Assessment of Black Raspberries for Prostate Cancer: Product Development for Clinical Intervention and Preclinical Study for Mechanism Investigation

DISSERTATION

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By

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Abstract

A diet rich in fruits, vegetables and whole grains can help reduce the risk of many cancers (AICR, 2009). Black raspberries (BRB) (*Rubus Occidentials*) rich in a variety of bioactive compounds may contribute to the preventive effects of BRB in certain cancers with both preclinical and clinical studies. Utilizing BRB in a non-food delivery system has been studied in several cancers with promising results. However, for long term prevention, a dietary approach of utilizing novel BRB food products that are fully characterized, consistent in composition, with high compliance, and excellent bioavailability of phytochemicals may prove more effective. The primary objective of this study is to design, select and fully characterize BRB food products suitable for human cancer prevention studies. In order to better understand cancer preventive effects of BRB on systematic cancers such as prostate cancer, this study also investigates BRB dietary intervention on gene expression changes in early prostate carcinogenesis with transgenic adenocarcinoma of the mouse prostate (TRAMP) model.

Three forms of confections containing whole BRB freeze-dried powder were developed with varying release rates of phytochemicals. Successful formulations yielded hard candies (22% BRB) with fast release, pectin based confections (22% BRB) with intermediate release, and starch based confections (40% BRB) with slow release rate as determined by dissolution studies. Pectin confections were selected for scale-up
production for clinical trials due to results from sensory, texture, and storage stability studies. Nectars with optimized texture and sensory acceptance were selected. The processing, scale-up and storage effect on the consistency, quality, bioactive stability and sensory acceptability of two BRB delivery systems of varying matrices were studied. Confections and nectars retained high amount of anthocyanins and ellagitannin after processing. Texture of products remained unchanged during storage for two months at 4 ºC. BRB products were well accepted by 32 clinical subjects in a phase II dietary intervention trial. In animal study, 4-week old TRAMP mice and their non-transgenic (WT) littermates were fed with control diet or 10% BRB diet for 6 weeks. As a result, 10-week-old TRAMP mice have developed early prostatic intraepithelial neoplasia (PIN) that leads to distinct changes in prostate lobe weight, histological features, Ki67 protein expression, and prostate mRNA expression regardless of diet. Urolithins were detected and quantified in plasma, liver and prostate of mice fed with BRB by HPLC-MS/MS. Interestingly, BRB diet significantly modulated gene changes in TRAMP mice during 6 weeks of feeding which are related to cell morphology and proliferation.

In conclusion, food delivery matrix could modulate the phytochemical release rate from BRB confection and also influence sensory preference. Two different BRB foods can be formulated to meet quality standards with a consistent bioactive pattern and successfully scaled up for a large human clinical trial focusing on cancer risk and other health outcomes. As to BRB intervention on mouse prostate carcinogenesis, diet has effects on pathways and disease related networks through modulating gene expression
which may impact prostate carcinogenesis. Further efforts will focus on the connection and comparison of gene expression changes between TRAMP and human prostate.
Dedication

I dedicate my dissertation work to my parents who always love, support and motivate me to pursue my academic dream. They set up an example through their own lives to let me learn the importance and meaning of honesty, diligence and independence. I also dedicate this to my sister Jun Gu and my boyfriend Peng He, who love and support me to be the best of myself.
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Fields of Study

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Chapter 1: Introduction

It is estimated that approximately 35% cancer cases in the US are preventable though healthy dietary and lifestyle patterns (AICR, 2009). Prevention of cancer through the use of synthetic or natural compounds, foods, and food products enrich natural compounds, to delay or even suppress carcinogenesis has the potential to significantly impact the burden of cancer. Notably compounds in fruit, vegetables and whole grains are widely investigated due to their rich sources of bioactive components and large daily consumption in Asian populations who have much lower incidence of certain cancers, including prostate cancer (PCa) than Western populations (Khan et al., 2010; Siegel et al., 2015). In the United States, PCa is currently the most common cancer in men comprising 26% of all cancers and it is also the second leading cause of male cancer death after lung cancer (Siegel et al., 2015). Various dietary components have shown anticancer activity against PCa in both pre-clinical and clinical studies, including but not limited to (-)-epigallocatechin-3-gallate (EGCG) from green tea, genistein from soy, lycopene from tomato, resveratrol from grape, ellagitannins from pomegranate and curcumin from turmeric (Khan et al., 2010; Pantuck et al., 2006).

Among fruits, black raspberries (BRB) (Rubus occidentalis) have gained much attention due to their distinct antioxidant, anti-inflammatory, anti-angiogenesis and other anticancer effects demonstrated in both in vitro and in vivo studies (Kresty et al., 2001; Casto et al., 2002; Rodrigo et al., 2006; Ozgen et al., 2008; Moyer et al., 2002; Mallery
et al., 2007 & 2008), making BRB a promising candidate food used in cancer prevention. However, investigation of BRB in PCa prevention is limited both in preclinical and clinical studies.

The logic and theoretical advantages of a food-based approach for disease prevention and health are several. Our goal is to define specific food-products prepared from fruits and vegetables that are fully chemically characterized, highly desirable and easily incorporated into a diet, and will be stable over time and storage conditions. These would represent complex mixtures of bioactive phytochemicals that when consumed, may impact multiple targets representing defective signaling pathways in mammalian carcinogenesis and thus may have additive and/or synergistic activity to enhance anti-cancer efficacy. Additionally, active compounds, each provided at lower dose, may reduce risk of toxicity. Thus, the overreaching goal for current project is to prevent cancer, including PCa, through food based intervention that can be incorporated over a lifetime, where bioactive compounds found in BRB are controlled in delivery for maximal absorption and distribution, and effectively inhibit the mechanisms of carcinogenesis in the prostate.

1.1 Incidence and PCa chemoprevention

PCa is the most common non-cutaneous cancer and the second leading cause of cancer mortality in men living in developed countries. An estimated 220,800 new cases and, of these cases, 27,540 men would die in the US in 2015 (Siegel et al., 2015). Incidence of PCa in the US varies with ethnicity: African Americans being the highest (219.8 per 100,000), followed by Caucasian Americans (133.2 per 100,000), and Asian Americans being the lowest (72.5 per 100,000) (Siegel et al., 2015). In addition, death rates from PCa
in African Americans remain more than twice as high as any other groups. The well-established risk factors for PCa include age, African ancestry, a family history and certain inherited genetic conditions (American Cancer Society, 2015).

Recent studies indicate that diet plays an important role in PCa development. Asian populations with much lower incidence of PCa consume a more plant-based diet that is lower in fat compared to a western counterpart (Khan et al., 2010). The increased incidence of PCa in Asian populations may be associated with the westernized lifestyle with increased consumption of fat (Pu et al., 2004). Typical westernized diets that contain high fat, high protein and low fiber lack important components of Asian diets including plant-derived antioxidants, soy isoflavone, and tea polyphenols which may prevent cancer development (Lee et al., 2007).

PCa represents an ideal disease condition for diet based prevention due to its long latency, high incidence rates, available tumor marker, and identifiable preneoplastic lesions and risk groups (Khan et al., 2010). Thus, the administration of synthetic or natural compounds to delay or suppress carcinogenesis has garnered much attention. The application of dietary nutrients or supplements is considered the simplest way for primary prevention (Poppel & Tombal, 2011). Various protective compounds derived from fruits and vegetables have demonstrated preventive effects against PCa development or progression in cell culture, animal models and human clinical trials including lycopene, resveratrol, ellagitannins, genistein, (-)-epigallocatechin-3-gallate, and curcumin (Khan et al., 2010). Incorporation of such compounds in the diets may provide new strategies for PCa prevention.
1.2 Bioactive compounds in BRB

BRB has two closely related species: *Rubus leucodermis* and *Rubus occidentalis*. Both species are native to North America. BRB from *Rubus occidentalis* single variety Jewel claims to contain the highest concentrations of anthocyanins and ellagitannins and demonstrates stronger antioxidant activity compared with other commercialized berries (Ozgen et al., 2008; Moyer et al., 2002). In addition, BRB contain a wide and complete range of compounds such as fiber, vitamin A, C and E, folic acid, calcium, selenium, β-sitosterol, ellagic acid, ferulic acid, bioflavonoids, ellagitannins and anthocyanins that are hypothesized to contribute to the bioactivities of BRB (Stoner, 2009). Among them, anthocyanins and ellagitannins are the most widely studied for their cancer preventive effects (Cooke et al., 2005; Ross et al., 2007; Wang et al., 2009) and found in higher concentrations in BRB compared to other berries (Stoner, 2009; Landete, 2011). Other compounds also studied for potential anticancer activity include sitosterol, ferulic acid and quercetin glycoside (Han et al., 2005; Erlund, 2004).

1.2.1 Anthocyanins in BRB

There are five anthocyanins reported in BRB and their relative quantities in Ohio berries (Jewel) were cyanidin 3-glucoside (7.5%), cyanidin 3-sambubioside (3.5%), cyanidin 3-xylosylrutinoside (36.8%), cyanidin 3-rutinoside (51.8%) and pelargonidin 3-rutinoside (0.8%) (Tian et al., 2006a). Their structure and HPLC profile are shown in Figure 1.1. Cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside are considered the primary phenolic antioxidants in BRB (Tulio et al., 2008).
Figure 1.1 HPLC profile of anthocyanin extract of BRB (Rubus Occidentalis) at 520 nm. 1. Cyanidin 3-glucoside, 2. Cyanidin 3-sambubioside, 3. Cyanidin 3-xylosylrutinoside, 4. Cyanidin 3-rutinoside, 5. Pelargonidin 3-rutinoside (Tian et al., 2006 a, b).

Anthocyanin concentration varies in different BRB seedling populations, and differences between the lowest and highest was more than two fold (244.8 and 541.3 mg/100 g fresh weight) (Dossett et al., 2010). BRB (Jewel cultivar) obtained from Ohio Stoke Raspberry Farm was reported to have 32 mg/g anthocyanins in dry BRB powder (Stoner et al., 2005) which was 479.8 mg/100 g fresh if water content is 85% according to
USDA reported data on raspberries. The contents of anthocyanins in different berries are shown in Table 1.1.

<table>
<thead>
<tr>
<th>Berries</th>
<th>Total anthocyanins (mg/100 g fresh weight)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black raspberry</td>
<td>244.8-541.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Dossett et al., 2010</td>
</tr>
<tr>
<td>Red raspberry</td>
<td>79.2; 92.1; usually &lt; 100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Rao &amp; Snyder, 2010</td>
</tr>
<tr>
<td>Blackberry</td>
<td>114.4-241.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cho et al., 2004</td>
</tr>
<tr>
<td>Strawberry</td>
<td>15-93.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Giampieri et al., 2012</td>
</tr>
<tr>
<td>Blueberry</td>
<td>143.5-822.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cho et al., 2004</td>
</tr>
</tbody>
</table>

<sup>a</sup>Quantified in each category of anthocyanins. <sup>b</sup>Quantified as cyanidin 3-glucoside equivalents. <sup>c</sup>Quantified as cyanidin 3-rutinoside and pelogonidin 3-glucoside equivalents.

1.2.2 Ellagitannins and ellagic acid in BRB

Raspberries contain appreciable levels of ellagitannins which is a major source of dietary ellagic acid (Gasperotti et al., 2010) and the relative amount of free ellagic acid was < 6% for all fruits (Rao & Snyder, 2010). Ellagic acid, sanguin H-6 and lambertianin C are the three major types of ellagitannins in the raspberries and the structures are shown in Figure 1.2.
Figure 1.2 Structures of ellagic acid (1), sanguin H-6 (2) and lambertianin C (3) (Gasperotti et al., 2010).

Ellagitannins are found in different fruits and nuts. Contents of ellagitannins in several berries measured by hydrolyzed ellagic acids are shown in Table 1.2. Rao & Snyder (2010) converted total ellagic acid in mg/100 g fresh weight from what was done in Daniel et al. (1989). However, the accurate quantification of ellagitannins and ellagic acid conjugates in berries was hindered by the lack of pure standard compounds. The widely used method for quantification of these compounds is by HPLC analysis of ellagic acid or gallic acid after ellagitannins hydrolysis (Mullen et al., 2003), but this method loses molecular structure information and does not accurately determine the intrinsic concentration due to difference in their structures.
Table 1.2 Concentrations of hydrolyzed ellagic acid in fruits

<table>
<thead>
<tr>
<th>Berries</th>
<th>Total ellagic acid (mg/100 g fresh weight)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black raspberry</td>
<td>33.8</td>
<td>Stoner, 2009</td>
</tr>
<tr>
<td>Red raspberry</td>
<td>21.4</td>
<td>Rao &amp; Snyder, 2010</td>
</tr>
<tr>
<td>Blackberry</td>
<td>21.4</td>
<td>Rao &amp; Snyder, 2010</td>
</tr>
<tr>
<td>Strawberry</td>
<td>9.0</td>
<td>Rao &amp; Snyder, 2010</td>
</tr>
<tr>
<td>Walnuts</td>
<td>8.4</td>
<td>Rao &amp; Snyder, 2010</td>
</tr>
</tbody>
</table>

Gasperotti et al. (2010) purified sanguin H-6 and lambertianin C from raspberry (Rubus Ideaeus L.) with high degree of purity (log ε_{220} nm for lambertianin C is 5.29 and log ε_{265} nm is 5.02). It was shown in their study that ellagitannins comprised more than 80% of total content of ellagitannins and ellagic acid; lambertianin C (32.2% - 38.3%) and sanguin H-6 (42.9% - 49.6%) were the two primary ellagitannins detected in six raspberries cultivars. Thus far, this is the first and only reported accurate quantification of ellagitannins by DPLC-Q-TOF HDMS and HPLC-DAD analysis.

1.3 Digestion, absorption and metabolism of anthocyanins and ellagitannins

The absorption and metabolism of anthocyanins are illustrated in Figure 1.3 (McGhie & Walton, 2007). BRB anthocyanins were reportedly absorbed within 2 h of ingestion and excreted in urine mainly as the intact form including a minor amount in methylated derivatives. However, less than 1% of the ingested dose appeared in the urine (Stoner et al., 2005; Tian et al., 2006b). This absorption of the intact form makes the bioavailability of anthocyanins different from other flavonoids which are absorbed as aglycones (McGhie & Walton, 2007).
Figure 1.3 Absorption and metabolism of anthocyanins based on current research (McGhie & Walton, 2007).
Absorption of anthocyanins begins in the stomach, and then occurs significantly in the jejunum and slightly in the duodenal tissue, with none detected in the ileum and colon (Matuschek et al., 2005). Anthocyanins are not stable in the small intestine due to pH change and are further converted to a combination of hemiketal, chalcone, and quinonoidial forms through hydration, tautomerization and proton loss, respectively. These compounds then undergo metabolism and excretion and cannot be detected by HPLC (McGhie & Walton, 2007). Further degradation of anthocyanins by colonic microflora results in the formation of aglycone and finally various phenolic acids (González-Barrio et al., 2011). $^{13}$C-labeled cyaniding-3-glucoside ($^{13}$C$_5$-C3G) was fed to eight healthy human subjects and C3G degradation products, protocatechuic acid (PCA) and phloroglucinaldehyde (PGA), and 14 their derived metabolites were detected in serum with HPLC-ESI-MS/MS analysis (Ferrars et al., 2014). Possible pathway for C3G metabolized into various phenolic acids in human was proposed in Ferrars et al. (2014). These metabolites may contribute to the overall health effects of anthocyanins (McGhie & Walton, 2007; Kay et al., 2009; Larrosa et al., 2009).

Ellagitannins belong to the hydrolysable tannin class and can be hydrolyzed into ellagic acid during enzymatic digestion in the GI tract, where low concentration of ellagic acid is absorbed due to its low solubility in plasma (Saura-Calixto & Pérez-Jiménez, 2009). Ellagic acid can be further degraded into urolithin A, B and C through microbial transformation and further absorption into the plasma as glucuronides derivatives (Cerdá et al., 2005). The proposed pathways are shown in Figure 1.4 (González-Barrio et al., 2011). Urolithins can reach micromolar (mM) concentrations in the bloodstream; much
greater than ellagitannins and ellagic acid (Cerdá et al., 2005). Tomás-Barberán et al. (2014) reported three phenotypes for urolithin production were observed in different human intervention trial: only urolithin A conjugates (25-80% of volunteers), iso-uro lithin A and/or urolithin B in addition to urolithin A (10-50%) and no urolithin (5-25%) groups. This phenotype was independent of ellagitannin food source, age and health status of volunteers, which could show human microbiota difference.

**Figure 1.4** Proposed pathways for the conversion of ellagitannins to ellagic acid and urolithins in anaerobic fecal suspensions (González-Barrio et al., 2011).

### 1.4 Integrate BRB into food products for cancer prevention studies

1.4.1 Whole food application to improve chemoprevention

The logic and theoretical advantages of a food-based approach for disease prevention and health are several. However, studies of specific foods have been limited by the
variation in bioactive content and incomplete chemical characterization. Specific developed food-products derived from fruits and vegetables can be fully chemically characterized, highly desirable, easily incorporated into a diet, and stable over time and storage conditions for long-term human trials of health and disease outcomes. These would represent complex mixtures of bioactive phytochemicals that when consumed, may impact multiple targets representing defective signaling pathways in mammalian carcinogenesis and thus may have additive and/or synergistic activity to enhance anti-cancer efficacy. Additionally, active agents, each provided at lower dose, may reduce risk of toxicity.

1.4.2 Controlled food matrix for BRB delivery

Food matrix is a key factor that influences the release and absorption of bioactive components (Yang et al., 2011). However, most studies focused on the absorption of bioactive extracts in water or bioactives as part of a meal with other food components (Yang et al., 2011), with limited studies investigating the effects of food matrix on the absorption of bioactive compounds (Walton et al., 2009). Nonspecific bioactive delivery and uncertainty of the true bioactive agents are some of the hurdles slowing the clinical application of foods in cancer prevention (Scalbert & Williamson, 2000; Manach et al., 2004; Hollman, 2004). Two forms of BRB have been used in clinical trials thus far: freeze-dried BRB powder with added water and a non-food based bioadhesive BRB gel (Stoner et al., 2005; Mallery et al., 2007). Compared to the freeze-dried BRB powder used in previous clinical trials, the bioadhesive gel improved the delivery of bioactive components to the local oral tissue with efficient penetrative properties. Although this
pharmaceutical form of bioactive delivery is promising, a dietary means of BRB delivery is more attractive for a prevention regiment in a long term clinical trial.

i. Confection as the solid food matrix for BRB delivery

Solids in the field of food technology are generally classified into crystalline and amorphous. Amorphous solids, including glassy and rubbery forms, are preferred matrices in pharmaceutical agents due to their higher solubility and rate of release (Yu, 2001; Leuner & Dressman, 2000; Craig et al., 1999). A confection is an ideal food matrix for controlled BRB delivery because it can be formulated into different amorphous states by capitalizing on the physical properties of diverse food polymers while achieving high sensory acceptability and adequate shelf life. Confections vary widely in their physical properties from gummy candies (rubbery solid) to hard candies (glassy solid) which will influence the release rate of bioactive components of BRB.

In rubbery confections, a gelling agent must be chosen such as starch, pectin, gelatin, agar and gums (Burey et al., 2009). Polymer gels form a three-dimensional network that can entrap and transport bioactives, and accordingly protect them from their proximate environment, favoring targeted delivery (Yu, 2001). This controlled and continuous release of bioactives may contribute to their absorption without saturating the transport system which has yet to be defined. Other major components in confections are sucrose, glucose syrup, and water (Burey et al., 2009) which act as plasticizers affecting the amorphous state of confections and thereby altering the release of bioactives. In glassy confections, these plasticizers are the major components and no gelling agents are included, thus they may exhibit different dissolution properties from the rubbery
confections in release of bioactives. Moreover, added whole BRB freeze-dried powder may influence confection texture by changing concentrations of solid composition, changing pH and interacting with other ingredients. Therefore, the preparation of the gel requires a combination of food science and pharmacology.

ii. Nectar as the liquid food matrix for BRB delivery

Compared to solid matrix, pure liquid delivers bioactives faster to the gastrointestinal tract (Ahn-Jarvis et al., 2012). Previous clinical trials with pomegranate and BRB were all delivered as juice, and results showed that ellagic acid was detected in plasma and urine (González-Sarrías et al., 2010; Stoner et al., 2005). It is believed that the addition of some stabilizer (such as pectin, xanthan and agar) to the nectar would increase viscosity and thus aid in suspension of particles (Neidhart et al., 2002).

Currently, the influence of food matrix on the delivery and metabolism of bioactive compounds has not been well investigated. Walton et al. (2009) reported that blackcurrant anthocyanins in a more viscous oatmeal matrix had delayed and also decreased the absorption and excretion of anthocyanins but did not impact the metabolism in a rat model compared with anthocyanins in water. Ahn-Jarvis et al. (2012) studied the excretion of microbial metabolites from soy isoflavone with men and women consuming soy bread versus soy beverage. They found that females consuming soy bread excreted significantly higher isoflavone microbial metabolites than the ones consuming soy beverage while men have not shown distinct difference between two matrices. This may be due to the longer transit and contact time with microbial in colon. Whether the difference in absorption and excretion of bioactive compounds from food matrices has an influence on the cancer
prevention is still unknown. Thus, the difference of confection and nectar matrix on bioactives’ retention after processing, their absorption *in vivo* and their metabolites’ deposition in tissue is a critical factor that may affect the downstream anticancer activity of a BRB based intervention. This study will focus on the retention of bioactives after processing with different food matrices.

1.4.3 Effects of processing and storage on phenolics of BRB food products

Processing methods and storage can influence the levels of berry phenolics. Loss of anthocyanins may occur during processing due to the presence of oxygen, high temperature, light, enzymes, and pH fluctuations (McGhie & Walton, 2007). Significant losses of monomeric anthocyanins were found in different berry products: BRB puree (37%), clarified and nonclarified juices (73% and 69%). Storage at 25 ºC of all these berry products resulted in marked loss of monomeric anthocyanins but not antioxidant capacity, which may indicate that anthocyanins were extensively polymerized with other phenolics (Hager *et al.*, 2008).

