edgar, 2013) method

(http://www.drive5.com/uparse/) was used to both clusters contigs with 97% identity and remove chimeras. Taxonomy was assigned to selected OTUs using BLAST (Altschul et al., 1997) against the SILVA database v132 (Pruesse et al., 2007) of 16S rRNA gene sequences and taxonomy.

The 16S rRNA-derived OTUs were mapped to fusionDB (C. Zhu et al., 2018a). The microbial functionalities of the mouse fecal microbiome samples were assessed by the pan-functional-repertoire of each OTU (i.e. the functions that exist in at least one member of bacterial corresponding OTU). Then, NMDS (Non-metric multidimensional scaling) analysis (Kruskal, 1964) and the subsequent PERMANOVA test (Oksanen, J. et al., 2016) using the Vegan R package were performed.

2.3.10 Statistical Analysis

SPSS statistical software (IBM SPSS Statistics, version 23; SPSS, Inc.) was used for statistical analysis. The distribution of the data was determined by the Shapiro-Wilk test, and normally distributed data were analyzed by Student's t test or a two-way analysis of variance (ANOVA). Non-normally distributed data were analyzed by Mann-Whitney U test or a Kruskal-Wallis test. Values are presented as the mean ± standard deviation (SD), and $p < 0.05$ was the cutoff for significance.

Table 2.1: Primer sequences used for real-time PCR analysis			
Gene	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Reference**
Tbp	CAAACCCAGAATTGTTCTCCTT	ATGTGGTCTTCCTGAATCCCT	Harvard Primer Data Bank
Muc2	GTCTGCCACCTCATCATGGA	CAGGCAAGCTTCATAGTAGTGCTT	This Study
Muc3	GTGGGACGGGCTCAAATG	CTCTACGCTCTCCACCAGTTCCT	This Study
RegIIIβ	GGCTTATGGCTCCTACTGCTATG	ATGGAGGACAAGAATGAAGCC	PMID: 27183576
RegIIIγ	GCTCCCGTGCCTATGGCTC	ATCATGGAGGACAGGAAGGAAG	PMID: 27183576
Zo-1	TGGGAACAGCACACAGTGAC	GCTGGCCCTCCTTTTAACAC	PMID: 25107366
Occludin	ACCCGAAGAAAGATGGATCG	CATAGTCAGATGGGGGTGGA	Harvard Primer Data Bank
Cldn-2	AGTACCCTTTTAGGACTTCCTGC	CCCACCACAGAGATAATACAAGC	Harvard Primer Data Bank
Cldn-3	ACCAACTGCGTACAAGACGAG	CGGGCACCAACGGGTTATAG	Harvard Primer Data Bank
Cldn-4	ATGGCGTCTATGGGACTACAG	GAGCGCACAACTCAGGATG	Harvard Primer Data Bank
Cldn-5	GCAAGGTGTATGAATCTGTGCT	GTCAAGGTAACAAAGAGTGCCA	Harvard Primer Data Bank
Cldn-7	GGCCTGATAGCGAGCACTG	TGGCGACAAACATGGCTAAGA	Harvard Primer Data Bank
Cldn-8	GCAACCTACGCTCTTCAAATGG	TTCCCAGCGGTTCTCAAACAC	Harvard Primer Data Bank
Il-1β	CAACCAACAAGTGATATTCTCCATG	GTGCCGTCTTTCATTACACAG	Harvard Primer Data Bank
Il-6	TCGGAGGCTTAATTACACATGTTC	TGCCATTGCACAACTCTTTTCT	This Study
Tnf-a	TCGGAGGCTTAATTACACATGTTC	TGCCATTGCACAACTCTTTTCT	Harvard Primer Data Bank
Il-22	CGAGGAGTCAGTGCTAAGGATCAGTG	GATTGCTGAGTTTGGTCAGGAAAGG	PMID: 27183576
Il-22(R)	AGCTCGTGTATCTCTGACGC	TTGGCTCTGTCCATACATCTTGT	This Study
Il-23	AATAATGTGCCCCGTATCCAGT	GCTCCCCTTTGAAGATGTCAG	This Study
Il-23(R)	TGCATGTGGTGATAGCCCTTT	AGGCTCAACCCACATGTCAC	This Study
Il-17	CCCATGGGATTACAACATCACTC	CACTGGGCCTCAGCGATC	PMID: 20434372
Harvard Primer Data Bank: http://pga.mgh.harvard.edu/primerbank/			

2.4 RESULTS

2.4.1 *Lrat-/-Rbp-/-* mice as a tunable model of VAD

The *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice are unable to store vitamin A (due to the lack of LRAT) and unable to mobilize hepatic retinol towards the peripheral tissues (due to the lack of RBP) (Kim et al., 2008; Wassef et al., 2013). Thus, these mutant mice rely exclusively on dietary vitamin A to support retinoid-dependent functions and display an extremely tenuous vitamin A status already when fed retinoid-containing diets (Kim et al., 2008; Wassef et al., 2013). We exploited this genetic model of VAD, together with WT control mice, to discriminate between the effects of the dietary vitamin A and vitamin A status of the host on intestinal retinoid homeostasis, fecal dysbiosis and gut barrier functions. To confirm VAD in this model under the above-described experimental conditions (**Fig. 2.1**), we first measured retinoid levels in serum and liver by HPLC (Kim et al., 2008; Kim and Quadro, 2010) and LC-MS/MS (Zhong et al., 2019a). Serum retinol was significantly lower in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice compared to WT, throughout the experiment, due to the absence of RBP (Kim et al., 2008; Quadro et al., 1999) (**Fig. 2.3A**). *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice at T1 VA-def showed the lowest circulating retinol levels (**Fig. 2.3A**). Given the lack of LRAT (Batten et al., 2004; Liu and Gudas, 2005; O'Byrne et al., 2005), serum retinyl esters were undetectable in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice, at all time points, regardless of the dietary regimen (**Fig. 2.3B**). At T1, retinoic acid levels were also reduced in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice compared to the WT groups, within the same dietary regimen (**Fig. 2.3C**). Moreover, retinoic acid concentration showed a trend ($p = 0.07$) towards a reduction in the marginal (*Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-suf) *vs.* the severe VAD group (*Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-def) (**Fig. 2.3C**). In contrast but as expected (Kim et al., 2008; Quadro et al., 2005, 1999), this relatively short regimen of VA-def diet did not decrease circulating

retinol levels in WT mice (**Fig. 2.3A**). In the WT strain serum retinyl ester concentrations were significantly higher at T2 (**Fig. 2.3B**), perhaps reflecting the higher food intake at this time point (**Fig. 2.2A**). Overall, at T1 serum retinol levels in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice were reduced ~80% on VA-suf and ~98% on VA-def compared to WT mice on the same dietary regimen (**Fig. 2.3A**). Thus, serum retinoid concentrations at T1 confirmed that the dietary vitamin A deprivation of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice further compromised the already vitamin A deficient status of this strain.

Hepatic retinol concentration was significantly lower in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice compared to WT at all time points (**Fig. 2.3D**), and retinyl esters were undetectable (**Fig. 2.3E**), as previously reported (Kim et al., 2008). At T1 VA-def, liver retinol (**Fig. 2.3D**) and retinoic acid concentration (**Fig. 2.3F**) in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice were the lowest among all groups and time points. Four weeks of VA-def decreased hepatic retinyl ester in WT mice (**Fig. 2.3E**). Notably, in WT mice, one week of the regular chow diet, following four weeks of the VA-def regimen (**Fig. 2.3E**, T2 chow in pink), was not sufficient to restore hepatic retinoid stores to those of age-matched WT mice never deprived of dietary vitamin A (**Fig. 2.3E**, T2 chow in green). Overall, hepatic total retinol (retinol + retinyl esters) levels were dramatically depleted in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice already on the VA-suf diet, whereas retinoic acid deficiency developed in the liver only under the VA-def dietary regimen.

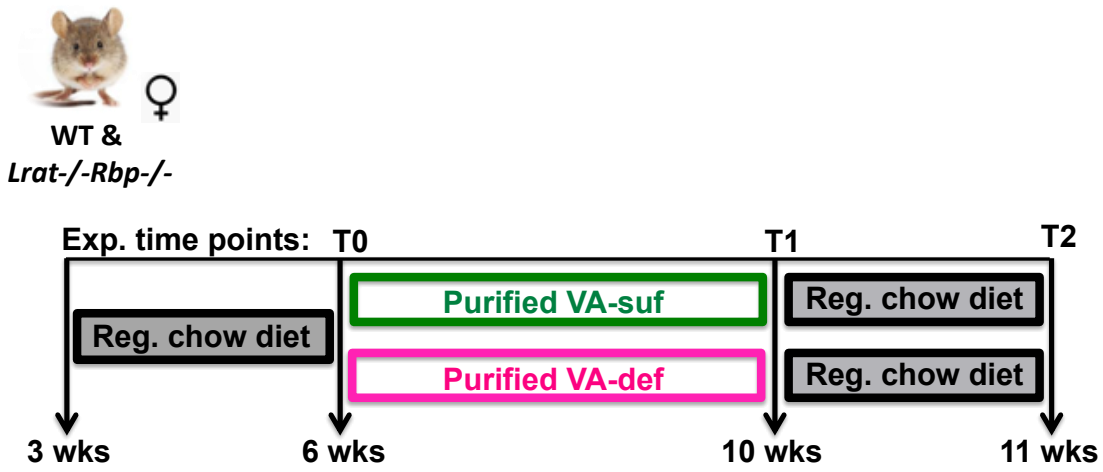

Figure 2.1: Experimental scheme

The number of weeks (wks) indicates the age of the mice. Wild type and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ female mice were maintained on regular chow (18IU VA/g diet) from weaning (3 weeks of age). At 6 weeks of age [the beginning of the experiment (T0), mice were randomly placed either on a purified vitamin A sufficient (VA-suf; 20 IU VA/g diet) or purified vitamin A deficient (VA-def; < 0.2 IU VA/g diet) diet for four weeks, e.g., until 10 weeks of age (T1). Mice were then returned to the regular chow diet for 1 week, e.g., until 11 weeks of age (T2). At each time point (T0 to T2), after collection of fresh feces, subgroups of mice were sacrificed to harvest serum and tissues (liver, small intestine, colon and adipose tissue) for further analysis. Chow, regular rodent diet; VA-d, vitamin A-deficient diet; VA-s, vitamin A-sufficient diet.

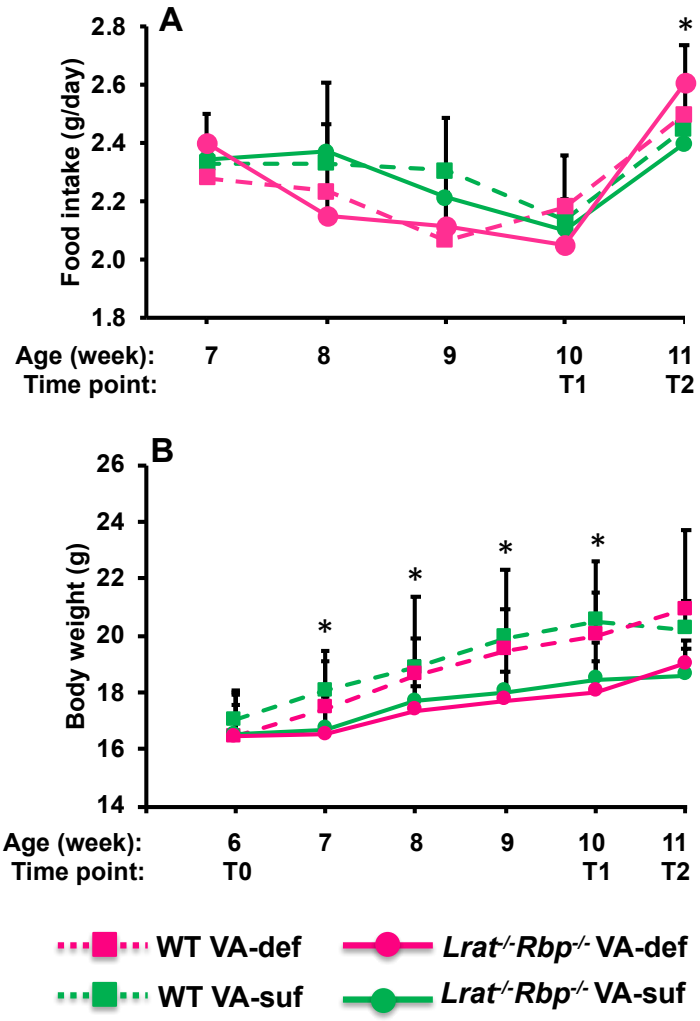

Figure 2.2: Food intake and body weight change in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice throughout the experiment.

(**A**) Food intake and (**B**) body weight throughout the experiment. T0, T1 and T2 indicate the experimental time points as in **Fig. 2.1**. Week indicates the age of the mice. Values are mean ± SD. Statistical analysis by two-way ANOVA at each time point; *, $p < 0.05$ indicates significant differences between *Lrat*$^{-/-}$*Rbp*$^{-/-}$ (VA-suf and VA-def) *vs.* the reference group WT VA-suf.

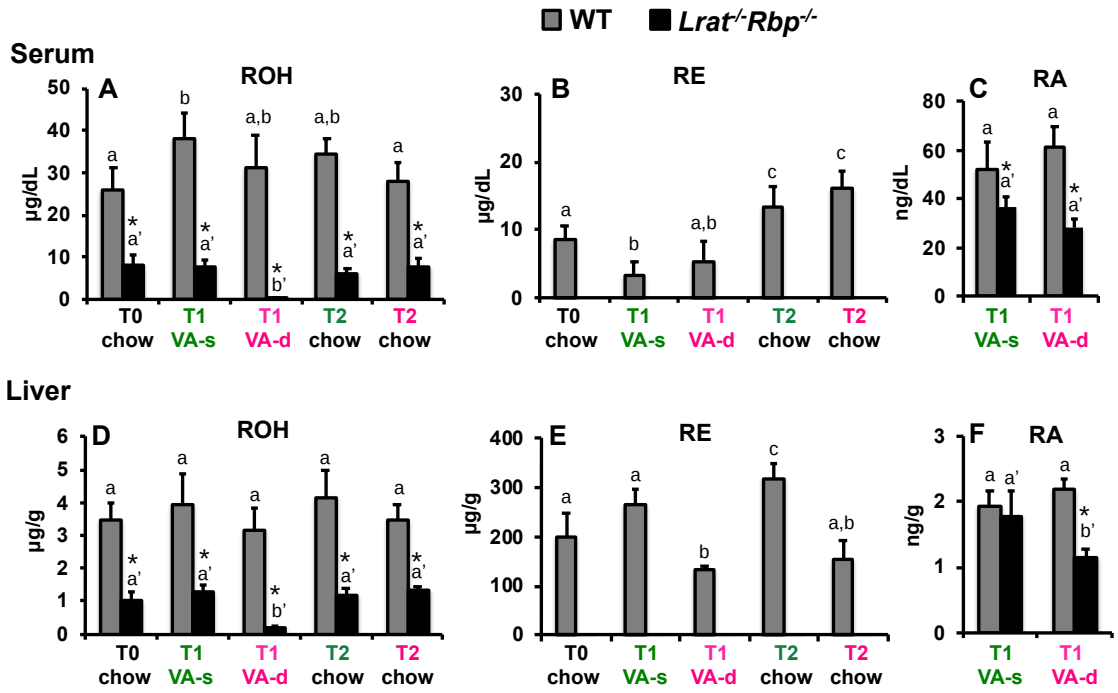

Figure 2.3: Serum and liver retinoid concentrations in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice.

Serum (A and B) and liver (D and E) retinol (ROH) and retinyl ester (RE) levels were determined by reversed-phase HPLC. Serum (C) and liver (F) retinoic acid (RA) levels were determined by LC-MS/MS. Data are mean ± SD; n = 3 - 5 mice/group. Statistical analysis by two-way ANOVA for normally distributed data and by Mann–Whitney test for non-normally distributed data. Different letters without (‘) indicates significant differences among the WT groups throughout the experiment. Different letter with (‘) indicates significant differences among *Lrat*$^{-/-}$*Rbp*$^{-/-}$ groups throughout the experiment. *, indicates $p < 0.05$ between WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ at each individual time point. Chow, regular rodent diet; VA-d, vitamin A-deficient diet; VA-s, vitamin A-sufficient diet. Red arrow indicates the percent reduction in serum ROH between WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ at T1 VA-s and T1 VA-d.

Previous findings pointed to a compensatory mechanism that enables mice to build retinoid stores in the adipose tissue in the absence of LRAT (Liu and Gudas, 2005; O'Byrne et al., 2005; Wongsiriroj et al., 2008b). Indeed, compared to WT mice, retinol (**Fig. 2.4A**) and retinyl ester (**Fig. 2.4B**) concentrations were elevated in the adipose tissue of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice maintained on regular chow or purified VA-suf diet. Dietary vitamin A deprivation (T1 VA-def) decreased adipose retinoids in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ but not in the WT mice (**Fig. 2.4A and B**), confirming that this is indeed the storage site the mutant mice utilize to overcome the lack of vitamin A in the diet. One week of regular chow diet after four weeks of the VA-def regimen was not sufficient to restore adipose retinoid stores in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice (T2 chow in pink) to those of age-matched *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice never deprived of dietary vitamin A (T2 chow in green) (**Fig. 2.4B**).

Altogether, liver and adipose retinoid concentrations indicated that the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice have very limited retinoid stores already under the vitamin A sufficient regimen. These stores become nearly exhausted in the absence of dietary vitamin A, consistent with what appears to be a more severe VAD status of the mutant mice on the VA-def regimen, based on their serum retinol levels.

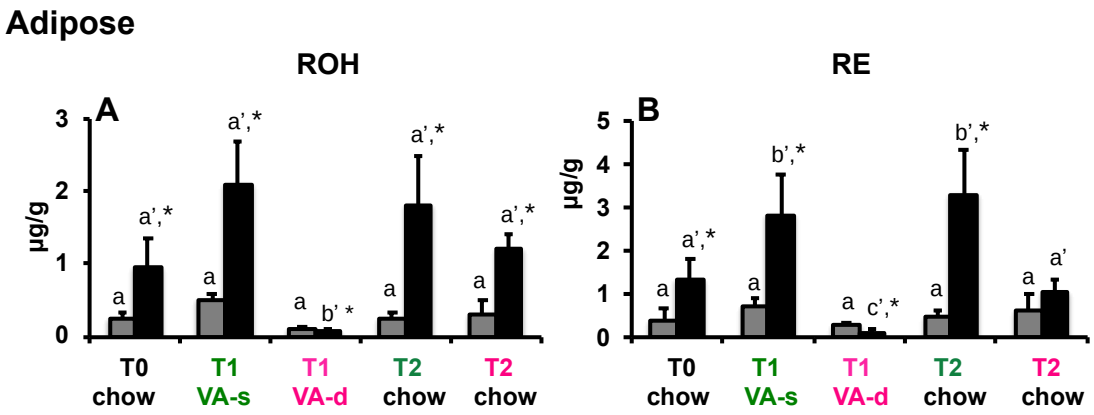

Figure 2.4: Retinol and retinyl ester levels in adipose tissue in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice throughout the experiment.

(**A**) retinol (ROH) and (**B**) retinyl ester (RE) levels were determined by reversed-phase HPLC analysis. Data are mean ± SD; n = 3 - 5 mice/group. Statistical analysis by two-way ANOVA for normally distributed data and by Mann–Whitney test for non-normally distributed data. Different letters without (‘) indicates significant differences among the WT groups throughout the experiment. Different letter with (‘) indicates significant differences among *Lrat*$^{-/-}$*Rbp*$^{-/-}$ groups throughout the experiment. *, indicates $p < 0.05$ between WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ at each individual time point. Chow, regular rodent diet; VA-d, vitamin A-deficient diet; VA-s, vitamin A-sufficient diet.

2.4.2 Vitamin A homeostasis in the gastrointestinal tract (GI) of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice

Vitamin A homoeostasis in the GI tract of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice was never investigated. In order to assess the retinoids content of the intestine in the presence and absence of the dietary vitamin A during VAD *vs.* VAS in our mouse model, we measured retinoid levels by HPLC (Kim et al., 2008; Kim and Quadro, 2010) and LC-MS/MS (Zhong et al., 2019b) in small intestine and colon of the mice from all the groups (**Fig. 2.1**). In the small intestine (duodenum), at each specific time point on the regular chow or purified VA-suf diet, retinol levels were similar between genotypes (**Fig. 2.5A**). Lack of dietary vitamin A (T1 VA-def) significantly diminished small intestine retinol content in both genotypes with a further significant reduction in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ *vs.* WT mice **(Fig. 2.5A**). Retinyl esters were also significantly diminished in the small intestine of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice compared to WT at all time points (**Fig. 2.5B**), confirming that LRAT is the main enzyme that esterifies retinol in the duodenum (O'Byrne et al., 2005; Wongsiriroj et al., 2008b). Furthermore, retinyl ester levels dropped significantly in the WT duodenum upon dietary vitamin A deprivation, pointing to the dietary origin of the majority of the retinoids in this GI segment (**Fig. 2.5B**). Moreover, retinoic acid levels were significantly reduced only in the duodenum of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on the VA-def (**Fig. 2.5C**).

Surprisingly, retinol **(Fig. 2.5D)** and retinyl ester **(Fig. 2.5E)** levels in the colon were similar among genotypes at each experimental time point, except at T1 VA-def when the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice showed the lowest concentration (**Fig. 2.5D**). Notably, dietary vitamin A deprivation (T1 VA-def) reduced colon retinyl ester levels in both strains (**Fig. 2.5E**). Interestingly, in the WT strain the magnitude of the retinyl ester reduction induced by the lack of vitamin A in the diet was greater in the small intestine than in the colon, suggesting

a less pronounced contribution of the diet to the overall retinoid content of the colon (compare **Fig. 2.5B and E)**.

At T1 VA-suf, retinoic acid levels were similar between the two genotypes (**Fig. 2.5F**). Lack of dietary vitamin A (T1 VA-def) however significantly diminished retinoic acid levels in the colon of both WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice. The latter also displayed significantly reduced retinoic acid concentration compared to WT VA-def (**Fig. 2.5F**).

These data clearly demonstrate that, like in other tissues of this mouse model of VAD, dietary vitamin A deprivation of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice further exacerbates the retinoid-deficiency of the GI tract.

Altogether, our results confirm that the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ regardless of the dietary vitamin A represent a model of VAD, based on their reduced serum retinol and retinoic acid levels. Under a regimen of sufficient vitamin A intake, however, tissues maintained normal retinoic acid concentrations, despite their lower total retinol levels. Furthermore, the dietary vitamin A deprivation exacerbated the VAD status of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice based on the further reduction of circulating retinol and retinoic acid levels, as well as the attenuation of tissues retinoic acid and total retinol (retinol and retinyl ester) concentrations.

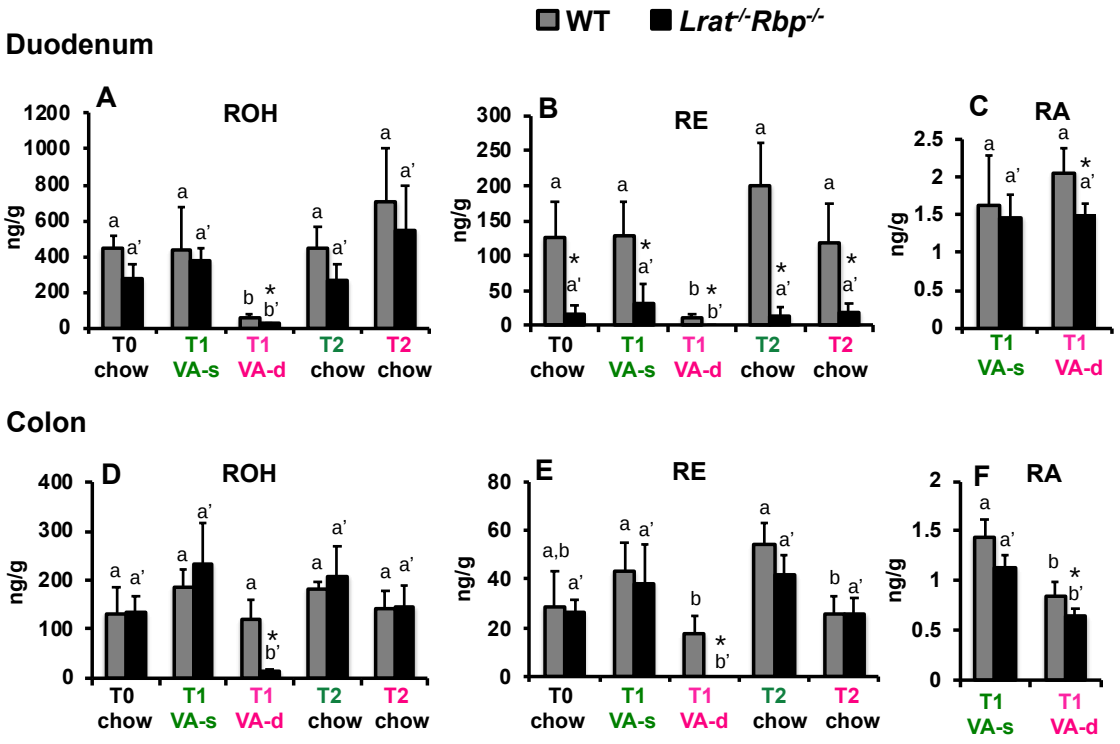

Figure 2.5: Retinoid concentrations in duodenum and colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice.

Small intestine (duodenum) (**A and B**) and colon (**D and E**) retinol (ROH) and retinyl ester (RE) levels were determined by reversed-phase HPLC. Retinoic acid (RA) levels in duodenum (**C**) and colon (**F**) were determined by LC-MS/MS. Data are mean ± SD; n = 3–5 mice/group. Statistical analysis by two-way ANOVA and by Mann–Whitney test for non-normally distributed data. Different letters indicate difference within genotype at each time point. Different letters without (‘) indicates significant differences among the WT groups throughout the experiment. Different letter with (‘) indicates significant differences among *Lrat*$^{-/-}$*Rbp*$^{-/-}$ groups throughout the experiment. *, indicates $p < 0.05$ between WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ at each individual time point. Chow, regular rodent diet; VA-d, vitamin A-deficient diet; VA-s, vitamin A-sufficient diet.

2.4.3 Fecal microbiome is altered in *Lrat-/-Rbp-/-* mice

To gain more insights into the interaction between vitamin A status, dietary vitamin A intake and gut microbial community, DNA was extracted from the feces collected at each time point (as in **Fig. 2.1**), the 16S rRNA genes were amplified and sequenced. The sequences were annotated using the SILVA database (Pruesse et al., 2007).

α-diversity was assessed based on the Shannon diversity index and showed no significant differences among the experimental groups, regardless of genotype and/or diet (**Table 2.2**). Annotation of sequence data to the level of phyla revealed *Actinobacteria, Bacteroidetes, Deferribacteres, Firmicutes* and *Proteobacteria* as the most abundant phyla in the fecal microbial population, regardless of genotype and/or diet (**Fig. 2.6A**), as reported in mice and humans (Nguyen et al., 2015). The ratio between *Firmicutes* and *Bacteroidetes* was not significantly different among the experimental groups at T1 (**Fig. 2.6B**) or at any other experimental time point (data not shown). By contrast, *Actinobacteria* were significantly reduced in the marginal and severe VAD mice compared to the WT groups on both diets at all time points (**Fig. 2.6A and C**). Note that, compared to the regular chow diet, the purified diet promoted a relative higher abundance of the *Actinobacteria*, regardless of the mouse genotype and dietary vitamin A content (**Fig. 2.6A**). This increased abundance of *Actinobacteria* likely reflects the difference in fibers composition between the two diets, given that these bacteria are known to be proficient at degrading complex carbohydrate, such as cellulose, which was more abundant in the purified diets (Pellizzon and Ricci, 2018). Furthermore, a significant reduction of *Bifidobacterium* sp. (phylum of *Actinobacteria*) and *Allobaculum* sp. (phylum of *Firmicutes*), as well as a higher abundance of the *Prevotella* sp. (phylum of *Bacteroidetes*) was observed in the marginally

and severely VAD mice compared with the WT groups on both diets (**Fig. 2.6D-F**).

	T0 (Reg chow)	**T1 (VA-suf)**	**T1 (VA-def)**	**T2 (Reg chow)**
WT	3.7 ± 0.3	3.9 ± 0.1	3.7 ± 0.2	3.9 ± 0.1
Lrat$^{-/-}$*Rbp*$^{-/-}$	3.7 ± 0.6	4.1 ± 0.1	3.9 ± 0.2	3.9 ± 0.3
Measured by Shannon diversity index; values are mean ± SD, n= 3-5 mice per group.				

