

# Potentials and Limitations of Bile Acids in Type 2 Diabetes Mellitus: Applications of Microencapsulation as a Novel Oral Delivery System

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**Abstract:** Type 2 diabetes (T2D) is a chronic metabolic disorder resulting from genetic and environmental factors that bring about tissue desensitization to insulin and consequent hyperglycemia. Despite strict glycemic control and the fact that new and more effective antidiabetic drugs are continuously appearing on the market, diabetic patients still suffer from the disease and its complications. Recent findings present a strong link between diabetes, inflammation, altered gut microbiota and bile acid (BA) disturbances. BAs are naturally produced in humans and are gaining an appreciable interest as an adjunct treatment for T2D due to their endocrine signalling and anti-inflammatory properties. However a significant limitation to their efficacy is their low oral absorption, poor targeted delivery, gut metabolism and inter- and intra-individual dose variations. Thus there is a need for a novel and robust formulation that will encapsulate the BAs and protect them until they reach the lower intestine allowing them to be clinically beneficial. Artificial Cell Microencapsulation (ACM) is a novel oral delivery system for biologically active molecules and has been used significantly in the delivery of various cells and therapeutics. ACM-BA formulation has the potential to optimise BA efficacy and safety profiles and may have a place in the treatment of diabetes. This review aims to investigate the applications of BAs in T2D and the use of ACM as a novel delivery system for their optimum delivery.

**Keywords:** Microencapsulation, type 2 diabetes, bile acids, inflammation.

## INTRODUCTION

### Diabetes Mellitus

Diabetes Mellitus is a polygenic disorder indicative of multiple aetiologies of which type 1 and type 2 are the most common subgroups [1, 2]. Type 1 diabetes (T1D) is an autoimmune disease that develops upon the destruction of the pancreatic  $\beta$ -cells, thus resulting in a total lack of insulin production and uncontrolled glucose homeostasis [3]. Conversely, T2D is caused by both genetic and environmental factors and is characterized by a poor response to insulin such that insulin is no longer able to stimulate the uptake of glucose into peripheral tissues such as fat and muscle, hence leading to insulin resistance [3]. Both diabetic forms have prominent disturbances of the gut with altered BA pools, which can significantly impact on the drug therapies available, potentially altering their absorption [4]. This review will focus on such alterations specific to T2D.

### Type 2 Diabetes

T2D is becoming an increasing epidemiological problem of the 21<sup>st</sup> century with approximately 346 million people suffering with this disease worldwide [1, 5]. Such a metabolic disorder will continue to increase in prevalence due to environmental factors including

Western diet, obesity and sedentary lifestyles [1, 5]. Alongside environmental factors, genetic factors can also result in dysregulation of glucose metabolism mediated *via* glucose transporter type 4 (GLUT4) [6, 7]. GLUT4, a protein transporter located in adipose and muscle tissues, is defective in the transport of insulin in diabetic patients, resulting in a constant hyperglycaemic state [6, 7]. This  $\beta$ -cell failure and insulin resistance has been associated with BA imbalances and chronic inflammation [8, 9].

Current therapies aimed at treating T2D focus on ameliorating hyperglycaemia *via* enhanced insulin production (e.g. sulphonylureas) or improving insulin sensitivity (e.g. metformin) [2, 10, 11]. However there are limitations to such therapies as they fail to address the dysbiosis, BA dysregulation and inflammation which all perpetuate the hyperglycaemic state and pancreatic cell damage [2, 12]. As a direct result of these inflammatory processes and physiological disturbances, T2D complications such as cardiovascular disease, metabolic disturbances and tissue necrosis still remain largely inevitable unless the underlying causes of inflammation, dysbiosis and BA disturbances are properly addressed [13, 14].

With continuous overstimulation of the  $\beta$ -cells in the pancreas from anti-diabetic drug therapy and chronic hyperglycemia, tissue exhaustion and subsequent destruction of the  $\beta$ -cells occurs which may lead to complete lack of insulin production, mimicking T1D

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(many T2D patients eventually revert to insulin therapy) [4].

## METHODOLOGY

The aim of this review was to examine the literature on the effects BAs instigate on pancreatic  $\beta$ -cells using a novel technique of oral delivery.

Initially, several databases and various search engines were analyzed using the primary outcome measures (key words used in the literature search strategy) deemed most relevant to the topic and included type 2 diabetes, glucose homeostasis, insulin resistance, inflammation, obesity, diet and gut disturbances. The search strategy was limited to the English language and filtered for articles published post year 2000.

In total 122 potentially relevant articles were found. Narrowing the search down *via* secondary outcome measures which included BAs, enterohepatic recirculation, homeostasis and microencapsulation resulted in a total of 78 publications being incorporated for the purpose of this review.

Figure 1 below details the process in which the primary and secondary outcomes were obtained.

Search engines utilized for the purpose of this review were as follows: PubMed, ProQuest, IPA, Google scholars, Science Direct, Embase and SciFinder.

## T2D: AN INFLAMMATORY DISEASE

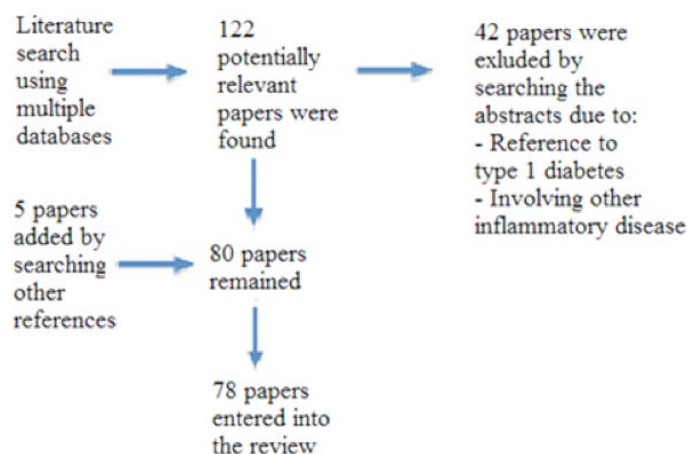
### GIT Disturbances

The gastrointestinal tract (GIT) microflora greatly contributes to energy balance and limits the access of

