TESTING OF ANTIDIABETIC FEATURES OF FORMULATED FOOD PRODUCTS IN ENTEROCYTE CELL CULTURE MODEL

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ABSTRACT

TESTING OF ANTIDIABETIC FEATURES OF FORMULATED FOOD PRODUCTS IN ENTEROCYTE CELL CULTURE MODEL

In recent years, there has been an increasing interest in designing low calorie functional foods that might have health benefit against development of obesity and T2D. However, researches on developing low calorie foods in Turkey are quite insufficient. Even if the products are developed, *in vitro* and *in vivo* approaches to design low calorie foods are not carried out properly. Thus, we aimed to test diabetic features of newly formulated low calorie and no-added sugar containing cake, almond paste and pudding in intestinal cell culture system. Cake, almond paste and pudding were developed by “Egepak Gıda ve Ambalaj San.” and same type foods were chosen from market to use as reference. We modeled human carbohydrates digestive systems in test tube and relative glucose efflux were measured from rat enterocyte cells that were grown on bicameral cell culture system. Bioaccessibility of glucose was found at least 61%, 89% and 64% lower in cake, almond paste and pudding respectively relative to references. The levels of cellular glucose efflux were significantly lower from 30min (for cake 1.9 fold; for almond paste 4.3; for pudding 3.3 fold) to 120 min (for cake 2.2 fold; for almond paste 3.7 fold) to 180 min (for pudding 2.0 fold) in products than their references. To the best of our knowledge, this is the first exemplary study in Turkey to develop newly formulated foods by analyzing their diabetic features in *in vitro* model of human intestine system.

ÖZET

FORMÜLE EDİLMİŞ GIDA ÜRÜNLERİNİN ANTİ-DİYABETİK ÖZELLİKLERİNİN BAĞIRSAK HÜCRE MODELİNDE TESTİ

Son yıllarda, obezite ve T2D gelişimine engel olabilecek, düşük kalorili fonksiyonel gıda tasarımlarına olan ilgi artmaktadır. Bununla birlikte, Türkiye'de bu tür ürünleri geliştirmeye yönelik araştırmalar oldukça yetersizdir. Geliştirilen ürünlerin ise *in vitro* ve *in vivo* çalışmalar gibi deneysel prosedürlerinin yürütülmesinde çok önemli eksiklikler bulunmaktadır. Bu tezin amacı; yeni geliştirilmiş düşük kalorili, şeker ilavesiz kek, badem ezmesi ve puding ürünlerinin antidiyabetik özelliklerini bağırsak hücre kültürü sisteminde test etmektir. Kek, badem ezmesi ve puding ürünleri "Egepak Gıda ve Ambalaj San." tarafından geliştirilmiştir. Referans olarak marketlerdeki aynı tip ürünler seçilmiştir. Bu amaçla, insan karbonhidrat sindirim sistemini test tüplerinde modelledik. Daha sonra glikoz geçişini göreceli olarak enterosit hücre siteminde ölçümledik. Tüpte insan karbonhidrat sindirim sisteminin modellenmesi sonucunda elde edilen bio-erişilebilirlik sonuçlarına göre kek, badem ezmesi ve puding ürünlerinin %61, %89 ve %64 daha az glikoz içerdikleri bulunmuştur. Ürünlerdeki glikozun hücresel salınımı ise kek ve badem ezmesi için 30. dakikadan 120. dakikaya, puding için ise 30. dakikadan 180. dakikaya kadar referanslarına göre belirgin olarak düşüktür (30 dakika: kek için 1.9 kat; badem ezmesi için 4.3 kat; puding için 3.3 kat. 120 dakika: kek için 2.2 kat; badem ezmesi için 3.7 kat ve 180 dakika: puding için 2.0 kat). Bildiğimiz kadarıyla bu çalışma Türkiye'de ürün geliştirme ve geliştirilen bu ürünlerin diyabetik özelliklerinin *in vitro* olarak insan sindirim sisteminde analiz edilmesi adına yapılan ilk örnek çalışmadır.

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CHAPTER 1

INTRODUCTION

In the first part, general information about obesity and Type 2 Diabetes (T2D) will be presented and then the possible risks on health of these diseases will be discussed. Chapter will then talk about sugar replacements and dietary fiber used in newly formulated cake, almond paste and pudding products for diabetes patients, obese patients and individuals in the risk group.

1.1. Obesity

Obesity is the storage of excess energy as fat in adipose tissue (van Dam & Seidell, 2007). Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health (WHO 2000). Because of changes in lifestyle and eating habits, people are rapidly gaining weight. According to estimation of the World Health Organization (WHO), 33.3% of women and 35.3% of men are obese, in the world. Also the prevalence of obesity in children and adolescents is increased. According to the National Health and Nutrition Examination Survey (NHANES), 16.3% of children and adolescents aged 2-19 are obese in US (Ogden, C. L., Carroll, M. D., & Flegal, K. M., 2008). In 1990, 18.8% of the adult population was obese in Turkey and the prevalence increased to 36% in 2010 (Erem, C. 2015).

Classification of overweight and obesity is achieved through the use of a valid method: Body Mass Index (BMI) (Swinburn, Caterson, Seidell & James, 2004). The BMI is calculated with the following equation:

$$[BMI = Weight\ (kg) / Height^2\ (m^2)]$$

Table 1.1. Body mass index scala. BMI defined as a person's weight in kilograms divided by the square of their height in meters and the scala.

Classification of overweight and obesity		
Classification	Subclassification	Criteria
Overweight		BMI 25-29.9 kg/m^2
Obesity		BMI 30 kg/m^2
	Obesity grade I	BMI 30-34.9 kg/m^2
	Obesity grade II	BMI 35-39.9 kg/m^2
	Obesity grade III	BMI 40 kg/m^2

Obesity is a chronic systemic disease requiring a multidisciplinary approach for prevention and alleviation. Genetic and nutritional factors are associated with the development of obesity (Vogler et al., 1995). Despite some genetic and epigenetic influences, obesity is subsequent disease that depends on lifestyle factors such as overeating and low physical activity (Swinburn et al., 2011).

The function of adipose tissue varies depending on the storage position of fat in the body. There are two types of adipose tissue in human, including visceral adipose tissue and subcutaneous adipose tissue. Increased visceral adipose tissue has been reported to be associated with a higher risk of obesity, type 2 diabetes (TD2) and cardiovascular disease more than subcutaneous fat (Srinath Reddy & Katan, 2004).

Obesity is a major global health challence and affects almost every organ system of the body including the endocrine, gastrointestinal (GI), cardiovascular (CV), and central nervous systems. A multidisciplinary approach is therefore warranted to prevent and manage obesity. The lack of comprehensive and multidisciplinary approaches might be one explanation why we still fail to control the obesity and TD2 risk.

1.2. Type 2 Diabetes (T2D)

Obesity and T2D are associated with each other. Weight gain increases the risk of T2D (Henry, Chilton & Garvey., 2013). T2D is more common in obese individuals. In obese individuals, fat tissue releases factors that play a role in the development of insulin resistance. When the insulin-releasing cells become dysfunctional, these cells can not control the blood sugar level and insulin resistance develops and researches showed that the most patients with T2D are obese (Kahn et al., 2006; Eckel et al., 2011).

The International Diabetes Federation (2015) predicts that by 2040 there will be 642 million diabetics in the world. If precaution is not taken, this figure will increase much more. 90-95% of diabetic cases are T2D. Increased rates of T2D, especially among adolescents and young adults, have the potential to become a public health crisis if measures are not taken (Pinhas-Hamiel and Zeitler, 2005). Early detection of people at risk will provide early intervention to delay the onset of T2D. It is accepted that the prevalence of T2D in adolescent younger than 20 years will increase fourfold in 40 years (Imperatore et al., 2014).

Currently, there is no treatment for diabetes, but lifestyle modification can prevent or delay the onset of T2D (Schellenberg et al., 2013). Prevention plays a vital role in preventing T2D, but initial screening in adolescents for the disease and its pre-stages has yet to be resolved. The American Diabetes Association recommends that people who are overweight, those with T2D stories in first- or second-degree relatives, people in high-risk ethnic groups, and those with insulin resistance markers are tested. The risk of developing T2D is considered high if people has a BMI higher than 85 percent and has two risk factors (ADA, 2015).

Consumption of processed food has risen considerably over the last century. Very high glycemic index value of products sold in the supermarket, at a very affordable price, instead of this, products that can be consumed by diabetic patients should be increased for sales.

1.3. Sugar Replacements: Sugar Alcohols

Sugar alcohol is a hybrid between the sugar molecule and the alcohol molecule. They are structurally similar with sugar but less digestible and metabolized in human (Back et al., 1979). They have been presented as a popular sweeteners alternative to sugar in the food industry (Akinterinwa et al., 2008). Sugar alcohols are similar in appearance and taste like sugar but they have fewer calories and less negative health effects (Bieleski., 1982). There are many sugar alcohols naturally in fruits and vegetables. However, most are industrially produced from other sugars such as sugar in corn starch. Sugar alcohols activate sweet taste receptors on the tongue. And they feel like eating sugar. These sugar alcohols are different from each other in terms of taste, calorie content and health effects.

The most important advantage of sugar alcohols compared to sugar is that they have lower glycemic index values. The glycemic index is a measure of how quickly food is involved in the blood and how quickly you increase blood sugar levels. Consuming high-glycemic index foods can cause obesity, T2D and numerous metabolic health diseases (Esfahani et al., 2011; Marsh et al., 2008: Brand-Miller et al., 2002). Figure 1.1 shows the glycemic index for several sugar alcohols when compared to sugar and pure glucose:

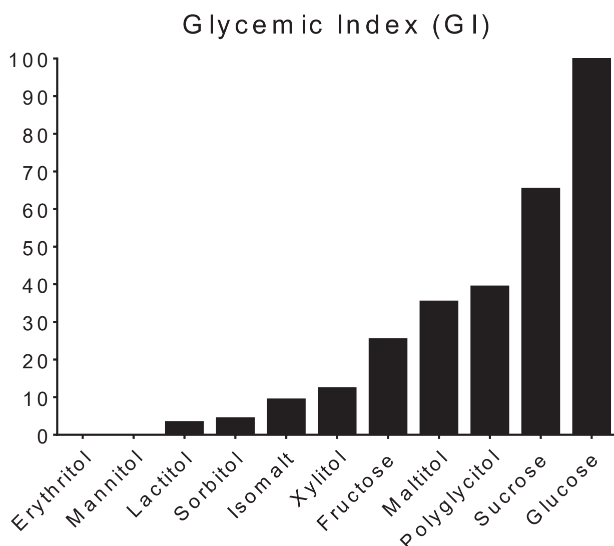

Figure 1.1. Glycemic index (GI) for sugar alcohols when compared to sucrose and glucose.

Most sugar alcohols have a negligible effect on blood sugar levels. The erythritol and mannitol glycemic index is zero. As seen in the figure, maltitol has a glycemic index of 35. Even this value is still very low compared to glucose. Many sugar alcohols have little or no effect on blood sugar and insulin levels except for maltitol. For people with metabolic syndrome, pre-diabetes or diabetes, sugar alcohols (except possibly maltitol) can be considered as the perfect alternative to sugar. Sugar alcohols also have a number of other beneficial effects. It is known that tooth decay is caused by excessive consumption of sugar. Sugar feeds bacteria in the mouth and these bacteria multiply and secrete acids that attack the protective layer in the teeth. In contrast, sugar alcohols such as xylitol, erythritol and sorbitol provide protection against tooth decay (Mäkinen, 2010). This is one of the main reasons why they are so popular in many gums and toothpastes. In addition, the positive effects of xylitol on dental health have been examined and these effects have been proven (Bahador et al., 2012; Mäkinen, 2011).

Sugar alcohols feed the good bacteria in the intestine, having a prebiotic effect like dietary fiber (Ur-Rehman et al., 2015; Tamura et al., 2013; Mäkivuokko et al 2010). They also have effects on bone health. Many studies on rats have shown that xylitol can enhance bone mass and bone mineral content; which should help protect against osteoporosis (Mattila et al., 1998; Mattila et al., 2001). Collagen is the main structural protein in skin and connective tissue. Studies on rats have shown that xylitol can increase collagen production (Mattila et al., 2005; Knuuttila et al., 2000).

Apart from many positive effects, one issue that needs to be mentioned is the digestibility of sugar alcohols. Sugar alcohols can cause some digestive problems in human physiology when consumed in large quantities. The body can not digest most of the sugar alcohols; they are metabolized by the intestinal bacteria in the large intestine. In the short term, excessive amounts of sugar alcohol can cause symptoms such as flatulence, bloating and diarrhea. Erythritol from sugar alcohols leads to the least known symptoms, while excessive consumption of sorbitol and maltitol can cause such symptoms (Livesey, G., 2001).

In general, it can be said that many beneficial effects of sugar alcohols have been researched and found. However, when consumed in large quantities, most sugar alcohols can cause significant digestive problems. This varies depending on the type of , sugar alcohol and person. When all sugar alcohols are evaluated, erythritol is the sugar alcohol from which the best results are obtained. Because it has almost no calories, it

has no effect on blood sugar and causes less digestive problems than others. It also has a nice taste.

1.3.1. Erythritol

Erythritol is processed by fermenting sugar from corn starch. It has 70% sugar sweetness, but only 6% of the calories. If too much sugar alcohol is consumed it can cause digestive problems. However, erythritol has not side effects like other sugar alcohols. Since the important amount of erythritol does not reach the large intestine. Erythrole is absorbed into the body before reaching the colon. It is absorbed in the small intestine and is absorbed into the bloodstream and removed from the body unchanged with the urine. Approximately 90% of the erythritol is expelled in this way (Arrigoni et al., 2005). Table sugar contain 4 calories per gram however erythritol contain 0.24 calories per gram. It contains only 6% of the calorie content of the sugar. Due to the chemical structure of erythritol, it is not broken down in the body. The human digestive system does not have the enzymes to digest erythritol. When erythritol was given to healthy people, no change in blood sugar or insulin levels was observed. In addition, there is no effect on cholesterol, triglycerides or other biomarkers (Noda et al., 1994). In individuals with diabetes, overweight and other metabolic syndromes, erythritol appears to be a very good alternative to sugar. Nutritional studies with 1 gram per kilo (0.45 g / lb) show that erythritol is well tolerated (Tetzloff et al., 1996; Oku et al., 1996). However another study showed that when erythritol consumed 50 grams in one go, it caused nausea and abdominal pain (Storey et al., 2007).

