A CytoSolve Testing Division January 2018

White Paper Commissioned by Anagenix IP Limited

Livaux™ as a Prebiotic on Gut Motility

An In Silico Efficacy Analysis

cytosolve®

ABOUT CYTOSOLVE PRODUCT TESTING DIVISION

CytoSolve Product Testing Division provides independent and objective research to help manufacturers in their research and development efforts. Ranging in scope from a short single ingredient reports to detailed multi-combination analyses, CytoSolve's Product Testing Division services enable manufacturers to leverage the CytoSolve's patented platform for in silico computational modeling of complex biological pathways and processes to understand the efficacy and toxicity of their products.

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KEY TAKEAWAYS

- CytoSolve® analysis identified three major physiological processes governing gut motility:
  - Mucus production
  - Prebiotic activity leading to fecal bulking
  - Inflammation via
    - Oxidative stress
    - Nitric oxide

- Bioactive compounds in Livaux™ were found to have a positive synergistic effect on all three physiological processes involved in gut motility.

- Livaux™ improved gut motility by increasing mucus production, increasing intestinal transit time which aided fecal bulking and reducing inflammation.

- Even at **low dose levels**, Livaux™ was very efficient at increasing the mucus production and gut transit time to the maximum possible levels.

- Livaux™ can be described as a holistic prebiotic since, in addition to having a substantial amount of fiber, Livaux™ also contains leucine, which helps increase mucus, and luteolin, which helps increase smooth muscle cell relaxation in the gut thereby significantly aiding bowel movement, gut motility and increases gut transit time which aids fecal bulking.

- Other prebiotics available in the market such as inulin, psyllium, FOS, GOS, etc., comprise of only fiber and none of the other components provided by Livaux™.

- Anagenix has proven in a recent clinical trial that Livaux™ boosts levels of the beneficial gut bacterium *Faecalibacterium prausnitzii* (*F. prau*) by more than 100% (Blatchford et al., 2017).

- Livaux™'s bioactive compounds affect gut motility as follows:

- **Amino acid Leucine** increased expression of Mucin 2 gene which enhanced mucus production in enterocytes.
- **Polyphenolic** bioactive compound **Luteolin** enhanced smooth muscle cell relaxation, consequently gut transit, via downregulation of protein kinase c – α (PKC- α).
- **Fiber** in Livaux™ acts as an effective **prebiotic** for the gut microflora that converts the fiber into short chain fatty acids such as propionate.
- **Short chain fatty acid (SCFA) propionate**, a fermentation product of **prebiotic fiber** from Livaux™, increased expression of peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) which play a significant role in delaying gastric emptying, increasing gut transit time and consequently affecting fecal bulking.
- **Antioxidant** bioactive compounds **Vitamin E, Vitamin A and Vitamin C**, and **polyphenolic** bioactive compound **Epicatechin** reduced inflammation by reducing oxidative stress biomarker reactive oxygen species (ROS) in the enterocytes.
- **Antioxidant** bioactive compounds **Vitamin C and Vitamin A** reduced inflammation by lowering amount of nitric oxide produced in the enterocytes.

- At recommended dose levels of 600 mg/day over a 30-day period, Livaux™ consumption led to similar amounts of Mucin 2 production as that of two gold kiwifruit over the same period of time.

- At recommended dose levels of 600 mg/day over a 30-day period, Livaux™ consumption led to similar amounts of PYY and GLP-1 production as that of two gold kiwifruit over the same period of time.

Efficacy Analysis of Livaux™ as a Prebiotic on Gut Motility

ABSTRACT

A systematic literature review is conducted to identify the molecular pathways affecting gut motility. The molecular pathways of gut motility are converted to individual mathematical models; each model is validated; and, the plurality of models are integrated with the CytoSolve® computational systems biology platform to produce an integrative model of gut motility. CytoSolve provides for the dynamic integration of molecular pathway models, *in silico* (through mathematical modeling on a computer), to understand synergistic effects of multi-ingredient dietary supplements on molecular pathways of biological processes. Livuax™ is a clinically proven prebiotic and was shown to boost levels of the beneficial gut bacterium *Faecalibacterium prausnitzii* by more than 100% in recent clinical trial by Anagenix. Combination of all the bioactive molecules at the recommended dose levels is tested in silico to elucidate the mechanism behind the beneficial effects of Livaux™ on gut motility. The results from the systematic review reveal three major biological systems that govern gut motility: 1) Mucus production; 2) Fecal bulking; and, 3) Inflammation. The results from the CytoSolve in silico modeling demonstrate that Livaux™ bioactive compounds synergistically enhance gut motility by: 1) increasing mucus production; 2) assisting fecal bulking and, 3) reducing inflammation.

Keywords: in silico modeling, Livaux™, systems biology, molecular pathways, Anagenix, prebiotic, gut motility, inflammation, mucus production, CytoSolve, polyphenols, short chain fatty acids, fiber, antioxidants.

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1.0 INTRODUCTION

Clinical and experimental studies have demonstrated the positive effect of gold kiwifruit and Livaux™, a wholefood-based nutritional supplement derived from gold kiwifruit, on digestive health. However, the molecular mechanistic understanding of the effect of Livaux™ bioactive components, individually and in combination needs to be fully understood. In this study, a systematic literature review is conducted to identify the molecular pathways affecting gut motility. The molecular pathways of gut motility are converted to individual mathematical models; each model is validated; and, the pluralities of models are integrated with the CytoSolve® computational systems biology platform to produce an integrative model of gut motility. CytoSolve provides for the dynamic integration of molecular pathway models, *in silico* (through mathematical modeling on a computer), to understand synergistic effects of multi-ingredient dietary supplements on molecular pathways of biological processes.

1.1 Background

Microorganisms in the intestine act on substrates like carbohydrates such as resistant starch, unabsorbed sugars, non-starch polysaccharides, gums and cellulose, proteins from the diet and endogenous sources such as mucin. They break down these complex macromolecules to organic acids (lactic and acetic acids), bacteriocins, reuterin. These organic acids are the most abundant end-products of microbial fermentation in the human colon and their production lowers pH and directly inhibits the growth of pathogens. Consumption of kiwifruit has an impact on intestinal microorganisms. Kiwifruit as well as Livaux™ has highly indigestible polysaccharides like hemicellulose that can act like a prebiotic. These are digested by microorganisms in the gut like

Lachnospiraceae members to short chain fatty acids (SCFA) like acetate, propionate and butyrate. These simple molecules are used by intestinal bacteria like *Bifidobacteria* thereby enhancing their growth, aiding in digestion and also competitively inhibiting the association of pathogenic bacteria to the host (Rosendale et al., 2012). The bacterial fermentation of prebiotic fiber may aid gastrointestinal motility through the SCFA.

Studies on the consumption of gold kiwifruit substrates showed an increase in lactic acid bacteria like *Lactobacillus* and *Bifidobacteria*, with succinate and lactate production in the first several hours post inoculation before dropping back by the 24 h and 48 h time points. These intermediates can serve as substrates for other bacteria and may have been further converted to acetate or propionate. *Lactobacillus* and *Bifidobacteria* are regarded as beneficial bacteria, which are considered to be important for a well-balanced intestinal microbiota, displaying several health-promoting effects such as prevention of diarrhea and intestinal infections. In addition to the increase in these probiotic microorganisms, kiwifruit consumption also lead to a relative decrease in other bacteria such as *Bacteroides*, and *Clostridia*. This reduction might be induced by end-products of dietary fiber fermentation, such as butyrate, or by different metabolites produced by other beneficial bacteria (Bayer et al., 2017a).

A recent clinical trial conducted by Blatchford et al. sought to examine the prebiotic potential of Livaux™. One of the gut microbiome bacteria they studied was *Faecalibacterium prausnitzii (F. prau)* which has been recently identified to be critical for gut flora balance. *F. prau* is one of the most abundant bacteria in the human gut ecosystem. Low numbers have been associated with irritable bowel syndrome (IBS), inflammatory bowel diseases (IBD, including ulcerative colitis

(UC), and Crohn's disease) coeliac disease and chronic constipation. Livaux™ was shown to boost levels of this beneficial gut bacterium by more than 100% in the clinical trial (Blatchford et al., 2017) indicating a strong prebiotic potential for Livaux™.

Gastrointestinal motility is an essential function of digestive and absorptive processes of the gut, required for propelling intestinal contents, mixing them with digestive juices, and preparing unabsorbed particles for excretion. Gut motility involves 1) segmentation - non-propulsive annular contraction of the circular muscle layer that is predominantly found in the small and large intestines; (its major function is to mix intestinal chyme through its squeezing action); 2) tonic contractions; and, 3) peristalsis describes a highly integrated, complex motor pattern marked by sequential annular contractions of gut segments that produce a sweeping, propulsive wave forcefully moving luminal contents distally. Segmentation and peristalsis involve oscillatory, alternating contractions and relaxations of the small intestine's smooth muscle (Chang & Leung, 2014).

Some of the major gastrointestinal motility dysfunctions include esophageal dysmotility, gastric dysmotility, small intestinal dysmotility and colonic and anorectal dysmotility based on the part of GI system where the dysfunction occurs. These dysfunctions often lead to delayed gastric emptying, nausea, vertigo, severe pain (Wingate, Hongo, Kellow, Lindberg, & Smout, 2002). Current therapeutic options are limited and have side-effects. Muscarinic agonist- bethanechol, carbachol, cholineesterase inhibitor niostigmine enhance tone and peristaltic activity of small intestine and colon. Domperidone, enhances gastric emptying. Metoclopramide and Bromopride enhances both resting tension and contraction evoked by acetylcholine (Kilbinger & Weihraunch,

1982). Cisapride increases lower oesophageal sphincter pressure (Malagelada & Distrutti, 1996). Erythromycin treats gastroparesis and also increases bowel movements. Octreotide is a long-acting somatostatin analogue which initiates ectopic fronts of motor activity in the intestine.

Some of the major physiological processes that affect include mucus production, fecal bulking and inflammation. These processes are discussed in detail in section 2 of this white paper. Briefly, mucus serves as a protective barrier for the gut lining and as a lubricant to facilitate the passage of stool (Matsuo et al. 1997). Lack of fecal bulking is a prominent feature in gut motility dysfunctions such as diarrhea (Bayer et al. 2017). Inflammation is a major pathophysiological feature in gut motility dysfunctions such as inflammatory bowel disease, Crohn's disease, etc. (Bayer et al. 2017). Intestinal inflammation aggravates gut dysmotility by the contractility of the smooth muscle cells. In addition, it also disturbs the microbiome by causing abnormal growth in the intestinal microflora and mucosal inflammation resulting in pain (Ohama 2007).

Livaux™ is a food-quality ingredient derived entirely from New Zealand grown gold kiwifruit (*Actinidia chinensis* var Gold3/Zesy002), sourced from Zespri-approved growers. This particular cultivar of gold kiwifruit has a special geographic advantage that helps its nutritional profile as it is grown in New Zealand. As New Zealand is located in the southern hemisphere, it gets – at least 37% higher UV light than similar latitudes in the northern hemisphere. This high exposure to UV lights helps the kiwifruit plant accumulate more polyphenols in the leaves and fruit (http://legacy.biotechlearn.org.nz/news_and_events/news/2010_archive/uv_effect_on_grapes). In additional to having a substantial amount of fiber, Livaux™ also contains leucine, which helps increase mucus, and luteolin, which helps increase smooth muscle cell relaxation in the gut thereby

significantly aiding gut motility as well as fecal bulking, whereas, other prebiotics available in the market such as inulin, psyllium, FOS, GOS, etc., comprise only fiber and none of the other components provided by Livaux™. The proprietary pharmaceutical grade processing and drying techniques used to manufacture Livaux™ ensure levels of key nutrients and bioactives are maintained in the powdered form (private correspondence from Anagenix).

1.2 Research Aim

Understanding the complexity of multi-combination ingredients on biological processes is non-trivial. In this study, the research aim is to understand the singular and synergistic effect of bioactive components of Livaux™ on molecular pathways of gut motility. As an alternative to *in vitro* and *in vivo* study, the CytoSolve technology platform is used to facilitate in silico computational modeling and analysis of Livaux™ bioactive components on gut motility.

1.3 Organization of White Paper

This manuscript is organized as follows: Section 2.0, provides the systematic literature review to identify the molecular pathway systems of gut motility; Section 3.0 itemizes the bioactive components of Livaux™ that will be tested individually and in combination through in silico modeling of gut motility pathways; Section 4.0 summarizes the CytoSolve methodology for in silico testing of Livaux™ bioactive component; Section 5.0 presents the results from the individual and combination testing of bioactive components of Livaux™ on the gut motility pathways; Section 6 summarizes concluding remarks from this study; and, Section 7 contains the bibliography of references used in this study.