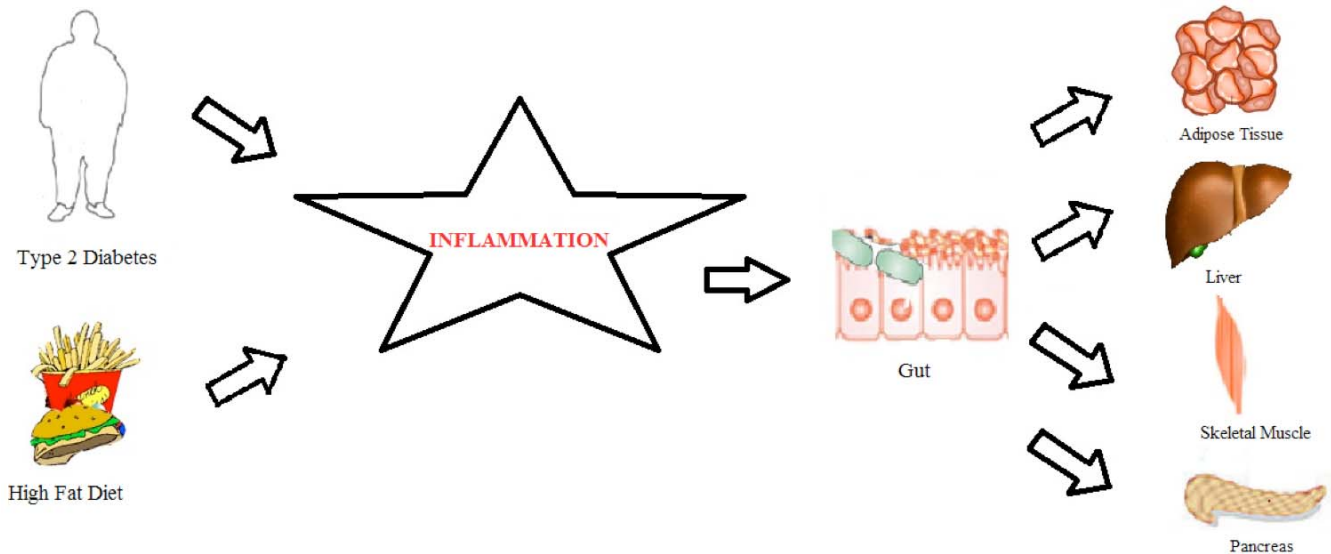
pathogenic bacteria [15, 16]. The concentration of bacteria varies greatly along the GIT with low concentrations in the stomach and duodenum ( $10\text{-}10^3$  colony forming units (cfu/ml) and increasing levels in the colon ( $10^{11-12}$ cfu/ml) [17, 18]. The microbial composition in the GIT harbours two main phyla; Lactobacillus and Bifidobacteria, while the colon represents mainly Bacteroidetes [17, 19]. In T2D, there is an alteration in the GIT flora with experimental studies of obese rats and humans revealing a 50% reduction of Bacteroidetes in the colon and an increase in Firmicutes proportionally [17, 20]. Interestingly, dietary modifications consisting of reduced caloric intakes of saturated fats and carbohydrates result in increasing levels of beneficial bacteria along the GIT (i.e. the less the intake, the greater the levels of beneficial microbiota) [17].

Dysbiosis (a term used to describe alterations and imbalances in the gastrointestinal microbial environment) arising from high fat diets in numerous animal studies has been shown to result in increased gut permeability with reduced protein expressions of ZO-1 and occludens [21]. The resulting reduction leads to a loss of beneficial bacteria to harmful strains and an activation of the innate immune system (*via* lipopolysaccharide (LPS) induced endotoxaemia) [21]. The resulting inflammatory burden perpetuates  $\beta$ -cell loss, insulin resistance and various metabolic sequelae (i.e. increased obesity and hepatic lipid accumulation) [21]. It is worth noting that the translocation of bacteria from diabetic to germ free mice under laboratory conditions results in profound hyperglycaemia mimicking the diabetic state [21-23].

The relationship interconnecting obesity, diet and inflammation to pathophysiological disturbances in



**Figure 1:** Literature search process.



**Figure 2:** Inflammation as the hallmark of pathological disturbances affecting multiple organs.

diabetes concerning the various organs discussed herein is depicted in Figure 2.

Recently, it has become evident that inflammation plays a pivotal role in the development and progression of T2D [24]. As illustrated in Figure 2, inflammation results from high fat diet and obesity which ultimately leads to an alteration of the gut epithelium and also results in widespread systemic inflammation affecting various organs including but not limited to the pancreas, muscle, liver and adipose tissue. This inflammatory cascade has been closely associated with tissue insensitivity to drugs, increasing levels of circulating reactive radicals and poor enterohepatic recirculation which cumulatively manifest as inadequate response by tissues to administered therapeutics, creating a cycle of add-on therapies and polypharmacy [15]. Thus it is necessary to investigate the underlying causes to the development of this inflammation which is the hallmark in the development and progression of T2D and its multiorgan complications [25]. Failure to properly appreciate, understand and target the inflammation of diabetes will result in worsening disease outcome and ineffective, suboptimal polypharmacy. Factors attributed to the inflammatory burden associated with T2D are many and varied but predominately include obesity, high fat diet and gut disturbances [26].

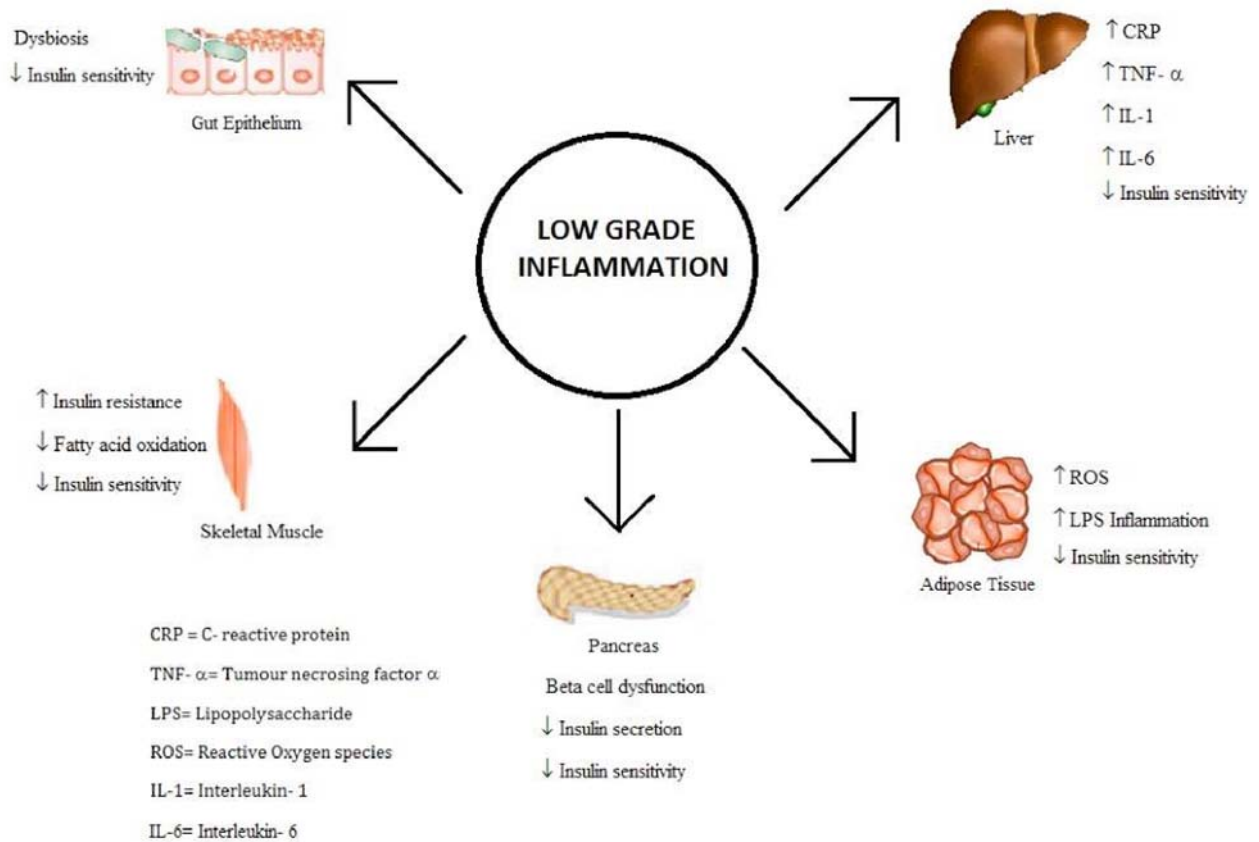
### Obesity

A large body of evidence is suggesting that there is a strong correlation between T2D and obesity, characterised by a low-grade inflammation with an unknown molecular origin [22, 27]. In both diet induced

and transgenic models of T2D, the enlargement of adipose tissue results in high levels of pro-inflammatory cytokines due to enhanced macrophage activity [25]. These mediators include but are not limited to C reactive protein, (CRP) interleukin 1 (IL-1), interleukin 6 (IL-6) and Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ) which further result in  $\beta$ -cell damage, increased insulin resistance and greater lipid accumulation by the liver and adipose tissue [27-29].