1.3.2. Sorbitol

Sorbitol is a sugar alcohol with a pleasant taste that gives a soft feeling to the mouth. It has about 40% less calories than sugar and 60% of the sweetness of sugar. It is used in many products including sugar-free foods, beverages, frozen desserts, chewing gum and soft candies. It also found naturally in many fresh fruits and strawberry. There is little effect on blood sugar and insulin. Sorbitol significantly reduces blood sugar and insulin secretion. For this reason, it can be a good alternative for diabetic patients. It can also be used for weight control. It has a reduced calorie value of approximately 2.54

kcal / g. However sorbitol has some side effects also. It can cause significant digestion problems. Also excess consumption can cause diarrhea problems (Brunzell, 1978).

1.3.3. Maltitol

Maltitol is a sugar alcohol that contains 90% of the sweetness of sugar and has 2.1 calories per gram. Maltose obtained from starch and maltitol is produced by the hydrogenation of maltose. It is used in low-calorie foods, sugar-free foods, and foods recommended for diabetics. Human body can only absorbed half of the carbohydrates in maltitol (Langkilde et al., 1994). As with many sugar alcohols, if maltitol is consumed too much, it has some side effects such as abdominal pain, intestinal gas, cramping and diarrhea. The usage of maltitol can alter the regularity of bowel movements. These side effects vary depending on age and dose. Glycemic index of table sugar is 60. Maltitol has a glycemic index of 36, this value higher than most other sugar alcohols and all artificial sweeteners. Almost all sugar alcohols are at least slightly better than maltitol if a high glycemic index value is considered. Maltitol is a good alternative to use with artificial sweeteners such as erythritol or sucralose.

1.4. Dietary Fiber

Dietary fiber is the edible portion of plants or similar carbohydrates, which are not digestible and absorbable in the human small intestine and partially or completely fermantable in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin and related plant substances. Dietary fibers support beneficial physiological effects like laxation, and/or blood cholesterol attenuation (Report of the dietary fiber definition committee to the board of directors of the American association of cereal chemists, 2001). In addition, dietary fiber intake has beneficial effects on the risk factors of developing various chronic diseases. Consumption of 14 g dietary fiber per 1000 kcal or 25 g for adult women and 38 g for adult men is recommended. Eating fiber content foods gives a feeling of full also provides weight control. Children and adults should provide adequate dietary fiber intake by increasing the variety of the daily diet. To increase the consumption of high fiber foods such as whole grains, legumes, fruits and vegetables, nutritional messages should be given by food and nutrition experts

(Slavin, 2008). Beside other foods, dietary fiber intake can lower blood pressure, improve the serum lipid levels and reduce inflammation indicators.

1.4.1. Inulin

Inulin is a fructose polymer found in many vegetables such as onion, garlic, leek, chicory and artichoke. In recent years it has been used in food industry due to its gel, thickening and sweetening properties (Moshfegh, A. J., et al., 1999). Inulin contains less energy than other carbohydrates. The most important function known is to stimulate the development of bifidobacteria in the intestines. In addition, inulin reduces the risk of diabetes, heart disease, cancer and osteoporosis. Inulin can be referred to as a functional product because it has a positive effect on health when taken in the body besides nutritional effects (Roberfroid, 2005). These positive effects of inulin on health will be explained in detail in the next section in common with oligofructose.

1.4.2. Oligofructose

Oligofructose is obtained by enzymatic hydrolysis of inulin. Oligofructose is also used as gelling, thickener and sweetener agent. Oligofructose contain less energy than many other carbohydrates. The most important function known is to stimulate the development of bifidobacteria in the intestines. In addition, oligofructose reduce the risk of diabetes, heart disease, cancer and osteoporosis like inulin. Oligofructose has many beneficial effects on health so it is a functional food (Roberfroid, 2005). Inulin and oligofructose are prebiotics because they stimulate the development of bifidobacteria in the large intestine. They affect the absorption of many minerals, especially calcium, increasing bone-mineral density and reducing the risk of osteoporosis. They stimulate the immune system, reduce fat production in the liver, and reduce the risk of cardiovascular disease by preventing hyperinsulinemia. They prevent constipation by increasing bowel motility. They prevent reproduction of bifidobacteria, gram-negative and positive bacteria so Also prevent diarrhea formation. By inhibiting or reducing the development of malignant tumors, the risk of large bowel cancer will be reduced (Roberfroid, 1999).

Oligofructoses commonly participate in cereals, fruit yogurts, ice creams and dairy products. The use of inulin and oligofructose in food industry is spreading rapidly (Niness, 1999). As consumers begin to learn about health and nutrition relationship, they are taking advantage from various technologies to develop healthy nutrition products in food industry. The food industry can control the chemical composition and physical structure of foods with effective methods and scientific equipment, and even develop new products (Richardson, 1999). Inulin and oligofructose are today synthesized from sucrose or extracted from chicory root in the food industry also as a dietary fiber are added to newly developed products for positive health effects. They have also participated in the products because of the health effects mentioned in the new formulated products in which the diabetic characteristics will be evaluated in this study.

CHAPTER 2

MATERIALS AND METHODS

2.1. General Overview of Test Tube Carbohydrate Digestion Methods

In the first part, it was mentioned that T2D and obesity are burden metabolic diseases in the world. The occurrence rate of global T2D requires the need for communication on the possible glycemic effects of foods by labeling foods (Woolnough, J. W., 2011). Many metabolic studies have shown that the effects of carbohydrate food sources on the rate of absorption, blood glucose and insulin concentrations vary greatly (Willett et al., 2002). Glycemic index (GI) is a good way of quantifying this variation, which is due to the diet carbohydrate. The GI is an index calculated from the postprandial glucose response produced by carbohydrate-containing foods (Jenkins et al., 1981). GI is defined as the area under the glucose response curve after consumption of 50 g carbohydrate from a test food divided by the area under the curve after consumption of 50 g carbohydrate from a control food, either white bread or glucose (Wolever et al., 1991). Glucose is a standard reference worldwide and most current methods use glucose as the reference food in the measurement of GI having a glycemic value of 100. However, some studies have used white bread as a reference because white bread is regarded as a basic food item. The disadvantage of using white bread is that the available carbohydrate content and portion size is not standardized (Foster-Powell et al., 2002). There are three ratings for GI : GI value of 55 or less is a Low-GI foods, Moderate-GI foods have score from 56 to 69, GI value of 70 or more is a High-GI food (Chen et al., 2010). Although these three GI ratings caused some controversy in the beginning, GI is widely accepted as a physiologically reliable classification of foods based on postmeal glycemic effects (Foster-Powell et al., 2002).

However, the *in vivo* method of measuring GI values for foods is relatively time consuming, costly and hence unsuitable for routine food analysis. Therefore, test tube enzymatic assays that estimate carbohydrate digestion and glucose uptake rates in the small intestine have been investigated instead of *in vivo* methodology as an easier and cheaper method of measuring GI values of foods. Carbohydrate digestion test tube

methods, provides an alternative to *in vivo* tests (Woolnough, J. W., 2011; Brand-Miller, J., & Holt, S., 2004).

2.1.1. Background Information

Available carbohydrate was identified as 'carbohydrate released from a food by digestion and is absorbed as monosaccharides and metabolised by the body' in 2006 by the Glycemic Carbohydrate Definition Committee of the American Association for Clinical Chemistry (AACC). The *in vivo* measurement of GI has several limitations, such as interpersonal variation, ethical concerns, and systemic factors of body. Because of these reasons it is unsuitable for routine food analysis. GI measurement is also costly and time-consuming. As a result, there has been increased interest in developing an alternative test tube carbohydrate digestion method to measure GI value. The members of AACC point out some concern about the use of *in vivo* method to determine the GI value of foods by the reason of inter-individual and individual changes. For this reason, the committee proposed a robust *in vitro* method to estimate GI values to mimic *in vivo* conditions (Brooks et al., 2006).

On the other hand, there was a lack of a standardized test tube carbohydrate digestion method that has been approved in the food industry in order to provide glycemic control with the help of nutritional labels and adapted to today's technology (Brooks et al., 2006). The test tube carbohydrate digestion methods that are commonly referred to must be correctly evaluated and the experimental conditions that indicate the differences between the methods should be determined for the development of a reliable and standardized test tube carbohydrate digestion method for the analysis of carbohydrate-containing foods on the market.

Test tube carbohydrate digestion methods are cheap and convenient for routine functional food analysis. They give high efficiency and precise results. However, the lack of standard methodology prevents the application of test tube digestion technology. Numerous test tube digestion methods have been described in the literature. Because of the increment of test tube conditions, there are differences in all of these approaches. In order to characterize the relative influence of the variables considered in the method, a systematic investigation was undertaken using 5 standard test foods. Variables examined include mode of comminution, pepsin inclusion versus omission, amylolytic

enzyme concentration, incubatum pH and stirring method. Comminution and stirring were the most effective method of *in vitro* starch digestion kinetics. For this reason, standardized *in vitro* methods have been proposed, and the proposed methods have included different approaches to comminution and stirring depending on the structural characteristics of the food to be analyzed.. (Woolnough, J. W., 2011).

When a new test tube digestion method is developed, firstly this new method should be performed with *in vivo* studies, using the same test foods. The aim of this is to compare the GI values of the new test tube digestion method with the GI values of the *in vivo* method. A strong correlation between the two methods indicates that this new method is reliable and can be used as an alternative to the *in vivo* method (El-Fahkhri, R., 2017).

2.1.2. Current Test Tube Carbohydrate Digestion Methods

The first carbohydrate digestion method in test tube was performed at the end of the 1960s. In 1969, Southgate used pullulanose and amyloglucosidase enzymes to hydrolyze the starch in test food and measured available and unavailable carbohydrates. However, the method had deficiencies such as incomplete removal of the starch (Southgate, D. A. T., 1969). In 1982, Englyst and colleagues introduced a more effective and specific starch digestion protocol. In this method, readily digestible starch (RDS) was hydrolyzed with α-amylase and pullulanase, while the resistant starch part was subjected to extensive hydrolysis with amyloglucosidase (Englyst et al., 1982). The same year Jenkins and colleagues investigated the effect of the lentil processing on the digestibility of the lentil and its blood sugar results. In this study, human saliva was used for starch digestion. The correlation between test tube digestion and *in vivo* results was positive, although it was a rudimentary method (Jenkins et al., 1982). In 1986, Berry presented a method of resistant starch based on the method of Englyst et al. In contrast to the Englyst method, Berry removed the 100˚C heating step in his own method. That is why it mimics the actual physiological conditions better. As a result, yields of resistant starch were higher (Berry, 1986),

The main purpose of test tube digestion methods published until early 1990s was not to mimic physiological conditions but the methods published since the 1990s are more successful in mimicing *in vivo* studies. In 1991, Granfeldt and Bjorck focused on

mimicking physiological conditions of digestion and established a new method that involved three *in vivo* digestion stages: oral, gastric, and intestinal (Granfeldt, Y., & Björck, I., 1991). Other methods involving these three stages of physiological digestion were also published in 1992 (Brighenti et al., 1992; Englyst et al., 1992, Muir & O'Dea, 1992). These experiments are usually carried out in parallel with *in vivo* experiments. From the early 1990s to the present day, the methods reported to analyze the digestibility of carbohydrate-containing foods are simulated in physiological conditions in each case, but the ways in which these conditions are simulated on methods vary considerably.

2.1.2.1 Oral Phase

The digestion of food first begins in the mouth which is called oral phase. During the oral phase; the teeth make a major breakdown in the structure of the food. Thus, salivary impregnation occurs and α-amylase enzyme, which found in saliva provides partial disintegration of starch. As a result of chewing the food become a bolus and the bolus passes easily through the esophagus and descend into the stomach.

In the literature, there are many variations in oral phase applications test tube digestion. Some of the researchers used an actual chewed food sample in the oral phase. An example of this, Muir and his colleagues used volunteers to chew food samples then the samples were taken and weighed; results indicated that the samples contained an average of 0.1 g of carbohydrates (Muir et al., 1995). A similar study was performed by Akerberg and his colleagues. In this study, the volunteers cleaned their mouths and brushed their teeth then they chewed samples containing approximately 1 g of available carbohydrate 15 times (approximately 15 seconds), and the samples were used for test tube carbohydrate digestion (Åkerberg et al., 1998). However, actual chewing in the oral phase is not a sufficient standardized method because of the variation in chewing time, different amount of accessible carbohydrates in each of the chewed sample. Chewing is subjective process, which may vary from person to person. In view of this, researchers used test tube digestion techniques to mimic the chewing process. Sieves with different pore sizes were used in some of the studies (Karkalas, 1985; Brighenti et al., 1998), also mincers were used with specific pore sizes which were then compared with actual chewed samples (Englyst et al., 1999; Araya et al., 2002), and choppers

were preferred to prepare the samples (Brennan et al., 1996). Some other methods did not consider food particle size in sample preparation, instead of using samples that were ground, milled or homogenized (Champ, 1992; Goni et al., 1997; Weurding et al., 2001)

The differences in chewing technique cause different particle sizes and food particle size plays a critical role in the digested starch and GI values in both *in vivo* and test tube digestion (Holt & Brand Miller, 1994; Kim et al., 2004; Read et al., 1986). Most test tube carbohydrate digestion methods aimed to standardise particle size of the food samples and used some alternatives to chewing, such as sieving, mincing or chopping to standardise the oral phase. These techniques are easier to standardise and provide more accurate results (Brighenti et al.,1998; Champ, 1992; Weurding et al., 2001).

In test tube carbohydrate digestion methods using actual chewing, food samples are treated with salivary amylase for a period of time and partial breakdown of the starch is provided (Granfeldt et al., 1992, Muir et al., 1992). Various other methods applied saliva *in vitro* to mimic the conditions of the oral phase. Researchers incubated food samples in human salivary α-amlyase at 37˚C with pH 6.9 for 5 and 15 minutes, respectively (Brighenti et al., 1995; Lebet et al., 1998). Urooj and Puttraj (1999) incubated food samples in actual human saliva, while Monro used α-amylase (Mishra et al., 2008). In some other methods, oral phase was omitted which means researchers did not use any α-amylase or saliva during the oral phase (Englyst et al., 1992; Brennan et al., 1996; Goni et al., 1997).