2.0 GUT MOTILITY MECHANISMS

Three major biological processes that govern gut motility were identified using CytoSolve methodology: 1. Inflammation; 2. Mucus Production; and, 3. Fecal Bulking.

2.1 Inflammation

Intestinal inflammation directly affects the gut motility and is a prominent pathological feature in several motility related bowel diseases (Bassotti et al. 2014). Intestinal inflammation promotes gut dysmotility by increasing the contractility of the smooth muscle cells. In addition, it also disturbs the microbiome by causing abnormal growth in the intestinal microflora and mucosal inflammation resulting in pain (Ohama 2007). Mechanistically, there are two major inflammatory molecular pathways that are closely associated with gut motility including: 1) Oxidative stress pathway; and, 2) TNF-α induced nitric oxide synthesis from inducible nitric oxide synthase (iNOS).

2.1.1 Oxidative stress pathway: High levels of reactive oxygen species (ROS) induce activation of the redox-sensitive nuclear transcription factor kappa-B (NF-κB) which, in turn, triggers the inflammatory cytokines. The reactive species include superoxide anions (O_2^-, hydroxyl radicals ($OH^\cdot$) and hydrogen peroxide (H_2O_2) (Wang et al., 2015). Higher levels of inflammatory biomarker ROS indicates poor gut motility, whereas lower levels of ROS indicate optimal gut motility. A schematic of the oxidative stress pathways is shown in Figure 1.

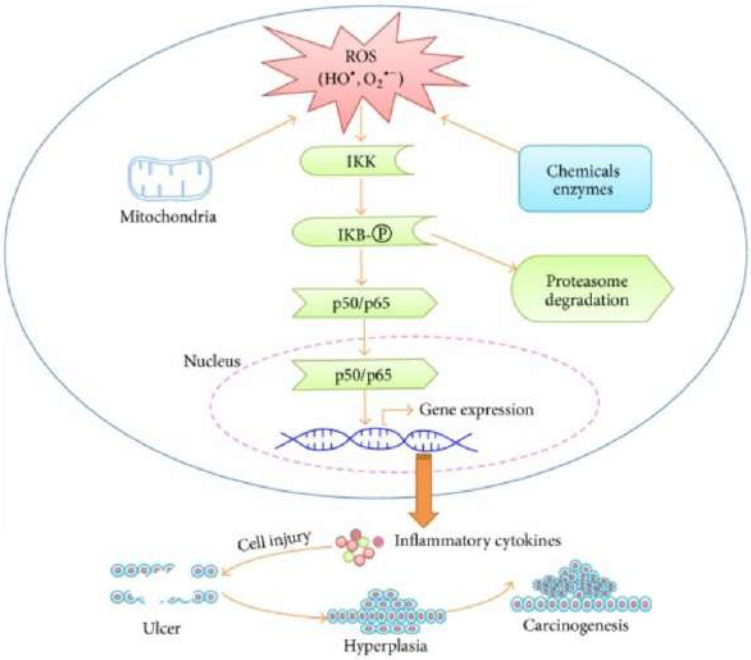

Figure 1: Oxidative stress leading to expression pf inflammatory cytokines. ROS: Reactive oxygen species; $HO^{\cdot}$: Hydroxyl radical; $O_2^{-\cdot}$: Superoxide; IKB: Inhibitor of KB; IKK: IKB Kinase; p50/p65: Nuclear factor kB subunits (Wang et al. 2015)

2.1.2 TNF-α induced nitric oxide synthesis from inducible nitric oxide synthase (iNOS): Oxidative stress can lead to the activation of inflammatory genes like IL-8, IL-1, TNF-α, Mcp-1 (Wang et al., 2015). The expressed TNF-α binds to its receptor TNFR1 and recruits TRAF2-RIP-TRADD complex. The association of this complex leads to activation of NIK leading to IKK activation. IKK causes activation of NF-kB and causes its nuclear translocation leading to iNOS expression (Dijkstra et al., 2002). The increased iNOS can lead to the increased nitric oxide (NO) expression. NO can interact with superoxide leading to the formation of peroxynitrite. The peroxynitrite can cause tyrosine nitration, lipid peroxidation, DNA strand breakage, DNA mutation leading to cell damage. The schematics for this pathway is shown in Figure 2.

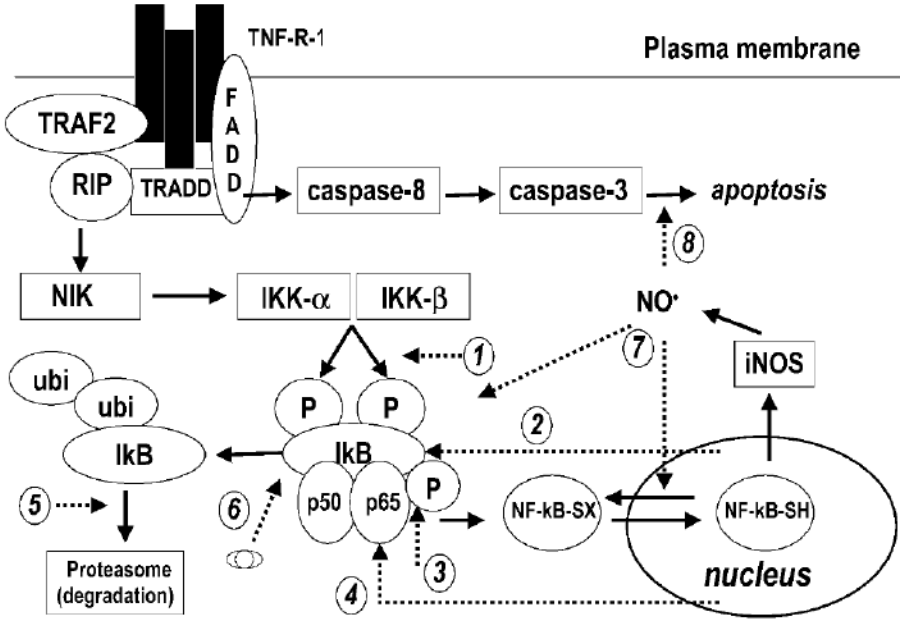

Figure 2: TNF Induced iNOS Expression. TNF-□: Tumor nexcrosis factor-□□□□NF-R-1: TNF□□ Receptor 1; TRAF2: TNF receptor-associated factor 2; RIP: Receptor-interacting protein; TRAAD: TRAF-1 -associated DEATH domain protein; FADD: Fas-associated protein with death domain; IKB: Inhibitor of KB; IKK: IKB Kinase; iNOS: inducible nitric oxide synthase (Dijkstra et al., 2002).

Higher levels of inflammatory biomarkers NO indicate poor gut motility, whereas lower levels of ROS and NO indicate optimal gut motility.

2.2 Mucus Production

Leucine is an essential amino acid for human health. Livaux™ contains leucine that can activate PI3K-Akt-mTOR pathway in intestinal epithelial cell leading to the expression of mucin-2. Mucin 2, an important component of the mucus gel layer in the intestine, is mainly synthesized and secreted by the goblet cells, which is the important component of non-specific barrier mechanisms in the intestinal mucosa (Mao et al., 2011a). Mucin 2 coats the intestinal epithelial cell providing a protective, lubricating barrier against particles and infectious agents at mucosal surfaces (Mao et al., 2016). A detailed schematic diagram for this pathway is shown in Figure 3.

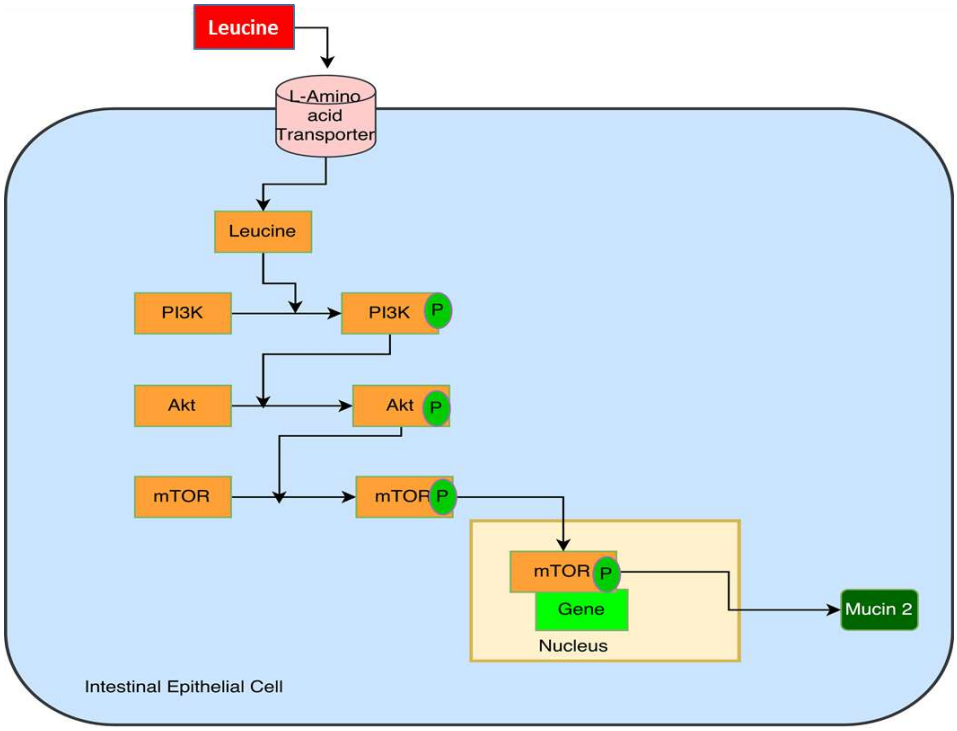

Figure 3: Leucine induced Mucin 2 production in intestinal epithelial cells. PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B; mTOR: Mammalian target of rapamycin (Mao, 2016)

2.3 Fecal Bulking

Fecal bulking is affected by two major pathways systems: 1) Gut transit pathways; and, 2) acetylcholine induced smooth muscle cell contractility.

2.3.1 Gut Transit Pathways: Undigested carbohydrates are fermented by gut microbiota into short-chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate. SCFAs affect the host metabolism in several ways. SCFAs can signal through G protein-coupled receptor 41 (GPR41) on enteroendocrine cells, inducing the secretion of peptide YY (PYY) which increases intestinal transit time, and reduces the harvest of energy from the diet. Engagement of G protein-coupled receptor 43 (GPR43) by SCFAs has been shown to trigger the glucogon-like peptide 1 (GLP-1) to increase insulin sensitivity. Gut microbiota efficiently suppresses fasting-induced adipose factor (Fiaf) expression in the ileum, which inhibits lipoprotein lipase (LPL) activity and fat storage in white adipose tissue. SCFAs-mediated activation of GPR43 results in suppression of insulin signaling in the adipose tissue and sub- sequent prevention of fat accumulation (Hur and Lee, 2015). Increase in gut transit time facilitates absorption of water and other nutrients, and facilitating fecal bulking (Rambaud et al 1988). This mechanism is very critical, especially in pathologies such as diarrhea.

SCFAs also activate intestinal gluconeogenesis (IGN) via a gut-brain neural circuit, which can improve glucose metabolism and reduce food intake (Hur and Lee, 2015). SCFA are associated with a number of beneficial effects in the GIT, including intestinal modulation of GIT contractility, increased numbers of beneficial bacteria and reduced numbers of

pathogenic bacteria. In addition, SCFA are the main energy source for epithelial cells (Montoya et al., 2015b). Since both PYY and GLP-1 increase the gut transit time, they have a positive effect on fecal bulking. Schematics for SCFA pathways is given in Figure 4.