This inflammation associated enlargement of adipose tissue in diabetic animals is also capable of altering the natural microbiota of the GIT, which under healthy conditions would play an essential role in harvesting energy from the diet and increasing lipogenesis (through the expression of enzymes such as acetyl coA carboxylase and fatty acid synthase) [20]. A proposed cause for this alteration in the GIT involves the activation of LPS (derived from a Gram negative bacteria) that is present in the GIT and is capable of triggering the secretion of pro-inflammatory cytokines, hence leading to liver insulin resistance, adipose tissue macrophage infiltration and hyperinsulinaemia [20, 22, 27].

The underlying cause to the development of T2D in conjunction with obesity, is high fat concentrations with circulating free fatty acids (due to increased lipid intakes), leading to a state known as endotoxaemia which is defined as the accumulation of toxic metabolic by-products (end products of oxidative stress) capable of stimulating the immune system and worsening the inflammatory burden [20]. With continued exposure to high glucose and non-esterified fatty acids (NEFA) that



**Figure 3:** Low grade inflammation impacting various organs.

are stored in adipose tissue, pancreatic  $\beta$ -cells become desensitized, leading to a reduction in glucose-stimulated insulin secretion and  $\beta$ -cell mass, eventually resulting in apoptosis [30]. This can be explained in terms of NEFA's slowing down metabolism, glucose uptake and oxidation [30]. As a consequence of this oxidation, glucose metabolism becomes impaired with the generation of free radicals, further enhancing inflammation and the progression of T2D [30]. In addition to high fat diets, high carbohydrate based diets have also been shown to negatively impact the glycaemic control in T2D [24]. Conversely, a low carbohydrate diet can significantly improve glycaemic control and reduce body weight, the latter being an important risk factor for the development and progression of T2D and its complications [24].

The relationship between high fat feeding in animal studies and an alteration in the gut microbiota has also demonstrated the imbalance between gram negative to gram positive bacteria [21]. This phenomenon, termed dysbiosis, leads to elevations of LPS levels creating a state of endotoxaemia characterized by enhanced macrophage activity and the release of pro-inflammatory cytokines [17]. Owing to poor antioxidant defense mechanisms of the pancreatic  $\beta$ -cells, this

inflammatory burden leads to gradual  $\beta$ -cell failure and worsening hyperglycaemia with subsequent insulin resistance and worsening obesity [17].

### Oxidative Stress

In T2D, pancreatic  $\beta$ -cell dysfunction occurs due to oxidative stress, which has the potential to initiate tissue damage [31]. This oxidative stress originates from the inflammatory burden of dysbiosis, lipid peroxidation and BA disturbances [21]. Probuocol, an anti-hyperlipidaemic agent, has been shown to preserve  $\beta$ -cell function and mass *via* potent antioxidant effects, resulting in an indirect improvement in the primary end points of glucose and insulin levels [9].

Probuocol's profound effects in diabetes are further confirmation that oxidation from radical oxygen species (ROS) play a crucial role in diabetes induction and development. It is well known that the pancreas has very little antioxidant defense mechanisms, thus portraying its vulnerability to oxidative damage [32]. Mechanistically, probuocol's high lipophilicity and strong radical scavenging effects (double aromatic rings in the chemical structure) allow cellular penetration and

reductions in ROS of pancreatic islets [31, 32]. Probucol has also been shown to decrease thioredoxin interacting protein (TXNIP) expression, compensated by increases in thioredoxin (TRX) expression [31]. This allows the reduction of oxidative stress *via* enhancements in the  $\beta$ -cell's cellular redox status [31].

### Bile Acids

BA synthesis from cholesterol is the primary pathway involved in the catabolism of cholesterol [13]. The synthesis of BA is a complex, highly regulated process constituting multistep and multi-organelle processes [11, 13, 33].

BAs are amphipathic molecules that are produced in the liver and secreted into bile [34]. In the liver, 75% of the BA synthesis occurs *via* the oxidation of cholesterol through a major enzyme known as CYP7A1 [35, 36]. The end product of this pathway results in the formation of two principal BAs in humans; namely cholic acid (CA) and chenodeoxycholic acid (CDA) [37, 38]. In the intestinal lumen, particularly in the colon, primary BAs are converted into secondary BAs known as deoxycholic acid (DCA) and lithocholic acid (LCA) *via* GIT microflora biochemical modifications namely deconjugation, oxidation and dehydroxylation [11, 33, 35]. Before the excretion of BA from the hepatocytes, they are conjugated to either taurine (mice) or glycine (humans) which ultimately converts them into more hydrophilic, zwitterionic compounds at physiological pH *via* the reduction of their  $pK_a$  [39]. These fully ionized species are now termed bile salts, which are highly effective detergents involved in lipid absorption due to micelle formations in the small intestine owing to their enhanced, beneficial amphoteric nature [4, 11, 33].

The BA pool of a human and mice differ in composition, with hydrophilic BAs muricholic acid (MCA) and CA predominating in mice and the more hydrophobic BAs CDA, CA and DCA predominating in humans [36, 40]. Importantly rats do not contain a gall bladder so cannot store BAs as such, rather bile is instantaneously made *via* a post-prandial mechanism after ingestion of a meal [4]. This has important ramifications in the extrapolation of animal studies concerning BAs to the human population.

In addition to dietary lipid absorption and cholesterol metabolism, BAs play a significant physiological role which includes functioning as potent signaling molecules and interestingly several BA signaling pathways have recently been identified [13, 41]. Thus,

BAs encompass crucial multi-organ functions and are vital for the body's overall homeostasis. They have pivotal roles in the modulation of the immune system (*via* their anti-inflammatory effects resulting in the down regulation of the synthesis of pro-inflammatory cytokines such as TNF- $\alpha$  from monocytes and macrophages through nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) inhibition), energy balance (BA receptors regulate glucose and lipid metabolism and energy expenditure) as well as key roles in dietary absorption of lipids previously alluded to [2, 13, 39, 41].

### Enterohepatic Circulation

After the ingestion of a meal, bile flows into the duodenum aiding the solubilisation of dietary lipids and their digestion [11, 33]. However, the BA pool is recycled *via* a complex system interconnecting the liver and intestine [2, 13, 39, 41]. BAs are reabsorbed *via* a combination of passive diffusion and active transport from the terminal ileum and transported back to the liver *via* the portal vein [42-44]. This process is termed enterohepatic circulation and constitutes an essential role in maintaining a constant BA pool as BAs are toxic to tissues at high concentrations [2, 13, 39, 41]. The enterohepatic circulation process occurs approximately twelve times a day with high efficiency of 95% [42-44].