Table 2.2: Alpha-diversity by Shannon index

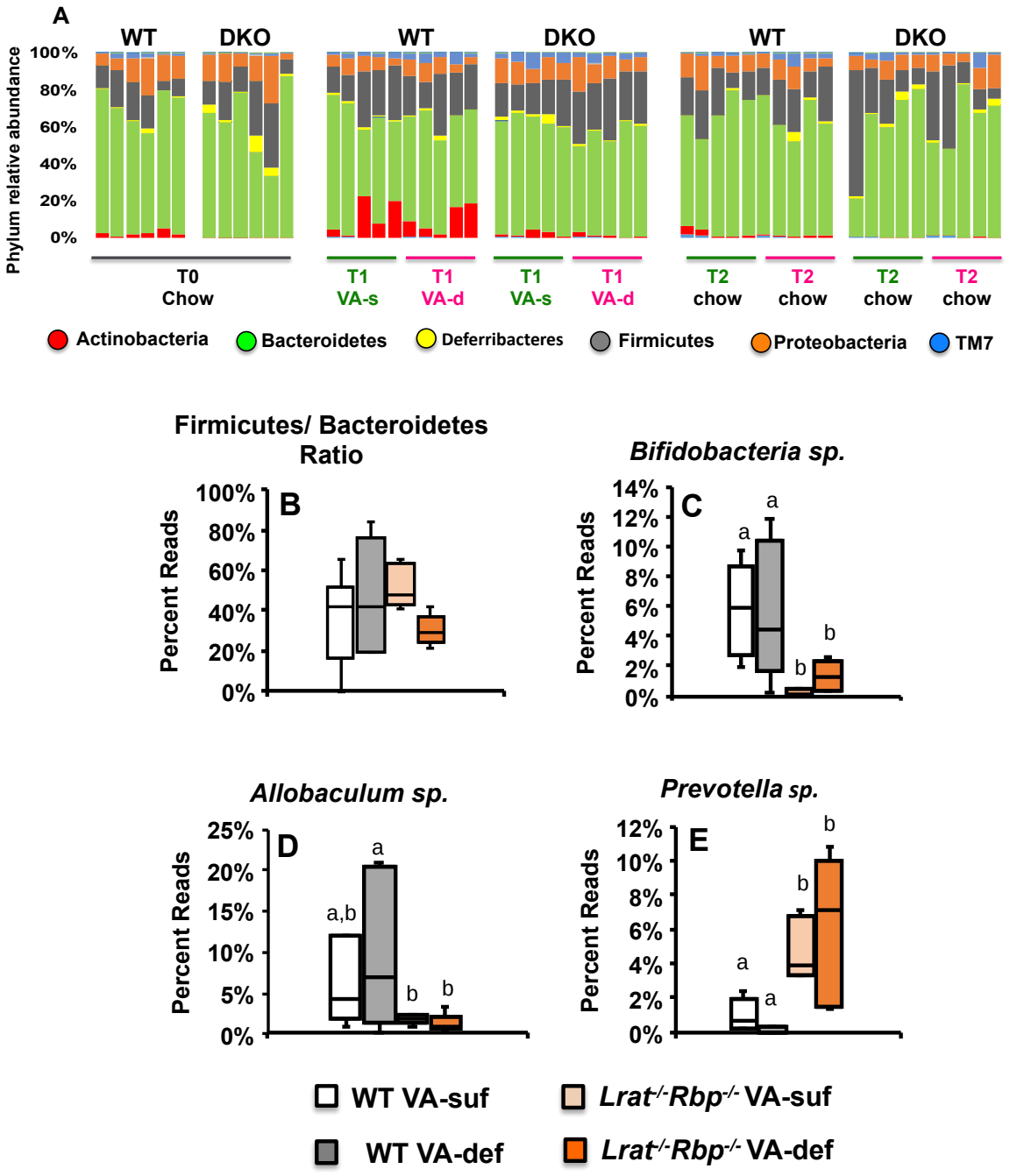

Figure 2.6: Impact of vitamin A deficiency status on fecal microbial composition. (**A**) Relative abundance of bacterial phyla in mouse fecal samples at the various time points (T0, T1 and T2) throughout the experiment. Percent ratio reads of (**B**) Firmicutes/Bacteroidetes and (**C-E**) *Bifidobacteria* sp. *Allobaculum* sp. and *Prevotella* sp. in the fecal microbiota at T1. Statistical analysis by two-way ANOVA. Different letters indicate significant difference ($p < 0.05$) among the groups.

Differences in community composition (i.e., β-diversity) among the four groups at T1 were analyzed by principal component analysis (PCA). **Figure 2.7** shows that the samples clustered based on genotype, i.e., based on the vitamin A status (PC1, accounting for 37.63% of variance) and not the vitamin A content of the diet. Moreover, the taxonomic footprint of the fecal bacteria community dietary seems to be independent from dietary vitamin A modulation of both WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice.

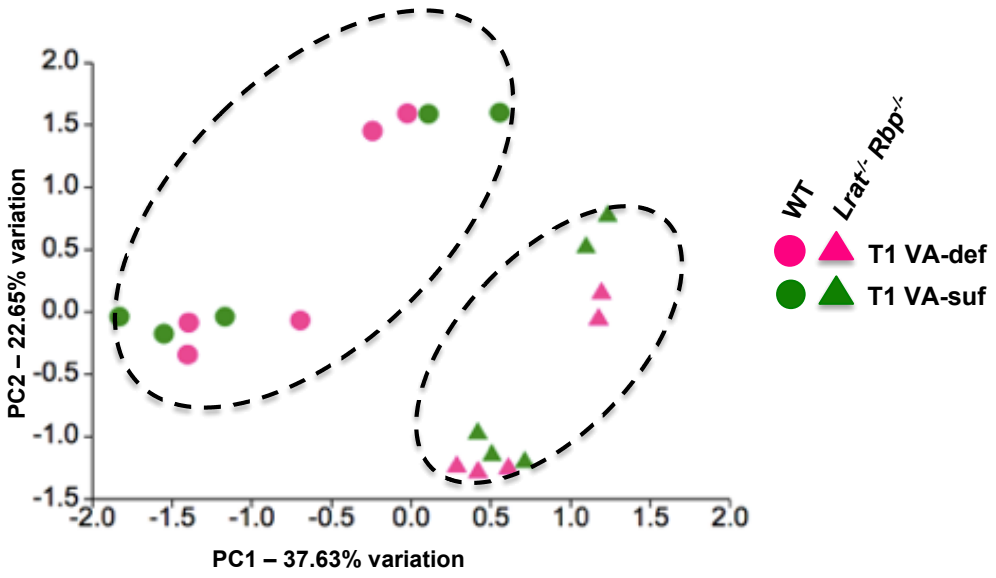

Figure 2.7: Impact of vitamin A deficiency status on B-diversity of fecal microbial composition.

Principal component analysis (PCA) of the 16S rRNA genes in fecal samples at T1. PC1 separated samples by status and accounted for 37.63% of total variance; PC2 separated samples by time points and accounted for 22.65% of total variance; n = 5 mice/group.

To determine which OTUs were the major contributors to the differences associated with the vitamin A status, a loading plot was generated from the same dataset used for the PCA analysis (**Fig. 2.8**). VAD status was associated with higher abundance of bacteria such as *Bacteroides* spp., *Sutterella*, *Helicobacter apodemus*, and *Paraprevotella* spp. and the family *Mogibacteriaceae*, while the vitamin A sufficient status was linked to a greater abundance of *Allobaculum* spp, *Bifidobacterium* and *Rikenella* spp., as well as of the family *Coriobacteriaceae* (**Fig. 2.8**). Overall, in agreement with the literature, our findings indicate that VAD correlates with lower fecal abundance of bacteria with known antioxidant and anti-inflammatory properties, including *Bifidobacterium* sp. (Huda et al., 2019), and a greater abundance of bacteria with known pro-inflammatory activities, including *Bacteroides* sp. (Hibberd et al., 2017).

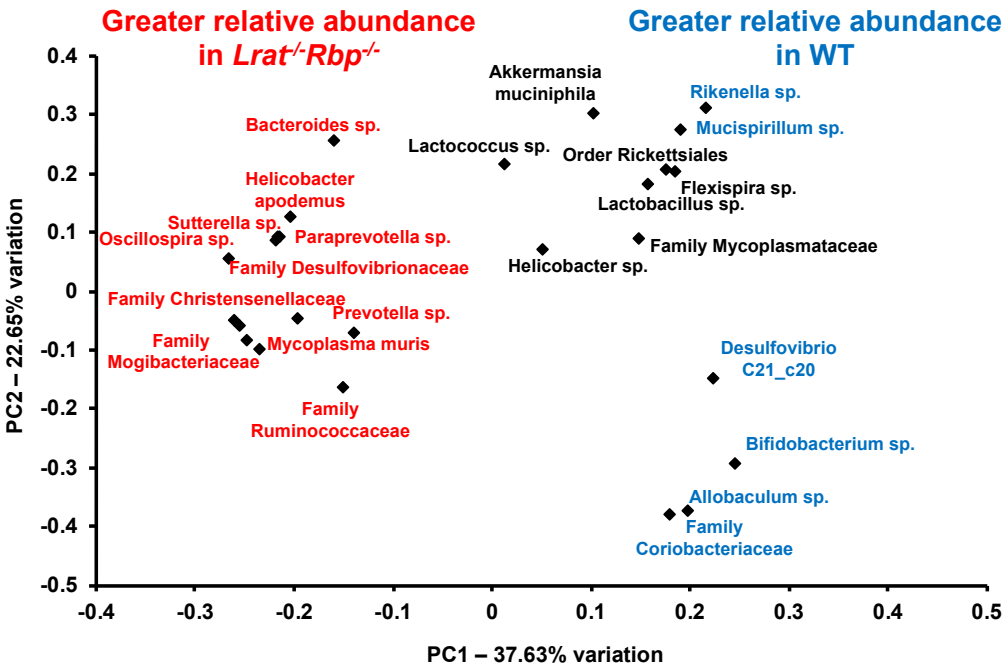

Figure 2.8: Contribution of individual OTUs to the detected variation in PC analysis. Loadings plot of the PCA data on **Fig. 2.7** showing the relative contribution of all operational taxonomic units (OTUs) to principal component 1 (PC1) and PC2. OTU highlighted in blue color indicate a significant higher abundance in the WT, and OTU highlighted in red indicate a significance higher abundance in *Lrat*$^{-/-}$*Rbp*$^{-/-}$, regardless of the diet.

Since VAD has been associated with fewer butyrate-producing gut bacteria in children (Lv et al., 2016) and mice (Tian et al., 2018), we also investigated whether dietary vitamin A intake impacted butyrate-producing gut bacteria and their products during VAD. Based on 16S rRNA gene sequence analysis, butyrate-producing bacteria, such as *Roseburia* sp., were undetectable in the feces during VAD (*Lrat*$^{-/-}$*Rbp*$^{-/-}$) regardless of the dietary vitamin A intake (**Fig. 2.9A**), whereas *Clostridium* sp. were undetectable exclusively in fecal samples from VAD mice maintained on dietary vitamin A (data not shown). In agreement with these findings, direct measurements of short chain fatty acids (SCFA) in fecal samples from the various groups of mice at T1, confirmed that VAD status is associated with significantly lower levels of all SCFA, including butyrate, whereas the dietary vitamin A intake content does not affect their concentration, at least in the feces (**Fig. 2.9B-G**).

Taken together these data suggest that vitamin A status (sufficient *vs.* severely deficient), rather than the amount of vitamin A in the diet *per se*, might be a major discriminant of the taxonomic of fecal microbiota.

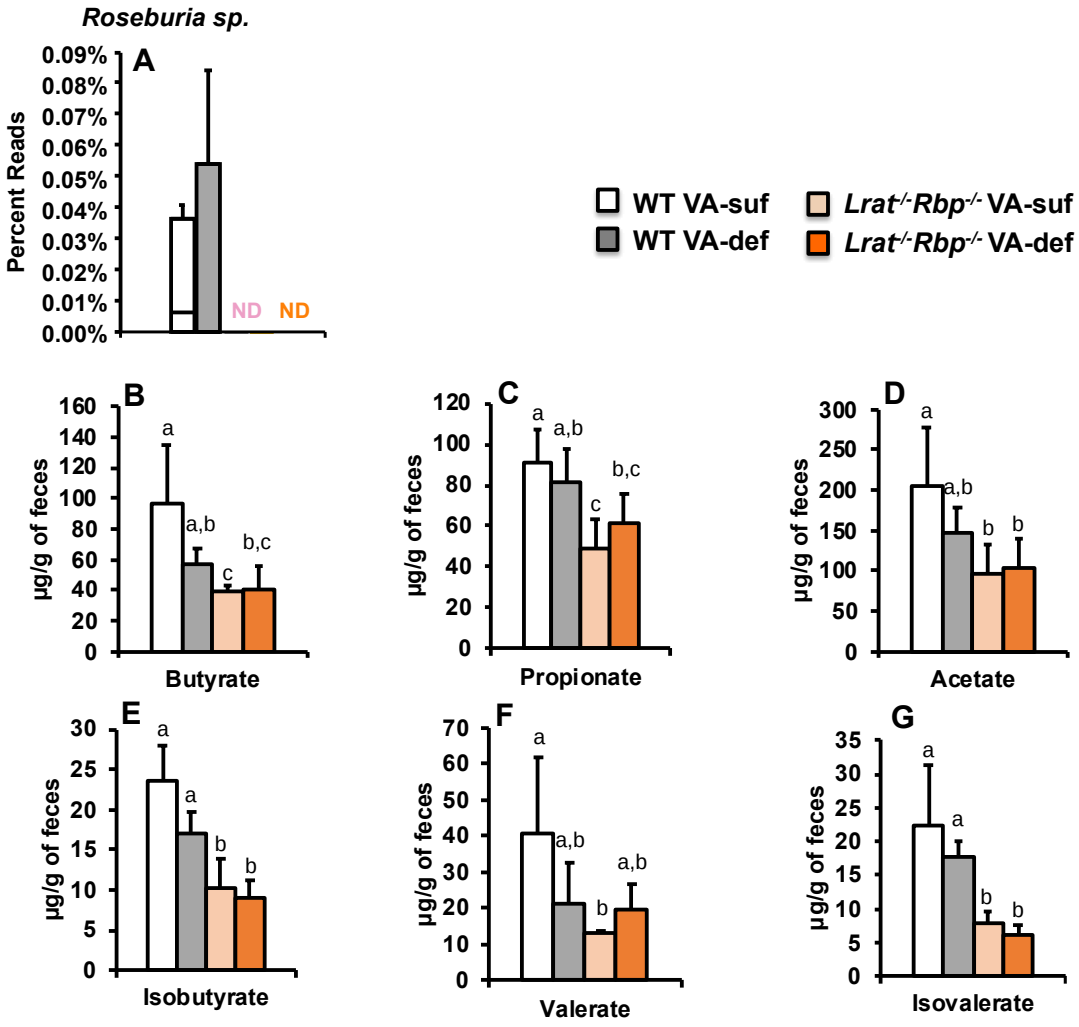

Figure 2.9: Vitamin A deficiency reduce butyrate production.

(**A**) Percent genus reads of *Roseburia* sp. in the fecal microbiota of the various groups of mice at T1. (**B-G**) Concentration (µg/g wet feces) of the short-chain fatty acids (SCFAs) in fecal samples from mice at T1. Values are mean ± SD; n = 3 - 4 samples/group; statistical analysis by Mann–Whitney test. Different letters indicate significant differences among the groups ($p < 0.05$). ND, not detected; VA-def, vitamin A deficient diet; VA-suf, vitamin A sufficient diet.

2.4.4 Preliminary functional profile of the fecal microbiome of VAD

To further extend our knowledge, we sought to predict the functional profile of the fecal microbiome of our model of VAD by annotating the 16S rRNA-based OTUs of the fecal microbiome using QIIME (Caporaso et al., 2010). We mapped the detected taxonomies to fusionDB (C. Zhu et al., 2018a), a database that represents bacteria with all the molecular functions encoded in their genomes. Interestingly, we observed a significant separation of microbial functionalities between VAS (WT) and VAD (*Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-suf) mice at T0 (PERMANOVA test, $p < 0.01$; **Fig. 2.10A**) and between VAS (WT VA-suf, WT VA-def), VAD (*Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-suf), and severe VAD (*Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-def) mice at T1 (PERMANOVA test, $p < 0.001$; **Fig. 2.10B**). In an effort to identify accurate microbial functional signals between VAD and VAS, we further curated the fusionDB functional profiles with a recently compiled protein database, in which all the functions are experimentally verified, and observed in bacteria (C. Zhu et al., 2018b). Notably, we found that enzymes involved in butanoate metabolism, which is associated with VAD status (Tian et al., 2018), were differentially represented in VAD as compared to the microbiome of the vitamin A sufficient mice ($p < 0.01$, *t*-test).

Thus, despite the fecal bacteria taxonomic similarities between *Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-suf and VA-def, a clear phenotypic separation based on vitamin A status of the host emerged when microbial functionalities were evaluated.

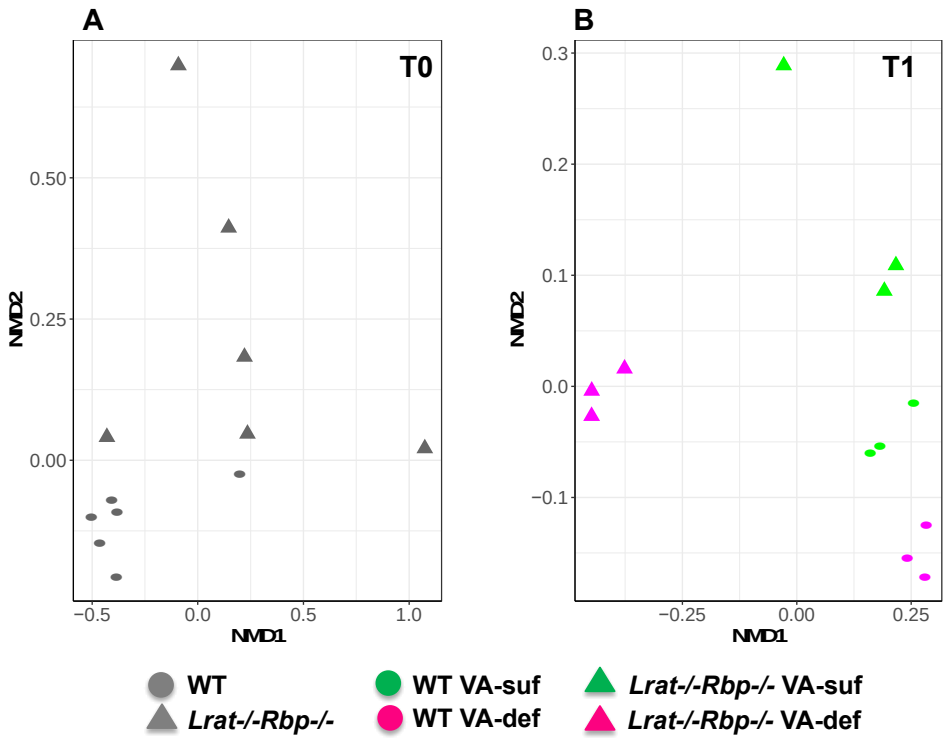

Figure 2.10: FusionDB-derived microbiome functional profile associates with vitamin A status and dietary vitamin A.

NMDS plots of the pan-functional profiles of mouse fecal microbiome at (**A**) T0 (grey circle: WT mice VA-suf; grey triangle: *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice VA-suf) and (**B**) T1 (green circle: WT mice VA-suf; pink circle: WT mice VA-def; green triangle: *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice VA-suf; pink triangle: *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice VA-def diet). VA-def, vitamin A-deficient diet; VA-suf, vitamin A-sufficient diet.

2.4.5 VAD compromised the intestinal barrier in *Lrat-/-Rbp-/-* mice

VAD has been associated with several morphological and functional retinoid-dependent alterations in the intestinal barrier that protects the body against microbes and antigens present in food (Amit-Romach et al., 2009; Cha et al., 2010; Rubin et al., 2017; Sirisinha, 2015). We therefore wondered to what extent, in our mouse model, VAD altered the intestinal barrier in the colon, where most of the bacteria reside. Our experimental set up enabled us to investigate the extent to which vitamin A status and intake would regulate the above-mentioned functions.

The intestinal mucus layer, the first physical line of defense of the GI tract, is mainly composed of MUC2, produced specifically by the goblet cells, and MUC3, expressed in both goblet cells and enterocytes (Johansson and Hansson, 2016). In agreement with the notion that VAD affects mucin expression (Amit-Romach et al., 2009; Fan et al., 2015), we found a significant reduction in the number of goblet cells, identified by PAS staining, in both *Lrat*$^{-/-}$*Rbp*$^{-/-}$ groups compared to WT VA-suf (**Fig. 2.1A and B**). Moreover, *Muc2* and *Muc3* expression was downregulated in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice regardless of their dietary regimen compared to WT mice (**Fig. 2.1C**). Dietary vitamin A deprivation of WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice did not affect either the number of goblet cells (**Fig. 2.11A and B**) or the expression of the mucin genes (**Fig. 2.11C**).

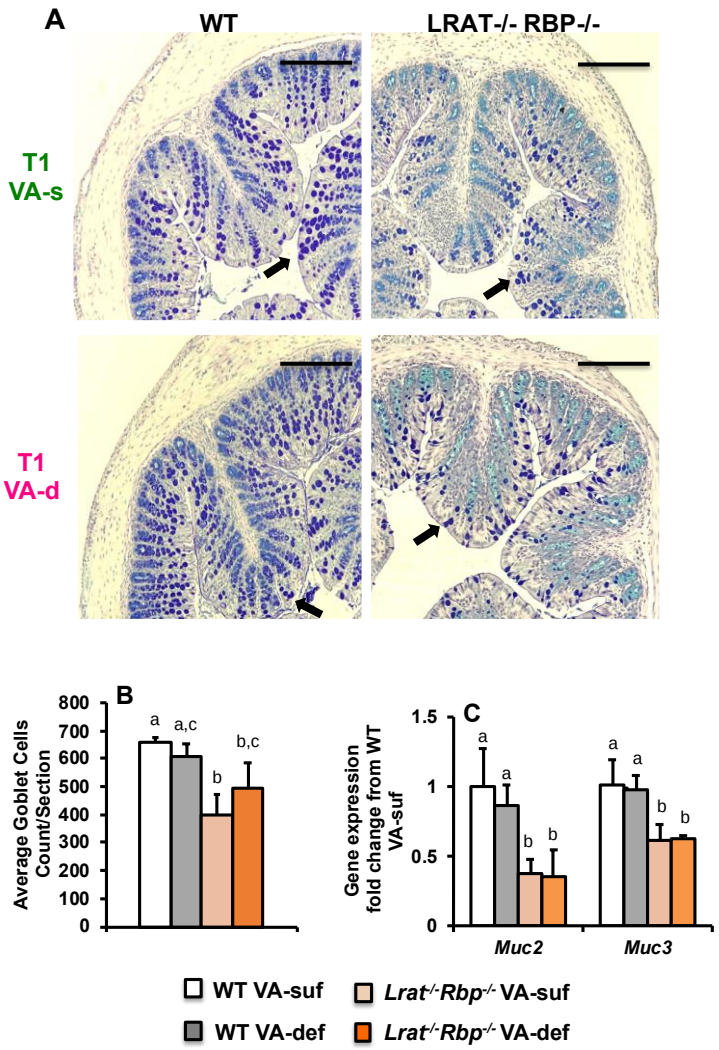

Figure 2.11: Goblet cells and mucin in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1.
For each mouse, tissue embedding and sections were performed as described in Materials and Methods. (**A**) Alcian blue-periodic acids-Shiff (AB-PAS) staining; scale bar = 220 µm; 100X magnification arrows point to goblet cells. (**B**) Quantification of the number of goblet cells using three different depths with an average of 400 µm apart of six colon sections/mouse; n= 3 mice/group; data as mean ± SD. (**C**) Real-time RT-PCR analysis of mRNA expression levels of mucins (*Muc2* and *Muc3*), data are mean ± SD, calculated using the $2^{-\Delta\Delta CT}$ method; n = 5-6 mice/group. Different letters indicate significant differences among the groups ($p<0.05$). Statistical analysis by two-way ANOVA. VA-def, vitamin A-deficient diet; VA-suf, vitamin A-sufficient diet.

VAD is also associated with reduced production of antimicrobial proteins (such as RegIIIβ and RegIIIγ) that, synthesized throughout the intestine by different cell types, control the number of luminal bacteria (Goverse et al., 2016). The expression of both *RegIIIβ* and *RegIIIγ* genes was significantly attenuated in the colon of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice compared to WT mice on the VA-suf (**Fig. 2.12**). Vitamin A deprivation significantly reduced *RegIIIβ* and *RegIIIγ* mRNA levels in both genotype (**Fig. 2.12**) and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on VA-def showed the lowest expression levels of these two genes compared to all other groups **(Fig. 2.12**).

Membrane tight-junction proteins, including occludin, claudins (CLDNs) and junctional complex proteins such as zonula occludens (ZO), play a critical role in the maintenance of both integrity and permeability of the gut barrier (Krug et al., 2012; Niessen, 2007; Van Itallie and Anderson, 2004). The mRNA expression of key genes of tight-junction proteins, including *occludin*, *Cldn-2* (pore forming protein) and *Cldn-3*, *-7* and *-8* (tightening proteins), was significantly attenuated in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice, regardless of the vitamin A content of the diet (**Fig. 2.13A**). However, immunohistochemical analysis did not show any change in CLDN-2 protein between groups (**Fig. 2.13B**). Instead, CLDN-2 seemed to be localized more towards the luminal surface in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ rather than at the crypt as in WT mice. Re-distribution of the CLDN-2 in the GI tract had been associated with barrier dysfunction and colitis (Ridyard et al., 2007; Yuan et al., 2015) (**Fig. 2.13B**). In contrast, the VA-def diet resulted in increased expression of *Cldn-5* and *-8* only in the colon of WT mice, while *Cldn-4* increased in the colon of both genotypes (**Fig. 2.13A**). Immunohistochemical analysis for CLDN-5 also confirmed the mRNA expression results (**Fig. 2.13D**).

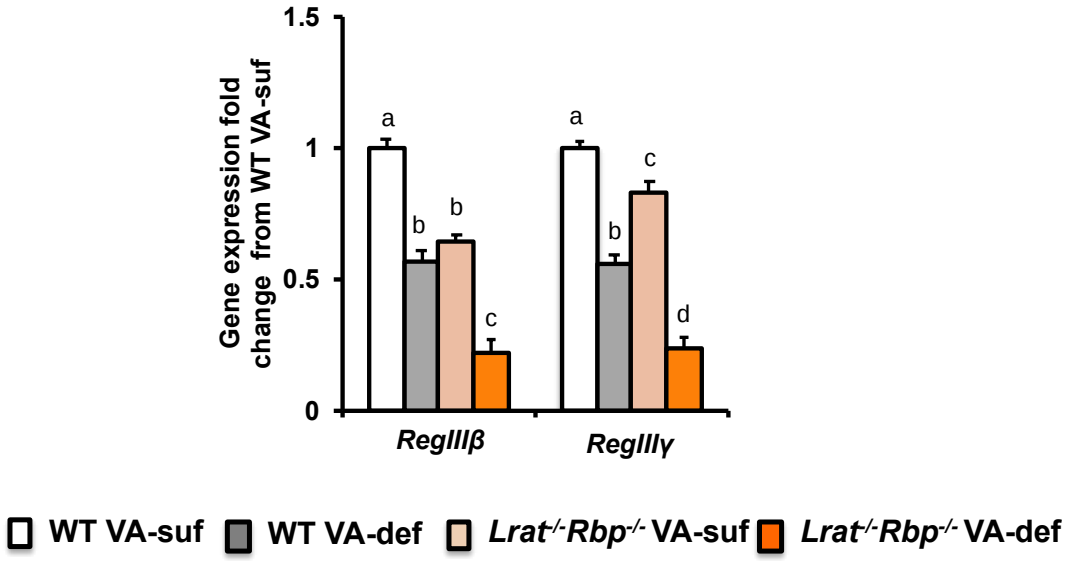

Figure 2.12: Antimicrobial peptide expression in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1.

Real-time RT-PCR analysis of mRNA expression levels of antimicrobial peptides (*RegIIIβ* and *RegIIIγ*). Values are mean ± SD calculated using the $2^{-\Delta\Delta CT}$ method; n = 3 individual sample pools/group (6 animals per pool). Statistical analysis by two-way ANOVA within each gene. Different letters indicate significant difference ($p < 0.05$) within each gene; VA-def, vitamin A-deficient diet; VA-suf, vitamin A-sufficient diet.

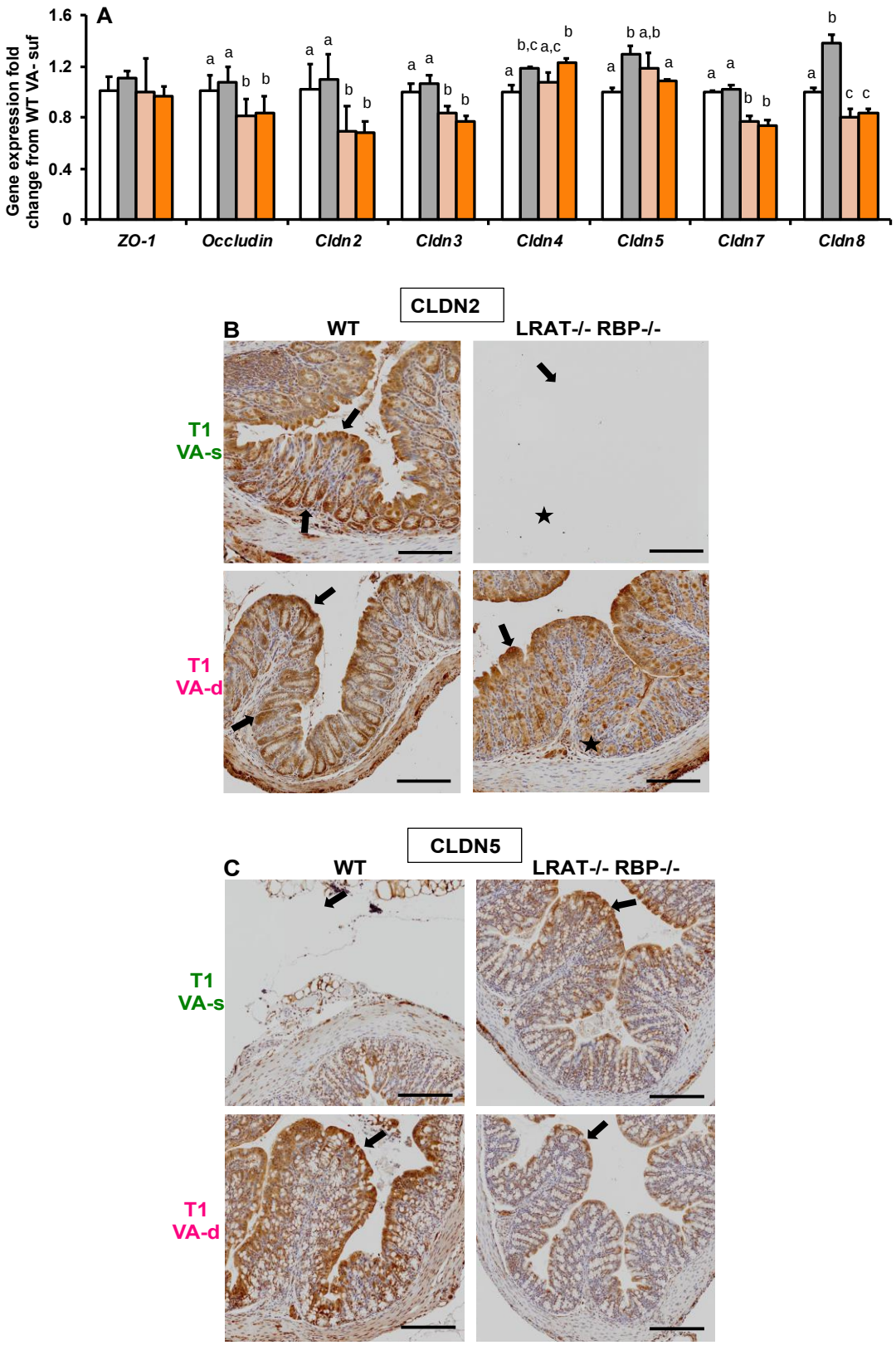

Figure 2.13: Tight-junction proteins in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1.

