Diplomarbeit

Prevalence of trehalose malabsorption and trehalose intolerance in symptomatic patients of the Medical University Graz

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ZUSAMMENFASSUNG

Fragestellung: Blähungen, Bauchschmerzen und Stuhlunregelmäßigkeiten lassen an Intoleranz von Laktose und Fruktose denken. Trehalose, ein Disaccharid, das vor allem in Pilzen und Kleinlebewesen vorkommt, ist seit 2001 in der EU als Lebensmittelzusatzstoff registriert und wird industriell eingesetzt; es kann bei verminderter Aktivität des Enzyms Trehalase zu Beschwerden der Kohlehydratintoleranz führen (1). Zur Prävalenz der Trehalosemalabsorption und Trehaloseintoleranz gibt es kaum Daten - sie beträgt in der Bevölkerung laut Literatur bis zu 8 % (2).
Ziel dieser Studie war es die Prävalenz der Trehalosemalabsorption und Trehaloseintoleranz bei Patienten zu ermitteln die zur Abklärung von Blähungen, Bauchschmerzen und Stuhlunregelmäßigkeiten zur Durchführung eines H_2 Atemtests (Laktose, Fruktose) zugewiesen worden waren.

Methodik: 30 PatientInnen (8 männlich, 22 weiblich, medianes Alter 32 a, min.-max.: 20-72 a) erhielten 50g Trehalose. Bei 8 PatientInnen war zuvor eine Laktosemalabsorption (LM), bei 11 eine Laktoseintoleranz (LIT), bei 6 eine kombinierte Laktosemalabsorption und Intoleranz (LM + LIT) bei 1 eine Fruktosemalabsorption (FM) und bei 3 eine Fruktoseintoleranz (FIT) festgestellt worden. Der endexspiratorische H_2-Gehalt wurde mittels H_2-Atemtest gemessen, sowie Symptome auf einer Skala von 0 bis 5 aufgezeichnet. Pathologisch war ein Anstieg des endexspiratorischen H_2- Gehaltes über 20 ppm (TM), sowie der Symptome um 2 (TIT) über den Basalwert.

Ergebnisse: Bei 9 PatientInnen (30%) konnte durch einen H_2-Anstieg eine Trehalosemalabsorption (TM) diagnostiziert werden. 9 PatientInnen (30%) waren symptomatisch und konnten als trehaloseintolerant (TIT) klassifiziert werden. Zwischen Patientinnen mit und ohne Trehalosemalabsorption oder Trehaloseintoleranz bestand kein signifikanter Unterschied hinsichtlich der Häufigkeit einer zusätzlichen LM und/ oder FM, LIT und/oder FIT, einer Zöliakie oder einer Pilzunverträglichkeit.

Schlussfolgerung: Bei PatientInnen mit Blähungen, Bauchschmerzen und Stuhlunregelmäßigkeiten bei denen an eine Kohlehydratintoleranz gedacht wird, liegt in 30 % eine Trehalosemalabsorption und in 30 % eine Trehaloseintoleranz vor; Es besteht kein Zusammenhang mit Malabsorption oder Intoleranz von Laktose oder Fruktose, oder einer anamnestischen Pilzunverträglichkeit. Aufgrund der hohen Prävalenz der Trehalosemalabsorption und/ oder Trehaloseintoleranz in dieser Studie sollte die Verwendung dieses Zuckers in Lebensmitteln genauer deklariert werden.

ABSTRACT

Introduction: Symptoms as bloating, abdominal pain and diarrhea are often caused by malabsorption of lactose or fructose. Trehalose, a disaccharide occurring in mushrooms, seafood, baker's and brewer's yeast was approved as Novel Food and Food ingredient in the European Union in 2001 and is increasingly used in the food industry. Trehalose is metabolized by the trehalose specific disaccharidase trehalase. Reduced trehalase levels might result in malabsorptive symptoms (1). Only few studies concerning the prevalence of trehalase deficiency and malabsorption or intolerance of trehalose exist. According to literature trehalase deficiency occurs in 8 % of the population (2). To date no studies exist concerning the prevalence of trehalose malabsorption and intolerance in a symptomatic population.

Materials and Methods: A mixture of 50 g trehalose and water was administered to thirty patients with the clinical suspicion of carbohydrate malabsorption (8 males, 22 females, median age 32 years, min.-max.: 20-72 years). Lactose malabsorption (LM) was documented in 8 patients (27 %), lactose intolerance (LIT) in 11 patients (37 %), combined malabsorption and intolerance of lactose (LM +LIT) in 6 patients (20 %), fructose malabsorption (FM) in 1 patient (3 %) and fructose intolerance (FIT) in 3 patients (10 %). There was no patient with combined malabsorption and intolerance of fructose (FM + FIT). End-expiratory breath hydrogen was measured using hydrogen breath test and symptoms were documented on a scale from 0 (no symptoms) to 5 (most severe symptoms). Trehalose malabsorption (TM) was defined by an increase of $H_2 \geq 20$ parts per million (ppm) over basal hydrogen levels, trehalose intolerance (TIT) was defined by an increase in symptoms ≥ 2.

Results: Trehalose malabsorption, defined by an increase in hydrogen ≥ 20 parts per million (ppm) was diagnosed in 9 patients (30 %). Trehalose intolerance, defined by an increase in symptoms ≥ 2 was diagnosed in 9 patients (30%). Referring to lactose- and fructose malabsorption no relation between trehalose malabsorption and either of these conditions could be observed. Equally no relation between trehalose intolerance and intolerance of lactose or fructose could be demonstrated. In addition no correlation

between a history of mushroom intolerance and malabsorption or intolerance of trehalose could be observed.

Discussion: It can be concluded that 30 % of patients with symptoms suggestive of carbohydrate intolerance suffer of trehalose malabsorption and 30 % of patients suffer of trehalose intolerance. No significant correlation between malabsorption and intolerance of trehalose and malabsorption or intolerance of lactose or/and fructose or a history of mushroom intolerance could be observed. In consideration of the high prevalence of trehalose malabsorption and intolerance the addition of trehalose to foods has to be declared more accurate.

ABBREVIATIONS

C	carbon
CH_4	methane
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
e.g.	for example (“exempli gratia”)
ET- kyoto	extracellular-type-trehalose containing kyoto
FIT	fructose intolerance
FM	fructose malabsorption
FODMAPS	fermentable oligosaccharides disaccharides monosaccharides and polyols
g.	gram
GDP- glucose	guanosindiphosphat- glucose
GRAS	generally recognized as safe
H_2	hydrogen
i.v.	intravenous
kb	kilobytes
LIT	lactose intolerance
LM	lactose malabsorption
MCM6	minichromosome maintenance
ml	milliliter
MTSase	Maltooligosyl-trehalose-synthase
MTHase	Maltooligosyl-trehalose-trehalohydrolase
OVX/DW	ovariectomized group treated with distilled water
OVX/TH	ovariectomized group treated with trehalose
ppm	parts per million
SIBO	small intestinal bacterial overgrowth
SNP	single nucleotide polymorphism
TIT	trehalose intolerance
TM	trehalose malabsorption
UDP- glucose	uridindiphosphat- glucose
α_D^2	optical rotation

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1 INTRODUCTION

1.1 TREHALOSE

1.1.1 Structure

Trehalose, also referred to as mycose or mushroom sugar, is a disaccharide that consists of two D-glucose molecules which are linked in an α, α-1,1-glycosidic bond (3) (1). Its systematic name is 1-α-D-glucopyranosyl-1-α-D-glucopyranoside (4). Figure 1 shows the structure of the disaccharide. There exist three isomers of trehalose α, β-trehalose (neotrehalose), β, β-trehalose (isotrehalose) and the most commonly α, α-trehalose (1). It is the only form found in nature and can be synthesized by various forms of plants and animals (3). These three isomers differ in their linkages; α, α- glycosidic linkage for α, α-trehalose, α, β-glycosidic linkage for α, β- trehalose (neotrehalose) and β, β-glycosic linkage for β, β-trehalose (isotrehalose) (3). α,- and β- glycosidic linkages differ from each other in the conformation of the hydroxyl group (OH) at the anomeric C_1atom (5).

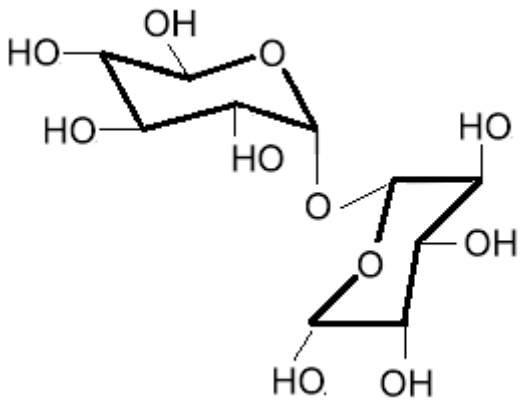

Figure 1: α, α- trehalose

α, β– and β, β-trehalose are rarely found in nature and have not been found in any living organisms; nevertheless these two forms can be produced by chemical synthesis (3).
In nature α, β– trehalose, also referred to as neotrehalose, can be found in koji extract and honey (6). It can be synthesized chemically by the Koenigs Knorr reaction (3).

The two anomeric C_1 atoms are linked with each other in an α- and β- glycosidic linkage.

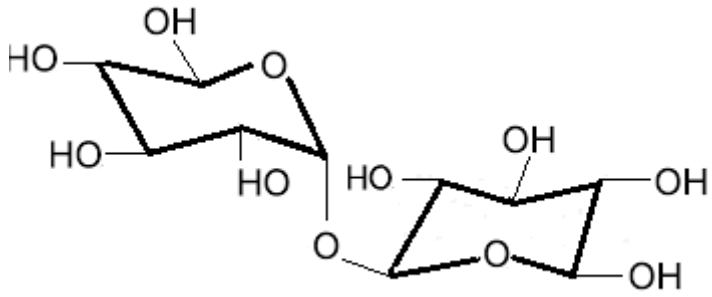

Figure 2: α, β- trehalose

β, β-trehalose, also referred to as isotrehalose, can be naturally found in hydrolysates of starch. The Koenigs Knorr reaction and the dehydration reaction can be used for its chemical synthesis (3). The two anomeric C_1 atoms are linked in a β-glycosidic linkage.