BA homeostasis is tightly regulated by the intestinal microbiota which play a crucial role in the biochemical modifications of the BAs (deconjugation, epimerization and oxidation) [45]. Such modifications are pivotal for BA reabsorption and their hydrophobic properties [45]. Of all the biochemical modifications by the gut microbiota, deconjugation is the primary catabolic step required for BA enterohepatic circulation [43]. Many bacteria located in the GIT are capable of deconjugating BA especially *Lactobacillus* and *Bifidobacteria* [13, 45-47].

In T2D, the dysbiosis in the gut results in the reduction of levels of beneficial bacteria (*Lactobacillus* and *Bifidobacteria*) which are replaced by pro-inflammatory strains such as *Bacteroides* [48]. This reduction in *Lactobacillus* contributes to an overall net pool of toxic conjugated BAs and their accumulation as described in T2D has been associated with further inflammatory damage to the pancreas and other organs [46]. Hence, the accumulation of these secondary BAs perpetuates the already widespread inflammation noted in T2D, compromising glycaemic and metabolic dysregulation even further [46].



Additionally, T2D has been associated with a disruption in the enterohepatic circulation of BAs and a higher rate of total BA synthesis [49]. This results in enhanced CA synthesis and subsequent conversion to DCA [49]. Thus, reductions of beneficial Bifidobacteria species as is noted in T2D lead to an increase in intestinal endotoxin levels that participate in low-grade inflammation and decrease the mucosal barrier function [20].

BAs, as previously described, are potent signaling molecules with multiorgan functions throughout the body [11, 33]. There are two prominent receptors responsible for BA signaling pathways, farnesoid X receptor (FXR) and the G-protein coupled membrane receptor TGR5 [11, 33]. Both these pathways are discussed herein, but it should be stressed that receptor independent pathways of cell signaling networks (such as the mitogen-activated protein kinase pathway) can also be activated by BAs, the scope of which is beyond this review [13].

The nuclear receptor FXR is primarily expressed in the liver and intestine; principle sites of BA biosynthesis, metabolism and enterohepatic cycling [50]. However, it has also been noted in the pancreas and adipose tissue and plays a central role in the body's energy balance [13, 35].

The activation of intestinal FXR by absorbed BA in the terminal ileum results in the expression of enterocytic fibroblast growth factor 19 (FGF-19, and FGF-15 in mice) [13]. BAs returning from the enterohepatic recirculation in the liver also stimulate FGF-19, which binds to its receptor complex FGF receptor 4 (FGFR4) on nearby hepatocytes [13]. This signaling interaction between FGF-19 and FGFR4 results in the inhibition of CYP7A1 *via* the c-Jun N-terminal kinase (JNK)-mediated pathway, avoiding the accumulation of excessive BA that are hepatotoxic [51]. In addition to the induction of FGF-19, basolateral transporters such as organic solute transporter alpha and beta (OSTa, OSTb) are essential in the transport of BA into the portal vein [52].

An increase in BA pool size results in negative feedback mechanisms governed by nuclear receptors that will suppress the transcription of CYP7A1 (involved in BA synthesis) and CYP8B1 (which controls the production of CA) [53]. As BAs reach a significant level in the liver, FXRa induces the expression of the small heterodimer partner, (SHP) which inhibits the activity of nuclear factor 4a (NR2A1/ HNFa) and liver receptor

homolog-1 (NRGA2/LRH-1) in the hepatocyte [36, 53]. The repression of these transcription factors and CYP7A1 results in the suppression of BA synthesis and therefore accumulation of BAs [36]. Both LRH-1 and HNF-4a are regulated by CYP8B1 which is an important modulator of BA pool size and composition [36, 54]. BAs have also been shown to activate FXR-independent signaling pathways to repress CYP7A1 gene expression [11]. Specifically, it has been shown that feeding FXR knockout mice a cholic acid diet still results in CYP7A1 repression independent of the FXR or SHP pathways [11]. These findings suggest that super-physiological levels of BAs result in redundant pathways being activated to repress BA synthesis and restore the BA pool size [11].

It is important to appreciate that the expression of several key transporters are altered in T2D. For example, the expression of mammalian transporters multidrug resistance-associated protein (MRP) 2, MRP 3 and MRP 4 located in the liver and renal tubules are altered resulting in the accumulation of toxic BAs [55-57]. Additionally, it has been shown *via* recent animal studies that T2D also alters the expression of ABC transporters and the FXR receptor, resulting in disturbances to the BA pool and manifesting in worsening inflammation, hyperglycemia and obesity [4, 11, 13, 33, 41]. Data from human studies showed that T2D patients have an increase in BA pool size and fecal BA excretion that can be decreased *via* insulin therapy [13]. Subsequently, animal studies revealed that insulin inhibits CYP7A1 and CYP27A1 expression in rat hepatocytes, enzymes involved in BA synthesis [13]. The dysbiosis noted in the gut of diabetic patients also has adverse metabolic sequelae; with pro-inflammatory bacterial strains converting cholic acid to deoxycholic acid, a potent toxic BA contributing to local inflammation and tissue necrosis [13].

## Energy Expenditure

It is now well documented that BAs regulate hepatic glucose metabolism *via* the FXR-mediated pathways [35]. These metabolic regulatory pathways are involved in hepatic glucose production, hepatic lipoprotein and cholesterol mechanism, lipogenesis, insulin sensitivity and adipocyte functions and muscle energy expenditure [11].

Additionally, it has recently been discovered that the BA receptor, TGR5 (a G-protein coupled receptor) also plays a key role in the body's energy balance and it is expressed in the GIT, skeletal muscle, adipose tissue

and immune cells as illustrated in Figure 4 [36]. TGR5 mediated signaling pathways are particularly involved in muscle energy expenditure and intestinal GLP-1 secretion [36].

Upon BA activation, TGR5 leads to an induced intracellular cAMP production, converting thyroxine (T4) into the active T3, thus causing weight loss *via* enhanced energy expenditure [11].