(**A**) mRNA expression of tight-junction proteins (*Zo-1, Occludin, Claud-2, Claud-3, Claud-4, Claud-5, Claud-7 and Claud-8*). Representative colon sections stained with (**B**) anti-Claudin 2 antibody (scale bar = 220 μm; 200X magnification; arrows point to positive staining, star points to the reduction) or (**C**) anti-Claudin 5 antibody (scale bar = 220 μm; 200X magnification); n = 3 mice/group; six sections for each mouse were stained. For panels A, data are mean ± SD, calculated using the $2^{-\Delta\Delta CT}$ method; n = 5-6 mice/group or 3 individual sample pools/group (6 mice/pool). Statistical analysis by two-way ANOVA within each gene. Different letters indicate significant difference ($p < 0.05$) within each gene. Cldn, claudin; VA-def, vitamin A-deficient diet; VA-suf, vitamin A-sufficient diet.

To further assess whether these VAD-dependent changes in tight-junction protein expression resulted in increased intestinal permeability, we measured serum concentrations of microbial lipopolysaccharide (LPS). Only *Lrat*$^{-/-}$*Rbp*$^{-/-}$ on VA-def mice had significantly higher concentrations of circulating LPS **(Fig. 2.14A)** and the lack of dietary vitamin A did not result in the translocation of LPS into the circulation in the case of WT mice. These data suggest that the dietary vitamin A restriction alone does not lead to intestinal permeability, unless the host has already a tenuous vitamin A status. Furthermore, the expression of *Alppl2* and *Alpi*, two enzymes synthesized by the enterocytes that modulate LPS toxicity (Yang et al., 2015), was attenuated in the colon of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice, regardless of their degree of VAD **(Fig. 2.14B).**

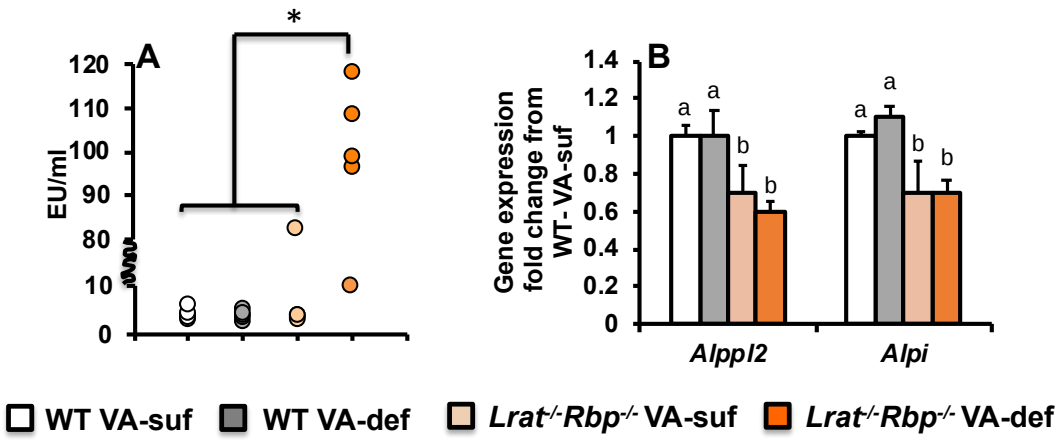

Figure 2.14: Serum LPS concentration of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1.

(A) Serum LPS concentrations measured by a commercial kit (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit); n = 5 mice/group. Statistical analysis by Mann–Whitney test. *, indicates $p < 0.05$ between *Lrat*$^{-/-}$*Rbp*$^{-/-}$ on VA-def and the other groups. (**B**) Real-time RT-PCR analysis of mRNA expression levels of alkaline phosphatases (*Alppl2* and *Alpi*). data are mean ± SD, calculated using the $2^{-\Delta\Delta CT}$ method; n = 5-6 mice/group Statistical analysis by two-way ANOVA within each gene. Different letters indicate significant difference ($p < 0.05$) within each gene. EU, endotoxin unit; VA-def, vitamin A-deficient diet; VA-suf, vitamin A-sufficient diet.

2.4.6 Oxidative stress status in the intestine of *Lrat-/-Rbp-/-* mice

VAD is associated with oxidative stress (Arruda et al., 2009; Chiu et al., 2008; Sohlenius-Sternbeck et al., 2000; Spiegler et al., 2018) which in turn, results in increased generation of reactive oxygen species (ROS) that, together with oxygen, could diffuse into the gut lumen from a structurally compromised intestinal epithelium (Kuhn et al., 2018) and disturb bacterial population (Yang et al., 2016). Thus, we measured intestinal ROS levels by optical in vivo imaging using near-infrared fluorescence (NIRF) light generated by cyanine-based fluorescent dyes in WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice at T1. ROS were significantly increased in the intestinal lumen of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-def mice compared to other groups (**Fig. 2.15A and B**) as measured by the enhanced intensity of the fluorescent signal. Interestingly, lack of dietary vitamin A slightly increased basal luminal ROS in the WT animals relative to VA-suf diet of the same genotype.

Next, to understand whether ROS originated from intestinal cell could contribute to the elevated ROS observed in the lumen, we did a survey of the genes involved in oxidative stress regulation in the colon. The mRNA levels of the antioxidant genes *Sod1/2*, *Gpx1/4* and *PrxIII* were significantly reduced in the colon of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice, compared to WT animals, regardless of the dietary vitamin A content (**Fig. 2.16**), suggesting a genotype effect on the expression of these antioxidant genes. In contrast, dietary vitamin A restriction resulted in increased expression of antioxidant genes only in the WT, specifically *Sod-2* and *Gpx-1* (**Fig. 2.16).** Overall, these data suggest that dietary vitamin A restriction triggers the oxidative defense system in the colon. This system however seems impaired in the mutant mice likely resulting in higher accumulation of ROS in their intestinal lumen under VA-def.

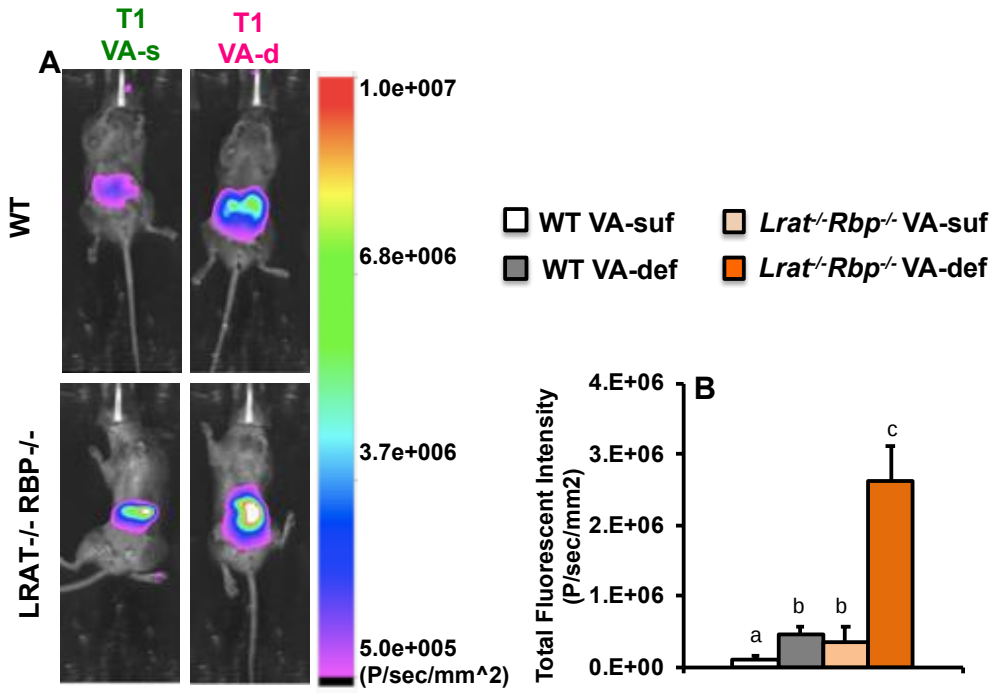

Figure 2.15: ROS levels in the intestinal lumen of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1.

(**A**) Overlay of ROS-associated NIRF image and corresponding brightfield image of WT T1-VA-d mice (top) and T1 VA-d mice (bottom). Near-infrared fluorescence (NIRF) intensity scale shown on the right. Image was normalized accordingly using Carestream MI software. (**B**) ROS-associated NIRF in WT mice and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ T1 mice after administration of hydro-indocyanine green (H-ICG). n = 4 mice/group. Values are mean ± SD. Statistical analysis by two-way ANOVA; *, $p < 0.05$. Different letters indicate significant difference ($p < 0.05$) within each gene. VA-def, vitamin A-deficient; VA-suf, vitamin A-sufficient.

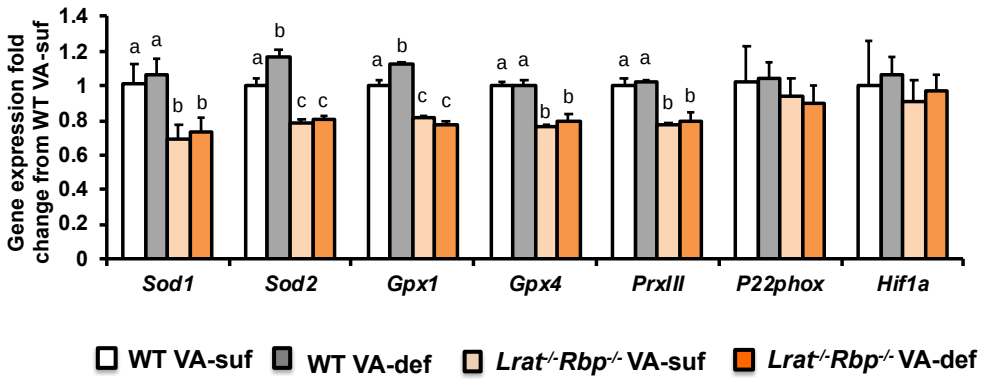

Figure 2.16: Oxidative stress in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1.

mRNA expression levels of oxidative stress response genes (*Sod-1, Sod-2, Gpx1, Gpx2, PrxIII, P22phox, Hif1a*) measured by real-time RT-PCR in mouse colon. Values as mean ± SD calculated using the $2^{-\Delta\Delta CT}$ method; 3 individual pools/group (6 animals per pool). Statistical analysis by two-way ANOVA within each gene. Different letters indicate significant difference ($p < 0.05$) within each gene. VA-def, vitamin A-deficient; VA-suf, vitamin A-sufficient.

2.4.6 Inflammatory and immunomodulatory cytokines in the intestine of the *Lrat-/-Rbp-/-* mice

VAD impairs mucosal immunity, and retinoic acid regulates this intestinal function at multiple levels (Goverse et al., 2016; Hall et al., 2011; Spencer et al., 2014; Veldhoen and Brucklacher-Waldert, 2012; Wilhelm et al., 2017). Interestingly, we observed larger lymphoid follicles in the colon of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice, independent of their dietary regimen (**Fig. 2.17**). We therefore tested the mRNA expression of key inflammatory markers. As shown in **Fig. 2.18A**, *Il-6* and *Tnfα* were generally elevated in the colon of the VAD mice (*Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-suf and VA-def) compared to both WT groups. Surprisingly, *Tnfα*, *Il-1β* and *Il-6* were significantly upregulated in the colon of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-suf, not only compared to WT on both diets but also compared to *Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-def, suggesting a paradoxical increased inflammatory tone in the colon of the mutant mice fed the retinoid-containing diet.

Both Interleukin (IL)-22 and -17 are key regulators of intestinal immune function and inflammation (Wilhelm et al., 2017; Zenewicz, 2018; B. Zeng et al., 2019). Their expression is modulated by other cytokines such as IL-23, IL-6 and TNFα (Parks et al., 2015; B. Zeng et al., 2019), as well as by retinoic acid in the case of *Il-22* (Oliveira et al., 2018). *Il-22* expression was significantly attenuated in the colon of WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice VA-def (**Fig. 2.18B**), suggesting the dependence of this phenotype on the dietary vitamin A intake. Surprisingly, though, this gene and the one encoding its receptor (Il-22R) were significantly upregulated in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on VA-suf diet (**Fig. 2.18B**). Moreover, expression levels of Il-23 increased in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on both diets, whereas a slight but significant upregulation of its receptor (Il-23R) could only be observed in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ VA-suf group **(Fig. 2.18B**). The *Il-17* mRNA levels were also

significantly increased exclusively in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ on the VA-suf compared to all other groups (**Fig. 2.18B**). Overall, the aberrant expression of *Il-22* and *Il-17* in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ on VA-suf diet agrees with the paradoxical increased inflammatory tone in their colon.

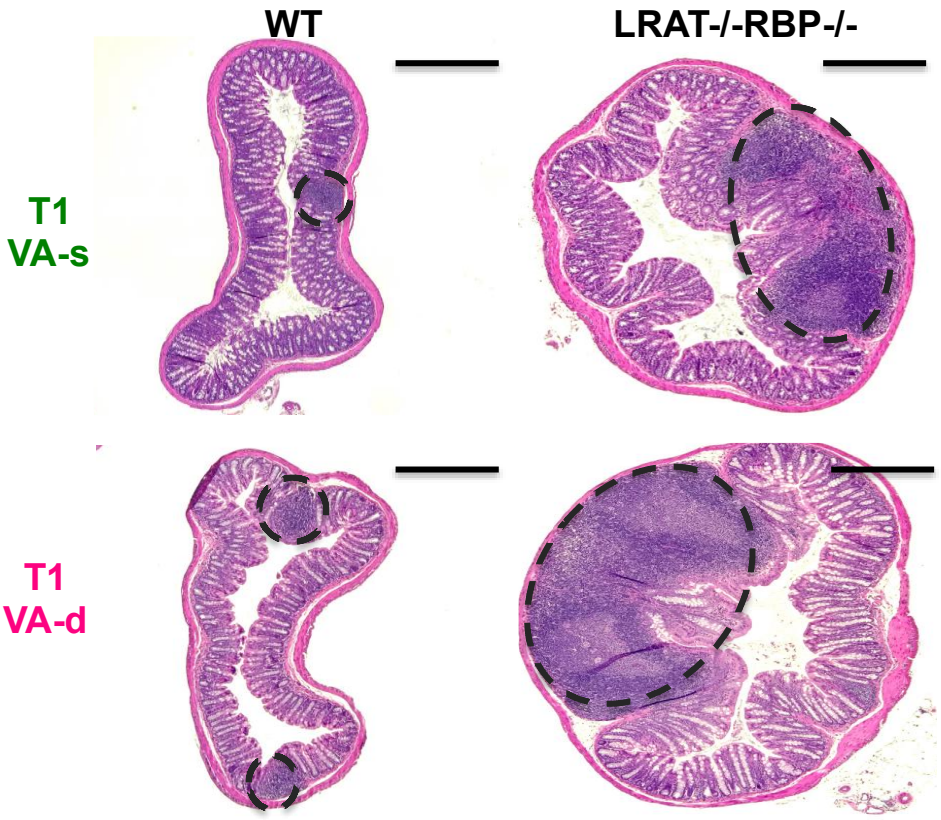

Figure 2.17: Lymph node in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1.

Representative H&E-stained cross sections of the colon. Scale bar = 550 μm; 40X magnification, n = 3 mice/group, six sections for each mouse were stained. Dark grey circle indicates lymphoid organs.

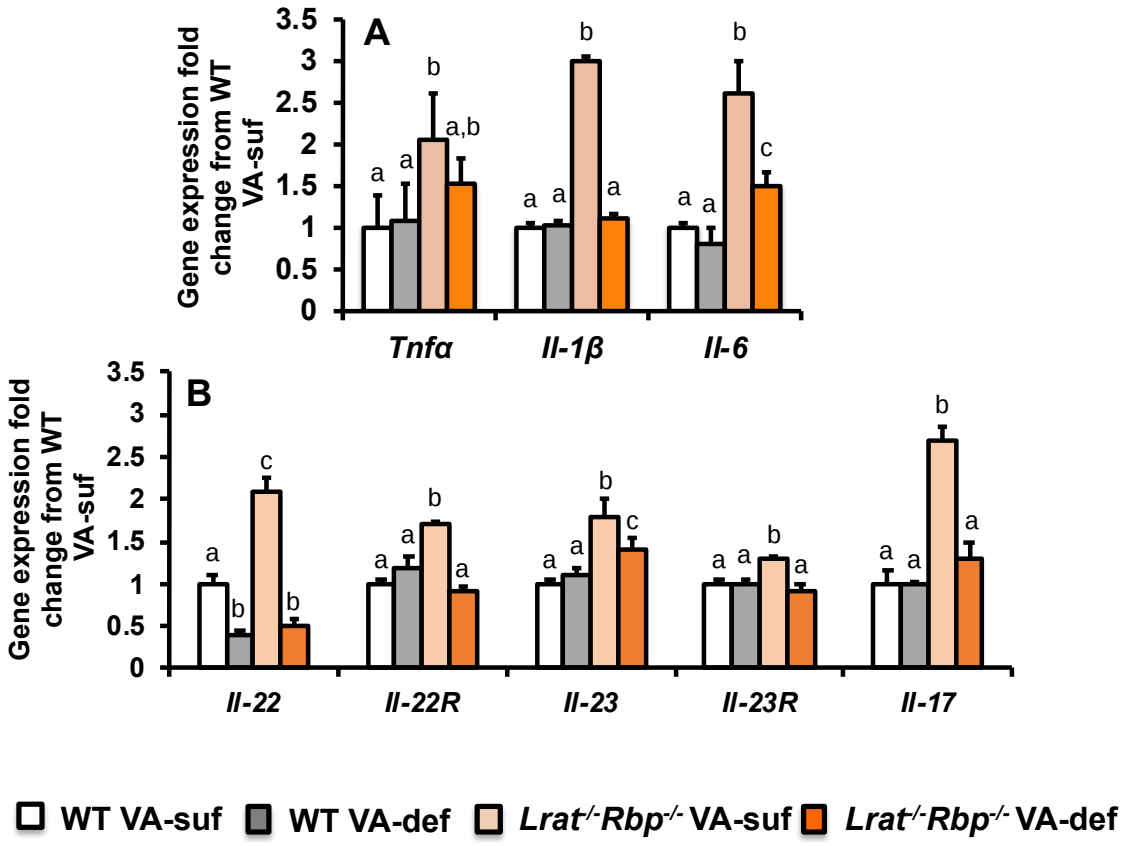

Figure 2.18: Innate immunity and inflammation markers in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ *and WT* mice at T1.

Real-time RT-PCR analysis of mRNA expression levels of (**A**) inflammatory cytokines (*Tnfα*, *Il-1β* and *Il-6*) and (**B**) interleukins and their receptors (*Il22*, *Il22R*, *Il23*, *Il23R*, *Il17*). Values are mean ± SD calculated using the $2^{-\Delta\Delta CT}$ method; n = 3 individual sample pools/group (6 animals per pool). Statistical analysis by two-way ANOVA within each gene. Different letters indicate significant difference ($p < 0.05$) within each gene; VA-def, vitamin A-deficient diet; VA-suf, vitamin A-sufficient diet.

2.5 DISCUSSION

The goal of this study was to gain insights into the impact of host dietary vitamin A intake and status on fecal microbiome diversity and intestinal retinoid homeostasis as well as barrier functions by using a unique genetic mouse model of VAD (*Lrat*$^{-/-}$*Rbp*$^{-/-}$ strain). These mice have a tenuous vitamin A status already on vitamin A-containing diets that is further compromised upon depriving the food of the vitamin (Kim et al., 2008; Wassef et al., 2013).

We provided a comprehensive analysis of the intestinal retinoid homeostasis depending upon the host vitamin A intake and status. Our data confirm that LRAT is the major enzyme that esterifies retinol in the small intestine (O'Byrne et al., 2005; Wongsiriroj et al., 2008b). This is not the case in the colon, despite *Lrat* expression in this segment of the GI tract (by RT-PCR analysis, *Lrat* CT values were similar in WT colon and duodenum, i.e., 28.3 ± 0.3 and 27.5 ± 1.2, respectively; n = 6/group). Indeed, in the colon, retinyl ester levels were found to be similar in the mutant and WT mice on the vitamin A-containing diets (chow or purified) (**Fig. 2.5E**). Perhaps, DGAT also functions to esterify retinol in this GI segment, but this remains to be unequivocally proved. As most of the vitamin A absorption occurs in the small intestine (Goodman and Blaner, 1984), it was not surprising that retinyl ester levels dropped significantly in the WT duodenum upon dietary vitamin A deprivation (**Fig. 2.5B**). In contrast, the contribution of the newly absorbed dietary vitamin A to the total retinol content in the colon was smaller than in the duodenum (percent reduction of total retinol upon dietary vitamin A deprivation of the WT mice was 88% in the duodenum and 35% in the colon), in agreement with the limited role of the colon in the absorption of dietary vitamin A (Goodman and Blaner, 1984). Perhaps

this is the reason why the colon appeared to be more resistant to develop retinoid deficiency than the small intestine. STRA6, the receptor for the complex retinol-RBP (Kawaguchi et al., 2015, 2007), has been shown to be expressed *in vivo* in the small intestine (Grizotte-Lake et al., 2018), as well as in a human colonic cell line (Berry et al., 2014). Thus, it is reasonable to hypothesize that the vitamin A mobilized from the hepatic stores as retinol-RBP could be delivered to both segments of the gut, hence the presence of retinol and retinyl esters - although at reduced levels - in duodenum and colon of WT mice deprived of dietary vitamin A (**Fig. 2.5**). In the mutant mice, i.e., in the absence of RBP, retinoids could still be delivered to the GI tract by retinyl ester-containing chylomicrons (Li et al., 2014), as 25% of these postprandial lipoprotein particles is cleared from the circulation by extrahepatic tissues (Li et al., 2014). Indeed, retinol and retinyl esters become undetectable in the GI of the mutant mice on the vitamin A deficient diet (**Fig. 2.5**). Clearly, dietary vitamin A maintains retinoic acid homeostasis in the tissues, including small intestine, of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ on the VA-suf regimen, despite their extremely low levels of serum retinol and tissue retinoid stores. Notably, these mice are viable and fertile when maintained on vitamin A-containing diets (Kim et al., 2008). The dietary vitamin A restriction however resulted in retinoic acid deficiency in all the tissues of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice, including duodenum and colon, exacerbating their vitamin A deficient status. Note that even smaller fluctuations in the concentration of a transcriptional regulator, such as retinoic acid, can have a large impact of overall cell and tissue functions.

It is becoming evident that VAD compromises the intestinal microbiome (Hibberd et al., 2017; Lv et al., 2016; Tian et al., 2018), although the distinction between the primary

effects of the dietary micronutrient content *vs.* the host health-related effects has remained difficult to elucidate. By using a gnotobiotic mouse model of human gut microbiome development, Hibberd and colleagues (Hibberd et al., 2017) proposed that micronutrient deficiencies (few weeks of dietary vitamin A deprivation) disrupt microbiome development directly. However, in this study, the vitamin A status of the mice was not rigorously assessed, making it a challenge to broadly interpret the effects of the dietary manipulation, especially in a model with a compromised gastrointestinal tract, like the gnotobiotic mice (Nicklas et al., 2015). Recently, Tian and colleagues (Tian et al., 2018) induced VAD in WT mice by maintaining the mothers on the VA-def during pregnancy and the weanlings on the same diet up to 12 weeks of age. In agreement with other reports in mice and humans (Hibberd et al., 2017; Lv et al., 2016), and based on cecal 16S rRNA sequencing, these authors showed that fewer *Bacteroidetes* and fewer butyrate-producing bacteria were present in the intestine of the VAD mice, and proposed that "changes in microbiome are caused by VAD" (Tian et al., 2018). Our findings support this relationship. Our experimental scheme (**Fig. 2.1**) enabled us to ask whether the taxonomic footprint of the fecal bacteria community was dependent upon i) the vitamin A content of the diet and/or ii) the vitamin A status. Neither the PCA nor the functional analysis of the 16S rRNA gene sequences separated the two WT groups based on the vitamin A concentration in their diet (**Fig. 2.7 and 10**). Likewise, the two WT groups were, for the most part, undistinguishable when intestinal structure and functions were assessed. Similarly, the fecal profile of the two *Lrat*$^{-/-}$*Rbp*$^{-/-}$ groups could not be separated by PCA based on the diet (**Fig. 2.7**). These findings indicate that the bacterial populations of the GI tract are not sensitive to the amount of vitamin A in the diet (see further below). This is reasonable if

one considers that the absorption of dietary vitamin A is not a regulated process; in other words, the more vitamin A is ingested, the more is absorbed (Harrison, 2012). Thus, the amount of unabsorbed vitamin A that travels from the small intestine (where most of the absorption occurs) through the colon to be ultimately excreted with the feces is normally negligible (Liu et al., 2008). In our study, the PCA separated the fecal microbial taxa based on the genotype of the mice, which reflects their vitamin A status (vitamin A sufficient *vs.* deficient) (**Fig. 2.7)**. This work was conducted with female mice. However, given the influence of sex hormones and estrous cycle on the microbiome (Org et al., 2016), it still needs to be understood whether the effects of VAD may be sex-dependent. Also, it remains to be established whether these changes in fecal microbial community mirror those in the bacteria within the intestine, including its mucosa, which is emerging as a critical factor in defining bacteria-host interactions (Parker et al., 2018).

Interestingly, the analysis of the fecal microbial functionalities with fusionDB (C. Zhu et al., 2018a) not only confirmed the distinction of the groups of mice based on genotype, i.e., vitamin A sufficient *vs.* deficient status, but also clearly distinguished the two groups of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice (**Fig. 2.10A and B**). This latter separation is not due to the vitamin A content of the diet *per se,* as it was not seen in the WT mice. Therefore, we reasoned that it is linked to the exacerbated VAD of the mutant mice secondary to the dietary vitamin A restriction. Despite the relatively small sample size used in this preliminary functional analysis, our findings argue in favor of the impact of the VAD status rather than the genetic background of the mutant strain *per se* on the fecal microbiome. Furthermore, this analysis demonstrates the potential of this approach in identifying

microbiome functional signatures that in the future could offer diagnostic value in assessing vitamin A status, e.g. tracking specific microbiome metabolites.

Barrier function(s) depends primarily on the mucus layer that separates the epithelial cells from the luminal content (Johansson et al., 2011, 2008) while providing a niche for the intestinal bacteria to colonize the gut (Johansson et al., 2011). Previous studies have shown that mucin expression (*Muc2* and *Muc5*) is regulated by retinoic acid, at least in human epithelial cells *in vitro* (Choi et al., 2003; Koo et al., 1999). In the colon of VAD rats, *Muc2* expression was reduced by 75% while *Muc3* was increased (Amit-Romach et al., 2009) In contrast, in the small intestine of VAD mice, *Muc2* mRNA levels increased (Cha et al., 2010), suggesting region-specific differences within the GI tract. In accord with their vitamin A deficient status, the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice displayed attenuated expression of *Muc2/3* in the colon regardless of dietary vitamin A content (**Fig. 2.11C**), whereas in the WT colon *Muc2/3* remained steady under dietary vitamin A restriction (**Fig. 2.11C**). Given that colon retinoic acid concentrations were not significantly different between WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on the VA-suf, it seems unlikely that the reduced expression of the mucin genes in the mutant mice arose from a retinoic acid-dependent transcriptional repression of *Muc2/3*. Instead, it could be secondary to the reduced number of goblet cells (**Fig. 2.11A**). The changes in mucins could result in alterations of the intestinal mucus layer (Schroeder, 2019) in the mutant mice that could have favored the shift in microbial populations observed in their fecal microbiome. For instance, it could have inhibited the growth of mucin-dependent bacteria such as *Allobaculum* (**Fig. 2.8**), and conversely favored the abundance of bacteria less dependent on mucins to thrive, such as *Prevotella* (**Fig. 2.8**) (Jakobsson et al., 2015; Rho et al., 2005)

Tight-junction proteins maintain intestinal permeability, so that alterations in expression and cellular distribution of these proteins is often associated with leaky gut (Reuter and Pizarro, 2009; Zeissig et al., 2007). Our data indicate that the dietary vitamin A restriction triggered gene and protein expression increase of the tightening claudins (4, 5 and 8) in the WT colon (**Fig. 2.13**), suggesting a cellular response aimed at protecting the integrity of the gut barrier. Indeed, serum concentrations of LPS, the major outer surface membrane component of Gram-negative bacteria that translocate from the intestinal lumen to the bloodstream when the intestinal barrier is compromised (Raetz and Whitfield, 2002), remained steady in WT mice deprived of dietary vitamin A (**Fig. 2.14A**). However, the modest but significant increase in luminal ROS in the vitamin A-restricted WT (**Fig. 2.15**) implies that structural and functional changes might have begun to take place in the intestine of the WT mice under this regimen. After all, retinoic acid levels were slightly but significantly reduced in the colon of the WT VA-suf vs WT VA-def mice (**Fig. 2.5F**). The true physiological significance of these changes, however, remains to be established.