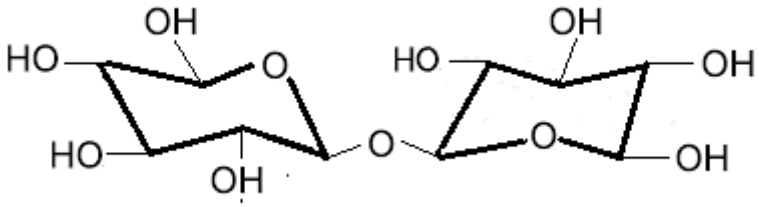

Figure 3: β, β-trehalose

1.1.2 Chemical and physical properties

Trehalose $(C_{12}H_{22}O_{11})$ is a non-reducing disaccharide consisting of two glucose molecules tightly bond in a α-1, 1- linkage (3). The sugar does not have inner hydrogen bonds and cannot be split by α-glucosidase, an enzyme which cleaves most sugars (1) (7). Because of its non-reducing qualities it does not participate in the Maillard reaction, a non-enzymatic browning process between reducing sugars and amino acids, which normally results in the denaturation of proteins (3) (8). Trehalose has a thermostability of over 99 % at temperatures up to 120 °C per 90 minutes and a freezing point at approximately -197 °C for 100 mg/ ml water (7). Due to the tight bonding and its special physico- chemical properties trehalose is a very stable disaccharide resistant to heat, desiccation, freezing, hydrolysis, osmopressure and ranges of pH (1) (9). The sugar has the ability to alternate

between the anhydrous and the dihydrate form. Trehalose dihydrate is the commercial product and forms white orthorhombic crystals (7). The molar mass for the anhydrous form is approximately 342.3 g/mol^{-1} and 378.3 g/mol^{-1} for the dihydrate (7). Trehalose has a low hygroscopic profile. Experiments showed that the dihydrate form remains stable with a water content of 9.54 % up to a humidity of 92 % (1) (10). The sugar is soluble in water and ethanol with a solubility in water of 46,6 g/ 100 g solution at 20 °C (10). Further it has special melting properties and a high optical rotation, α_D^2 = +178 °C. The melting point of trehalose-dihydrate is at about 97 °C, the anhydrous form melts at about 203 °C (1). In addition trehalose has kosmotropic properties (water structuring qualities) giving it the ability to split water clusters and position the water molecules around itself. That way it prevents the formation of ice and has a good cryoprotectant biological activity (7).

1.1.3 Biological properties

Figure 4 shows three possible explanations for the stabilizing effects of trehalose on proteins.

A. Vitrifaction theory

It is assumed that during dehydration trehalose turns into a glassy state in place of crystallizing. The highly viscous trehalose- glass is supposed to encase proteins and lipids and to protect these biomolecules from stresses such as dehydration. Thus it results in a preservation of the cellular integrity. In addition the glass of trehalose has the special property to switch between the anhydrous and dihydrate form. Trehalose has a high glass transition temperature of about 117 °C and builds a non-hygroscopic glass (1) (7) (10) (11).

B. Exclusion theory

When trehalose is added to macromolecules such as proteins, the water molecules, that normally encase biomolecules, are moved away and trehalose is positioned around them. This decreases the hydrated radius and increases the stability of the molecules (7).

C. Water replacement theory

The theory may clarify the role of trehalose in anhydrobiosis, the ability of organisms to survive periods of desiccation and readopt normal activity when rehydrated. It suggests that trehalose substitutes water molecules, which normally stabilize biologic macromolecules. It is supposed that the sugar forms hydrogen bonds with the irregular polar groups of the molecules such as proteins or lipids (1) (7) (10).

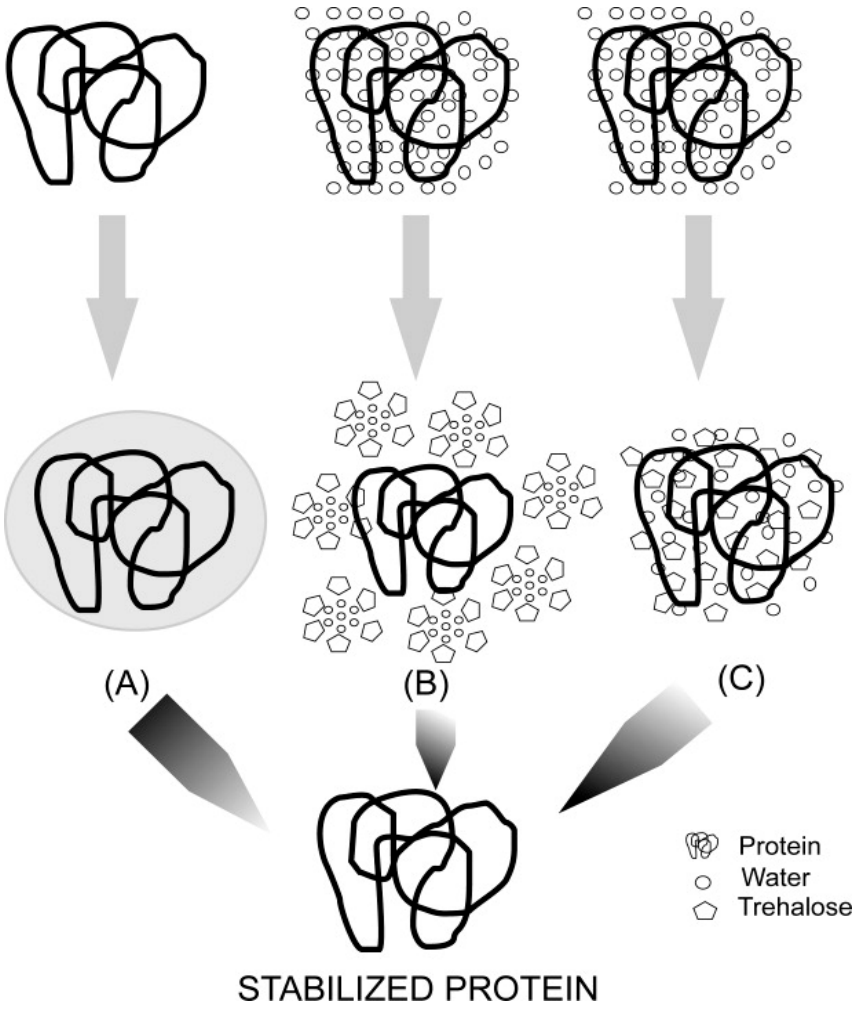

Figure 4: Possible effects of trehalose on stabilization of proteins (7)

1.1.4 History

Trehalose was first discovered in 1832 by H.A. Wiggers in a solution of ergot of rye (1). In 1858 it was found in mushrooms and in trehala manna, a cocoon produced by Syrian weevils (Larinus) on shoots or leaves of echinops sphaerocephalus (1) (12). In figures 5 to 7 the snout beetle larinus, echinops spaerocephalus, also known as glandular globe thistle and the ergot of rye are demonstrated (13). After several unsuccessful attempts, Koch and Koch finally extracted the sugar from an alcoholic extract of baker's yeast in 1925 (14). In the past only a few organisms were identified to contain trehalose (the resurrection plant, trehala manna and mushrooms), but in the early nineties scientists discovered that the sugar was a consistent part of various plants, animals and microorganisms around the world (1).

Figure 5: Larinus (51)

Figure 6: Echinops spaerocephalus (52)

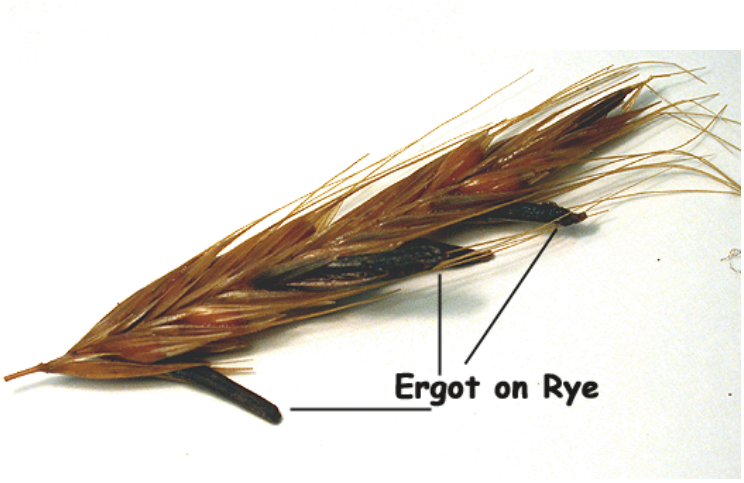

Figure 7: Ergot of rye (53)

1.1.5 Natural occurrence

Trehalose is widely distributed throughout the biological world. It is present in the cytosol of a variety of organisms of the flora and fauna (3). Natural sources that contain the disaccharide include higher and lower plants, fungi, algae, ferns, lichens, yeast, bacteria, insects and invertebrate animals (1). Trehalose does not occur in higher vertebrates, although the trehalose degrading enzyme trehalase is present in these organisms. In insects trehalose is the major sugar and is found in the haemolymph and in the thoracic muscles (3) (1). In specific evolutionary stages of insect development the sugar can make up to 20 % of all carbohydrates (1). It occurs in larvae, pupae, in tardigrades and in the roundworm ascaris lumbricoides, where especially the eggs possess high amounts of the sugar (up to 9 % of the dry weight) (1) (7). In yeast and fungi trehalose can be found in fruiting bodies or vegetative cells (3). The amount of trehalose depends on the age, growth condition and nutritional state of the cells and on its surrounding conditions (3). For instance the yeast Saccharomyces cerevisiae synthesizes high amounts of trehalose during specific stationary growth periods. In general yeast cells accumulate the disaccharide when exposed to noxious conditions such as elevated temperatures or desiccation (11). Many bacteria have the ability to synthesize trehalose as well. Some examples include Eubacteria, like Escherichia coli, Mycobacteria (including Mycobacterium tuberculosis and Mycobacterium smegmatis), Corynebacteria, Sulfdolobus acidocaldarius, Pimelobacter species R48, Arthrobacter species Q36 and Bacillus subtilis (3) (7). Trehalose captures different functions depending on the bacterium. Mostly it is used as a storage form for carbon and energy. In E. coli trehalose seems to be a protectant against osmotic stress. In Mycobacteria it acts as a structural component of the cell wall and is responsible for the survival in humans (7).