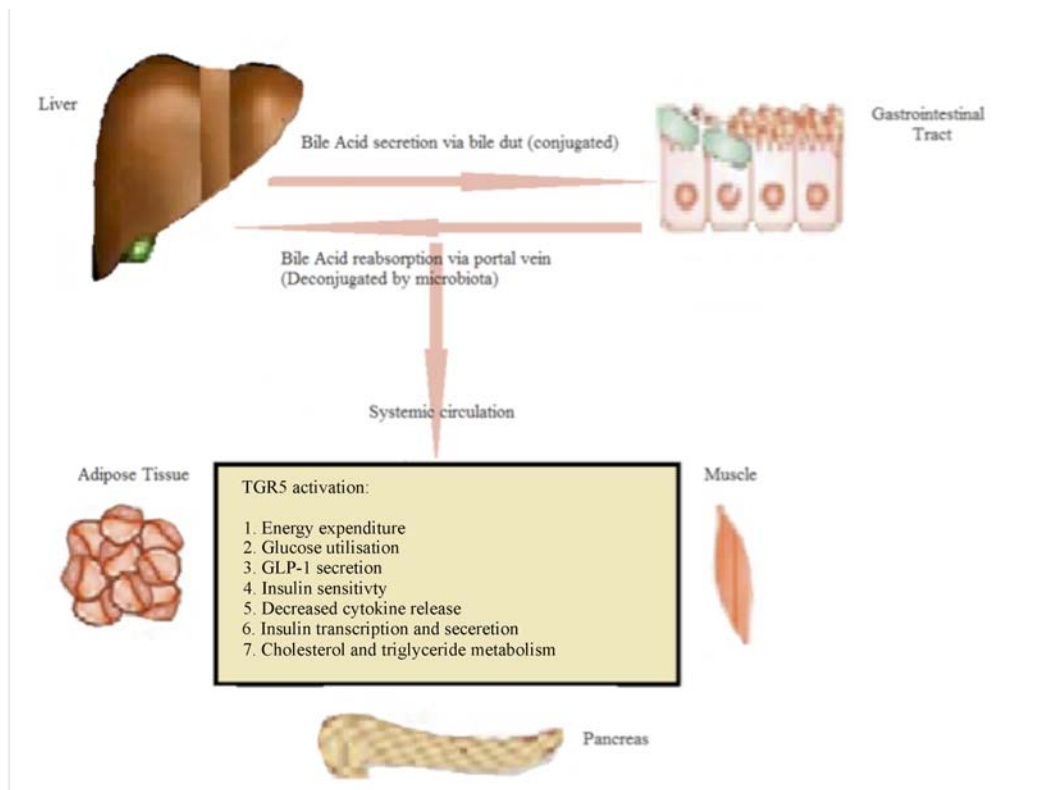
In addition, TGR5 is also involved in immunoregulatory properties of BA [35]. TGR5 is very relevant to BA metabolism as chronic inflammation is now known to contribute to the metabolic dysfunction evident in T2D [36]. BAs capable of TGR5 activation were found to increase cAMP in alveolar macrophages which inhibited LPS induced pro-inflammatory cytokines TNF $\alpha$ , IL-1, 6 and 8 [36]. TGR5's anti-inflammatory effects are therefore useful for protecting the liver against lipid peroxidation and injury induced by BAs [36]. The identification of these pathways provides the definitive proof of BAs role in immunosuppression, glucose homeostasis and energy balance [36].

Furthermore, FXR expression itself is reduced in rat models of T2D. In its absence, mice showed an increased level of inflammation, (induced by LPS) insulin resistance and hyperglycemia [35, 42].

Furthermore they developed pro-inflammatory and pro-fibrotic phenotypes in the intestine, leading to bacterial overgrowth and exacerbated colitis [50]. Therefore the prospective for BA as a potential source for the treatment of diabetes stems from the idea that BAs inhibit the macrophage release of LPS-induced pro-inflammatory cytokines and therefore play an immunosuppressive effect [42]. Given that inflammation appears to be an important "cause and effect" mediator in T2D, the potential anti-inflammatory and immunosuppressive effects of BAs as biotherapeutic adjuncts in the therapy of diabetes hold great promise. The concern is how to best deliver such potent yet labile agents; fortunately artificial cell microencapsulation offers such an avenue, as discussed below.

### ARTIFICIAL CELL MICROENCAPSULATION

Biodegradable polymers have been the major focus to developing efficacious delivery systems for pharmaceutical research [58, 59]. Therefore microencapsulation is a novel technique that can be useful in terms of protecting a labile drug or as a means to control drug release [60]. This method aims to envelope solids or liquids into a capsule as a means to isolate the inside from the host immune system and



**Figure 4:** The physiological roles of bile acids.

environment [61, 62]. However at the same time, once it reaches the site of action, allows the exchange of nutrients and waste; thus releasing the encapsulated ingredients in a sustained, constant manner (zero-order release of contents at the site of action) [63].

Furthermore, the semipermeable membrane prevents antibodies, immunologic moieties and high molecular weight molecules from destroying the encapsulated material as foreign invaders [64]. Ideally the system should achieve an effective concentration of the material entrapped at the site of action for an extended period of time, whilst minimizing systemic exposure [63]. As a consequence, artificial cell microencapsulation is gaining favour in providing a constant drug delivery system (with zero order kinetics of drug release) at an affordable cost [64]. This zero order drug release is made possible using several polymers such as alginate and provides an advantage over conventional oral delivery which would otherwise have erratic rise and fall in plasma concentrations, rendering many therapies suboptimal or even ineffective. For the drug encapsulated inside the capsule to be released at the targeted site of action, it must undergo several steps [65]. Firstly, the outer layer absorbs water resulting in swelling of the microcapsule and gradual loss of bead surface integrity, making the outer surface more porous. This is followed by erosion which involves the breakdown of the porous outer layer (due to dissolution of the alginate matrix at alkaline pH) resulting in zero order kinetics whereby diffusion of the encapsulated material occurs in a time independent manner [58]. Thus it has been shown and is well documented in the literature that at adequate alginate concentrations and optimal viscosity, microcapsules containing alginate can at the desired site of action release encapsulated drugs in a zero order manner due to an equilibrium in swelling and erosion at alkaline pH, resulting in a constant diffusion taking place whereby the drug is liberated and achieves steady concentrations at the site (with no fluctuations) independent of time or the drug's half-life [58, 65, 66]. This technique could be a potential avenue for the delivery of BAs such as deoxycholic acid, into the colon, by promoting a sustained release system so that the BA can act as an anti-inflammatory agent and aid in the treatment of T2D [61]. A recent interest in providing a sustained release formulation was conducted on the sulphonylurea gliclazide [67]. This oral hypoglycemic agent has an unpredictable and highly varied absorption from the gastrointestinal tract partly due to the poor dissolution of drug from the formulation and

poor permeability across the GI membrane [59]. A more effective drug delivery system was attained following microencapsulation of gliclazide, which displayed enhanced in-vitro and in-vivo characteristics compared to conventional delivery [67].

Throughout the literature there are several commonly used polymers as coating materials required for encapsulation including sodium alginate (SA), pectin, chitosan and methylcellulose with majority of studies employing sodium alginate [64, 68, 69]. This polymer has gained high popularity due to its favourable properties such as high stability, non-toxicity, solubility and excellent biocompatibility and biodegradability [12, 64, 70].

SA is the salt of alginic acid, which is a natural polysaccharide derived from seaweed that consists of two sugars known as D-mannuronate and L-guluronate [71, 72]. SA has well established pH-release kinetics and is significantly influenced by the viscosity of its matrix [73]. Recent studies have demonstrated a faster and well controlled drug release from the low viscosity SA (LVSA) compared with high viscosity (HVSA) suggesting more superior performance when targeting distal sites of the GIT such as the cecum [74]. In gastric fluid, the hydrated LVSA is converted into a porous, insoluble structure, called an alginic acid matrix [58].

The combination of hydrophilic alginate with calcium chloride is commonly employed to form a calcium-alginate system (calcium alginate beads sized 300-800um diameters) due to its easiness in gel formation [75, 76]. The production technique used to prepare the polyelectrolyte complex gel includes the vibration nozzle method (VNM) [60, 77].