In contrast to WT mice, vitamin A restriction did not alter thigh junctions' gene expression in the colon of the mutants (**Fig. 2.13**). Instead, *occludin, claudin-3, -7* and *-8* were downregulated in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice already on the vitamin A-containing diet, suggesting a genotype (i.e., vitamin A status)-dependent impaired integrity of the barrier (**Fig. 2.13**). The abnormal cellular localization of claudin-2 in the colon of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice supports this interpretation (**Fig. 2.13B**) (Bardenbacher et al., 2019; Yuan et al., 2015). However, only when VAD was exacerbated secondary to the dietary vitamin A restriction, serum LPS concentration increased in the mutant mice, suggesting the

development of leaky gut (**Fig. 2.14A**). This interpretation is further supported by the modest but significant rise in intestinal luminal ROS levels in the VA-suf *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice (compared to WT on the same dietary regimen) that were further increased upon vitamin A restriction (**Fig. 2.15**). Likely, these ROS "leaked" from the compromised intestinal cells, given the imbalance in the mucosa expression of key oxidative stress genes under VAD (**Fig. 2.15**). Although increased levels of ROS are normally associated with higher expression of antioxidant genes (Newsholme et al., 2007), a number of studies have also shown that oxidative stress may increase as a result of impaired antioxidant activity of enzymes such as superoxide dismutase and glutathione peroxidase (Esworthy et al., 2014; Gargouri et al., 2020; Meng et al., 2020; Newsholme et al., 2007; Wang et al., 2018). Therefore, lower expression of the antioxidant-related genes in the VAD mutants supports the hypothesis that ROS leaked from their compromised intestinal mucosa.

In both WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice, lack of dietary vitamin A and VAD significantly attenuated the expression of *RegIIIβ* and *RegIIIγ* (**Fig. 2.12**), two antimicrobial proteins that by controlling bacterial load in the gut constitute another critical intestinal barrier function (Lehotzky et al., 2010). While it is unknown whether these genes are directly regulated by retinoids, it is established that the mucosal antimicrobial response of the intestinal epithelial cells is modulated by IL-22, an immunomodulatory cytokine synthesized by various cells of the immune systems, including innate lymphoid cells (ILCs), T lymphocytes and dendritic cells (Zenewicz, 2018). Interestingly, retinoic acid promotes maturation and proliferation of IL-22-producing RORγt+ ILC3s, directly binds to the II-22 promoter and upregulates IL-23 from dendritic cells that acts as a potent inducer of IL-22 (DePaolo et al., 2011b; Goverse et al., 2016; Grizotte-Lake et al., 2018; Mielke et

al., 2013; Oliveira et al., 2018). Thus, the lower expression of *Il-22* in the colon of both mutant and WT mice on VA-def (**Fig. 2.18B**) is in agreement with the compromised antimicrobial response of these two groups of mice (**Fig. 2.12**) and is likely linked to the attenuated retinoic acid concentrations in the colon mucosa of these mice secondary to the vitamin A restriction (**Fig. 2.5F**).

Surprisingly, though, the colon of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on VA-suf showed enhanced expression of *Il-22* and its inducer, *Il-23*, as well as *Il-17*, potential drivers of chronic gastrointestinal inflammation (Huang et al., 2018; Mielke et al., 2013). Although IL-22 has been shown to positively regulate intestinal barrier functions (Hernández et al., 2015; Liang et al., 2006; Sonnenberg et al., 2011), its induction is not always beneficial for the host, as it could favor infections by pathogens such as *Salmonella* (Behnsen et al., 2014), or lead to pathological inflammation (Andoh et al., 2005; Boniface et al., 2005). The colon of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on VA-suf also presented enhanced expression of inflammatory cytokines (**Fig. 2.18A**), implying a paradoxical higher inflammatory tone of the gastrointestinal tract. Interestingly, It has been shown that under a condition of altered microbial load, retinoic acid can promote, rather than prevent, the inflammatory response (Rampal et al., 2016). We therefore hypothesize that this genotype-dependent inflammatory status could be sustained by a synergistic effect of the relatively normal levels of retinoic acid in the colon and microbially driven signal(s) in the GI tract of mutant mice on VA-suf. Note that the inflammatory status of other organs from these mice was not investigated, and we cannot exclude a more widespread inflammatory condition.

In conclusion, our work establishes the suitability of our experimental setup to study intestinal dysfunctions and dysbiosis depending upon the dietary vitamin A intake

and status. Key components of the intestinal barrier functions are modulated by dietary deprivation also in WT mice, but the true physiological significance of these changes is unknown. Importantly, the two groups of WT mice were indistinguishable based on fecal microbial taxonomy and function, in accord with the fact that they maintain a vitamin A sufficient status on both dietary regimens. We interpreted the fact that some of the intestinal functions were already compromised in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on the vitamin A-containing diet as a result of their VAD. However, the lack of vitamin A in the food exacerbated some of these phenotypes in agreement with their overt VAD under this regimen. Importantly, the fecal microbial population of the VAD mutants was clearly distinguished from the one of the WT group and further discriminated the mutants based on their dietary vitamin A intake. Not only we could distinguish the mice based on VAS and VAD, but also based on severity of VAD secondary to the dietary vitamin A restriction.

CHAPTER 3 :

EFFECTS OF β-CAROTENE ON INTESTINAL DYSFUNCTIONS AND FECAL DYSBIOSIS OF VITAMIN A DEFICIENCY

3.1 ABSTRACT

Intake of β-carotene (βC), the most abundant dietary precursor of vitamin A, and also known for its antioxidant activity, has been associated with a wide range of health benefits. Supplementation and/or biofortification with βC are common strategies to combat vitamin A deficiency (VAD), still common in developing countries. VAD has a profound impact on intestinal health. Indeed, it is often associated with pathological conditions of the gut and with taxonomic and functional changes of the intestinal microbiome. However, the effects of βC on intestinal health during VAD have not been investigated. Here we addressed this question by using WT (vitamin A sufficient) and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ (vitamin A deficient) mice deprived of dietary preformed vitamin A and supplemented with βC as the sole source of the vitamin. We showed that that β-carotene not only improved the vitamin A status of the vitamin A deficient mice but also improved intestinal dysfunctions and modified the taxonomic profile of the fecal microbiome of VAD. Insights about the mechanisms whereby βC exerts these functions are discussed.

3.2 INTRODUCTION

β-carotene from fruits and vegetables is the most abundant dietary vitamin A precursor (von Lintig, 2012) and a food component with potential antioxidant capacity as well (Mueller and Boehm, 2011; Tapiero et al., 2004).

Upon ingestion and absorption within the enterocytes, βC is converted to vitamin A by the enzyme β-carotene 15-15' oxygenase (BCO1), which cleaves βC symmetrically giving rise to two molecules of retinaldehyde (von Lintig et al., 2019). Both intestinal βC's absorption, mediated by the scavenger receptor SR-B1, and conversion into retinoids (vitamin A and its metabolites), mediated by BCO1, are regulated *via* an intestine specific

homeodomain transcription factor, ISX, through a negative feedback regulatory mechanism (Bouis et al., 2011). *Isx* is positively regulated by retinoic acid (Lobo et al., 2010b). When tissue vitamin A levels are adequate or elevated, retinoic acid binds to the retinoic acid receptors RARs/RXRs and increases the expression of *Isx* that in turn represses the expression of *Bco1* and *Srb1* (or Scarb1) in the enterocytes (Lobo et al., 2010b). Therefore, unlike preformed vitamin A, βC is considered a "safe" source of vitamin A as the above-mentioned mechanism prevents toxicity from excessive tissue accumulation of βC and its conversion to retinoids (Grune et al., 2010). The bioavailability of βC varies from 10 to 90% (Grune et al., 2010) depending on many factors including food matrix and processing, dosage, dietary fat and fibers, vitamin A status and sex (Grune et al., 2010; Moran et al., 2018). Overall, the majority of βC is poorly bioavailable and transits to the large intestine to be excreted (Grune et al., 2010; La Frano et al., 2014; Van Loo-Bouwman et al., 2014).

The antioxidant capacity of βC has also been well documented (Mueller and Boehm, 2011; Young and Lowe, 2018) as it quenches single oxygen mostly through electron transfer to free radicals and formation of βC radical cation (Böhm et al., 1998; Levin and Mokady, 1994). In general, consumption of food-containing βC has been linked to beneficial health effects in humans due to both its provitamin A and antioxidant activities.

βC is the most abundant source of vitamin A worldwide, often representing the sole source of the vitamin in developing countries (Li et al., 2014). Vitamin A modulates intestinal morphology and functions by maintaining epithelial barrier integrity and by regulating gut immunity and inflammation (Cha et al., 2010; Li et al., 2017; Sirisinha,

2015). The vitamin A status has a profound impact on intestinal health. Indeed, VAD is often associated with pathological conditions of the gut (Barbalho et al., 2019b; de Medeiros et al., 2018; Sirisinha, 2015). A "healthy" intestine is also critical to support a "healthy" microbiome, a complex and large microbial community residing in the gastrointestinal tract (GI) (Byndloss et al., 2018). Indeed, a specific taxonomic profile of the microbiome of vitamin A deficiency (VAD) has begun to emerge. Supplementation and/or biofortification with βC are common strategies to combat VAD in developing countries (Dubock, 2017). However, the effects of βC on intestinal health during VAD have not been clarified.

We *hypothesized* that dietary βC may improve intestinal health and microbiome fitness during VAD through its retinoid-generating capacity and antioxidant activity, acting both on the intestine and on the microbial populations residing in the GI tract. We addressed this hypothesis by using WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice deprived of dietary preformed vitamin A and supplemented with βC as the sole source of the vitamin. We showed that dietary βC improved intestinal vitamin A status, barrier integrity and inflammation associated with VAD. βC supplementation also selectively modified the taxonomic profile of the fecal microbiome of VAD. Antioxidant activity of βC attenuated the excessive ROS in the lumen during vitamin A deficiency. βC supplementation on WT mice under restricted vitamin A intake had similar consequences on intestine functions, although to a lesser extent.

3.3 MATERIAL AND METHODS

3.3.1 Animals and Dietary supplementation

Both wild type (WT) and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ (model of VAD) mice on a mixed genetic background (C57Bl/6J × Sv/129) were maintained on a standard vitamin A-sufficient chow diet containing 18 IU vitamin A/g diet (Prolab Isopro RMH3000 5p75). At six-weeks of age (T0; **Fig. 3.1**), *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT female mice, raised on the above-described regular chow diet, were placed on vitamin A deficient diet (Research Diets, VA-def: < 0.02 IU vitamin A/g diet) for four weeks until the time of sacrifice (T1; **Fig. 3.1)**. Foods and water were provided *ad libitum*. A 10 μg/mL stock solution of βC (Type II, Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving βC crystalline with >98% purity into vehicle (corn oil) by vortexing, and the concentration of the resulting solution was determined using spectrophotometer at 450 nm. The solution was protected from light the entire time.

For the βC supplementation study, at eight weeks of age (after two weeks on VAD diet) mice from both genotypes were randomized to two groups and received either com oil as vehicle (Veh) or βC (50 mg/kg/day), every other day via feeding tube (gavage) between 9-10 AM for two weeks. Food intake and body weight was measured weekly throughout the experiment. At T1, fresh feces were collected and 4hrs after the last gavage mice were sacrificed by CO_2 inhalation and serum and tissues (liver, adipose tissue (perigonadal), small intestine (mucosa) and colon), were collected and immediately snap frozen on dry ice and stored at −80 °C until further processing.

3.3.2 HPLC Analysis of retinoids

Reversed-phase HPLC analysis was performed as previously described to measure retinol and retinyl ester and βC (Kim and Quadro, 2010) concentrations in serum and tissues, including 100 mg small intestine and colon. To measure βC concentration in fecal samples, 100 mg of feces sample was weighted and followed as described previously (Kim and Quadro, 2010).

3.3.3 RNA extraction and Real-Time PCR

RNA extraction and Real-Time PCR were performed as described previously (Spiegler et al., 2018). Briefly, total mRNA was extracted from mouse colon (groups at T1) using RNABee according to the manufacturer instructions (Tel-test Inc. Friendswood, TX). The concentration and purity of RNA were determined by Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA). 1 µg of RNA was reverse transcribed to complementary DNA (cDNA) using Verso cDNA synthesis kit according to the manufacturer's instructions (ThermoFisher Scientific, Dallas, TX). To quantify mRNA, real time PCRs were performed using an Applied Biosystems QuantStudio 3 Applied Biosystem instrument (ThermoFisher Scientific, Dallas, TX). Primers used for the RT-PCR were validated for PCR efficiency and a unique melt curve with SYBR Green chemistry. For the RT-PCR experiments, 300 nM of each specific primer (final concentration) were mixed with 25-50 ng of cDNA equivalent of the total RNA, and 7.5 µL of SYBR Green Master Mix (ThermoFisher Scientific, Dallas, TX) in a total volume of 15 µL. Each sample was run in duplicates or triplicates. Relative quantification of mRNA expression was calculated using $2(-\Delta\Delta Ct)$ method, normalized to the TATA-binding

protein (TBP) gene. Gene expression changes were expressed as mRNA fold change from the control group (WT on VA-suf).

3.3.4 Preparation of hydro-indocyanine green (H-ICG) and in vivo ROS imaging and analysis

H-ICG was prepared from the cyanine dye, indocyanine green, by reduction with $NaBH_4$ as previously described (Kuhn et al., 2018). WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice at T1 VA-def, were gavaged with H-ICG reconstituted in water (0.5 mg/mL) at a dose of 2 mg/kg. *In-Vivo* ROS imaging and analysis was performed by using a Bruker In-Vivo Multispectral (MS) FX PRO imaging system (Bruker, Ettlingen, German) (Kuhn et al., 2018). Briefly, after mice were anesthetized with 2% isoflurane, they were laid down in the abdomen-down position directed towards the camera into the MARS system. Following excitation illumination at 760 nm, emission at 830 nm was recorded using a filter equipped high sensitivity cooled charged coupled device camera. Acquisition time was 30s for near infrared fluorescent (NIRF) images, followed by a bright-field light photograph (0.5 s exposure). Both NIRF and bright-field images were optically superimposed to visualize anatomical information. Fluorescence was quantified as photons/s/mm^2 using Carestream MI software v5/0.529 (Carestream Health Inc., Rochester, NY). The background intensity of each image was set to zero and identical elliptical regions of interest were drawn on each image (137x145 pixels; interior area = 15605). The mean fluorescence intensity within the ellipse was recorded for each animal.

3.3.5 Fecal sample collection and DNA extraction

Before the last oral gavage of either βC or vehicle, at T1, each mouse was placed in an empty cage without bedding for 10–15 min to allow collection of fresh stool samples that were snap frozen in dry ice and kept at -80°C until further processing. Fecal genomic DNA was extracted at the University of Missouri DNA Core facility using PowerFecal kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with the exception that the samples were homogenized in the provided bead tubes using a TissueLyser II (Qiagen, Venlo, Netherlands) for three minutes at 30/sec. DNA yields were quantified via fluorometry (Qubit 2.0, Invitrogen, Carlsbad, CA) using quant-iT BR dsDNA reagent kits (Invitrogen, Carlbad, CA).

3.3.6 16S rRNA library construction and sequencing

Library construction and sequencing was performed at the University of Missouri DNA Core facility (Ericsson et al., 2015). Bacterial 16S rRNA amplicons were generated via amplification of the V4 hypervariable region of the 16S rRNA gene using dual-indexed universal primers (U515F/806R) flanked by Illumina standard adapter sequences and the following parameters: 98°C(3:00)+[98°C(0:15)+50°C(0:30)+72°C(0:30)] × 25 cycles +72°C(7:00). PCR was performed in 50 µL reactions containing 100 ng DNA, primers (0.2 µM each), dNTPs (200 µM each), and Phusion high-fidelity DNA polymerase (1U; ThermoFisher Scientific, Waltham, MA). Amplicon pools (5 µL/reaction) were combined, thoroughly mixed, and then purified by addition of Axygen Axyprep MagPCR clean-up beads (ThermoFisher Scientific, Waltham, MA) to an equal volume of 50 µL of amplicons and incubated for 15 minutes at room temperature. Products were then washed multiple times with 80% ethanol and the dried pellet was re-suspended in 32.5 µL elution buffer,

incubated for 2 minutes at room temperature, and then placed on the magnetic stand for 5 minutes. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system (Agilent, Santa Carla, CA), quantified using quant-iT HS dsDNA reagent kits (Invitrogen, Carlsbad, CA), and diluted according to Illumina's standard protocol for sequencing on the MiSeq instrument (Illumina, San Diego, CA), using the V2 chemistry to generate 2×250 bp paired-end reads. Samples returning greater than 10,000 reads were deemed to have successful amplified.

3.3.7 Informatics analysis

Read merging, clustering, and annotation of DNA sequences was performed at the University of Missouri Informatics Research Core Facility. Paired DNA sequences were merged using FLASH software, and removed if found to be far from the expected length of 292 bases after trimming for base quality of 31. Cutadapt (Martin, 2011) (https://github.com/marcelm/cutadapt) was used to remove the primers at both ends of the contig and cull contigs that did not contain both primers. The u-search (Edgar, 2010) fastq_filter command (http://drive5.com/usearch/manual/cmd_fastq_filter.html) was used for quality trimming of contigs, rejecting those for which the expected number of errors was greater than 0.5. All contigs were trimmed to 248 bases and shorter contigs were removed. The Qiime2 (Kuczynski et al., 2011) command split_libraries_fastq.py was used to demultiplex the samples. The outputs for all samples were combined into a single file for clustering. The uparse (Edgar, 2013) method (http://www.drive5.com/uparse/) was used to both cluster contigs with 97% identity and remove chimeras. Taxonomy was assigned to selected ASVs using BLAST (Altschul, 1997) against the SILVA database v132 (Pruesse et al., 2007) of 16S rRNA sequences and taxonomy.

3.3.8 Statistical analysis

SPSS statistical software (IBM SPSS Statistics, version 23; SPSS, Inc.) was used for statistical analysis. The distribution of the data was determined by the Shapiro-Wilk test, and normally distributed data were analyzed by Student's t test or a two-way analysis of variance (ANOVA). Non-normally distributed data were analyzed by Mann-Whitney U test or a Kruskal-Wallis test. Values are presented as the mean ± standard deviation (SD), and $p < 0.05$ was the cutoff for significance. Association of variables was analyzed by the p value-assigned Spearman rank correlation coefficient.

3.4 RESULTS

3.4.1 β-carotene as the sole source of vitamin A improves the vitamin A status of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice.

The *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice are unable to store vitamin A (due to the lack of LRAT) and unable to mobilize hepatic retinol towards the peripheral tissues (due to the lack of RBP) (Kim et al., 2008; Wassef et al., 2013). Thus, they rely exclusively on dietary vitamin A to support retinoid-dependent functions. *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice rapidly develop severe retinoid deficiency when deprived of dietary vitamin A (Kim et al., 2008; Wassef et al., 2013). We showed that, in contrast to WT mice, 4 weeks of dietary vitamin A restriction of the *Lrat*$^{-/-}$ *Rbp*$^{-/-}$ mice generated a severe VAD status, as based on low circulating retinol and retinoic acid levels and on reduced tissues total retinoids (retinol and retinyl ester) as well as retinoic acid concentrations (**Chapter 2**).

In order to evaluate the effects of dietary βC supplementation on intestinal health during VAD, we orally supplement *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice fed the VA-def diet with a daily oral gavage of 1 mg of βC (or vehicle, i.e., corn oil). Specifically, after two weeks

of dietary vitamin A deprivation, both *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice underwent two weeks of βC supplementation prior to sacrifice (**Fig. 3.1)**. No significant changes in food intake (**Fig. 3.2A**) and body weight (**Fig. 3.2B**) were observed throughout the study among the four experimental groups.

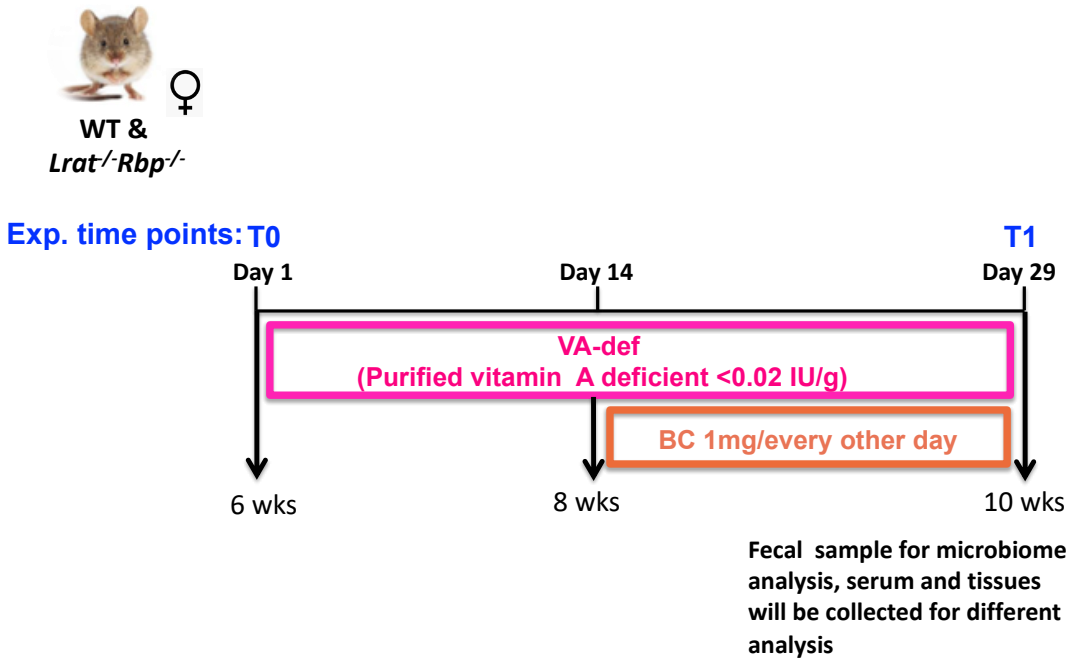

Figure 3.1: Experimental scheme

The number of weeks (wks) indicates the age of the mice. Wild type and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ female mice were maintained on regular chow (18IU VA/g diet) from weaning (3 weeks of age). At 6 weeks of age [the beginning of the experiment (T0)], mice were placed on a purified vitamin A deficient (VA-def; < 0.2 IU VA/g diet) diet for four weeks, e.g., until 10 weeks of age (T1). At 8 weeks of age mice were gavaged either with β-carotene (BC) or vehicle (control), every other day for the following 2 weeks. At T1, after collection of fresh feces, mice were sacrificed to harvest serum and tissues (liver, small intestine, colon and adipose tissue) for further analysis. VA-def, vitamin A-deficient diet.

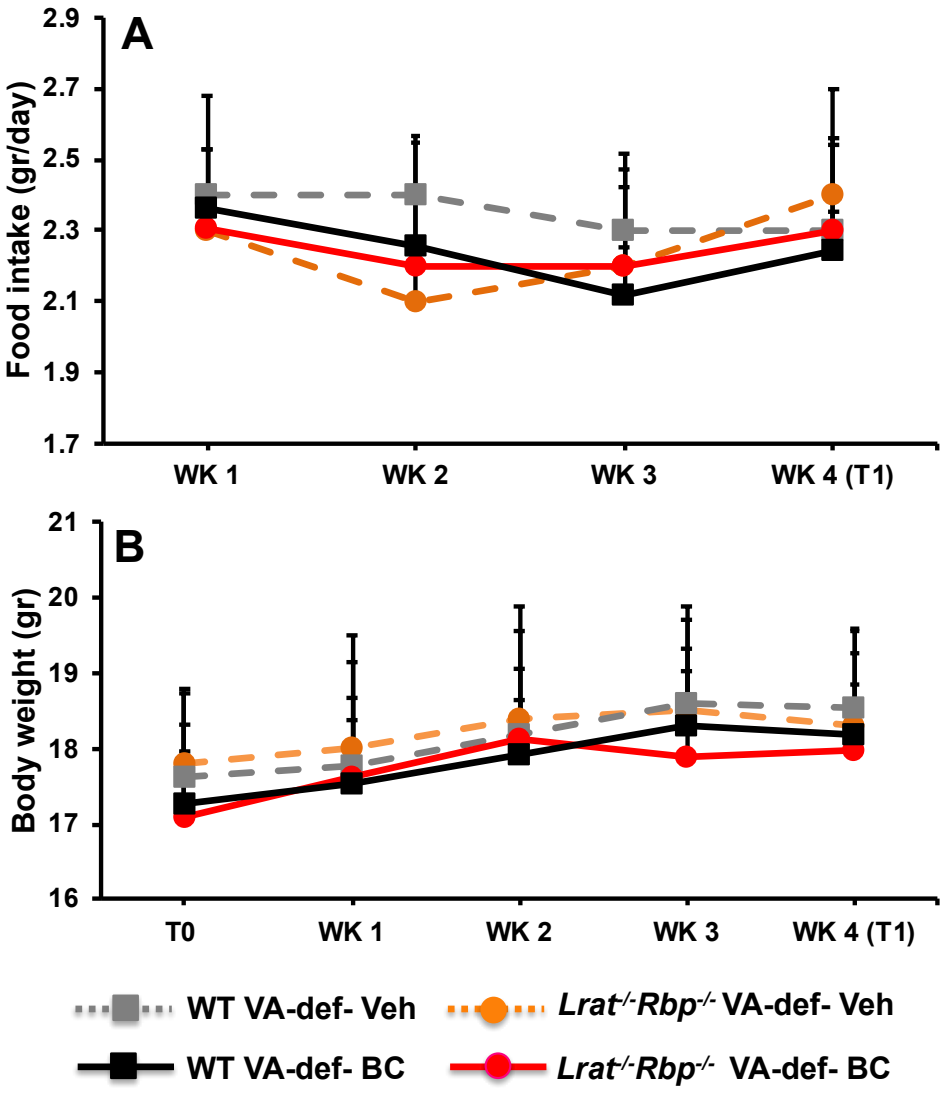

Figure 3.2: Food intake and body weight changes in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice throughout the experiment

(**A**) Food intake and (**B**) body weight. T0 and T1 indicate the experimental time points as in **Fig. 3.1**. Week indicates the age of the mice. Values are mean ± SD. Statistical analysis by two-way ANOVA at each time point; VA-def, vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

With the exception of the adipose tissue, intact βC was detected in serum, liver, small intestine (duodenum) and colon with no differences among genotypes **(Table 3.1**). Also, regardless of the genotype, the highest concentration of βC was found in fecal samples, implying that the majority of ingested βC was not absorbed (**Table 3.1**).

In order to assess the provitamin A activity of βC, we first measured retinoid levels in serum, liver, adipose tissue, small intestine (duodenum) and colon by HPLC at T1 (**Fig. 3.1)**. As expected due to the lack of RBP (Kim et al., 2008; Wassef et al., 2013), serum retinol was significantly reduced in Veh-treated *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice compared to WT under the same regimen (**Fig. 3.3A)**. Remarkably, in the mutant's serum retinol concentration was restored to the WT levels upon βC administration (**Fig. 3.3A**). In contrast, βC supplementation did not alter neither serum retinol nor retinyl ester levels in WT mice (**Fig. 3.3A and B)**. Also, βC administration did not result in detectable retinyl ester levels in the mutant mice (**Fig. 3.3B)**. βC supplementation did not alter hepatic retinol levels neither in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ nor in the WT mice relative to their respective Veh-treated group (**Fig. 3.3 C and D**). In contrast, the supplementation significantly elevated retinyl ester levels in the liver of WT mice (**Fig. 3.3D**).

		Veh gavage	βC gavage
WT	Serum (μg/dL)	ND	8.4 ± 0.5
	Liver (μg/g)	ND	0.04 ± 0.01
	Adipose (μg/g)	ND	ND
	Duodenum (μg/g)	ND	5.6 ± 3.9
	Colon (μg/g)	ND	1.8 ± 1.2
	Feces (μg/g)	ND	791.6 ± 104
LRAT$^{-/-}$RBP$^{-/-}$	Serum (μg/dL)	ND	8.2 ± 0.7
	Liver (μg/g)	ND	0.04 ± 0.01
	Adipose (μg/g)	ND	ND
	Duodenum (μg/g)	ND	12 ± 8.4
	Colon (μg/g)	ND	0.7 ± 0.6
	Feces (μg/g)	ND	619.5 ± 182.5

Data are shown as mean ± SD; n = 3–5 mice/group; ND, not detected; Veh, vehicle; βC, β-carotene.

Table 3.1: HPLC analysis of serum and tissues βC concentration

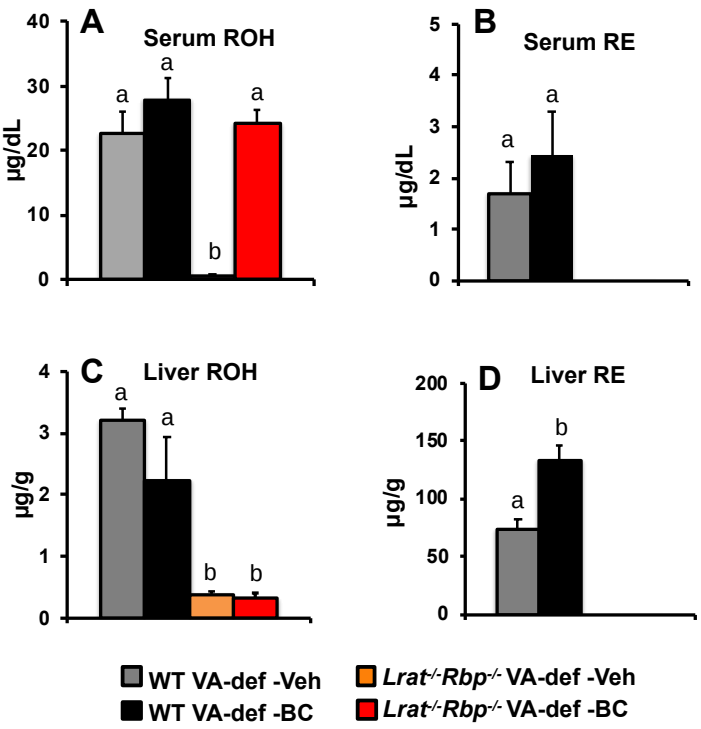

Figure 3.3: Serum and liver retinoid concentrations in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1

Serum (**A and B**) and liver (**C and D**) retinol (ROH) and retinyl ester (RE) levels were determined by reversed-phase HPLC. Data are mean ± SD; n = 3 - 4 mice/group. Statistical analysis by two-way ANOVA for normally distributed data and by Mann–Whitney test for non-normally distributed data. Different letters indicate significant difference ($p < 0.05$) among the groups. VA-def, vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

Overall, this regimen of βC supplementation increased serum retinol concentration of the VAD mice and hepatic retinoids stores in WT mice.