In lower plants trehalose is quite common, where it occurs in many types of Selaginella plants, desert plants with the ability to survive desiccation. For instance Selaginella lepidophylla also referred to as ‘rose of Jericho’ accumulates the disaccharide in amounts of about 10 % of its dry weight when exposed to dry conditions. When this spike-moss is rehydrated the trehalose content decreases subsequently to about 1 % of its weight (4). In figures 8 and 9 the ‘rose of Jericho’ is demonstrated. In higher plants trehalose is rare and can only be found in two particular angiosperms, also referred to as resurrection plants. The first of these angiosperms is called myrothamnus flabelifollius and is demonstrated in figure 10. It is a plant found in dry regions in South Africa that contains the sugar in the

leaves in amounts up to 3 % of its dry weight. The second is a grass called sporobolus stapfianus (4). Other plants containing the disaccharide are the ferns Botrychum lunaria and Ophioglossum vulgatum, some species of apiaceae, the sedge Carex brunnescens, and the beech tree Fagus sylvatica (4) In animals the sugar is present in invertebrates such as lobster, crabs and prawns (1).

Figure 8: Rehydrated rose of Jericho

Figure 9: Dehydrated rose of Jericho

Figure 10: Myrothamnus flabellifolius (15)

1.1.5.1 Biosynthesis

The natural production of trehalose may be divided into three pathways.

1. **Enzymatic synthesis by trehalose-6-phosphat-synthase and trehalose-6-phosphate-phosphatase**

The most commonly and widely distributed mechanism of trehalose production in the biological world is catalyzed by the enzyme trehalose-6-phosphate-synthase. This enzyme helps to form the intermediate product trehalose-6-phosphate by the transfer of glucose from UDP- or GDP-glucose to glucose-6-phosphate. The intermediate trehalose-6-phosphate is dephosphorylated by the enzyme trehalose-6-phosphate-phosphatase providing trehalose. The two key enzymes are present in a complex in the cytosol. This synthetic path occurs in yeast, insects and in bacteria e.g. in mycobacterium tuberculosis. In Escherichia coli the enzyme Ots A adopts the function of trehalose-6-synthase and Ots B acts instead of trehalose-6-phosphate-phosphatase (3).

2. **Transformation of maltose**

Some bacteria, such as Pimelobacter species R48 have the ability to produce trehalose from maltose. These bacteria contain a catalytic enzyme named trehalose-synthase that modifies the 1,4-linkage of maltose to a 1,1-bond to form trehalose (3).

3. **Transformation of oligosaccharides and starch**

Another way of trehalose synthesis in bacteria is the modification of starch and various oligosaccharides. This pathway consists of two reactions and two different catalytic enzymes, known as maltooligosyl-trehalose-synthase and maltooligosyl-trehalose-trehalohydrolase, are involved. First the enzyme maltooligoysl-trehalose-synthase transforms maltodextrin into maltooligosyltrehalose. Then the intermediate maltooligosyltrehalose is hydrolyzed by the second enzyme maltooligosyl-trehalose-trehalohydrolase producing trehalose (3).

Some mushrooms contain the catalytic enzyme trehalose-phosphorylase, which is responsible for the hydrolysis of trehalose. It produces glucose-1-phosphat and free glucose by connecting one glucose moiety from trehalose with an inorganic phosphate. In vitro the reaction is reversible. Thus the enzyme catalyzes the reaction of glucose-1-phosphat and glucose backwards to release trehalose and an inorganic phosphate (3).

1.1.6 Occurrence in foods

Trehalose can be found in many modern food sources including yeast products such as bread, pastries and beer, honey and invertebrates such as lobster, crabs and prawns. It is widely distributed in mushrooms (e.g. boletus, ergot) in amounts up to 17 % and can also be found in sunflower seeds (1) (16). Further the disaccharide is present in a variety of spirituous beverages, including the Japanese rice wine mirin and the Spanish wine sherries (1).
In the following table 1 some food sources and their trehalose content are listed.

Foods	Trehalose content
Honey	0,1-1,9 %
Mirin	1,3-2,2 %
Sherries	<10-391 mg/l
brewer's yeast	0,01- 5 %
baker's yeast	15- 20 %
mushrooms	8-17 %
Lobster	2,5 mg/ 100 ml blood
Crab	1,5 mg/100 ml blood
Prawns	0,5 %

Table 1: Trehalose content of different food sources (1)

1.1.7 The biological role of trehalose and its beneficial effects

The biological function of trehalose seems to be species dependent (1). In lower species such as prokaryotes, trehalose seems to be a storage compound and a source of carbon (3) (1). In insects trehalose supplies energy needed to fly (1). Generally the disaccharide is produced in response to stress conditions (3). Trehalose has the capacity to protect organisms and cell components, such as phospholipid-bilayers and proteins from adverse stresses such as dehydration, heat, freezing, osmotic and oxidative stress (3) (1). The sugar is supposed to be a free radical scavenger and prohibits changes of biomolecules such as denaturation or protein aggregation (17). Trehalose confers anhydrobiotic properties, the ability to survive long periods without water and turn to normal activity upon rehydration (3) (1). In some bacteria as in mycobacteria and corynebacteria trehalose is a structural constituent and is integrated into glycolipids of the bacterial cell wall (3) (7). In mycobacterium tuberculosis this glycolipid is called cord factor also referred to as trehalose dimycolate. This specific element results in an impenetrability of the cell wall and in a drug resistance of the bacterium (3). In plants the sugar acts as a stress protectant and a growth regulator (7). In vitro experiments have demonstrated that trehalose is able to protect plant cells against injuries which can occur through heat, drought or freezing (4).

Further trehalose seems to have regulatory properties, connecting trehalose metabolism with the glycolytic path (3).

1.1.8 Manufacture

In the past manufacturing of trehalose has been difficult and costly because of the complex production process (9). Previous methods have included extraction from yeast, chemical synthesis, enzymatic reorganization of maltose and transgenetic processes (1) (9). In 1995 the Hayashibara Company in Okayama, Japan discovered an inexpensive method to produce trehalose from starch using two enzymes produced by the bacterium Arthrobacter species Q 36 (1) (10). Due to this process, using the enzymes Maltooligosyl-trehalose-synthase (MTSase) and Maltooligosyl-trehalose-trehalohydrolase (MTHase), it is possible to produce trehalose for commercial use (9).

1.1.8.1 Production process

At first a slurry-suspension is created by mixing starch and water, further the suspension is scalded and α-amylase is added for liquefaction. Then the slurry is reheated to inactivate the α-amylase. Afterwards the slurry is refrigerated and the enzymes isoamylase, cyclodextrin glucanotransferase, α-amylase and glucoamylase are mixed into it for further processing, such as the debranching of amylopectin and the reduction of the chain length. The resulting intermediates are processed by the two key enzymes maltooligosyl-trehalose-synthase and maltooligosyl-trehalose-trehalohydrolase. MTSase is responsible for the transformation of the α-1.4-bond of amylose into a α-1.1-linkage. The intermediate produced by this intramolecular transglycosilation is called amylosyl trehalose, which is an amylose molecule with a terminal trehalose residue. MTH-ase then hydrolyses the α-1.4-linkage releasing trehalose. The continuing processes include the decolourization of the solution with activated carbon, the removal of carbon, insoluble parts, salts and proteins by filtration and ion-exchange. In addition the solution is evaporated and the resulting crystals are centrifuged (1).

1.1.9 Regulatory Status

In 1991 trehalose was granted novel food status in the UK for use as cryoprotectant for freeze-dried foods at concentrations up to 5 %. In Japan it was approved as food additive in 1995. In Japan the sugar is widely distributed and present in various products (e.g. confectionary products, pastries). In 1998 trehalose received approval as food ingredient in Korea and Taiwan and in 2000 the disaccharide was attested as safe ("generally recognized as safe" (GRAS)) in the US. In Europe trehalose was approved as novel food or food ingredient in 2001 (1). Several in vitro and in vivo animal safety studies concerning the intake of trehalose were performed, but no harmful effects, mutagenicity or genotoxicity have been observed (1).

1.1.10 Applications

Due to its versatile properties trehalose has been used in many applications including the food, cosmetic, pharmaceutical, medical and biotechnological industry (1). In the field of biotechnology it is used for the preservation of bacteria and yeast. In vitro experiments have shown that trehalose has positive effects on the viability of plant and mammalian cells after freezing and thawing when the sugar is added to the inside and outside of the cell. De Carlos et al. demonstrated that the addition of trehalose to biologic samples prepared for cryo-electron microscopy results in a better stability and contrast as compared to samples treated with other sugars (1). The disaccharide has also been used for cryopreservation of bovine embryos and mammalian insulin producing cells. Both experiments have shown a better viability of the cells compared to cells that were not preserved with trehalose (1).