This technique deploys vibration, which causes a break up of laminar liquid resulting in equally sized droplets, at a selected frequency (higher frequency produces smaller beads). The formation of these spherical droplets occurs due to surface tension [71]. Parameters involved in the droplet formation via the VNM technique includes vibration frequency, electrode tension, nozzle size and jet flow rate [60]. This novel and promising technique is effectively utilized to produce homogeneous and stable beads using the Buchi-B390 Encapsulator (Buchi Instruments, Switzerland) equipped with various nozzle sizes [60]. However, the VNM methodology is not without its limitations. This method does not entail high production yields as only a single droplet forms after another at any given time. Adjusting the production flow rate with



increasing diameters or increasing vibration frequency results in greater production volumes and represents a way of balancing production yields with beads size control [60, 71].

## CONCLUSION

Throughout this literature review, it is evident that the underlying cause of developing insulin resistance and therefore T2D is inflammation (beginning with low-grade inflammation that precedes widespread systemic inflammation and oxidative stress evident in overt hyperglycaemia and chronic T2D). There are several factors that contribute to this low-grade inflammation including obesity, high fat diets and a change in gut microflora. This inflammatory cascade results in major changes in the gut, which in turn initiates an alteration in the bile flow and feedback mechanisms required for enterohepatic circulation. Therefore future diabetes therapy should not only focus on rectifying glucose imbalance but also in targeting the disturbances in BA composition and the inflammation cascade initiated in the gut. This can be achieved by using small concentrations of BA (known to inhibit the growth of bacteria) to reach the colon as the site of action to normalize the composition of BAs and microflora, gut immune- response and microflora-epithelial interactions towards maintaining normal biochemical reactions and healthy body physiology [78]. A novel delivery system with promising results such as artificial cell microencapsulation could allow for targeted delivery of labile therapeutic agents such as BAs with sustained release kinetics, ensuring effective delivery to the gut to alleviate inflammation and provide a better means of improving glycaemic control in T2D as an adjunct to mainstream drug therapy.

## REFERENCES

- [1] Patel D, Kumar R, Laloo D, Hemalatha S. Diabetes mellitus: An overview on its pharmacological aspects and reported medicinal plants having antidiabetic activity. *Asian Pac J Trop Biomed* 2012; 2: 411-20. [http://dx.doi.org/10.1016/S2221-1691\(12\)60067-7](http://dx.doi.org/10.1016/S2221-1691(12)60067-7)
- [2] Zarrinpar A, Loomba R. Review article: the emerging interplay among the gastrointestinal tract, bile acids and incretins in the pathogenesis of diabetes and non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2012; 36: 909-21. <http://dx.doi.org/10.1111/apt.12084>
- [3] Barnett R. Historical keyword: diabetes. *Lancet* 2010; 375: 191. [http://dx.doi.org/10.1016/S0140-6736\(10\)60079-7](http://dx.doi.org/10.1016/S0140-6736(10)60079-7)
- [4] Mikov M A-SH, Golocorbin-Kon G. Potentials and Limitations of Bile Acids and Probiotics in Diabetes Mellitus. 2012. p. 365- 402.
- [5] Nolan CJ, Damm P, Prentki M. Type 2 diabetes across generations: from pathophysiology to prevention and management. *Lancet*; 378: 169-81. [http://dx.doi.org/10.1016/S0140-6736\(11\)60614-4](http://dx.doi.org/10.1016/S0140-6736(11)60614-4)
- [6] Kouidhi S, Berrhouma R, Rouissi K, *et al.* Human subcutaneous adipose tissue Glut 4 mRNA expression in obesity and type 2 diabetes. *Acta Diabetol* 2013; 50: 227-32. <http://dx.doi.org/10.1007/s00592-011-0295-8>
- [7] James DE, Piper RC. Insulin resistance, diabetes, and the insulin-regulated trafficking of GLUT-4. *J Cell Biol* 1994; 126: 1123-6. <http://dx.doi.org/10.1083/jcb.126.5.1123>
- [8] Goldfine AB, Fonseca V, Shoelson SE. Therapeutic approaches to target inflammation in type 2 diabetes. *Clinl Chem* 2011; 57: 162-7. <http://dx.doi.org/10.1373/clinchem.2010.148833>
- [9] Kolb H, Mandrup-Poulsen T. An immune origin of type 2 diabetes? *Diabetologia* 2005; 48: 1038-50. <http://dx.doi.org/10.1007/s00125-005-1764-9>
- [10] Fan MY, Lum ZP, Fu XW, Levesque L, Tai IT, Sun AM. Reversal of diabetes in BB rats by transplantation of encapsulated pancreatic islets. *Diabetes* 1990; 39: 519-22. <http://dx.doi.org/10.2337/diab.39.4.519>
- [11] Li T, Chiang JY. Bile Acid signaling in liver metabolism and diseases. *J Lipids* 2012; 2012: 754067.
- [12] Al-Kassas RS, Al-Gohary OM, Al-Faadhel MM. Controlling of systemic absorption of gliclazide through incorporation into alginate beads. *Int J Pharm* 2007; 341: 230-7. <http://dx.doi.org/10.1016/j.ijpharm.2007.03.047>
- [13] Prawitt J, Caron S, Staels B. Bile acid metabolism and the pathogenesis of type 2 diabetes. *Curr Diab Rep* 2011; 11: 160-6. <http://dx.doi.org/10.1007/s11892-011-0187-x>
- [14] Taylor R. Type 2 diabetes: etiology and reversibility. *Diabetes Care* 2013; 36: 1047-55. <http://dx.doi.org/10.2337/dc12-1805>
- [15] Al-Salami H CR, Golocorbin-Kon S, Mikov M. Probiotics application in autoimmune diseases. *Intech* 2012: 325-66.
- [16] Martinez-Moya P, Romero-Calvo I, Requena P, *et al.* Dose-dependent antiinflammatory effect of ursodeoxycholic acid in experimental colitis. *Int Immunopharmacol* 2013; 15: 372-80. <http://dx.doi.org/10.1016/j.intimp.2012.11.017>
- [17] Festi D, Schiumerini R, Birtolo C, *et al.* Gut microbiota and its pathophysiology in disease paradigms. *Dig Dis* 2011; 29: 518-24. <http://dx.doi.org/10.1159/000332975>
- [18] Varbanova M, Malfertheiner P. Bacterial load and degree of gastric mucosal inflammation in *Helicobacter pylori* infection. *Dig Dis* 2011; 29: 592-9. <http://dx.doi.org/10.1159/000333260>
- [19] Firouzi S, Barakatun-Nisak MY, Ismail A, Majid HA, Azmi KN. Role of probiotics in modulating glucose homeostasis: evidence from animal and human studies. *Int J Food Sci Nutr* 2013; 64: 780-6. <http://dx.doi.org/10.3109/09637486.2013.775227>
- [20] Cani PD, Delzenne NM. The role of the gut microbiota in energy metabolism and metabolic disease. *Curr Pharm Des* 2009; 15: 1546-58. <http://dx.doi.org/10.2174/138161209788168164>
- [21] Cani PD, Bibiloni R, Knauf C, *et al.* Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008; 57: 1470-81. <http://dx.doi.org/10.2337/db07-1403>
- [22] Cani PD, Neyrinck AM, Fava F, *et al.* Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia* 2007; 50: 2374-83. <http://dx.doi.org/10.1007/s00125-007-0791-0>
- [23] Chikai T, Nakao H, Uchida K. Deconjugation of bile acids by human intestinal bacteria implanted in germ-free rats. *Lipids* 1987; 22: 669-71. <http://dx.doi.org/10.1007/BF02533948>