Compared to anthocyanins, ellagitannins levels are not affected by pasteurization but by pressing during juice production. This may be due to removal of seeds where ellagitannins are predominantly located (Howard *et al.*, 2012). Total ellagitannins were reported to be retained well in purees and canned BRB products and also during their storage over 6 months at 25 ºC. During juice storage, total ellagitannins level changed little while ellagic acid increased (Hager, 2008). Increased ellagic acid in raspberry juice after heat processing may result from better extraction from the cell matrix (Rommel & Wrolstad, 1993). Free ellagic acid was increased during processing and storage mainly
due to the hydrolysis of ellagitannins. However, ellagic acid derivatives were not affected by thermal processing and remained stable during processing and storage in red raspberry jams (Zafrilla et al., 2001).

In addition to processing and storage, other factors such as growing location, cultivar, maturity and post-harvest storage all influence the bioactive compounds’ levels. Previous studies have shown that the concentrations of ellagic acid and anthocyanins in BRB vary on a per-farm basis in Ohio (Tulio et al., 2008). Freezing and subsequent frozen storage was shown to have little effect on red raspberry phytochemicals (Mullen et al., 2002). Stoner (2009) reported that the levels of 26 nutrients in BRB remained within 10% - 20% of the original measurements for at least 2 years in the freeze-dried form stored at -20 ºC. All of these factors should be taken into consideration in the functional BRB product development in order to retain maximum and consistent levels of the bioactives for the clinical trial.

1.5 BRB as promising PCa prevention agent

1.5.1 Gastrointestinal-tract anticancer studies of BRB

As a fruit highly yielded in Ohio, studies of BRB on gastrointestinal tract related cancers, such as oral, esophageal and colon cancer, have been well studied by Stoner group at The Ohio State University in cell cultures, animal models and several human clinical trials (Kresty et al., 2001; Casto et al., 2002; Mallery et al., 2007 & 2008; Wang et al., 2009, 2010 & 2011). These studies showed the promising preventive effects of BRB in cancer models through anti-proliferative, apoptosis inducing, anti-inflammatory, cell growth inhibition, oxidative stress reduction and angiogenesis inhibition effects. Figure
1.5 shows the effects of berry bioactives on some central cellular functions and signal transduction pathways (Stoner et al., 2008). Besides these pathways, Wang et al. (2011) reported that BRB could modulate matrix metalloproteinases related to tissue invasion/metastasis and some proteins involved in cell-cell adhesion in rat preneoplastic esophagus and esophageal papillomas tissue. Moreover, BRB were shown to demethylate promoter genes related to Wnt pathway (CDKN2A, SFRP2, SFRP5, and WIF1) in colon cancer cells or colon cancer tissues towards a protective direction through reducing expression of DNA methyltransferase 1 (DNMT1) that regulates methylation status of promoter regions of tumor suppressor genes (Wang et al., 2011 & 2013).

**Figure 1.5** The effects of berry bioactives on cellular functions and signal transduction pathways. (Stoner et al., 2008)
1.5.2 Promising PCa preventive effects of BRB

Localized absorption of BRB compounds appears to be important for their preventive effects in the oral cavity, esophageal and colon (Stoner et al., 2008). For systemic cancers such as (breast, prostate, bladder and cervical cancers), there are a few in vitro cell culture studies exploring the anticancer role of BRB extract (Seeram et al., 2006; Keatley, 2008; Zhang et al., 2012). Additionally, for in vivo studies, there is a concern that with the low uptake of the bioactive compounds into the bloodstream shown in pharmacokinetic studies (Ravoori et al., 2012; Stoner et al., 2005) could have sufficient protective effects on cancer development. Lyophilized strawberries in 10% of chow diet were not effective in preventing lung cancer in mice (Carlton et al., 2000); however, Ravoori et al. (2012) reported that daily administration of 5% freeze-dried BRB to rats with mammary tumors delayed breast tumor latency and inhibited ERα (Estrogen Receptor alpha) expression. Thus addressing factors such as the BRB bioactive compound concentration in circulation and the deposition of different bioactive metabolites or derivatives in various tissues are crucial to understand their role in systemic cancer prevention/treatment.

Studies on pomegranate enriched in punicalagin (one type of ellagitannins, > 2g/L) showed anticancer effects on PCA in cell and animal models which may provide promising evidence that BRB, a fruit also rich in ellagitannin (2mg/g dry weight) and anthocyanins (30 mg/g dry weight) (Stoner, 2009), may have preventive effects against PCa. Ellagitannin microbial metabolites, urolithins and their derivatives, showed lower IC50 values than pomegranate ellagic acid tested in LNCaP, LNCaP-AR, DU145, and 22RV1 prostate cancer cells. Urolithins were detected in mouse prostate, intestinal, and colon
tissues (Seeram et al. 2007) after consumption of urolithin A, but not detected from mice administered pomegranate extract. However, urolithin A glucuronide, urolithin B and dimethyl ellagic acid were detected in prostate tissue of small percentage of patients with benign prostatic hyperplasia (BPH) or PCa, after intake of pomegranate (200 ml/day) for 3 days (González-Sarrías et al., 2010). Notably, a phase II clinical study demonstrated mean PSA doubling time increased significantly from 15 to 54 months after 46 PCa patients consumed 8 ounces of pomegranate juice enriched with punicalagin (> 2 g/L) daily for an average of 13 months (Pantuck et al., 2006).

No animal or clinical studies have investigated the effects of BRB bioactive compounds on PCa. Therefore, such studies are needed to help further elucidate the PCa prevention potential of this berry.

1.5.3 Prostate carcinogenesis and TRAMP mouse model

Both mouse and human prostatic epithelium contain three cell types: luminal, basal and neuroendocrine which can be differentiated by their morphology, function and relevance to carcinogenesis (Abate-Shen & Shen, 2000; Roy-Burman et al., 2004). Prostatic intraepithelial neoplasia (PIN) is widely accepted as a precursor for PCa even though this is not conclusive, and characteristics of PIN include the appearance of luminal epithelial hyperplasia, basal cell reduction, nuclei and nucleoli enlargement, cytoplasmic hyperchromasias, and nuclear atypia at histological level (Shen & Abate-Shen, 2010). High grade PIN is considered to represent the immediate precursor of early invasive carcinoma. However, PIN differs from invasive carcinoma due to the characteristics such as intact basement membrane, not producing high levels of prostate specific antigen
(PSA), and only detected in biopsy samples (Abate-Shen & Shen, 2000). In the initial stages, prostate carcinoma is confined to the prostate tissue and when confined to the prostate, likely curable. In the aggressive forms, prostate carcinoma advances to stages characterized by local invasion, followed by metastasis primarily to the bone resulting, and ultimately a lethal disease progressing with castrate levels of circulating testosterone.

Regarding prostate anatomy and morphology, the human prostate gland is a single lobular tissue around the urethra at the base of the bladder and produces critical components of the seminal fluid. In addition to an anterior fibromuscular stroma, the human prostate has three architectural zones including central, periurethral transition and peripheral (McNeal, 1969, 1978 & 1988). The peripheral zone occupies the most volume where majority of prostate carcinomas occur (Shen & Abate-Shen, 2010). Different from human prostate, mouse prostate consists of four paired lobes - ventral, lateral, dorsal and anterior lobes. For analysis, the dorsal and lateral lobes are often combined as the dorsolateral lobes and showed the most similar gene expression profile to the peripheral zone of the human prostate, which is the zone in which most carcinomas occur (Berquin et al., 2005; Xue et al., 1997). Besides this anatomy and morphology difference, mice do not spontaneously develop PCa, thus using a mouse model for PCa investigation has considerable controversy. However, mice have been widely used in PCa studies due to several advantages including easy to genetically modify, relative short gestation time, and reasonably easy and affordable to house and breed (Valkenburg & Williams, 2011). Moreover, mouse models of prostate carcinogenesis provide an approach to study gene
expression signatures due to the challenges of procuring premalignant human tissue samples.

Current mouse models in PCa research include xenograft and genetically engineered models. With comparatively shorter timeframe, xenograft models have used different PCa cell lines implanted into immunodeficient mice to investigate molecular mechanisms of prostate carcinogenesis especially for chemotherapeutic studies (Shen & Abate-Shen, 2010). Aimed to better recapitulate genetic events occurring in human PCa, mouse models with gain or loss of gene functions were generated. Transgenic models with viral oncogenes, such as transgenic adenocarcinoma of the mouse prostate (TRAMP) and LADY models, and knockout models based on tumor suppressor genes including p53, pRB, PTEN and Nkx3.1 specific/conditional deletion have represented the major approaches for PCa investigations (Wu et al., 2013).

TRAMP is one frequently used mouse model of PCa. In this model, expression of the simian virus (SV40) early-region genes (T and t antigen, Tag) are driven by the prostate-specific promoter probasin that leads to cell transformation within the prostate (Greenberg et al., 1995). The SV40 large tumor T antigen (Tag) performs as an oncoprotein through interacting with retinoblastoma and p53 pathways, and small t antigen interacts with a protein phosphatase (Greenberg et al., 1995). One-hundred percent of male TRAMP mice develop PCa without any chemical or hormonal treatment. The TRAMP model of PCa progresses through normal, to pre-cancerous PIN (8-10 weeks of age), to micrometastatic adenocarcinoma (near 18 weeks of age), and ultimately may progress with distal metastases with the potential for neuroendocrine phenotypes (28-32 weeks of age) (Abate-
Shen & Shen, 2000). TRAMP was originally produced in C57BL/6 inbred background strain although tumors developed more quickly in C57BL/6 × FVB F1 mice, which indicates that genetic background may have a strong effect on tumor initiation or progression in the TRAMP model. Chiaverotti et al. (2008) have shown that TRAMP mice with FVB background had significantly shorter survival time compared with C57BL/6 TRAMP mice due to rapid development and progression of neuroendocrine carcinoma.

The TRAMP model has the ability to induce prostate specific adenocarcinoma as a consequence of probasin-directed oncogene expression, and some advantages in this model include: 1). Recapitulate many salient aspects of human PCa and significantly advanced the knowledge in molecular pathways of PCa early development and progression; 2). Androgen depletion by castration results in variably decreased tumor incidence, but ultimately progresses to androgen-independent disease, which might be beneficial to study the molecular changes as to androgen-insensitive growth of human PCa; 3). Develop metastasis to distant sites such as lung and lymph node. Additionally, research in transgenic mice has contributed to the identification of biomarkers that can predict disease recurrence, investigate novel therapies and evaluate chemopreventive agents (Shen & Abate-Shen, 2010).

Several limitations for this TRAMP model might constrain its application in studies. Although the transition from low-grade PIN to high-grade PIN to invasive carcinoma is uniform in the TRAMP model, the pace of progression is rapid and prevalently develops cancer with characteristics of neuroendocrine differentiation in the later stages of tumor.
progression, which occurs at a lower percentage in human PCa cases (Chiaverotti et al., 2008). Moreover, this model develops metastasis primarily to lungs and lymph nodes but less often to bone while metastatic PCa almost invariably metastasizes to bone in humans. There is no autochthonous model that reliably displays bone metastasis, which represents a major limitation in the study of advanced prostate cancer. In addition, the overt histological appearance of prostate carcinoma in most genetically engineered mouse models often differs from that of typical human PCa.

1.5.4 Dietary intervention in early prostate carcinogenesis

PCa mainly is a disease of older men, while prostate specimens from young men aged \( \leq 55 \) years have shown early-onset PCa (Salinas et al., 2014), indicating that cancer initiation has already started at an early age. The widespread use of PSA testing has resulted in an extensive increase in PCa diagnoses, many of which might be in latent or indolent forms of disease. At this time, there is no way to differentiate the likely latent forms from the cancers that develop into an aggressive form. Thus, it is critical to have improved measurement with molecular markers and/or other methods for more effective diagnosis and management. Regarding that PCa has a long latency, delaying its onset or progression is likely to have an invaluable impact on PCa outcome.

Epidemiological evidence suggests that dietary and lifestyle patterns may contribute to the considerable differences in PCa incidence between Asian and American populations. As to dietary factors, notably many types of plant-based food components have been studied for their cancer-preventive efficacy in the TRAMP mouse model and results have shown decreased proliferation index, reduced tumor incidence and lowered metastasis,
and these including but not limited to pomegranate fruit extract, green tea polyphenols extract, tomato/lycopene and soy genistein (Adhami et al., 2012; Gupta et al., 2001; Wang et al., 2007; Zuniga et al., 2013). While these studies are informative and supportive of a cancer preventive activity in the prostate, the molecular mechanisms of action are not yet well defined. Wan et al. (2014) evaluated the impact of dietary tomato and lycopene feeding on 200 PCa-related genes that are involved in androgen signaling and carcinogenesis in both wild type and TRAMP mice at 10 weeks of age using NanoString nCounter technology. This was the first study to investigate the murine PCa-related gene expression differences influenced by genotype, testosterone status and diet type. They found that 30 out of 189 detectable genes were significantly affected by tomato or lycopene which are associated with stem cell features (whole tomato) and neuroendocrine differentiation (lycopene). Moreover, this study indicated that gene changes initiated early prostate carcinogenesis in TRAMP mice and dietary food components could target some potential genes that might influence the PCa outcome. However, to date, there are no studies examining the effect of a BRB intervention on the transcriptional signature of the prostate while cancer is developing.

There are a number of approaches available to examine gene expression in tissues. When analyzing a pre-defined and limited number of genes, approaches such as qRT-PCR, pathway-focused PCR Arrays (Tan et al., 2014), or custom designed NanoString codesets (Wan et al., 2014) are useful options. Lately, various technologies have been developed for untargeted, large scale transcriptional analysis including hybridization- or sequence-based approaches. Microarray is a hybridization-based method involving
incubation of fluorescently labeled probe DNA to hybridize a cDNA sample for quantification of relative abundance of transcripts. However, this high throughput and relatively inexpensive approach relies on existing knowledge about genome sequence, has high levels of background due to cross-hybridization, and additionally has difficulties to compare expression levels across different experiments (Wang Z. et al., 2009).

RNA-Seq is a technology that uses the deep-sequencing to reveal a snapshot of the whole transcriptome, providing the ability to measure differences in gene expression, gene spliced transcripts, post-transcriptional modifications, gene fusion, and mutations/single nucleotide polymorphisms (SNPs) (Oshlack et al., 2010). Total or fractionated pure RNA is first converted to a library of cDNA fragments with adaptors attached to one or both ends. High-throughput platform, such as Illumina GA/HiSeq, ABI SOLiD and Roche 454 Life Science, generates millions of short reads sequenced from one end (single-end sequencing) or both ends (pair-end sequencing) of those cDNA fragments. Resulting reads with 30-400 bp depending on the used platform are then aligned to a reference genome or assembled without the genomic sequence to generate expression profile with transcriptional structure and abundance of each gene (Wang L. et al., 2009; Wilhelm & Landry, 2009). With high sensitivity and accuracy, RNA-Seq does not depend on existing genome sequence, but provides more sequence/structural variations, has a wide range of detection as well as requires low RNA sample quantity for analysis. As a technique to allow the whole transcriptome to be sequenced in a high-throughput approach, vast data storage and complex analysis often bring challenges. Specifically, detection of difference in gene level expression between groups such as wild type and transgenic strains, and
untreated versus treated has been studied with RNA-Seq (Oshlack et al., 2010). This untargeted approach allows for the examination of genes and pathways for which there is evidence from the literature of a potential impact, as well as the identification of novel pathways that may be impacted by the intervention.

This project aims to optimize the quality and bioactives’ stability of food products containing freeze-dried BRB intended for a phase I/II PCa clinical trial. Moreover, the effects of BRB on molecular changes in early stages of PCa with TRAMP model will be investigated.

The long-term research goal is to formulate functional food products containing BRB of high quality that are optimized in delivery of bioactives for PCa prevention trials. The hypotheses are that the release of bioactives from BRB to targeted sites can be modulated through different food matrices and specific BRB components have cancer-preventive effects on PCa. Based on these hypotheses, two specific objectives are proposed:

**Aim # 1**: Develop and optimize BRB functional food products as a targeted system for the delivery of BRB bioactive components and to obtain clinical compliance and sensory acceptance in a human clinical trial with men suffering from resectable PCa.

*Sub aim # 1: Develop BRB food products with stable and optimized BRB bioactive compounds that deliver a known concentration of these compounds while maintaining overall sensory acceptability and quality.*

*Sub aim # 2: Assess consistency, bioactive retention and shelf-life due to scale-up production of BRB food products utilized in a phase I/II prostate cancer clinical trial.*
**Aim # 2:** Investigate dietary BRB intervention on early prostate carcinogenesis with TRAMP model.

*Sub aim # 1:* Investigate transcriptional changes in the prostate of wild type and TRAMP mouse after BRB intervention.

*Sub aim # 2:* Investigate changes in prostate proliferation biomarkers of TRAMP mouse after BRB intervention.

*Sub aim # 3:* Investigate biodistribution of BRB bioactive compound metabolites in mouse liver, plasma, and prostate with HPLC-MS/MS analysis.
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Chapter 2: Development and characterization of different black raspberry confection matrices designed for delivery of phytochemicals

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Abstract

Three forms of confections containing black raspberries (BRB) powder were developed to provide controlled release of phytochemicals for oral disease prevention. Our objective was to investigate the impact of varying confection matrices on the release rate of BRB phytochemicals. Confections were developed and prepared. Textural properties of confections were analyzed, compared and correlated with the release rate of phytochemicals from BRB confections with *in vitro* dissolution test. In the results, BRB content reached 22% in hard candy and pectin based confections and 40% in starch based confections, respectively. Pectin and starch based confections retained > 93% of its original anthocyanins after processing while hard candy had 59%. Starch confections showed higher G’ in rheological analysis and higher hardness but lower cohesiveness and springiness in textural profile analysis than pectin confections (*P* < 0.05). The confection types showed different microstructure with scanning electronic microscopy (SEM). Corresponding to their physicochemical properties, confections showed fast (hard candy), intermediate (pectin confections), and slow (starch confections) release rates with a final releasing time of 90, 150 and 540 min in dissolution studies. Three confections were rated between neither like nor dislike to like slightly (n=60). Pectin confections had the highest overall acceptance (like slightly) and 62% of subjects rated this type of confection as the most liked ones. These results indicate that delivery matrix could modulate the phytochemical release rate from BRB confection and also influence sensory preference. **Key words:** Confection matrix; Anthocyanins retention; Physicochemical properties; Modulated delivery of phytochemicals
1. Introduction

Among fruits, black raspberries (*Rubus occidentalis*) (BRB) have shown distinct antioxidant, anti-inflammatory and anti-cancer properties as demonstrated in oral cancer cells, animal models with induced oral cavity tumors and patients with premalignant oral lesions (Han *et al.*, 2005; Casto *et al.*, 2002; Shumway *et al.*, 2008). Various bioactives are found in BRB including anthocyanins, ellagitannins, ellagic acid, flavonoids, ferulic acid, fiber, vitamins, and minerals (Stoner 2009), among which anthocyanins are one of the major group of compounds contributing to the antioxidant and anticancer activities (Cooke *et al.*, 2005). Although BRB showed promising effects in oral diseases prevention, there is significant concern that many of the bioactive components consumed from fruit and vegetable are poorly absorbed (Epriliati & Ginjom, 2012), which forms an obstacle to systemic bioactivity.

Controlled and sustained release and deposition of bioactive compounds (synthetic and natural) via the oral mucosa has become a critical approach in the development of pharmaceuticals (Jones *et al.*, 2004; Dhiman *et al.*, 2008). Previous work utilized a non-food based bioadhesive gel containing 5 - 10% freeze-dried whole BRB to patients with premalignant oral lesions (Mallery *et al.*, 2007, 2008) and found this gel to be an effective delivery system of anthocyanins to saliva and oral tissues (Ugalde *et al.*, 2009). Translation of these findings from a pharmaceutical setting to food formulation is a viable alternative (Knobloch *et al.*, 2011) and a dietary means of BRB delivery is more attractive for extended use and prevention regimen. Yet additional hurdles such as organoleptic acceptability need to be addressed.
A confection is the ideal food matrix for controlled BRB delivery because it dissolves slowly in the mouth, it can be formulated with high sensory acceptability, and it possesses adequate shelf life (Sessler et al., 2013; Gu et al., 2014). More importantly, the physical properties of confections can be manipulated through different ingredients and processing conditions, from which the matrices can be yielded and exhibit unique bioactive release rate kinetics. Polymer gels composed of starch, pectin, gelatin, agar or gums form a three-dimensional network that can entrap and transport bioactives, therefore, protecting the compounds from their proximate environment and favoring targeted delivery (Yu, 2001). Other major components in confections are sucrose, glucose syrup, and water which act as plasticizers affecting the amorphous state and may therefore alter the release of bioactives (Burey et al., 2009). The use of whole BRB rather than an extract has been recommended since a variety of bioactives may impart benefits at multiple stages of carcinogenesis of oral cells (Knobloch et al., 2011).