For medical purposes trehalose might be useful in the therapy of osteoporosis. Nishizaki et al. examined the biological role of trehalose on bone resorption in ovariectomized mice. In this study it was demonstrated that a deficiency of estrogen due to ovariectomy leads to an increase in bone turnover, resulting in a decrease of bone weight, calcium and phosphorus contents of the femur and reduced tibial trabeaculae in mice. However the oral administration of 100 mg/kg trehalose to ovariectomized mice for four weeks demonstrated suppression in the decrease of bone weight and in calcium and phosphorus

contents in the femur (18). The oral administration of trehalose to ovariectomized mice also had positive effects on the preservation of tibial trabecuale when compared to mice that were not treated with trehalose. Figure 11 demonstrates tibiae of a healthy control group (control), an ovariectomized group treated with distilled water (OVX/DW) and an ovariectomized group treated with trehalose (OVX/TH). You can see the reduction of the tibial trabecuale in the OVX/DW- group, in contrast the OVX/TH- group shows nearly normal trabecular structure (18).

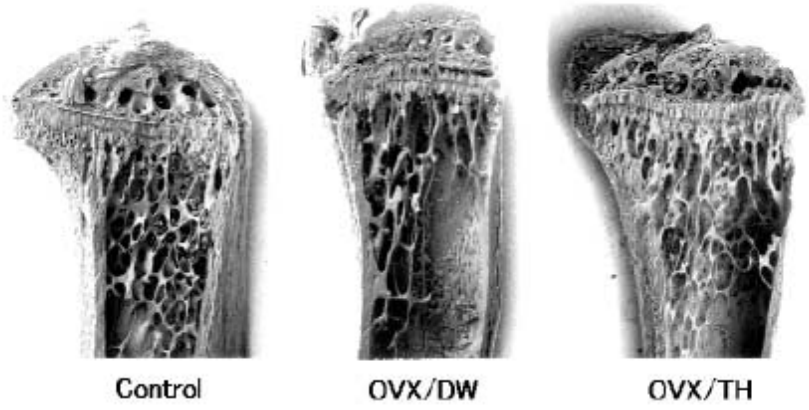

Figure 11: Trabeculae of the tibia of a control-group, the OVX/DW-group and the OVX/TH-group (18)

Trehalose can also be used to cryopreserve fibroblasts, oocytes, sperm cells, platelets, hepatocytes, pancreatic endocrine tissue and to preserve grafts (1) (19). In vivo experiments have shown that the preservation of grafts with trehalose results in a more viable tissue and reduced transplant rejections. In Japan scientists of the University of Kyoto developed a new graft preservation solution with trehalose that was effectively used for lung transplantations. The trehalose containing organ preservation solution, called extracellular-type-trehalose-containing Kyoto (ET-Kyoto), was compared with the two other solutions, which are usually used for the preservation of grafts. In comparison to the solutions without trehalose ET-Kyoto demonstrated equal and better effects on preservation of grafts. Equally trehalose containing solutions demonstrated an improvement in the stability of pulmonal tissue after transplantation which might be due to the sugar's ability to stabilize cell membranes (19).

Trehalose is under investigation for ophthalmologic use because it protects mammalian eyes from oxidative injury and desiccation. At present the disaccharide is used to treat the

dry eye syndrome, as data suggest that it has positive impacts on the desiccation tolerance of mammalian corneal cells (20). In Austria the drug Thealoz Duo® containing trehalose is registered for this use (21).

In addition trehalose might be useful for the protection of neurodegeneration. Animal studies have shown that trehalose has positive effects on neurodegenerative diseases like Huntington's, Alzheimer's and Parkinson's disease by stabilizing proteins, reducing protein aggregations and inducing autophagy (22). However these positive effects could not be observed in vivo, because trehalose is already hydrolyzed into two glucose moieties in the small intestine (19).

In the pharmaceutical industry the disaccharide is used to stabilize drugs such as the monoclonal antibodies Ranibizumab ($Lucentis^{®}$), Bavacizumab ($Avastin^{®}$) and Trastuzumab ($Herceptin^{®}$) (20).

Trehalose acts as stabilizing agent for many other drugs as well. These pharmaceuticals are listed in table 2.

product name	**agent(s)**
Pentiro®	Levodopa, Carbidopa, Entacapon
Sastravi®	Levodopa, Carbidopa, Entacapon
Adcetris®	Brentuximab Vedotin
Avastin®	Bavicizumab
Gazyvaro®	Obinutuzumab
Cosentyx®	Secukinumab
Lucentis®	Ranibizumab
Blincyto®	Blinatumomab
Herceptin®	Trastuzumab
Mabthera®	Rituximab
Advate®	Octocog-Alpha (human factor VIII)
Optivate®	Human Factor VIII, von Willebrand Factor
Epercan®	Albiglutid
Raplixa®	Fibrinogen and Thrombin

Table 2: Pharmaceuticals with trehalose

1.1.11 Presence in the food industry

Since approval as food additive and novel food in Europe in 2001, trehalose has been increasingly used in the food industry (1). Due to its beneficial properties (e.g. expansion of the shelf life of products, improvement of the food consistency, preservation of flavors and taste, cryoprotective properties, resistance to heat, drought, cold, pH-ranges and oxidative stress, less caries producing) it has been incorporated into many products (10) (23) (24). Trehalose containing products include pastries, sweets (e.g. Maoam®), chocolate, breakfast cereals, frozen products (cake coverings, fruit sorbets, ice cream) refrigerated goods, dried and freeze-dried products, juices, meat products, dairy products and Surimi (10) (24) (25) . Trehalose is a component of FortiCare®, a nutrition supplement for cachectic oncologic patients (26).

Figure 12: Maoam ® (27)

Figure 13: FortiCare® (26)

Positive aspects of trehalose containing products

High processing stability

The resistance of trehalose against heat, drought, cold temperatures and ranges of pH improves the quality and the storage life of frozen and dried food (3) (23). Previous studies have shown that the addition of trehalose prior to air drying has protective and stabilizing effects on products and results in normal consistence, color and taste after rehydration (19).

Sweetening power

The disaccharide is often used as an alternative sweetening agent. In comparison to sucrose trehalose is less sweet (45 % of the sweetness of sucrose) but has a longer persistence in sweetness (19) (28). Trehalose has the same caloric value as sucrose (400 kcal per 100 gramm) (29). The sugar is less caries producing and has a reduced insulinemic response compared to sucrose (28) (10).

Preservation of crispness

Trehalose- dihydrate has a low hygroscopic profile (low ability to bind moisture). In experiments the content of moisture of trehalose- dihydrate remained stable up to a humidity of 92% (19). When added to cake coverings or crackers the sugar appears to build a moisture barrier resulting in more crispy products (10).

Preservation of flavors and taste because of protein stabilizing effects

The adding of trehalose before air drying protects proteins from denaturation and results in the preservation of flavors and taste (10). A former experiment showed that the addition of trehalose to mixed fresh eggs before drying results in odorless powder. Upon rehydration the eggs turned back to normal with the same odor and taste and were hardly distinguishable from fresh eggs (30). Furthermore the addition of trehalose increases the taste of a specific product (e.g. increases the taste of salt) (10).

Cryoprotective effects

Trehalose is used as a cryoprotectant for baker's yeast and Surimi (10). The disaccharide is also used for products undergoing phase transitions, such as frozen pastries, frozen desserts, dried fruits and vegetables, cake coverings and for spray-dried products (10).

Retardation of the retrogradation of starch

In the bakery industry trehalose has been used as an adjuvant, because it retards the retrogradation of starch more effectively than other disaccharides. This specific property results in a longer shelf life and improved consistency of pastries. For this reason trehalose has been incorporated into many other starch based products as pastries (10) (31). The disaccharide improves the dough raise, the fermentative ability during the production process and inhibits browning reactions (3) (32).

In addition trehalose has protein stabilizing and moisture regulating effects in meat and contributes to the preservation of juiciness (10) (24).

The advantageous effects of trehalose are shown in figure 14– 16.

Figure 14 shows the comparison of bread rolls with sucrose (left one) and a mixture of sucrose and trehalose (right one). The right bread roll, in which forty percent of sucrose was replaced by trehalose, shows a more homogenous pattern (24).

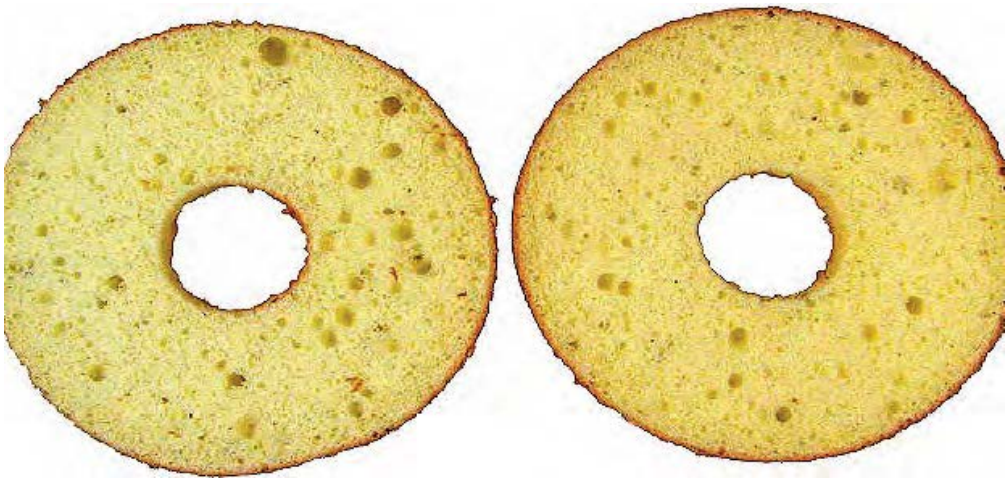

Figure 14: Bread rolls with sucrose (left one) and a mixture of sucrose and trehalose (right one) (24)

Figure 15 demonstrates the comparison of chocolate cakes with sucrose (left one) and a mixture of sucrose and trehalose (right one). The right chocolate cake, in which thirty percent of sucrose was replaced by trehalose, shows increased volume despite reduced weight (385 g) compared to the sucrose cake (407 g) (24).