Adipose tissue stores both vitamin A and βC (Coronel et al., 2019; Lobo et al., 2010a). In mice lacking LRAT enzyme, the liver cannot store vitamin A, but the adipose tissue serves as an alternative storage site of retinoids (Liu and Gudas, 2005; O'Byrne et al., 2005; Wongsiriroj et al., 2008c). Therefore, we wondered whether the βC-derived retinoids accumulated in the adipose tissue of the supplemented mutant mice. Indeed, adipose retinol and retinyl esters concentrations increased significantly in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ animals following βC supplementation similar to WT mice (**Fig. 3.4A and B)**.

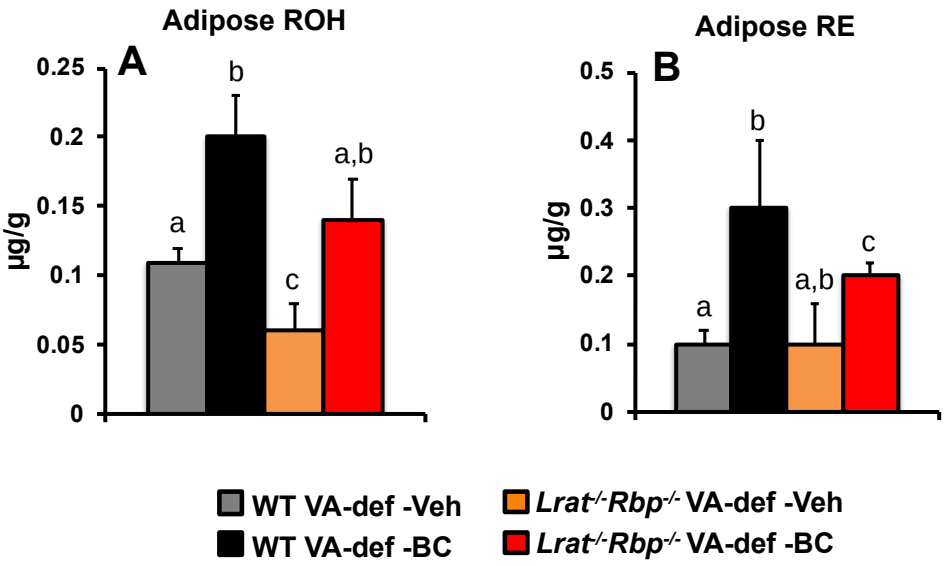

Figure 3.4: Retinoid concentrations in adipose tissue of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1

(**A**) retinol (ROH) and (**B**) retinyl ester (RE) levels were determined by reversed-phase HPLC analysis. Data are mean ± SD; n = 3 - 5 mice/group. Statistical analysis by two-way ANOVA. Different letters indicate significant difference ($p < 0.05$) among the groups. VA-def, vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

Next, we measured retinoids levels in the GI tract. On the VA-def diet, Veh-treated *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice showed significantly reduced retinol concentration in the duodenum compared to WT mice under the same regimen (**Fig. 3.5A)**. These findings are also in agreement with the data shown in **Chapter 2**. Interestingly, regardless of genotypes, dietary βC supplementation significantly increased retinol levels compared to the vehicle-treated group of the same genotype (**Fig. 3.5A and B).** Furthermore, the βC supplementation increased retinyl ester concentration in the duodenum of WT as well as of the mutant mice, even though not to the same level of the WT mice (**Fig. 3.5B)**. These lower levels of retinyl esters in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ βC-treated group further support the notion that LRAT is the key enzyme that esterifies retinol in the small intestine (O'Byrne et al., 2005; Wongsiriroj et al., 2008c).

Similar to the small intestine, Veh-treated *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice showed the lowest retinol concentration in the colon compared to WT Veh-treated group (**Fig. 3.5C).** However, in both genotypes dietary βC supplementation significantly increased retinol levels compared to the respective vehicle-treated group (**Fig. 3.5C).** Upon βC supplementation retinyl esters were still undetectable in the colon of the mutant mice and were significantly reduced in the WT mice compared to the vehicle-treated group of the same genotype (**Fig. 3.5D**). We also assessed vitamin A metabolism in the colon upon βC supplementation by measuring the mRNA expression of key enzymes of the pathway that are known to be regulated by tissue retinoic acid levels (Belyaeva et al., 2017; Grenier et al., 2007; Topletz et al., 2012) . Dietary βC significantly increase in the expression of the *Rdh1* in both WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$, *Raldh1* in the WT and *Raldh2* in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice while significantly reduced the expression of the *Rdh10* in both genotypes. Also, in the mutant

mice, *Raldh1* expression was significantly attenuated, regardless of the βC supplementation **(Fig. 3.6A)**. These results suggest that dietary βC increases retinoic acid synthesis, at least in the colon. However, it seems that the biosynthesis may be carried out by a different retinaldehyde dehydrogenase in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice. In line with increase in the retinoic acid synthesis, βC supplementation resulted in a dramatic increase in expression of *Cyp26b1,* a key enzyme that is responsible for the catabolism of retinoic acid (Isoherranen and Zhong, 2019; Topletz et al., 2012), in both genotypes **(Fig. 3.6B)**. Note that we were not able to detect expression of *Cyp26a1* in the colon (data not shown). Dietary βC also increased the expression of the retinoic caid receptor *Rarb,* but only in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice compared with the vehicle treated group of the same genotype **(Fig. 3.6B)**. Also, note that the lower expression levels of *Rarb* and *Cyp26b1* in the *Lrat*$^{-/-}$ *Rbp*$^{-/-}$ vehicle group compared to WT under the same treatment, confirm the tenuous vitamin A status of the colon of the mutant mice.

Altogether, these data indicate that the βC supplementation improved retinoid concentrations in the colon in both genotypes.

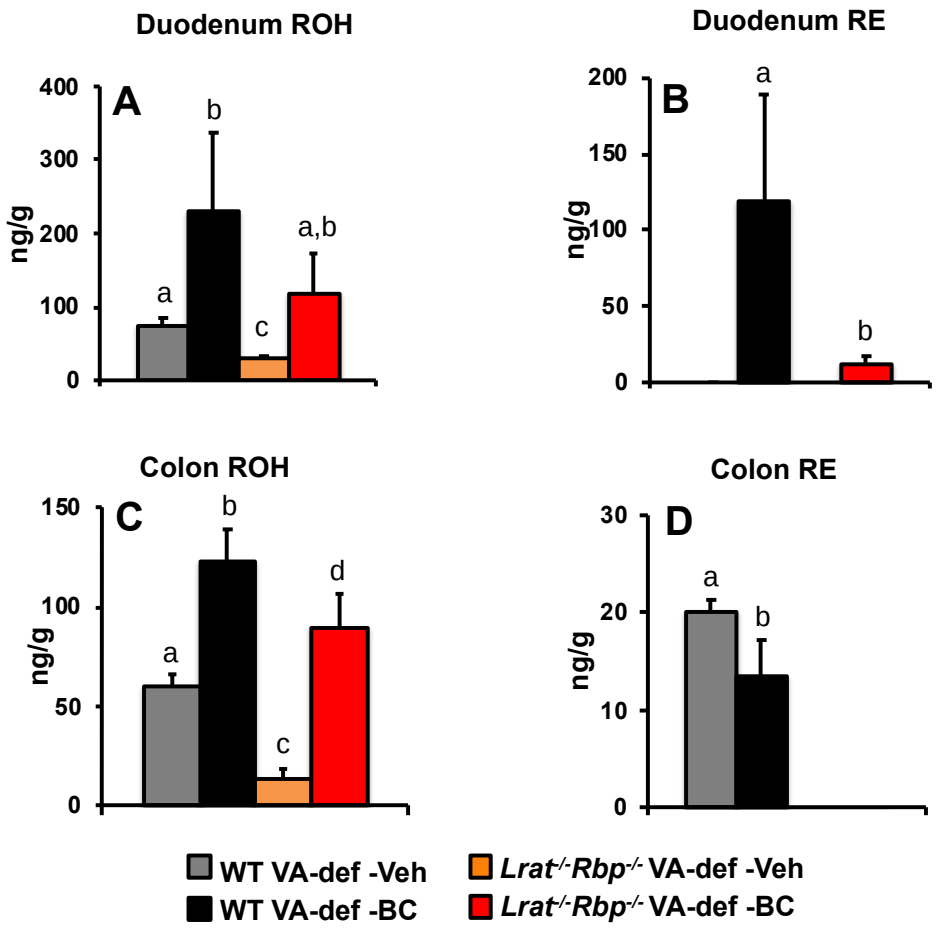

Figure 3.5: Retinoid concentrations in duodenum and colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1

Small intestine (duodenum) (**A and B**) and colon (**D and E**) retinol (ROH) and retinyl ester (RE) levels were determined by reversed-phase HPLC. Data are mean ± SD; n = 3–5 mice/group. Statistical analysis by two-way ANOVA and by Mann–Whitney test for non-normally distributed data. Different letters indicate significant difference ($p < 0.05$) among the groups. VA-def, vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

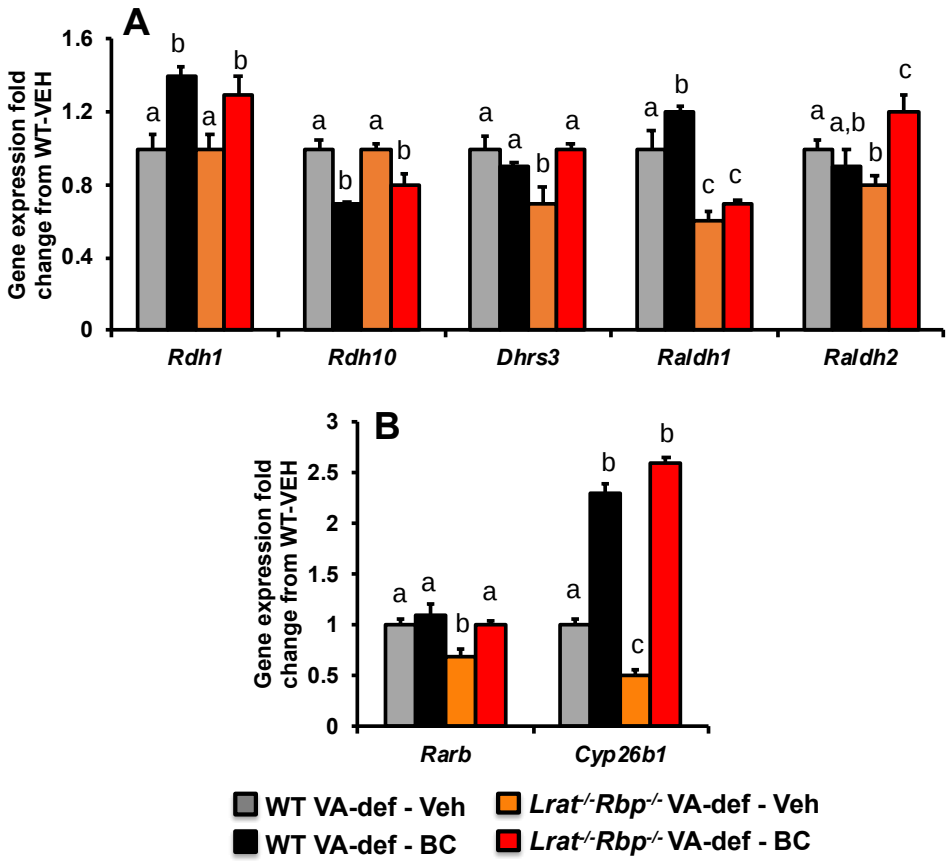

Figure 3.6: mRNA expression of key genes involved in retinoid homeostasis in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice

(**A**) Real-time RT-PCR analysis of mRNA expression levels of *Rdh1*, *Rdh10*, *Dhrs3*, *Raldh1*, *Raldh2*, (**B**) *Rarb* and *Cyp26b1*. Data are mean ± SD, calculated using the $2^{-\Delta\Delta CT}$ method; n = 3 individual sample pools/group (3 mice/pool). Statistical analysis by two-way ANOVA within each gene. Different letters indicate significant difference ($p < 0.05$) within each gene; BC, β-carotene; VA-def, vitamin A-deficient diet; Veh, vehicle.

3.4.2 β-carotene supplementation altered the taxonomic profile of the fecal microbiome of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice.

We next examined whether dietary βC supplementation impacted the fecal microbiome profile of under VAD and vitamin A sufficiency (VAS). We performed a high throughput sequencing of bacterial 16S rRNA V4 region by Illumina Miseq platform of fecal samples from the four experimental groups at T1. The rarefaction curves for the four groups of mice reached a plateau (**Fig. 3.7**), indicating adequate sequencing depth and species richness in our samples. β-diversity in the fecal samples' bacteria community was measured by Shannon Index, that takes into consideration both richness and abundance of the bacteria; total amplicon sequence variance (ASV), that measures the richness or the number of the species; and Faith's phylogenetic diversity (PD) which considers species richness based on the phylogenetic distances in the samples (Cadotte et al., 2010). Both Shannon Index and Faith's PD were not significantly different among the four experimental groups, regardless of genotype or treatments **(Table 3.2)**. Moreover, there was no significant difference in the total number of amplicon sequence variance (ASV) in the βC-treated groups compared to the Veh-treated ones of the same genotype **(Table 3.2)**.

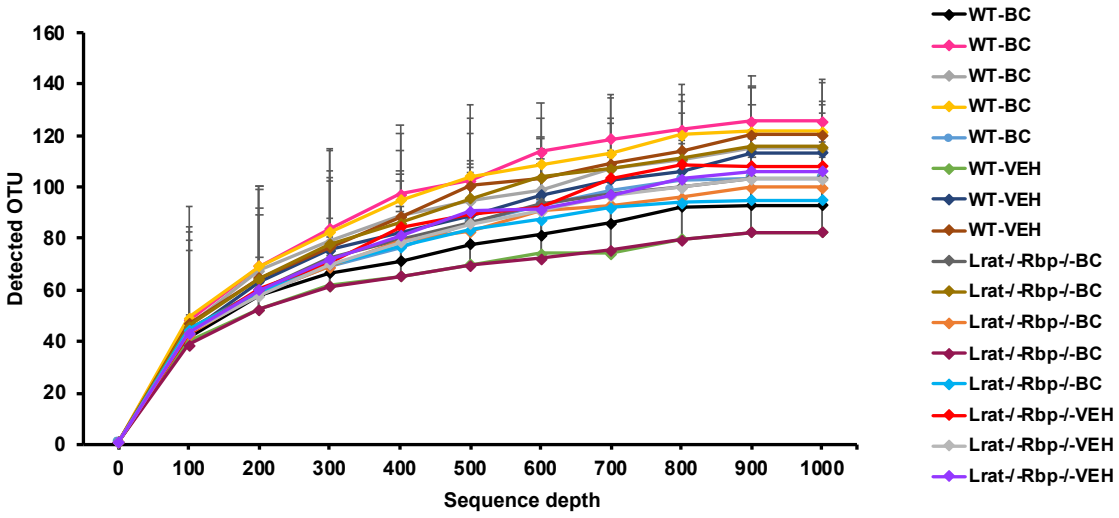

Figure 3.7: Rarefaction curve of fecal samples of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1

n = 3–5 mice/group. VA-def, vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

	WT-Veh	WT-BC	*Lrat*$^{-/-}$*Rbp*$^{-/-}$Veh	*Lrat*$^{-/-}$*Rbp*$^{-/-}$BC
Shannon	5.3 ± 0.04	5.5 ± 0.02	5.3 ± 0.02	5.3 ± 0.03
Total ASV	83.3 ± 23.3^{ab}	90.3 ± 23.5^{a}	84.9 ± 20.7^{ab}	81 ± 19.9^{b}
Faith's PD	6.4 ± 2.3	6.5 ± 2.3	6.3 ± 2.3	5.9 ± 2.1

Table 3.2: Alpha-diversity indexes of fecal samples of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice at T1

As shown in **Figure 3.8A** the fecal microbial community of both genotypes was mainly composed of *Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria*. Consistent with the data shown in **Chapter 2**, fecal samples of the vitamin A deficient *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice showed lower abundance of *Actinobacteria* compared to WT mice, regardless of the βC supplementation (**Fig. 3.8B)**. Moreover, fecal samples from the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice also showed a significant lower abundance of *Firmicutes* ($p < 0.001$) (**Fig. 3.8C)** and a higher abundance of *Bacteroidetes* ($p = 0.002$) (**Fig. 3.8D)** compared to the WT groups and irrespective of the βC treatment. Thus, the Firmicutes/Bacteroidetes ratio (F/B ratio) was significantly reduced in the mutant mice irrespective of the supplementation with βC (**Fig. 3.8E)**. Notably, dietary βC did not alter the fecal bacterial communities at the level of phylum in any genotype, relative to its respective vehicle-treated group (**Fig. 3.8B-E)**.

Principle component analysis (PCA) was performed to analyze the differences in community composition, i.e., β-diversity, among the bacterial genera. As shown in **Fig. 3.9A**, PC1 accounted for 53.4% of the variation and separated the fecal samples by genotype, regardless of the βC supplementation. These results further support our previous findings (**Chapter 2**) that vitamin A status (VAS *vs.* VAD) has a dramatic impact on fecal bacterial diversity. Notably, the WT mice on the VA-def for four weeks do not develop VAD (**Chapter 2**). Unlike WT mice (data not shown), PCA of the fecal microbiome of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ groups showed distinct clusters depending on the treatment (**Fig. 3.9B**). Specifically, PC1 accounted form 41% of the variation and separated the fecal microbiota of the mutant mice by treatment (βC, red circle *vs.* vehicle, orange circle) (**Fig. 3.9B**).

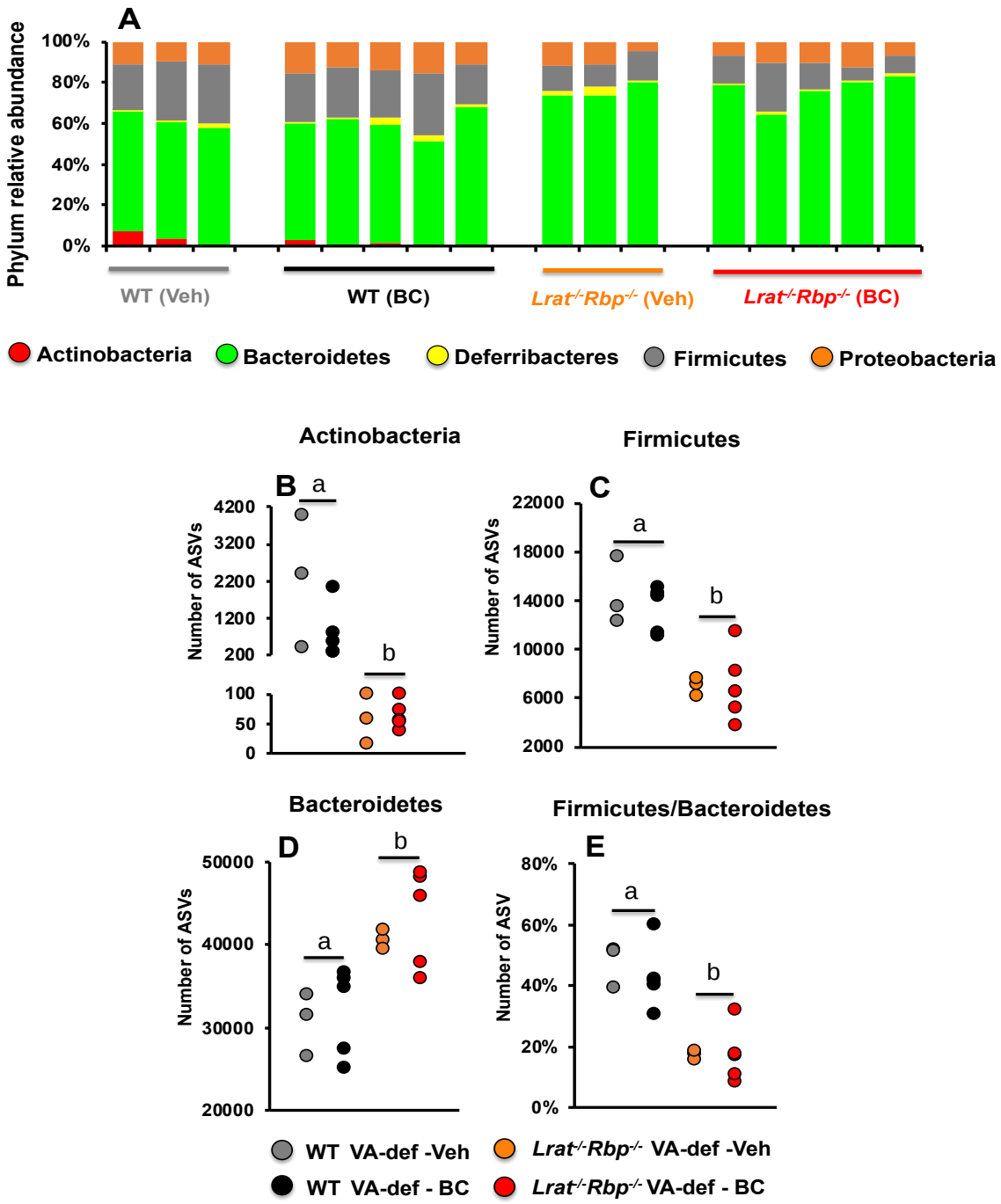

Figure 3.8: Impact of βC treatment on fecal microbial composition

(**A**) Relative abundance of bacterial phyla in mouse fecal samples at T1. Total number of the ASVs **(B)** Actinobacteria and **(C)** Firmicutes (**D**) Bacteroidetes (**E**) Firmicutes/Bacteroidetes ratio. Statistical analysis by two-way ANOVA. Different letters indicate significant difference ($p < 0.05$) among the groups. n = 3-5 mice/group. VA-def,

vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

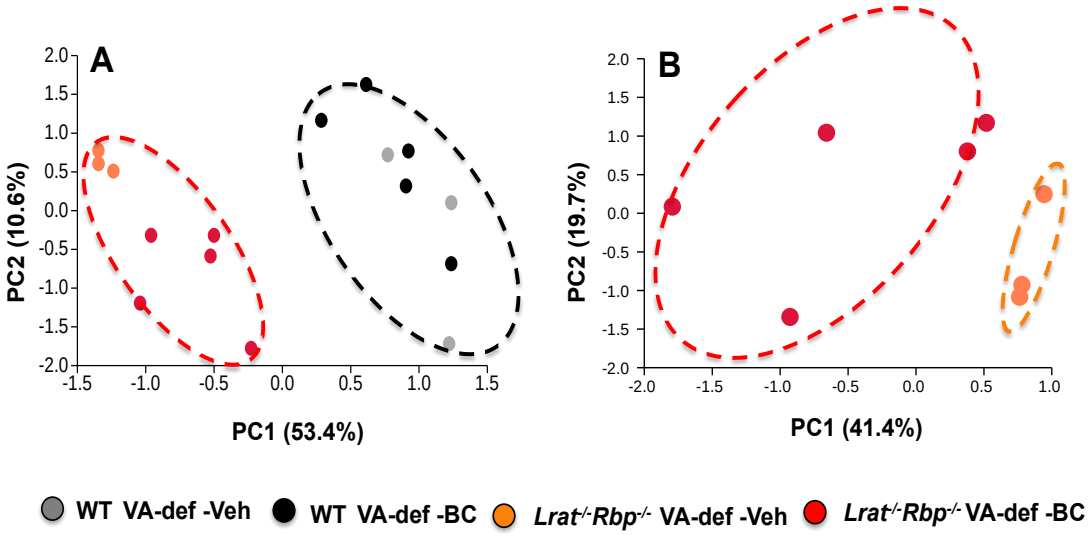

Figure 3.9: Principal component analysis (PCA) of the 16S rRNA genes in fecal samples at T1

(**A**) PC1 separated samples by status, i.e., genotype, and accounted for 53.4% of total variance. (**B**) PC1 separated samples by the treatment (BC vs. vehicle) in *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice time points and accounted for 41.4% of total variance; n = 3-5 mice/group. VA-def, vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

Only 8 genera displayed a difference in their relative abundance between Veh- and βC-treated *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice: *Parabacteroides, Alistipes, Millionella, Paraprevotella* spp. (Bacteroidetes phylum), *Lachnoclostridium and Marvinbryantia* spp. (Firmicutes phylum), *Brachyspira* sp. (Spirochaetes phylum) and *Desulfovibrio* (Proteobacteria phylum) (**Fig. 3.10A-H)**. Notably, higher taxonomic ranks of these genera were similar between vehicle- and βC-treated mutant groups. Next, we compared the relative abundance of these 8 differentially represented genera in the feces of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice with those of the WT animals. Compared to WT mice, vitamin A deficient *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice showed a significantly higher abundance of *Paraprevotella* and *Alistipes* genera (**Fig. 3.11A and B)**, which was attenuated by the dietary βC treatment (**Fig. 3.11A and B)**. While βC did not impact the abundance of the *Parabacteroides* and *Brachyspira* between Veh-treated *vs.* βC-treated WT, βC supplementation significantly lowered the abundance of these genera in the βC-treated *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice (**Fig. 3.11C and D**). Dietary βC supplementation did not alter the abundance of *Alistipes, Paraprevotella, Parabacteroides* and *Brachyspira* genera in the feces of WT mice relative to its respective vehicle-treated group (**Fig. 3.11A-D)**.

All together these data suggest that this regimen of dietary βC may modify the fecal taxonomic profile only under VAD.

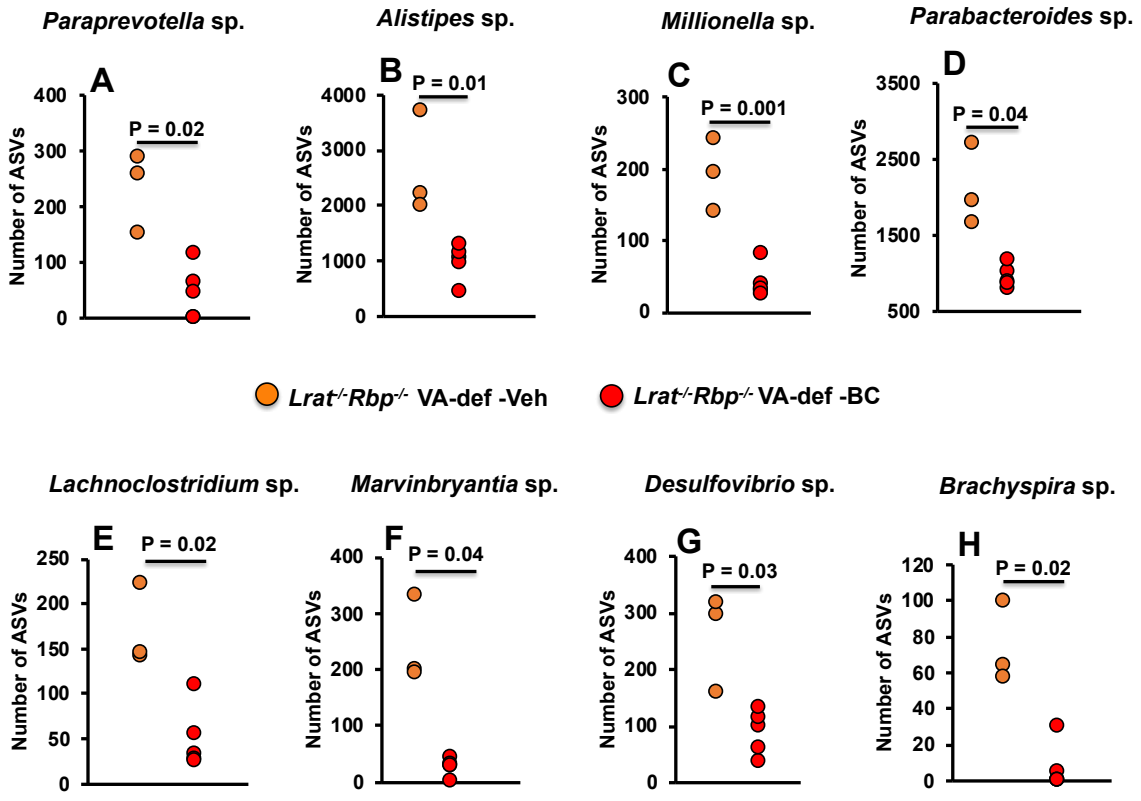

Figure 3.10: Impact of βC treatment on fecal microbial genera of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice

Relative abundance of bacterial genera (**A**) *Paraprevotella* sp. (**B**) *Alistipes* sp. (**C**) *Millionella* sp. (**D**) *Parabacteroides* sp. (**E**) *Lachnoclostridium* sp. (**F**) *Marvinbryantia* sp. (**G**) *Desulfovibrio* sp. (**H**) *Brachyspira* sp. in the fecal microbiota at T1. Statistical analysis by *T-test*; n =3- 5 mice/group. VA-def, vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

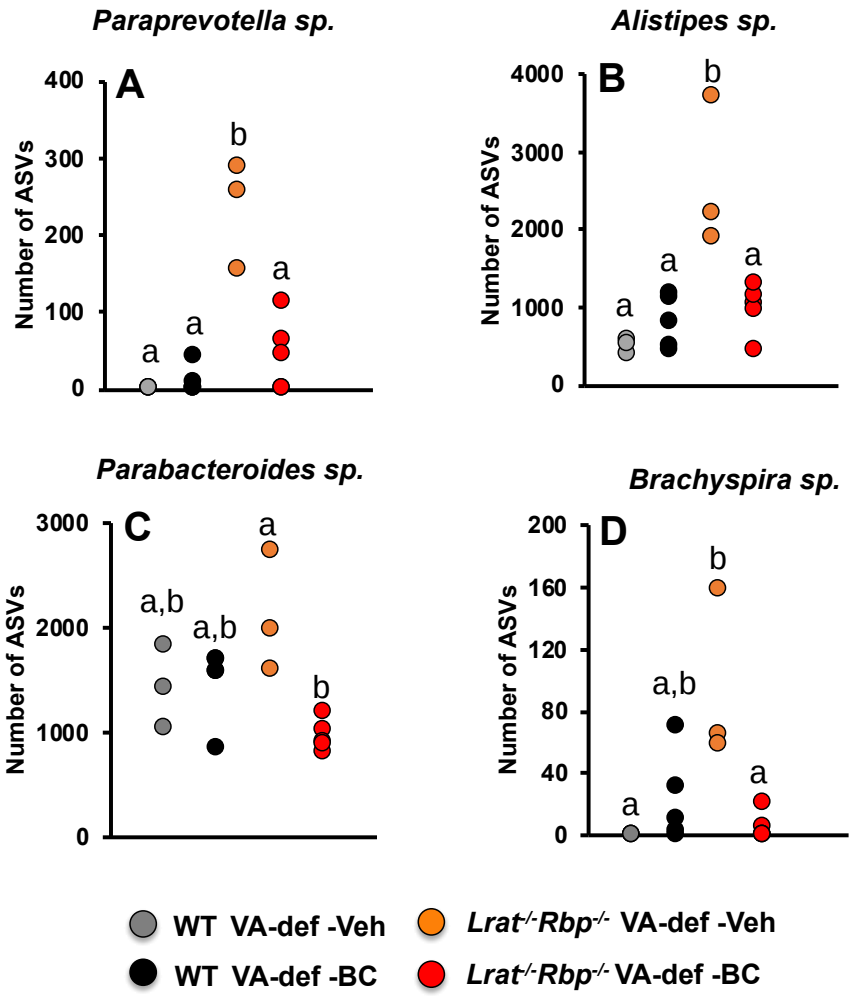

Figure 3.11: Impact of βC treatment on fecal microbial composition of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice

Relative abundance of bacterial genera (**A**) *Paraprevotella* sp. (**B**) *Alistipes* sp. (**C**) *Parabacteroides* sp. (**D**) *Brachyspira* sp. in the fecal microbiota of both WT and *Lrat-/-Rbp-/-* mice at T1. Statistical analysis by two-way ANOVA; n =3- 5 mice/group. VA-def, vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

3.4.3 β-carotene supplementation attenuate the excess of intestinal luminal ROS associated with VAD.