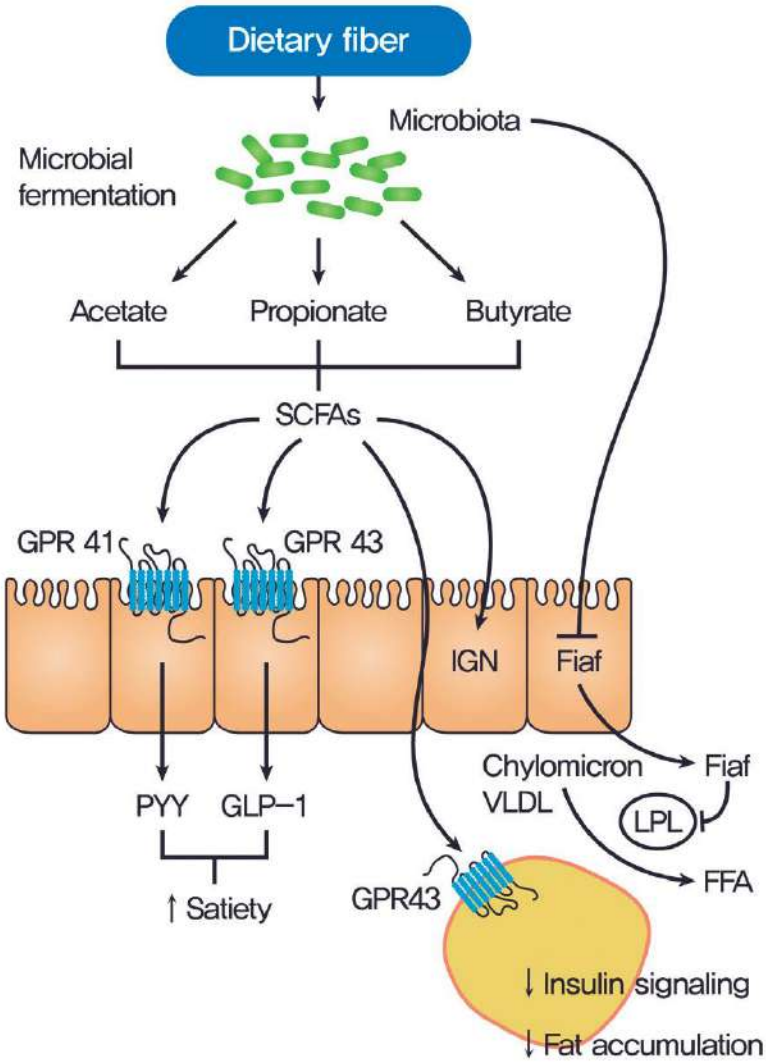

Figure 4: Gut transit mechanisms in enteroendocrine cells. Short chain fatty acids SCFAs can signal through G protein-coupled receptor 41 (GPR41) and G protein-coupled receptor 43 (GPR43) inducing the secretion of peptide YY (PYY) and glucagon-like peptide 1 (GLP-1), respectively. PYY and GLP-1 aid in fecal bulking by increasing the intestinal transit time (Hur and Lee, 2015).

2.3.2 Acetylcholine induced smooth muscle cell contractility: Acetylcholine (Ach) is an excitatory neurotransmitter and is responsible for inducing intestinal smooth muscle contraction (Uchiyama and Chess-Williams, 2004). Two types of muscarinic receptors, M2 and M3, on gastrointestinal smooth muscle cells participate in the Ach induced signaling. M3 receptors are more commonly found on the gastrointestinal smooth muscle cell surface. Once Ach binds to M3 receptors on the cell surface, it initiates a signaling cascade that activation of secondary messengers such as inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). These second messengers in turn initiate Ca^{2+} signaling necessary for myosin fibers to shorten and cause the whole smooth muscle cell to contract (Uchiyama and Chess-Williams, 2004). Prolonged contraction can restrict the bowel movement and lead to gut dysmotility (De Mari et al 2005). The schematics for this pathway is shown in Figure 5.

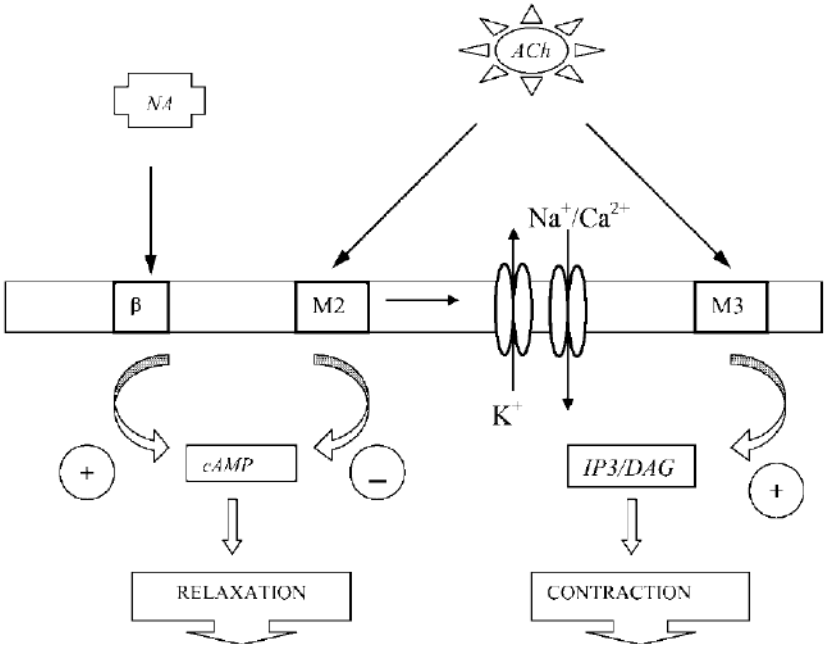

Figure 5: Acetylcholine (Ach) stimulates M3 receptor via inositol 3-phosphate (IP3)/ diacylglycerol (DAG) leading to smooth muscle cell contraction (Uchiyama and Chess-Williams, 2004).

3.0 BIOACTIVE COMPOUNDS OF LIVAUX™

Livaux™ contains several bioactive compound classes including antioxidant vitamins, polyphenols, and **prebiotic** fiber. They are discussed in detail as follows.

3.1 Antioxidant Vitamins

Livaux™ and fresh gold kiwifruit contain antioxidant vitamins C, E and β-carotene (Beck et al., 2011, Dias, 2014, Rizvi et al., 2014). All three vitamins play a major role in reducing the oxidative stress in the enterocytes as well as lowering the amount of NO produced by iNOS. Intestinal inflammation promotes gut dysmotilty by increasing the contractility of the smooth muscle cells. In addition, it also disturbs the microbiome by causing abnormal growth in the intestinal microflora and mucosal inflammation resulting in pain (Ohama 2007).

Additionally, vitamin C also enhances iron (Fe) absorption by reducing ferric Fe to ferrous Fe, for transport by divalent metal transport protein 1 into the intestinal mucosal cell. Ascorbic acid also forms a soluble chelate with Fe, preventing it being precipitated as insoluble compounds, such as ferric hydroxide or ferric phosphate, or binding to inhibitory ligands such as phytate (Beck et al., 2011).

3.2 Polyphenols

Livaux™ and fresh gold kiwifruit contain nine (9) identified phenolic compounds of which epicatechin and luteolin had a significant effect on gut motility. Epicatechin is a member of a group of polyphenolic compounds collectively known as catechins, belonging to flavonoid family

(Sabarimuthu et al. 2005). Epicatechin acts as an antioxidant and has a beneficial effect in reducing the effect of oxidative stress (Lee et al., 2010). Luteolin (3′,4′,5,7-tetrahydroxy-flavone) is one of the most bioactive flavonoids with many beneficial effects on human health, including cardiovascular protection, anticancer activity, anti-ulcer effects, cataract prevention, antiviral activity, anti-inflammatory effects and anti-allergic properties. High consumption of luteolin is inversely related to risk of cardiovascular diseases (Zeng et al. 1994). Luteolin has been shown to inhibit the cytosolic PKC activity (Kang and Liang, 1997). Activation of PKC is necessary to induce intestinal smooth muscle cell contraction. Prolonged contraction can restrict the bowl movement and lead to gut dysmotility. Luteolin also increases PYY and GLP-1 in the enterocytes in the gut. These two molecules are directly related to increasing the gut transit time that allows optimal absorption of nutrients and water leading to fecal bulking.

3.3 Fiber

Dietary fiber is a major constituent of Livaux™. Dietary fiber is categorized into soluble and insoluble fiber. Soluble fiber is digested and is used as a direct source of energy for humans whereas microorganisms in the intestine break down the insoluble fiber to short chain fatty acids like acetate, propionate and butyrate via fermentation (Slavin 2013). These simple molecules are used by microorganisms in the intestines like *Bifidobacteria* for enhancing their growth, aiding in digestion and also competitively inhibiting the association of pathogenic bacteria to the host (Rosendale et al., 2012). These SCFAs are essential nutrient sources for colonic epithelium, and in addition can provide up to 500 cal/day of overall nutritional needs. They are passively and actively transported into the cell where they become an important energy source for the cell through the oxidation pathway (Montoya et al., 2015a). SCFAs exert multifaceted effects in

polymorphonuclear cells (PMNCs), including the alteration of cytoplasmic pH, calcium concentration, oxygen metabolism, phagocytosis, cell proliferation, cytoskeletal actin distribution, granulocyte motility, and chemotaxis (Yonezawa et al., 2013). They also initiate the GPR41 and GPR43 signaling cascade leading to PYY and GLP-1 expression, which in turn aid in increasing the gut transit time (Yonezawa et al., 2013). Increase in gut transit time facilitates optimal absorption of water and other nutrients, and facilitating stool forming and fecal bulking (Rambaud et al., 1988).

In additional to having a substantial amount of fiber, Livaux™ also contains leucine, which helps increase mucus, and luteolin, which helps 1) increase in smooth muscle cell relaxation that affects bowel movement in the gut thereby significantly aiding gut motility, and 2) increase gut transit time that helps optimal absorption of water and nutrients thereby enhancing fecal bulking. Livaux™ can be a holistic prebiotic as compared to the commonly available prebiotics in the market such as inulin, psyllium, FOS, GOS, etc., which comprise only fiber and none of the other components provided by Livaux™. Luteolin also increases PYY and GLP-1 in the enterocytes in the gut. These two molecules are directly related to increasing the gut transit time that allows optimal absorption of nutrients and water leading to fecal bulking.

4.0 METHODOLOGY

Computational systems biology approaches such as CytoSolve can provide insights to understand complex molecular phenomena and effect of Livaux™ on biological phenomenon such as gut motility. The CytoSolve technology applies a six-step process to understand the effect of Livaux™

at the molecular mechanistic level: 1. conducting and archiving search results from disparate data sources including PubMed, Google Scholar, and multiple online databases; 2. managing and annotating the identification of the molecular pathway diagrams; 3. integrating molecular pathway diagrams to create large scale molecular systems; 4. managing and identifying modelling parameters such as rate constants, initial conditions, etc.; 5. creating and simulating component molecular pathway models; and, 6. integrating component models to create large scale functional and predictive models of biological phenomena.

4.1 CytoSolve Background

CytoSolve is a proven, scalable computational systems biology technology for the dynamic integration of complex and large-scale molecular pathway models (Ayyadurai et al., 2011a, Ayyadurai, 2011b, Koo et al., 2013, Al-Lazikani et al., 2012). Among its various applications, CytoSolve provides a framework for performing methodological analyses of the efficacy and toxicity of combinations of one or more ingredients relative to a particular biological process.

The CytoSolve technology abstracts complex cellular functions as a plurality of molecular pathway models, as illustrated in Figure 6, that span multiple spatial and temporal scales, across compartments, across cell types and across biological domains.

CytoSolve aggregates existing peer-reviewed scientific literature, and mines this literature to extract complex molecular pathways of biological processes. Mathematical models derived from these pathways are integrated to create a validated and integrative model. The platform provides an inherent scalability for integrating multiple molecular pathways to create integrative models of

complex biological phenomena. These integrative models can then be exploited for in silico computational analysis of individual or multiple ingredients relative to a particular biological process.