Figure 15: Comparison of chocolate cakes with sucrose (left one) and a sucrose-trehalose-mixture (right one) (24)

Figure 16 compares freeze- dried banana purée, in which different sugars were added as sweetening agents. The banana mouse which was sweetened with trehalose showed a reduced browning reaction, did not agglutinate and remained powdery even after the drying process. This is because of the low hygroscopic profile of trehalose. On the contrary the banana purées with glucose or sucrose showed a tough consistency after the same drying process (25).

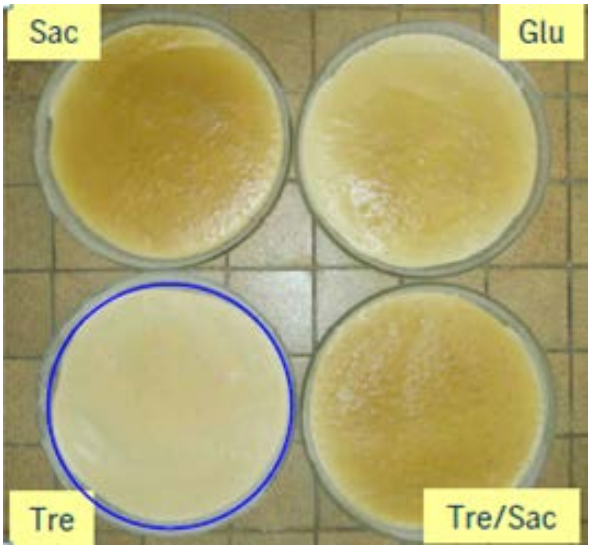

Figure 16: Freeze-dried banana purée with different sugars added as sweetening agents (25)

1.1.12 Digestion of trehalose by humans

When trehalose is ingested it is hydrolyzed by the disaccharidase trehalase in the brush border of the small intestine (23). Trehalase is a trehalose-specific β-galactosidase with catalytic properties and is responsible for the splitting of trehalose into two glucose moieties making them ready for absorption (23). About 80 % of the D- glucose molecules require carrier molecules to traverse the cell membrane actively, the remaining 20 % are absorbed through passive diffusion (1). In humans trehalase is tightly bound to the external surface of microvilli of enterocytes with highest concentrations measured in the proximal and mid small intestine (1). In addition the enzyme is found in the proximal tubules of the kidneys, in the renal cortex, in the urine, bile, liver, in the lymphocytes and in the human serum (1) (33). The biological role of trehalase in these organs has not been established (1).

It has been reported that plasma trehalase is elevated in diabetics compared to healthy individuals and decreased in rheumatoid arthritis (33). Because of these influences, the measurement of plasma trehalase activity cannot be utilized for the determination of the activity of trehalase in the small intestine (33).

Trehalase was first discovered in the mould fungus Aspergillus niger in 1893 and in the yeast Saccharomyces cerevisiae in 1895 (1). Since then the disaccharidase has been found in many organisms including humans. The development of intestinal trehalase occurs approximately 10-14 weeks after conception and achieves adult levels at birth (1).

In yeast, as in Saccharomyces cerevisiae, two types of trehalase are present. The first, neutral trehalase, is present in the cytosol and has a pH optimum of 7 (3). It is activated by protein-phosphorylation and inhibited by Validamycin A (4). The second trehalase, acid trehalase, has a pH optimum of 4.5 and can be found in the extracellular plasma and in the vacuoles (3) (4). Trehalase activity is also present in many organisms of the plant kingdom, although most of them lack the capacity to produce trehalose (4).

1.2 DIAGNOSTIC EVALUATION OF CARBOHYDRATE MALABSORPTION

1.2.1 H2-Breath Test

Hydrogen breath test is a non-invasive diagnostic technique to detect functional gastrointestinal disorders such as small intestinal bacterial overgrowth (SIBO) and malabsorption of different sugars (e.g. lactose, fructose, glucose or trehalose). Because of its non-invasiveness this particular method has become clinical standard to detect these conditions (34). The principle of this test is to measure end-expiratory H_2- concentrations after the oral administration of fermentable carbohydrates utilizing an electrochemical sensor (35) (36). Due to malabsorptive diseases (e.g. coeliac disease, exocrine pancreatic insufficiency) or disaccharidase deficiency the ingested sugar cannot be absorbed in the small intestine and passes into the colon, where bacterial fermentation takes place (37). In the colon malabsorbed sugars are metabolized by bacteria, predominantly gram negative and anaerobic bacteria, into gases (hydrogen, methane, carbon dioxide) and organic acids (short chain fatty acids and lactate) (34) (37). Hydrogen is absorbed through the colonic mucosa into the blood stream, transported to the lungs and exhaled (35).
Figure 17 represents the functional principle of the hydrogen breath test.

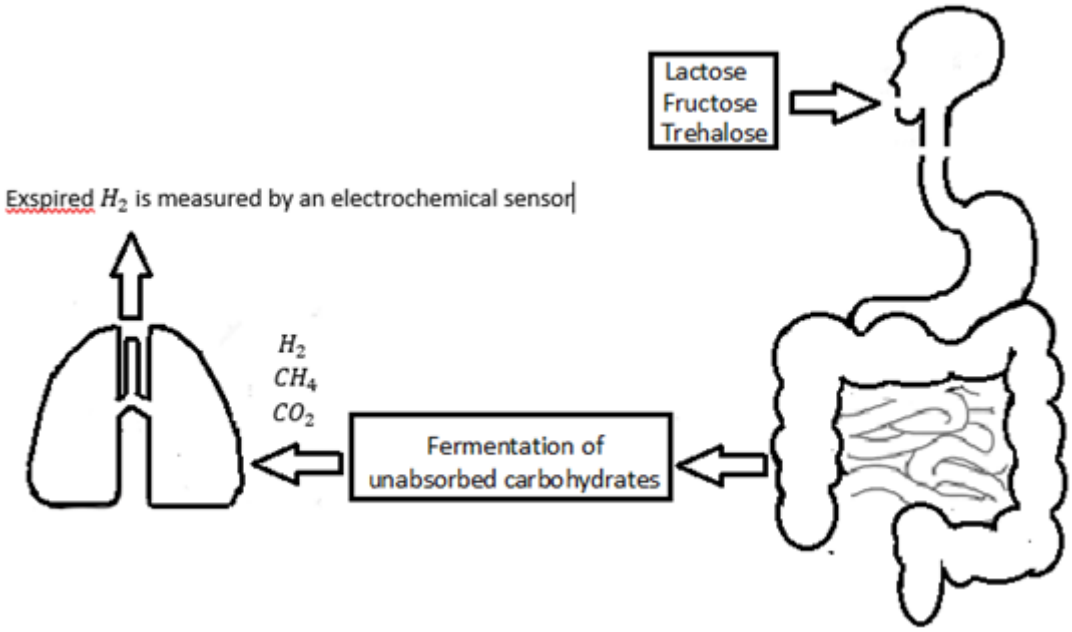

Figure 17: Functional principle of H_2 breath test -modified after (38)

Applications

As mentioned above hydrogen breath tests are used to diagnose carbohydrate malabsorption or small intestinal bacterial overgrowth and to determine oroceacal transit time (39). Simultaneous assessment of symptoms allows diagnosis of carbohydrate intolerance (38).

Below the different substrates for the diagnosis of the above mentioned conditions are listed

- Carbohydrate malabsorption: H_2-breath test with lactose (50 g lactose), fructose (25- 30 g fructose), sucrose, trehalose (50 g trehalose) (University hospital Graz)
- Small bacterial overgrowth: H_2-breath test with 33 g glucose (University hospital Graz)
- Determination of the orocaecal transit time: H_2-breath test with lactulose or inulin (39) (40)

Limitations

- **No standardization**

There is no international standardization respective to the dose of the administered sugar, the frequency of measurements, the period over which H_2 should be measured and the interpretation of the test (34) (35) (38).

- **False negative results**

False negative test outcomes can be due to delayed gastric emptying, the intake of antibiotics or hydrogen nonexcretion (35) (38). For this reason it has to be kept in mind that about 15-30 % of people have colonic methane producing bacterial flora, among them Methanobrevibacter smithii, that metabolizes hydrogen into methane (35). These subjects, also referred to as hydrogen nonexcretors, may have a negative hydrogen breath test in spite of having carbohydrate malabsorption (38). The rate of hydrogen nonexcretion in the area around Graz is 18 % (41). False negative test results can also be generated by physical training before the test which may cause hyperventilation (35).

- **False positive test results**

False positive test results may occur in the presence of small intestinal bacterial overgrowth (SIBO), rapid small bowel transit, as a result of smoking or non-adherence to fasting before the test (35) (38).

Patient preparation

Hydrogen breath test is performed after fasting for at least twelve hours (34). One day prior to the test patients should avoid eating slowly absorbable foods such as bread, potatoes, beans and fibers because these might result in high basal H_2- levels (35). Two hours before and during the test smoking and exercise should be avoided (42). Medications like antibiotics, pro- and anti-motility drugs have to be documented (35). Antibiotics have to be discontinued four weeks and poorly absorbable lactulose three days prior to the test (34). In addition colonoscopies should be avoided four weeks before the test, in order to allow reconstitution of colonic bacterial flora (34). Some authors suggest that patients should brush teeth prior to the test in order to avoid an early hydrogen peak caused by bacterial fermentation of the test sugar by the oral bacterial flora (35).