In order to better understand the relationship between structure and function of delivery, physicochemical properties of the confection delivery system need to be measured at different structural strata, beginning from molecular level up to micro and macro levels, this includes: differential scanning calorimetry, microscopic, and rheology and texture analysis, respectively (Benshitrit et al., 2012; Sessler et al., 2013; Fisher et al., 2014). The goal of this study was to develop and characterize BRB confections with varying matrices that result in different phytochemical release rate while maintaining organoleptic and textural acceptability.
2. Materials and Methods

2.1 Confection ingredients

Three amorphous confectionaries ranging from glassy to elastic were prepared and evaluated. The glassy form (hard candy) contained corn syrup (Gordon Food Service, Springfield, OH, USA), sugar (sucrose) (US food service, Cincinnati, OH, USA), and freeze-dried BRB powder (Stokes Raspberry Farm, Wilmington, OH, USA); whereas the two elastic confections (pectin and starch based confections) were composed of sugar, corn syrup, freeze-dried BRB powder and gelling agents - Grindsted pectin (70% esterification, CF 130B, Danisco USA Inc., New Century, KS, USA) and Confectioners G food starch (Tate & Lyle, Decatur, IL, USA), respectively. BRB of a single variety (Jewel) was obtained from the farm in Southern Ohio (Stokes Raspberry Farm, Wilmington, OH, USA). BRB to be used for this trial were harvested, washed and frozen at -20 ºC within 2 - 3 hours of picking. Berries were shipped frozen to Van Drunen Farms (Momence, Illinois) and subsequently freeze-dried under anoxic conditions and finally ground into BRB powder. Freeze-dried powder was stored at -20 ºC without light until use. The nutrient composition in 1 g BRB powder based on initial moisture content of 85% in fresh BRB (Stoner 2009) was 0.8 g carbohydrate (0.43 g fiber and 0.30 g sugar), 0.08 g protein, 0.04 g lipid, 0.017 g minerals and vitamins (USDA National Nutrient Database).

Confection preparation

Pectin based and starch based confections were prepared by mixing water, sugar, corn syrup and gelling agent, and stirring the mixture on a stirrer/hot plate (PC-620D, Corning,
Tewksbury, MA, USA) to a final temperature of 95 °C and °Brix of 67 ± 2, determined by a hand-held refractometer (range 58 - 90 °Brix, Fisher Scientific Japan Ltd., Tokyo, Japan). Optimal consistency confections was achieved within 15 ± 1 min and 20 ± 1 min for pectin and starch confections, respectively. Upon cooling to 70 °C at room temperature, freeze-dried BRB powder was added and mixed into the gel. The mixture was deposited with a pastry bag at 50 °C onto a Silpat (Demarle, Inc., Cranbury, NJ, USA). Confections were equilibrated at room temperature (21 ± 2 °C) in the dark for 24 hours before placing each into 2 oz plastic portion cups with lids (Gordon Food Service, Springfield, OH, USA). Hard candy was prepared by heating mixed corn syrup, sugar and water on the Coning PC-620D stirrer/hot plate to 150 °C. This temperature was reached within 19 ± 1 minutes. Freeze-dried BRB was directly added into the mixture and evenly blended before candy pieces were deposited on a Silpat. Hard candies were set and packaged the same as described for the previous two confections. Developed formulations of three confections are shown in Table 2.1. To minimize batch to batch variation, all preparations were performed under room relative humidity (22% - 42%) and temperature (19 - 22°C). Samples were randomly collected in three different batches for further analysis.

2.2 Physicochemical properties analysis

Thermogravimetric analysis (TGA)

Analysis of total water content and distribution within the confections was conducted with a high-resolution Thermogravimetric Analyzer (Model Q5000, TA Instruments, New Castle, DE, USA). Total of 15 to 20 mg samples removed centrally from the interior
of the confections were loaded into platinum TGA pans immediately before analysis. Samples were heated from 25 to 160 °C with a heating rate of 5 °C/min. Both percentage weight loss (%) and derivative weight loss as a function of temperature (%/°C) were analyzed with TA Universal Analysis software (TA Instruments, New Castle, DE, USA). Moisture content was calculated as the mass loss from initial temperature to the temperature of the first derivative peak weight loss (Siegwein et al., 2011). Derivative weight loss was the rate of weight loss (%) as a function of temperature and the peaks indicated temperature ranges of accelerated water loss (Fessas & Schiraldi, 2001). Each type of confection was run in triplicates.

**Differential scanning calorimetry (DSC)**

Calorimetric measurements were performed using a Differential Scanning Calorimeter (Model Q100, TA Instruments) equipped with a refrigerated cooling system (TA Instruments, New Castle, DE, USA). The instrument was calibrated using Indium standard. Samples with a weight between 15 to 20 mg were obtained from the center of the confections immediately before analysis. The samples were placed in hermetically sealed aluminum pans (PerkinElmer Instruments, Waltham, MA, USA) with O-rings to avoid moisture loss during thermal scanning. An empty pan was used as a reference. Each sample was cooled inside the chamber from room temperature to - 80 °C, held isothermally for 3 minutes, and then heated to 160 °C at a scanning rate of 5 °C/min. The transitions observed in the DSC thermogram were analyzed using Universal Analysis™ (TA Instruments, New Castle, DE, USA). The glass transition temperature (Tg) was reported as a range of the onset and the endpoint temperature. The enthalpy of transition
was estimated from the integrated heat flow over the temperature range of the transition, and expressed as joules per gram sample (J/g).

**Rheological analysis**

Rheological properties of the pectin and starch confections were measured using a stress-controlled AR 2000ex rheometer (TA Instruments, New Castle, DE, USA) with a 20 mm steel parallel plate geometry and a fixed gap of 2 mm. Preliminary oscillatory strain sweeps (0.01 to 10% strain, 1 Hz, 25 °C) were conducted for pectin and starch based BRB confections to determine the linear viscoelastic region (LVR) and 0.1% was within LVR for all confections. Confections were cut in 2.5 cm diameter round disk that weighed approximately 1.0 g. Excess sample was removed after adjusting to the 2 mm gap. Dynamic oscillatory testing was conducted to assess the storage modulus (G’), the loss modulus (G’’), the loss factor (tan δ) and complex viscosity (η*) as a function of frequency (0.1 to 100 Hz, 25 °C) at 0.1% of strain with Rheology Advantage Data Analysis v5.7.0 software from TA Instruments. Oscillatory temperature-dependent changes were assessed with a temperature ramp from 20 to 40 °C, 1 Hz and 0.1% of strain to mimic changes from room to oral temperature. Changes of apparent viscosity (η) with increasing shear rates was determined from flow analysis within a range of 0.01 - 1000 s⁻¹ and 1 Hz at 37 °C to mimic mouth shearing process. Oscillatory analyses and flow analysis were both performed in triplicate.

**Texture analysis**

Texture profile analysis (TPA) was conducted using 35 mm compression parallel plate geometry on an Instron 5542 Universal Testing Machine (Instron Corp., Canton,
MA, USA) with Bluehill 2 Materials Testing Software (Instron Corp., Norwood, MA, USA). To simulate mastication, TPA with 40% compression at a rate of 1 mm/s was used (Daubert & Foegeding, 2003). Pectin and starch confections were prepared in 20 mm³ cubes and the following textural parameters were obtained: hardness, cohesiveness, springiness, gumminess and chewiness (Peleg, 1976). Due to the limited load cell capacity (500 N) of Instron 5542, Instron 5569 (Instron Corp., Canton, MA, USA) with a 50 KN load capacity was used to analyze the texture properties of hard candy with the same test speed and compression as those for pectin and starch based confections. One compression cycle rather than two cycles in TPA was used in this measurement due to the hard texture of this confection. Ten replicates of all three confections were analyzed.

2.3 Scanning electron microscopy (SEM)

SEM of confections followed procedure described by Yoon et al. (2009) with some modifications. Confection samples were cut into 7 mm small cubes leaving one naturally fractured surface, and then freeze-dried. After freeze-drying, samples were coated with Au/Pd alloy via a Cressington 108 Auto Sputter Coater (Watford, UK) for 180 seconds. Fractured surface was observed via a scanning electron microscopy at an accelerating voltage of 5 kV (Nova Nano SEM 400, FEI Company, Hillsboro, OG, USA).

2.4 Total phenolics and anthocyanins in confections

Confections containing 1 g of BRB were extracted with 80% methanol (v/v) till a pale-colored extract was obtained (Gansch and others 2009). All samples were colorimetrically evaluated at a fixed wavelength of 765 nm using Folin-Ciocalteau Reagent (Sigma-Aldrich, 2N) utilizing methods described by Waterhouse (2002). Gallic
acid (Sigma-Aldrich, St. Louis, MO, USA) standard curve with four concentration levels
(50, 100, 250, 500 mg/L) was used to quantify total phenolic content and results were
reported in gallic acid equivalents (GAE) using units of mg/L (Waterhouse, 2002).
Retention of total phenolics in confections after processing was calculated by comparison
to total phenolic content in BRB powder.

Anthocyanins were analyzed and compared among confections. Confections
containing 1 g of BRB powder was extracted by acetone/water (80/20, v/v) containing 5%
formic acid for three times until the extract color was pale, which was referred to the
method in Gu et al. (2014). Extracted samples were dried in Speedvac Concentrator (SPD
131DDA-115, Thermo Fisher Scientific, Waltham, MA, USA) and re-dissolved into
acetone/water (20/80, v/v) for HPLC analysis. All samples were filtered through PTFE
filters (Fisher Scientific, Pittsburgh, PA, 0.2 μm, 13 mm dia.) before injection.
Chromatographic separation was performed on a symmetry C18 Reverse Phase column
(75 mm × 4.6 mm i.d, 3.5 μm particle size) from Waters (Milford, MA, USA) with
Agilent 1100 (Agilent, Waldbronn, Germany) controlled by the ChemStation software
(Agilent, Waldbronn, Germany). The mobile phase consisted of 1% formic acid in water
(A) and 1% formic acid in acetonitrile (B) with a gradient, flow rate and column
condition shown in Gu et al. (2014). Absorbance at 520 nm wavelength was recorded and
anthocyanins were characterized by their retention time and external standard. Cyanidin-
3-glucoside with concentrations of 3.125, 6.25, 12.5, 25, 50 μg/g was used as a standard
for quantifying anthocyanins in BRB (Mullen and others 2002). Anthocyanins were
quantified and their retention (%) in the confections was calculated as previously
described by Gu et al. (2014). Random samples (n = 3) from three batch (50 pieces/batch) were obtained to evaluate their total phenolic and anthocyanin content.

2.5 *In vitro* dissolution study

Dissolution testing is typically used in the pharmaceutical industry to evaluate the release rate of a compound for its quality or development under standardized conditions (Yuksel *et al*., 2000). Confections intended to deliver bioactive compounds to the oral cavity can be evaluated with an *in vitro* dissolution apparatus using artificial saliva. Release rates of total phenolics from three types of confections were analyzed with a VK7000 Dissolution Apparatus (Varian, Cary, NC, USA) in 250 ml 0.01 M sodium phosphate buffer (pH at 6.5) at 37 ± 1 °C controlled by the VK 750 D heater/circulator (Varian, Cary, NC, USA). Buffer was prepared with double deionized water. Experiments were conducted under dim lighting conditions to prevent anthocyanins from degrading. One piece of confection (6 g for hard candy and pectin confections, 3.3 g for starch confections) with equivalent BRB content (1.3 g) was placed in the basket of dissolution apparatus, submersed in the solution and dissolved while subjected to a rotation speed of 100 rpm. Sampling of 5 ml of the dissolution medium occurred at every 3 min for the first 15 min, followed by every 10 min until reaching one hour, every 30 min in the first two hours, and then every 60 min until the confection dissolved fully. Fresh 5 ml medium was replaced every time at sampling. Samples were acidified with formic acid (final concentration reached to 5% v/v), and then the samples were frozen at -25 °C prior to analysis.
Dissolutions performed at 21 ± 2 °C (room temperature) in the pre-acidified sodium phosphate buffer (pH=2) were used as controls. Release percentage of total phenolics in confections during dissolution was determined by total phenolics released at specific time point divided by that of the final control sample. Release percentage of anthocyanins in confections during dissolution was also determined. Three replicates of each confection were used to determine the release of total phenolics.

2.6 Sensory evaluation of three confections

In order to obtain the overall acceptability of the three BRB confections, sensory evaluation survey was developed and approved by The Ohio State University (OSU) human subjects Institutional Review Board (IRB) exemption (2013E0546). Sensory acceptance was conducted using a 9-point hedonic scale (1-dislike extremely, 2-dislike very much, 3-dislike moderately, 4-dislike slightly, 5-neither like nor dislike, 6-like slightly, 7-like moderately, 8-like very much, 9-like extremely) to evaluate the overall acceptability (Lawles & Heymann, 1998). In addition, hardness of three confections was rated in a 0 - 10 line scale (0 - not hard, 5 - medium hard, 10 - very hard) and a medium reference sample was provided (Twizzlers twisted licorice candy, strawberry flavor, Hershey Co., PA, USA). Drinking water and Premium crackers with unsalted tops (Nabisco, East Hanover, NJ, USA) were used to cleanse the palate between samples. In total 60 healthy people (28 male and 32 female, average age range: 18 - 25) were recruited and the sensory test was conducted in Sensory Lab in Parker Food Science Building at OSU.
2.7 Statistical analysis

A one-way analysis of variance (ANOVA) and Tukey’s post-hoc test were used with SPSS 17.0 (Chicago, IL, USA) to determine significant differences in frequency sweep, TGA, dissolution and sensory among different confections. Independent t-test compared differences between optimized starch and pectin confections in temperature sweep and TPA with Minitab 15 statistical software (Minitab Inc, State College, PA, USA). A $P \leq 0.05$ was considered to be significantly different.

3. Results and Discussion

3.1 Confection Composition

Compositions of pectin and starch based confections, and hard candy were optimized by selecting appropriate concentrations of water, sugar, and pectin or starch that would allow for the highest concentrations of BRB powder while maintaining confection formation. The amount of BRB powder reached to 22.0% for hard candy and pectin confections and 40.0% for starch confections. Variances of the BRB amount were due to the processing difference among each confection, polymer gelling conditions and some water loss during the processing. Final pectin and starch concentrations increased to 1.7% and 8.7% after processing. Gel formation of high methoxyl pectin depends on high soluble solids content (> 62%) and low acid environment (pH 2.8 - 3.8), which requires high sugar amount but restricts BRB content. Starch based confections require the starch granules to swell and form the gel structure and thus it is moisture and temperature dependent (Remsen & Clark, 1978). In the preparation of hard candy, evaporative heating is employed to decrease water content to the desired range of 0 - 5% (Engel & Knechtel,
Freeze-dried BRB is well suited for hard candy manufacturing since its low water content (around 4%).

3.2 Physicochemical properties of BRB confections

Physical state of confection components

Water content in the three confections was determined as weight loss percent (Figure 2.1A) according to Fessas and Schiraldi (2001). As expected, hard candy contained the least amount of water 4.7%, followed by starch confections with 18.5%, and pectin confections with 26.0%. During hard candy processing, water evaporation is key so that the sugar concentration is high enough to form the glassy state (Nowakowski & Hartel, 2002). Derivative weight loss percentages versus temperature of the confection samples in TGA analysis are also shown in Figure 2.1A. The accelerated weight loss in all three confection types occurred at high temperatures, with hard candy resulting in the highest temperature peak of weight loss (140.7 ± 5.9 °C), followed by starch (112.7 ± 1.6 °C) and pectin confections (107.9 ± 0.7 °C). The high temperature range may be due to the tight water association with carbohydrates in the confection (Siegwein et al., 2011).

Thermal dynamic changes of three confections during heating with DSC are shown in Figure 2.1B. All samples showed two endothermic peaks at -40 °C and -20 °C in Figure 2.1B, which were also observed in freeze-dried BRB powder (Figure 2.1C) indicating the powder was likely their origin. Additionally, the methanol extracting residue of BRB powder (fiber components) also showed two peaks at the same temperatures as those in the confections. Moreover, the peak at -20 °C was observed to increase when BRB powder was mixed with 20% water. Thus, the endothermic peak at -20 °C shown in all
confections and BRB powder is speculated to be the water that is bound to fiber. There were no water melting peaks around 0 °C, which indicated there was no “freezable” water in all three confection types and this was consistent with the TGA results that temperatures of derivative weight loss peaks of three confections showed at high temperature (> 100 °C).

A glass transition with a Tg (temperature range of glass transition) of 20 - 40 °C and Tg midpoint of 30 °C was observed in the hard candy (Figure 2.1B). This wide range may be attributed to the mixture of sugars in the system (Saavedra-Leos et al., 2012). Nowakowski and Hartel (2002) reported Tg (midpoint of the glass transition) of the sugar glasses (corn syrup/sugar = 37/63) around 35 °C at 5% of moisture content, encompassing the temperature range observed in this study. Due to the BRB addition and complexity of the hard candy system, a glass transition temperature range rather than a single midpoint of glass transition zone was reported in our study as recommended by Reinheimer et al. (2010). Glassy candies stored above the Tg and in high humidity environment are unstable resulting in increased molecular mobility leading to structural transformations during storage such as stickiness and crystallization (Nowakowski & Hartel, 2002). Thus, hard candy was at the onset of glass transition, and pectin and starch based confections were in the rubbery state at room temperature.

**Varied physical state of confection components yield differences in rheological and textural properties**

Characterization of elastic modulus (G’), viscous modulus (G’’) and complex viscosity (η*) of optimized pectin and starch based confections were recorded and
compared in oscillatory frequency sweep with a range from 0.1 to 100 Hz. In addition to the optimized starch confection (6.5% of starch), a 1.5% alternative was used which contained equivalent polymer concentration to the pectin confection and served as a direct comparison of rheological properties (Figure 2.2). Increased frequency resulted in an increased $G'$ and $G''$ for all samples, indicating the confections were weak gels (Tabilo-Munizaga & Barbosa-Cáovas, 2004). $G'$ was higher than $G''$ and no $G'$ - $G''$ cross-over points were observed in the range of frequency studied in both optimized confections, suggesting that confections exhibited solid-like properties and were comparatively stable (Figure 2.2A). Complex viscosity ($\eta^*$) decreased with increasing frequency for all confection types (Figure 2.2B). $G'$, $G''$ and $\eta^*$ of optimized starch confections (6.5% of starch) were higher than those of the pectin confections, which indicated a stronger structure of the starch confections. Confections with 1.5% concentration of starch showed a $G'$ - $G''$ crossover at 0.4 ± 0.1 Hz (Figure 2.2A), which indicated that this system was more liquid-like and not stable at higher frequency. Moreover, the difference in $G'$, $G''$ and $\eta^*$ between two confections with the same concentration of the gelling agents (1.5%) may indicate the variance in gel formation process and gel structure between pectin and starch. $G'$ of optimized starch confections, pectin confections and lower starch concentration confections showed significant difference ($P < 0.05$) at 1.00 Hz with $3.4 \times 10^5$ ($\pm 1.8 \times 10^4$), $4.2 \times 10^4$ ($\pm 3.6 \times 10^3$), and $2.0 \times 10^2$ ($\pm 1.7$), respectively. The same significant difference at 10 and 100 Hz frequency were also observed ($P < 0.05$).
The behavior observed for Apparent viscosity ($\eta$) with increasing shear rate measured at 37 °C reflected three different regions: constant at low shear rate ($< 0.005$ s$^{-1}$), decreasing in the middle range (0.005 - 100 s$^{-1}$) and constant at high shear rate ($> 100$ s$^{-1}$) (Figure 2.2C). This indicated non-Newtonian shear-thinning behavior of the confections (Steffe, 1996). Oral perception of thickness was reported to have a correlation with instrumental measurements at shear rates ranging from 10 to 1000 s$^{-1}$, such as 5 s$^{-1}$ for hard margarine, 37 s$^{-1}$ for more fluid products such as ketchup (Shama & Sherman, 1973) and approximate 60 s$^{-1}$ for fluid foods (Steff, 1996). Therefore, a shear rate of 5 s$^{-1}$ was chosen to compare the pectin and starch confections and the results indicated that viscosities ($\eta$) were 440.9 and 1756.0 Pa.s, respectively. Both confection types showed distinct changes in viscosity at a shear rate of 21.5 ± 4.9 s$^{-1}$ and subsequently reached to very low viscosity ($< 1$ Pa.s) around 65.3 s$^{-1}$ potentially caused by structure breakage. It should be noted that under high shear rates ($> 100$ s$^{-1}$) slippage may occur as both confections showed low and constant viscosity. The link between oral perception and mechanical measurement is complicated and Malone et al. (2003) suggested that it was still not clear what shear and stress conditions were active in the mouth and how many other non-rheological factors contribute to sensory textural assessment.

Results of temperature sweeps (20 - 40 °C) of optimized pectin and starch confections are shown in Figure 2.2D. This encompasses the temperature range from room storage to exposure in the oral cavity. It was found that both $G'$ and $G''$ decreased with increased temperature. Starch confections and pectin confections both showed significantly higher $G'$ and $G''$ at 20 °C than those at 37 °C ($P < 0.05$). This suggests the structures of
confections are temperature dependent and confections may become softer with increased temperature. Temperature increase is one part of the changes that confections experience in oral cavity, while continuous secretion of saliva leading to confection dissolution in the mouth plays an important role but is hard to mimic and measure.

Mean and standard deviation of the textural properties of each confection are shown in Table 2.2. The hardness of the glassy hard candy, measured as the force at first peak in the compression, was 606.9 ± 154.3 N, much higher than those of the elastic confections due to the structure difference and very low water content (4.7%). Starch confections showed significantly higher hardness, but lower cohesiveness and springiness ($P < 0.05$) compared to the pectin confections. Higher BRB powder content and lower moisture content in the starch based confections were possibly related to the higher hardness, lower cohesiveness and lower springiness. There were no significant difference in gumminess and chewiness between two rubbery confections samples ($P = 0.391$ and 0.359, respectively).