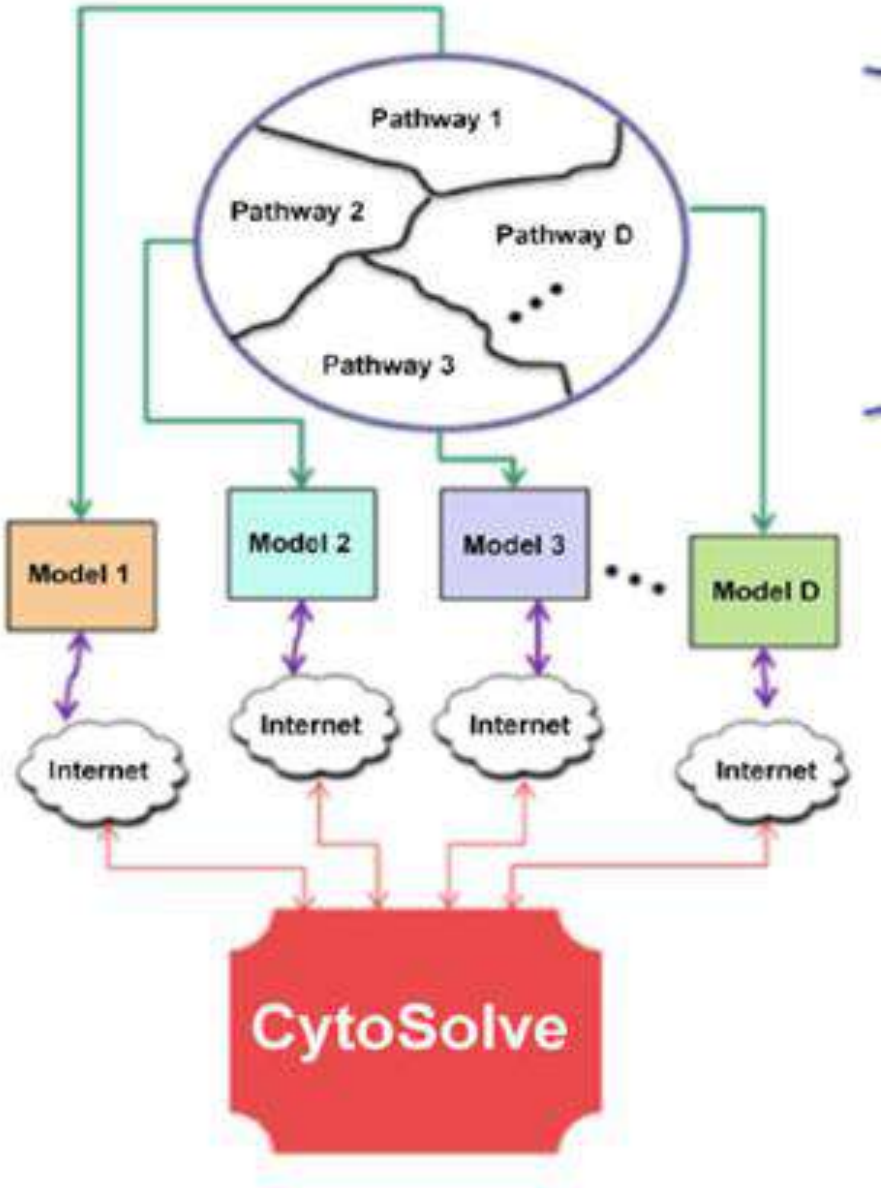

Figure 6: CytoSolve provides a framework for integrating systems of systems of molecular pathway models (Ayyadurai, 2011).

4.2 Deployment of CytoSolve Technology

The CytoSolve platform is deployed in the following step-by-step manner:

(1) collaborative review, organization, and prioritization of relevant literature

(2) identification and extraction of key molecular pathways from literature

(3) translation of diagrammatic molecular pathway representations to molecular pathway models

(4) validation of individual molecular pathway models

(5) integration of validated molecular pathway models to create integrative models

(6) identification of key ingredients and the relevant molecules as well as their molecular interactions with the particular species in the computational models

(7) execution of in silico experiments using the CytoSolve integrative models to test individual bioactive compounds of Livaux™ as well as their combination

(8) production of in silico modeling results to understand the efficacy of the individual and multi-combination ingredients

4.3 Setup of Integrative Models in CytoSolve for Gut Motility

Based on the pathways identified in Section 2.0, and following steps 3-5 from Section 4.2, an integrative model of gut motility was setup, tested and validated in CytoSolve. Figure 7 represents the setup of multiple models integrated in CytoSolve and the interactions of bioactive compounds with these models.

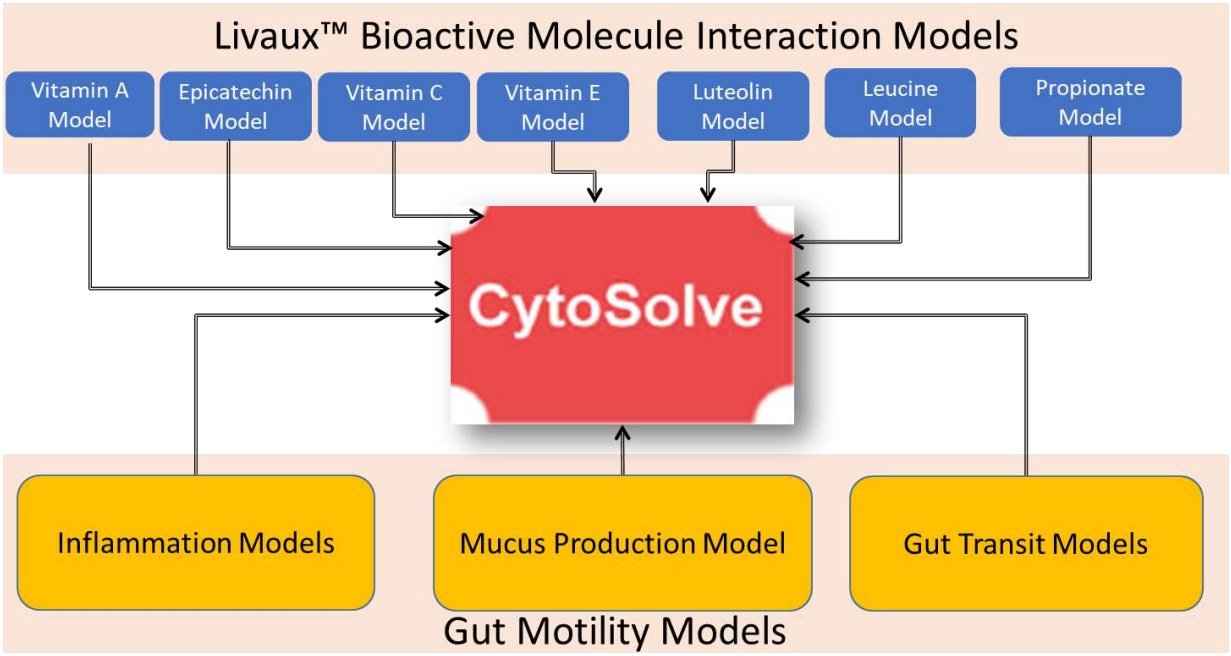

Figure 7: Integration of CytoSolve models of gut motility to test the individual and combination effect of bioactive compounds in Livaux™.

4.4 Setup of Bioactive Compounds in CytoSolve for In Silico Testing

Based on the ingredients and key molecules identified in Section 3.0, initial concentrations of the key molecules in each of the ingredients were obtained based on the Cmax (maximum plasma concentrations for a given dose) data available from the pharmacokinetic studies in the literature. The concentration values were converted into molar units by using following formula:

$$\text{Concentration of active ingredient} = \frac{\text{Weight in grams}}{\text{Molecular Weight}} \times \frac{1}{\text{volume of plasma}}$$

The calculations of concentrations of all the bioactive compounds in Livaux™ were based on dose levels of 600 mg/day and 2,400 mg/day. For the whole gold kiwifruit, the dosage was two fruits per day.

4.5 Setup of Simulations and Key Biomarkers of Measure for Gut Motility

Based on the initial conditions of all the key bioactive compounds of Livaux™ in Section 4.3, the models developed in Section 4.4 were simulated

The following **five (5)** in silico experiments were conducted. Below, the details of each experimental setup are provided.

a. Effect of single dose of 600 Livaux™ mg, 2,400 mg Livaux™ and two (2) gold kiwifruit over a period of one (1) day
b. Effect of 600 mg/day Livaux™ over a period of 30 days
c. Effect of 2,400 mg/day Livaux™ over a period of 30 days
d. Effect of two gold kiwifruit per day over a period of 30 days
e. Effect of 2,400 mg/day Livaux™ for three days followed by 600 mg/day Livaux™ for 27 days

The effect of all bioactive molecules in Livaux and gold kiwifruit was assessed by estimating the following biomarkers for corresponding molecular pathway system. For oxidative stress pathway, reactive oxygen species was identified as the biomarker. For TNF-α induced nitric oxide synthesis, the biomarker was identified as NO. For mucus production, the biomarker was identified as Mucin 2. For fecal bulking, the biomarkers identified were PKC-α, PYY and GLP-1.

5.0 RESULTS

A selected literature set of peer-reviewed articles on gut motility and the Livaux™ bioactive components to setup CytoSolve models of gut motility was reviewed. Simulations were performed to test the synergistic effect of bioactive compounds in Livaux™ on the gut motility pathways. The results section is organized as follows. Section 5.1 summarizes the results from the systematic review. Section 5.2 summarizes the synergistic effect of bioactive compounds in Livaux™ on inflammation and comparison with that of whole gold kiwifruit. Section 5.3 summarizes synergistic effect of bioactive compounds in Livaux™ on mucus production and comparison with that of whole gold kiwifruit. Section 5.4 summarizes synergistic effect of bioactive compounds in Livaux™ on fecal bulking and comparison with that of whole gold kiwifruit. Section 5.5 summarizes the estimation of minimum amount of Livaux™ required to achieve the maximum possible Mucin 2, PYY and GLP-1 production.

5.1 Literature Review Results

An exhaustive scientific literature review from online databases such as PubMed and Google Scholar was performed, which yielded 104 most relevant research articles. These research articles were thoroughly reviewed for identification of bioactive compounds in Livaux™, molecular pathway systems, bimolecular species concentrations and kinetic parameters used for computational modeling. The 104 research articles comprising the systematic review are enumerated with complete citation in the Section 7. The systematic bioinformatics review process for literature and pathway identification is shown in Figure 8.

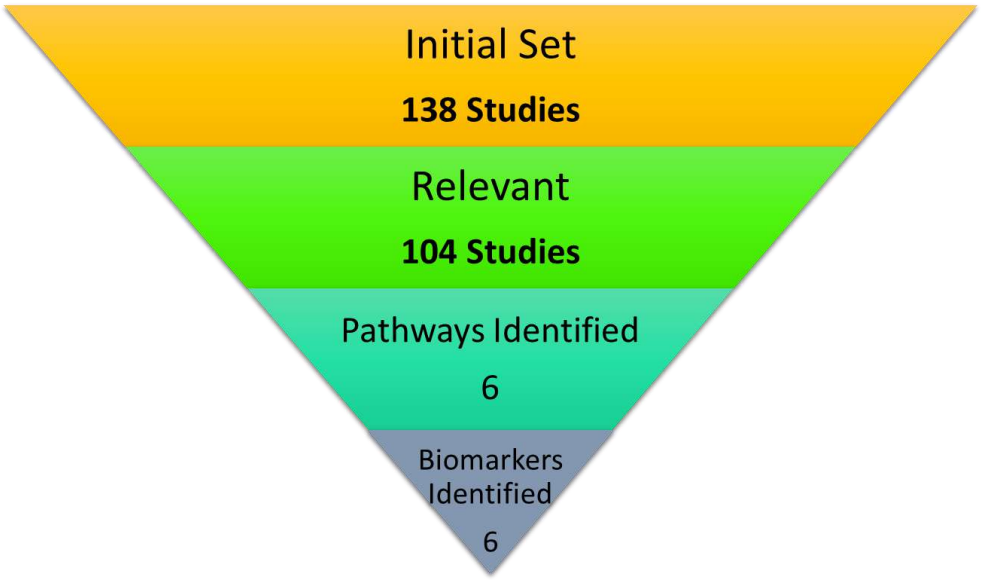

Figure 8: Results from the systematic bioinformatics review.

A total of six (6) key molecular pathways involved in the three biological processes of inflammation, mucus production, and gut transit/fecal bulking were extracted from the most relevant 104 studies. Six (6) biomarkers were identified from these pathways on which the effect of seven (7) bioactive compounds from Livaux™ was tested. The pathways, key biomarkers and the bioactive molecules are listed in Table 1.

Table 1. Pathway, biomarker and bioactive compound information obtained from the systematic bioinformatics literature review.

Physiological Function	Molecular Pathway	Biomarker	Livaux™ Bioactive Compound Associated with Biomarker
Inflammation	TNFα induced iNOS Oxidative Stress Pathway	Nitric Oxide (NO) Reactive Oxygen Species (ROS)	Vitamin E, β Carotene, Vitamin C, Epicatechin
Mucus Production	mTOR signalling	Mucin 2	Leucine
Gut Transit/Fecal Bulking	GPR-43 Signaling GPR-41 Signaling Acetylcholine induced smooth muscle relaxation	PYY GLP-1 PKC-α	Propionate Luteolin

5.2 Synergistic Effect of Bioactive Components in Livaux™ on Inflammation

Of all the bioactive compounds present in Livaux™, three vitamins (vitamin E, C and □) and one polyphenolic compound (epicatechin) were found to have direct targets in the oxidative stress pathway and TNF-α induced NO production pathway. Inflammation is negatively correlated with the gut motility. Higher levels of inflammatory biomarkers of ROS and NO indicated poor gut motility, whereas lower levels of ROS and NO indicate optimal gut motility.