In 2008, a study was conducted to compare sample preparation procedures between different test tube carbohydrate digestion methods and to examine the effects of the procedures on the final amount of starch and glucose digested in the same food. At the same time, the results of the test tube digestion method were compared to the results of the test tube digestion method with the actual chewed sample of the corresponding food. In the study, many common starchy foods were prepared for the oral phase via sieving, mincing or chopping, and applied digestion in test tube. Results showed that the preparation method seriously affects the amount of digestible pasta and wheat starch. Wheat prepared via sieving, mincing, or chopping was evaluated as having a lower glucose release compared with the actual chewed samples. In addition, depending on the method of preparation, significant amounts of digestible starch were observed in the first 60 minutes of digestion (Woolnough et al., 2008). These results showed that even for the same foods, the variation between preparation techniques

could affect the amount of starch digested and change the final results. The studies showed the importance of standardizing the oral phase of test tube carbohydrate digestion methods and demonstrated the importance of standardizing test tube carbohydrate digestion methods to produce more accurate results when compared to *in vivo* methods.

2.1.2.2. Gastric Phase

Gastric phase is the second step of food digestion. It occurs in the stomach. The factors affecting of food digestion during the gastric phase are: gastric enzyme (i.e., pepsin), acidity (i.e., pH), viscosity, food quantity, incubation temperature and time, mechanical motion of the stomach and sample mixing method (shaker water bath, magnetically stirred incubated containers or periodical manual mixing etc.) (Turnbull et al., 2005). Test tube carbohydrate digestion results can be influenced by all these parameters. These parameters need to be standardized.

Information about the available methods in the literature with and without pepsin proteolysis step is as follows: A method for the analysis of cereal starches comprises a pepsin incubation for 60 minutes at pH 1.5 and 37 ° C on a magnetic stirrer (Holm et al., 1986). In 1992, chewed food samples were spit into a pepsin solution and pepsin digestion was carried out at pH 1.5 and 37 ° C for 30 minutes. The blend was gently mixed three times during the incubation (Granfeldt et al., 1992). These methods have found that treatment of the samples with pepsin degraded the starch-protein interaction and increases the α-amylase sensitivity to starch. In another study, spaghetti samples prepared by extrusion were acidified to pH 2.0 with HCl and they were incubated with 575 U of hog pepsin/g of starch for 1 hour (Brighenti et al., 1992). In the 1992 method of Muir and O'Dea, a pepsin incubation was carried out in a shaking water bath at 37 ° C, pH 2.0 for 30 minutes (Muir, J. G., & O'dea, K., 1992).

The 1992 method of Englyst for determining the nutritionally major starch fractions did not include a pepsin proteolysis step (Englyst et al., 1992). However, in subsequent Englyst methods, the samples were incubated in a shaking water bath with pepsin for 30 minutes at pH 2.0 (Silvester et al., 1995; Englyst et al., 1999). Englyst method also considers the effect of viscosity. Viscosity slows down the duration of gastric emptying, reduces access of digestive enzymes to food and affects glycemic

response. Viscosity is a difficult factor to imitate test tube conditions. For this reason, most test tube carbohydrate digestion methods do not consider for the effect of viscosity. Englyst and colleagues have added guar gum to the media to mimic the viscosity conditions of *in vivo* digestion, also enhanced the suspension of enzymes in the media. This step was considered important in the development of the test tube digestion methodology and allowed researchers to measure the effect of viscose on food digestion rate and glucose release. The duration of the pepsin digestions used in the methods ranges from 10 minutes to one hour. The ideal duration of test tube pepsin digestion varies depending on the rate of gastric emptying *in vivo*, type of food, digestible amount and form (Santangelo et al., 1998). The period of test tube pepsin incubation may need to be determined specifically for a food group or a food form (Frost et al., 2003).

Gastric phase has been omitted in some test tube carbohydrate digestion protocols. However, in some other methods include a pepsin proteolysis step, particularly in total and resistant starch assays, in order to achieve complete starch digestion through disruption of the starch-protein interactions. Studies suggest that pepsin incubation is necessary when the analyzed food contains an important amount of protein component that can interfere with starch and amylolytic enzymes in the compound (Woolnough, J. W., 2011). In this kind of foods, pepsin is a key factor in the completion of starch digestion because it breaks down the protein-starch interaction in the food. Woolnough and colleagues conducted atest tube digestion study with or without pepsin in the gastric phase. In the experiment, various starchy foods were incubated with either pepsin for 30, 60 minutes or without pepsin. Most of the tested samples showed a non-significant difference between the "pepsin" and "no pepsin" conditions. However, for macaroni, which is higher than other foods in terms of protein, the starch digestion rate is increased by incubation with pepsin. In other words, the digestion rate of samples incubated with pepsin for 60 minutes had higher digested starch content than those incubated with pepsin for 30 minutes. These results suggest that the incubation of the samples with pepsin during the gastric phase is more important for protein-containing foods in significant quantities. If starch protein interactions are high in a food, the duration of incubation with pepsin is important and in this condition starch digestion was increased by incubation with pepsin (Woolnough et al., 2008).

2.1.2.3. Intestinal Phase

The last stage of the digestion is the intestinal phase. In this stage, chyme is poured into duodenum. Here, chyme is mixed with α-amylase-containing secretions of the pancreas. This mixture passes through the intestinal lumen via peristaltic contractions. Brush border enzymes in the small intestine hydrolyze starch to oligosaccharides, disaccharides (maltose, sucrose and lactose) and also monosaccharides (glucose and fructose). These monosaccharides then pass to the blood and glycemic reaction occurs (Guyton & Hall, 2000). In intestinal phase, the main purpose is the hydrolysis of the sample with enzymes within the constraints of physiological parameters. Depending on the method they are attempting to measure, samples may be taken at specific time points for the carbohydrate digestion rate. Samples may only be taken at the end of the hydrolysis period for the determination of the carbohydrate. Also, samples may not be taken at all. In which case hydrolysis is carried out to remove digestible carbohydrates for isolation of resistant starch and dietary fiber fractions (Woolnough, 2011).

In 1984, 10 mL of saliva was mixed with different food samples (bread, cornflakes, potatoes and legumes). The mixtures placed in the dialysis bags were suspended in a stirred water bath containing distilled water and incubated at 37 ° C for 3 hours. After 3 hours, 5 mL aliquots were taken for glucose measurement. The concentration of 3-hour glucose in the aliquots represented the digestibility of starch in the sample. The percentage of total digested starch was compared with the digested starch of the reference food, which was white bread. The results of the test tube digestion GI values were positively correlated with the *in vivo* GI results.

Englyst and colleagues digested chopped food samples with an enzyme mixture containing pancreatin, amyloglucosidase and invertase. To better mimic the test tube digestion conditions, guar gum was added to standardize viscosity. Also, glass balls were added and the tubes were shaken horizontally in a shaking water bath. Hydrolysis proceeded over 2 hours at pH 5.2 and 37 ° C and 0.5 mL aliquots were taken at 20 and 120 minutes. Englyst determined digestable starch fractions according to digestion ratio. Rapidly digestible starch (RDS) is amount of digested starch after 20 minutes digestion, slowly digestible starch (SDS) is amount of digested starch between 20 - 120 minutes and resistant starch (RS) measured as indigestible part after 120 minutes. Englyst's

1990 and 1992 methods, has contributed significantly to the evolutionary development of the test tube carbohydrate digestion method, because these methods are the most commonly used methods to determine the glycemic index of digestible starch and since then (Woolnough et al., 2008).

The buffer is another important element in the intestinal phase and is used in this phase to mimic the optimum pH for pancreatic enzymes. If methods involving a stomach phase, the intestinal phase digestion is initiated after the buffer is added to raise the incubation pH. In Muir's method, samples were incubated in a shaking water bath for up to 21 hours at 37 ° C. Prior to this procedure, the samples are neutralized with 4 M NaOH and pH was raised to 5.0 with acetate buffer (Muir etal., 1995). Test tube digestion method by Goni and coleguages, a Tris-Malate buffer (pH 6.9) was added to adjust the pH of the intestinal phase. Food sample was mixed with α-amylase for 3 hours at 37˚C in a shaking water bath and aliquots were taken every 30 minutes. The aliquots were then mixed with 3 mL of sodium acetate buffer and 60 μL of amyloglucosidase to degredation of starch. This method was performed with several starchy foods and glucose measurement was applied at different time points. The measurements express the percentage of starch hydrolysis at different time points. The area under the curve (AUC) of the digested foods was calculated compered with the reference food (white bread). The results was expressed as the hydrolysis index (HI) of the foods. The results were compared with the *in vivo* glycemic responses of these foods. The digestibility rate of the test tube carbohydrate digestion method showed a strong correlation with the *in vivo* GI of the same foods within 90 minutes (Goni et al., 1997).

The most important points affecting the intestinal phase of test tube digestion methods are enzymes and pH values. Even if most of the previous methods used pancreatin or α-amylase as the primary hydrolytic enzyme, there were changes in the concentrations of these enzymes from method to method. In addition, the methods differed in whether or not they used amyloglucosidase and at what stage of digestion amyloglucosidase should be added. pH is another factor that varies between methods. The optimum pH value for amyloglucosidase is between 4-5, while the optimum pH value for α-amylase and pancreatin is between 6-7. Thus, in most methods amyloglucosidase and α-amylase or pancreatin were incubated at about pH 5. The change in pH can lead to different estimates of the amount of starch digested between test tube carbohydrate digestion methods. To investigate the effect of pH on enzyme

activity, Woolnough and colleguages used the most commonly used test tube carbohydrate digestion methods to test the stability of pancreatin. In this study, samples were incubated at a pH ranging from 4-9. Results showed that pH 5-7 was sufficient for pancreatin activity during digestion in test tube studies (Woolnough et al., 2008). Another important factor affecting the intestinal phase is enzyme concentration. The usage of enzymes at low concentrations causes more changes in the amount of digestible starch. For this reason, more accuracy is required when measuring the enzyme at small concentrations to reduce the change. Also, incubation time is another factor affecting starch digestion. For this reason, the duration of incubation is particularly critical for methods that measure RDS, SDS and GI. Finally, in addition to the mentioned factors, the intestinal phase of *in vitro* methods is influenced by some other factors such as temperature, mixing method and viscosity.

GI measurement of food is very important in terms of food preferences of diabetic individuals. The usage of test tube carbohydrate digestion methods has increased in the last 20 years. The newest methods have always tried to simulate the stages of physiological digestion (ie, oral phase, gastric phase and intestinal phase). However, the differences between methods is very high. There is no robust, reliable and standard test tube carbohydrate digestion methods accepted. These differences in test tube techniques lead to differences in the amount and deviation of starch digested in test tube against the *in vivo* GI values of the same foods. For this reason, studies should be continued to establish a standard and robust test tube carbohydrate digestion methods.

2.2. Test Tube Digestion Protocol Applied in the Experiments

Newly formulated, low calorie and no-added sugar cake, almond paste and pudding have been developed by Takita Company (Egepak Gıda ve Ambalaj San. A.Ş., İzmir). These foods are sensually analyzed and produced according to the most popular formulations. As a reference, same types of foods already placed on the market were selected. Reference samples have not any functional properties. Takita cake, almond paste, pudding samples and their references were digested in the test tube. Then, these digested samples were used to measure glucose efflux in the intestinal cell culture model. Test tube carbohydrate digestion method used in the experiments was followed similar to Englyst and colleagues (Englyst et al., 2000). The following information is

given about the general characteristics of the protocol and its application. The next section includes changes of the protocol that are appropriate for the cell culture studies.

This analytical procedure quantifies various fractions of starch by enzymatic hydrolysis and measurement of released glucose. The samples were analyzed "as eaten" and treated with a protease enzyme to disrupt the starch-protein interaction. Previously, it was mentioned that to treat with a protease enzyme would be more important if the protein content was high in the food sample. The protocol continues with incubation with amylolytic enzymes under controlled conditions for temperature, pH, viscosity, and mechanical stirring rate. In the protocol, colorimetry and HPLC procedures are summarized. The method we used is calorimetric so the application of the calorimetric method is mentioned.

Carbohydrate digestibility is largely influenced by food processing and samples should be analyzed as eaten. Dry edible foods should be analyzed dry, hot and cooked edible foods should be cooked just before being analyzed and stored at 70-80 ° C and used in this way. Foods that normally have a structure that requires chewing (eg, pasta, rice, corn) are passed through the sieves, mincers or choppers. Sample weights should be selected to contain 500-600 mg of starch and sugar. These quantities can be estimated from food tables. Examples of suitable sample weights are given in the table below.

Table 2.1. Suitable sample weights for digestion.

Dry Matter (%)	Examples	Weight (g)
75 – 100	Starch	0.6
75 – 100	Flours, breakfast cereals	0.8
55 – 75	Bread, cakes	1.0 – 2
35 – 55	Beans, pasta, rice	1.5 – 3
15 – 35	Canned foods, sauces	3.0 - 4

The steps in the protocol are as follows: Weigh 500 to 600 mg of starch and sugar containing food sample (0.6 – 4 g). Add 10 mL pepsin – guar gum solution. For 18 samples, add 1 g of pepsin powder to 200 mL of 0.05 mol L^{-1} hydrochloric acid and mix with a magnetic stirring bar. Just before use, add 1 g of guar gum and mix well. The pepsin–guar gum solution should be prepared immediately before use. Incubate at 37 °C for 30 minutes. Add 10 mL 0.25 M sodium acetate, 5 glass ball and 5 mL enzyme mixture (pancreatin, amyloglucosidase, invertase), respectively. For 18 samples /

standards, into each of six centrifuge tubes weigh 3.0 g of pancreatin and suspend in 20 mL of water using a vortex-mixer. Add a magnetic stirring bar and mix for 10 min. Centrifuge at 1500 *g* for 10 min, then remove 15 mL of the cloudy supernatant from each tube and combine (90mL total). Add 4mL of amyloglucosidase and 6mL of invertase. Mix well. The enzyme mixture should be prepared immediately before use. Incubate with shaking at 37 °C. After 20 min remove 0.5 mL. Place in 20 mL ethanol. Centrifuge and hold 10 min at 100 °C and measure glucose released after 20 min (G20). After 100 minutes have elapsed, 0.5 mL sample is taken and the same procedures are repeated (G120). After taking the G120 samples, vortex remainder mixture in test tube. Hold at 100 ° C for 30 min. Cool to 0°C. Add 10 mL 7 M KOH. and vortex mixture. Shake 30 min at 0 ° C. Vortex mixture again and transfer 1 mL into 10 mL 0.5 M acetic acid. Add 0.2 mL amyloglucosidase. Hold at 70 ° C for 30 min. Add 40 mL water. Centrifuge and measure total glucose (TG).

As a result of various experiments, this method has been revised to make it specific for the Takita and reference products and the cell culture study, in accordance with the purpose of the study to be performed.