The fractural surface of the three confections was observed with SEM after freeze-drying and the results are shown in Figure 2.3. Hard candy (Figure 2.3A) showed a smooth surface with particulates (BRB powder) dissolved in it. Reinheimer et al. (2010) showed the similar smooth microstructure of hard candy with SEM. Pectin and starch based confections showed rougher structures compared to hard candy and BRB was distributed evenly in both gel matrices. Compared to pectin based confections (Figure 2.3B), starch confections (Figure 2.3C) exhibited more continuous and compact gel structure, with less pores confirming TPA and rheology results of harder/firmer texture.
3.3 Retention of total phenolics and anthocyanins in BRB confections

Retention of total phenolics and anthocyanins in BRB confections was obtained through comparison with those in freeze-dried BRB powder. Freeze-dried BRB used in this study contained 48.4 ± 2.3 mg GAE/g of total phenolics (equivalent to 725.4 ± 34.5 mg GAE/100g fresh BRB) and 26.6 ± 1.7 mg/g of anthocyanins (equivalent to 398.6 ± 25.7 mg/100 fresh BRB). Total phenolic content was within the range (489.3 - 875.3 mg GAE/100 g fresh) reported by Gansch et al. (2009) and variations were observed among cultivars. Total phenolics were highly retained after processing in three types of confections with 88.2% in hard candy and > 96% in pectin and starch based confections. Anthocyanins were 94.3% and 93.7% retained in pectin and starch based confections after processing separately, while 59.4% retained in hard candy. This significant decrease is likely due to the high mixing temperature (150 °C) of BRB powder with the sugar syrup in the preparation of hard candy compared to 70 - 75 °C in pectin and starch based confections. Anthocyanins are heat sensitive and high temperature exposure causes degradation (Sadilova et al., 2007). Hager et al. (2008) suggested that other factors in processing including the number of processing steps and heat duration can also markedly affect the anthocyanin content and antioxidant capacity of fruit. Oxygen, enzyme, light, pH and metal existence in food processing and some food components have been found to have marked effects on anthocyanin stability (Jackman et al., 1987).

3.4 Release rate of total phenolics and anthocyanins from BRB confections

Dissolution of BRB confections was determined and results are shown in Figure 2.4. The three confection types showed different release rates of total phenolics in sodium
phosphate buffer (Figure 2.4A). Hard candy exhibited the fastest release (75 min) with 93.5% of total release, while starch confections showed the slowest release (540 min) with 78.7%. Pectin confections showed intermediate release rate within 180 min reaching to 94.5% of total release. These differences were likely caused by the gelling polymer addition and the confection structure differences. As mentioned previously, variances of the BRB amount in confections (40% in starch confections and 22% in hard candies and pectin confections) were due to the processing differences required to manufacture each confection type. In order to more directly compare the effect of confection matrix on dissolution kinetics, starch confections with 22% BRB were also run and the release rate was not significantly faster than starch confections with 40% BRB ($P > 0.05$). Miller-Chou and Koenig (2003) indicated that non-polymeric materials were different from polymers since they dissolved instantaneously and the dissolution was generally controlled by the external mass transfer resistance through a liquid layer adjacent to the solid-liquid interface. Hard candy in this study with no added gelling agent underwent a fast dissolution rate compared to pectin and starch based confections. Since the pectin and starch based confections were in a rubbery state (as indicated by DSC analysis), a gel layer containing swollen polymer material and a liquid layer were formed during dissolution as reported by Miller-Chou and Koenig (2003). They suggested that solvent diffusion and chain disentanglement were two transport processes in the dissolution of a polymer. Solvent diffusion results in gel layer formation followed by an increase of gel layer thickness, which finally lead to disentanglement (decrease of gel layer thickness) and dissolution (Miller-Chou & Koenig, 2003; Narasimhan & Peppas, 1996). The slow
dissolution of starch confections was likely due to the more compact structure and lower moisture content compared with pectin confections (Figure 2.3B & C) resulting in a higher $G'$, complex viscosity ($\eta^*$) (Figure 2.2) and hardness (Table 2.2). Thus, solvent diffusion may need longer time for more compact structure to reach to the point of disentanglement.

Release of anthocyanins in the three types of confections showed a similar trend to that of total phenolics (Figure 2.4B). Compared to each corresponding control performed in room temperature and an acidic environment, hard candy reached 94.8% anthocyanin delivery in 75 min, pectin had 92.5% in 180 min while starch confections lost 20% anthocyanins during 540 min release. Longer exposure time at neutral pH may cause the loss of anthocyanins. Thus, with the consideration of anthocyanin retention after processing, pectin confections showed the highest final release of anthocyanins.

Dissolution behavior of these three different confections were examined through in vitro dissolution tests which provide objective and consistent conditions to evaluate the effects of structural/textural difference on the release rates of bioactives. Pectin based confections showed the highest release of total phenolics in an intermediate rate. Possible differences in dissolution behavior may exist in the human oral cavity compared to in vitro dissolution system due to the differences such as saliva incorporation, oral dynamics and swallowing as discussed in Chen (2014). The accomplished three confections with different release rate are ideal for testing in a human clinical trial.
3.5 Sensory evaluation of three confections

The overall liking of three confections was evaluated by 60 recruited subjects resulting in a score of 6.0 ± 1.6 for pectin confections, which was significantly higher than that of hard candy (5.0 ± 1.9) and starch confections (5.2 ± 1.5) \((P < 0.05)\). Hardness of three confections in the sensory evaluation was rated in a 0 - 10 hardness line scale (not hard to very hard) and significant difference was found among three confections \((P < 0.05)\): 9.7 ± 0.5 for hard candy, 1.0 ± 0.7 for pectin confections, and 3.3 ± 1.0 for starch confections.

Due to the wide textural difference among the three confections, subjects who liked rubbery type of confections tended to dislike hard candy and vice versa. Among the 60 subjects, 62% chose pectin confections as the most liked ones with a rating of 6.8 ± 1.1 for the overall liking, 23% chose hard candy and scored it 6.6 ± 0.9 as the overall liking, and 15% chose starch based confections with an overall liking score of 6.6 ± 1.0. These overall liking scores were between 6 (like slightly) and 7 (like moderately) among the most liked ones and were not significantly different \((P = 0.860)\). The test subjects commented that seeds in the whole black raspberry powder were found to cause the rougher texture and thus were found less desirable.

4. Conclusions

In our study, hard candy, pectin based and starch based confections containing 22.0%, 22.0% and 40.0% of freeze-dried BRB, respectively, were developed. Physicochemical analysis showed that textural and microstructural properties were distinctly different among hard candy, pectin based and starch based confections. Pectin and starch based
confections showed high retention of anthocyanins (> 93%) after processing, while hard candy lost 40.6% of anthocyanins. The final release percentage of the total phenolics reached 93.5% for hard candy, 94.5% for pectin confections and 78.7% for starch confections in sodium phosphate buffer after total dissolution and these confections showed fast, intermediate, and slow release rates, respectively. BRB content in starch confections did not significantly change release rates of total phenolics and anthocyanins. This indicated that the release rate of phenolics from BRB could be modulated by confection matrix with different physicochemical properties. All three confections were scored between 5 to 6 (5 - neither like nor dislike, 6 - like slightly) and pectin confection had the highest overall acceptance. A well-characterized confection system designed for controlled release of phytochemicals was achieved in this study.

5. Acknowledgements

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**Table 2.1** Formulations of three types of confections

<table>
<thead>
<tr>
<th>Composition (g)</th>
<th>Hard Candy</th>
<th>Pectin confection</th>
<th>Starch confection</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRB powder</td>
<td>17</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Sugar</td>
<td>43</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>Corn syrup</td>
<td>30</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td>Water</td>
<td>10</td>
<td>29.5</td>
<td>28.5</td>
</tr>
<tr>
<td>Starch/Pectin</td>
<td>-</td>
<td>1.5 (Pectin)</td>
<td>6.5 (Starch)</td>
</tr>
<tr>
<td>50 % (w/w) Citric acid</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 2.2 Texture analysis of three types of confections

<table>
<thead>
<tr>
<th>Properties</th>
<th>Hard candy</th>
<th>Pectin confection</th>
<th>Starch confection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardness (N)</td>
<td>606.9 ± 154.3 (^a)</td>
<td>32.3 ± 3.1 (^i)</td>
<td>52.9 ± 5.8 (^b)</td>
</tr>
<tr>
<td>Cohesiveness</td>
<td>-</td>
<td>0.3 ± 0.03 (^a)</td>
<td>0.2 ± 0.01 (^b)</td>
</tr>
<tr>
<td>Springiness (mm)</td>
<td>-</td>
<td>3.0 ± 0.2 (^a)</td>
<td>1.0 ± 0.2 (^b)</td>
</tr>
<tr>
<td>Gumminess (N)</td>
<td>-</td>
<td>8.2 ± 1.6 (^a)</td>
<td>8.8 ± 1.0 (^a)</td>
</tr>
<tr>
<td>Chewiness (N)</td>
<td>-</td>
<td>45.7 ± 8.6 (^a)</td>
<td>42.1 ± 4.7 (^a)</td>
</tr>
</tbody>
</table>

Values represent means ± standard error (n = 10). Means with different letters in the same row are significantly different (p ≤ 0.05).
Figure 2.1 Thermogravimetric analysis (A) and differential scanning calorimetry (B & C) of black raspberry confections.

A
Figure 2.1 continued

B

Temperature (ºC)
-60 -40 -20 0 20 40 60 80 100
Heat flow (W/g)
-0.28
-0.24
-0.20
-0.16
-0.12
-0.08
Hard candy
Pectin confection
Starch confection

Continued
Figure 2.1 Continued

C
Figure 2.2 Rheological analysis of elastic confections. (A) $G'$ (black) and $G''$ (grey) in frequency sweep; (B) Complex viscosity $\eta^*$ in frequency sweep; (C) Apparent viscosity $\eta$ in flow shear rate sweep; (D) $G'$ (black) and $G''$ (grey) in temperature sweep.

A
Figure 2.2 Continued

B

![Graph showing the relationship between frequency (Hz) and modulus (|n|) with data points for Starch-6.5%, Pectin-1.5%, and Starch-1.5%.

Continued

Starch-6.5%
Pectin-1.5%
Starch-1.5%

Frequency (Hz)
Figure 2.2 Continued

C

Shear rate (s\(^{-1}\))

Viscosity (Pa.s)

10\(^{-1}\)
10\(^0\)
10\(^1\)
10\(^2\)
10\(^3\)
10\(^4\)
10\(^5\)
10\(^6\)

0.001 0.01 0.1 1 10 100 1000

Shear rate (s\(^{-1}\))

Starch

Pectin

Constant

Decreasing

Constant

Continued
Figure 2.2 Continued

D

Temperature (°C)

$G'$, $G''$ (Pa)

10^4

10^5

10^6

Starch

Pectin

Temperature (°C)
Figure 2.3 Scanning electron micrographs (SEM) of hard candy (A), pectin confections (B) and starch confections (C). Magnifications are 1000 × and 2500 ×.

A

B

Continued
Figure 2.3 Continued

C
Figure 2.4 Dissolution of hard candy (diamond), pectin confections (triangle) and starch confections (circles) in sodium phosphate buffer. (A) Release rate of total phenolics; (B) Release rate of anthocyanins.

A
Figure 2.4 Continued

B

![Graph showing release percentage of anthocyanins over time for different types of confections: Hard candy, Pectin confection, Starch confection - 40% BRB, Starch confection - 22% BRB. The x-axis represents time in minutes (0 to 600), and the y-axis represents release percentage of anthocyanins (%).]
Chapter 3: Characterization of black raspberry functional food products for cancer prevention human clinical trials

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Abstract

Our team is designing and fully characterizing black raspberries (BRB) food products suitable for long-term cancer prevention studies. The processing, scale up and storage effect on the consistency, quality, bioactive stability and sensory acceptability of two BRB delivery systems of varying matrices is presented. BRB dosage, pH, water activity and texture were consistent in the scale-up production. Confections retained > 90% of anthocyanins and ellagitannin after processing. Nectars had > 69% of anthocyanins and > 66% of ellagitannin retention which varied with BRB dosage due to the processing difference. Texture remained unchanged during storage. BRB products consumed in a prostate cancer clinical trial were well accepted in sensory tests. Thus, this study demonstrates that two different BRB foods can be formulated to meet quality standards with a consistent bioactive pattern and successfully scaled up for a large human clinical trial focusing on cancer risk and other health outcomes.

Keywords: black raspberry; scale-up production; consistency in characteristics; retention of phenolics; storage stability; sensory acceptance
1. Introduction

Black raspberries (BRB) (*Rubus occidentalis*) have gained much attention due to their distinct antioxidant, anti-inflammatory, anti-angiogenesis and other anti-cancer bioactivities demonstrated in both *in vitro* (Keatley, 2008; Mallery *et al*., 2007; Seeram *et al*., 2006 & 2007) and *in vivo* (Mallery *et al*., 2008; Wang *et al*., 2009, 2010 & 2011; Ravoori *et al*., 2012; Stoner *et al*., 2007) studies. BRB chemopreventive properties are partially attributed to the wide range of phytochemicals including anthocyanins, ellagitannins, ferulic acid, β-sitosterol, bioflavonoids, fiber, vitamins and minerals (Stoner, 2009). Among them, anthocyanins and ellagitannins are considered as the most potent anticancer components (Wang *et al*., 2009 & 2010; Cooke *et al*., 2005; Ross *et al*., 2007) and are found in higher concentrations in BRB compared to other berries (Stoner, 2009; Landete, 2011). These bioactive compounds may be involved to inhibit chronic inflammatory processes that are now increasingly associated with the initiation and promotion of cancer in various organs (Wang *et al*., 2009; Stoner *et al*., 2009; Coussens & Werb, 2002).

The logic and theoretical advantages of a food-based approach for disease prevention and health are several. However, studies of specific foods have been limited by the variation in bioactive content and incomplete chemical characterization. Our goal is to define specific food-products derived from fruits and vegetables that are fully chemically characterized, highly desirable and easily incorporated into a diet, and will be stable over time and storage conditions for long-term human trials of health and disease outcomes. These would represent complex mixtures of bioactive phytochemicals that when
consumed, may impact multiple targets representing defective signaling pathways in mammalian carcinogenesis and thus may have additive and/or synergistic activity to enhance anti-cancer efficacy. Additionally, active agents, each provided at lower dose, may reduce risk of toxicity.

Food matrix is a key factor that influences the release, absorption and thus bioavailability of bioactive components (Yang et al., 2011). However, most studies focused on the absorption of bioactive extracts in water or bioactives as part of a meal with other food components (Yang et al., 2011). Walton et al. (2009) reported that blackcurrant anthocyanins in a more viscous oatmeal matrix had delayed and decreased absorption and excretion of these bioactives but did not change the metabolism in a rat model compared with anthocyanins in water. Ahn-Jarvis et al. (2012) showed that significantly higher soy isoflavone microbial metabolites were excreted in female subjects consuming soy bread compared to soy beverage. However, the effects of food matrix on cancer prevention are largely unknown.

We have developed two different matrices, a pectin based confection and a nectar (viscous juice), to be used in delivering BRB bioactives in human clinical trials and tested them in a cohort of men with prostate cancer. Food products containing freeze-dried BRB powder have been developed to increase BRB acceptability in clinical trial compared to the BRB powder with water added and a non-food bioadhesive gel used previously (Mallery et al., 2008; Stoner et al., 2005). Pectin confections have been found to be an appropriate solid matrix to deliver BRB bioactive compounds and characterized for their in vitro dissolution rate, retention of bioactive compounds during processing and
high sensory acceptability (Gu & Vodovotz, 2012). Compared to a solid matrix, liquids deliver bioactives faster to the gastrointestinal tract (GIT) hastening digestion and absorption (Ahn-Jarvis et al., 2012). Significantly, ellagic acid was detected in plasma and urine of subjects consuming pomegranate juice or BRB powder in water (Stoner et al., 2005; González-Sarrías et al., 2010). For nectar formulation, the addition of stabilizer (such as pectin, xanthan and agar) to the juice would increase viscosity and thus aid in suspension of particles such as fruit pulp and seeds (Neidhart et al., 2002).

Aside from the formulation, the processing and storage need to be considered carefully to assure minimal effect on the levels of berry bioactives in products. For example, the manufacturing and storage of berry jam, juice, and puree as well as canning yielded a decrease in anthocyanins and flavonoids while ellagitannins content remained heat stable and more influenced by the processing related to seeds exclusion (Zafrilla et al., 2001; Hager et al., 2008; Hager et al., 2010). Formulation and processing of a food delivery system for a human clinical trial and subsequent scale up requires consistency of ingredients and final product as well as quality and bioactive retention during storage. As such, the objective of the current study was to assess the consistency, quality and shelf stability of BRB pectin confections and nectar developed for clinical trials and demonstrate their acceptability amongst men enrolled in a prostate cancer clinical trial.

2. Materials and Methods

2.1 Standardization of All Ingredients and Adjustment of Formulations from Lab-Scale to Large-Scale Production To ensure the homogeneity of the food products for the clinical trial, all ingredients were purchased in a single lot: corn syrup (Gordon Food
Service, Springfield, OH), sugar (US food service, Cincinnati, OH) and pectin (RS 400, Danisco, USA Inc., New Century, KS). Pectin (CF 130 B) from Danisco (New Century, KS) was previously used in the lab scale for both confections and nectars; however, due to the lack of the availability for the scale-up production, RS 400 pectin was applied. Freeze-dried BRB powder was obtained from both Stoke Raspberry Farm (Wilmington, OH) (cultivar: Jewel) and BerriProducts LLC (Corvallis, OR) (cultivar: Munger). Mixed BRB powder (Ohio:Oregon = 2:3) was blended using a mixer (Day mixing, 1608, Federal Equipment Co., Cleveland, OH) in the Food Pilot plant in Parker Food Science Building at The Ohio State University (OSU). After mixing, the powder was separated into 4 kg/bag, sealed by using a Vacuum sealer (MC-30 computer control, Sipromac, Houston, TX) and kept at -40 °C avoiding light until use. According to Stoner et al. (2009), the levels of 26 nutrients in BRB remained within 10% - 20% of the original measurements for at least 2 years in the freeze-dried form stored at -20 °C. Scale-up production of confections and nectars required reformulation due to ingredients’ change and processing parameters adjustments with the goal of retaining bioactives and quality. Final selected formulations are shown in Table 1.

2.2 Scale-up Production of BRB Products

Confection Production. Confections were prepared in 1 kg batches and formulation adjusted from lab scale is shown in Table 1. In the preparation, water, sugar, corn syrup and pectin were mixed, and then stirred and heated on a hot plate (PC-620 D, Corning, Tewksbury, MA) until a final temperature of 95 °C and °Brix from 65 to 68 reached, which was determined by a hand-held Refractometer (Fisher Scientific Japan Ltd.,
Tokyo, Japan). These processes were the same as lab scale and achieved within 45 ± 3 min. Upon cooling to 78 °C at room temperature, freeze-dried BRB powder was added and mixed into the gel. The mixture was deposited at 65 °C with a pastry bag into molds (2115-1521 Truffles, Wilton, Columbus, OH). Confections equilibrated at room temperature (21 ± 2 °C) avoiding lights for 24 hours before packaging. All of the products was prepared in OSU Pilot Plant in Parker Food Science Building with Relative humidity (RH) (0.1 - 3.5%) and Temperature (22 ± 1 °C). Random samples (n = 3) from each batch (83 ± 4 pieces) were obtained to evaluate their water activity, water content and pH. Four random batches were chosen to track the stability of bioactives with HPLC (n = 6 per batch), rheological (n = 6 per batch) and textural properties (n = 10 per batch).

**Low Dose BRB Nectar Production.** Nectar with 10 g BRB dose was produced in MicroThermics, Inc. (Raleigh, NC) in a 120 kg batch according to the large-scale formulation shown in Table 3.1. In the preparation, pectin, sugar, corn syrup and water were first mixed and heated to 95 °C with UHT/HTST Lab Direct & Indirect Processing System (MicroThermics Inc., Raleigh, NC) and then the pre-mix was kept at an extended hold cabinet to maintain a temperature above 60 °C. BRB powder was added into the pre-mix and an adjustable speed drive (Leeson Electric Corporation, Grafton, Wisconsin) was used for mixing. The mixture was then pumped into sterilized UHT/HTST Lab Direct & Indirect Processing System with 3 L/min flow rate and pasteurized at 75 °C for 15 min adapted from Silva and Gibbs to inactivate enzymes and majority of molds (Silva & Gibbs, 2004). Nectar was then cooled to 25 °C and filled into 250 mL Nalgene PETG sterilized bottles (Thermo Fisher Scientific Inc.) in a Clean Fill Hood with sterile product
outlet (MicroThermics Inc., Raleigh, NC). Nectars were stored at 4 °C until use. Random bottles (n = 9) were selected to evaluate the pH, Brix, water activity, viscosity and bioactive compounds with HPLC.

**High Dose BRB Nectar Production.** Nectar with 20 g BRB dose was produced in OSU Pilot Plant in Parker Food Science Building since Microthermics equipment was unable to process such high viscosity nectar. A 100 kg batch of nectar was prepared with the large-scale formulation shown in Table 3.1. In the preparation, pectin, sugar, corn syrup and water were first mixed well and heated in a steam-jacketed kettle (A5132-1 ATMOS, Hamilton Kettles, Cincinnati, OH). Once the temperature reached 95 °C, BRB powder was added and mixed well. Temperature was kept at 95 °C for 15 min to pasteurize, a method adopted from the production of BRB puree and strawberry nectar (Hager et al., 2008; Gössinger et al., 2009). After heating, nectar was cooled to 75 °C and filled into 250 mL Nalgene PETG sterilized bottles (Thermo Fisher Scientific Inc.) with a Volumetric Piston Filler (Simplex, AS-1, Napa, CA). A fixed volume of nectar (8 oz) was controlled to fill into bottles. After cooling, nectars were stored at 4 °C until use. Random bottles (n = 9) were selected to evaluate the pH, Brix, water activity, viscosity and bioactive compounds with HPLC.

### 2.3 Phenolic Analysis of BRB Products

**Extraction.** One gram of BRB powder or confection/nectar containing 1 g of BRB was fully dispersed into water containing 5% (v/v) of formic acid and adjusted to a final volume of 50 mL. Aliquots of 2 mL were immediately removed from the well-mixed solution and then mixed with 8 mL of acetone containing 5% (v/v) of formic acid,
followed by bath sonication for 1 min in a FS3OH Sonicator (Fisher Scientific, Fair Lawn, NJ) and then centrifuged at $2000 \times g$ for 10 min with a IEC HN-SII centrifuge (Damon Corp., Needham Heights, MA). Supernatant was removed to a clean 22 mL glass vial (Fisher Scientific, Pittsburgh, PA, USA) with a disposable glass pipette (Fisher Scientific, Pittsburgh, PA). Solvent of acetone/water (80/20, v/v) containing 5% formic acid was used to extract the pellet twice more until pellet was colorless. A speedvac concentrator (SPD 131DDA-115, Thermo Fisher Scientific, Waltham, MA) was used to dry sample extracts.