Table 2 lists the amounts of these bioactive compounds present in various dose levels of Livaux™ and two whole gold kiwifruit.

Table 2: Quantification of bioactive compounds in Livaux™ and gold kiwifruit

Bioactive Compound	Livaux™ Dose Levels		Two Gold Kiwifruit
	600 mg	2400 mg	150 g
Epicatechin (mg)	0.0066	0.026	0.68
Vitamin A (mg)	0.0081	0.032	0.098
Vitamin C (mg)	1.84	7.16	160
Vitamin E (mg)	2.26	0.07	0.3

Source: Anagenix Data

Synergistic effect of bioactive molecules in Livaux™ on the reactive oxygen species, a biomarker of oxidative stress pathway, and on NO, a biomarker of TNF-α induced NO production pathway was measured and the results are discussed in detail below.

5.2.1 Effect of bioactive compounds in Livaux™ on oxidative stress pathway

Three sets of results from the in silico experiments on the effects of bioactive compounds in Livaux™ on oxidative stress pathway were obtained:

1. Comparison of 600 mg/day Livaux™ versus 2,400 mg/day Livaux™ versus two whole gold kiwifruit on ROS for a period of one (1) day (Figure 9A);

2. Comparison of 600 mg/day Livaux™ versus 2,400 mg/day Livaux™ versus two whole gold kiwifruit on ROS for a period of 30 days (Figure 9B); and,

3. Comparison of 600 mg/day Livaux™ versus 2,400 Livaux™ versus 2,400 mg/day Livaux for three days followed by 600 mg/day Livaux™ for 27 days on ROS (Figure 9C).

For these simulations, the system was assumed to be under inflammatory state with no Livaux™ or gold kiwifruit dose.

In the first set of results, under control conditions, in absence of Livaux™ and gold kiwifruit, the levels of ROS were estimated to be 122 nM under the inflammatory conditions. At the end of 1-day period, Livaux™ at 600 mg/day, Livaux™ at 2,400 mg/day, and two gold kiwifruit downregulated the ROS levels to 105 nM, 104 nM and 58 nM,, respectively, as shown in Figure 9A. These results indicate that although the bioactive compounds of Livaux™ reduced the ROS levels compared to the control, the reduction at the end of a single dose was not very significant.

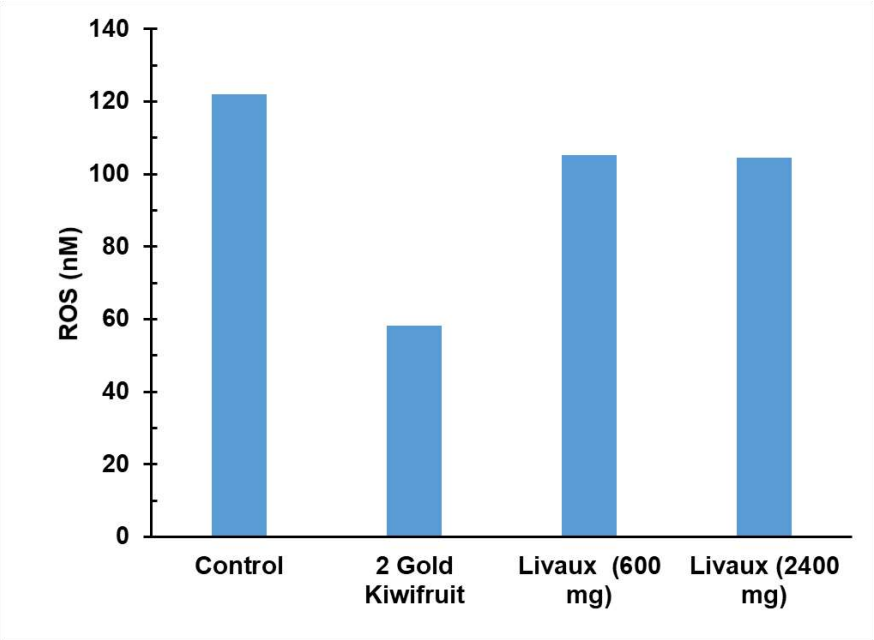

Figure 9A: Synergistic effect of bioactive compounds in Livaux™ and in gold kiwifruit on reactive oxygen species (ROS) at a single dose over 1-day period

In the second set of results, under control conditions, in absence of Livaux ™ and gold kiwifruit, the levels of ROS were estimated to be 409 nM under the inflammatory conditions (in Figure 9B, the bar and value for control is not shown). At the end of 30-day period, Livaux™ at 600 mg/day and 2,400 mg/day brought down the ROS levels to 1.2 nM and 0.2 nM, respectively, as shown in Figure 9A. A dose of two kiwifruit per day performed slightly better than Livaux™. These results indicate that the bioactive compounds of Livaux™ have a significant and positive effect in reducing inflammation via lowering ROS at the end of 30-day dose regimen compared to the single dose as shown in Figure 9A.

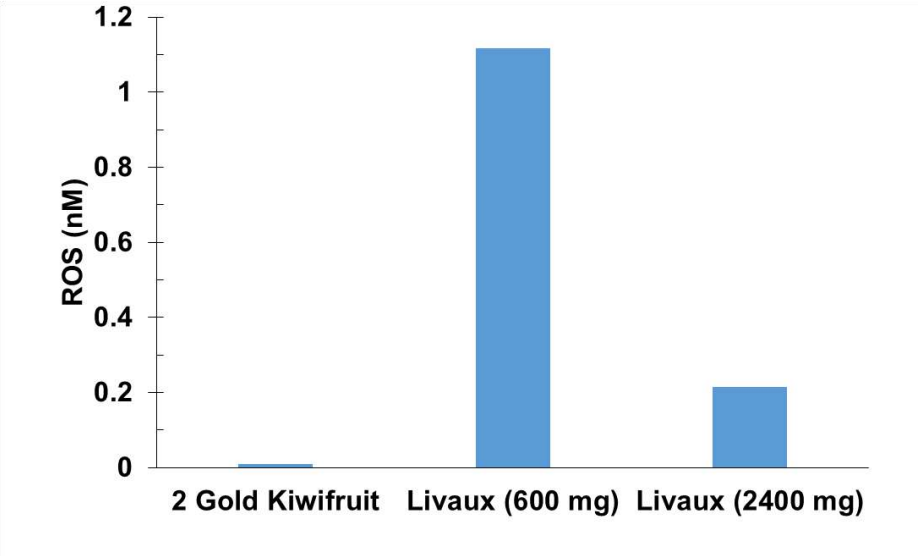

Figure 9B: Synergistic effect of bioactive compounds in Livaux™ and in gold kiwifruit on reactive oxygen species (ROS) at a fixed dose over 30-day period

In the third set of results, the dose regimen included Livaux™ at 2,400 mg/day for three days followed by 600 mg/day for 27 days and results were compared to that of fixed dosing of Livaux™ at 600 mg/day and 2,400 mg/day for 30 days. As shown in Figure 9C, the modified dose regimen was able to significantly lower the ROS levels; however, it had no improvement on ROS levels compared to the fixed dose levels of 600 mg/day and 2,400 mg/day for 30 days.

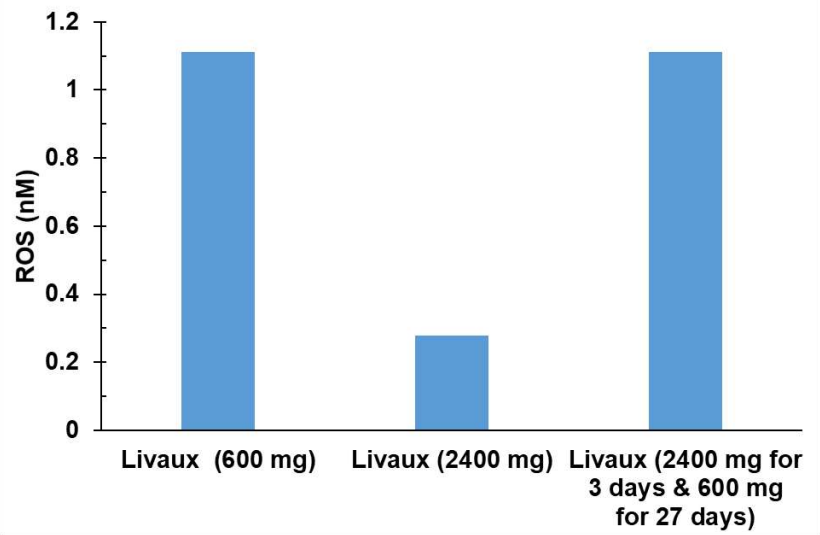

Figure 9C: Effect of Livaux™ regimen of 2,400 mg/day for three days followed by 600 mg/day for 27 days on reactive oxidative species (ROS)

5.2.2 Effect of bioactive compounds in Livaux™ on TNF-α induced NO production pathway

Three sets of results from the simulation of bioactive compounds in Livaux™ and whole gold kiwifruit at recommended dose levels on TNF-α induced NO production pathway were obtained:

1. Comparison of 600 mg/day Livaux™ versus 2,400 mg/day Livaux™ versus two whole gold kiwifruit on NO for a period of one (1) day (Figure 10A);

2. Comparison of 600 mg/day Livaux™ versus 2,400 mg/day Livaux™ versus two whole gold kiwifruit on NO for a period of 30 days (Figure 10B); and,

3. Comparison of 600 mg/day Livaux™ versus 2,400 Livaux™ versus 2,400 mg/day Livaux for three days followed by 600 mg/day Livaux™ for 27 days on NO (Figure 10C).

For these simulations, the system was assumed to be under inflammatory state with no Livaux™ or gold kiwifruit dose.

In the first set of results, under control conditions, in absence of Livaux™ and gold kiwifruit, the levels of NO were estimated to be 18.8 µM under the inflammatory conditions. At the end of 1-day period, Livaux™ at 600 mg/day, Livaux™ at 2,400 mg/day, and two gold kiwifruit downregulated the NO levels to 0.09 µM, 7.8 µM, and 7.3 µM, respectively, as shown in Figure 10A. These results indicate that the bioactive compounds of Livaux™ reduced the NO levels significantly at both dose levels by a similar extent.

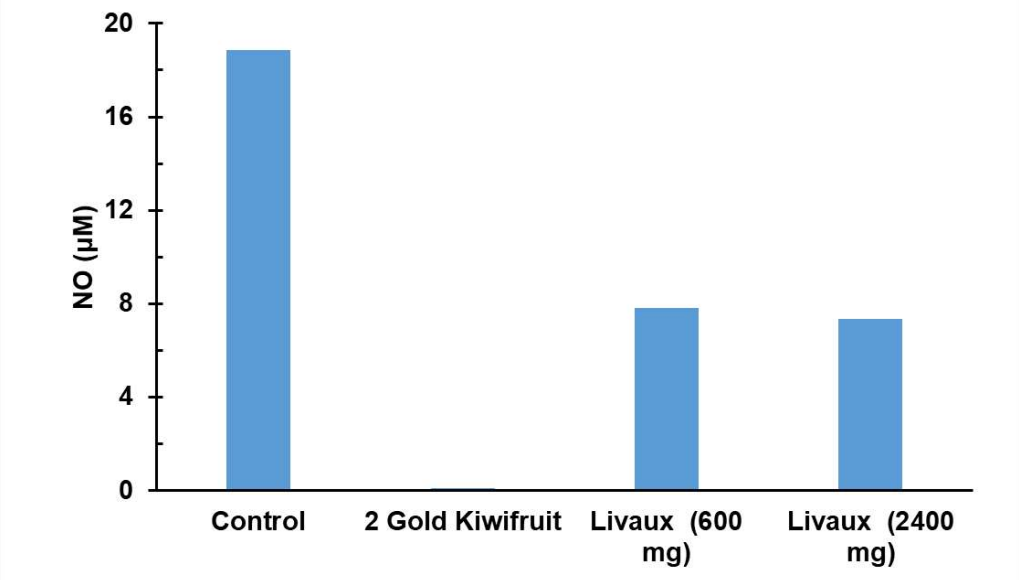

Figure 10A: Synergistic effect of bioactive compounds in Livaux™ and in gold kiwifruit on NO at a single dose over 1-day period.

The second set of results are shown in Figure 10B. Under control conditions, in absence of Livaux™ and gold kiwifruit, the levels of NO were estimated to be 533 µM under the inflammatory conditions. At the end of 30-day period, a dose of Livaux™ at 600 mg/day reduced the NO levels to 532 µM and for 2,400 mg/day reduced the NO levels down to 146 µM. A dose of two kiwifruit per day performed brought the NO levels down to 6.8 µM, which was significantly better than Livaux™ at both dose levels. These results indicate that the Livaux™ has a better effect on lowering inflammation via reducing NO levels only at high dose level of 2,400 mg/day.