2.3. Protocol Implementations for Cell Culture Studies

In this study, the main purpose of the test tube carbohydrate digestion was to compare the glucose levels of newly formulated low calorie and no-added sugar cake almond paste and pudding samples with the glucose levels of reference samples. In the protocol, after 120 minutes step, the remaining starch is completely disintegrated and a value is obtained for total glucose (TG). The difference between this TG and slowly available starch (G120) value is also calculated as the value of the resistant starch (RS). However, resistant starch does not break down and is thrown out of the body in human physiology. Due to these two main reasons, the steps of after the 120 min enzymatic incubation were not applied for the products. Instead, enzymatic incubation was performed for 2 hours and 4 hours.

Firstly, determination of enzymatic incubation time and suitable sample weight analysis were performed for test tube carbohydrate digestion of cake, almond paste and pudding. Reference samples have more digestable carbohydrates than Takita samples. For this reason, enzymatic incubation time and initial amount of sample experiments

were carried out for reference samples. Dry material analyses were done for each Takita product and its reference to determine the initial sample weights to be taken. Initial amount of samples are determined by using Table 2.1. The protocol volumes were reduced by half and therefore sample quantities were tested in two different weights, half and quarter. Specific product quantities and digestion times for each product were determined and experiments were conducted accordingly.

According to above mentioned parameters, test tube carbohydrate digestion experiment was applied for cake, almond paste and pudding as follows. Firstly, dry material analyses of Takita and reference products were done. The dry material content of the Takita cake, almond paste and pudding are 69.46%, 89.94% and 28.43%, respectively. Also dry material content of the reference cake, almond paste and pudding are 74.10%, 92.26% and 26.98%, respectively. The amount of initial sample weight was selected 1.0 g for cake, 0.8 g for almond paste and 3.0 g for pudding by using Table 2.1. However since the protocol volumes were reduced by half, sample quantities were tested in half and quarter (for cake samples: 0.50 g and 0.25 g; for almond paste samples: 0.40 g and 0.20 g; for pudding samples: 1.50 g and 0.75 g). Also reference samples were tested for 2 hour and 4 hour enzyme incubation times.

The results of these experiments were evaluated statistically and suitable time points and initial portion sizes were determined. After that, test tube carbohydrate digestion was performed for Takita samples and reference samples as follows: Takita and reference samples were weighed and put into falcon tubes. Next, 5 mL of pepsin-guar gum solution (P7000-Sigma Aldrich, G4129-Sigma Aldrich) was added to each tube (solution preparation was expressed the previous part). The samples were kept in water bath for 30 minutes at 37 ° C. After that 5 mL of 0.25 M sodium acetate (S8750-Sigma Aldrich), 5 glass balls and 2.5 mL of enzyme mixture containing pancreatin (P7545-Sigma Aldrich), amyloglucosidase (A7095-Sigma Aldrich) and invertase (I4504-Sigma Aldrich) were added respectively to the samples (enzyme mixture preparation was expressed the previous part) and enzyme incubation was performed at 37 ° C (for cake 2 h; for almond paste and pudding 4 h). At the end of incubation, the enzyme inactivation is maintained at 100 ° C for 30 minutes. Glucose contents were measured with the glucose assay kit (GAGO-20 – Sigma Aldrich).

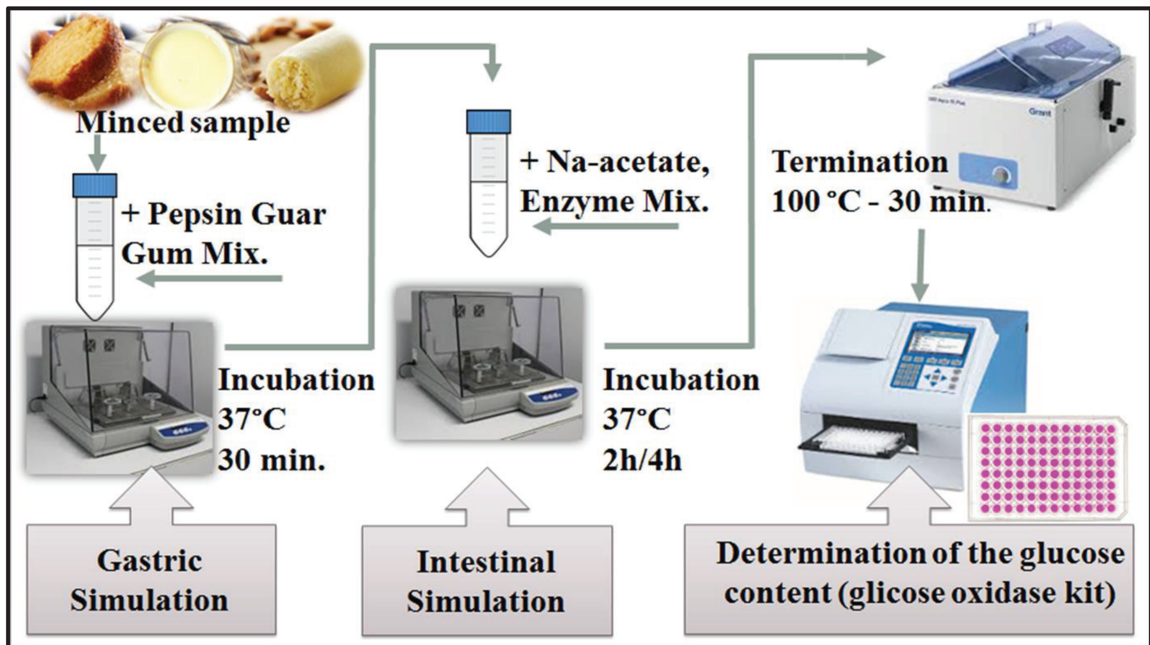

Figure 2.1. Schematic presentation of digestion protocol applied to products.

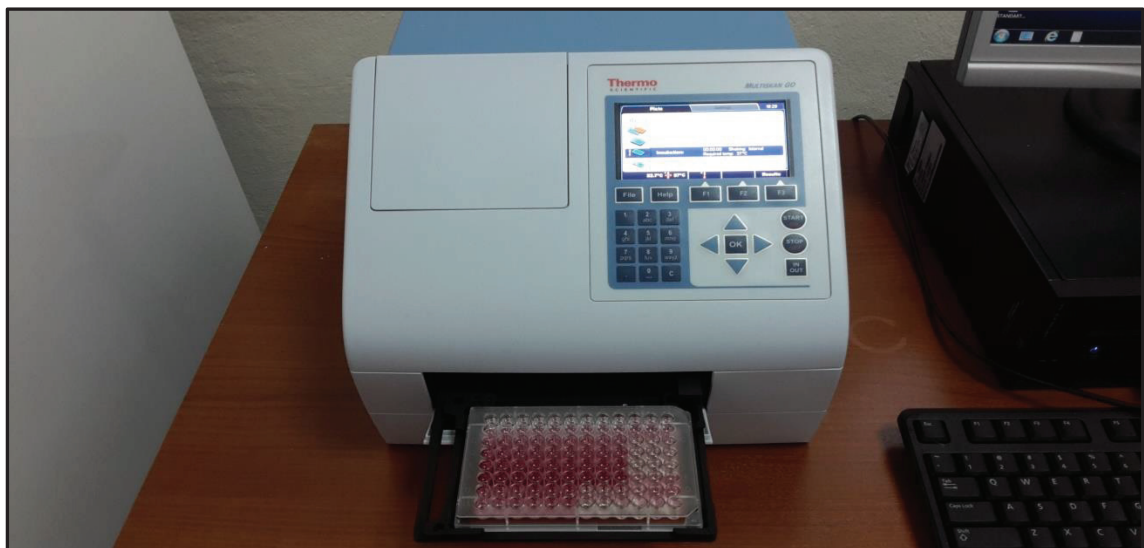

Figure 2.2. The photos of 96 well plate and microplate spectrophotometer (Thermo Fisher - Multiskan™ GO).

The most important reasons for using this enzyme kit in determining glucose is that the kit are specific, sensitive and fast. At the same time, it has high specificity and sensitivity also it provides sample preparation in small quantities, so it was preferred in this study. The working principle of the glucose assay kit is as follows: The kit contains the glucose oxidase enzyme. This enzyme allows oxidation of glucose in the products, to gluconic acid and hydrogen peroxides. When hydrogen peroxide reacts with reduced

o-dianisidine, the peroxidase enzyme converts it to oxidized o-dianisidine, which is a brown colored. This oxidized O-dianisidine reacts with sulfuric acid to form a stable pink color, which is measured at 540 nm on a spectrophotometer. This pink color intensity is directly proportional to the glucose concentration. The sample should be diluted to a concentration of approximately 20-80 μg glucose / ml in order to be able to measure with this kit. Data were normalized dry weight of samples.

In these experiments, in order to be able to make another comparison, digestion for white bread was performed with the same protocol. Firstly, dry material analysis of white bread was carried out. The dry material content of the white bread was founded as 54%. The initial amount of sample was determined as 1.0 g according to the Table 2.1., but since the protocol volumes were reduced by half, sample quantities were tested in half (0.5 g) and quarter (0.25 g) and enzymatic incubation time were tested for 2 hour and 4 hour enzyme incubation times.

2.4. Dry Material Analysis

The reason for the dry material analysis in these experiments, to determine how much of the initial sample taken is water so how much less initial amount of sample was weighted and from there to correct the calculation mistakes. Also, in general terms, it is necessary to determine moisture content in order to determine the shelf life, quality and price, nutrient content, transportability and storability of the food. In oven drying methods, the sample loses weight due to temperature. The drying time varies depending on the oven used and the conditions in the oven. Other factors include particle size, particle size distribution, sample size, and surface. During drying, the area influences the dehumidification rate and efficiency. The methods were approved by AOAC International. The method is simple and suitable for performing a large number of analyzes.

When dry matter analysis of carbohydrate-containing foods is done, it is necessary to use a vacuum oven so the process ends in a shorter time. The process can be carried out for a long time at lower temperatures. If there is no vacuum oven, it is necessary to work longer times at lower temperatures. In the experiments, the weighing vessels were dried in oven, cooled in desiccator and weighed as long as the weights are fixed. Then 1 gram sample was weighed into each weighing vessel. Aftter that the

products were dried at 60 ° C overnight without vacuum and the next day cooling and weighing process was applied. Then the dry material content (DMC) calculated by the calculation shown below:

$$DMC\ (\%) = 100 - \left[\frac{[S - (W1 - W0)]}{S} \times 100\right]$$

S = Quantity of test sample taken (g)
W0= Weight of receptacle (g)
W1= Weight of receptacle after has been dried (g)

2.5. Freeze Drying (Lyophilisation) Process

Freeze drying is a method of removing water from a frozen product by sublimation. In this method frozen water passes directly into the gas phase without passing through the liquid phase. The product must be pre-frozen before starting the freeze drying process. In order to obtain the maximum amount of ice, the product should be frozen at temperatures between the glass transition temperature of maximally freeze concentrated material (Tg') and the onset temperature of ice melting in maximally freeze concentrated material (Tm'). Table below shows glass transition temperatures and temperature of ice melting of maximally freeze-concentrated matrix for various sugars. Process of freeze-dry is more difficult for sugar containing materials because their onset temperatures of ice melting in maximally freeze concentrated systems are very low.

Table 2.2. T g' and T m' values for various sugars.

Material (Reference)	T g' (°C)	T m' (°C)
Fructose (Roos, 1993)	–57	–46
Glucose (Roos, 1993)	–57	–46
Lactose (Roos, 1993)	–41	–30
Maltose (Roos, 1993)	–42	–32
Sucrose (Roos & Karel, 1991a)	–46	–34

After pre-freezing, ice can be removed from the frozen product by sublimation via controlling the temperature and pressure of the drying system. In freeze-drying, conditions must be constituted to assist free flow of water molecules from the product. For this purpose, a vacuum pump is used to reduce the pressure of the environment around the product and a condenser is used to condense and collect the moisture away from the frozen product. The vacuum pump also removes all gases that non-condensible (Hyvönen, L., & Jouppila, K., 2008).

In this study, freeze-drying technique is used to adjust concentrations of samples before using for cell culture study. Maximum glucose concentration should be 25 mM for the cell culture study which is used. If the concentration is above 25 mM, it causes damage to the physiological structure of the cell. This condition may lead to incorrect results. Freeze drying technique gives the opportunity to allow the concentration value to be adjusted for cells.

Our freeze drying procedure was carried out as follows: The digestion process was completed in the test tubes for the Takita and reference cake, almond paste and pudding samples. These samples were taken in 2 mL eppendorf tubes and frozen at -20 ºC overnight. The condenser is cooled to about -80 °C before giving the samples so freeze dryer should be activated 2-3 hours before starting the treatment. During the lyophilization process, to avoid loss of sample, aluminum foils are placed in the mouth parts of the eppendorf tubes and holes are made with the help of a sterile needle. Frozen products are placed on the shelves of the freezer dryer. The pressure is adjusted to the appropriate values. Incoming heat causes the ice to transition phase. Water vapor condenses on the condenser in the form of solid ice. The operator decides whether the drying process is sufficient. The products were kept in the freeze-dryer for 2 days. Vacuum is released after the process is completed. Aluminum foils are removed from the cover parts and the covers of the tubes are immediately closed to prevent moisture transfer from the air. Freeze-dried products were stored at -20 °C and solubilized by adjusted amount of water for using cell culture studies.

2.6. IEC-6 Cell Culture Studies

The first part of this thesis is that bioaccessibility part which is the digestion of the carbohydrate in test tube. Cell culture study is the second part which was performed for the bioavailability of glucose. In this part IEC-6 cells were used. The ability of a cell model to be used for *in vitro* digestion methods requires that these cells must be intestinal origin and have functional uptake and efflux transporters (Thomas et al., 2002). IEC-6 cells are nontransformed rat intestinal epithelial cell lines (Puthia, M. K., et al., 2006). The cell lines derived from rat jejunal crypts (Park, J. H. et al., 1992). IEC-6 cells are frequently used in *in vitro* studies (Gulec & Collins, 2014, Thomas et al., 2002). IEC-6 cells are characterised as a homogenous population of epithelial-like cells. They have microvilli in a perinuclear area also Golgi with membrane-limited granules, numerous mitochondria and ribosomes. These mitochondria and ribosomesare are both free and attached to the endoplasmic retireticulum (Wood et al., 2003). There is no any noncancerous human intestine cell line so this cell line is the best alternative for this project. Also this cell line constitutes tight junction and polarized monolayer forms at short notice. This is an important advantage for routine studies.