**Identification.** Waters 2695 HPLC with Waters 996 photodiode array detector (Waters, Milford, MA) combined with a Waters Q-Tof Premier (Micromass MS Technologies, Manchester, UK) was used for the identification of phenolic compounds. Dried extracts were dissolved in acetone/water (20/80, v/v) containing 5% of formic acid and filtered through PTFE filters (Fisher Scientific, Pittsburgh, PA, 0.2 μm, 13 mm dia.) before injection. Separation was carried out on a Symmetry C18 (75 mm × 4.6 mm i.d, 3.5 μm particle size) Reversed Phase column (Waters Corp., Milford, MA). The mobile phase for separation consisted of 1% (v/v) formic acid in water (A) and 1% (v/v) formic acid in acetonitrile (B). Initial mobile phase composition, 100% A and 0% B, was followed by a linear gradient to 80% A at 10 min, 70% A at 12 min, 50% A at 14 min, 0% A at 16 min, and returned to 100% A at 16.1 min and re-equilibrated through 20 min. Column temperature was kept at 35 °C and flow rate was 1.3 mL/min. HPLC flow was split 1:10 prior to MS. The MS analysis was performed in positive and negative ion modes and calibrated with sodium formate in the range of 50 - 3000 m/z. Leucine
enkephalin was used as lockSpray mass with $m/z$ at 556.2771$^+/554.2615^-$. Capillary voltage was at 2.8 kV in negative mode and 3.2 kV in positive mode. Dry gas flow was at 700 L/h, cone voltage at 35 V and desolvation gas temperature was at 480°C. For MS/MS analysis, the same instrumental parameters were used except that collision energy has been set to 25 eV. Standards of cyanidin 3-gucoside, cyanidin 3-sambubioside, cyanidin 3-rutinoside, ellagic acid and rutin were run to support peak identification.

**Quantification.** BRB confections and nectars were analyzed by HPLC to quantify anthocyanins, ellagittannins and ellagic acid content. Freeze-dried BRB powder was quantified to determine the changes of BRB bioactives during production and subsequent storage in products. Quantification in all BRB samples was carried out using Agilent 1100 series (Agilent, Waldbronn, Germany): G 1322A degasser, G 1328A Manual injector, G 1311A Quat Pump and G 1365A MWD controlled by the ChemStation software (Agilent, Waldbronn, Germany). Chromatographic separation was performed with the same column and method as those in the identification step. Samples were injected with a volume of 20 µL. Absorbance at 260, 355, and 520 nm of wavelength was recorded. Anthocyanins were quantified as one peak at 520 nm with cyanidin 3-glucoside as external calibrant$^{24}$ with concentrations ranging from 3.125 to 100.0 µg/mL. Ellagic acid was used as the external standard to quantify free ellagic acid and ellagic acid derivatives under 260 nm with a range of 0.25 to 4.0 µg/mL. Ellagic acid and ellagic acid derivatives were represented as total ellagic acid. Ellagittannin quantification was following the method of Gasperotti et al. (2010), in which (sanguiin H-6) $\varepsilon_{260 \text{ nm}} = 63615$ M$^{-1}$ cm$^{-1}$ and (ellagic acid) $\varepsilon_{260 \text{ nm}} = 28266$ M$^{-1}$ cm$^{-1}$ in 88% of acetonitrile and 12% of 1% acetic acid.
formic acid in water (v/v) were reported. Standard curve of Sanguin H-6 was derived from ellagic acid standard curve using a calculated factor (28266/63615 = 0.44).

2.4 Retention of Anthocyanins, Ellagitannins and Total Ellagic Acid in BRB Products

Retention rate of these compounds from BRB confections and nectars was calculated using the following formula:

\[
\text{Retention(\%)} = \frac{C_{\text{phenolics}}}{C_{\text{BRB}} \times C'_{\text{phenolics}}}
\]

Where \( C_{\text{phenolics}} \) = concentration of anthocyanins or ellagitannins in products, \( C_{\text{BRB}} \) = percentage of BRB in products after processing, \( C'_{\text{phenolics}} \) = concentration of anthocyanins or ellagitannins in freeze-dried BRB powder. \( C_{\text{phenolics}} \) and \( C'_{\text{phenolics}} \) were obtained from HPLC quantification, and \( C_{\text{BRB}} \) was obtained from products’ preparation.

2.5 Characteristic Consistency of the BRB Products

BRB delivery dosage, water activity (Aqua Lab Water Activity Meter Series 3, Pullman, WA), pH and soluble solid (°Brix) were determined to check the consistency of BRB products needed for the clinical trials. SevenMulti pH conductivity meter (Mettler Toledo Inc., Columbus, OH) was used to measure pH of the confection (mashed into a paste) and nectar. °Brix was measured by hand-held Refractometers with ranges of 0 - 32 ° and 58 - 90 ° (Fisher Scientific Japan Ltd., Tokyo, Japan). Water content of confections was obtained from thermogravimetric analysis (TGA) (Q5000, TA Instrument, New Castle, DE) by heating 15 - 20 mg samples at 5 °C/min from 25 to 160 °C. Moisture content was calculated as described in Siegwein et al. (2011).
2.6 Rheological Consistency of BRB Products

Confections were analyzed with an AR 2000ex Controlled Stress Rheometer (TA Instruments, New Castle, DE) with a 20 mm diameter probe. Dynamic strain sweep (0.01 - 10%) at 25 °C and different frequencies (0.1, 1, 10, 100 Hz) were first tested to determine the linear viscoelastic region (LVR) and all samples exhibited a linear response for strain values up to 0.15%. Dynamic frequency sweep (0.1 - 100 Hz) tests were then carried out at 25 °C with 0.1% of strain to obtain viscoelastic behavior of confections. Storage modulus (G'), loss modulus (G''), and complex viscosity (η*) were recorded and compared. Changes of apparent viscosity (η) in nectars with a 40 mm diameter probe were recorded with increasing flow shear rate (0.001 - 1000 s⁻¹) at 1 Hz and 25 °C. Random samples from scale-up production were also analyzed. Texture profile analysis (TPA) of confections were obtained with Instron 5542 Universal Testing Machine (Instron Corp., Canton, MA, USA) with Bluehill 2 Materials Testing Software (Instron Corp., Norwood, MA, USA) with 40% of compression at 1 mm/s rate by 35 mm diameter probe. Hardness, cohesiveness, springiness and chewiness were obtained according to Peleg (1976).

2.7 Two-month Storage of BRB Products

A two-month shelf life required for BRB products to allow for recruitment and clinical intervention was studied. Both confections and nectars were packaged, plastic 2 oz cups with lids (Gordon Food Service, Springfield, OH) for confections and amber bags for each nectar bottle, and kept in the dark at 4 °C for 2 months. Samples at fresh, 4, 6 and 8 weeks were collected to evaluate the storage stability of bioactive compounds and
textural properties in the products. The number of samples used for analysis was the same as those in fresh samples described earlier.

2.8 Scale-up Production Design for a Prostate Cancer Clinical Trial

This clinical trial was approved by OSU Clinical Scientific Review Committee (CSRC) first and subsequently by the Institutional Review Board (IRB) with approval numbers of OSU-12125 and 2012C0096, respectively. In total, 56 patients newly diagnosed with resectable prostate cancer (an average age = 61.6 ± 1.02 yrs) were recruited into this study, 32 of which were in BRB functional food intervention groups and 24 in dietary assessment groups (control). Only BRB intervention groups were focused and discussed in this study. Design of intervention groups in this clinical trial, BRB dosage in products and total amount of processed products are shown in Figure 3.1. All patients signed written informed consent forms before participation. The BRB intervention period for each patient lasted for 20 - 30 days depending on the scheduled surgery day. Extra products were given to participants in case of a need to delay surgery. Only the sensory acceptability of the products tested in this study will be discussed herein.

2.9 Sensory Evaluation

In order to obtain the overall acceptability of BRB products, sensory evaluation survey was developed and approved by IRB (2012C0096). At the end of clinical trial, sensory tests were conducted using a 9-point hedonic scale (1-dislike extremely, 2-dislike very much, 3-dislike moderately, 4 dislike slightly, 5-Neither like nor dislike, 6-like slightly, 7-like moderately, 8-like very much, 9-like extremely) to evaluate the overall
acceptability and acceptability of aroma, flavor, sweetness, texture and grittiness (Lawless & Heyman, 1998).

2.10 Statistical Analysis

A one-way analysis of variance (ANOVA) and Tukey’s post-hoc test were used with SPSS 20.0 (Chicago, IL, USA) to determine the significance in the characteristics between confection batches. The means of rheological properties and contents of bioactive compounds in BRB confections and nectars during storage were compared as well. Independent t-test compared differences between lab-scale and large-scale products with Minitab 15 statistical software (Minitab Inc, State College, PA, USA). A p ≤ 0.05 was considered to be significantly different.

3. Results and Discussion

3.1 Formulation adjustment of BRB products from lab-scale to large-scale production

Formulations of pectin based confection and nectar (Table 3.1) were adjusted from the lab scale to achieve similar textural properties as well as a lower caloric content for clinical subjects. Pectin RS 400 used in large-scale production had a higher degree of esterification (70%) compared to CF 130 B used in lab scale (31% esterification and 19% of amidation), therefore pectin was decreased from 1.5% to 1.2% in BRB confection to reach similar rheological properties as indicated by similar G’, G” and η* values (Figure 3.2). Sugar in BRB confection and citric acid were both decreased in scale-up versions to achieve desired lower calorie content (29.3 to 23.1 kcal/piece confection). Calorie control was essential so as not to induce weight gain during intervention. For the nectar, calorie
adjustment was not required but pectin addition decreased from 1.8% in lab scale to 0.8% for clinical trial so as to reach similar rheological endpoints during scale up (Figure 3.3).

3.2 Identification and quantification of phenolics

Phenolics in BRB powder were identified with a combination of HPLC-MS/MS, accessible standards, UV-Vis and reported mass. HPLC chromatograph and peak identification information for phenolics are shown in Figure 3.4 and the accompanying table. Peak 1a,b and 1c,d coeluted in the HPLC but were identified by MS as cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-xyosylrutinoside and cyanidin 3-rutinoside with a characteristic fragment of cyanidin aglycon [M-H]⁻ at m/z 285 and other reported fragment ions (Tian et al., 2006). In this study, pelargonidin 3-rutinoside was detected in a very low content with MS, thus only the four major cyanidin glycosides were quantified as anthocyanins in BRB powder and the processed products. This HPLC method was not developed to separate each anthocyanin well due to other phenolics including ellagitannins and quercetin glycoside. Peaks shown with a retention time of 7.4 - 8.6 min were unknown but considered as possible anthocyanin related compounds due to absorbance at 480 nm and MS [M-H]⁻ m/z 651 and MS/MS m/z at 285 and 593.

Peak 2 was tentatively identified as quercetin xyosylrutinoside with [M-H]⁻ m/z at 741 and MS/MS m/z 301.036 and 355 nm UV-vis. Peak 3 was identified as sanguin H-6 with MS [M-H]²⁻ m/z at 934; MS/MS m/z at 935, 633, 301 with UV-Vis featured at 260 nm and comparison with previous reports (Mullen et al., 2003; Seeram et al., 2006; Hager et al., 2008b). Lambertianin C with MS [M-H]²⁻ m/z at 1401 and a true mass of 2803 was identified according to Hager et al. (2008b), but it was detected in a minor peak.
at 8.57 min and coeluted with some unknown anthocyanins degradation compounds in this HPLC method. However, lambertianin C was reported as one of the major ellagitannins (> 32%) besides sanguin H-6 (> 42%) in six raspberry (Rubus idaeus L.) cultivars (Gasperotti et al., 2010). Several ellagitannins eluted before anthocyanins with MS of 783, 933, and 633, which were likely pedunculagin isomer, castalagin/vescalagin isomer and galloyl-HHDP glucose isomer according to Hager et al. (2008b). In this study, sanguin H-6 was the most abundant ellagitannins detected and thus quantified as ellagitannin and compared during processing and storage. In Peak 4 - 6, rutin, ellagic acid and quercetin hexuronic acid were identified with a combination of available standards (rutin and ellagic acid), accurate MS, MS/MS, UV-Vis and reported data (Paudel et al., 2013). Methyl-ellagic acid-pentose, acetyl-ellagic acid-pentose, malonyl-methyl-ellagic acid-pentose, and acetyl-methyl-ellagic acid-pentose were identified according to the accurate masses of their molecular ions and fragment m/z (MeEA [M-H]− m/z at 315, EA at 301) by MS/MS in Peak 7 – 10 (Gasperotti et al., 2010; Mullen et al., 2003).

3.3 Changes of phenolics during lab-scale and large-scale processing

HPLC profiles of BRB confections and nectars were the same as that of BRB powder, but in some cases were present at different concentrations due to the effect of processing. Anthocyanins, ellagitannin and total ellagic acid in BRB products were quantified according to the identification above and then were compared to that of lab scale in order to assure similar delivery of these bioactives for the clinical trial. Retention rates of phenolics in both lab-scale and large-scale products are shown in Table 3.2. Confections contained 6.8 mg/g of anthocyanins and proved to have the highest anthocyanins
retention rates (94%) after processing. In addition, there were no distinct changes of
anthocyanins and ellagitannin between lab-scale and large-scale confections
demonstrating consistency in the processing steps (p > 0.05). This was mainly due to the
low mixing temperature of the pectin gel with freeze-dried BRB powder (78 °C). For the
nectars with 10 g BRB dosage, 86% of anthocyanins were retained after large-scale
processing while only 69% was left in the 20 g BRB dose. This was related to the
different processing conditions of the low and high dose of BRB nectars: 75 °C for 15
min and 95 °C for 15 min, respectively. Higher temperature and longer heating time were
required to pasteurize the 20 g BRB dose nectars. Both anthocyanins retention rates in
large scale were significantly lower than those in the lab scale (p < 0.05), mainly due to
the longer mixing time of 10 g BRB powder with pre-mix at 60 °C in the hold cabinet
and higher heating temperature for 20 g dose nectar in the large-scale production.
Anthocyanin degradation is known to follow first-order reactions kinetics and processing
at lower temperature for shorter time improves the retention rates (Kechinski et al.,
2010). In addition, multiple processing steps influence the retention of anthocyanins.
Significantly lower retention of monomeric anthocyanins was found in nonclarified and
clarified juices (31% and 27%) than BRB puree (63%) and berries canned in water (58%)
(Hager et al., 2008a). The comparatively higher retention rates of anthocyanins in BRB
confections and nectars (69 - 94%) may be due to the use of whole BRB powder and
single mixing and/or heating steps. Other factors such as the presence of oxygen, light,
enzymes, and pH fluctuations may also influence the loss of anthocyanins (McGhie et al.,
2007).
Ellagitannin were retained at 95% and 91% after processing of confection and nectar with the 20 g BRB dose in large-scale production. Nectar with lower BRB dose had 66% of ellagitannin retention rate, which was significantly lower than those of confection and nectar with 20 g BRB as well as that of lab scale (p < 0.05). This may be due to partial seeds sedimentation in the Microthermics flow processing system since higher BRB dose nectar would cause blockage of the system, which resulted in the higher BRB dose nectar being processed in OSU pilot plant. This was consistent with previous results where BRB ellagitannins were retained in higher quantities in purees (65%), berries canned in water or syrup (79%) but not juice (only 31-33% retained) where the exclusion of seeds accounted for the significant loss (Hager, 2008). Similar results were also found in blackberry processing where 30% and 18% of ellagitannin were recovered in nonclarified and clarified juices, respectively, while canning and pureeing had little effect (Hager et al., 2010).

All BRB products demonstrated above 100% of ellagic acid retention rates. Ellagic acid increased in raspberry juice after heat processing due to better extraction from the cell matrix (Rommel & Wrolstad, 1993). It was possible that the heat treatment of BRB products in this study may have improved ellagic acid extraction compared to the BRB powder (Hager et al., 2010). Additionally, increased ellagic acid may result from hydrolysis of ellagitannins during processing.

3.4 Consistency of scale-up production of BRB products

Characteristics of BRB confections from different batches and nectars from randomly selected bottles were measured to evaluate the production consistency (Table 3.3).
Confections were prepared within 4 weeks and a total 50 batches (83 ± 4 pieces per batch) totaling 4162 pieces. The average weight of the confections was 8.42 ± 0.35 g with a BRB concentration of 24.95 ± 0.48%. Dosage of BRB was within 5% of desired concentration (Table 3.3). Physical properties such as pH and water activity also showed consistency in the products (Table 3.3).

Rheological properties of confections from different batches (randomly selected) were evaluated (Figure 3.5 A & B). There were no distinct differences in G’, G’’ and η* between different batches at 0.1, 1, 10 and 100 Hz (p > 0.05), suggesting the confections were made with consistent textural properties. Viscosity of samples from 10 g and 20 g of BRB nectar were measured with shear flow tests and results are shown in Figure 3.5 C. With increased shear rate, viscosity showed smaller difference between replicates. Nectars with 10 g and 20 g of BRB had 0.15 ± 0.02 Pa.s and 0.72 ± 0.13 Pa.s viscosity at 1.00 s⁻¹ of shear rate, respectively.

3.5 Stability of phenolics during storage

Concentration and daily delivery amount of anthocyanins, ellagitannins and total ellagic acid in fresh products are shown in Table 3.4. Confections delivered higher daily amount of anthocyanins and ellagitannins than nectars for equivalent BRB doses due to higher initial value of these bioactives in confections (Table 3.2). The retention rate of these phenolics in BRB products during storage are shown in Table 3.5. During storage, anthocyanins in confections decreased significantly from 94% to 87% in 4 weeks but were fairly constant (no statistically significant change) up to 8 weeks of storage. A similar trend was observed for the anthocyanins in the low nectar dose while the 20 g
dose had no significant change until 8 weeks of storage. The depletion of anthocyanins in the confections may be a consequence of residual enzymatic activity, as was observed in processing of blueberry and storage of strawberry nectar (Skrede et al., 2000; Gössinger et al., 2010). Gössinger et al. (2010) reported that enzymes were not fully inactivated even after a heat treatment of 90 °C for 60 min (Gössinger et al., 2010). Therefore, to promote retention of anthocyanins, a balance may need to be reached between initial heat processing to achieve enzyme inactivation (high heat for longer time results in lower anthocyanin concentration) and stability during storage (higher heat initially applied, less degradation during storage). Additionally, storage conditions may also play an important role in the stability of anthocyanins. Refrigerated storage resulted in lowering of the polymeric color percentage of blueberry juice (Howard et al., 2012) and reducing rate of anthocyanin degradation (Srivastava et al., 2007) compared to room temperature.

Retention of ellagitannins was not significantly changed in confection and 20 g BRB nectar during storage (p > 0.05). Ellagitannins in the 10 g BRB nectar decreased significantly but the change was less than 10% after 8 weeks of storage. The high stability of ellagitannins in confections and nectar during storage was consistent with those previously reported in other berries (Hager et al., 2010; Hager, 2008). Total ellagic acid concentration remained stable during storage of all products (p > 0.05). This was consistent with previous reports where ellagic acid derivatives were not affected by thermal processing and remained stable during storage in red raspberry jams (Zafriella et al., 2001).
3.6 Textural stability during storage

Rheological properties of BRB confections showed no significant change in complex viscosity $\eta^*$ (Figure 3.6) during storage with similar trends for $G'$ and $G''$ (data are not shown). Texture profile analysis (TPA) showed significant decrease ($p < 0.05$) in cohesiveness and springiness but not in hardness and chewiness after 2 months of storage (data not shown). Chewiness is the combining results of hardness, cohesiveness and springiness. This indicated that minor changes in cohesiveness and springiness of BRB confections did not influence chewiness. Water content was 27.85 ± 0.55% at day 1 and did not significantly change during 8 week of storage ($p > 0.05$). However, water activity (Aw) increased significantly from 0.72 ± 0.02 to 0.76 ± 0.02 after 8 weeks of storage ($p < 0.05$). This increase in water activity indicated water migration in the gel structure, which resulted in changes of cohesiveness and springiness but no other textural properties. Nectars with both BRB doses showed no significant changes in viscosity during storage at 4 °C ($p > 0.05$).

3.7 Sensory acceptability of BRB products during dietary intervention

Sensory evaluation was obtained from 15 out of 16 subjects from BRB nectar group and 12 out of 16 in BRB confection group. Results are shown in Table 3.6. Nectar and confections scored 7.27 and 7.08 in overall acceptability (7-like moderately on a 9 point hedonic scale). Both products scored above 6 on flavor, aroma, sweetness, and texture. Grittiness was below 6 (like slightly) in nectars, likely due to the seeds in the whole freeze-dried BRB. Grittiness may influence the overall liking score of both products. When subjects were asked in the surveys what factors contribute to or hinder the
consumption of BRB products, 33% in nectar group and 20% in confection group mentioned grittiness. Other factors such as dose frequency in confection may have influenced the overall liking (40% mentioned high dose frequency, 10 pieces per day, was a hindrance).

In this study, both BRB confections and nectars were formulated successfully to deliver a specific dose of bioactives in a consistent manner. After scale up, rheological properties did not vary within a product category and ellagitanin and ellagic acid proved to be more heat stable and consistent than anthocyanins. During 8 weeks of storage, high dose nectar showed the least changes in bioactives. Final products were well accepted (>
like moderately) by men enrolled in a prostate cancer clinical trial. Thus, this study demonstrates that two different BRB delivery vehicles consisting of different matrices can be formulated to meet quality standards and bioactive stability and successfully scaled up for a human clinical trial. The use of these food products can prove to be instrumental in studying the efficacy of BRB bioactives in the prevention or as adjuvant therapy in a variety of cancers (prostate, esophageal, oral etc).