Procedure:

Initially basal breath hydrogen is measured to verify the fasting state. This basal breath value has to be below 10 ppm (parts per million) in order to use the test. A fixed amount of carbohydrate is administered orally (50 g lactose dissolved in 250 ml water for the evaluation of lactose malabsorption, 25-30 g fructose dissolved in 250 ml water to measure incomplete fructose absorption or 50 g trehalose dissolved in 200 ml water to evaluate trehalose malabsorption) (34). Thereafter breath samples are collected every 15- 30 minutes for 2-4 hours and analysed using a H_2 measuring instrument (41).
Samples may also be analysed for methane if a specific instrument for this is available. However, collection of breath samples for methane measurement is more complex, requiring a large duration of a steady expiratory air flow, which makes it less applicable in children and elderly patients. In addition, the instrument for combined methane and hydrogen measurement is considerably more expensive than the analyzer.
A positive H_2 breath test is defined by a rise of at least 20 ppm over basal hydrogen concentration (35). A hydrogen peak indicating carbohydrate malabsorption usually occurs within 2- 4 hours (38). In contrast an early peak, occurring before 90 minutes after ingestion of the test sugar, is suggestive of rapid orocaecal transit, small intestinal bacterial

overgrowth or sugar fermentation by the oral bacterial flora. Positive symptom assessment allows the diagnosis of carbohydrate intolerance (38).

1.2.2 Other tests used to indicate defects in mechanisms of carbohydrate malabsorption

1.2.2.1 Mucosal biopsy of the small intestine for the detection of enzyme deficiencies

Endoscopic biopsy specimen are taken from the second part of the duodenum or the proximal part of the jejunum and are analyzed for disaccharidase activity (e.g. activity of lactase, maltase, sucrase, trehalase) by the Method of Dahlquist (23) (43). Additionally protein content can be measured by the technique of Lowry (23). Despite high cost, this method is referred to as the gold standard for the diagnosis of lactase deficiency. Positive aspects of this diagnostic technique are the concurrent possibility to exclude secondary causes of sugar malabsorption like coeliac disease. In contrast negative aspects are the invasiveness of the test, the lack of symptom assessment and the possibility to get false negative test results because of inconsistent enzyme expression (43).

1.2.2.2 Genetic testing in lactose intolerance

Primary lactase deficiency (adult-type hypolactasia) is an autosomal recessive inherited trait mostly concerning Asians, Afro- Americans, Latin Americans, Native Americans and Southern and Eastern European subjects such as Italians and Greeks (43). Affected subjects show normal lactase levels at time of birth, but have a decline in lactase activity during infancy which starts soon after weaning (37) (44). Hypolactasia is associated with two polymorphisms, LCT-13910 C/T and LCT-22018. These single nucleotide polymorphisms (SNP) are located in non-coding regions of the MCM6 gene (minichromosome maintenance deficient 6) of chromosome 2 (2q 21). SNP LCT-13910 T/C is located 14 kb upstream of the LCT gene within intron 13 of the MCM6 gene, LCT-22018 is located within intron 9 (45). Since identification of the DNA variant LCT 13910

CC by Enattah et al. in 2002 genetic testing is used for the evaluation of lactase non-persistence (45).
In genetic testing the polymorphism 13910 T/C is analysed for lactase persistence (TC or TT genotype) and lactase non-persistence (CC-genotype). This method can be exclusively used in white Caucasians, because in Africa, Saudi Arabia and Asia other polymorphisms are associated with lactase non-persistence. Unfortunately no secondary causes of lactose intolerance can be excluded and no symptoms can be assessed during the test (43).

1.2.2.3 Fecal carbohydrate analysis

Stool samples are anyalysed for short chain fatty acids and carbohydrates using a gas-phase chromatograph (37). Further pH is measured; a pH lower than 5,5 is indicative for carbohydrate malabsorption (46). Titration is used for the measurement for total organic acids. Additionally the content and weight of fecal carbohydrates are determined by the anthrone method and the osmotic activity of malabsorbed carbohydrates can be determined by the reducing sugar method (37).

2 MATERIALS AND METHODS

Objective

Gastrointestinal symptoms such as abdominal pain, bloating, distension and diarrhea are a frequent clinical condition. These complaints can be due to malabsorption of lactose or incomplete absorption of fructose or due to malabsorptive disease such as coeliac disease or exocrine pancreatic insufficiency (37). Another frequent cause of these symptoms is the irritable bowel syndrome (38). Nevertheless in some patients symptoms cannot be explained by these conditions. The disaccharide trehalose, mainly present in mushrooms, seafood, baker's and brewer's yeast was approved as novel food and food ingredient in the European Union in 2001 and is increasingly used by the food industry (1). For digestion trehalose requires the small intestinal brush border enzyme trehalase (1). If trehalase activity is low, trehalose cannot be absorbed by the intestinal mucosa and may lead to symptoms of carbohydrate intolerance (33). The prevalence of trehalase deficiency varies around the world, with the highest prevalence in Greenland. In this study Gudmand-Høyer et all analyzed small intestinal biopsy samples of 97 Greenlanders for trehalase activity and demonstrated an incidence of trehalase deficiency of 8 % (2). Less is known about the prevalence of trehalose malabsorption and trehalose intolerance and nothing at all has until know been reported on the prevalence of trehalose intolerance in a population presenting with symptoms suggestive of carbohydrate intolerance (43).

Aims

The aim of this study was to establish the prevalence of trehalose malabsorption and trehalose intolerance in Austrian patients with symptoms suggestive of carbohydrate malabsorption who were referred for a hydrogen breath test, to evaluate the clinical suspicion of lactose or fructose intolerance.

Study design

This study was approved by the ethics committee of the Medical University of Graz with the approval number: EK 26-162 ex 13/14. It is a controlled single center study that included 30 consecutive patients.

Hypothesis

In this prospective study the following hypotheses were studied:

1) Gastrointestinal symptoms such as bloating, abdominal pain, distension and diarrhea may be due to trehalose intolerance caused by malabsorption of trehalose.
2) Trehalose malabsorption leads to symptoms of trehalose intolerance.
3) There is a correlation between trehalose malabsorption (TM) and malabsorption of lactose (LM) or fructose (FM) and a correlation between trehalose intolerance (TIT) and intolerance of lactose (LIT) or fructose (FIT)
4) There is a correlation between a history of mushroom intolerance and trehalose intolerance.

Recording of clinical data

Informed consent and clinical data were obtained from all study participants during their stay at the outpatient clinic of the Division of Gastroenterology/ Hepatology and were documented.

Below the recorded clinical data are listed:

- Demographic Data: Age, Sex
- Abdominal symptoms: crampy abdominal pain, intestinal gas expulsion, abdominal distension, diarrhea
- Concomitant medication: e.g. antibiotics, lactulose
- Concomitant disease: known gastrointestinal disease as coeliac disease
- Mushroom intolerance
- Diagnosed lactose/ fructose malabsorption and diagnosed lactose/ fructose intolerance

In figures 18 and 19 documentation sheets, for a patient with TM and a patient without TM, are demonstrated.

Trehalose H_2-Atemtest: 50g Trehalose in 200ml Wasser gelöst

Durchfall: Ja/Nein

Blähungen: Ja/Nein

Winde: Ja/Nein

Bauchkrämpfe: Ja/Nein

Milchunverträglichkeit: Ja/Nein

Unverträglichkeit von Pilzen: Ja/Nein

Fruchtzuckerunverträglichkeit: Ja/Nein

Sehr geehrte(r) Patient(in):

Bitte umkreisen Sie bei Durchfall **JA**/Nein wenn Sie zu diesem Zeitpunkt Durchfall haben, und Ja/**NEIN** wenn Sie keinen Durchfall haben. Bitte umkreisen Sie **0** 1 2 3 4 5 wenn Sie keine Beschwerden haben und 0 1 2 3 4 **5** wenn sie heftigste Beschwerden haben; bei mittelgradigen Beschwerden kreuzen Sie bitte einen dazwischen liegenden Wert, je nach dem Schweregrad der Beschwerden, an. Vielen Dank!

Zeit	H_2/Atem (ppm)	Durchfall	Blähungen/Krämpfe
			Kein ------ heftigste
0	0	Ja/Nein	0 1 2 3 4 5
15	0	Ja/Nein	0 1 2 3 4 5
30	0	Ja/Nein	0 1 2 3 4 5
45	0	Ja/Nein	0 1 2 3 4 5
60	0	Ja/Nein	0 1 2 3 4 5
90	0	Ja/Nein	0 1 2 3 4 5
120	0	Ja/Nein	0 1 2 3 4 5
150	0	Ja/Nein	0 1 2 3 4 5
180	0	Ja/Nein	0 1 2 3 4 5

Beurteilung:

H_2- Atemtest: neg

Korrelation mit Symptomen: neg

Durchführung einer Diätberatung: Ja/Nein

Falls Ja: Termin am.....

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Telefon: 0316 / 385 - 12422

Figure 18: Documentation sheet of a patient without trehalose malabsorption (TM) and trehalose intolerance (TIT)

Trehalose H_2-Atemtest: 50g Trehalose in 200ml Wasser gelöst

Durchfall: Ja/Nein — **Blähungen:** Ja/Nein

Winde: Ja/Nein — **Bauchkrämpfe:** Ja/Nein

Milchunverträglichkeit: Ja/Nein — **Unverträglichkeit von Pilzen:** Ja/Nein

Fruchtzuckerunverträglichkeit: Ja/Nein

Sehr geehrte(r) Patient(in):

Bitte umkreisen Sie bei Durchfall **JA**/Nein wenn Sie zu diesem Zeitpunkt Durchfall haben, und Ja/**NEIN** wenn Sie keinen Durchfall haben. Bitte umkreisen Sie **0** 1 2 3 4 5 wenn Sie keine Beschwerden haben und 0 1 2 3 4 **5** wenn sie heftigste Beschwerden haben; bei mittelgradigen Beschwerden kreuzen Sie bitte einen dazwischen liegenden Wert, je nach dem Schweregrad der Beschwerden, an. Vielen Dank!