2.7. Modeling of Intestine System *In vitro*

IEC-6 cells were purchased from ATCC and were grown on collagen-coated inserts to create *in vitro* intestine system and test antidiabetic features of formulated food products. Figure 2.3. shows the insert system. In this system, membranes have 0.4 micron pore size and they can be placed on 12 mm diameter cell petri dishes. The growth medium consisted of Dulbecco's Modified Eagle's Medium (DMEM with 4.5 g/L of glucose) (Gibco 41965-039) with 5% heat-inactivated Fetal Bovine Serum (FBS) (Gibco 10500-064), 0.1 unit/ml Insulin (Sigma I9278), % 1 Penicillin-Streptomycin (Sigma P4333) and % 1 L-Glutamin (Gibco 25030-024). Cell culture study was carried out as follows: The membranes were kept with the medium for 1 night before the cells were given to the membranes. There were proteins in the FBS, and through this pre-treatment, cells could attached more easily to the insert. After that, cells were seeded on the membrane. 1×10^6 cell/well were plated on the upper side of collagen-coated insert. IEC-6 cells were humidified 37 °C incubator with 10% CO_2, and the passage number

was kept between 15-25. The culture medium was changed every 3 days. 700 µL and 1.500 µl medium were added to apical sides and basolateral sides, respectively. The growth of the cells was monitored by the microscope regularly. The cells covered the membrane within 3 day. After 3 day, the cells were needed 7 days incubation to differentiate and polarized. After observing that the cells were confluent, medium was changed every 2 days. At the end of 7 days, lyophilized samples were dissolved at a certain concentration with DMEM (Gibco A14430-01). This medium did not include D-Glucose. In this way, it was known that detected glucose only came from the formulated food samples.

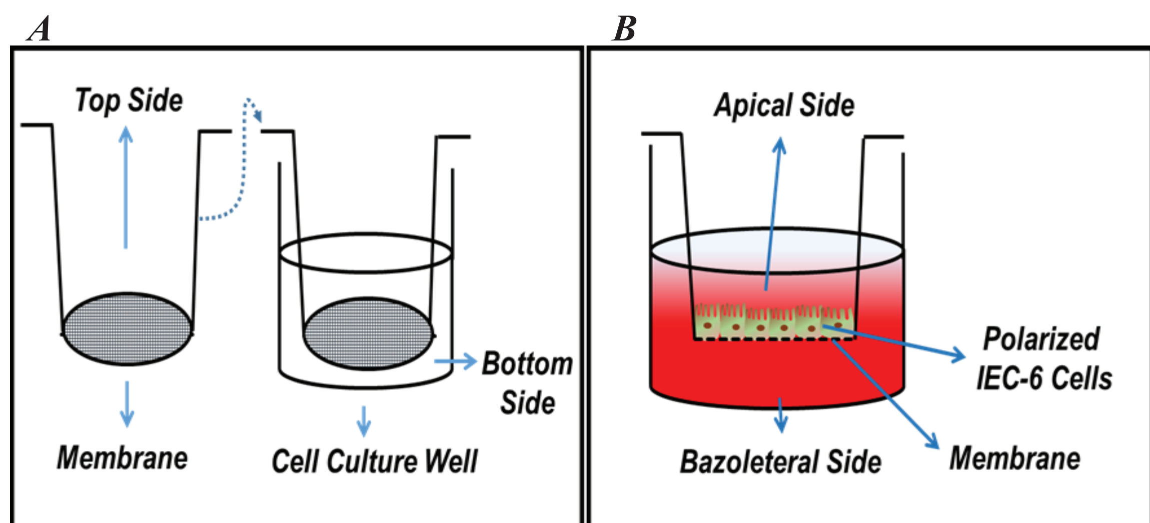

Figure 2.3. (A) Membrane and cell culture plate system and placement of the membrane on the plate (B) after polarization of the cells to apical sides and basolateral sides on membrane.

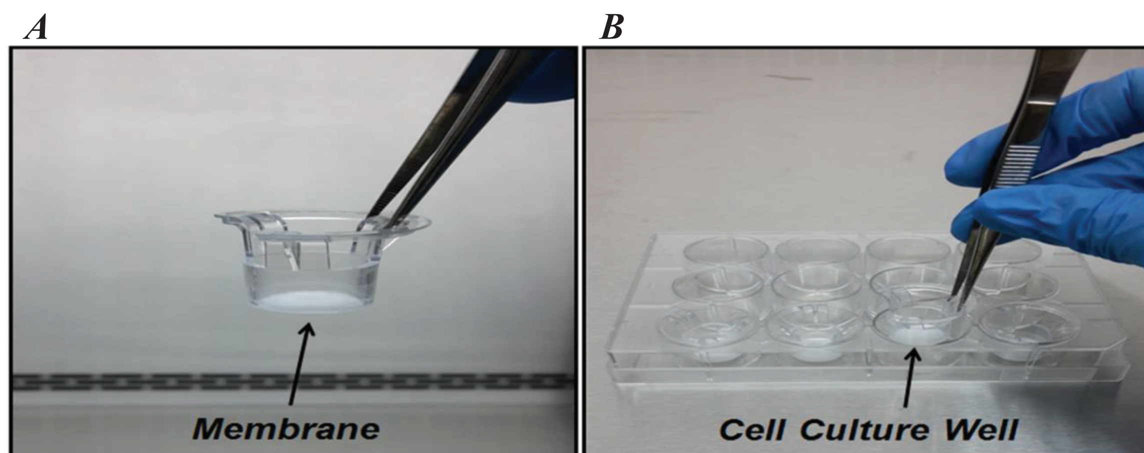

Figure 2.4. (A) The photos of membrane used in the experiments (B) The photos of cell culture wells used in experiments.

2.8. Transepithelial Electrical Resistance (TEER)

After 10 days, the IEC-6 cells constitute tight junction and polarized monolayer forms. At this stage they can create a transepithelial electrical resistance barrier (TEER). TEER values were to check if cells generate a monolayer form and to see if they tightly connect with each other by the EVOM instrument in terms of ohm/cm^2 (WPI, USA).

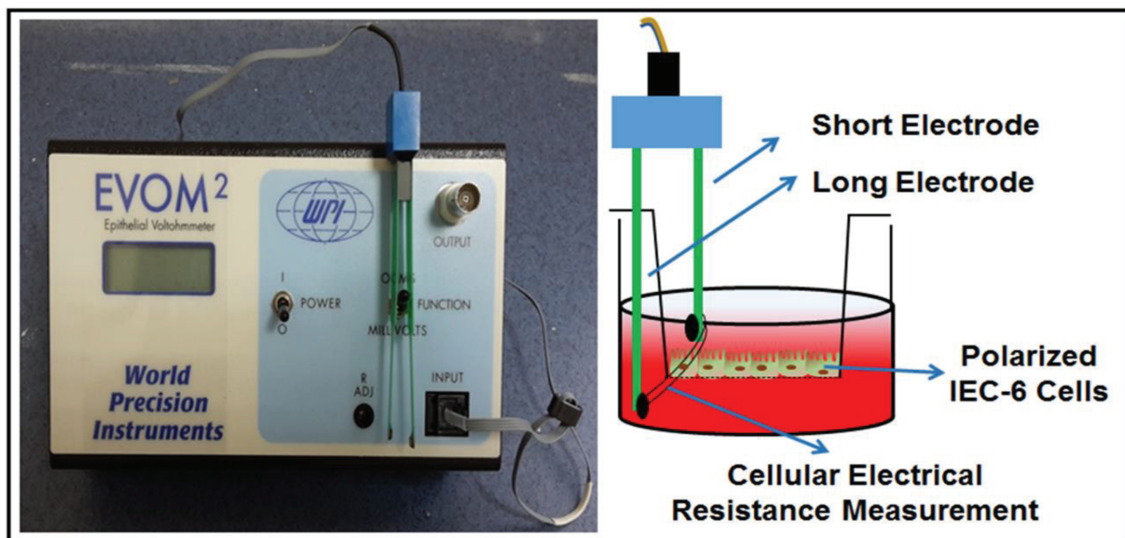

Figure 2.5. The photo of EVOM meter and demonstration of TEER measurements from fully differentiated IEC-6 cells.

The EVOM instrument has one short and one long electrodes. The short electrode is placed on the apical side; the long electrode is placed on the basolateral side. When the cells exhibit barrier resistance, this resistance is converted to a number per cm^2. IEC-6 cells have 30-40 Ω/cm^2 characteristic transepithelial electric resistance (TEER) values (Bastian et al., 1999; Puthia et al., 2006). Figure 2.6. shows that the first and tenth day of IEC-6 cells microscopic photos. When the cells become postconfluent, absorbable molecules go from the apical side through the cells to the basoleteral side. For this reason, TEER values were measured on the 10. day also cells were examined by using microscop and further experiments were carried out thereafter.

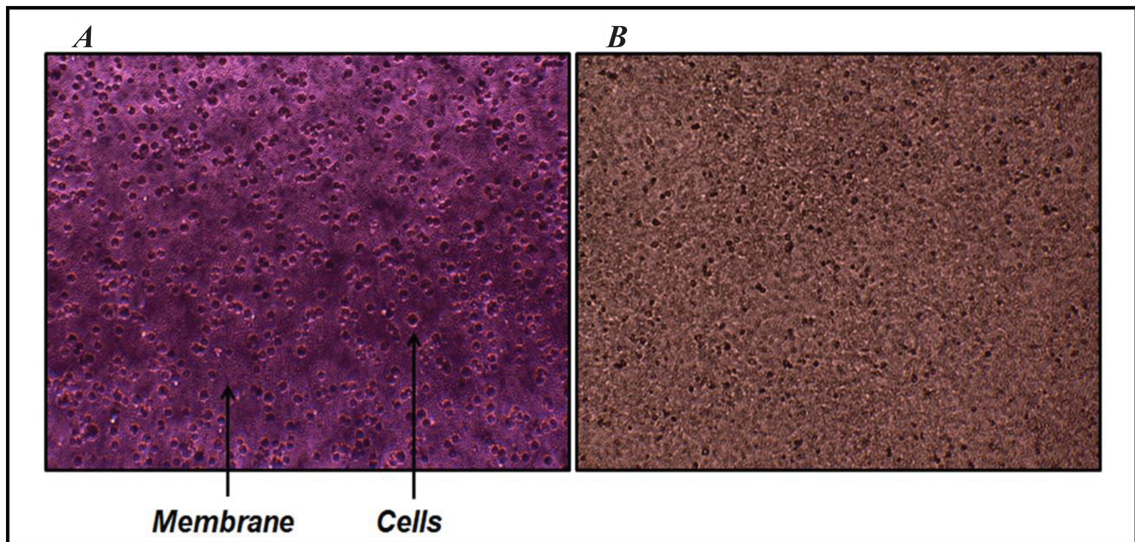

Figure 2.6. (A) Microscopic photos of IEC-6 cells on the membrane (1st day),
(B) Microscopic photos of IEC-6 cells on the membrane (10st day).

2.9. Measurement of Basolateral Glucose Level

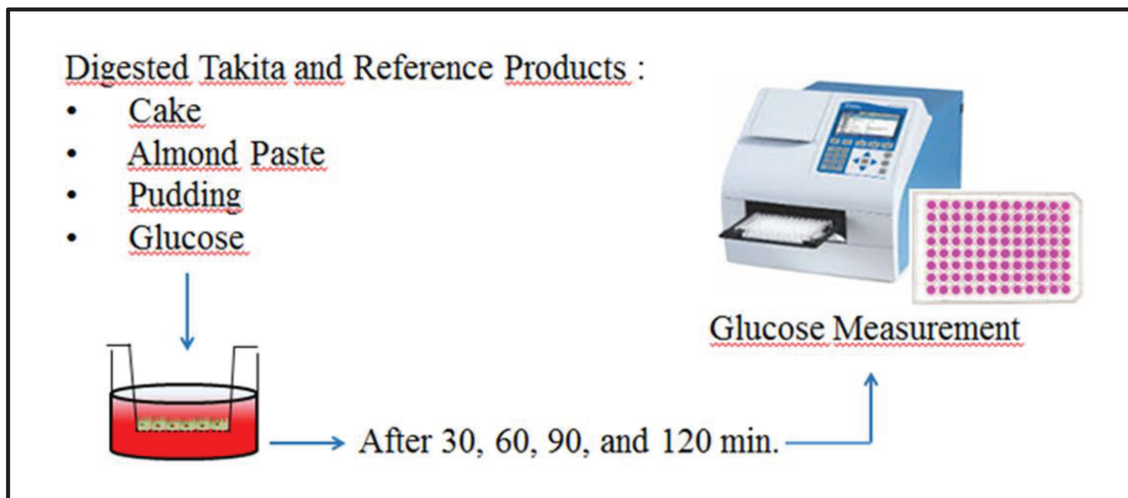

Figure 2.7. Experimental strategy to measure glucose efflux from cells.

In human physiology, the absorption of glucose occurs in the small intestine. The basic mechanism of glucose absorption is as follows. The glucose is taken from the apical side to the basolateral side of the intestinal cells and then the glucose molecules pass to other tissues. To model the same situation in *in vitro* system, experiments for individual samples were performed as decibrided on figure 2.7. After TEER measurement (values reached at least 30 Ω/cm2), digested cakes, almond paste and

puding samples and their references were given apical side of the bicameral cell culture system. Glucose was used as experimental control. After 30, 60, 90,120, 180 minutes (only for pudding), samples were taken from basolateral side and glucose levels were measured by using glucose oxidase assay kit (GAGO-20, Sigma Aldrich).

2.10. Statistical Analysis

The results were expressed as mean ± SE. All analyses were performed and figures were made in GraphPad Prism (version 6.0 for Windows, GraphPad). Initial sample weight and enzyme digestion time point analysis were analyzed by two-way ANOVA. Test tube digestion measurement results and basolateral glucose results were analyzed by one-way ANOVA. The results of initial sample weight & enzyme digestion time point analysis and test tube carbohydrate measurement results were given as mg/g sample. Basolateral glucose results were normalized to 1 and the changes of high glucose group were given as fold change.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Comparison of Experimental Groups with Their Control Groups for Cake, Almond Paste and Pudding Samples

In this experiments, reference samples were used to optimize experimental conditions because reference samples have more digestable carbohydrates than the Takita samples. Figure 2.8. shows that description of experimental group and control groups which were used for the experiments. It was necessary to know the purpose of these groups in order to evaluate the results correctly.