**Abbreviations**

References


Hager A, Howard RL, Brownmiller C. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed black raspberry products. J Food Sci 2008a; 73 134-140.


Keatley KE. Chemoprevention of bladder cancer with anthocyanin-rich extract from black raspberries. Thesis, The Ohio State University, Columbus, 2008.


Mullen W, Yokota T, Lean MEJ, Crozier A. Analysis of ellagitannins and conjugates of ellagic acid and quercetin in raspberry fruits by LC-MS/MS. Phytochem 2003; 64: 617-624.


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Table 3.1 Adjusted formulations of BRB confections and nectars from lab-scale to large-scale production

<table>
<thead>
<tr>
<th>Composition (%)</th>
<th>Confections</th>
<th>Nectars (2 BRB doses)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lab scale</td>
<td>Large scale</td>
</tr>
<tr>
<td>BRB powder</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Sugar</td>
<td>35.0</td>
<td>29.5</td>
</tr>
<tr>
<td>Corn syrup</td>
<td>12.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Water</td>
<td>29.5</td>
<td>37.8</td>
</tr>
<tr>
<td>Pectin</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>50 % (w/w) Citric acid</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 3.2 Retention of phenolics in BRB products after processing

<table>
<thead>
<tr>
<th>Products</th>
<th>Processing scale</th>
<th>Anthocyanins (%)</th>
<th>Ellagitannins (%)</th>
<th>Total ellagic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confection</td>
<td>Lab</td>
<td>94.3 ± 0.3a</td>
<td>93.4 ± 1.5a</td>
<td>101.3 ± 2.3a</td>
</tr>
<tr>
<td>n</td>
<td>Large</td>
<td>94.4 ± 0.98a</td>
<td>95.6 ± 1.9a</td>
<td>106.4 ± 3.3a</td>
</tr>
<tr>
<td>Nectar - 10 g</td>
<td>Lab (75 °C, 10 min)</td>
<td>92.5 ± 0.3a</td>
<td>95.1 ± 1.0a</td>
<td>105.2 ± 3.2a</td>
</tr>
<tr>
<td>BRB</td>
<td>Large (75 °C, 15 min)</td>
<td>86.1 ± 2.7b</td>
<td>66.4 ± 3.5b</td>
<td>113.6 ± 6.8b</td>
</tr>
<tr>
<td>Nectar - 20 g</td>
<td>Lab (85 °C, 10 min)</td>
<td>81.7 ± 0.3a</td>
<td>89.5 ± 0.8a</td>
<td>110.4 ± 4.2a</td>
</tr>
<tr>
<td>BRB</td>
<td>Large (95 °C, 15 min)</td>
<td>69.0 ± 2.8b</td>
<td>91.0 ± 3.6a</td>
<td>127.9 ± 6.2b</td>
</tr>
</tbody>
</table>

a Values represent means ± standard error (n = 24 for confections and n = 9 for nectars). Means with different letters in the same column within each BRB product are significantly different (p ≤ 0.05).
Table 3.3 Characteristics of BRB confections and nectars in scale-up processing$^a$

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>BRB confections (per piece)</th>
<th>Nectar - 10 g BRB (per bottle)</th>
<th>Nectar - 20 g BRB (per bottle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRB dosage (g)</td>
<td>2.10 ± 0.09</td>
<td>10.48 ± 0.31</td>
<td>20.22 ± 0.45</td>
</tr>
<tr>
<td>pH</td>
<td>3.44 ± 0.02</td>
<td>3.64 ± 0.02</td>
<td>3.56 ± 0.02</td>
</tr>
<tr>
<td>Aw</td>
<td>0.72 ± 0.02</td>
<td>0.95 ± 0.00</td>
<td>0.95 ± 0.00</td>
</tr>
</tbody>
</table>

$^a$ Values represent means ± standard error (n = 3 per batch for confections and n = 9 for nectars).
Table 3.4 Concentration and daily delivery of phenolics in fresh BRB products

<table>
<thead>
<tr>
<th></th>
<th>Products for BRB delivery&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Confections</td>
<td>Confections</td>
<td>Nectar -10 g</td>
<td>Nectar -20 g</td>
</tr>
<tr>
<td></td>
<td>(5 pieces/day)</td>
<td>(10 pieces/day)</td>
<td>BRB (one bottle/day)</td>
<td>BRB (one bottle/day)</td>
</tr>
<tr>
<td>Concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthocyanins (mg/g)</td>
<td>6.8 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>1.0 ± 0.03</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Ellagitannins (µg/g)</td>
<td>593.0 ± 12.1</td>
<td>593.0 ± 12.1</td>
<td>66.4 ± 3.5</td>
<td>182.2 ± 9.3</td>
</tr>
<tr>
<td>Total Ellagic acid (µg/g)</td>
<td>113.7 ± 3.5</td>
<td>113.7 ± 3.5</td>
<td>19.6 ± 3.1</td>
<td>44.1 ± 2.1</td>
</tr>
<tr>
<td>Estimated daily amount of delivery&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthocyanins (mg)</td>
<td>285.6</td>
<td>571.2</td>
<td>253.7</td>
<td>399.2</td>
</tr>
<tr>
<td>Ellagitannins (mg)</td>
<td>24.9</td>
<td>49.8</td>
<td>16.8</td>
<td>45.5</td>
</tr>
<tr>
<td>Total Ellagic acid (mg)</td>
<td>4.8</td>
<td>9.6</td>
<td>5.0</td>
<td>11.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average weight of products: confections were 8.4 g/piece, nectars - 10 g BRB were 253.7 g/bottle; nectars - 20 g BRB were 249.5 g/bottle. <sup>b</sup> Estimated daily amount of delivery was obtained from the average weight of confections/nectars, thus no SD was provided.
**Table 3.5 Retention of phenolics in BRB products during two-month storage at 4 °C**

<table>
<thead>
<tr>
<th>Products</th>
<th>Storage time (Weeks)</th>
<th>Anthocyanins (%)</th>
<th>Ellagitannins (%)</th>
<th>Total ellagic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confection</td>
<td>0</td>
<td>94.4 ± 1.0a</td>
<td>95.6 ± 1.9a</td>
<td>106.4 ± 3.3a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>87.0 ± 1.0b</td>
<td>94.4 ± 2.7a</td>
<td>101.4 ± 7.3a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>84.5 ± 1.6b</td>
<td>95.7 ± 4.8a</td>
<td>100.0 ± 1.4a</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>83.4 ± 2.5b</td>
<td>93.6 ± 4.5a</td>
<td>106.9 ± 6.9a</td>
</tr>
<tr>
<td>Nectar - 10 g BRB</td>
<td>0</td>
<td>86.1 ± 2.7a</td>
<td>66.4 ± 3.5a</td>
<td>113.6 ± 6.8a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>78.9 ± 3.6b</td>
<td>59.6 ± 1.3b</td>
<td>105.0 ± 2.3a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>77.9 ± 3.2b</td>
<td>57.2 ± 2.6b</td>
<td>103.2 ± 7.0a</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>76.3 ± 2.7b</td>
<td>60.2 ± 1.7b</td>
<td>104.1 ± 4.8a</td>
</tr>
<tr>
<td>Nectar - 20 g BRB</td>
<td>0</td>
<td>69.0 ± 2.8a</td>
<td>91.0 ± 4.6a</td>
<td>128.0 ± 6.2a</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>68.5 ± 3.2ab</td>
<td>91.4 ± 3.7a</td>
<td>129.4 ± 3.0a</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>66.3 ± 1.0ab</td>
<td>89.9 ± 3.1a</td>
<td>125.3 ± 2.5a</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>65.1 ± 3.3b</td>
<td>93.3 ± 2.4a</td>
<td>127.8 ± 3.3a</td>
</tr>
</tbody>
</table>

*Values represent means ± standard error (n = 24 for confections and n = 9 for nectars). Means with different letters in the same column within each BRB product are significantly different (p ≤ 0.05).*
Table 3.6 Sensory acceptance of BRB confections and nectars with 9-point hedonic scale

<table>
<thead>
<tr>
<th>Products</th>
<th>Overall liking</th>
<th>Flavor</th>
<th>Aroma</th>
<th>Sweetness</th>
<th>Hardness or Thickness</th>
<th>Grittiness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confections</td>
<td>7.08 ± 1.68</td>
<td>7.08 ± 1.78</td>
<td>6.25 ± 1.36</td>
<td>6.92 ± 1.98</td>
<td>6.83 ± 1.75</td>
<td>6.08 ± 1.78</td>
</tr>
<tr>
<td>Nectars</td>
<td>7.27 ± 1.44</td>
<td>7.00 ± 1.60</td>
<td>6.93 ± 1.44</td>
<td>6.53 ± 1.77</td>
<td>6.47 ± 1.60</td>
<td>5.33 ± 1.76</td>
</tr>
</tbody>
</table>

Values represent means ± standard error (n = 12 for confections and n = 15 for nectars).
**Figure 3.1** BRB delivery dosage and total BRB products in a prostate cancer clinical trial.

<table>
<thead>
<tr>
<th>Intervention Groups</th>
<th>Products</th>
<th>Total products for 40 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nectar, 10 g BRB/day</td>
<td>10 g BRB in one bottle ~ 8 oz (92.5 kcal)</td>
<td>320 bottles</td>
</tr>
<tr>
<td>8 men</td>
<td></td>
<td>320 bottles</td>
</tr>
<tr>
<td>Nectar, 20 g BRB/ day</td>
<td>20 g BRB in one bottle ~ 8 oz (145 kcal)</td>
<td>320 bottles</td>
</tr>
<tr>
<td>8 men</td>
<td></td>
<td>320 bottles</td>
</tr>
<tr>
<td>Confection, 10 g BRB/day</td>
<td>10 g BRB in 5 pieces ~ 8 g/piece (23.1 kcal)</td>
<td>1600 pieces</td>
</tr>
<tr>
<td>8 men</td>
<td></td>
<td>1600 pieces</td>
</tr>
<tr>
<td>Confection, 20 g BRB/day</td>
<td>20 g BRB in 10 pieces ~ 8 g/piece (23.1 kcal)</td>
<td>3200 pieces</td>
</tr>
<tr>
<td>8 men</td>
<td></td>
<td>3200 pieces</td>
</tr>
</tbody>
</table>
Figure 3.2 Frequency sweep of lab-scale confection (circle) and large-scale confection (triangle). Storage modulus $G'$ - solid fill; loss modulus $G''$ - blank fill; complex viscosity $|\eta^*|$ - gray fill.
Figure 3.3 Viscosity of nectar premix with different concentrations of large-scale RS 400 pectin compared to CF 130 B pectin used in lab scale.
**Figure 3.4** HPLC profile of freeze-dried BRB powder and the tentative identification of each peak.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Retention Time (min)</th>
<th>HPLC-UV (nm)</th>
<th>MS [M-H]-</th>
<th>MS/MS [M-H]-</th>
<th>Tentative identification¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>6.601</td>
<td>520</td>
<td>447.094</td>
<td>285.040</td>
<td>Cy 3-glu</td>
</tr>
<tr>
<td>1b</td>
<td>6.601</td>
<td>520</td>
<td>579.137</td>
<td>285.040</td>
<td>Cy 3-sam</td>
</tr>
<tr>
<td>1c</td>
<td>6.885</td>
<td>520</td>
<td>725.194</td>
<td>285.040</td>
<td>Cy 3-xyrut</td>
</tr>
<tr>
<td>1d</td>
<td>6.885</td>
<td>520</td>
<td>593.151</td>
<td>285.040</td>
<td>Cy 3-rut</td>
</tr>
<tr>
<td>2</td>
<td>8.923</td>
<td>355</td>
<td>741.118</td>
<td>301.036</td>
<td>Q-xyrut</td>
</tr>
<tr>
<td>3</td>
<td>8.941</td>
<td>260</td>
<td>934.090²</td>
<td>935.068, 633.066, 300.999</td>
<td>ET (Sanguin H-6)</td>
</tr>
<tr>
<td>4</td>
<td>9.869</td>
<td>360</td>
<td>300.998</td>
<td>300.998</td>
<td>EA</td>
</tr>
<tr>
<td>5</td>
<td>10.066</td>
<td>355</td>
<td>609.146</td>
<td>301.036</td>
<td>Rutin</td>
</tr>
<tr>
<td>6</td>
<td>10.403</td>
<td>355</td>
<td>477.065</td>
<td>301.036</td>
<td>Q-hexA</td>
</tr>
<tr>
<td>7</td>
<td>11.167</td>
<td>360</td>
<td>447.057</td>
<td>315.012, 300.998</td>
<td>Me-EA-pentose</td>
</tr>
<tr>
<td>8</td>
<td>11.319</td>
<td>360</td>
<td>475.059</td>
<td>300.998</td>
<td>Ac-EA-Pentose</td>
</tr>
<tr>
<td>9</td>
<td>11.511</td>
<td>360</td>
<td>533.059</td>
<td>315.016, 300.998</td>
<td>Mal-Me-EA-Pentose</td>
</tr>
<tr>
<td>10</td>
<td>12.541</td>
<td>360</td>
<td>489.066</td>
<td>315.015, 300.998</td>
<td>Ac-Me-EA-Pentose</td>
</tr>
</tbody>
</table>

¹Continued
Figure 3.4 Continued

Figure 3.5 Rheological consistency of BRB products in the scale-up production. Confection $G'$ (black fill) and $G''$ (blank fill) (A), confection $|\eta^*|$ (gray fill) (B), nectar - 10 g (solid fill) and 20 g (blank fill) $\eta$ (C).
Figure 3.5 Continued

B

Frequency (Hz)

|n*| (Pa.s) 

0.1 1 10 100

Continued
Figure 3.5 Continued

C

[Diagram showing a graph with shear rate on the x-axis and viscosity (n, Pa.s) on the y-axis. The graph illustrates the relationship between shear rate and viscosity across different ranges.]
Figure 3.6 Viscosity of BRB scale-up products during storage at 4 °C. Confections $|\eta^*|$ (A), nectar - 10 g BRB dosage $\eta$ (B), nectar - 20 g BRB dosage $\eta$ (C).

A
Figure 3.6 Continued

B

![Graph showing viscosity (n in Pa.s) vs. shear rate (1/s) for Nectar - 10 g BRB over 8 weeks.](image)

- **Day 1**
- **4 weeks**
- **6 weeks**
- **8 weeks**

Continued
Figure 3.6 Continued

C

Nectar - 20g BRB

Shear rate (1/s)

0.01 0.1 1 10 100 1000 10000

n (Pa.s)

0.01

0.1

1

10

Day 1

4 weeks

6 weeks

8 weeks
Chapter 4: Dietary Intervention of Black Raspberry on Early TRAMP Prostate Carcinogenesis

Authors:

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Abstract

Black raspberries (BRB) (*Rubus Occidentialis*) are rich in a wide range of bioactive compounds that may contribute to the preventive effects of BRB in gastrointestinal-tract related cancers. To investigate molecular mechanism changes in early prostate carcinogenesis modulated by dietary BRB, four-week old transgenic adenocarcinoma of the mouse prostate (TRAMP) mice and their non-transgenic (WT) littermates were fed with control AIN-93 G diet or 10% BRB diet for 6 weeks. Body weights were recorded weekly throughout the study and plasma and prostate lobes were collected at final necropsy for metabolite biodistribution and biological analysis. Prostate transcriptomic changes in mice with different diet were analyzed by RNA-Seq.

Ten-week-old TRAMP mice have developed early prostatic intraepithelial neoplasia (PIN) with histological analysis and they have significantly higher urogenital tract (UGT) and prostate weights regardless of dietary treatment ($P < 0.05$). Around 2018 and 3129 genes have been distinctly changed with genotype (TRAMP vs. WT) in control and BRB diet, separately (adj $P \leq 0.01$ & FoldChange $\geq 2$). Urolithins were detected and quantified in plasma, liver and prostate of mice fed with BRB by HPLC-MS/MS and prostate urolithins were moderately correlated with liver urolithins ($r=0.6425$). BRB diet significantly modulated gene changes (adj $P \leq 0.05$) in TRAMP mice during 6 weeks of feeding which are related to cell morphology and proliferation demonstrating that dietary exposure has an impact on the prostate warranting further investigation into the mechanisms of action and cancer preventive potential in the prostate.
1. Introduction

Epidemiological evidence suggests that dietary and lifestyle patterns may contribute to the considerable differences in prostate cancer (PCa) incidence between Asian and American populations. As to dietary factors, notably many types of plant-based food components have been studied for their PCa-preventive efficacy and results have shown decreased proliferation index, reduced tumor incidence and lowered rates of metastasis. These investigations include, but are not limited to, pomegranate fruit extract, green tea polyphenols extract, tomato/lycopene and soy genistein (Adhami et al., 2012; Gupta et al., 2001; Wang et al., 2007; Zuniga et al., 2013).

As a fruit rich in bioactive compounds, black raspberries (BRB) have been well studied in gastrointestinal tract related cancers, such as oral, esophageal and colon cancer, by Stoner group at The Ohio State University in cell culture systems, animal models and several human clinical trials (Kresty et al., 2001; Casto et al., 2002; Mallery et al., 2007 & 2008; Wang et al., 2009, 2010 & 2011). These studies showed the promising preventive effects of BRB in cancer models through anti-proliferative, apoptosis inducing, anti-inflammatory, cell growth inhibition, oxidative stress reduction and angiogenesis inhibition effects (Stoner et al., 2008). Localized absorption of BRB compounds appears to be important for their preventive effects in the oral cavity, esophageal and colon (Stoner et al., 2008). For systemic cancers, there is a concern that whether the low uptake of the bioactive compounds into the bloodstream shown in pharmacokinetic studies (Ravoori et al., 2012; Stoner et al., 2005) could have sufficient protective effects on cancer development in vivo. Addressing factors such as the BRB bioactive compound
concentrations in the circulation and the deposition of different bioactive metabolites or derivatives in various tissues are crucial to understand their potential role in systemic cancer prevention/treatment.

Studies on pomegranate enriched in punicalagin (one type of ellagitannins) showed anticancer effects on PCa in cell culture and xenograft mouse model (> 50% inhibition in tumor volume) which may provide promising evidence that BRB, a fruit also rich in ellagitannins (2mg/g dry weight) and anthocyanins (30 mg/g dry weight) (Stoner, 2009), may have preventive effects against PCa. Notably, a phase II clinical study demonstrated mean PSA doubling time increased significantly from 15 to 54 months after 46 PCa patients consumed 8 ounces of pomegranate juice enriched with punicalagin (> 2 g/L) daily for an average of 13 months (Pantuck et al., 2006). Ellagitannin microbial metabolites, urolithins and their derivatives, showed lower IC50 values than pomegranate ellagic acid tested in LNCaP, LNCaP-AR, DU145, and 22RV1 prostate cancer cells. Urolithins were detected in mouse prostate, intestinal, and colon tissues (Seeram et al. 2007) after consumption of urolithin A. Moreover, urolithins and ellagic acid derivatives were detected in prostate tissue of small percentage of patients with benign prostatic hyperplasia (BPH) or PCa after intake of pomegranate (200 ml/day) for 3 days (González-Sarrías et al., 2010) indicating that urolithins could reach to prostate tissue and may directly modulate disease progression. Besides ellagitannins, BRB have a wide range of bioactive compounds which could impact multiple signaling pathways in mammalian carcinogenesis and thus may have additive and/or synergistic activity to enhance anti-cancer efficacy. No animal or clinical studies have investigated the effects
of whole BRB with varieties of bioactive compounds on PCa. Therefore, such studies are needed to help further elucidate the PCa prevention potential of this berry.

PCa mainly is a disease of older men, while prostate specimens from young men aged ≤ 55 years have shown early-onset PCa (Salinas et al., 2014), indicating that cancer initiation has already started at an early age. Regarding that PCa has a long latency, delaying its onset or progression is likely to have an invaluable impact on PCa outcome. The mechanisms associated with the initiation of the carcinogenesis process in the prostate are not well defined and subsequently the molecular mechanisms of action of preventive dietary intervention are not well understood. Wan et al. (2014) evaluated the impact of dietary tomato and lycopene feeding on 200 PCa-related genes that are involved in androgen signaling and carcinogenesis in TRAMP mice at early stage using NanoString nCounter technology. They found that 30 out of 189 detectable genes were significantly affected by tomato or lycopene which are associated with stem cell features (whole tomato) and neuroendocrine differentiation (lycopene), indicating that gene changes initiated early prostate carcinogenesis in TRAMP mice and dietary food components could target some potential genes that might influence the PCa outcome. To date, there are no studies examining the effect of a BRB intervention on the transcriptional signature of the prostate while cancer is developing. The primary objective of this current study is to evaluate the impacts of dietary BRB on prostatic transcriptional changes during early prostate carcinogenesis.
2. Materials and Methods

2.1 Animals, diets and study design

All animal protocols and procedures were in compliance with The Ohio State University (OSU) Institute Animal Care and Use Committee. Male C57BL/6 TRAMP\(^{+/-}\) x FVB F1 (TRAMP) and their non-transgenic male littermates (WT) were generated at the Ohio State University animal facility and genotyped at 3 weeks of age for the probasin-SV40 T-antigen (PB-Tag) transgene from tail tip DNA using the REDExtract-N-Amp tissue PCR kit (Sigma-Aldrich, St. Louis, MO, USA). TRAMP and WT mice were weaned at 4 weeks of age and randomly divided into groups fed with control AIN-93 G diet (#110700, Dyets, Bethlehem, PA, USA) or 10% BRB powder diet separately using a 2 × 2 factorial study design (\(n = 20-24\)/group, total of 4 groups). Freeze-dried BRB powder was the same batch used in our previous human clinical study (Gu et al., 2014), which was reported containing 1.35 mg ellagittannins/g. Assuming 3 g as the average mouse daily intake, 10% BRB diet provides an average of 0.4 mg ellagittannins/day/mouse. This ellagittannin dose was similar to a reported study which used 0.3 mg ellagittannins from pomegranate extract (Seeram et al., 2007). Compositions of the control and 10% BRB diets were listed in Table 4.1. Both diets were in powdered form and stored at -20 °C in the dark, provided freshly every other day to mice in sufficient amount for 6 weeks.