VAD is associated with oxidative stress caused by increased generation and/or accumulation of reactive oxygen species (ROS) (Arruda et al., 2009; Chiu et al., 2008; Sohlenius-Sternbeck et al., 2000; Spiegler et al., 2018), which could diffuse into the gut lumen from a structurally compromised intestinal epithelium (Kuhn et al., 2018). In the studies described in **Chapter 2**, we showed that the vitamin A deficient *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice displayed leaky gut and high ROS luminal concentrations. To investigate whether βC supplementation could attenuate the concentration of the luminal ROS in our vitamin A deficient model, we measured intestinal ROS in our four experimental groups of mice by optical *in vivo* imaging using near-infrared fluorescence (NIRF) light generated by cyanine-based fluorescent dyes. Consistent with our previous observations (**Chapter 2**), *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on the VA-def diet displayed significantly increased ROS levels in the intestinal lumen compared to WT mice (**Fig. 3.12A and B**) as measured by the enhanced intensity of the fluorescent signal. Interestingly, βC supplementation significantly reduced ROS levels in the intestine of the mutants ($p < 0.05$). Only a trend toward ROS reduction ($p = 0.05$) was observed in the βC-treated WT mice compared to their corresponding Veh-supplemented group (**Fig. 3.12A and B)**. This effect may be due to the antioxidant activity of the unabsorbed βC in the GI tract

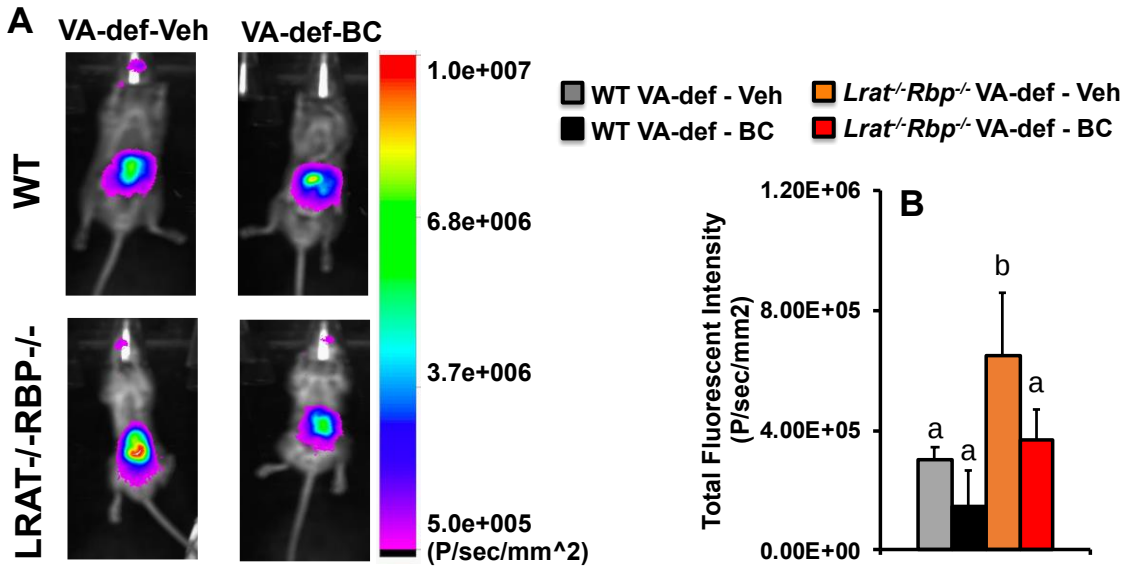

Figure 3.12: ROS in the lumen of the intestine of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice

(A) Representative overlay of ROS-associated NIRF image and corresponding brightfield image of Veh-treated WT mice (top, left), βC-treated WT mice (top, right), Veh-treated *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice (bottom, left) and βC-treated *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice (bottom, right). Near-infrared fluorescence (NIRF) intensity scale shown on the right. Images were normalized accordingly using Carestream MI software. (**B**) Quantification of ROS-associated NIRF image. n=4 mice/group; Statistical analysis by two-way ANOVA. Different letters indicate significant difference ($p < 0.05$) among the groups. VA-def, vitamin A-deficient diet; BC, βC-carotene; Veh, vehicle.

3.4.4 β-carotene supplementation improved intestinal barrier functions in the colon of the vitamin A deficient *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice.

3.4.4. 1 Mucins and tight junctions

In the studies described in **Chapter 2** we showed that VAD dysregulates the intestinal physical barrier of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice. We therefore asked whether βC supplementation could improve intestinal barrier integrity. Confirming our previous findings, the colon of the Veh-treated *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice showed the lowest mRNA expression of *Muc2 and 3*, that was only modestly increased by the βC availability (**Fig. 3.13A)**. Notably, this regimen also modestly increases *Muc3* mRNA expression in the colon of WT mice (**Fig. 3.13A**).

Tight junction proteins regulate both barrier and permeability of the intestine (Krug et al., 2012; Niessen, 2007; Van Itallie and Anderson, 2004), and we previously showed that VAD in our mouse model affects the expression of some of the tight junction proteins **(Chapter 2)**. Upon dietary βC supplementation only the expression of *Occludin* and *Claudin-3* increased significantly in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice to reach the WT levels (**Fig. 3.13B).** βC treatment did not have any effect on the expression of the tight junction proteins in the βC-treated WT mice **(Fig. 3.13B).**

To further assess whether dietary βC supplementation resulted in reduced intestinal permeability, we measured serum concentrations of microbial lipopolysaccharide (LPS). Consistent with the data shown in **Chapter 2**, only *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on VA-def diet treated with VEH had significantly higher concentrations of circulating LPS. However, βC supplementation attenuated circulating LPS concentrations in the mutant mice to levels similar to those of the WT groups (**Fig. 3-14**).

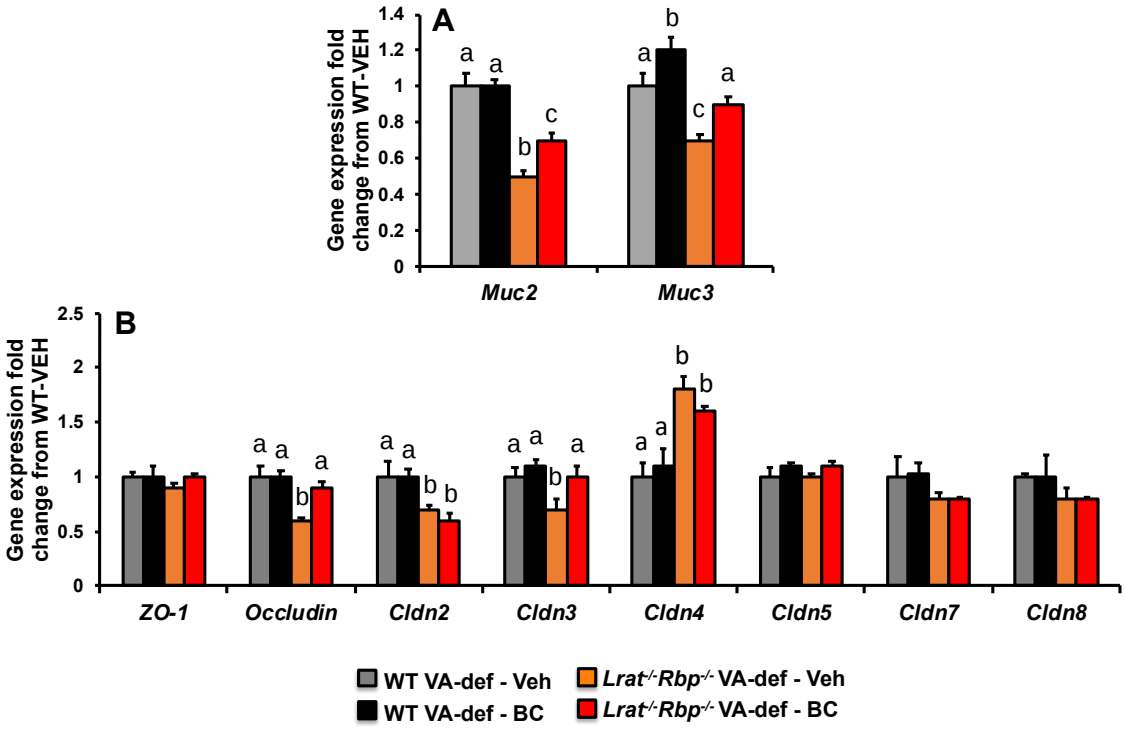

Figure 3.13: Mucins, and tight-junction proteins in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice

(**A**) Real-time RT-PCR analysis of mRNA expression levels of mucins (*Muc2* and *Muc3*) and (**B**) Tight-junction proteins (*Zo-1*, *Occludin*, *Claud-2*, *Claud-3*, *Claud-4*, *Claud-5*, *Claud-7*, *Claud-8*). Data are mean ± SD, calculated using the $2^{-\Delta\Delta CT}$ method; n = 3 individual sample pools/group (3 mice/pool). Statistical analysis by two-way ANOVA within each gene. Different letters indicate significant difference ($p < 0.05$) within each gene. Cldn, claudin; VA-def, vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

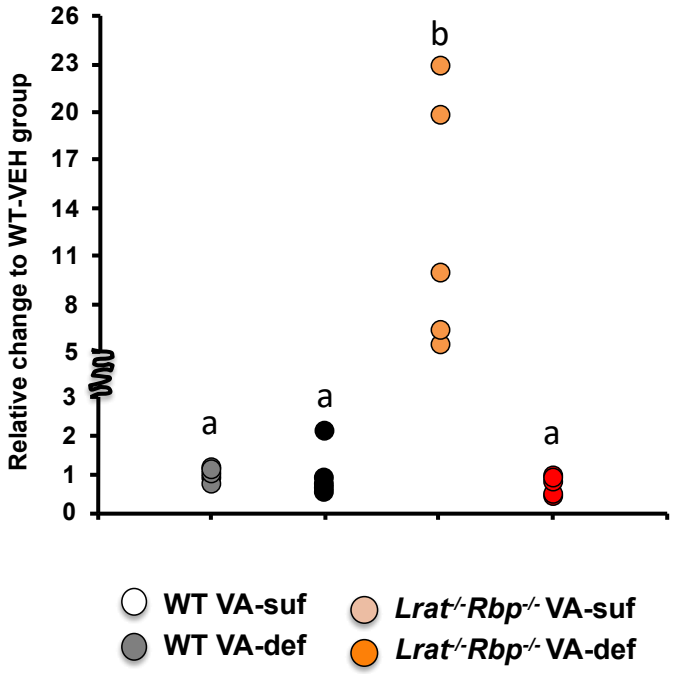

Figure 3.14: Serum LPS concentration of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice.

Serum LPS concentrations measured by a commercial kit (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit); n = 5 mice/group. Statistical analysis by Mann–Whitney test. Different letters indicate significant difference ($p < 0.05$) among the groups.

3.4.4. 2 Inflammatory and immune-modulatory cytokines.

Since we previously showed (**Chapter 2**) that our model of VAD displayed altered inflammatory and immune-modulatory cytokine profile in the colon, we wondered whether dietary βC supplementation would alleviate these VAD-related phenotypes. We performed RT-PCR analysis to test the mRNA expression of key inflammatory markers. As shown in **Figure 3-15A**, Veh-treated *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice had significantly elevated mRNA expression levels of the pro-inflammatory cytokines *Il-6*, *Tnfα* and *Il-1β* in the colon compared to the Veh-treated WT group. Interestingly, βC administration significantly reduced *Il-6*, *Tnfα* and *Il-1β* expression in the colon of the mutant mice, but it also attenuated the expression of *Tnfα* and *Il-1β* in WT mice (compared to the Veh-treated group of the same genotype) (**Fig. 3.15A).**

Both Interleukin (IL)-22 and -17 are key mediators of intestinal immunity and inflammation (Wilhelm et al., 2017; Zenewicz, 2018; B. Zeng et al., 2019) and their expression is in turn, regulated by other cytokines, such as IL-23, IL-6 and TNFα (Parks et al., 2015; B. Zeng et al., 2019). As shown in **Fig. 3.15B** as well as in **Chapter 2**, four weeks of VA-def diet significantly increased the expression of *Il-22*, *Il-23 and Il-17* in the colon of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice compared to WT. βC supplementation attenuated the expression of the above-mentioned three genes in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice compared to the vehicle-treated group of the same genotype (**Fig. 3.15B)**. Similarly, in the WT mice, βC supplementation attenuated the expression of *Il-17* and *Il-22* compared their respective veh-treated controls **(Fig. 3.15B)**.

These data suggest that dietary βC modulates the expression of inflammatory markers and immune cytokines in the colon independent of the vitamin A status but with a

more pronounced effect under VAD.

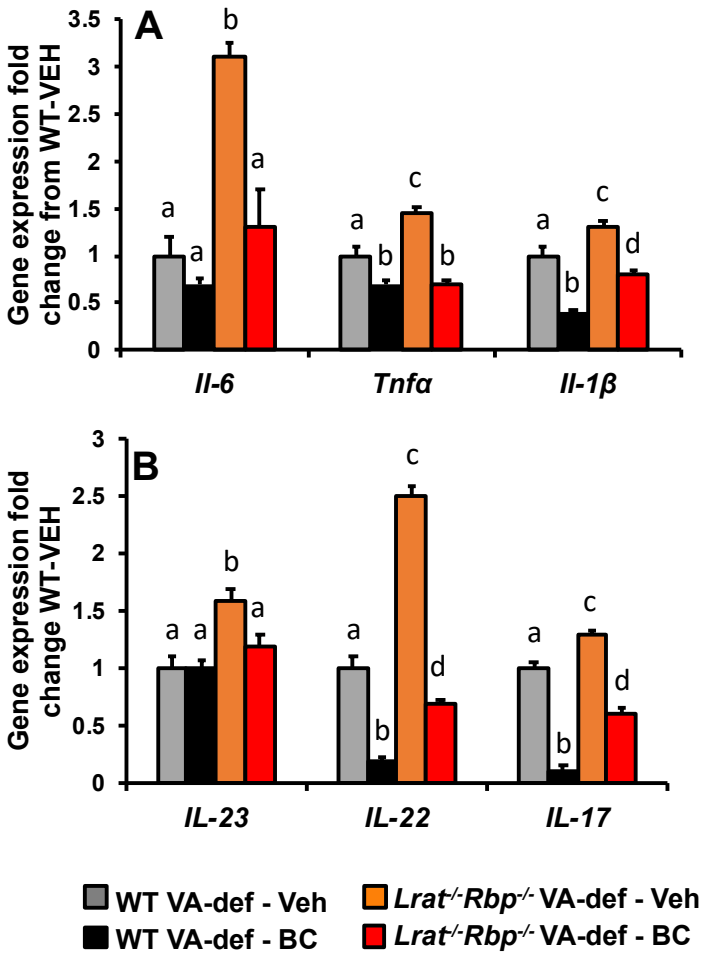

Figure 3.15: Inflammatory and immunomodulatory cytokines in the colon of *Lrat*$^{-/-}$ *Rbp*$^{-/-}$ and WT mice

Real-time RT-PCR analysis of mRNA expression levels of (**A**) Inflammatory cytokines (*Il-6*, *Tnfα* and *Il1-β*); and (**B**) immunomodulatory cytokines (*Il22, Il23* and *Il17*). Values are mean ± SD calculated using the $2^{-\Delta\Delta CT}$ method; n = 3 individual sample pools/group (3 animals per pool). Statistical analysis by two-way ANOVA within each gene. Different letters indicate significant difference ($p < 0.05$) within each gene; VA-def, vitamin A-deficient diet; BC, β-carotene; Veh, vehicle.

3.5 DISCUSSION

Beneficial effects of βC have been associated with its retinoid-generating capacity and its antioxidant activities in the various pathological conditions and chronic diseases (Hui et al., 2020; Lyu et al., 2018; Mudronová et al., 2018). Here we assessed whether βC could ameliorate intestinal health and microbiota fitness in a mouse model of VAD, the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice (Kim et al., 2008; Wassef et al., 2013). As mentioned above, these mutants rely exclusively on dietary vitamin A to support retinoid-dependent functions and display an extremely tenuous vitamin A status already when fed retinoid-containing diets (Kim et al., 2008; Wassef et al., 2013). In contrast to WT, when deprived of dietary vitamin A for four weeks *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice develop severe VAD associated with intestinal dysfunctions and dysbiosis (**Chapter 2**).

In this study, mice were treated for 2 weeks with an oral gavage of βC at 50 mg/kg body weight (~0.9 mg/dose considering an average body weight of ~ 18g) every other day. Our dose was modeled on previous studies where mice were chronically fed a vitamin A deficient diet supplemented with 0.15 mg βC/g diet (~ 0.5 mg of βC daily) (Lobo et al., 2010b). Additionally, Trivedi and Jena (Trivedi and Jena, 2015) supplemented mice with βC (up to 20 mg/kg body weight) daily for 28 days to reduce the inflammation associated with ulcerative colitis.

Intestinal absorption of βC was expected to be enhanced under VAD (Lobo et al., 2010b). However, no differences were observed in βC concentrations in serum and other tissues. Interestingly, despite the lack of statistically significant differences in the amount of βC in the feces of the two genotypes, perhaps due to the rather small samples size, our data suggest that βC could have been absorbed more efficiently in the intestine of the *Lrat*

$^{-/-}$Rbp$^{-/-}$ mice (they excreted ~60% of the initial βC dose) relative to WT mice (they excreted ~80% of the initial βC dose). Also, the duodenum of the *Lat$^{-/-}$Rbp$^{-/-}$* mice showed a trend towards higher levels of βC compared to WT mice. Finally, given that βC has been proposed as the primary source of the retinoids in adipocyte (Lobo et al., 2010a), it is not surprising that intact βC was undetectable in adipose, likely due to its cleavage into retinoids. Similarly, when *Lrat$^{-/-}$* mice were fed a vitamin A deficient diet and gavaged daily with 0.5 mg of βC for 10 days, only trace amounts of βC (~10 pmol/g) were seen in the adipose tissue whereas retinol levels increased (Lobo et al., 2010a).

Due to the lack of RBP, circulating retinol levels were significantly lower in the *Lrat$^{-/-}$Rbp$^{-/-}$* mice compared to WT, as previously shown (Kim et al., 2008; Wassef et al., 2013) and **Chapter 2**). Interestingly, βC supplementation dramatically increased the concentration of serum retinol to the same level of the WT mice (**Fig. 3.2A)**. In the absence of RBP and LRAT, postprandial-derived "free" retinol can be incorporated into chylomicrons (O'Byrne et al., 2005). Since the mice in this study were sacrificed 4 hrs after the last dose of βC, it is likely that the retinol detected in the circulation of the supplemented mutants is associated with postprandial lipoprotein particles.

βC supplementation increased hepatic retinoid concentrations in the WT but not in the mutant mice (**Fig. 3.2A)**. Perhaps βC-derived retinol was rapidly metabolized into retinoic acid in the severely vitamin A deficient *Lrat$^{-/-}$Rbp$^{-/-}$* mice. This hypothesis however needs to be unequivocally proved.

Dietary βC supplementation increased retinol levels in the adipose tissue of both genotypes (**Fig. 3.3)**. This result is in agreement with the above-mentioned notion that βC is the primary source of retinoids in adipocyte (Lobo et al., 2010a). Indeed, Lobo and

colleagues (Lobo et al., 2010a) showed that βC supplementation during vitamin A deprivation of *Lrat*$^{-/-}$ mice increased the expression of *Bco1*, *Raldh1* and *Cyp26A1* expression, suggesting preferential cleavage of βC into retinoids in the adipose tissue. However, one would have expected more βC taken up or metabolized by this tissue under VAD. Our data do not suggest that this is the case, even though they cannot unequivocally exclude it. In the future, retinoid homeostasis should be assessed by gene expression and/or turnover studies to address this issue.

As most of the dietary vitamin A is absorbed in the small intestine (Goodman and Blaner, 1984), and as dietary vitamin A represents the majority of the retinoids in this GI segment (**Chapter 2**), it was not surprising that the βC supplementation enhanced retinoids content in the duodenum of both genotypes. Retinyl esters, however, remained lower in the mutant mice, as a result of the lack of LRAT (**Fig. 3.5A-B)** (O'Byrne et al., 2005; Wongsiriroj et al., 2008c) the main enzyme that synthesizes retinyl esters in the small intestine (O'Byrne et al., 2005; Wongsiriroj et al., 2008c). Overall, total retinoids (retinol + retinyl esters) content in the duodenum of the βC-treated *Lrat*$^{-/-}$*Rbp*$^{-/-}$ was lower than that of βC-treated WT mice (129 ± 52 ng/g vs. 347 ± 57 ng/g, respectively; $p = 0.01$) (**Fig. 3.5A-B**). This is likely due to the fact that βC cleavage by BCO1 is regulated by the vitamin A status (Lobo et al., 2010b) and expected to be more efficient in VAD. Indeed, It has been shown that βC supplementation during VAD significantly increased the expression of *Bco1* in adipose tissue (Lobo et al., 2010a) and placenta (Wassef et al., 2013), for instance.

Similar to the small intestine, βC supplementation significantly enhanced colon retinoids content in both *Lrat*$^{-/-}$*Rbp*$^{-/-}$ and WT mice (~6.5 and 1.7 folds, respectively) even though the total retinoid concentration in the colon of the mutants remained lower than that

of supplemented WT mice (**Fig. 3.5C-D)**. Retinol dehydrogenase (Rdh) and retinal dehydrogenase (Raldh) control the two oxidative steps toward retinoic acid production (Adams et al., 2014; Belyaeva et al., 2019, 2017; Kedishvili, 2016). Thus, the increased expression of these enzyme upon βC supplementation **(Fig. 3.6A)** suggested that the provitamin A carotenoid is being converted into retinoic acid, and that this conversion contributes to improve VAD in the tissues of the mutant mice. Interestingly, though, it seems that different members of the Rdh and Raldh family of enzymes are being recruited to generate retinoid from βC depending upon the genotype of the mice **(Fig. 3.6A)**. The fact that *Cyp26b1* expression was significant lower in the vitamin A-deficient *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice compared to other groups supports our earlier findings discussed in **Chapter 2** indicating that the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice maintained on the VA-def diet are indeed vitamin A-deficient in many tissues **(Fig. 3.6B)**. Moreover, that *Cyp26b1* expression significantly increased after βC supplementation **(Fig. 3.6B)** is in agreement with our interpretation that βC is being converted into retinoids, given that Cyp26b1 is known to be upregulated by retinoic acid (Topletz et al., 2012).

Overall, this regimen of dietary βC supplementation restored vitamin A sufficiency in the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice based on serum retinol levels. βC also enhanced to a certain extent the extrahepatic retinoid storage capacity of the VAD mutant mice in various tissues. However, due to the predominant function of LRAT in the liver (Golczak and Palczewski, 2010), hepatic vitamin A stores could not be restored.

Evidence suggests that dietary carotenoids may influence the taxonomic composition of the gut microbiota (Lyu et al., 2018; Xia et al., 2018). A recent study indicated that dietary carotenoids such as astaxanthin (200 μg/g diet) alters the

Firmicutes/Bacteroides (F/B) ratio and this shift in intestinal bacteria abundance was hypothesized to be one of the reasons as to why astaxanthin prevented high-fat diet induced obesity in mice (J. Wang et al., 2019). Furthermore, βC supplementation (60 mg/kg diet) of layer-type cockerels was shown to contribute to intestinal development by improving mucin and tight junction, as well as by increasing retinoic acid-derived IgA production, and alter cecal microbial composition by decreasing the levels of *Escherichia coli* and increasing the abundance of *Lactobacillus* (Hui et al., 2020; Mudronová et al., 2018).

Our study confirmed that VAD is concomitant with a distinct fecal taxonomic profile, characterized for example by a lower abundance on the phylum of Actinobacteria (as also shown in **Chapter 2**) as they play a major role in maintain the gut barrier homeostasis and they are involved in degradation of resistant starch (Binda et al., 2018). In contrast to other reports, (Tian et al., 2018; Zhou et al., 2020), a lower F/B ratio was observed in the mutant VAD mice relative to the WT controls **(Fig. 3.8E).** Similar results were obtained in the study described in **Chapter 2**, although the difference in F/B ratio between WT and *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on the VA-def diet did not reach statistical significance. Perhaps, different diets used by different laboratories are responsible for the discrepant F/B ratio between our studies and those of others.

ROS levels in the gut have been shown to perturb bacterial populations that rely on anaerobic to microaerophilic respiration (Imlay, 2013; Riggle et al., 2013; Wlodarska et al., 2015). Interestingly, dietary βC supplementation selectively modified the abundance of certain fecal bacteria taxa only in the vitamin A deficient *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice (**Fig. 3.9B**). Among these, *Alistipes* and *Paraprevotella* genera (Bacteroidetes Phylum) were more abundant under VAD, perhaps because the intestinal morphological and functional

dysfunctions of the VAD intestine provided a better niche for them to thrive (**Fig. 3.10A and B)**. Indeed, abundance of *Alistipes* has been shown to be associated with inflammation and colorectal cancer in human (Berni Canani et al., 2018; Dai et al., 2018) and animal models (Moschen et al., 2016; Y.-N. Wang et al., 2019; Ye et al., 2016). These conditions have been linked to a higher oxidative stress status (Mandal, 2017). *Alistipes* also positively correlated with the production of IL-6 and TNF-α in the intestine (Moschen et al., 2016; Ye et al., 2016). Indeed, higher levels of IL-6 and higher oxidative stress were observed in our mouse model of VAD. In addition, in *Sod1*$^{-/-}$ mice the abundance of the *Paraprevotella* bacteria was significantly increased most likely due to enhanced oxidative stress (Sagi et al., 2020). Moreover, treatment with pomegranate polyphenols, that are known as poorly absorbed antioxidants, reversed the high abundance of *Paraprevotella* associated with a high-fat diet in rats (Zhao 2019). Similarly, administration of the carotenoid astaxanthin significantly reduced the relative abundance of the *Parabacteroides* genus in mice (H. Liu et al., 2018).

Desulfovibrio, which belong to the family of *Desulfovibrionaceae*, can adapt to different oxygen and oxidative stress levels (Coulter and Kurtz, 2001; Scholten et al., 2007; Zhou et al., 2010). Thus, higher oxidative stress caused by VAD could also have favored the growth of *Desulfovibrio* (Proteobacteria phylum). However, given the above-mentioned features of these bacteria, one would have not expected the lower abundance of this taxon in the βC supplemented VAD mice. Perhaps, other factors modified upon βC treatment in the intestine, such as mucin (Li et al., 2019), can drive the changes in these bacteria's abundance.

Lachnoclostridium was also attenuated in the feces of the supplemented VAD mice. Higher abundance of *Lachnoclostridium* has been associated with inflammatory diseases, such as Ulcerative Colitis (UC) and high fat diet-induced obesity in mice (Sun et al., 2020; D. Zhu et al., 2018). Interestingly, administration of compounds with antioxidant properties, such as fucoxanthin or melatonin, to mice has been shown to reduce the abundance of *Lachnoclostridium* (Sun et al., 2020; D. Zhu et al., 2018).

Overall, it would be reasonable to hypothesize that some of the changes driven by βC in the VAD fecal bacterial communities may be, at least in part, mediated by the antioxidant properties of βC which mainly travels unabsorbed in the GI tract. This unabsorbed βC could act by modulating oxidative stress levels (as per ROS levels) in the lumen of the intestine, even though the exact mechanisms remain to be elucidated. As the supplementation did not modulate the abundance of these bacteria taxa in the WT, we do not favor the possibility the βC could act directly on the bacteria.

Given the ability of βC to increase retinoid concentration in the various segments of the intestine, it is also plausible that its provitamin A activity improved intestinal barrier dysfunctions associated with VAD (Groeger et al., 2016; Hui et al., 2020); and **Chapter 2**) thus contributing to reducing leakage of ROS from the intestinal cells (Keshavarzian et al., 1992; Kuhn et al., 2018). Indeed, βC supplementation of *Lrat*$^{-/-}$*Rbp*$^{-/-}$ improved colon mRNA levels of tight junction proteins (*Occludin*, and *Claudin-3*) **(Fig. 3.13B)**, whose expression is known to be regulated by retinoic acid (He et al., 2019). Perhaps increase in the expression of the tight junction proteins and mucin **(Fig. 3.13 A and B)**, could improve the leaky gut phenotype linked to VAD, resulting in lower circulating LPS **(Fig. 3.14)**.