Table 3.1. Schematic representation of the experimental group and control groups

Control 1 (Group 1) :	Control 2 (Group 2) :	Experimental Group (Group 3) :
Sample	Sample	Sample
+ 12.5 mL water	+ 5 mL Na-acetate	+ 5 mL Na-acetate
	+5 mL Pepsin-Guar Gum Solution	+ 5 mL Pepsin-Guar Gum Solution
	+ 2.5 mL water	+ 2.5 mL Enzyme Mixture
Total Volume 12.5 mL	Total Volume 12.5 mL	Total Volume 12.5 mL

Group 1 is a control group, which indicate that only soluble glucose from the samples. Grup 2 is also the other control group and it shows the effect of low Ph condition and pepsin enzyme to digestion of the carbohydrates. Group 3 is the experimental group and it contained all over the enzymes, which were used in the protocol.

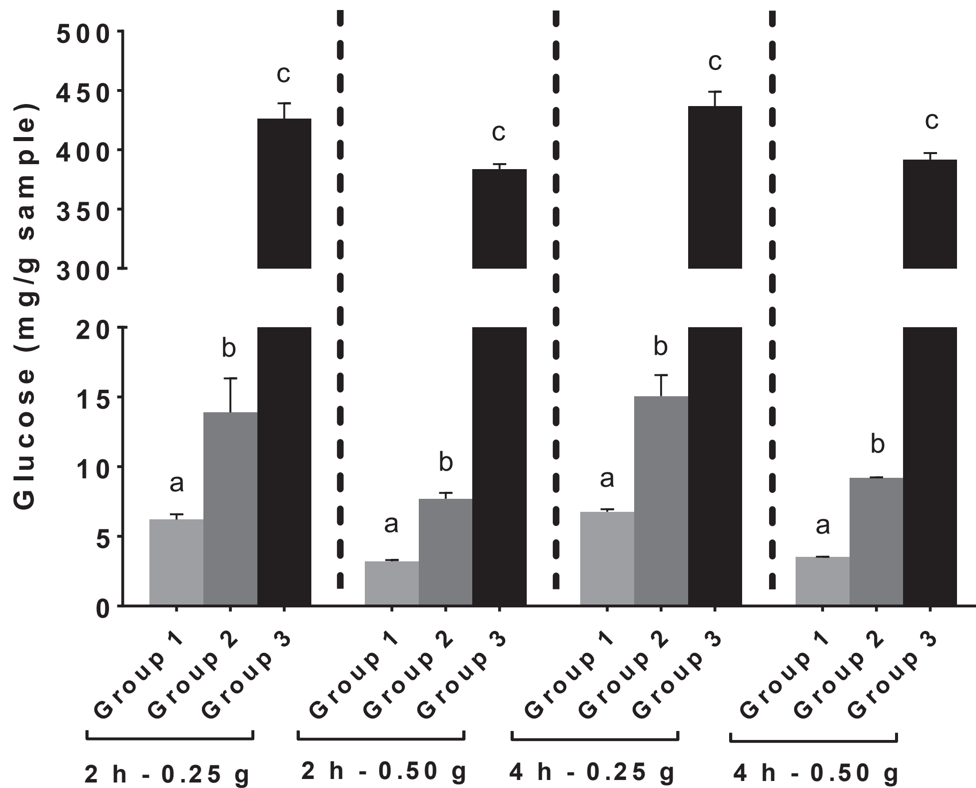

Figure 3.1.A. Comparison of experimental group with their control groups for reference cake. Group 1: 0.25/0.50 g reference cake sample + 12.5 mL water, Group 2: 0.25/0.50 g reference cake sample + 5 mL of pepsin-guar gum solution + 5 mL of sodium acetate + 2.5 mL of water, Group 3: 0.25/0.50 g reference cake sample + 5 mL of pepsin-guar gum solution + 5 mL of sodium acetate + 2.5 mL of enzyme mixture. Data are presented as the mean value ± SE (n = 3). Results were analyzed by one-way ANOVA (*P*<0.05) Tukey's post hoc test was performed.

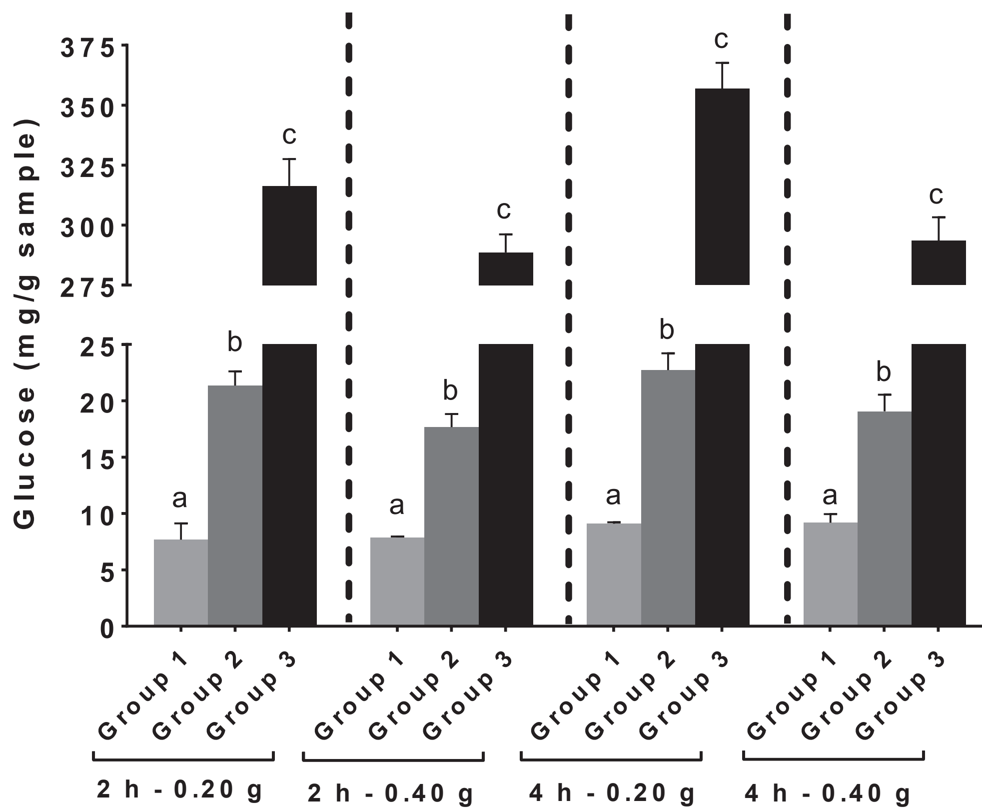

Figure 3.1.B. Comparison of experimental group with their control groups for reference almond paste. Group 1: 0.20/0.40 g reference almond paste sample + 12.5 mL water, Group 2: 0.20/0.40 g reference almond paste sample + 5 mL of pepsin-guar gum solution + 5 mL of sodium acetate + 2.5 mL of water, Group 3: 0.20/0.40 g reference almond paste sample + 5 mL of pepsin-guar gum solution + 5 mL of sodium acetate + 2.5 mL of enzyme mixture. Data are presented as the mean value ± SE (n = 3). Results were analyzed by one-way ANOVA ($P<0.05$) Tukey's post hoc test was performed.

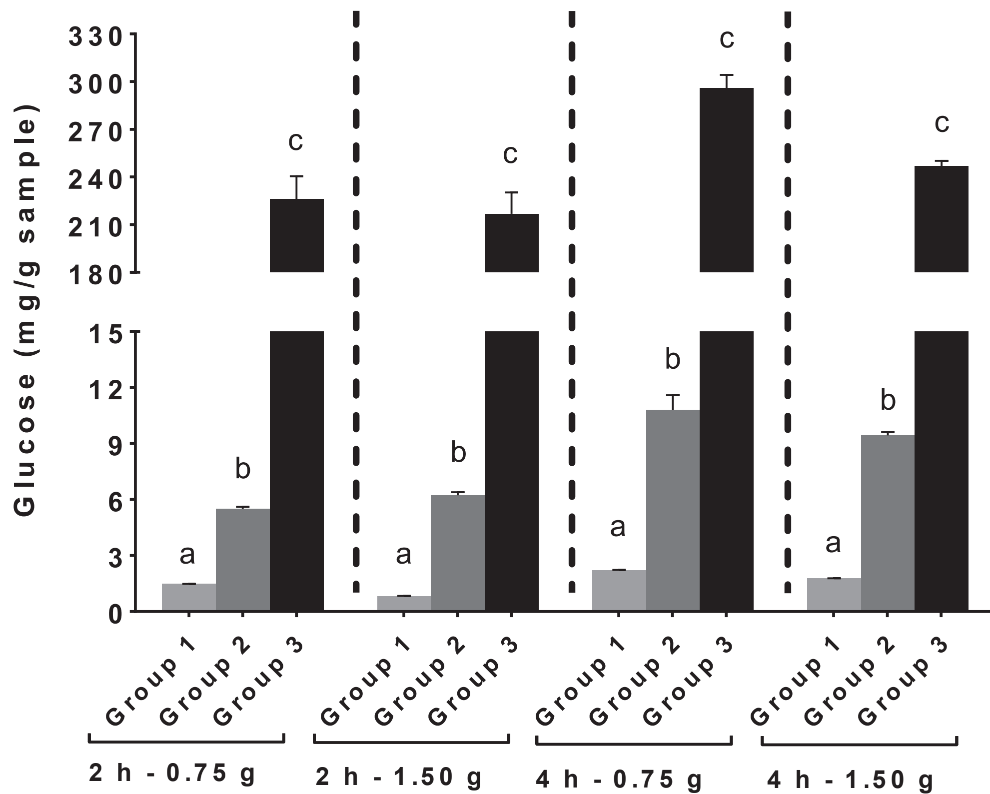

Figure 3.1.C. Comparison of experimental group with their control groups for reference pudding. Group 1: 0.75/1.50 g reference pudding sample + 12.5 mL water, Group 2: 0.75/1.50 g reference pudding sample + 5 mL of pepsin-guar gum solution + 5 mL of sodium acetate + 2.5 mL of water, Group 3: 0.75/1.50 g reference pudding sample + 5 mL of pepsin-guar gum solution + 5 mL of sodium acetate + 2.5 mL of enzyme mixture. Data are presented as the mean value ± SE (n = 3). Results were analyzed by one-way ANOVA ($P<0.05$) Tukey's post hoc test was performed.

Figure 3.1.A., Figure 3.1.B. and Figure 3.1.C. shows that the comparison of experimental groups with their control groups for reference cake, almond paste and pudding samples, respectively. In this digestion efficiency experiments, also different amount of samples and incubation time period were tested to optimize the experimental condition for the cell culture system. When we compare the control group 1 and control group 2, we can observe that the pepsin enzyme and low ph condition significantly increases the output of the glucose for all over the three different samples. This significant difference is due to the glucose that is released by degradation of the protein-carbohydrate linkages by the pepsin enzyme in the cake, almond paste and pudding samples.

But the most important part of this experiment when we use the all of the digestion enzymes and perform factors which are necessary for carbohydrate digestion we measured the very significant amount of glucose from the individual samples. The differences between control group 2 and the experimental group is the result of pancreatin, amyloglucosidase and invertase enzymes in the enzyme mixture. As a conclucion the digestion enzymes work efficiently.

3.2. Determination of Time Point and Portion Size for Test Tube Digestion of Cake, Almond Paste and Pudding

Enzymatic incubation with enzyme mixture (pancreatin, invertase and amyloglucosidase) was carried out for 2 hours and 4 hours for reference cake, almond paste and pudding samples. Also initial sample quantities were tested in two different weights. These two different sample quantities were determined according to the dry matter contents of the products. After the determination of specific product quantities and digestion time points for each producs, test tube carbohydrate digestion protocol was performed.

Experiments with 0.50 g and 0.25 g of reference cake samples, 0.40 g and 0.2 g of reference almond paste samples, 1.50 g and 0.75 g of reference pudding samples were carried out for 2 and 4 hours. Figures 3.2.A., 3.2.B. and 3.2.C. specifically show that the effect of different amount of samples and time points to glucose output.

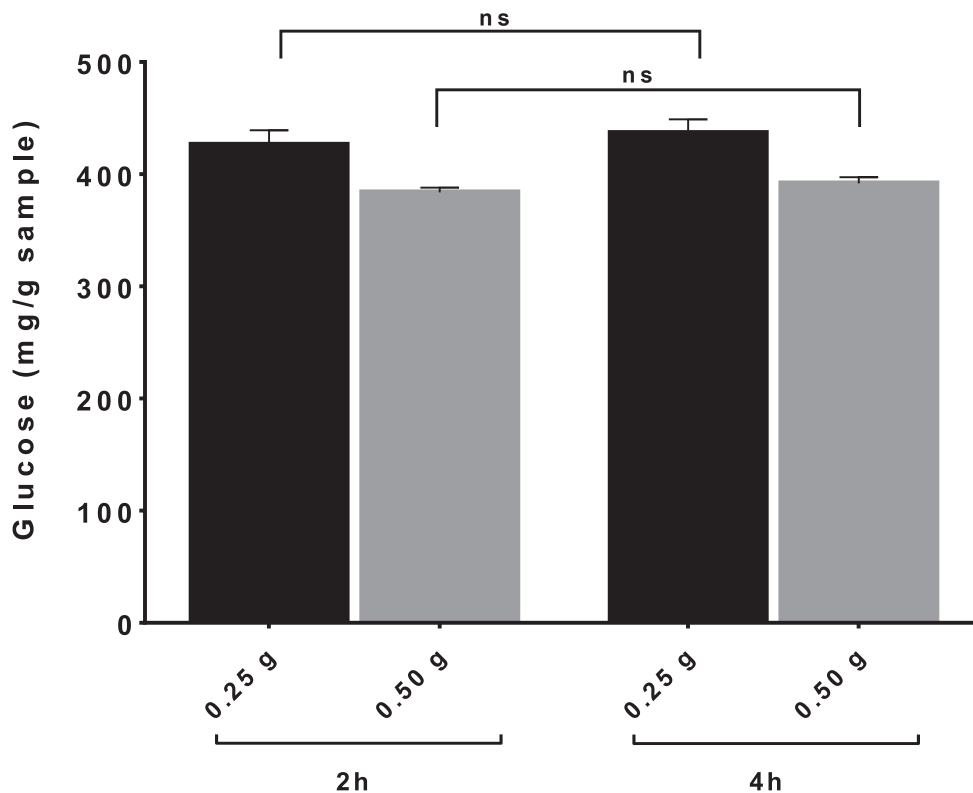

Figure 3.2.A. The differences between 0.25 g – 2 hour, 0.25 g 4 hour, 0.50 g – 2 hour and 0.50 g – 4 hour groups for reference cake samples.