Zeit	H_2/Atem (ppm)	Durchfall	Blähungen/Krämpfe
			Kein ------ heftigste
0	0	Ja/Nein	0 1 2 3 4 5
15	0	Ja/Nein	0 1 2 3 4 5
30	3	Ja/Nein	0 1 2 3 4 5
45	12	Ja/Nein	0 1 2 3 4 5
60	23	Ja/Nein	0 1 2 3 4 5
90	28	Ja/Nein	0 1 2 3 4 5
120	22	Ja/Nein	0 1 2 3 4 5
150	22	Ja/Nein	0 1 2 3 4 5
180	18	Ja/Nein	0 1 2 3 4 5

Beurteilung:

H_2- Atemtest: pos Trehalosentoleranz

Korrelation mit Symptomen: ja

Durchführung H_2-Atemtest mit **25g** Trehalose: Ja/Nein

Falls Ja: Termin am..... (mind. 1 Woche Abstand)

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Figure 19: Documentation sheet of a patient with trehalose malabsorption (TM) and trehalose intolerance (TIT)

Selection and withdrawal of subjects

Inclusion criteria

In this study female and male subjects with an age range of 18-90 years were included. Patients were referred from their primary care physicians for the evaluation of the clinical suspicion of lactose intolerance or fructose intolerance or for suspected mushroom intolerance. The suspicion was based on the presence of gastrointestinal symptoms like bloating, crampy abdominal pain, flatulence, abdominal distension and diarrhea. After the test, requested by the referring physician, had been performed, patients were offered to participate in this study. Written informed consent was obtained from all study participants and they were informed about the test procedure and about the aims, purposes and risks of the study.

Exclusion criteria

Subjects with known gastrointestinal diseases, antibiotic treatment or colonoscopy during the preceding four weeks, basal breath hydrogen concentration greater than 10 ppm or non-compliance with fasting or avoiding to smoke for at least 12 hours prior to the test were excluded.

Hydrogen breath test

Trehalose hydrogen breath test was performed after an overnight fast. Patients were instructed to fast for at least 12 hours. Additionally chewing gum, smoking and stressful exercises had to be avoided during that time. Prior to the test patients were instructed to brush teeth with water. Concomitant medication had to be documented. Antibiotics had to be discontinued four weeks and lactulose (Laevolac®) three days prior to the test. Colonoscopies had to be avoided four weeks before the test. There had to be an interval of at least one week between two hydrogen breath tests.

Before the test the basal hydrogen concentration was measured in order to validate the fasting state and to get the baseline value against which the hydrogen concentrations after ingestion of trehalose were compared. That value had to be below 10 ppm in order to continue the test. If basal hydrogen concentration was greater than 10 ppm the test could not be used and the participant had to repeat it with proper preparation (no smoking, no dietary fibers on the previous evening). After oral administration of 50 g trehalose dissolved in 200 ml water end-expiratory breath samples were taken for 3 hours and were

analyzed electrochemically for hydrogen using the portable measuring instrument 'Gastrolyzer' which is demonstrated in figure 20 and figure 21.

Figure 20: Breath H_2 monitor 'Gastrolyzer' – front view

Figure 21: Breath H_2 monitor 'Gastrolyzer' – back view

During the first hour hydrogen concentration was measured every 15 minutes and afterwards every 30 minutes. Bloating, abdominal cramps and pain occurring before and after ingestion of trehalose were scored on a scale from 0 (no symptoms) to 5 (most severe symptoms), at the same time points when hydrogen measurements were obtained. The presence of diarrhea was scored yes or no.
Trehalose malabsorption was defined by an increase in H_2-concentration of at least 20 ppm over basal concentration within three hours. Trehalose intolerance was defined by an increase in symptoms by more than two over baseline, or the occurrence of diarrhea after trehalose.

Concerns of safety

Trehalose is a naturally occurring disaccharide that is present in many living organisms around the world (e.g. plants, insects, mushrooms, yeast, seafood) (1). Due to its stability and remarkable physical and chemical properties it is increasingly used in the food industry. In 2000 the disaccharide was attested as "generally recognized as safe" (GRAS) in the US and in Europe it was approved as food ingredient and novel food in 2001 (1). Several in vitro and in vivo animal safety studies concerning the intake of trehalose have been performed but no harmful effects, mutagenicity or genotoxicity have been observed and there are no limitations of its use (1). For this particular study a maximal dose of 50 g trehalose was administered, which is the same as the amount of lactose administered for lactose hydrogen breath test.
In case intolerant subjects developed severe symptoms oral Simethicone (Antiflat®) or oral or intravenous Scopalamine (Buscopan®) was offered. Despite symptom development in some subjects there were no organic complications.

Statistics

Clinical data was analyzed using IBM SPSS Statistics 22. Data was analyzed descriptively utilizing mean and standard deviation and with cross-classified tables

3 RESULTS

Study population

Thirty consecutive patients with suspected carbohydrate malabsorption were recruited from the outpatient clinic of the Department of Internal Medicine, Division of Gastroenterology and Hepatology of the Medical University of Graz. Eight patients were male and twenty-two female; the median age was 32 ± 15,5 years (range 20-72 years, mean age 38,2 years).

Patients were referred for a lactose- or fructose hydrogen breath test in order to evaluate gastrointestinal symptoms such as abdominal pain, intestinal bloating, flatulence and diarrhea. 33 % of patients had abdominal cramps, 67 % had bloating and 27 % had diarrhea. In eight patients (27%) no symptoms were recorded (Figure 22). Lactose malabsorption (LM) was documented in 8 patients (27 %), lactose intolerance (LIT) in 11 patients (37 %), combined malabsorption and intolerance of lactose (LM +LIT) in six patients (20 %), fructose malabsorption (FM) in one patient (3 %) and fructose intolerance (FIT) in 3 patients (10 %). There was no patient with combined malabsorption and intolerance of fructose (FM + FIT) (Figure 23).