It has been shown that intestinal inflammation *per se* could also contribute to reducing the expression of both mucins (Heazlewood et al., 2008; Johansson et al., 2014) and tight junction proteins (Prasad et al., 2005; Trivedi and Jena, 2015) through a variety of mechanisms. Therefore, improved expression of *Mucin2/3* and/or O*ccludin* and *Claudin-3* in the VAD mice (**Fig. 3.13A and B)** might be also linked to the anti-inflammatory function of βC (Trivedi and Jena, 2015). Trivedi and colleagues showed that βC administration (5, 10 and 20 mg/kg body weight) for 28 days in mice attenuated inflammation associated with the Ulcerative Colitis (UC) by reducing the levels of the IL-17, IL-6 and TNFα proteins in the colon (Trivedi and Jena, 2015). The authors proposed that the anti-inflammatory action of βC could be associated with the decrease in expression of NF-kB, a key regulator of proinflammatory cytokines (Trivedi and Jena, 2015), in tum linked to the direct regulatory function of ROS on NF-KB signaling (Chew and Park, 2004; Kawata et al., 2018; Morgan and Liu, 2011; Park et al., 2019).

The provitamin A activity of βC generates retinoic acid **(Fig. 3.6A)**, a well known immune modulatory molecule (Erkelens and Mebius, 2017), which in turn inhibits the differentiation of naïve T cells to Th17 cells by blocking IL-6, IL-21, and IL-23 production in mice (Xiao et al., 2008). Also, through retinoic acid production, βC supplementation in both mice (50 mg/kg diet for 21 days) (Nishida et al., 2014) and chickens (60 mg/kg body weight for 28 days) has been reported to induce intestinal mucosal IgA production which prevents bacterial translocation to the intestinal mucosa and suppress host inflammation (Hui et al., 2020; Nishida et al., 2014).

Our findings indicated that dietary βC attenuated the expression levels of intestinal inflammatory cytokines in VAD and to a certain extent also in VAS (**Fig. 3.15A).** Also,

βC regulated the expression of immunomodulatory cytokines, such as *Il-17* and *Il-22*, potential drivers of chronic GI tract inflammation (Huang et al., 2018; Mielke et al., 2013), regardless of the vitamin A status **(Fig. 3.15B).** We cannot discriminate whether these effects of βC on the immune system are due to its provitamin A or antioxidant activity or both.

In conclusion, we demonstrated that βC can restore intestinal dysregulation and fecal dysbiosis in our mouse model of VAD. Specifically, βC improved intestinal retinoids content, physical barrier integrity, reduced oxidative stress and inflammation and selectively modified the abundance of certain microbial component. Thus, our data provide strong evidence to further this area of research with potential large impact on human health.

CHAPTER 4 :

GENERAL CONCLUSION AND FUTURE DIRECTIONS

Vitamin A deficiency (VAD) remains a worldwide problem in both developed and developing countries. Among the many health implications of VAD there is the impairment of intestinal physiology and functions (Cha et al., 2010). Intestinal damage and, thus, inadequate absorption of vitamin A could also reduce the effectiveness of intervention programs to defeat VAD. On the other hand, a "healthy" intestine is critical to support a "healthy" intestinal microbiome, which in turn helps maintaining proper intestinal functions in a feedback loop (Conlon and Bird, 2014; Quigley, 2013). The work presented here provided insights into the relationship between different forms of dietary vitamin A, host vitamin A status intestinal health and microbiome fitness.

- ***Why is it important to understand the microbiome of vitamin A deficiency?***

Given that intestinal bacteria play major roles in the fermentation of residual food, modulation of gut immune functions, and protection against pathogens and diseases, changes in bacterial populations or their metabolites may contribute to disease development. Intestinal bacterial dysbiosis have been associated with increased in gut permeability, endotoxemia, pro-inflammatory cytokines, as well as increased adiposity which ultimately can cause metabolic conditions, including Type 2 diabetes, cardiovascular diseases (Boulangé et al., 2016) and inflammatory bowel disease (IBD) (Kaakoush et al., 2012; Sobhani et al., 2011).

We demonstrated that VAD status of the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice dysregulated the intestinal barrier at different levels. We showed disrupted intestinal permeability in *Lrat*$^{-/-}$ *Rbp*$^{-/-}$ (VA-def) resulting in increased bacterial LPS in the circulation (**Fig. 3.14**). It was demonstrated that elevated plasma LPS is associated with metabolic endotoxemia and

systemic inflammation (Danenberg et al., 2002; de Punder and Pruimboom, 2015; Saramago et al., 2019). Therefore, future studies should consider assessing systemic inflammation in our mouse model of VAD by measuring markers of endotoxemia such as LPS/LBP (LPS binding protein) and serum IgA/IgM in the circulation (de Punder and Pruimboom, 2015).

Additionally, several comprehensive studies (Grizotte-Lake et al., 2018; Lv et al., 2016; Tian et al., 2018) as well as ours (**Fig. 3.9**) demonstrated that VAD is associated with lower butyrate and butyrate producing bacteria (*Clostridia* and *Roseburia*). Butyrate, the product of microbial fermentation by anaerobic gut bacteria, serves as an important immunomodulatory compound, enhances the integrity of the intestinal epithelium and modulates enteric tolerance against microbial communities (Bui et al., 2015; Lv et al., 2016). In addition, butyrate can systemically influence distant organs, including the lungs (Haak et al., 2018). A recent study has demonstrated that reduction of butyrate-producing bacteria, and consequently of butyrate, is associated with higher risk of respiratory viral infections in human by modulating the immune system (Haak et al., 2018). It has been shown that intestinal commensal microbiota regulates the generation of virus-specific CD4 and CD8 T cells and antibody responses following respiratory influenza virus infections (Ichinohe et al., 2011). Interestingly, VAD is also associated with increased risk of respiratory infectious diseases (Chen et al., 2018, 2014; Marquez and Chen, 2020; Sommer et al., 1984; Timoneda et al., 2018). Thus, it is possible that the changes induced by VAD on intestinal microbiota may impact the development of extra-intestinal disease.

- ***Does bacterial dysbiosis contribute to the intestinal dysfunction caused by VAD?***

Evidence supports that VAD alters the diversity of the intestinal bacterial populations (Amit-Romach et al., 2009; Lv et al., 2016). Our 16S rRNA gene survey showed that the fecal taxonomic microbial profile is not influenced directly by the dietary vitamin A restriction, but rather by the host vitamin A status (**Fig. 2.7)**. For instance, VAD status was associated with a significant lower relative abundance of anti-inflammatory *Bifidobacteria*, and increased abundance of proinflammatory *Bacteroides*, independent from dietary vitamin A intake (**Fig. 2.8**). It is likely that changes in the bacterial intestine further exacerbate inflammation (**Fig. 2.17 and Fig. 2.18**) caused by VAD *per se* in the intestine (Cha et al., 2010; Sirisinha, 2015).

A recent study shows the critical role of commensal bacteria (*Clostridium*) in modulating the host vitamin A metabolism to protect the host from enteric pathogen colonization (Grizotte-Lake et al., 2018). This finding supports the notion that intestinal bacteria and their metabolites can induce changes in the host. Therefore, future studies should aim at clarifying if and how intestinal bacterial dysbiosis caused by VAD affects host physiology and functions, both locally and systemically. Perhaps this question can be addressed by fecal microbiome transplant (FMT) from VAD to VAS mice. These studies can be performed in germ-free mice or conventionally raised animals with or without microbiota depletion before the transfer. Since germ-free mice have showed immune system deficits, metabolic alterations and reduced nutrient absorption (Belkaid and Hand, 2014), they might not be an ideal model. Therefore, using conventionally raised mice

treated with antibiotics to favor the colonization of the donor microbiome could be more useful (Le Roy et al., 2018).

- ***What are the knowledge gaps in fecal microbial dysbiosis caused by VAD?***

Many factors including, but not limited to, genetic background, fiber composition of the diet, type of samples collected (e.g., fecal, cecal, intestinal mucosa), as well as the techniques employed to survey the bacteria (e.g., 16S rRNA gene or metagenomic shotgun sequencing) make the microbiome analysis across studies hard to interpret (Hasan and Yang, 2019). Comparing microbiomes from different regions of the intestine, showed that bacteria are different among fecal, cecal, small and large intestine, as well as from lumen and mucosa of the various region of the GI tract (Kozik et al., 2019; Tang et al., 2015).

Interestingly, we showed that in the VAD *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice, the expression of both mucins (*Muc2/3*) **(Fig. 2.11)** and antimicrobial **(Fig. 2.12)** are significantly reduced. Mucins and antimicrobial primarily regulate mucosa-associated bacteria. While selective intestinal bacteria can degrade mucus glycans as an energy source, (Schroeder, 2019), antimicrobial peptides limits translocation and penetration of microbes across the mucosal barrier with limited impact on luminal microbial numbers (Vaishnava et al., 2008). Therefore, lack of both mucins and antimicrobial peptides has the most impact on the bacteria that are closely associated with mucosa rather than luminal or fecal. Future studies should focus mostly on understanding the taxonomy and functionalities of the mucosal bacterial community, rather than luminal or fecal bacteria.

The 16S rRNA gene survey is commonly used to study bacterial communities (Jo et al., 2016). However, this methodology has its own limitations that needs to be considered

when the data is interpreted. For instance, the annotation of the bacteria is based on similarities that are more precise at higher taxonomic levels, i.e., phyla or genera, and less accurate at the species level (Ranjan et al., 2016). Alternatively, shotgun metagenomic sequencing does not involve the biased amplification of 16S rRNA genes sequenced. Therefore, it can identify differences at the species levels more accurately, even though this technique is more expensive and thus is less commonly used (Ranjan et al., 2016). In the future, it is desirable to investigate both fecal and intestinal microbial populations with a shotgun metagenome sequencing approach.

The prediction of bacterial functions in a given biological sample constitutes another critical challenge. Despite the similar taxonomic footprints of the fecal microbiota between the *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice on VA-suf and VA-def, analysis of the microbial functionalities with fusionDB (C. Zhu et al., 2018a) clearly separated the groups of mutant mice, based on the dietary vitamin A content, i.e., the severity of VAD (**Fig. 2.10**). Notably, fusionDB database contains ~1500 taxonomic distinct bacteria and ~4.5 million protein as a reference to predict the function of the unknown bacteria. Thus, while fusionDB database can provide some preliminary result of the possible function of unknown sample, it has limited annotated functional references. Additionally, the current functional analysis, was generated based on the 16S rRNA gene of the fecal bacterial DNA which provides limited information on the true functional properties on the microbiome. Since this method just target small region of 16S rRNA gene, therefore many genes are not sequenced and rather will be predicted based on the operational taxonomic unit (OTU). Also, given that bacteria exchange genes through horizontal gene transfer, the lack of direct gene sequencing and identification potentially limits understanding of a microbiome function (Ranjan et al.,

2016). Therefore, to get insight into the function of intestinal bacteria in response to dietary vitamin A and/or vitamin A status we could perform, for instance, proteomic analysis that provide accurate results in terms of the function of the intestinal bacteria. Metabolomics studies that measure small metabolites produced by the bacteria and impact the host physiology could also be highly informative in the study of the microbiome of VAD and its functions (Chen et al., 2019).

- ***What drives the bacterial changes in the intestine upon βC treatment?***

Despite the wide range of health benefits of βC, the mechanisms underlying the impact of dietary βC on intestinal microbiome and health during VAD remains elusive. Our findings showed that βC supplementation reduced the abundance of several genera in the vitamin A deficient *Lrat*$^{-/-}$*Rbp*$^{-/-}$ mice with no impacts on WT mice (**Fig. 3.10**). As stated in the discussion of chapter 3, it has been shown that antioxidant such as astaxanthin, fucoxanthin or melatonin can alter the intestinal bacteria (H. Liu et al., 2018; Sun et al., 2020; D. Zhu et al., 2018). Therefore, it is possible that changes in bacterial abundance is associated with antioxidant property of βC.

On the other hand, it has been shown that in isolated murine thymocyte cell (Thymoma), βC is converted into radicals, e.g., βC peroxyl radical (β-COO$^{\bullet}$) or carbon-centered radical (β-C$^{\bullet}$) in the presence of ROS (Palozza et al., 1997). The newly formed radicals may, in turn, function as potential pro-oxidant agents and interact with other biological molecules, such as protein or lipids, at least in mammalian cells and tissues (Fiedor and Burda, 2014). While there is no evidence whether this βC radicals can have any detrimental effect on bacteria, the formation of alkyl peroxyl radicals has been shown

to have detrimental effect on both gram (+) and (-) bacteria with less pronounced effect on gram (-) bacteria due to the LPS membrane (Akaike et al., 1992). Therefore, that the formation of carotenoid radicals may have detrimental effects on the fitness of certain bacteria taxa it is a possibility that remains to be investigated.

It is also possible that the changes in morphology and functions of the gut driven by the βC supplementation may indirectly modulate the abundance of various bacteria taxa in the intestine. For instance, higher mucins expression, and hence enhanced mucus production, have been shown to reduce the abundance of *Desulfovibrio* (Li et al., 2019).

Our experimental setup does not unequivocally exclude the possibility that the effects of βC on the microbiome could be host-mediated. To clarify this issue, future studies should consider using an *in vitro* system, such as the Simulator Intestine Human Microbial Ecosystem (SHIME), that mimics only the physical intestinal conditions without the "biological component" of the host. This system allows the growth of microbiomes sampled from human subjects, for instance, in different compartments/vessels mimicking the various segments of the GI tract (based on oxygen tension, pH, temperature, etc). Moreover, through a "stomach-like" compartment the system can be "fed" various nutrients providing the ability to modify the concentrations and composition of the nutrient mix. Finally, samples of the bacteria growing in the small and large intestine compartments can be taken at any given time throughout the experiment (Van den Abbeele et al., 2010). By using this unique experimental system, one could evaluate the effect of βC at different concentrations (physiological and pharmacological doses) on the fecal microbiota collected from VAD and VAS individuals.

- ***Does provitamin A and/or antioxidant activities of βC regulates the intestinal function?***

In our model we could not unequivocally establish whether the improvements of the intestinal barrier functions observed upon βC treatment of the VAD mutant mice were the result of the provitamin A or antioxidant activities of βC or both. To address this issue, we could supplement the mutant VAD mice with retinoic acid, the active form of vitamin A, or with carotenoids with no provitamin A activity, such as lycopene, astaxanthin, lutein and zeaxanthin (Toti et al., 2018). Interestingly, astaxanthin, an oxygenated carotenoid (H. Liu et al., 2018), and lycopene (Kawata et al., 2018) has been shown to have strong antioxidant and anti-inflammatory properties. However, in designing these experiments one should also consider the different mechanisms whereby various carotenoids may exert their antioxidant activity to avoid drawing erroneous conclusions.

- ***Can βC-producing probiotic bacteria serve as an alternative source of βC to improve the microbial dysbiosis and intestinal dysfunction due to VAD?***

Probiotic supplementation is one of the commonly used strategies to improve intestinal health (Pace et al., 2015; Sánchez et al., 2017). Thus, probiotics supplementation with anti-inflammatory and antioxidant activities can improve the intestinal dysfunctions linked to VAD. Interestingly, bacteria have been exploited for their biotechnological potential in the large production of a variety of compounds such as different enzymes or vitamins (Hale et al., 2007; Hermann, 2003; Nigam, 2013). A few years ago, our laboratory showed that a strain of bacteria engineered to produce βC, *E.coli* MG1655*-pACβ, could colonize the mouse intestine and "locally" produce βC that could be absorbed by the

intestine and distributed to other organs (Wassef et al., 2014). Despite this approach, i.e., using bacteria as a source of βC rather than food, provided a successful proof of principle, it showed some limitations mainly in the relatively low amount of βC synthesized in the mouse intestine by the recombinant bacteria (Wassef et al., 2014). Follow up experiments in the lab led to the generation of a new strain of bacteria further engineered to significantly increase the yield of βC synthesis, at least under conditions of aerobic growth. Unfortunately, when administered to VAD mice we did not observe the same dramatic enhancement of the βC synthesis, clearly indicating that the limited O_2 level in the intestine was the main barrier that still needs to be overcome to rescue VAD *in vivo*. We did not confirm, however, whether this new strain of recombinant bacteria may improve the functions of the vitamin A deficient intestine in our VAD model, providing a moderate local source of βC for the enterocytes and other cells of the gut mucosa. This could be an important achievement *per se*. In this respect, one could envision to engineer bacteria such as *Lactobacillus* and *Nissle* 1917 that could provide beneficial effects not only through their known probiotic properties (Bull et al., 2013; Bures et al., 2011) but also through their acquired ability to synthesize βC. In addition, the knowledge we acquired through our studies of the microbiome of VAD could guide us to choose the most appropriate bacteria to engineer, the one(s) that could thrive under the intestinal conditions linked to VAD.

CHAPTER 5

APPENDIX:

ENGINEERING β-CAROTENE PRODUCING BACTERIA FOR *IN SITU* PRODUCTION OF β-CAROTENE IN THE MOUSE INTESTINE

5.1 INTRODUCTION

Vitamin A deficiency is one of the world leading micronutrient deficiency (Bailey et al., 2015; Wiseman et al., 2017). Current interventions to eradicate VAD include vitamin A supplementation (VAS) and fortification of staple food with preformed vitamin A, as well as implementation of dietary diversification and biofortification with provitamin A carotenoids, mainly β-carotene (Dubock, 2017; Tanumihardjo, 2015). In some instances, these programs, separately or in combination, have shown progress in improving vitamin A status and decreasing by 23% the rate of mortality in children (Benn et al., 2015). However, results of randomized controlled clinical trials of supplementation are surprisingly conflicting for reasons that are still unclear (Benn et al., 2015).

Bacteria have been exploited for their biotechnological potential in the large production of a variety of compounds, such as enzymes (amylase, proteases and etc.), vitamins (vitamin B12), drugs (artemisinin, human insulin), biofuels, etc. (Baeshen et al., 2014; Hale et al., 2007; Hermann, 2003; Nigam, 2013). Interestingly, bacteria also reside in the mammalian intestine, which hosts many different species that all together establish a complex community of such microorganisms, called the microbiota. This community has developed a symbiotic relationship that affects and maintains the host physiology, metabolism and overall health (Altenhoefer et al., 2004; DiBaise et al., 2012; Nguyen et al., 2015). Some of these microorganisms within the microbiota are also capable of producing micronutrients, including the vitamins K, folic acid, B2, B7 and B12 (Patterson et al., 2014). Notably, none of the microorganisms that reside in the mammalian intestine can synthesize vitamin A.

As shown in **Fig. 5.1**, βC is synthesized from the common precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which in turn are synthesized through either the mevalonate (MVA) pathway or non-mevalonate pathway (MEP). DMAPP and IPP are converted to geranyl geranyl pyrophospate GGPP by GGPP synthase (CrtE) (Lee et al., 2003; Yang and Guo, 2014) which will be converted to form the colorless carotenoid phytoene by phytoene synthase (CrtB). The introduction of additional double bonds into phytoene by phytoene desaturase (CrtI) produces the colored carotenoids called lycopene and by cyclization of the lycopene by lycopene cyclase (CrtY), β-carotene is produced **(Fig. 5.1)** (Lee et al., 2003). Bacteria (mainly *E.coli* k12 strains) have been engineered to synthesize the vitamin A precursor βC upon transformation with a low copy number plasmid (pAC-BETA or pACβ) containing the genes coding for the four key enzymes of the biosynthetic pathway (crtE, crtB, crtI and crtY) derived from the naturally βC producing *Erwinia herbicola* (Cunningham et al., 1996; Miller et al., 2013; Pitera et al., 2007).

We hypothesize that VAD could be alleviated if one could provide undernourished humans with a continuous source of βC such as βC producing bacteria, independent of the need to ingest foods or supplements. Our laboratory previously engineered the non-carotenogenic bacteria strain MG1655* (a mouse intestine adapted mutant of the *E. coli* MG1655) with the plasmid pACβ carrying the 4 essential genes of the βC biosynthetic pathway (MG1655*-pACβ). Upon colonization of the mouse intestine, βC synthesized "locally" by these bacteria was absorbed by the intestine and distributed to distant organs, such as liver, within the body through the bloodstream (Wassef et al., 2014). This study strongly supported the possibility that intestine-resident bacteria can serve as a source of

βC for various tissues in the mammalian body, even though it showed some limitations. In particular, the amount of βC synthesized in the mouse intestine by the recombinant bacteria was relatively low (5 ±1 ng/g in mucosa, 1.2 ± 0.2 μg/dL in serum and 52 ± 23 ng/g in liver; n=3). Therefore, this appendix provides preliminary results of experiments aimed at improving the yield of βC produced by engineered bacteria.

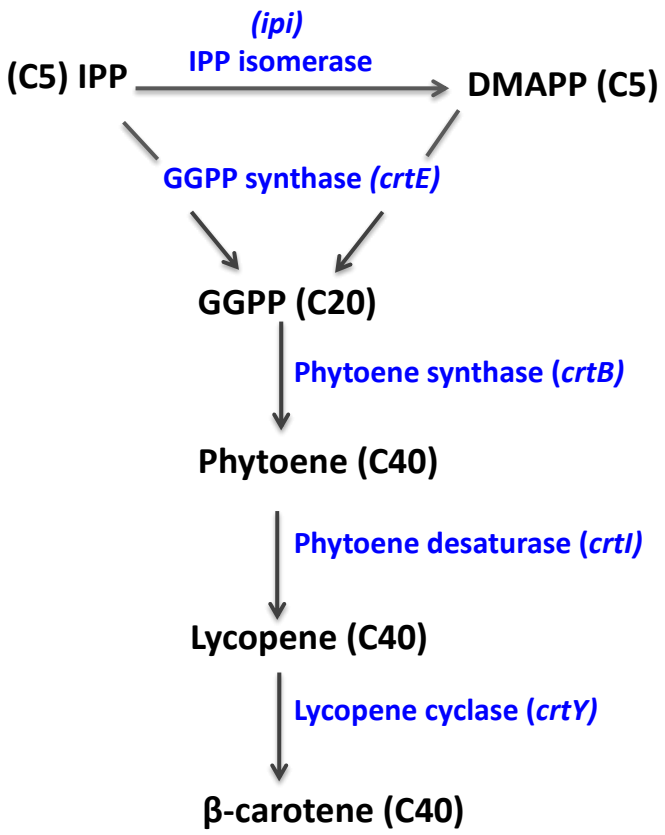

Figure 5.1: Biosynthetic pathways of βC production by bacteria

Four essential steps for the biosynthesis of the βC are shown in blue. Isopentenyl diphosphate, IPP; Dimethylallyl pyrophosphate, DMAPP; Geranyl geranyl pyrophosphate, GGPP.

5.2 MATERIALS AND METHODS

5.2.1 Engineering MG1655* pACβ-/ipi/dxs for higher yield of βC production

To improve the βC production, a second plasmid expressing the genes (ipi and dxs) for the two key enzymes that synthesize the five-carbon βC precursors was introduced to *E. coli* MG1655*-pACβ and generated the MG1655*-pACβ/ipi/dxs strain. To introduce the ipi/dxs plasmid into MG1655*-pACβ cells, 10beta bacterial cells containing ipi/dxs plasmid grown overnight to stationary phase in Luria Broth (LB) media containing chloramphenicol (50mg/L) and/or kanamycin (30mg/L), and the plasmids harvested using QIAGEN Plasmid Mini kit (QIAGEN, Valencia, CA). The concentration of the plasmid was quantified using a Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA). Isolated plasmids were transformed by electroporation into MG1655*-pACβ and plated on LB containing streptomycin (100mg/L), chloramphenicol (50mg/L) and/or kanamycin (30mg/L).

5.2.2 Engineering MG1655*-λDE3-Crt's for higher yield of βC production

DUET vector system carrying different genes required for the biosynthesis of βC pathway (pET-CrtE, pRSF-CrtB, pCDF-CrtI-IPI), were a kind gift from Dr. Ralf Welsch (Freiburg, Germany) (Welsch et al., 2010). To be able to produce βC using DUET vector system, first MG1655*-λDE3 bacteria were generated using λDE3 lysogenization kit (Novagen, MilliporeSigma) as recommended by manufacturer. This kit is designed for site-specific integration of λDE3 prophage into an *E. coli* host cell chromosome, such that the lysogenized host can be used to express target genes cloned in vectors under T7 promoters. Prior to co-transformation of the MG1655*-λDE3 cell with plasmids carrying βC pathway,

CrtY gene was inserted into the second multi cloning (MCS) site of the pRSF-CrtB vector. MG1655*-λDE3 was co-transformed with three vectors (pET-CrtE, pRSF-CrtB/Y, pCDF-CrtI-IPI) by electroporation and plated on LB containing kanamycin (30mg/L), Ampicillin (12mg/L) and streptomycin (100mg/L).

5.2.3 *In vitro* βC production by engineered βC producer bacteria in the presence or absence of oxygen

The recombinant bacteria MG1655*-pACβ and MG1655*-pACβ/ipi/dxs and MG1655*λDE3-Crt's were grown in liquid culture under aerobic and anaerobic condition in the presence of the antibiotics as mentioned above. For the anaerobic condition oxygen was depleted and nitrogen gas was replaced as a final electron acceptor. Cell pellets were obtained from aliquots (5 mL) of the bacteria culture every 2 h during the first 12 h of growth, and after 1 and 3, 6 and 9 days of culture at 37 °C. At the same time points, the number of cells was assessed by measuring OD at 595 nm and cell count. Harvested cells under anaerobic and aerobic conditions was used to evaluate the efficiency of βC production by HPLC.

5.2.4 Knock-out Transgenic LRAT-/-BCO1-/- Mice

LRAT-/-BCO1-/- knock-out mice were generated by crossing LRAT-/-RBP-/- (Kim et al., 2008) and BCO1-/- (Hessel et al., 2007) mice. The resulting double heterozygous mice (LRAT-/+RBP-/+BCO1-/+) of the F1 generation were crossed, and the double knock-out animals (LRAT-/-BCO1-/-) were obtained in the F2 generation at the expected Mendelian ratio. Both the LRAT and BCO1 genotype was confirmed by PCR

analysis. All mice employed for this study were from a mixed C57Bl/6 X sv129 genetic background.

5.2.5 Nutritional manipulation

5 week-old female mice, maintained on a regular chow diet (14IU vitamin A/g of diet, 2.6ppmm βC/g of diet and 26, 14, and 60% energy from protein, fat and carbohydrates respectively) were administered antibiotics starting from 2 days prior to the oral administration of the bacteria and maintained on this antibiotic regimen until sacrifice. Streptomycin (5 g/L) was administered to reduce the resident facultative anaerobe bacteria; Chloramphenicol (1.2 g/L) and Kanamycin (3 g/L) were administered to ensure replication and maintenance of pACβ and ipi/dxs plasmids. For the MG1655*λDE3-Crt's oral administration, Chloramphenicol was replaced with Ampicillin (5 g/L) in addition to Streptomycin and Kanamycin to maintain the replication of pET-CrtE, pRSF-CrtB and pCDF-CrtI-IPI plasmids. After 18 hrs starvation from food and water, mice were gavaged orally with single or multiple doses of 100 □l of (10^8 CFU/ml per dose) of each bacteria cell re-suspend in 20% sucrose. After the gavage, the mice were placed again on an *ad libitum* regimen of food and water intake and sacrificed 7 days after the bacteria administration. Feces were collected on the 1st and 3rd day post-gavage, as well as on the day of sacrifice when liver, mucosa, and serum were also collected.

5.2.6 HPLC analysis

Reversed-phase HPLC analysis was performed as previously described to measure retinol and retinyl ester and βC (Kim and Quadro, 2010) concentrations in serum and tissues, including 100 mg small intestine and colon. To measure βC concentration in fecal

samples, 100 mg of feces sample was weighted and followed as described previously (Kim and Quadro, 2010). To measure βC concentration in bacteria, 10ml of the bacterial liquid culture spin down and the pellet was resuspended in 1 ml of the suprenatant and homogenate for 30sec and 50 μl of the homogenate was targeted for the βC extraction as described previously (Kim and Quadro, 2010).

5.3 RESULTS

5.3.1 Enhancing bacterial βC synthesis

In order to improve βC synthesis in the previously generated MG1655*-pACβ strain in our laboratory (Wassef et al., 2014), we transformed the MG1655*-pACβ strain with a plasmid containing the *ipi* (isopentenyl pyrophosphate isomerase) and *dxs (deoxyxyloluse 5- phosphate)* genes from *Arabidopsis thaliana* and *Tagetes erecta*, respectively. The *ipi* and *dxs* genes carry the initial steps in the assembly of the backbone of carotenoids (Rodríguez-Concepción and Boronat, 2002). When grown overnight at 37 °C on agar plates, the resulting recombinant bacteria, MG1655*-pACβ/ipi/dxs, yielded higher level of βC compared to the MG1655*-pACβ and shown by the darker orange color of the colonies (**Fig. 5.2A**). Moreover, a time-course study in liquid media under aerobic conditions, revealed that the maximum production of βC occurred when the bacteria were in stationary phase (**Fig. 5.2B** and **5.2C**).

These results suggest that addition of the IPI and DXS can improve βC yield in the MG1655 strain.