Body weights were recorded weekly throughout the study. Mice were sacrificed at 10 weeks of age and blood was collected by cardiac puncture. Plasma was obtained after blood centrifuge at 1.3 rcf for 10 min and stored at -80 °C for urolithin measurement.
Liver, kidney and spleen were collected for both histology and protein analysis; for protein analysis, tissues were snap-frozen in liquid nitrogen and then stored at -80 °C. The urogenital tract (UGT) including bladder, urethra, seminal vesicles, ampullary gland and the prostate was excised, removed and weighed after mouse sacrifice. Prostate glands were excised and separated by microdissection into constituent lobe pairs: ventral (VP), lateral (LP), dorsal (DP) and anterior (AP). Weight of bladder and prostate lobes were recorded if the mouse was not designated for RNA analysis. Ten mouse prostates per experimental group were randomly assigned for RNA analysis and preserved overnight in RNAlater solution (Life Technologies, Grand Island, NY) before stored at -80 °C. Ten prostates in each group were assigned for both histology (one lobe pair) and protein/urolithin measurement (the other pair). Prostate lobes for protein/urolithin measurement were snap-frozen in liquid nitrogen and stored at -80 °C. For histological staining, tissues were immediately fixed in 10% neutral buffered formalin. After overnight fixation, the tissues will be transferred into 70% ethanol and further processed by dehydration and paraffinization in a tissue processor. All samples were embedded in paraffin by a tissue embedding system.

2.2 Measurement of plasma, liver and prostate urolithins by HPLC-ESI-MS/MS

Sample preparation. Plasma, liver and prostate from 11 mice in BRB group (5 WT & 6 TRAMP) were analyzed for urolithins. Three WT mice in control diet group were analyzed for liver and plasma urolithins. Plasma samples were thawed and extracted using probe sonication with acetonitrile added into plasma in a 2:1 ratio (v/v). Around 100 mg liver samples were weighed and finely minced and pulverized in liquid nitrogen,
and then sonicated in 0.5 ml water before acetonitrile was added in a 2:1 (v/v) ratio for extraction. Approximate 30 mg pooled prostate lobes were weighed and finely minced in liquid nitrogen and sonicated in 150µl water before adding acetonitrile in a 2:1 (v/v) ratio. After addition of acetonitrile, all samples were probe sonicated. Samples were centrifuged at 2000 ×g for 10 min and the supernatant was collected. The pellet was re-suspended in acetonitrile/water (2:1, v/v) then probe sonicated and centrifuged. Supernatants were pooled and dried under nitrogen. Dried samples were re-suspended in 0.5 ml water, then 0.5 ml 2 M sodium acetate buffer (pH 5.5) was added before 10 µl 54 mg/ml glucuronidase/sulfatase (S9296, Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 ºC for 2 hours. Samples were extracted twice with 3 volumes of diethyl ether and supernatants were pooled and dried under nitrogen. The dry liver residue was dissolved in 20 µl methanol, bath sonicated, then 180 µl 0.1% aqueous formic acid (v/v) added, bath sonicated and filtered through a 0.2 µm PTFE syringe filter before UPLC-MS/MS. Dry prostate and plasma residues were processed identically except redissolution solvent was 10 µl methanol plus 90 µl 0.1% aqueous formic acid (v/v).

**HPLC-ESI-MS/MS Analysis.** Filtered samples (5 µl) were injected into a UPLC system (ACQUITY, Waters, Milford, MA, USA) and separated on a BEH C18 Reverse Phase column (2.1 × 50 mm, 1.7 µm; ACQUITY, Waters). The mobile phase for separation consisted of 1% aqueous formic acid (v/v) (A) and 1% formic acid in acetonitrile (v/v) (B). Initial composition was held at 10% B for 0.5 min and increased linearly to 55.5% B by 5 min, then increased to 95% B by 6 min and re-equilibrated through 9 min. Column temperature was 40 ºC and flow rate was 0.3 ml/min. UPLC flow
was introduced to a triple quadrupole mass spectrometer (Quattro Ultima, Waters, Milford, MA, USA) via an electrospray probe without splitting. Standards of urolithin A, B, C, D (UA, UB, UC, UD), methyl urolithin A (mUA), and dimethyl urolithin A (DMUA) were used for external calibration. These standards were synthesized by the lab of Jim Fuchs (OSU Medicinal Chemistry Department) with identity and purity confirmed by NMR. Methyl urolithin C (mUC) and dimethyl ellagic acid (DMEA) were tentatively identified according to their accurate mass and fragmentation (loss of methyl groups) which determined separately on a Q-TOF instrument (6550, Agilent, Santa Clara, CA, USA). In addition, UV absorption was consistent with urolithin and ellagic acid structures, respectively. MS/MS transition included 261>171, 199 for UD, 245>155, 183 for UC, 229>128, 157 for UA, 259>183 for mUC, 213>115, 141 for UB, 243>171, 184 for mUA, and 257>198 for di-mUA, each monitored in positive ion mode. DMEA was monitored in negative ion mode during the same analysis and 329>299, 314 transitions were used for detection. MS source parameters included 500 °C desolvation temperature, 800 l/hr desolvation gas, and 3x10⁻³ mBar collision gas pressure (Ar).

2.3 Histological staining

Paraffin-embedded tissue blocks were cut with 5 µm thickness by an automated microtome (HM355S Microm, Thermo Scientific, Bellefonte, PA, USA) and then baked at 60 °C for 60 min before the slides were processed and stained with hematoxylin and eosin (H&E) in an automated slide stainer (AutoStainer XL, Leica Instrument, Nussloch, Germany). Bright field images were captured with a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY, USA) equipped with a Nikon DS-Ri digital camera.
2.4 Immunohistochemical (IHC) analysis

**Proliferation biomarker - Ki67.** Protein expression of the Mki67 gene in prostate lobes was evaluated using IHC. Tissue blocks were sectioned into 5 µm and baked at 60 °C for 60 min before performing rehydration antigen retrieval (1× Citra Plus Buffer, Biogenex, San Ramon, CA, USA) for 30 min in a steamer. After one wash of double deionized water, the tissue sections were incubated with endogenous H₂O₂ block for 15 min, monoclonal rat anti-mouse mAb Ki67 (TEC-3, Dako, Carpinteria, CA, USA) at 1:50 for 60 min, biotinylated rabbit anti-rat IgG (Dako) at 1:200 for 30 min, followed by streptavidin/HRP (1:200) for 30 min and 3,3’-diaminobenzidine (Dako) for 10 min to visualize the staining. Each step was followed by 5 min washing for 3 times with wash buffer (Biogenex). The stained tissue sections were finally counterstained with Mayer’s hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) for 2 min. Slide were scanned using Aperio Scancope XT Scanner (40X, Leica Biosystems, Buffalo Grove, IL, USA) and Ki67 staining was quantified by counting positive pixels as a percentage of total (positive and negative) pixels for the whole prostate lobe.

2.5 RNA isolation and RNA-Seq measurement

Prostate dorsal lobes were pulverized in liquid nitrogen with a mortar and pestle. Total RNA was extracted using Norgen RNA/Protein purification plus kit protocol (Norgen Biotech, ON, Canada). RNA integrity was verified by an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) and samples with RIN score higher than 7 were selected for further analysis. High quality RNA from WT and TRAMP mice in both control and BRB diet groups (n=5/group; 4 groups) were obtained and processed using an
Illumina RNA-Seq sample preparation kit. Final generated RNA-Seq libraries were validated and sequenced in paired-ended approach with 50 bp/sequence using an Illumina HiSeq 2500 sequencer at a depth of approximately 17 - 20 million sequences per sample. After alignment to GRCm38.p2 with STAR and QC analysis, RNA-Seq reads were uploaded to R package (edgeR) for RDESeq2 analysis. Gene reads were normalized and compared statistically for genotype, diet and genotype × diet effects with reported log2FoldChange (log2FC), pairwise P-values and adjusted P-values (False Discovery Ratio, FDR).

2.6 Pathway analysis

Genes were analyzed with Qiagen’s Ingenuity Pathway Analysis (IPA) for core and comparison analysis to illustrate transcriptomic changes by genotype and diet. Gene expression log2FC and FDR for TRAMP vs. WT in both diets and BRB vs. Control diet in both genotypes were used as data inputs. IPA Base (Genes Only) and mouse gene were selected for this study. Cutoff criteria set to do core and comparison analysis were determined and shown in Table 4.2.

2.7 Statistical analysis

Mouse body weight, tissue weight and Ki67 expression in prostate were compared by a two-way ANOVA model using SigmaPlot 13.0 (Systat Software Inc.) to define the main and interaction effects of genotype and diet. A one-way analysis of variance (ANOVA) and Tukey’s post-hoc test were used to determine the significance in urolithins among mouse plasma, liver and prostate. Independent t-test compared urolithin detection differences between WT and TRAMP mice with SigmaPlot 13.0. $P$-value ≤ 0.05 was considered to be significantly different.
3. Results

3.1 Mouse body and tissue weights

Mouse body weight during 6 weeks of feeding was not significantly affected by either diets or genotype \((P>0.05)\) with a final body weight of \(27.68 \pm 2.65\) g (Figure 4.1A). Weights of mouse liver, spleen and kidney were not substantially different by diets or genotype \((P >0.05)\). Mouse urogenital tract (UGT) weights were larger in TRAMP compared to WT UGT \((P<0.05)\) while diet did not significantly influence UGT mass (Figure 4.1B). Bladder and individual prostate lobe pairs were also weighed and compared within diet and genotype groups, and it is shown that VP, LP and DP weights were significantly higher in TRAMP mice compared to WT ones (Figure 4.2) \((P<0.05)\). There were no diet influence in these tissue weights \((P>0.05)\).

3.2 Plasma, liver and prostate urolithin concentrations

With HPLC-MS/MS based quantification, urolithins were detected in plasma, liver and prostate of BRB-fed mice while they were not detectable in liver or plasma of control-fed WT mice. UA were the main metabolites found in all samples and the average concentration in liver \((0.42 \pm 0.22 \mu\text{mol/kg})\) was significantly higher than that of plasma \((0.12 \pm 0.06 \mu\text{M})\) \((P<0.05)\); UA was detected in the prostate at a concentration \((0.21 \pm 0.17 \mu\text{mol/kg})\) not significantly different than either liver or plasma (Figure 4.3A). No significant differences were observed by genotype (WT vs. TRAMP) for plasma, liver or prostate \((P>0.05)\).

Besides UA, UC was also detected in all plasma and liver samples, and 3 out of 11 prostate samples. Averaged UC levels were \(0.0067 \pm 0.0063 \mu\text{M}\) in plasma, \(0.0154 \pm 0.0050 \mu\text{M}\) in liver, and \(0.0037 \pm 0.0032 \mu\text{M}\) in prostate.
0.0143 μmol/kg in liver and 0.0167 ± 0.0032 μmol/kg among the 3 with detectable levels in the prostate. Methyl UC and DMEA were also identified and detected in some but not all samples: mUC in one plasma and 2 prostates and DMEA in 6 plasma and 4 liver samples. Due to the lack of standards for mUC and DMEA, these detected compounds were not quantified.

Correlations between UA levels in prostate and liver or prostate and plasma demonstrate a moderate relationship between liver and prostate (r=0.64; Figure 4.4) while plasma UA and prostate UA show little correlation (r=0.13).

### 3.3 Histological staining and IHC analysis of Prostate

Genotype impacted prostatic morphology in mice of 10 weeks of age with TRAMP showed multifocal early PIN lesions in VP, LP, DP and AP (arrows indicated some PIN in Figure 4.5). This study was not designed to define a subtle dietary impact on histopathology. However, histologic images of WT and TRAMP prostate lobes in control diet were shown in Figure 4.5. Compared to WT mice with typical luminal morphology, TRAMP mice showed characteristics of luminal epithelium hyperplasia, nuclei and nucleoli enlargement and polymorphonuclear epithelium in all four lobes, but the extent of the phenotype is greatest in the VP, LP and DP compared to the AP.

As measured by IHC staining for Ki67 expression, TRAMP mice at 10 weeks of age had higher proliferation in all four lobes compared to WT mice fed with control diet (Figure 4.6). BRB diet groups showed the same trend (images not shown). Determined by quantification of positivity in scanned IHC images, genotype distinctly impacted the expression of Ki67 in TRAMP prostates compared to WT ones ($P = 0.001$ in DP with control diet; $P<0.05$ in four lobes with BRB diet). With two-way ANOVA, significant
interactions between genotype and diet were shown in the expression of Ki67 in VP ($P = 0.005$), LP ($P = 0.033$) and DP ($P = 0.030$), and BRB diet increased Ki67 expression in TRAMP mice verses control diet in these three types of lobes ($P<0.05$).

3.4 Molecular pathways and gene expression impacted by genotype

As shown in Table 4.2, RNAseq analysis reported total 38921 gene IDs in all mouse prostate, among them 25312 were mapped in the IPA Base analysis. Analysis-ready IDs are genes met the cutoffs and might be eligible for analysis of networks, functions, and canonical pathways and listed in Table 4.2. There were 5393 and 6456 genes for IPA core analysis in TRAMP vs. WT control diet and TRAMP vs. WT BRB diet, separately (FDR ≤ 0.01). Top 10 canonical pathways that most significantly regulated by genotype in control diet were graphed in Figure 4.7. Mismatch repair in eukaryotes, the most significantly regulated pathway by genotype, had 16 molecules significantly altered by genotype (1 down regulated and 15 up regulated) [Pathway $P$-value = 1.99E-08 or $-\log(P$-value) = 7.70]. Hereditary breast cancer signaling involved the highest number of molecules with 36 down regulated and 89 up regulated [Pathway $P$-value = 1.37E-07 or $-\log(P$-value) = 6.86]. The overall increased/decreased activity of canonical pathways is predicted based on a z-score algorithm. Four out of these 10 pathways had positive z-scores predicting increased activity and these were: role of BRCA1 in DNA damage response, p53 signaling, pancreatic adenocarcinoma signaling and role of CHK proteins in cell cycle checkpoint control.

IPA Disease Functions analysis was performed with the added threshold of $|\log2FC| \geq 1$ (Table 4.2). Sixteen related genes in the IPA Disease and Function of
“PIN and prostate cancer & tumor” and their log2FC and P-value for the effect of genotype in control diet are reported in Table 4.3. Twelve genes were strongly up regulated in TRAMP compared to WT including cyclin-dependent kinase inhibitor 2A (CDKN2A), prostate stem cell antigen (PSCA), baculoviral IAP repeat containing 5 (BIRC5), forkhead box M1 (FOXM1), cyclin-dependent kinase inhibitor 2C (CDKN2C), peroxisome proliferator-activated receptor gamma (PPARG), DNA (cytosine-5-)methyltransferase 1 (DNMT1), pim-1 proto-oncogene, serine/threonine kinase (PIM1), retinoblastoma-like 1 (RBL1), erb-b2 receptor tyrosine kinase 2 (ERBB2), XIAP associated factor 1 (XAF1), cyclin-dependent kinase inhibitor 1B (CDKN1B). Four genes down regulated in TRAMP compared to WT were insulin-like growth factor 1 (IGF1), acid phosphatase, prostate (ACPP), NK3 homobox 1 (NKX3-1), and transmembrane protease, serine 2 (TMPRSS2).

The Comparison Analysis was conducted to compare the genotype effect between the two different diets. Within this analysis, 14 IPA Diseases & Functions displayed z-scores in different directions, predictive of different activity in control and BRB diets (Table 4.4). Compared to control diet, genotype effect in BRB diet is associated with decreased cell differentiation, vasculogenesis, cell movement, quantity of cells, angiogenesis and survival of organism. Functions of increased cell death, apoptosis and necrosis were increased in BRB diet group. The “Proliferation of cells” Function demonstrated increased activity in both diets with a smaller z-score in BRB diet compared to control diet (1.128 vs. 3.179). The Function “Prostate cancer and tumors” had low z-scores in
both diets but with opposite predicting directions (0.485 in control diet and -0.355 in BRB diet).

### 3.5 Gene expression and top network impacted by dietary BRB

With IPA core analysis, 14 genes in TRAMP mice and 2 genes in WT involved in molecular networks were significantly impacted by BRB diet treatment (FDR ≤ 0.05) (Table 4.5). Out of 14 genes, 12 were up regulated including interleukin 31 receptor A (IL31RA), filamin A interacting protein 1-like (FILIP1L), cyclin-dependent kinase 8 (CDK8), rho GTPase activating protein 21 (ARHGAP21), Kv channel interacting protein 1 (KCNIP1), sarcospan (SSPN), jumonji, AT rich interactive domain 2 (JARID2), gephyrin (GPHN), spermatogenesis associated 5 (SPATA5), autophagy related 4A, cysteine peptidase (ATG4A), ribosomal protein L21 (RPL21), and karyopherin alpha 1 (importin alpha 5) (KPNA1). Histocompatibility 2, Q region locus 5 (H2-Q5) and Histocompatibility 2, Q region locus 2 (HLA-A or H2-Q2) were down regulated. In WT mice, there were only 2 genes involved in networks and distinctly impacted by BRB diet which were CDK8 and hemoglobin X, alpha-like embryonic chain in Hba complex (HBZ or Hba-x).

Gene expression was analyzed to identify cellular networks that may illustrate the action of BRB on TRAMP early carcinogenesis. Seven networks associated with the gene changes were observed and Network 1 included 8 out of 14 genes identified in TRAMP (Figure 4.8). These molecules were related to cell morphology, lymphoid tissue structure and development, and also infectious disease with TP53, RBL1, MYC, STAT1 and IFN-α/β as key molecules interacting with other molecules in this network.
4. Discussion

In this study, we investigated how BRB diet impacts early carcinogenesis of prostate with TRAMP model. Dietary BRB (10% w/w) was well tolerated with no evidence of toxicity as examined histological analysis of liver, kidney and spleen tissues (results not presented). As expected, regardless of diet, UGT and prostate lobe weights were significantly higher in TRAMP mice compared to WT ones while body weight were not distinctly impacted. Histologic analysis and gene expression were distinctly influenced by genotype. All this indicates that TRAMP mice started to show increased proliferation and PIN at this early stage (10 weeks). Interestingly, BRB interacted with genotype impacting Ki67 expression in prostate and also significantly influenced the expression of a number of genes in both WT and TRAMP mice.

4.1 Measurement of BRB metabolites in mouse plasma and tissues

BRB ellagitannin metabolites, urolithins, were found in mouse plasma, liver and prostate. UA and UC were detected and quantified in all three types of mouse samples and two more compounds, methyl UC and Dimethyl ellagic acid (DMEA), were identified in some of the samples. Urolithin profile in mice showed less variety and inter-variance when comparing to human clinical studies that presenting urolithins such as UA, UB, UC, UD and isoUA, detected in urine, plasma, prostate and colon tissues after consumption of ellagitannin enriched pomegranate juice/extract or walnut (González-Sarrías et al., 2010; Nuñez-Sánchez et al., 2014).

In our study, prostate UA reached to 48.17 ± 37.98 ng/g after ~0.4 mg ellagitannin/day from 10% BRB diet without fasting. Seeram et al. (2007) reported that UA was detected with a peak concentration of 1000 ng/g at 4 hours after 0.3 mg pure UA
compound administration. However, UA or its derivatives were not detectable in mouse tissues (including prostate) following pomegranate extract administration either through oral or intraperitoneal administration. The authors hypothesized that this may be due to the lack of microflora that necessary for urolithin production in mice. Interestingly, González-Barrio et al. (2011) found the occurrence of UA and UC in mouse feces after having pomegranate ellagitannins, which provided the evidence that mice like other measured mammals (rat, squirrels, beavers, pigs, sheep, bull calves and humans) have related microbiota to transform ellagitannins to urolithins. No other mouse tissue measurement was reported except mouse feces in this study. According to Stoner et al. (2005 & 2008), a dose of 5% BRB administered to mice (~ 25 g body weight) is the estimated equivalent of 45 g of BRB per day in human subjects (70 kg adult), from which our human equivalent dose would be 90 g BRB (121.5 mg ellagitannin/day). This present study reported for the first time on 1) urolithin detection in mouse prostate after consumption of ellagitannins from a food source that is achievable in human equivalent dose, 2) an optimized analytical protocol for urolithins in tiny mouse prostate (~ 30 mg), 3) urolithin observation in mouse plasma and tissues to support González-Barrio et al. (2011) that mice have correlated microflora to produce urolithins but more importantly urolithins could achieve detectable levels in tissues through absorption.

UA was reported to modulate cell cycle (G2/M arrest) through inducing cdc 2 phosphorylation at tyrosine-15 and an accumulation of cyclin B1 in advanced prostate cancer cell lines, PC-3 and DU-145, when treated at levels of 60 and 90 μM (Vicinanza et al., 2013). Moreover, UA and ellagic acid could synergistically inhibit PC-3 cell
proliferation. However, compared to the levels necessary to induce changes in this cell culture study, the achieved concentrations of UA after dietary intake in the murine prostate only reached 0.1 - 0.5 μM or μmol/kg. Thus, the effects of urolithins at physiological levels combined with other rich amount of BRB phytochemicals or their metabolites on prostate carcinogenesis are of great interest but not yet well defined.