- [24] Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol* 2011; 11: 98-107. <http://dx.doi.org/10.1038/nri2925>
- [25] Backhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc Natl Acad Sci USA* 2007; 104: 979-84. <http://dx.doi.org/10.1073/pnas.0605374104>
- [26] Duboc H, Rajca S, Rainteau D, *et al.* Connecting dysbiosis, bile-acid dysmetabolism and gut inflammation in inflammatory bowel diseases. *Gut* 2013; 62: 531-9. <http://dx.doi.org/10.1136/gutjnl-2012-302578>
- [27] Muccioli GG, Naslain D, Backhed F, *et al.* The endocannabinoid system links gut microbiota to adipogenesis. *Mol Syst Biol* 2010; 6: 392. <http://dx.doi.org/10.1038/msb.2010.46>
- [28] Amar J, Chabo C, Waget A, *et al.* Intestinal mucosal adherence and translocation of commensal bacteria at the early onset of type 2 diabetes: molecular mechanisms and probiotic treatment. *EMBO Mol Med* 2011; 3: 559-72. <http://dx.doi.org/10.1002/emmm.201100159>
- [29] Torpy JM, Lynn C, Glass RM. Diabetes. *JAMA* 2009; 301: 1620. <http://dx.doi.org/10.1001/jama.301.15.1620>
- [30] Keane DC, Takahashi HK, Dhayal S, Morgan NG, Curi R, Newsholme P. Arachidonic acid actions on functional integrity and attenuation of the negative effects of palmitic acid in a clonal pancreatic beta-cell line. *Clin Sci* 2011; 120: 195-206. <http://dx.doi.org/10.1042/CS20100282>
- [31] Liu JH, Liu DF, Wang NN, Lin HL, Mei X. Possible role for the thioredoxin system in the protective effects of probucol in the pancreatic islets of diabetic rats. *Clin Exp Pharmacol Physiol* 2011; 38: 528-33. <http://dx.doi.org/10.1111/j.1440-1681.2011.05545.x>
- [32] Parthasarathy S, Young SG, Witztum JL, Pittman RC, Steinberg D. Probucool inhibits oxidative modification of low density lipoprotein. *J Clin Invest* 1986; 77: 641-4. <http://dx.doi.org/10.1172/JCI112349>
- [33] Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. *Physiol Rev* 2009; 89: 147-91. <http://dx.doi.org/10.1152/physrev.00010.2008>
- [34] Kim GB LB. Biochemical and molecular insights into bile salt hydrolase in the gastrointestinal microflora - a review. Food Research & Development Centre, Agriculture and Agri- Food Canada 2005; 1505-15.
- [35] Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bile-acid signalling for metabolic diseases. *Nat Rev Drug Discov* 2008; 7: 678-93. <http://dx.doi.org/10.1038/nrd2619>
- [36] Pols TW, Noriega LG, Nomura M, Auwerx J, Schoonjans K. The bile acid membrane receptor TGR5: a valuable metabolic target. *Dig Dis* 2011; 29: 37-44. <http://dx.doi.org/10.1159/000324126>
- [37] Einarsson C, Hillebrant CG, Axelson M. Effects of treatment with deoxycholic acid and chenodeoxycholic acid on the hepatic synthesis of cholesterol and bile acids in healthy subjects. *Hepatology* 2001; 33: 1189-93. <http://dx.doi.org/10.1053/jhep.2001.23790>
- [38] Mosbach EH, Kalinsky HJ, Halpern E, Kendall FE. Determination of deoxycholic and cholic acids in bile. *Arch Biochem Biophys* 1954; 51: 402-10. [http://dx.doi.org/10.1016/0003-9861\(54\)90495-6](http://dx.doi.org/10.1016/0003-9861(54)90495-6)
- [39] Thomas C, Auwerx J, Schoonjans K. Bile acids and the membrane bile acid receptor TGR5--connecting nutrition and metabolism. *Thyroid* 2008; 18: 167-74. <http://dx.doi.org/10.1089/thy.2007.0255>
- [40] Bergstrom S, Lindstedt S, Samuelsson B. Bile acids and steroids. LXXXII. On the mechanism of deoxycholic acid formation in the rabbit. *J Biol Chem* 1959; 234: 2022-5.
- [41] Renga B, Mencarelli A, Vavassori P, Brancaleone V, Fiorucci S. The bile acid sensor FXR regulates insulin transcription and secretion. *Biochim Biophys Acta* 2010; 1802: 363-72. <http://dx.doi.org/10.1016/j.bbadis.2010.01.002>
- [42] Wagner M, Zollner G, Trauner M. Nuclear bile acid receptor farnesoid X receptor meets nuclear factor-kappaB: new insights into hepatic inflammation. *Hepatology* 2008; 48: 1383-6. <http://dx.doi.org/10.1002/hep.22668>
- [43] Roberts MS, Magnusson BM, Burczynski FJ, Weiss M. Enterohepatic circulation: physiological, pharmacokinetic and clinical implications. *Clin Pharmacokinet* 2002; 41: 751-90. <http://dx.doi.org/10.2165/00003088-200241100-00005>
- [44] Nervi FO, Severin CH, Valdivieso VD. Bile acid pool changes and regulation of cholate synthesis in experimental diabetes. *Biochim Biophys Acta* 1978; 529: 212-23. [http://dx.doi.org/10.1016/0005-2760\(78\)90064-4](http://dx.doi.org/10.1016/0005-2760(78)90064-4)
- [45] Morimoto K IH, Watanabe M. Developments in undersanding bile acid metabolism. *ProQuest* 2013; 8: 59-69.
- [46] Gilliland SE, Speck ML. Deconjugation of bile acids by intestinal lactobacilli. *Appl Environ Microbiol* 1977; 33: 15-8.
- [47] Ridlon JM, Kang DJ, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res.* 2006; 47(2): 241-59. <http://dx.doi.org/10.1194/jlr.R500013-JLR200>
- [48] Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev Immunol* 2008; 8: 411-20. PubMed PMID: 18469830. <http://dx.doi.org/10.1038/nri2316>
- [49] Bajjal PK, Fitzpatrick DW, Bird RP. Modulation of colonic xenobiotic metabolizing enzymes by feeding bile acids: comparative effects of cholic, deoxycholic, lithocholic and ursodeoxycholic acids. *Food Chem Toxicol* 1998; 36: 601-7. [http://dx.doi.org/10.1016/S0278-6915\(98\)00020-9](http://dx.doi.org/10.1016/S0278-6915(98)00020-9)
- [50] Renga B, Mencarelli A, Cipriani S, *et al.* The bile acid sensor FXR is required for immune-regulatory activities of TLR-9 in intestinal inflammation. *PLoS One* 2013; 8: e54472. <http://dx.doi.org/10.1371/journal.pone.0054472>
- [51] Inagaki T, Choi M, Moschetta A, *et al.* Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell* 2005; 2: 217-25.
- [52] Holzer A, Harsch S, Renner O, *et al.* Diminished expression of apical sodium-dependent bile acid transporter in gallstone disease is independent of ileal inflammation. *Digestion* 2008; 78: 52-9. <http://dx.doi.org/10.1159/000159379>
- [53] Houten SM, Watanabe M, Auwerx J. Endocrine functions of bile acids. *EMBO J* 2006; 25: 1419-25. <http://dx.doi.org/10.1038/sj.emboj.7601049>
- [54] Staels B, Fonseca VA. Bile acids and metabolic regulation: mechanisms and clinical responses to bile acid sequestration. *Diabetes Care* 2009; 32 (Suppl 2): S237-45. <http://dx.doi.org/10.2337/dc09-S355>
- [55] Vos TA, Hooiveld GJ, Koning H, *et al.* Up-regulation of the multidrug resistance genes, Mrp1 and Mdr1b, and down-regulation of the organic anion transporter, Mrp2, and the bile salt transporter, Spgp, in endotoxemic rat liver. *Hepatology* 1998; 28: 1637-44. <http://dx.doi.org/10.1002/hep.510280625>
- [56] Schou J, Tybjaerg-Hansen A, Moller HJ, Nordestgaard BG, Frikke-Schmidt R. ABC transporter genes and risk of type 2 diabetes: a study of 40,000 individuals from the general population. *Diabetes Care* 2012; 35: 2600-6. <http://dx.doi.org/10.2337/dc12-0082>