Data are presented as the mean value ± SE (n = 3). Results were analyzed by two-way ANOVA. Tukey's post hoc test was performed (ns: non-significant).

Referring to the statistical results of reference cake, significant differences were not observed for different initial sample weight and time point. In this case, 0.25 g – 2 h conditions were selected. Cake digestions are very common in the literature and it is confirmed that these parameters would be sufficient to degradation of carbohydrates to glucose for reference and Takita cake samples with this selection.

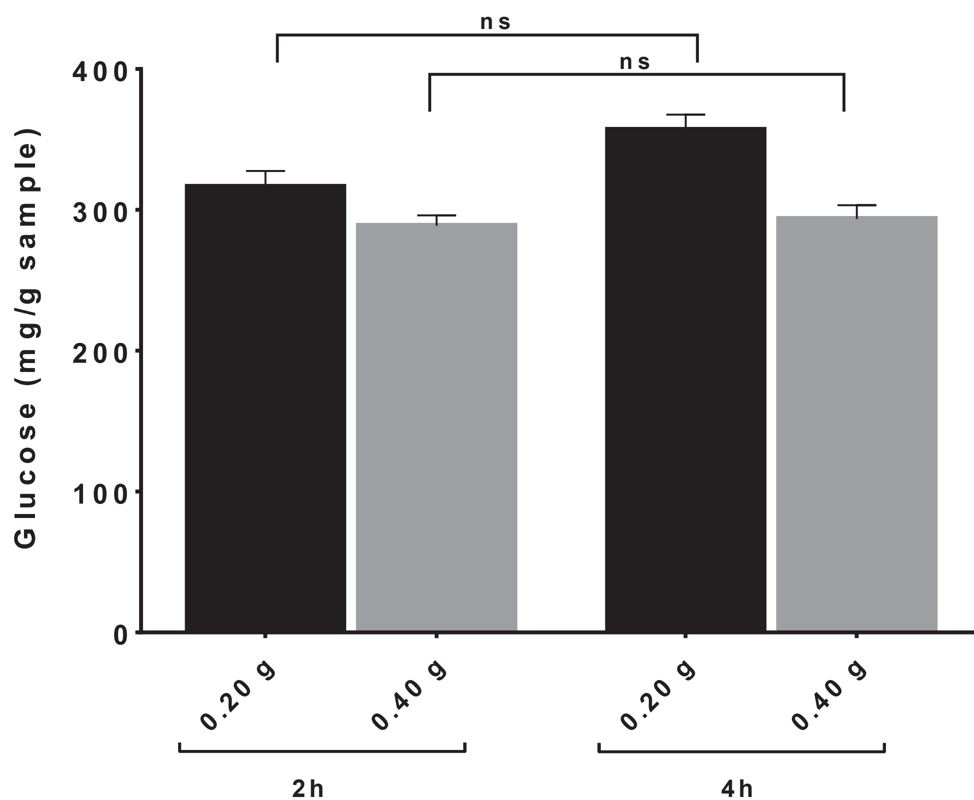

Figure 3.2.B. The differences between 0.20 g – 2 hour, 0.20 g 4 hour, 0.40 g – 2 hour and 0.40 g – 4 hour groups for reference almond paste samples. Data are presented as the mean value ± SE (n = 3). Results were analyzed by two-way ANOVA. Tukey's post hoc test were performed (ns: non-significant).

According to digesiton results of reference almond paste samples, there is no significantly difference was observed between the two different incubation times for almond paste samples. However there is a significant difference between 0.2 g – 4 h and 0.4 g – 4 h almond paste samples (P value < 0.05). Also it is known that Takita almond paste was enriched with dietery fibers to slow down the transition period of glucose to blood. Considering this factor, it would be correct to choose the 0.2 g – 4 h parameter. For the reference pudding, significantly difference was observed between 0.75 g – 2 h and 0.75 g – 4 h conditions and 0.75 g – 4 h were selected for the test tube carbohydrate digestion parameters.

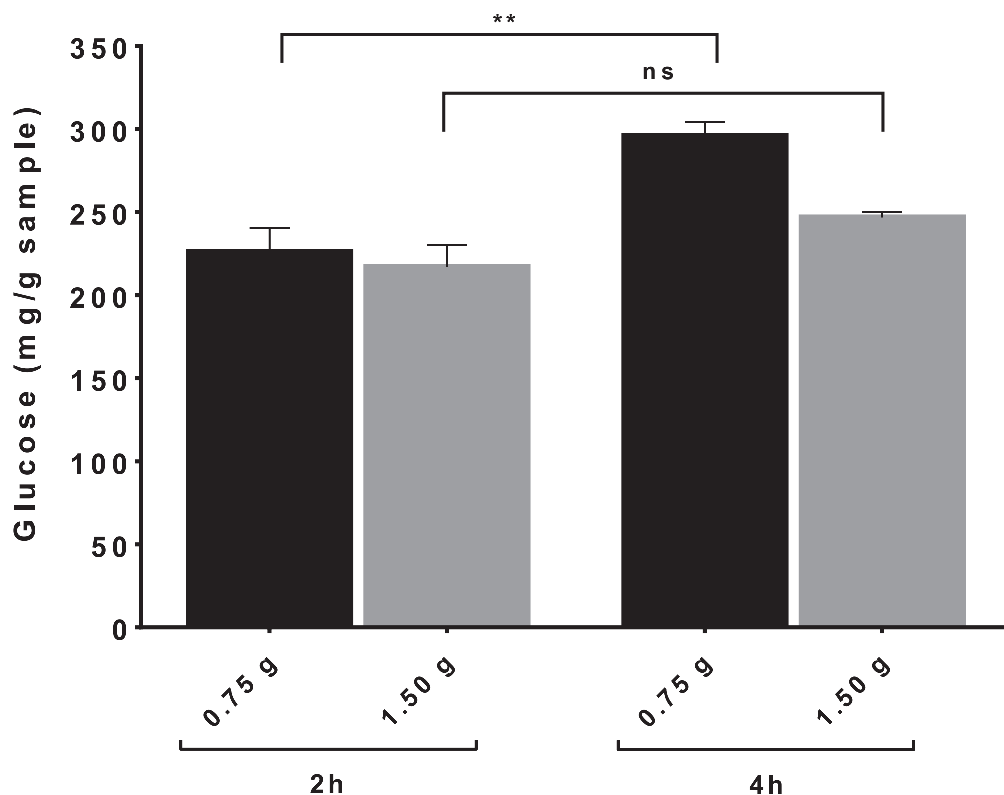

Figure 3.2.C. The differences between 0.75 g – 2 hour, 0.75 g 4 hour, 1.50 g – 2 hour and 1.50 g – 4 hour groups for reference pudding samples. Data are presented as the mean value ± SE (n = 3). Results were analyzed by two-way ANOVA. Tukey's post hoc test were performed (ns: non-significant).

3.3. Test Tube Digestion Results of Cake, Almond Paste and Pudding Samples

Takita and reference samples were digestied in the test tubes. In the literature, most of the times, bioaccessibility of the products is compared to white bread so white bread was digested and used as a control. When the test tube digestion results of white bread were evaluated statistically, it was found that the initial weighing amount and time point should be 0.25 g – 2 h for white bread. So, Takita cake, almond paste and pudding products were compared with both their reference products and white bread.

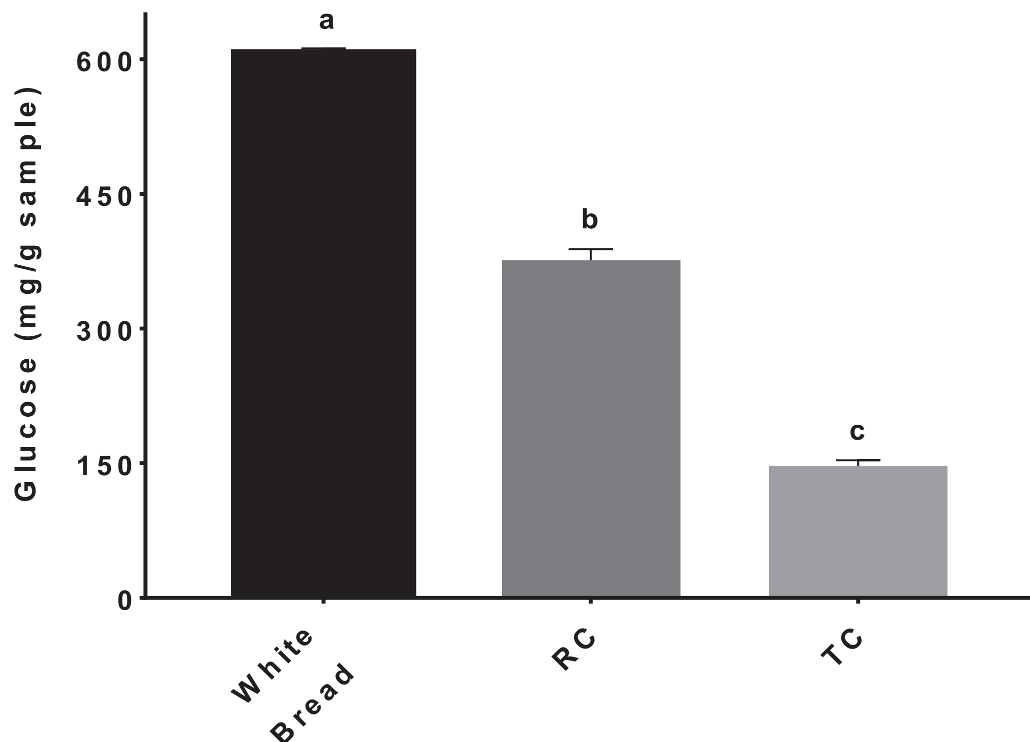

Figure 3.3.A. Test tube digestion results of cake. Data are presented as the mean value ± SE (n = 9). Results were analyzed by one-way ANOVA (*P*<0.05) Tukey's post hoc test was performed. RC= Reference Cake, TC= Takita Cake.

The results show that white bread, reference cake and Takita cake contain 607.3 mg/g sample, 375.9 mg/g sample and 147.3 mg/g sample, respectively. All three samples are significantly differences between each other (P value < 0.0001). Takita cake contains 61% less glucose than the reference and 76% less glucose than the white bread.

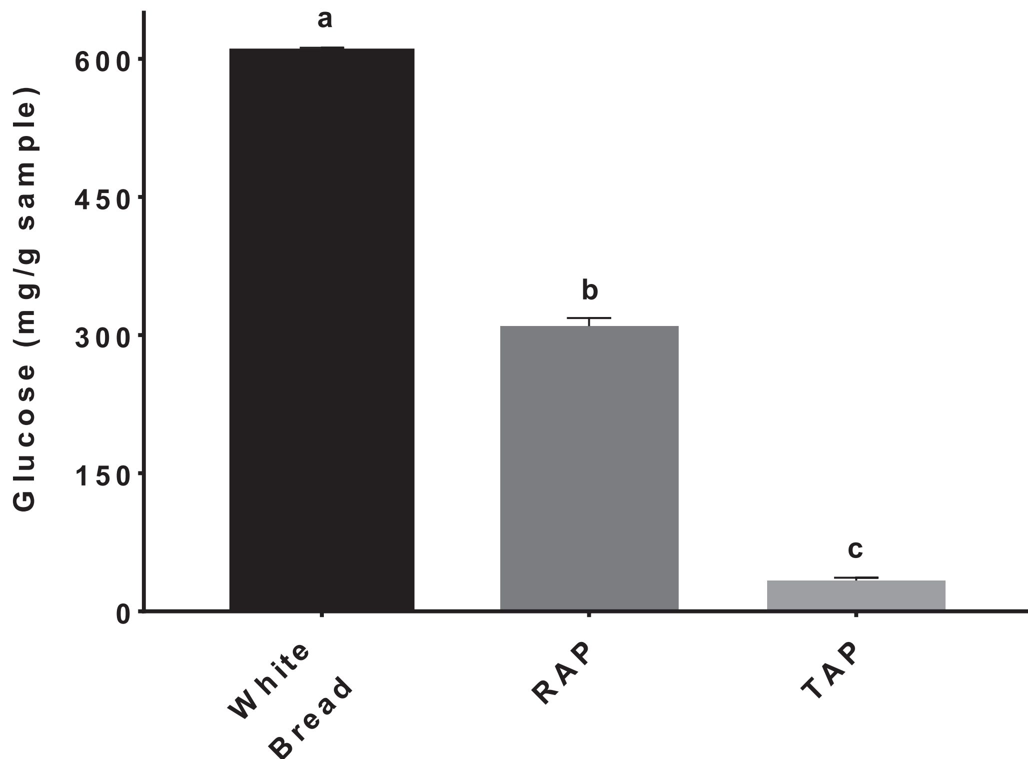

Figure 3.3.B. Test tube digestion results of almond paste. Data are presented as the mean value ± SE (n = 9). Results were analyzed by one-way ANOVA (*P*<0.05) Tukey's post hoc test was performed. RAP= Reference Almond Paste, TAP= Takita Almond Paste.

White bread, reference almond paste and Takita almond paste contain 607.3 mg/g sample, 309.8 mg/g sample and 33.56 mg/g sample, respectively. All three samples are significantly differences between each other (P value < 0.0001). The results show that Takita almond paste contain 89% and 94% less glucose than the reference and white bread, respectively.