4.2 Molecular pathways and gene expression impacted by genotype

In this study, 10-week old TRAMP mice have demonstrated RNA changes in pathways related to DNA mismatch repair, DNA damage, cell cycle control and apoptosis (Figure 4.7). The SV40 large tumor T antigen (Tag) in TRAMP mice performs as an oncoprotein through inhibitory effect of tumor suppressors: retinoblastoma (DeCaprio et al., 1988) and p53 (Greenberg et al., 1995), which may result in activated functions of DNA damage, p53 signaling, cell-cycle checkpoint control. Mismatch repair in eukaryotic pathway provides functions of genetic stabilization and inactivation of this pathway has a strong predisposition to tumor development (Modrich, 2006). Role of BRCA1 in DNA damage response has been activated with the highest z-score (4.536). BRCA1 have been demonstrated to promote DNA damage repair and participates in non-homologous end-joining and homologous recombination repair (Wu et al., 2010). Besides DNA repair, BRCA1 was reported as a human prostate tumor suppressor to modulate proliferation, chemosensitivity, apoptosis induction and expression of certain key cellular regulatory proteins in DU-145 cells (Fan et al., 1998). Cyclin D1, D2 and G1, cyclin-dependent kinase (CDKs), checkpoint kinase, RB1 and TP53 are the important genes modulating cell cycles which are closely related to increased
proliferation and tumor development in TRAMP mice. These molecules shared the roles in the regulation of pathways of DNA damage, p53 signaling, pancreatic adenocarcinoma signaling and cell cycle checkpoint control.

Specific genes related to Function “PIN and Prostate cancer & tumor” were identified by the IPA Disease and Functions analysis (Table 4.3). CDKN2A (p16) and CDKN2C (p18) are grouped into one of the cyclin dependent kinase inhibitors (CDKIs) families – INK4 family, which specifically inhibits cyclin D-associated kinases (CDKs 4 and 6). CDKN1B (p27) belongs to the CIP/KIP (kinase inhibitor protein) family that inhibits most CDKs (Sherr & Roberts, 1995). Inactivation of CDKN2A through deletion, point mutation, and silencing by hypermethylation is reported in prostate cancer (Cairns et al., 1995). Methylation-mediated inactivation of CDKN2A gene has been found in prostate cancer cell lines and tissues with primary cancer and metastatic tumor (Jarrard et al., 1997). In our study, these tumor suppresser genes were all up regulated in TRAMP mice at 10 weeks of age, which may indicate the distinct difference in CDKNs expression between early prostate carcinogenesis and prostate tumor. Similar situations may exist with RBL1 and IGF-1. Prediagnostic IGF-1 expression was reported to be associated with a high risk of prostate cancer (Cao et al., 2015). In our study, RBL1 was up regulated and IGF-1 and down regulated which were different from reports in prostate cancer/metastasis. Jiang et al. (2010) found that conditional knockout PPAR-gamma in double transgenic PBCre4^{Cre/0} and PPAR-gamma^{flox/flox} mouse prostate epithelial resulted in focal hyperplasia that developed into PIN, thus PPARG may protect PIN process. PPARG was up regulated in our TRAMP mice at PIN stage and this difference may be
due to the differences in applied mouse models and variations in molecular carcinogenesis pathways. Down regulation of TMPRSS2 in our TRAMP mice at 10 weeks of age seems to contradict to changes observed in prostate cancer cell cultures in which TMPRSS2 was shown to activate a proteolytic cascade mediating androgen-induced prostate cancer cell invasion, tumor growth and metastasis (Ko et al., 2015).

FOXM1, PIM1, and ERBB2 were up regulated in our study and consistent with other prostate cancer reports. FOXM1 expression is critical for prostate carcinogenesis and loss of FOXM1 is associated with decreased tumorigenesis with decreased cell proliferation and angiogenesis (Cai et al., 2013). Pim1 protein (PIM1) is one type of homologous protein serine/threonine kinase and the overexpression of this protein in prostate epithelium of conditional Pim1 transgenic mice increased the severity of mouse PIN moderately (Narlik-Grassow et al., 2013). Expression of ErbB-2 induced cell cycle progression in human prostate cancer cell lines and mouse PIN development (Casimiro et al., 2007). ACPP and NKX3-1 were down regulated in our study and consistent with changes reported in prostate cancer development. Prostatic acid phosphatase (PAP; ACPP) has been linked to prostate cancer and PAP knockout mouse showed slow growing non-metastatic prostate adenocarcinoma (Quintero et al., 2013). Down-regulation of NKX3-1 homeobox gene is critical in prostate cancer initiation and reduction of NKX3-1 expression has been shown throughout prostate cancer progression (Shen & Abate-Shen, 2010). All these consistent gene upregulation and downregulation could possibly be regarded as the early critical events in the carcinogenesis process.
Ten genes significantly impacted by genotype (TRAMP vs. WT) at early stage through NanoString have been reported (Wan et al., 2014): BIRC5, MKI67, AURKB, CCNB2, SPP1, FOXM1, CCNE2, E2F-1, EGF, and PBSN. Among these, BIRC5 and FOXM1 were listed in Table 4.3 with converted FC of 6.55 and 6.45 that were close to Wan et al. (2014). All other 8 genes have shown distinct changes by genotype in our study with FCs close to reported values in their study. This confirms the consistency of gene expression in TRAMP model and also validated RNAseq technique used in this TRAMP study.

4.3 Gene expression and top network impacted by dietary BRB

BRB diet significantly impacted 14 molecules in TRAMP mice (FDR ≤ 0.05), among which 8 genes were involved in a network related to cell morphology, lymphoid tissue structure and development, and infectious disease (Table 4.5 & Figure 4.8). IL31RA, ATG4A, JARID2 and KPNA1 were closely regulated through TP53, RBL1 and MYC which mediate cell cycle, proliferation and apoptosis. FILIP1L is an important mediator of cell proliferation and migration were down regulated in ovarian cancer cells compared with normal ovarian epithelial cells through DNA methylation (Burton et al., 2011). Up-regulated FILIP1L supports the observation of modest increases in the proliferative index with BRB diet as measured by IHC image analysis. CDK8 was reported to serve as a positive transcription regulator in multiple signaling pathways including p53 pathway, Wnt/β-catenin pathway, and TGFβ signaling pathway and regarded as an oncogene in colon cancer (Donner et al., 2007; Firestein et al., 2008). CDK8 has been shown to be upregulated by dutasteride, a clinically utilized anti-androgen for the treatment of prostate
cancer, in LNCaP prostate cancer cells suggesting that BRB treatment lead to some transcriptional changes consistent with inhibited androgen signaling (Schmidt et al., 2004). ARHGAP21 is a RhoGAP (Rho GTPase-activating protein), of which the expression has been associated with certain cancer. However, at this point, little is known about its role in prostate cancer beyond one report demonstrating that depletion of ARHGAP21 decreased proliferation in PC3 cells but not LNCaP cells (Lazarini et al., 2013).

5. Conclusion

In conclusion, prostate weight, histological features, and RNA expression were distinctly different with genotype (TRAMP vs. WT) at 10 weeks of age. BRB ellagitannin metabolites, urolithins, were absorbed and reached quantifiable concentrations in the plasma, liver and prostate as quantified by HPLC-MS/MS. BRB diet significantly modulated several gene changes in TRAMP mice which are related to cell morphology and proliferation demonstrating that dietary exposure has an impact on the prostate warranting further investigation into the mechanisms of action and cancer preventive potential in the prostate.
References


Mallery SR, Zwick JC, Pei P, Tong M, Larsen PE, Shumway BS, et al. Topical application of a bioadhesive black raspberry gel modulates gene expression and


Wu J, Lu L, Yu X. The role of BRCA1 in DNA damage response. Protein Cell 2010; 1: 117-123.

Table 4.1 Ingredient Composition of Control and 10% BRB diet

<table>
<thead>
<tr>
<th>Ingredient (g)</th>
<th>AIN-93G Diet</th>
<th>10% BRB Diet*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>200 (casein)</td>
<td>188</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>3</td>
<td>2.7</td>
</tr>
<tr>
<td>Corn starch</td>
<td>397.486</td>
<td>357.737</td>
</tr>
<tr>
<td>Malto dextrin 10</td>
<td>132</td>
<td>118.8</td>
</tr>
<tr>
<td>Sugar</td>
<td>100 (sucrose)</td>
<td>120</td>
</tr>
<tr>
<td>Fiber</td>
<td>50 (cellulose)</td>
<td>88</td>
</tr>
<tr>
<td>Fat</td>
<td>70 (soybean oil)</td>
<td>67.3</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35</td>
<td>33.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
<td>9.2</td>
</tr>
<tr>
<td>Choline bitatrate</td>
<td>2.5</td>
<td>2.25</td>
</tr>
<tr>
<td>Tert-butylhydroquione</td>
<td>0.014</td>
<td>0.126</td>
</tr>
<tr>
<td>BRB powder</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Total (g)</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Calories (Kcal)</td>
<td>4000</td>
<td>3947</td>
</tr>
</tbody>
</table>

*Diet was prepared with 10% BRB powder and 90% AIN-93G diet. Protein, sugar, fat, fiber, minerals and vitamins from BRB are added into the final diet. Calories of 10% BRB diet are calculated combining calories of 900 g AIN-93G diet and calories of 100 g BRB powder. Nutrient profile of BRB is referred to USDA nutrition database standard reference.
### Table 4.2 Number of genes obtained for IPA with different cutoff values

<table>
<thead>
<tr>
<th></th>
<th>TRAMP vs. WT</th>
<th>BRB vs. Control Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Diet</td>
<td>BRB Diet</td>
</tr>
<tr>
<td>Total IDs</td>
<td>38921</td>
<td>38921</td>
</tr>
<tr>
<td>Mapped IDs</td>
<td>25312</td>
<td>25312</td>
</tr>
<tr>
<td>Analysis-Ready IDs</td>
<td>17639</td>
<td>18723</td>
</tr>
<tr>
<td>FDR = 0.1</td>
<td>9515</td>
<td>10882</td>
</tr>
<tr>
<td>FDR = 0.05</td>
<td>8096</td>
<td>9345</td>
</tr>
<tr>
<td>FDR = 0.01</td>
<td>5393*</td>
<td>6456*</td>
</tr>
<tr>
<td>FDR = 0.01 &amp; FC = 2</td>
<td>2018^</td>
<td>3129^</td>
</tr>
</tbody>
</table>

* Genes for IPA Core Analysis, ^ Genes for IPA Comparison Analysis.
Table 4.3 Prostate cancer and tumor associated molecules that significantly impacted by genotype (TRAMP vs. WT) in control diet

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Entrez Gene Name</th>
<th>Log2FC</th>
<th>P-value</th>
<th>adj P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
<td>3.674</td>
<td>5.20E-06</td>
<td>1.08E-04</td>
</tr>
<tr>
<td>PSCA</td>
<td>Prostate stem cell antigen</td>
<td>3.464</td>
<td>9.93E-07</td>
<td>4.03E-05</td>
</tr>
<tr>
<td>BIRC5</td>
<td>Baculoviral IAP repeat containing 5</td>
<td>2.711</td>
<td>1.31E-07</td>
<td>1.75E-05</td>
</tr>
<tr>
<td>FOXM1</td>
<td>Forkhead box M1</td>
<td>2.690</td>
<td>3.46E-07</td>
<td>2.46E-05</td>
</tr>
<tr>
<td>CDKN2C</td>
<td>Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)</td>
<td>2.416</td>
<td>5.80E-08</td>
<td>1.47E-05</td>
</tr>
<tr>
<td>PPARG</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
<td>2.194</td>
<td>4.07E-05</td>
<td>4.44E-04</td>
</tr>
<tr>
<td>DNMT1</td>
<td>DNA (cytosine-5')-methyltransferase 1</td>
<td>2.144</td>
<td>4.26E-07</td>
<td>2.71E-05</td>
</tr>
<tr>
<td>PIM1</td>
<td>Pim-1 proto-oncogene, serine/threonine kinase</td>
<td>1.903</td>
<td>6.74E-06</td>
<td>1.27E-04</td>
</tr>
<tr>
<td>RBL1</td>
<td>Retinoblastoma-like 1</td>
<td>1.547</td>
<td>5.91E-07</td>
<td>3.21E-05</td>
</tr>
<tr>
<td>ERBB2</td>
<td>Erb-b2 receptor tyrosine kinase 2</td>
<td>1.310</td>
<td>5.08E-07</td>
<td>2.98E-05</td>
</tr>
<tr>
<td>XAF1</td>
<td>XIAP associated factor 1</td>
<td>1.244</td>
<td>3.09E-04</td>
<td>1.91E-03</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>Cyclin-dependent kinase inhibitor 1B (p27, Kip1)</td>
<td>1.031</td>
<td>8.02E-07</td>
<td>3.68E-05</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1 (somatomedin C)</td>
<td>-1.318</td>
<td>1.47E-05</td>
<td>2.18E-04</td>
</tr>
<tr>
<td>ACPP</td>
<td>Acid phosphatase, prostate</td>
<td>-1.762</td>
<td>5.86E-05</td>
<td>5.70E-04</td>
</tr>
<tr>
<td>NNX3-1</td>
<td>NK3 homeobox 1</td>
<td>-1.886</td>
<td>4.39E-08</td>
<td>1.37E-05</td>
</tr>
<tr>
<td>TMPRSS2</td>
<td>Transmembrane protease, serine 2</td>
<td>-2.246</td>
<td>6.65E-08</td>
<td>1.47E-05</td>
</tr>
</tbody>
</table>

*Cutoff value: FDR ≤ 0.01 & log2FC ≥ 1 or ≤ -1.*
### Table 4.4 Comparison analysis of diet effect on diseases and functions predicted in IPA

<table>
<thead>
<tr>
<th></th>
<th><strong>Control diet</strong></th>
<th></th>
<th><strong>BRB diet</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>TRAMP vs. WT</strong></td>
<td><strong>Molecule #</strong></td>
<td><strong>TRAMP vs. WT</strong></td>
<td><strong>Molecule #</strong></td>
</tr>
<tr>
<td></td>
<td><em>z</em>-score</td>
<td><em>P</em>-value</td>
<td><em>z</em>-score</td>
<td><em>P</em>-value</td>
</tr>
<tr>
<td>Organismal death</td>
<td>-6.283</td>
<td>2.1E-06</td>
<td>4.769</td>
<td>4.7E-15</td>
</tr>
<tr>
<td>Size of body</td>
<td>1.777</td>
<td>4.81E-04</td>
<td>-5.811</td>
<td>1.68E-11</td>
</tr>
<tr>
<td>Differentiation of cells</td>
<td>0.924</td>
<td>2.19E-05</td>
<td>-2.807</td>
<td>9.94E-06</td>
</tr>
<tr>
<td>Vasculogenesis</td>
<td>1.35</td>
<td>1.86E-03</td>
<td>-2.236</td>
<td>1.26E-14</td>
</tr>
<tr>
<td>Cell death</td>
<td>-2.122</td>
<td>3.41E-07</td>
<td>1.376</td>
<td>3.98E-08</td>
</tr>
<tr>
<td>Cell movement</td>
<td>1.163</td>
<td>5.94E-05</td>
<td>-2.282</td>
<td>3.44E-10</td>
</tr>
<tr>
<td>Quantity of cells</td>
<td>1.881</td>
<td>3.09E-07</td>
<td>-1.53</td>
<td>7.01E-11</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>-1.865</td>
<td>7.48E-11</td>
<td>1.37</td>
<td>3.23E-10</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>1.247</td>
<td>4.45E-04</td>
<td>-1.675</td>
<td>2.35E-14</td>
</tr>
<tr>
<td>Development of cardiovascular system</td>
<td>1.274</td>
<td>4.05E-05</td>
<td>-1.635</td>
<td>4.17E-14</td>
</tr>
<tr>
<td>Migration of cells</td>
<td>1.274</td>
<td>3.21E-04</td>
<td>-1.376</td>
<td>6.66E-09</td>
</tr>
<tr>
<td>Survival of organism</td>
<td>0.682</td>
<td>9.55E-05</td>
<td>-1.924</td>
<td>9.06E-05</td>
</tr>
<tr>
<td>Necrosis</td>
<td>-0.828</td>
<td>6.04E-04</td>
<td>1.649</td>
<td>1.65E-05</td>
</tr>
<tr>
<td>Quantity of leukocytes</td>
<td>1.427</td>
<td>3.66E-06</td>
<td>-0.44</td>
<td>7.7E-05</td>
</tr>
</tbody>
</table>

Any diseases and functions with z-scores in different directions in two datasets and z-score ≥ 1 in any one dataset were selected and compared.
### Table 4.5 Molecules significantly impacted by BRB diet in TRAMP and WT mice

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Entrez Gene Name</th>
<th>Log2 FC TRAMP mice</th>
<th>P-value TRAMP mice</th>
<th>adj P-value TRAMP mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL31RA</td>
<td>Interleukin 31 receptor A</td>
<td>5.37</td>
<td>4.14E-08</td>
<td>1.60E-04</td>
</tr>
<tr>
<td>FILIP1L</td>
<td>Filamin A interacting protein 1-like</td>
<td>3.56</td>
<td>4.03E-06</td>
<td>3.22E-03</td>
</tr>
<tr>
<td>CDK8</td>
<td>Cyclin-dependent kinase 8</td>
<td>3.36</td>
<td>4.07E-07</td>
<td>6.28E-04</td>
</tr>
<tr>
<td>ARHGAP21</td>
<td>Rho GTPase activating protein 21</td>
<td>2.66</td>
<td>2.07E-06</td>
<td>2.53E-03</td>
</tr>
<tr>
<td>KCNIP1</td>
<td>Kv channel-interacting protein 1</td>
<td>2.62</td>
<td>1.76E-06</td>
<td>2.29E-03</td>
</tr>
<tr>
<td>SSPN</td>
<td>Sarcospan</td>
<td>2.57</td>
<td>2.35E-05</td>
<td>1.31E-02</td>
</tr>
<tr>
<td>JARID2</td>
<td>Jumonji, AT rich interactive domain 2</td>
<td>2.23</td>
<td>9.87E-06</td>
<td>7.14E-03</td>
</tr>
<tr>
<td>GPHN</td>
<td>Gephyrin</td>
<td>1.60</td>
<td>1.78E-06</td>
<td>2.29E-03</td>
</tr>
<tr>
<td>SPATA5</td>
<td>Spermatogenesis associated 5</td>
<td>1.26</td>
<td>8.34E-05</td>
<td>3.27E-02</td>
</tr>
<tr>
<td>ATG4A</td>
<td>Autophagy related 4A, Cysteine peptidase</td>
<td>0.57</td>
<td>9.30E-05</td>
<td>3.47E-02</td>
</tr>
<tr>
<td>RPL21</td>
<td>Ribosomal protein L21</td>
<td>0.57</td>
<td>1.16E-04</td>
<td>4.02E-02</td>
</tr>
<tr>
<td>KPNA1</td>
<td>Karyopherin alpha 1 (importin alpha 5)</td>
<td>0.55</td>
<td>7.52E-05</td>
<td>3.17E-02</td>
</tr>
<tr>
<td>H2-Q5</td>
<td>Histocompatibility 2, Q region locus 5</td>
<td>-2.2</td>
<td>9.01E-05</td>
<td>3.42E-02</td>
</tr>
<tr>
<td>HLA-A (H2-Q2)</td>
<td>Histocompatibility 2, Q region locus 2</td>
<td>-0.77</td>
<td>2.05E-05</td>
<td>1.25E-02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Entrez Gene Name</th>
<th>Log2 FC WT mice</th>
<th>P-value WT mice</th>
<th>adj P-value WT mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK8</td>
<td>Cyclin-dependent kinase 8</td>
<td>2.76</td>
<td>1.16E-05</td>
<td>1.96E-02</td>
</tr>
<tr>
<td>HBZ (Hba-x)</td>
<td>Hemoglobin X, alpha-like embryonic chain</td>
<td>-5.00</td>
<td>2.60E-06</td>
<td>7.49E-03</td>
</tr>
</tbody>
</table>

Molecules involved in IPA networks and diseases & functions (Cutoff value: FDR ≤ 0.05).
Figure 4.1 Final body, UGT, and bladder weights of 10-week-age WT and TRAMP mice fed either control or 10% BRB diet. Bars represent the mean ± SE (Body weight: n=20/group; UGT weight: n=15/group; Bladder weight: n=10/group). * Denotes a significant main effect of TRAMP mice compared to WT (P<0.05).
Figure 4.2 Prostate lobe weights of 10-week-age WT and TRAMP mice fed either control or 10% BRB diet. Bars represent the mean ± SE (n=10/group with each lobe type). * Denotes a significant main effect of TRAMP mice compared to WT (P<0.05).
**Figure 4.3** Urolithin A in plasma, liver and prostate of 10-week-age WT and TRAMP mice fed 10% BRB diet (no urolithins detected in control-fed mice). Bars represent the mean ± SE (n=11/group: 5 WT & 6 TRAMP). Different letters indicates significant difference between groups.
Figure 4.4 Correlations of prostate urolithin A and liver urolithin A (solid circle) and correlations of prostate urolithin A and plasma urolithin A (open circle).
Figure 4.5 Representative H&E staining images of prostate ventral, lateral, dorsal and anterior lobes from 10-week-age WT (A-D) or TRAMP (E-H) fed with control diet. Arrows indicates any early PIN lesions.
Figure 4.6 Representative Immunohistochemical staining - Ki67 images of prostate ventral, lateral, dorsal and anterior lobes from 10-week-age WT (A-D) or TRAMP (E-H) mice fed with control diet.
Figure 4.7 Top 10 canonical pathways significantly regulated by genotype in control diet. Bars represent total molecules in each pathway and number of molecules was labeled above the bar.
Figure 4.8 Top network impacted by BRB diet in TRAMP mice. Biological networks affected by BRB were generated from IPA core analysis. A network related to cell morphology, lymphoid tissue structure and development, and infectious disease. Red color indicates upregulated genes and green color indicates downregulated genes.
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Keatley KE. Chemoprevention of bladder cancer with anthocyanin-rich extract from black raspberries. Thesis, The Ohio State University, Columbus, 2008.


Mullen W, Yokota T, Lean MEJ, Crozier A. Analysis of ellagitannins and conjugates of ellagic acid and quercetin in raspberry fruits by LC-MS/MS. Phytochem 2003; 64: 617-624.


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