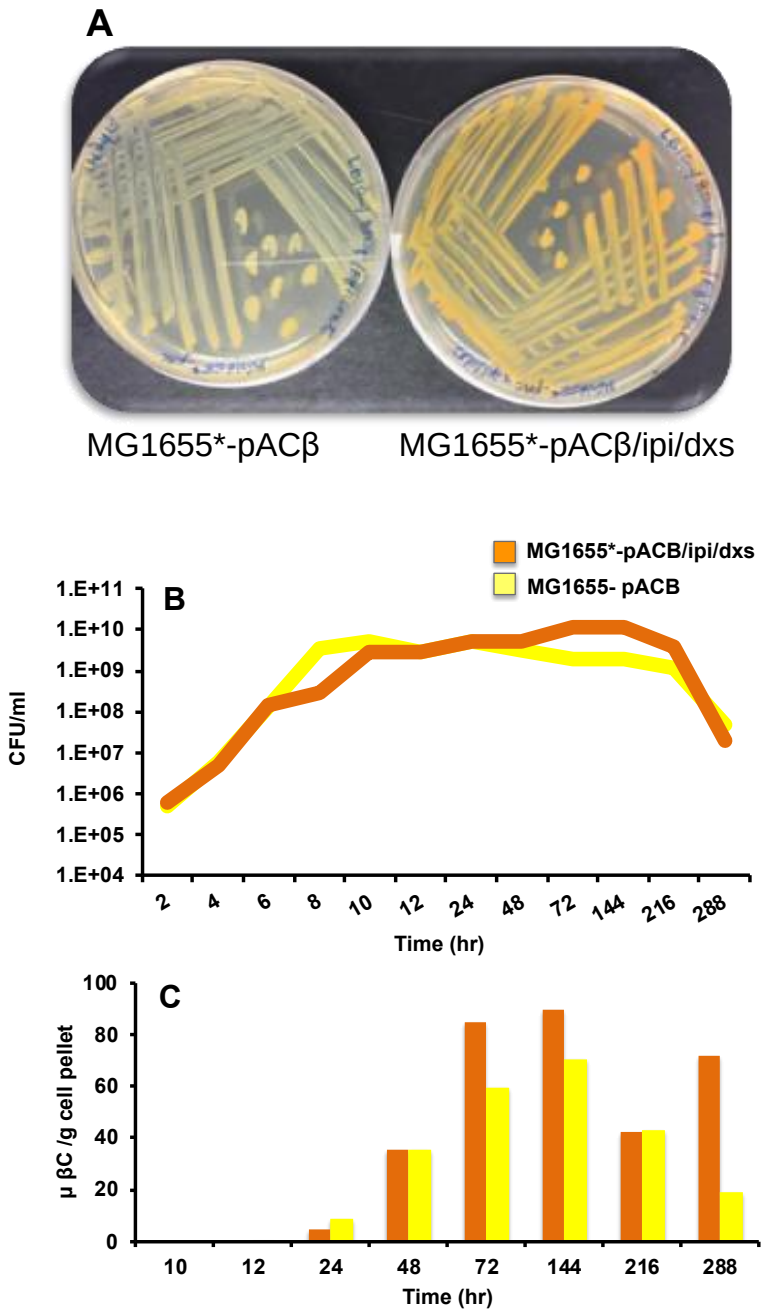

Figure 5.2: Aerobic βC production by βC-producing MG1655* bacteria

(**A**) streak plate of MG1655*-pAC (left) and MG1655*-pAC/ipi/dxs β (right) on LB plate supplemented with antibiotics. (**B**) Growth curve of the MG1655*-pACβ and MG1655*-pACβ/ipi/dxs bacteria over time. (**C**) HPLC analysis of βC in the pellet of bacteria harvested at various time points during the liquid growth; n=1 for each strain and time point.

5.3.2 In vivo βC production by MG1655*-pACβ/ipi/dxs

To establish whether the engineered MG1655*-pACβ/ipi/dxs strain was able to enhance βC production in the mouse intestine compared to the previously generated MG1655*-pACβ (Wassef et al., 2014), we administered either of these two strains to *Bco1*$^{-/-}$*Bco2*$^{-/-}$ mice as in **Fig. 5.3**. As these mice lack both βC cleavage enzymes, i.e., BCO1 and BCO2, it was expected that intact βC could be easily detected (by HPLC analysis) in their tissues, as it cannot be enzymatically cleaved (Amengual et al., 2013b). Upon gavage of a single dose of bacteria, they were detected (~10^7 CFU/ml) in fresh feces collected on day 6, 8 and 10 (fresh feces were resuspended in LB medium, serial diluted, plated and colony counted after overnight growth at 37C). Moreover, βC was measured by HPLC in mouse intestinal mucosa, liver and serum (**Table 5.1**), indicating that βC produced in the mouse intestine by the recombinant bacteria was absorbed and distributed to various tissues in the body, as previously reported (Wassef et al., 2014). However, unlike the results obtained from the LB culture (**Fig. 5.2**), there was no difference in the amount of βC in the tissues of the mice orally dosed with the bacteria carrying only pACβ vs. those carrying IPI and DXS as well (**Table 5.1**).

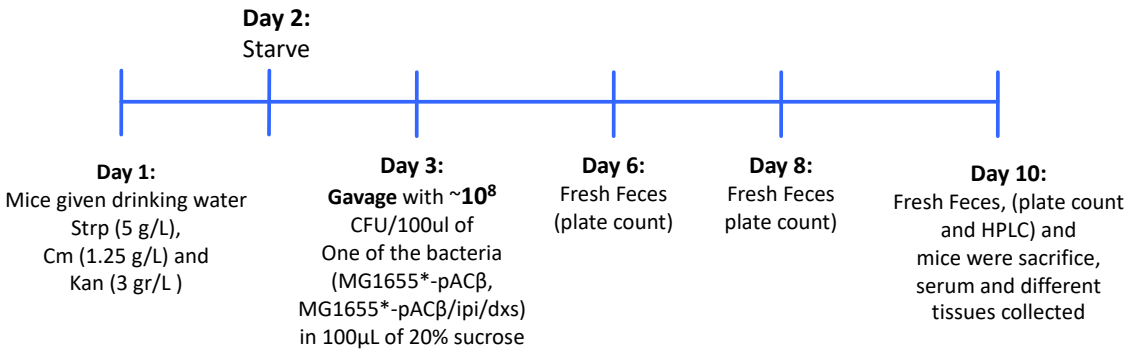

Figure 5.3: Experimental scheme

Bco1$^{-/-}$*Bco2*$^{-/-}$ female mice were maintained on regular chow (18IU VA/g diet) from weaning (3 weeks of age). At 6 weeks of age, from day 1, the mice (females) were maintained on water containing antibiotics throughout the experiment. On day 2, mice were food deprived ("starve") until day 3 when they were gavaged with either MG1655*-pACβ or MG1655*-pACβ/ipi/dxs bacterial suspension in sucrose. On day 6, 8 and 10 fresh feces were collected for the bacterial count. On day 10, after collection of fresh feces, mice were sacrificed to harvest serum, liver and small intestine for HPLC analysis. Value are mean ± SD; n=3 mice for each strain. Cm, Chloramphenicol; Kan, kanamycin; Strp, streptomycin.

MG1655*	Serum (µg/dL)	Liver (ng/g)	Mucosa (ng/g)	Feces (µg/g)
pACβ	1.8 ± 0.4	31.8 ± 7.8	21.4 ± 6.7	0.7
pACβ/ipi/dxs	2.4 ± 0.6	40.4 ± 10.6	24.2 ± 5.7	0.7
n=3 female mice (6 weeks old)/ bacteria strain				

Table 5.1: HPLC analysis of β-carotene content in *Bco1*$^{-/-}$/*Bco2*$^{-/-}$ mouse tissues

5.3.3 βC production by the facultative anaerobe *E.coli* strains under anaerobic condition

To test whether the reduced intestinal levels of oxygen might have contributed to the inability of our recombinant *E. coli* strains to efficiently synthesize βC *in vivo*, we set out to measure βC production form MG1655*-pACβ and MG1655*-pACβ/ipi/dxs under anaerobic respiration (Albenberg et al., 2014; Conway and Cohen, 2015). The recombinant bacteria MG1655*-pACβ and MG1655*-pACβ/ipi/dxs were grown in liquid culture under anaerobic condition in the presence of nitrate as the final electron acceptor (Martín-Mora et al., 2019). There was no difference in the growth rate between the two MG1655* strains under anaerobic growth conditions (**Fig. 5.4A**). HPLC analysis of these bacteria pellets showed that under anaerobic respiration the maximum production of βC occurred in the first 12 h and then gradually decreased (**Fig. 5.4B**). However, βC production under anaerobic growth conditions was reduced by 98% compared to the same strain under aerobic conditions (**Fig. 5.2B)**. Overall, these results confirm that the reduced tension of oxygen in the mouse intestine may be a critical factor that attenuates bacteria βC synthesis *in vivo*.

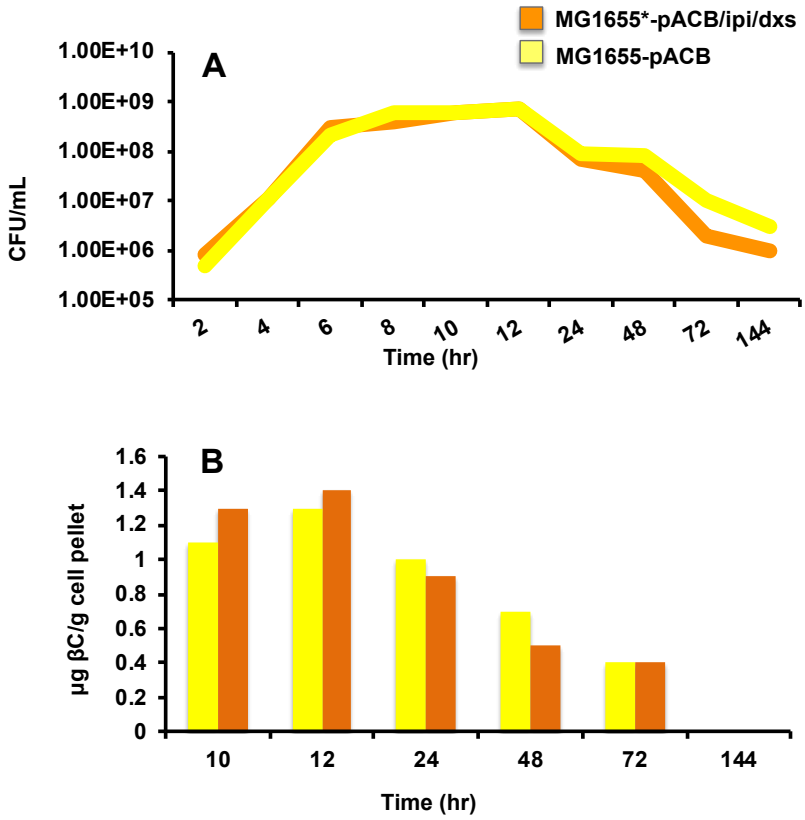

Figure 5.4: Anaerobic βC production by MG1655* βC producing bacteria

(**A**) Growth curve of the MG1655*-pACβ and MG1655*-pACβ/ipi/dxs bacteria over time. (**B**) HPLC analysis of βC producing bacteria pellets; n=1 sample for each strain.

5.3.4 Using the DUET vector system to increase the βC production

To increase the efficiency of βC synthesis in bacteria, we use the DUET vector system (Novagen), consisting of a set of individual plasmids that carry compatible replicons, thus allowing to maintain up to 4 vectors carrying the desirable genes in a single cell. These plasmids (pRSF-CrtB, pCDF-CrtI-IPI and pET-CrtE) were already engineered to express some of the key enzymes (CrtE, CrtB and CrtI) of the βC biosynthetic pathway and the precursor of the βC production under a strong/inducible T7 promoter (Welsch et al., 2010) **(Fig. 5.5 A-C)**. To complete the βC biosynthetic pathway, first, the *crtY* gene was inserted into the second multi cloning (MCS) site of the pRSF-CrtB/Y vector **(Fig. 5.5 B)** (Welsch et al., 2010). Then, the MG1655*λDE3 bacterial strain that is suitable to express the T7 RNA polymerase required for the expression of the DUET vector system, was co-transferred with all three plasmids previously generated by Welsch and colleagues (Welsch et al., 2010) (pRSF-CrtB/Y, pCDF-CrtI-IPI and pET-CrtE vectors). HPLC analysis of the bacterial pellets of MG1655*λDE3-Crt's and MG1655*pACB/ipi/dxs at different timepoints of the growth curve under aerobic and anaerobic conditions, suggest that indeed MG1655*λDE3-Crt's is capable of producing significantly higher amount of βC compared with MG1655*pACB/ipi/dxs regardless of presence (**Fig. 5.6A)** or absence of oxygen (**Fig. 5.6C**). Moreover, MG1655*λDE3-Crt's shows the highest pick of βC production after 20 hours, while MG1655*pACB/ipi/dxs peacked after 44 hours post inoculation (**Fig. 5.6A**). Under anaerobic growth conditions, the highest amount of βC was detected as early as 2hrs after inoculation in both MG1655*λDE3-Crt's and MG1655*pACB/ipi/dxs bacterial strains (**Fig. 5.6C**).

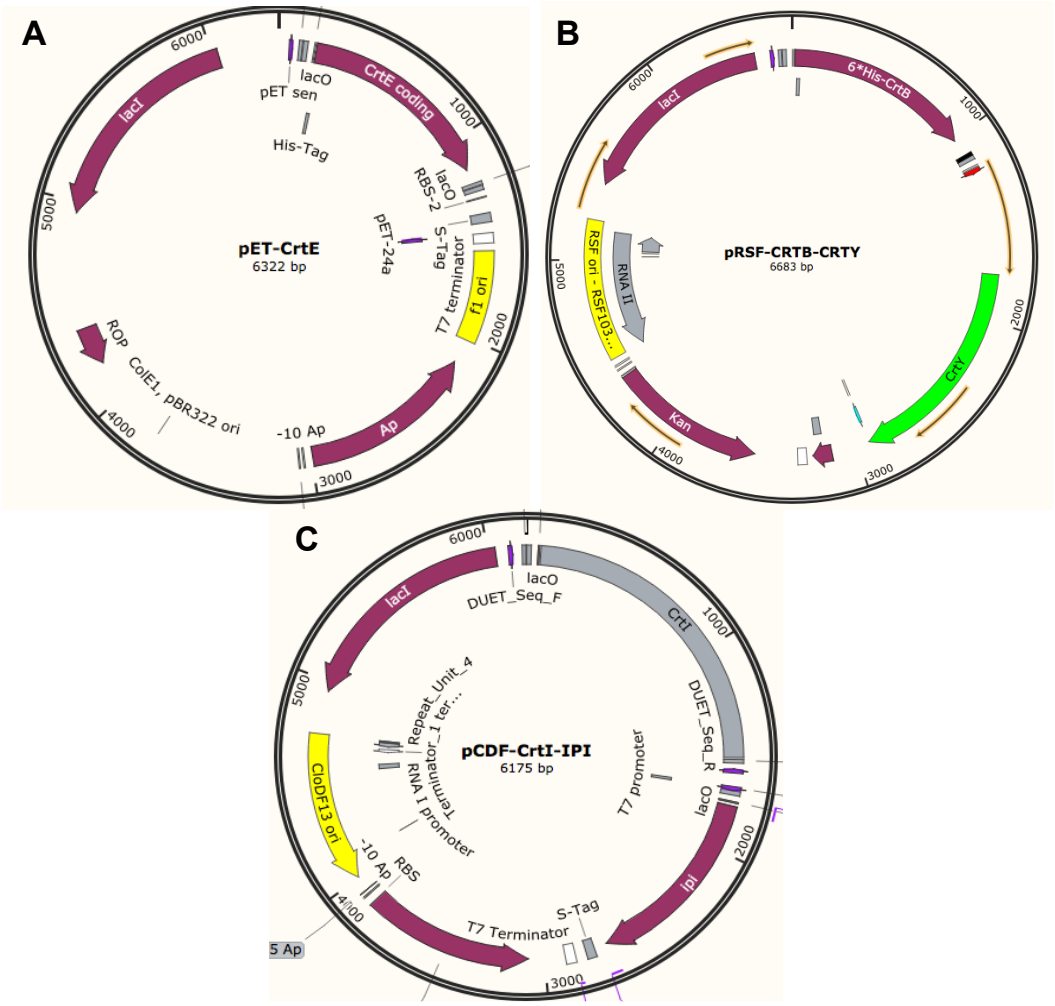

Figure 5.5: DUET vector plasmid carrying βC biosynthesis pathway

(**A**) pRSF-CrtE (**B**) pRSF-CrtB-Y (**C**) pCDF-CrtI-IPI.

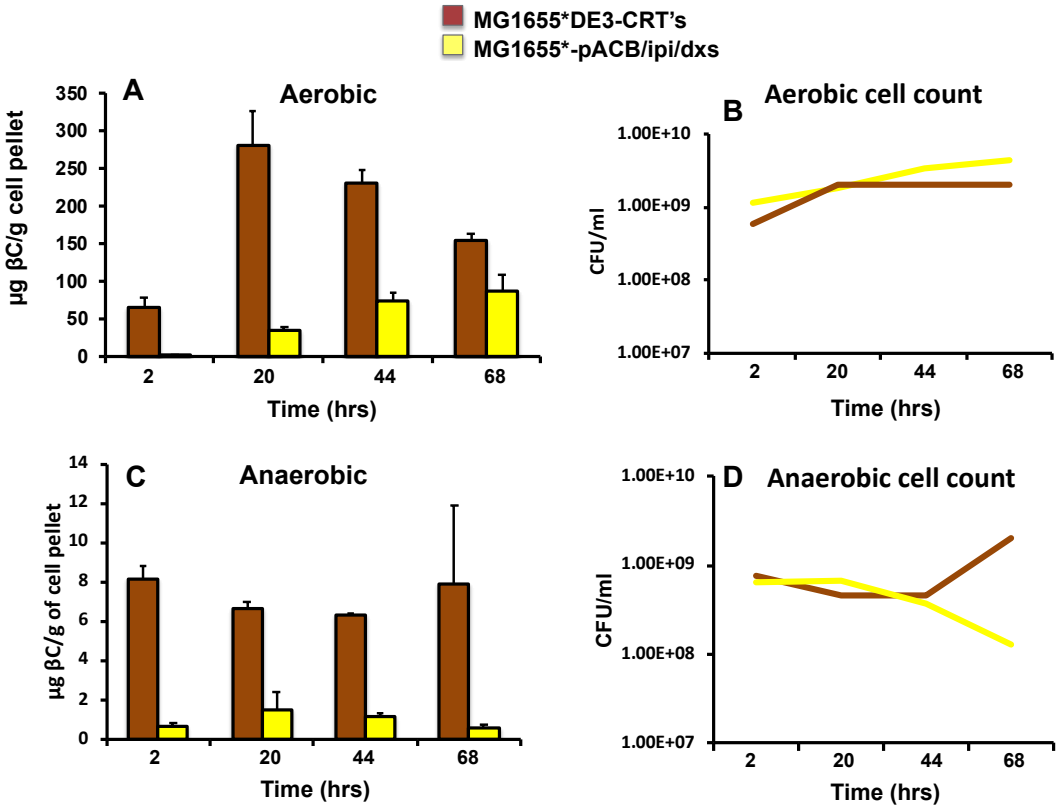

Figure 5.6: Anaerobic βC production by MG1655* βC producing bacteria

(**A**) Growth curve of the MG1655*-pACβ and MG1655*-pACβ/ipi/dxs bacteria over time.

(**B**) HPLC analysis of βC producing bacteria pellets; n=1 sample for each strain.

5.3.5 In vivo βC production by MG1655*λDE3-Crt's

To assess the efficiency of the MG1655*λDE3-Crt's bacterial strain *in vivo*, we used the *Lrat*$^{-/-}$*Bco1*$^{-/-}$ mice (**Fig. 5.7**). These mice have lack lecithin:retinol acyltransferase (LRAT), an enzyme responsible for the esterification of the retinol to retinyl ester (Blaner et al., 2016; O'Byrne and Blaner, 2013), and β-carotene 15-15' oxygenase (BCO1), symmetric βC cleavage enzyme (von Lintig, 2012). Therefore, these mice cannot store the vitamin A in the form of retinyl esters and cannot cleave βC to generate retinoids. Hence, these mutant mice are susceptible to develop VAD due to the lack of LRAT and should accumulate intact βC in their tissues due to the lack of BCO1.

After oral dosing, the bacteria were detected (~10^9 CFU/ml) in fresh feces collected on day 17, 19 and 20 post-gavage (**Fig. 5.7**). Surprisingly, βC concentrations in intestinal mucosa, liver and serum (**Table 5.2**), were not much different from those measured in earlier studies (**Table 5.1**), indicating that *in vivo* βC synthesis by the MG1655*λDE3-Crt's recombinant bacteria was still attenuated.

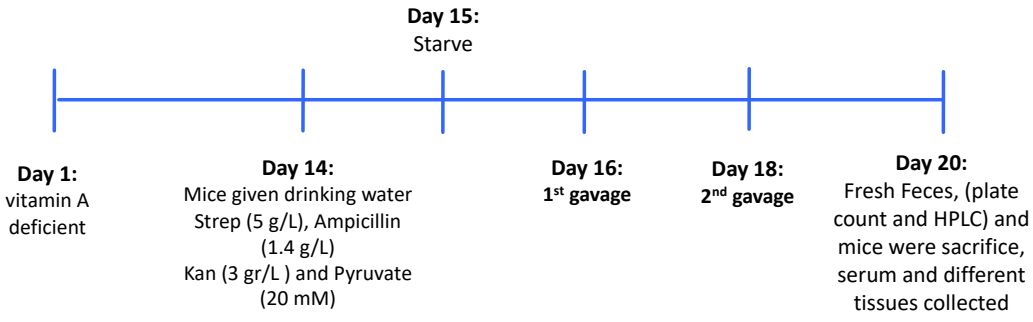

Figure 5.7: Experimental scheme

Lrat$^{-/-}$*Bco1*$^{-/-}$ female mice were maintained on regular chow (18IU VA/g diet) from weaning (3 weeks of age). At 6 weeks of age, i.e., from day 1, the mice (females) were maintained on purified vitamin A deficient diet until the end of the experiments. On day 14, mice were placed on antibiotic-containing until the end of the experiment. On day 15, mice were starved O/N and on day 16 and 18, they were gavaged with either MG1655*λDE3-Crt's bacterial suspension in sucrose or sucrose as a vehicle. On day 17 and 19, fresh feces were collected for the bacterial count. On day 20, after collection of fresh feces, mice were sacrificed to harvest serum, liver and small intestine for HPLC analysis. Value are mean ± SD, n=3 mice for each group. Kan, kanamycin; Strp, streptomycin.

	Serum (µg/dL)	Liver (ng/g)	Colon (ng/g)	Feces (µg/g)
MG1655*DE3-Crt's	0.6 ± 0.08	12.85 ± 8.4	ND	1.2
20% Sucrose (Vehicle)	ND	ND	ND	ND
n=3 female mice (6 weeks old)/ bacteria strain				

Table 5.2: HPLC analysis of β-carotene content in *Lrat*$^{-/-}$/*Bco1*$^{-/-}$ mouse tissues

5.4 DISCUSSION

β-carotene (βC), obtained exclusively from fruits and vegetables, is the most abundant dietary vitamin A precursor generated upon its enzymatic cleavage (von Lintig, 2012). The overall goal of this study was to improve *in vivo* βC production by recombinant βC-producing bacteria in mouse model of vitamin A deficiency (VAD). It was expected that βC produced by the bacteria residing in the mouse intestine would improve VAD in these mice. This work stemmed from earlier studies in our laboratory (Wassef et al., 2014) that provided the proof-of-principle of this concept but also showed the limitation of the first recombinant strain tested, that yielded a very low concentration of βC (Wassef et al., 2014). Our first attempt to engineer a "super βC-producer" bacteria strain (**Fig. 5.2** and **Table 5.1**) took advantage of two genes required for the synthesis of the precursors of the βC, *ipi* (isopentenyl pyrophosphate isomerase) and *dxs (deoxyxyloluse 5- phosphate).* Previous studies indeed showed that these genes can improve βC biosynthesis in *E. coli* (Cunningham et al., 2000; Yang and Guo, 2014). We confirmed these earlier studies *in vitro* (**Fig. 5.2**), but not *in vivo* (**Table 5.1**). Our data suggest that the low production of βC *in vivo* was not due to a low number of the bacteria in the intestine, as we showed successful colonization of the bacteria exogenously introduced (**Fig. 5.3**). Our data suggested that the intestine may not provide a favorable environment for the production of βC. Perhaps one critical factor is the levels of oxygen that ranges from low to almost complete depletion, with fluctuation from 30-40 pO_2 (mm Hg) in the small intestine to 3-11 pO_2 (mm Hg) in the large intestine, both in human and mice (Espey, 2013). Moreover, there is a gradient of oxygen levels decreasing from the epithelial cells towards the intestinal lumen, where almost anoxic conditions may exist (Albenberg et al., 2014). Therefore, this environment

favors the proliferation of mixed bacteria populations with different capability to tolerate different levels of oxygen (Albenberg et al., 2014; Ravcheev and Thiele, 2014). Importantly, a critical enzyme of the βC biosynthetic pathway, phytoene desaturase (CRTI) catalyzes an oxygen-dependent reaction (Dailey and Dailey, 1998; Schaub et al., 2012). In bacteria and fungi, CRTI is the only enzyme that desaturates and isomerizes 15-cis phytoene to produce all-*trans*-lycopene that, in turn, serves as a substrate for CRTY to produce all-*trans*-βC. By using bacteria engineered with the key genes for the main carotenoid biosynthetic pathway, it has been demonstrated that in the absence of oxygen CRTI is not ”enzymatically dead” but produces *cis*-lycopene (Schaub et al., 2012; Yu et al., 2011). However, this latter is not a suitable substrate for CRTY, and under this condition, this enzyme will preferentially produce other products, such as monocyclic γ-carotene, in addition to βC (Dailey and Dailey, 1998), thus decreasing the overall synthesis of βC (Dailey and Dailey, 1998). Similar to our findings, other studies also showed that βC production of engineered bacteria is decreased significantly when they are grown in anaerobic conditions compared to their aerobic growth (Miller et al., 2013). Therefore, one key factor that could inhibit βC biosynthesis *in vivo* in our model could be the overall limiting oxygen levels in the intestine.

Moreover, the plasmid pACβ that drives the expression of the four key genes of the βC biosynthetic pathway is a low copy number plasmid and the transcription of these genes is controlled by a weak promoter. Thus, we speculated that the low levels of oxygen in the mouse intestine together with a relatively low production of the CRTI enzyme, which is the limiting factor of the βC biosynthetic pathway (Schaub et al., 2012), could result in the production of cis-lycopene, rather than trans-lycopene, therefore attenuating βC production

We hypothesized that this hurdle could be overcame by enhancing the expression of *crtI*, and thus the expression of the phytoene synthase enzyme in the recombinant bacteria strains. Therefore, we used the DUET vector system in which each vector has two strong inducible T7/lacI promoters and 2 multi cloning sites (MCS) to help co-express two proteins in any given cell. Indeed, when we co-transformed the DUET vectors (**Fig. 5.5)** into the MG1655*-λDE3, βC production *in vitro* dramatically improved both aerobically and anaerobically (**Fig. 5.6A** and **5.6C**).

Intestinal βC's absorption, mediated by the scavenger receptor SR-B1, is regulated *via* an intestine specific homeodomain transcription factor, ISX, through a negative feedback regulatory mechanism (Bouis et al., 2011). *Isx* is positively regulated by retinoic acid (Lobo et al., 2010b). When tissue vitamin A levels are adequate or elevated, retinoic acid binds to the retinoic acid receptors RARs/RXRs and increases the expression of *Isx* that in turn represses the expression of *Srb1* (or Scarb1) in the enterocytes (Lobo et al., 2010b). Therefore, the *Lrat*$^{-/-}$*Bco1*$^{-/-}$ mice are supposed to be more efficient at absorbing βC from the intestinal lumen compared to the *Bco1*$^{-/-}$*Bco2*$^{-/-}$ mice, given that they have a tenuous vitamin A status already when fed a vitamin A sufficient diet. Despite we observed an improvement of βC synthesis *in vitro* in the bacteria engineered with the DUET vector system (**Fig. 5.5** and **5.6**), when the *Lrat*$^{-/-}$*Bco1*$^{-/-}$ mice where orally administered with the recombinant MG1655*-λDE3-Crt's, we still detected relatively low concentration of βC in the serum and liver of these mice compared to earlier experiments with the *Bco1*$^{-/-}$*Bco2*$^{-/-}$ mice and a different, less efficient, recombinant bacteria strain (compare data in **Table 5.1** and **5.2**). Clearly, the limited tension of oxygen in the intestine has continued to affect the biosynthetic efficiency of the recombinant bacteria. Also, slightly higher level of βC

was detected in the feces of the *Lrat*$^{-/-}$*Bco1*$^{-/-}$ (compare data in **Table 5.1** and **5.2**). Therefore, despite the MG1655*-λDE3-Crt's bacteria were more efficient at synthesizing βC, it is possible that the enterocyte absorption of the βC may be still unsatisfactory, at least under these experimental conditions. Further investigations are clearly needed in this direction.

Overall, both *in vitro* and *in vivo* results suggest that limited oxygen availability in the intestinal environment is one the key factors that may contribute to the inefficient βC production by the recombinant bacteria. How could we overcome this limitation? Unlike bacteria that carry out the conversion of phytoene to lycopene through the action of a single enzyme, CRTI, in plants lycopene synthesis is achieved in multiple steps of desaturation and isomerization carried out by different enzymes (Schaub et al., 2012; Yu et al., 2011). In particular, the last step of isomerization of *cis*-lycopene into all-*trans*-lycopene is performed by the enzyme CRTISO. CRTISO and CRTI have a certain degree of sequences homology, and both bind the FAD cofactor (Isaacson et al., 2004; Schaub et al., 2012). As mentioned before, under low level of oxygen, CRTI produces *cis*-isomers of lycopene, such a *tri-cis*- and *di-cis*-lycopene, which cannot be further isomerized to all-*trans*-lycopne. CRTISO has a wider range of substrate specificity, being able to isomerase tetra-, tri- and di-*cis*-lycopene to all-*trans*-lycopene (Schaub et al., 2012; Yu et al., 2011). Therefore, addition of the CRTISO into the DUET vector systems, together with the afore-mentioned βC biosynthetic genes, could be considered as an alternative solution to increase the levels of all-*trans*-lycopene. Moreover, the addition of other electron acceptors, such as the quinone, to the growth media, could help retain the desaturation activity of CRTI almost to the same level as under aerobic conditions (Mayer et al., 1990). Alternative approaches

to improve overall βC production in the intestine will entail gavaging the mice with a cocktail of βC-synthesizing bacteria, instead of individual strains, and/or multiple doses bacteria and/or extending the duration of the experiment to > 2 weeks. These strategies constitute future approaches that need to be undertaken to improve intestinal colonization and βC biosynthesis by the engineered bacteria.

CHAPTER 6 BIBLIOGRAPHY

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