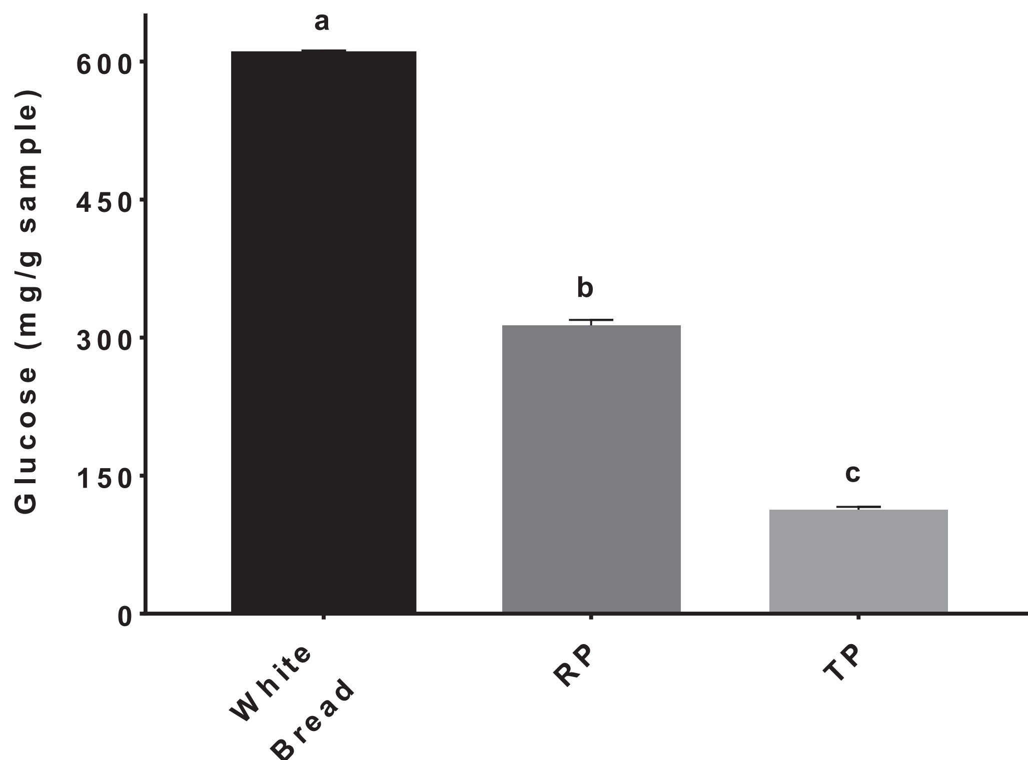

Figure 3.3.C. Test tube digestion results of pudding. Data are presented as the mean value ± SE (n = 9). Results were analyzed by one-way ANOVA (*P*<0.05) Tukey's post hoc test was performed. RP= Reference Pudding, TP= Takita Puding.

As a result of test tube carbohydrate digestion of pudding, white bread, reference pudding and Takita pudding contain 607.3 mg/g sample, 313.24 mg/g sample and 113.10 mg/g sample, respectively. There is a significant difference between white bread, Takita puddig and reference pudding (P value < 0.0001). The results indicate that Takita pudding contain 64% less glucose than the reference and 81% less glucose than the white bread.

3.4. Cellular Glucose Efflux for Cake, Almond Paste and Pudding

Digested Takita and reference cake, almond paste and pudding samples, also glucose as a control were given to the cells from the apical side (n=4, for each group). After 30, 60, 90, and 120. min. samples were taken and glucose levels were measured by using glucose oxidase assay kit (GAGO-20, Sigma Aldrich).

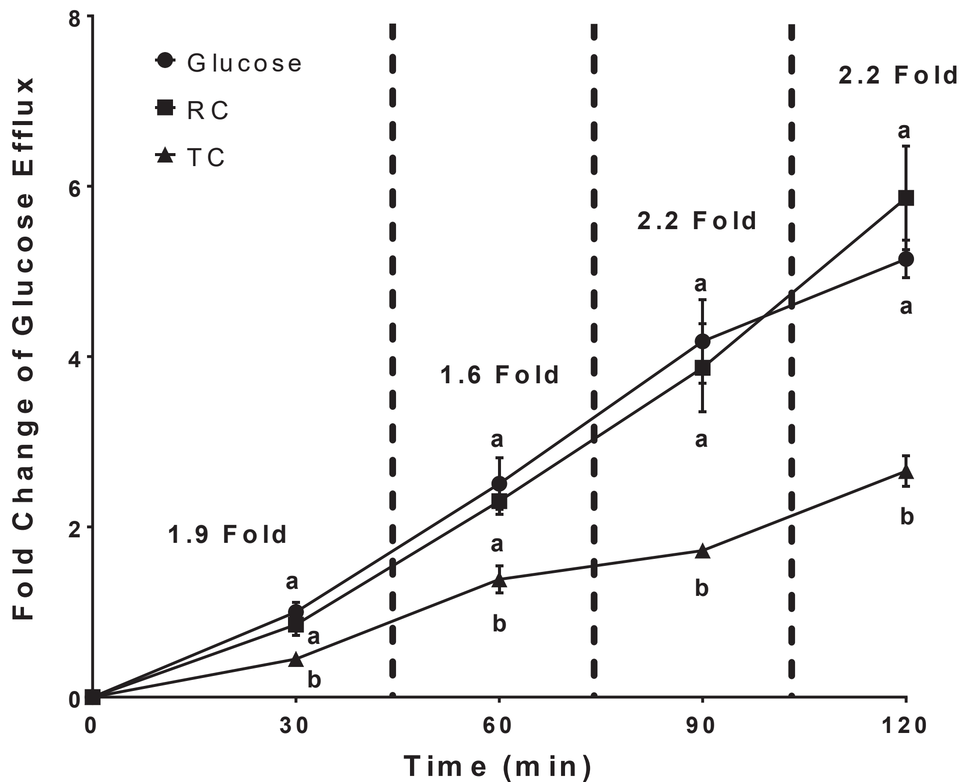

Figure 3.4.A. Measurement of cellular glucose efflux for cake. Data are presented as the mean value ± SE (n = 4). Results were analyzed by one-way ANOVA (*P*<0.05) Tukey's post hoc test was performed. RC: Reference Cake, TC: Takita Cake.

The results at the end of 120 min cell culture study show that newly formulated Takita cake have 2.2 fold changes of glucose efflux from their reference. Cell culture study results of cake are parallel to the results of test tube carbohydrate digestion results, considerably.

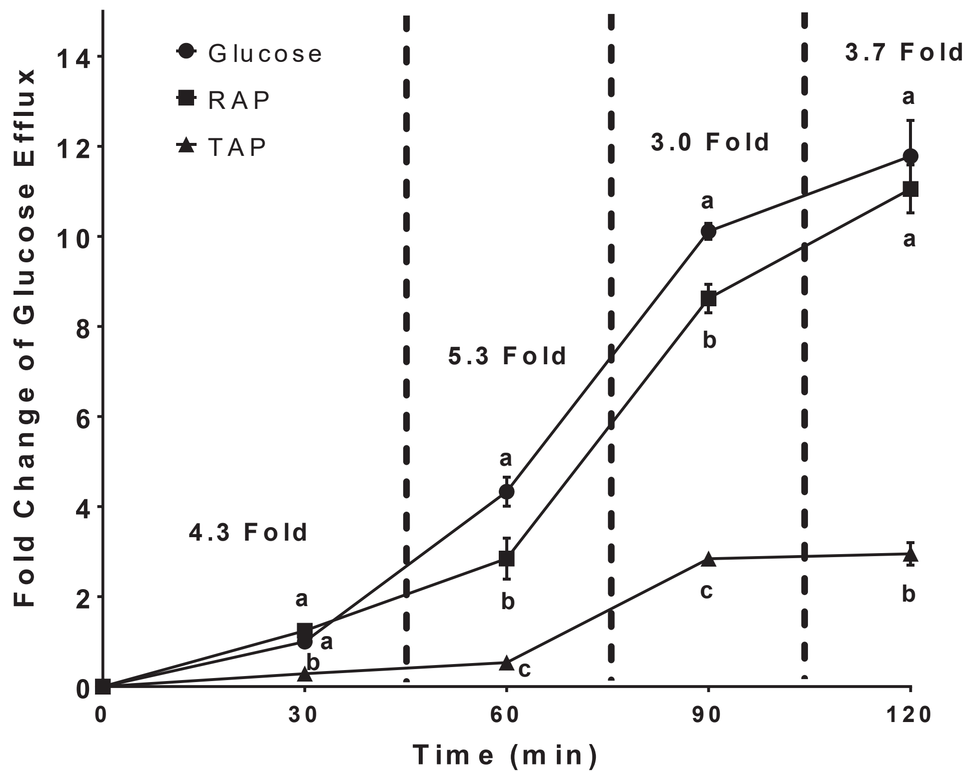

Figure 3.4.B. Measurement of cellular glucose efflux for almond paste. Data are presented as the mean value ± SE (n = 4). Results were analyzed by one-way ANOVA (*P*<0.05) Tukey's post hoc test was performed. RAP: Reference Almond Paste, TAP: Takita Almond Paste.

At the end of 120 min cell culture study, the level of cellular glucose efflux for almond paste was significantly lower for Takita almond paste, which is 3.7 fold. These results also highly compatible with test tube carbohydrate digestion results.

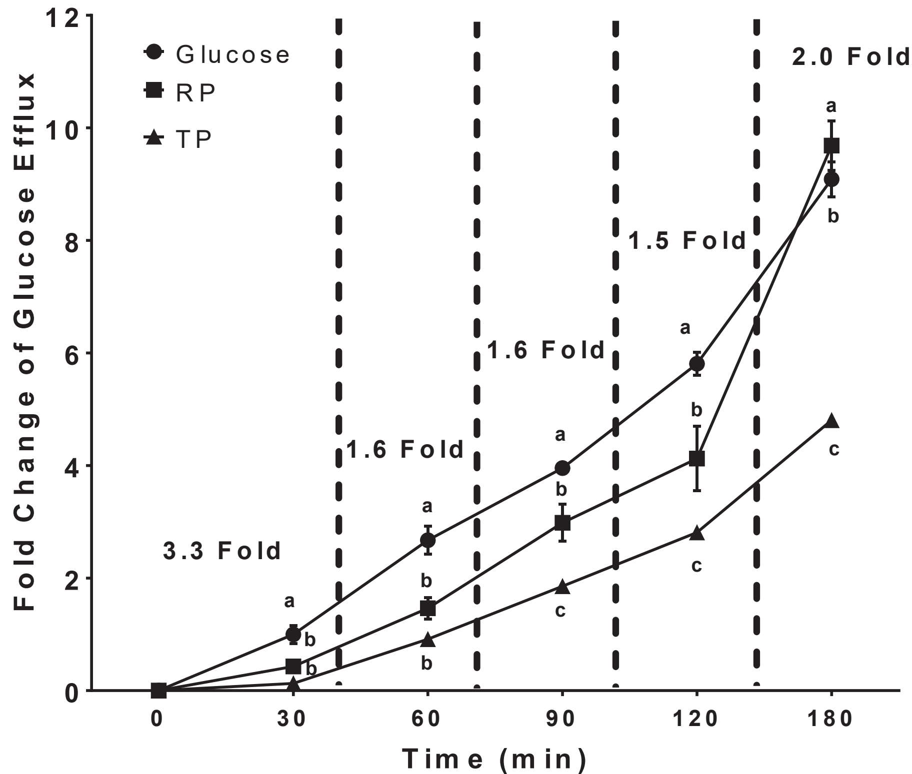

Figure 3.4.C. Measurement of cellular glucose efflux for pudding. Data are presented as the mean value ± SE (n = 4). Results were analyzed by one-way ANOVA ($P<0.05$) Tukey's post hoc test was performed. RP: Reference Pudding, TP: Takita Pudding.

Compared to cake and almond paste products, no difference was observed at 120 min cell culture study on the pudding products. Cell culture studies were performed as 180 min for the pudding products. The results at the end of 180 min cell culture studies indicate that newly formulated Takita pudding have 2.0 fold changes of glucose efflux from their reference. Cell culture study results are more important than the results of digestion in the test tube in terms of blood glucose modeling. Moreover, test tube carbohydrate digestion results and cell culture study results are quite matching for three products. These results prove that newly formulated low calorie and no-added sugar cake, almond paste and pudding products contain significantly less sugar than the their references.

CHAPTER 4

CONCLUSION

Obesity and Type 2 diabetes (T2D) are important metabolic diseases affecting human health. The increased prevalence of obesity and T2D is becoming a major medical challenge globally. As well as genetic factors, due to changing of life style, less physical activity, less sleeping hours, exposing mental stress in business & social life and high consumption of glucose and fat containing foods, food additives, and taste enhancers might lead obesity and T2D in all age groups. Obesity is associated with an increased risk of developing T2D because T2D is characterized by insulin resistance and altered glucose and lipid metabolism. In obese individuals, fat tissue releases factors that play a role in the development of insulin resistance. When the insulin-releasing cells become dysfunctional, these cells cannot control the blood sugar level and insulin resistance develops and studies showed that the most patients with T2D were obese. Glucose is also one of the nutritional factors to develop of obesity and T2D other than fat. At least half of the disaccharides formed by the breakdown of carbohydrates are glucose and glucose is mainly used as added sugar in food products. Moreover, high dietary glucose can be taken by adipose and muscle tissues and stored as fatty acids. Thus glucose behaves as fat in human body. Simple carbohydrate rich products can be replaced by newly formulated low calorie food products in the supermarket. This might be start point to reduce risk of the obesity and T2D. However, it is important how to design these low calorie foods and which approaches that are used to define low calorie foods on market. Thus, methods for testing the diabetic properties of these products have should be standardized. In the literature mostly carbohydrate digestion methods are available in the test tube. Bioaccessibility rate of glucose in test tube is used as diabetic definition. However, it is essential to know how much glucose is also absorbed in human intestine and how insulin secretion is affected by glucose in *in vivo*.

The food companies have a great impact on human health. Because we buy dietary needs from markets in modern world. In reality, food companies pay attention to produce cheap and most desirable foods instead of thinking about human health. Many unhealthy products are easily accessible at the markets with cheaper prices for all age groups and they have risk for development of obesity and T2D. There are serious problems in this legal arrangement to develop new functional foods in Turkey. The *in vitro* and *in vivo* approaches should be standardized to design of functional food by the government.

In this study diabetic features of the newly formulated low calorie and no-added sugar containing cake, almond paste and pudding products were tested in test tubes and in intestinal cell culture models. Many nutrient digestion protocols in the literature have been examined. A number of different tests have been made on chosen protocol to make them unique to the protocol for individual food products in this study. Moreover, we utilized rat enterocyte cell culture model to mimic human intestine system (Olson, Pysher & Bienkowski, 1991; Gulec & Collins 2014). There is no human noncancerous cell model for *in vitro* modeling of human intestine system. Thus, we utilized rat enterocyte cells in current project. Moreover, this cell type was used in different studies to mimic human intestine system. Using of cell culture system in this project provided a chain on current protocols that have been used to design functional foods in literature. Furthermore, human subjects should be involved, as a follow up study for current project because of human studies will provide more realistic data to evaluate diabetic features of functional foods. For instance insulin and HbA1c are important systemic markers to design low glucose containing food on market. We believe that this current study will be good example to develop low glucose containing functional foods for market. Another prominent feature of this project is that study was performed as a collaborative work between university and private food company. This is important to develop scientific interaction between universities and industry. This should lead food scientists and producers to do more interdisciplinary approaches to develop functional foods for human health.

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