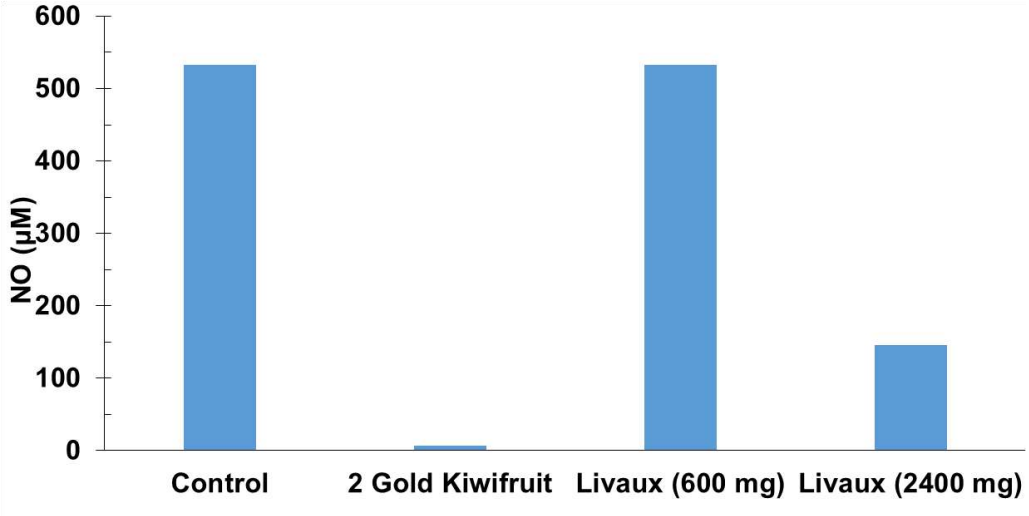

Figure 10B: Synergistic effect of bioactive compounds in Livaux™ and in gold kiwifruit on NO at a fixed dose over 30-day period.

In the third set of results as shown in Figure 10C, the dose regimen included Livaux™ at 2,400 mg/day for three days followed by 600 mg/day for 27 days and the results were compared to that of fixed dosing of Livaux™ at 600 mg/day and 2,400 mg/day for 30 days. As shown in Figure 10B, the modified dose regimen lowered the NO levels from

533 µM to 442 µM. These results indicate that the variable dose regimen was able to significantly lower the inflammation via reduced NO production compared to fixed dose regimen of 600 mg/day over a 30-day period.

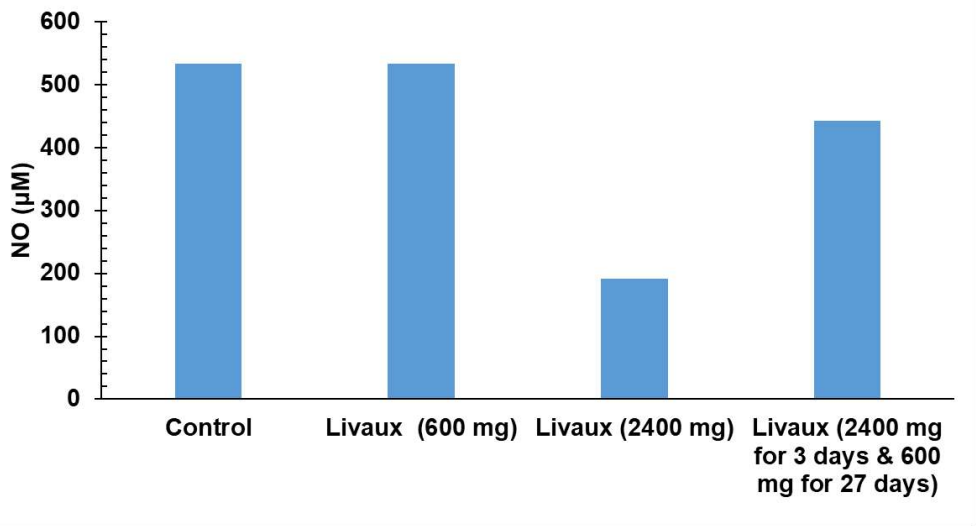

Figure 10C: Effect of Livaux™ regimen of 2,400 mg/day for three days followed by 600 mg/day for 27 days on NO production

5.3 Synergistic Effect of Bioactive Components in Livaux™ on Mucus Production

Of all the bioactive compounds present in Livaux™, only leucine was found to have direct targets in the mucus production pathway. Table 3 lists the amount of leucine present in various dose levels of Livaux™ and two whole gold kiwifruit.

Table 3: Quantification of bioactive compounds in Livaux™ and gold kiwifruit

Bioactive Compound	Livaux™ Dose Levels		Two Gold Kiwifruit (150 g)
	600 mg	2400 mg	
Leucine (mg)	1.38	5.52	85

Source: Anagenix Data

Effect of leucine in Livaux™ on the mucin 2, a biomarker of mucus production pathway was measured. Mucus production is positively correlated with gut motility, which means, higher amounts of mucin 2 production favors gut motility.

Three sets of results from the simulation of leucine in Livaux™ and in whole gold kiwifruit at recommended dose levels on mucus production were obtained:

1. Comparison of 600 mg/day Livaux™ versus 2,400 mg/day Livaux™ versus two whole gold kiwifruit on mucin 2 for a period of one (1) day (Figure 11A);

2. Comparison of 600 mg/day Livaux™ versus 2,400 mg/day Livaux™ versus two whole gold kiwifruit on mucin 2 for a period of 30 days (Figure 11B); and,

3. Comparison of 600 mg/day Livaux™ versus 2,400 Livaux™ versus 2,400 mg/day Livaux for three days followed by 600 mg/day Livaux™ for 27 days on mucin 2 (Figure 11C).

For these simulations, the system was assumed to be under dysfunctional state with no Livaux™ or gold kiwifruit dose.

In the first set of results, under control conditions, in absence of Livaux™ and gold kiwifruit, the levels of mucin 2 were estimated to be 0 nM under the dysfunctional conditions. At the end of 1-day period, Livaux™ at 600 mg/day, Livaux™ at 2,400 mg/day, and two gold kiwifruit increased the mucin 2 levels to 0.33 nM, 0.35 nM, and 0.36 nM, respectively, as shown in Figure 11A. These results indicate that the bioactive compounds of Livaux™ increased the mucin 2 levels significantly at both single dose levels by a similar extent.

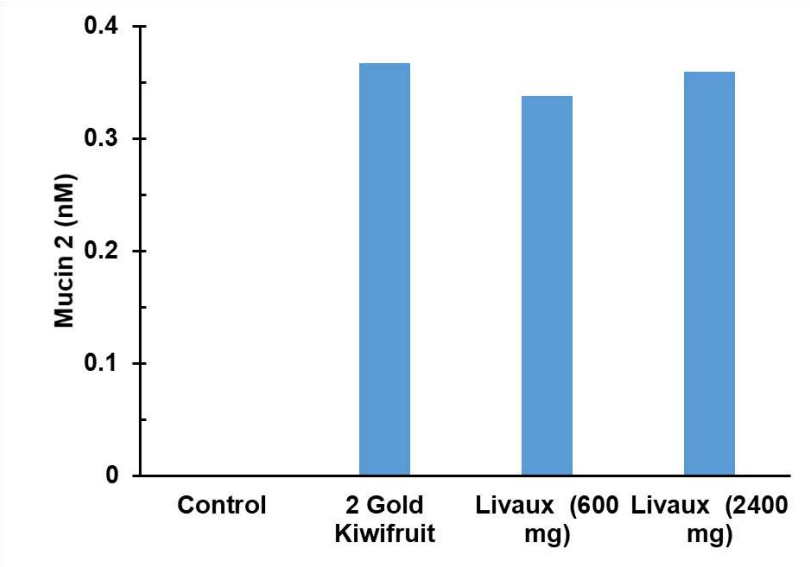

Figure 11A: Effect of leucine in Livaux™ and in gold kiwifruit on mucin 2 at a single dose over 1-day period.

The second set of results are shown in Figure 11B. Under control conditions, in absence of Livaux™ and gold kiwifruit, levels of mucin 2 were 2.94 nM under the dysfunctional conditions. At the end of 30-day period, the mucin 2 levels reached a maximum value of 4.54 nM for both doses of Livaux™ at 600 mg/day as well as for two kiwifruit per day. These results indicate that the Livaux™ is equally effective on mucus production as that of gold kiwifruit.

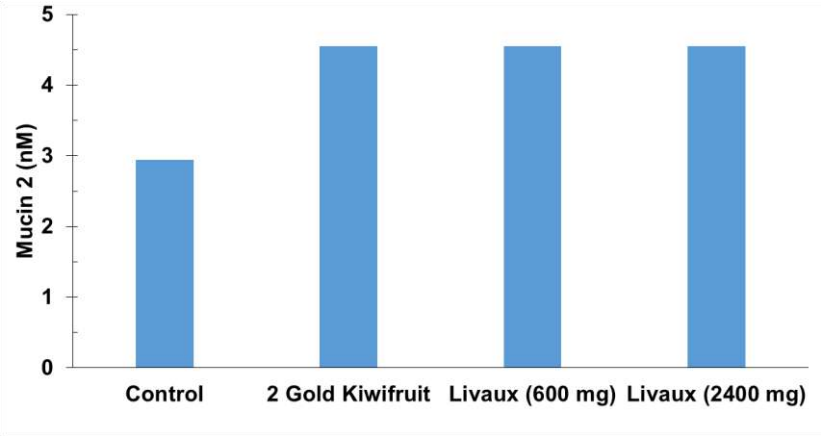

Figure 11B: Effect of leucine in Livaux™ and in gold kiwifruit on mucin 2 at a fixed dose over 30-day period.

In the third set of results as shown in Figure 11C, the dose regimen included Livaux™ at 2,400 mg/day for three days followed by 600 mg/day for 27 days and results were compared to that of fixed dosing of Livaux™ at 600 mg/day and 2,400 mg/day for 30 days. As shown in Figure 11C, the mucin 2 levels were same for the modified dose regimen as that of fixed dose regimen of 600 mg/day and 2,400 mg/day over a 30-day period.

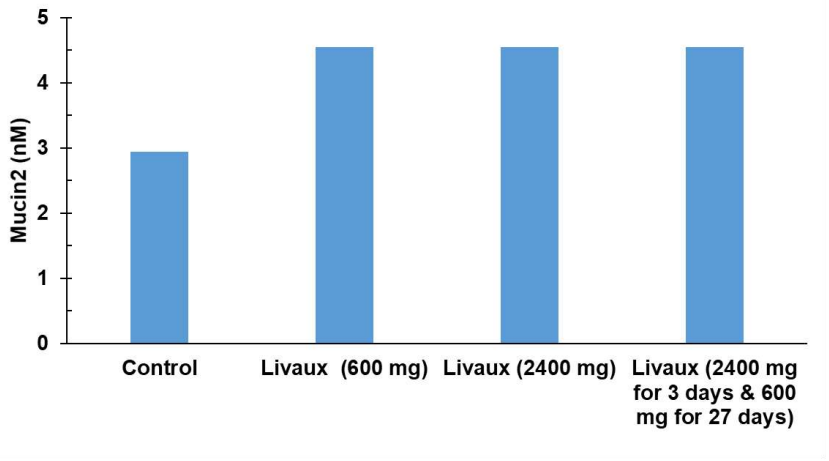

Figure 11C: Effect of Livaux™ regimen of 2,400 mg/day for three days followed by 600 mg/day for 27 days on mucin 2 production

5.4 Synergistic Effect of Bioactive Components in Livaux™ on Fecal Bulking

Fermented SCFAs such as propionate from the prebiotic fiber and polyphenolic compound luteolin in Livaux™ were found to have direct targets in the gut transit pathway and acetylcholine induced smooth muscle cell contractility pathway, respectively.

Table 4 lists the amounts of the bioactive compounds present in various dose levels of Livaux™ and two whole gold kiwifruit.

Table 4: Quantification of bioactive compounds in Livaux™ and gold kiwifruit

Bioactive Compound	Livaux™ Dose Levels		Two Gold Kiwifruit
	600 mg	2400 mg	150 g
Propionate (µM)	217	868	1033
Luteolin (mg)	0.006	0.024	3.38

Source: Propionate – Anagenix data; Luteolin - Lugasi and Takács, 2002

Synergistic effect of bioactive molecules in Livaux™ on PYY and GLP-1, biomarkers of gut transit pathway, and on PKC-α, a biomarker of acetylcholine induced smooth muscle cell contractility pathway was estimated and the results are discussed in detail below.