- [57] Mei D, Li J, Liu H, *et al.* Induction of multidrug resistance-associated protein 2 in liver, intestine and kidney of streptozotocin-induced diabetic rats. *Xenobiotica* 2012; 42: 709-18.  
<http://dx.doi.org/10.3109/00498254.2011.654363>
- [58] Efentakis M, Buckton G. The effect of erosion and swelling on the dissolution of theophylline from low and high viscosity sodium alginate matrices. *Pharm Dev Technol* 2002; 7: 69-77.  
<http://dx.doi.org/10.1081/PDT-120002232>
- [59] Barakat NS A-SG, Almedany AH. Development of Novel controlled release gliclazide-loaded poly (ε-caprolactone) microparticles: effect of polymer blends. *Pharm Anal Acta* 2012; 3: 1-9.  
<http://dx.doi.org/10.4172/2153-2435.1000150>
- [60] Dorati R, Genta I, Modena T, Conti B. Microencapsulation of a hydrophilic model molecule through vibration nozzle and emulsion phase inversion technologies. *J Microencapsul* 2013; 30: 559-70.  
<http://dx.doi.org/10.3109/02652048.2013.764938>
- [61] Ding WK, Shah NP. Acid, bile, and heat tolerance of free and microencapsulated probiotic bacteria. *J Food Sci* 2007; 72: M446-50.  
<http://dx.doi.org/10.1111/j.1750-3841.2007.00565.x>
- [62] Sliwka W. Microencapsulation. *Angew Chem* 1975; 14: 539-50.  
<http://dx.doi.org/10.1002/anie.197505391>
- [63] Murua A, Portero A, Orive G, Hernandez RM, de Castro M, Pedraz JL. Cell microencapsulation technology: towards clinical application. *J Control Release* 2008; 132: 76-83.  
<http://dx.doi.org/10.1016/j.jconrel.2008.08.010>
- [64] Orive G, Hernandez RM, Rodriguez Gascon A, *et al.* History, challenges and perspectives of cell microencapsulation. *Trends Biotechnol* 2004; 22: 87-92.  
<http://dx.doi.org/10.1016/j.tibtech.2003.11.004>
- [65] Freiberg S, Zhu XX. Polymer microspheres for controlled drug release. *Int J Pharm* 2004; 282: 1-18.  
<http://dx.doi.org/10.1016/j.ijpharm.2004.04.013>
- [66] Singh MN, Hemant KS, Ram M, Shivakumar HG. Microencapsulation: a promising technique for controlled drug delivery. *Res Pharm Sci* 2010; 5: 65-77.
- [67] Al-Salami H, Butt G, Tucker I, Golocorbin-Kon S, Mikov M. Probiotics decreased the bioavailability of the bile acid analog, monoketocholic acid, when coadministered with gliclazide, in healthy but not diabetic rats. *Eur J Drug Metab Pharmacokinet* 2012; 37: 99-108.  
<http://dx.doi.org/10.1007/s13318-011-0060-y>
- [68] Miura S, Teramura Y, Iwata H. Encapsulation of islets with ultra-thin polyion complex membrane through poly(ethylene glycol)-phospholipids anchored to cell membrane. *Biomaterials* 2006; 27: 5828-35.  
<http://dx.doi.org/10.1016/j.biomaterials.2006.07.039>
- [69] Chavarri M, Maranon I, Ares R, Ibanez FC, Marzo F, Villaran Mdel C. Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules improves survival in simulated gastro-intestinal conditions. *Int J Food Microbiol* 2010; 142: 185-9.  
<http://dx.doi.org/10.1016/j.ijfoodmicro.2010.06.022>
- [70] Mitrevej A, Sinchaipanid N, Rungvejhavuttivittaya Y, Kositchaiyong V. Multiunit controlled-release diclofenac sodium capsules using complex of chitosan with sodium alginate or pectin. *Pharm Dev Technol* 2001; 6: 385-92.  
<http://dx.doi.org/10.1081/PDT-100002247>
- [71] Whelehan M, Marison IW. Microencapsulation using vibrating technology. *J Microencapsul* 2011; 28: 669-88.  
<http://dx.doi.org/10.3109/02652048.2011.586068>
- [72] Kendall W, Darrabie M, Freeman B, Hobbs H, Collins B, Opara E. Effect of bead swelling on the durability of polylysine alginate microcapsules. *Curr Surg* 2000; 57: 636-7.  
[http://dx.doi.org/10.1016/S0149-7944\(00\)00354-8](http://dx.doi.org/10.1016/S0149-7944(00)00354-8)
- [73] Koch S, Schwinger C, Kressler J, Heinzen C, Rainov NG. Alginate encapsulation of genetically engineered mammalian cells: comparison of production devices, methods and microcapsule characteristics. *J Microencapsul* 2003; 20: 303-16.
- [74] Tapia-Albarran M, Villafuerte-Robles L. Effect of formulation and process variables on the release behavior of amoxicillin matrix tablets. *Drug Dev Ind Pharm* 2004; 30: 901-8.  
<http://dx.doi.org/10.1081/DDC-200034594>
- [75] Sugiura S, Oda T, Izumida Y, *et al.* Size control of calcium alginate beads containing living cells using micro-nozzle array. *Biomaterials* 2005; 26: 3327-31.  
<http://dx.doi.org/10.1016/j.biomaterials.2004.08.029>
- [76] Sanchez P, Hernandez RM, Pedraz JL, Orive G. Encapsulation of cells in alginate gels. *Methods Mol Biol* 2013; 1051: 313-25.  
[http://dx.doi.org/10.1007/978-1-62703-550-7\\_21](http://dx.doi.org/10.1007/978-1-62703-550-7_21)
- [77] Patil P CD, Wagh M. A review on ionotropic gelation method: novel approach for controlled gastroretentive gelispheres. *Int J Pharm* 2012; 4: 27-32.
- [78] Cook MT, Tzortzis G, Charalampopoulos D, Khutoryanskiy VV. Microencapsulation of probiotics for gastrointestinal delivery. *J Control Release* 2012; 162: 56-67.  
<http://dx.doi.org/10.1016/j.jconrel.2012.06.003>

Received on 05-12-2013

Accepted on 19-12-2013

Published on 21-01-2014

DOI: <http://dx.doi.org/10.12970/2310-9971.2013.01.02.4>© 2013 Negrulj *et al.*; Licensee Synergy Publishers.

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