5.4.1 Effect of propionate from fermented Livaux™ on gut transit pathway

Three sets of results from the simulation of propionate from fermented Livaux™ and from fermented whole gold kiwifruit at recommended dose levels on gut transit pathway were obtained:

1. Comparison of 600 mg/day Livaux™ versus 2,400 mg/day Livaux™ versus two whole gold kiwifruit on PYY and GLP-1 for a period of one (1) day (Figure 12 A and B);

2. Comparison of 600 mg/day Livaux™ versus 2,400 mg/day Livaux™ versus two whole gold kiwifruit on PYY and GLP-1 for a period of 30 days (Figure 12 C and D); and,

3. Comparison of 600 mg/day Livaux™ versus 2,400 Livaux™ versus 2,400 mg/day Livaux for three days followed by 600 mg/day Livaux™ for 27 days on PYY and GLP-1 (Figure 12 E and F).

For these simulations, the system was assumed to be under dysfunctional state with no Livaux™ or gold kiwifruit dose.

In the first set of results, under control conditions, in absence of Livaux™ and gold kiwifruit, the levels of PYY and GLP-1 were estimated to be 1.42 nM and 0 nM, respectively under the dysfunctional conditions. At the end of 1-day period, Livaux™ at 600 mg/day, Livaux™ at 2,400 mg/day, and two gold kiwifruit increased the PYY levels to 71.9 nM, 72.35 nM, and 72.43 nM, respectively, as shown in Figure 12A.

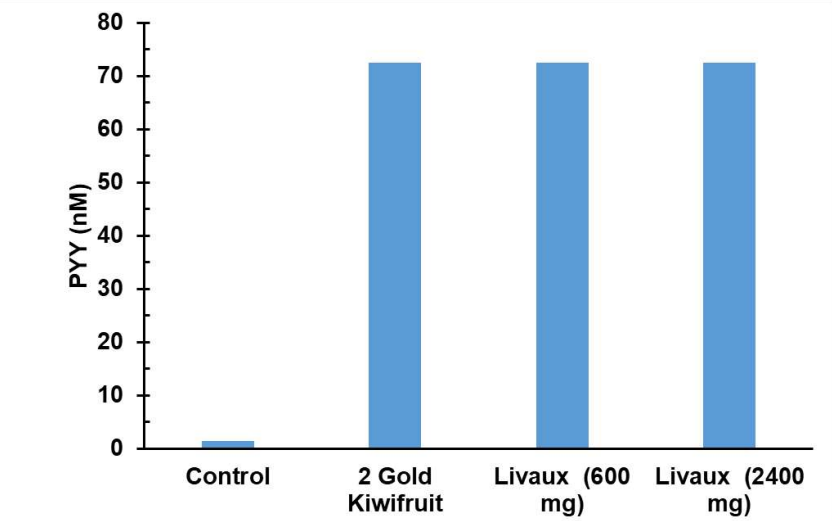

Figure 12A: Effect of propionate from fermented Livaux™ and fermented gold kiwifruit on PYY at a single dose over 1-day period.

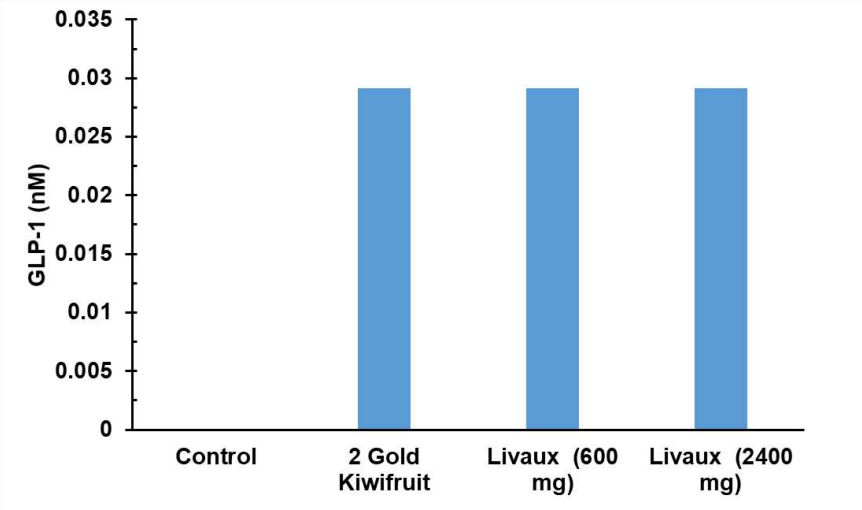

Figure 12B: Effect of propionate from fermented Livaux™ and fermented gold kiwifruit on GLP-1 at a single dose over 1-day period.

At the end of 1-day period, GLP-1 levels increased to 0.029 nM for both doses of Livaux™ and two gold kiwifruit, as shown in Figure 12B.

In the second set of results, under control conditions, in absence of Livaux™ and gold kiwifruit, the levels of PYY and GLP-1 were 1.4 nM and 0 nM, respectively, under dysfunctional state. At the end of 30-day period, Livaux™ for 600 mg/day, 2,400 mg/day and two gold kiwifruit, PYY levels reached a maximum value of 73 nM as shown in Figure 12C, and GLP-1 levels reached a maximum value of 0.029 nM, as shown in Figure 12D. The high amounts of propionate indicate the prebiotic potential of Livaux™, which has also translated into higher PYY and GLP-1 production. The increased PYY and GLP-1 formation means longer gut transit time and more efficient fecal bulking. These results indicate that fermented prebiotic fiber of Livaux™ is equally effective on increasing gut transit time and consequently fecal bulking, as that of gold kiwifruit.

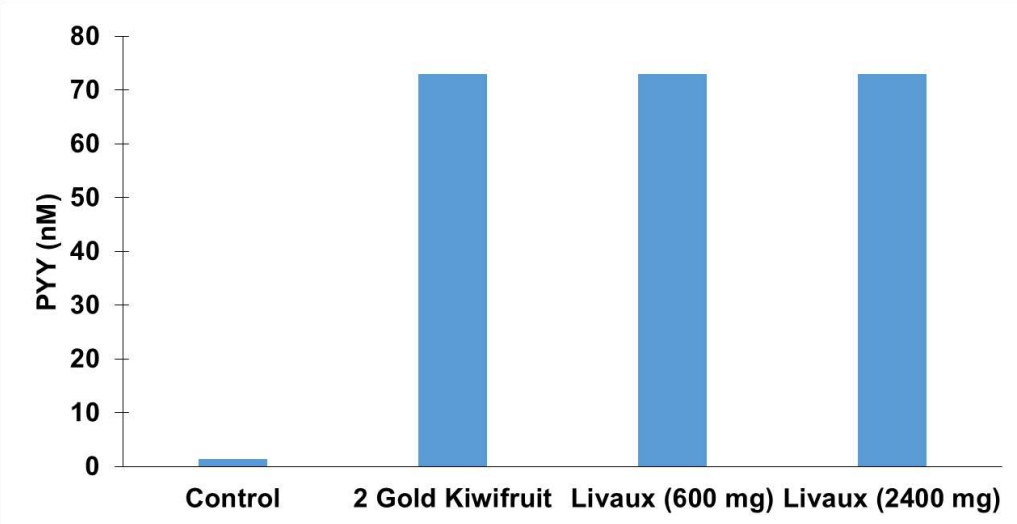

Figure 12C: Effect of propionate from fermented Livaux™ and fermented gold kiwifruit on PYY at a fixed dose over 30-day period.

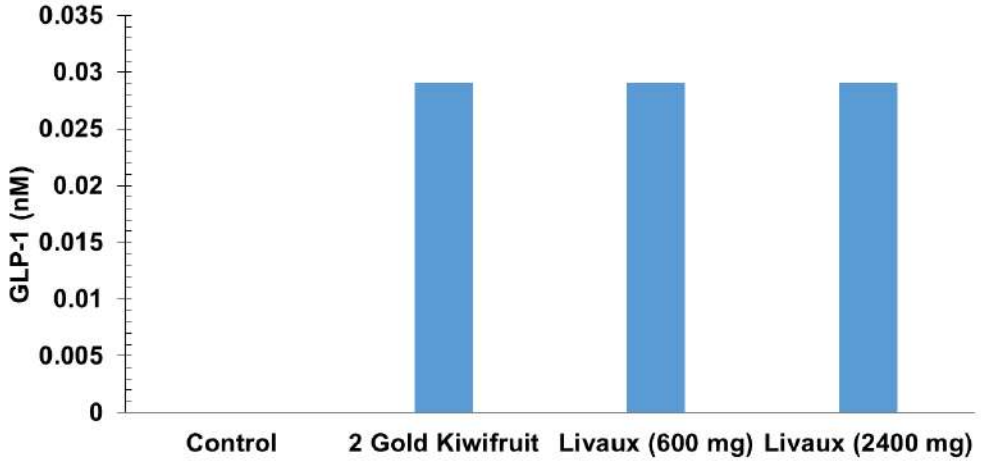

Figure 12D: Effect of propionate from fermented Livaux™ and fermented gold kiwifruit on GLP-1 at a fixed dose over 30-day period.

In the third set of results, the dose regimen included Livaux™ at 2,400 mg/day for three days followed by 600 mg/day for 27 days and results were compared to that of fixed dosing of Livaux™ at 600 mg/day and 2,400 mg/day for 30 days. The PYY and GLP-1 levels were same for the modified dose regimen, as shown in Figure 12E and 12F, respectively, as that of fixed dose regimen of 600 mg/day and 2,400 mg/day over a 30-day period.

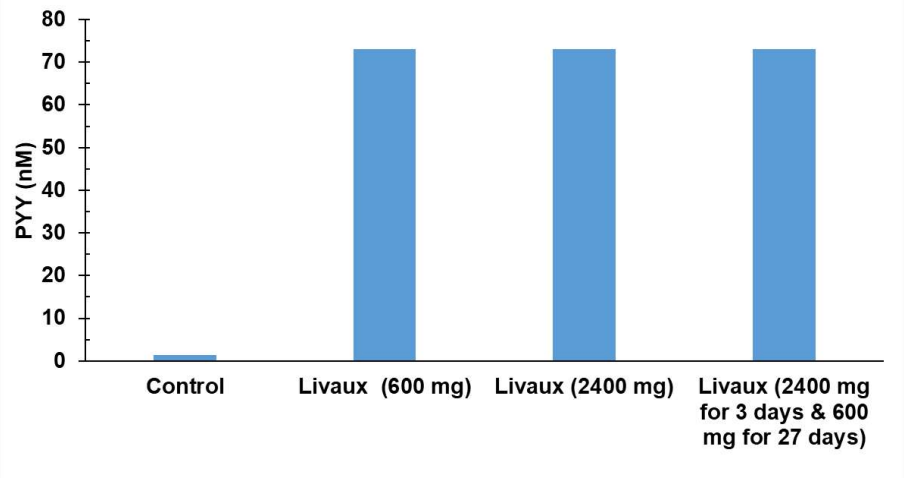

Figure 12E: Effect of Livaux™ regimen of 2,400 mg/day for three days followed by 600 mg/day for 27 days on PYY production.

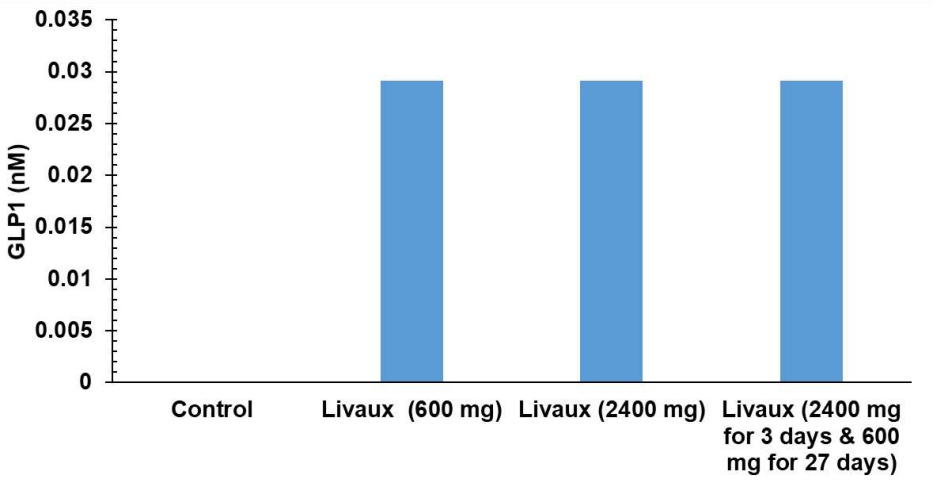

Figure 12F: Effect of Livaux™ regimen of 2,400 mg/day for three days followed by 600 mg/day for 27 days on PYY production.

5.4.2 Effect of luteolin on acetylcholine induced smooth muscle cell contractility pathway

Three sets of results from the simulation of luteolin in Livaux™ and in whole gold kiwifruit at recommended dose levels on acetylcholine induced smooth muscle cell contractility pathway were obtained:

1. Comparison of 600 mg/day Livaux™ versus 2,400 mg/day Livaux™ versus two whole gold kiwifruit on PKC-α levels for a period of one (1) day (Figure 13A);

2. Comparison of 600 mg/day Livaux™ versus 2,400 mg/day Livaux™ versus two whole gold kiwifruit on PKC-α levels for a period of 30 days (Figure 13B); and,

3. Comparison of 600 mg/day Livaux™ versus 2,400 Livaux™ versus 2,400 mg/day Livaux for three days followed by 600 mg/day Livaux™ for 27 days on mucin 2 PKC-α levels (Figure 13C).

For these simulations, the system was assumed to be under dysfunctional state with no Livaux™ or gold kiwifruit dose.

In the first set of results, under control conditions, in absence of Livaux™ and gold kiwifruit, the levels of PKC-α were estimated to be 0.13 nM under the dysfunctional conditions. At the end of 1-day period, Livaux™ at 600 mg/day, Livaux™ at 2,400 mg/day, and two gold kiwifruit downregulated the PKC-α levels to 0.025 nM, 0.0075 nM, and 0 nM, respectively, as shown in Figure 13A. These results indicate that the bioactive compounds of Livaux™ reduced the PKC-α levels significantly at both dose levels.

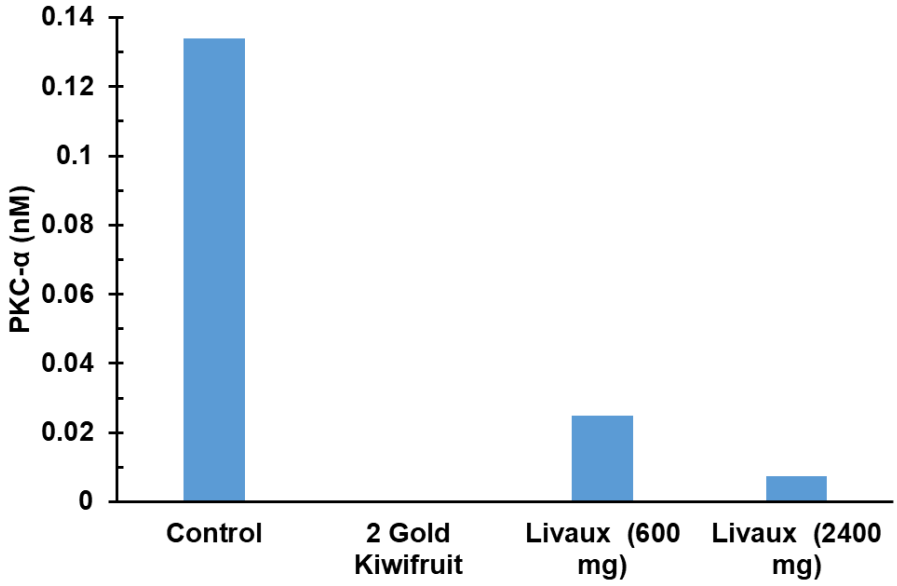

Figure 13A: Effect of luteolin in Livaux™ and in gold kiwifruit on PKC-α at a single dose over 1-day period.

The second set of results are shown in Figure 13B. Under control conditions, in absence of Livaux™ and gold kiwifruit, levels of PKC-α were at 3.46 nM under dysfunctional state. At the end of 30-day period, PKC-α levels were suppressed for Livaux™ at 600 mg/day

dose and at 2,400 mg/day dose. A dose of two kiwifruit per day was able to suppress PKC-α levels effectively. These results indicate that the Livaux™ is equally effective as gold kiwifruit in causing smooth muscle cell relaxation and consequently increasing the efficiency of fecal bulking.

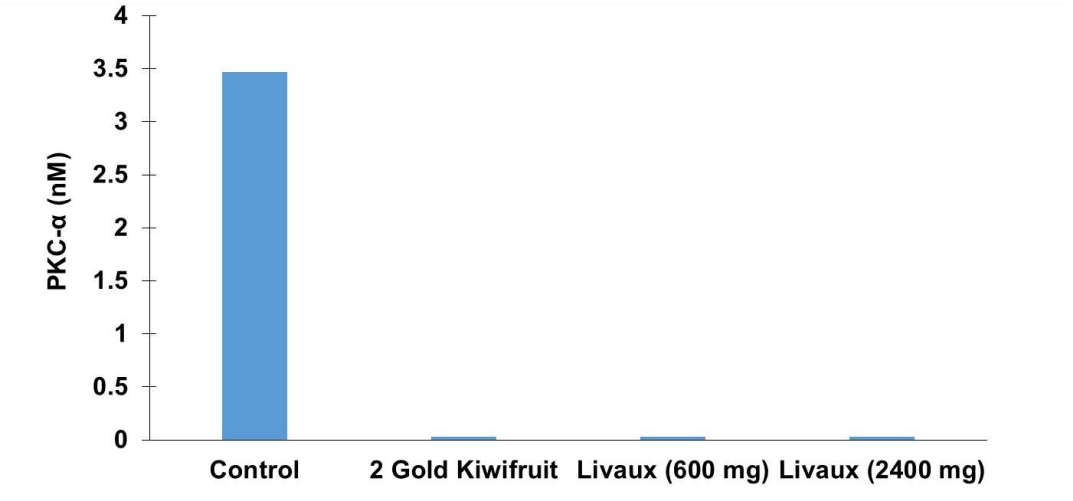

Figure 13B: Effect of luteolin in Livaux™ and in gold kiwifruit on PKC-α at a fixed dose over 30-day period.

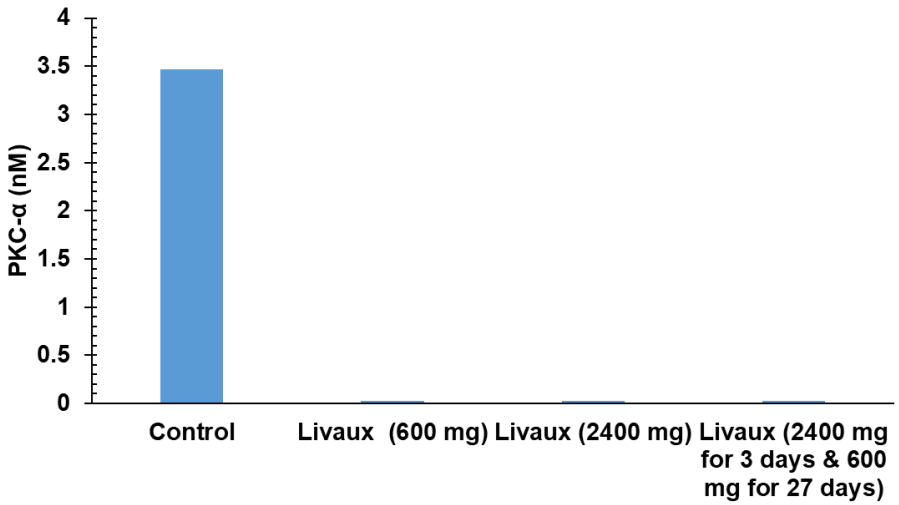

Figure 13C: Effect of Livaux™ regimen of 2,400 mg/day for three days followed by 600 mg/day for 27 days on PKC-α production

In the third set of results as shown in Figure 13C, the dose regimen included Livaux™ at 2,400 mg/day for three days followed by 600 mg/day for 27 days and results were compared to that of fixed dosing of Livaux™ at 600 mg/day and 2,400 mg/day for 30 days. As shown in Figure 13C, the PKC-α levels were same for the modified dose regimen as that of fixed dose regimen of 600 mg/day, indicating that there was no additional benefit of modifying the dose regimen.

5.5 Minimum Levels of Livaux™ Required to Achieve Significant Physiological Effect

In Figures 11B-C, 12A-F, it was observed that the biomarkers of Mucin2, PYY and GLP-1 reached the same value for both 600 mg/day and 2400 mg/day doses of Livaux™ and those levels were equal to that of two gold kiwifruit. This is a very significant result as it indicates that these biomarkers have reached the maximum possible value and the biomarkers may be very sensitive to lower concentrations of active biomolecules in Livaux™. To substantiate whether there is a minimum possible dose for Livaux™ that can elicit the maximum possible value for these biomarkers, we performed simulations at lower dose levels of Livaux™. The results are summarized in Table 5, based on the biomarker.

Table 5: Minimum amount of Livaux™ required to achieve maximum biomarker levels

Physiological Function	Biomarker	Livaux™
Gut Transit	Biomarker PYY	25 mg
Gut Transit	Biomarker GLP-1	25 mg
Mucin Production	Biomarker Mucin 2	0.5 mg

Gut transit biomarker PYY and GLP-1 reached maximum levels for Livaux™ supplementation of only 25 mg/day. Mucin production biomarker Mucin 2 was seen to be the most sensitive, as it reached its peak value at a low dose of 0.5 mg/day of Livaux™.

6.0 CONCLUDING REMARKS

A study was conducted to analyze the synergistic effects of bioactive compounds in Livaux™ on the gut motility molecular pathway systems. The conclusions of this study are as follows:

- CytoSolve literature review identified three major biological processes that affect gut motility:
    - Mucus production
    - Prebiotic activity leading to fecal bulking
    - Inflammation via
        - Oxidative stress
        - Nitric oxide
- Bioactive compounds in Livaux™ were found to have a positive synergistic effect on all three physiological processes involved in gut motility.
- Livaux™ improved gut motility by increasing mucus production, increasing smooth muscle relaxation, increasing intestinal transit time which aided fecal bulking and by reducing inflammation.
- Even at low dose levels, Livaux™ was very efficient at increasing the mucus production and gut transit time to the maximum possible levels.
- Livaux™ can be described as a holistic prebiotic since, in addition to having a substantial amount of fiber, Livaux™ also contains leucine, which helps increase mucus, and luteolin, which helps increase smooth muscle cell relaxation in the gut thereby significantly aiding bowel movement, gut motility and increases gut transit time which aids fecal bulking.

- Other prebiotics available in the market such as inulin, psyllium, FOS, GOS, etc., comprise of only fiber and none of the other components provided by Livaux™.
- Anagenix has proven in a recent clinical trial that Livaux™ boosts levels of the beneficial gut bacterium *Faecalibacterium prausnitzii* (*F. prau*) by more than 100% (Blatchford et al., 2017).
- Livaux™'s bioactive compounds affect gut motility as follows:
    - **Amino acid Leucine** increased expression of Mucin 2 gene which enhanced mucus production in enterocytes.
    - **Polyphenolic** bioactive compound **Luteolin** enhanced smooth muscle cell relaxation, consequently gut transit, via downregulation of PKC- α.
    - **Fiber** in Livaux™ acts as an effective **prebiotic** for the gut microflora that converts the fiber into short chain fatty acids such as propionate.
    - **Short chain fatty acid (SCFA) propionate**, a fermentation product of **fiber** from Livaux™, increased expression of PYY and GLP-1, which play a significant role in delaying gastric emptying, increasing gut transit time and consequently affecting fecal bulking..
    - **Antioxidant** bioactive compounds **Vitamin E, Vitamin A and Vitamin C**, and **polyphenolic** bioactive compound **Epicatechin** reduced inflammation by reducing oxidative stress biomarker ROS in the enterocytes.
    - **Antioxidant** bioactive compounds **Vitamin C and Vitamin A** reduced inflammation by lowering amount of nitric oxide produced in the enterocytes.

- As PYY and GLP-1 also play significant direct and/or indirect role in other biological processes in maintaining **glucose levels, appetite suppression, immune function**, etc. (Kuhre et al. 2016, Drucker et al. 2011), the role of Livaux™ can be further investigated for its efficacy on the aforementioned biological processes.
- At recommended dose levels over a 30-day period, Livaux™ consumption led to similar amounts of Mucin 2 production as that of gold kiwifruit over the same period of time
- At recommended dose levels over a 30-day period, Livaux™ consumption led to similar amounts of PYY and GLP-1 production as that of gold kiwifruit over the same period of time.

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