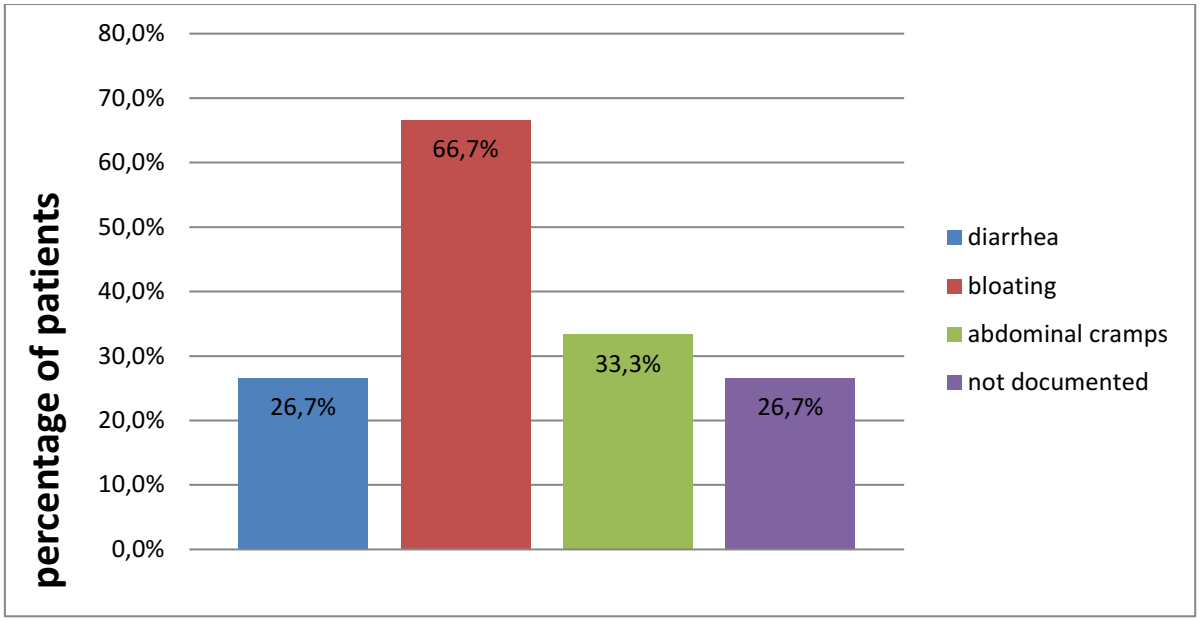

Figure 22: Symptoms in patients referred for hydrogen breath test

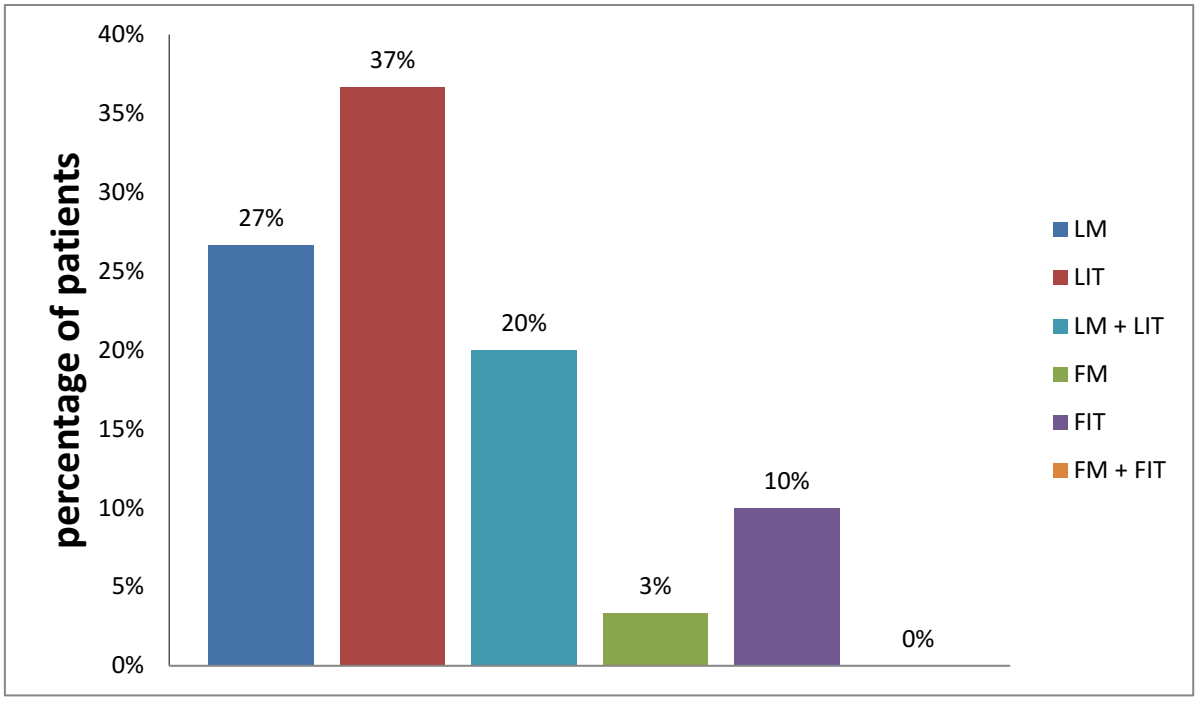

Figure 23: Percentage of patients with lactose malabsorption (LM), lactose intolerance (LIT), malabsorption and intolerance of lactose (LM + LIT), fructose malabsorption (FM), fructose intolerance (FIT) and malabsorption and intolerance of fructose (FM + FIT)

Of 30 patients who underwent hydrogen breath test with trehalose, nine (30 %) had an increase in hydrogen concentration greater than 20 ppm, suggesting trehalose malabsorption. Of nine patients with a positive test result (H_2 > 20 ppm) four reported symptoms during the test, suggesting trehalose intolerance; five persons reported no symptoms in spite of having trehalose malabsorption. Five of 21 patients with a negative hydrogen breath test result (H_2 < 20 ppm) reported an increase in symptom score of at least 2 or diarrhea (Table 3).

	TIT		**Total**
	yes	**no**	
TM yes	4	5	9
TM no	5	16	21
Total	9	21	30

Table 3: Number of patients with trehalose malabsorption (TM) and trehalose intolerance (TIT)

Hydrogen increase and symptom development

Figure 24 shows the time of H_2- increase greater than 20 ppm and the time of symptom development greater than Δ 2 in patients with one or both of those findings. The simple linear regression is represented by the green line; it is a method to illustrate the relationship between a dependent variable (time of symptom development > Δ 2) and one or more independent variables (time of H_2 – increase in this case) (47).

Referring to the H_2-increase greater than 20 ppm two groups can be identified. One group, consisting of four patients, has an early hydrogen peak within 60 minutes. This early increase in hydrogen concentration might be due to a bacterial overgrowth of the small intestine (SIBO). The second group, consisting of five patients, shows a later increase in hydrogen concentration which is most likely caused by colonic bacterial fermentation of unabsorbed trehalose.

Referring to symptom development greater than Δ 2, two groups can be identified as well. Six patients showed an early increase in symptoms at 30 minutes. Late symptom development, between 120 and 150 minutes was established in three patients.

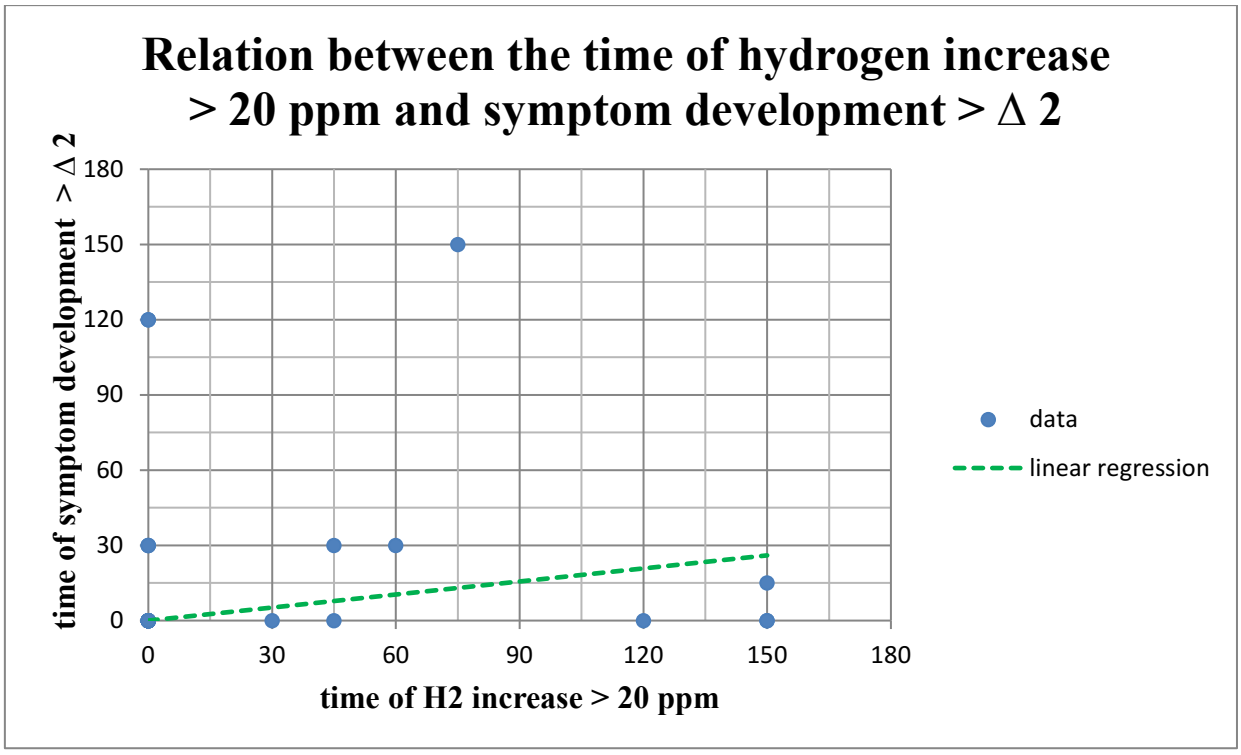

Figure 24: Visualization of hydrogen increase and symptom development

In the following figures 25-27 examples of different breath and symptom profiles of patients are domstrated.

Figure 25 shows hydrogen increase and symptom development in a patient suffering from trehalose malabsorption and trehalose intolerance. Hydrogen peaks occurred at 45 and 90 minutes and symptom peaks at 45 and 120 minutes.

Figure 26 shows hydrogen increase and time course of symptoms in a patient with TM but no TIT; this patient had an increase in H_2 concentration greater than 20 ppm but no symptom development greater than Δ 2.

Figure 27 shows symptom development and H_2 concentrations in a patient presenting with trehalose intolerance (symptoms > Δ 2) but without an increase in H_2- concentration > 20 ppm. Symptom development without an increase in hydrogen concentrations might be explained by hydrogen nonexcretion.

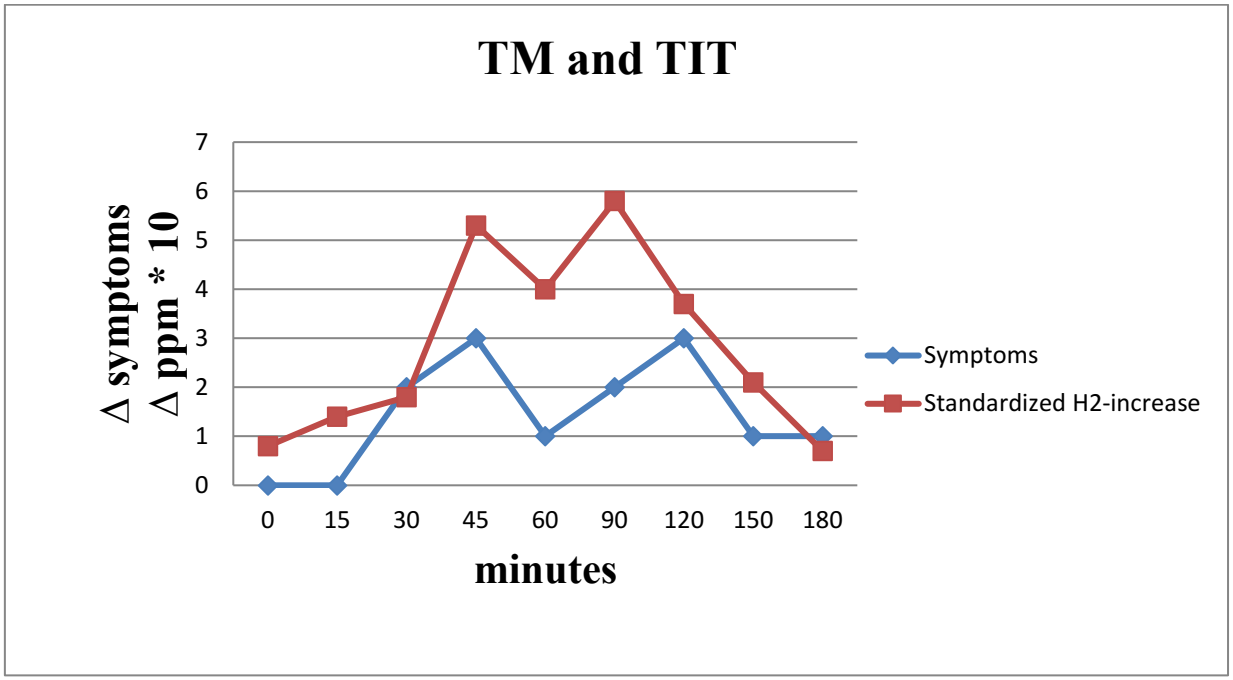

Figure 25: Hydrogen increase and symptom development in a patient suffering from TM and TIT

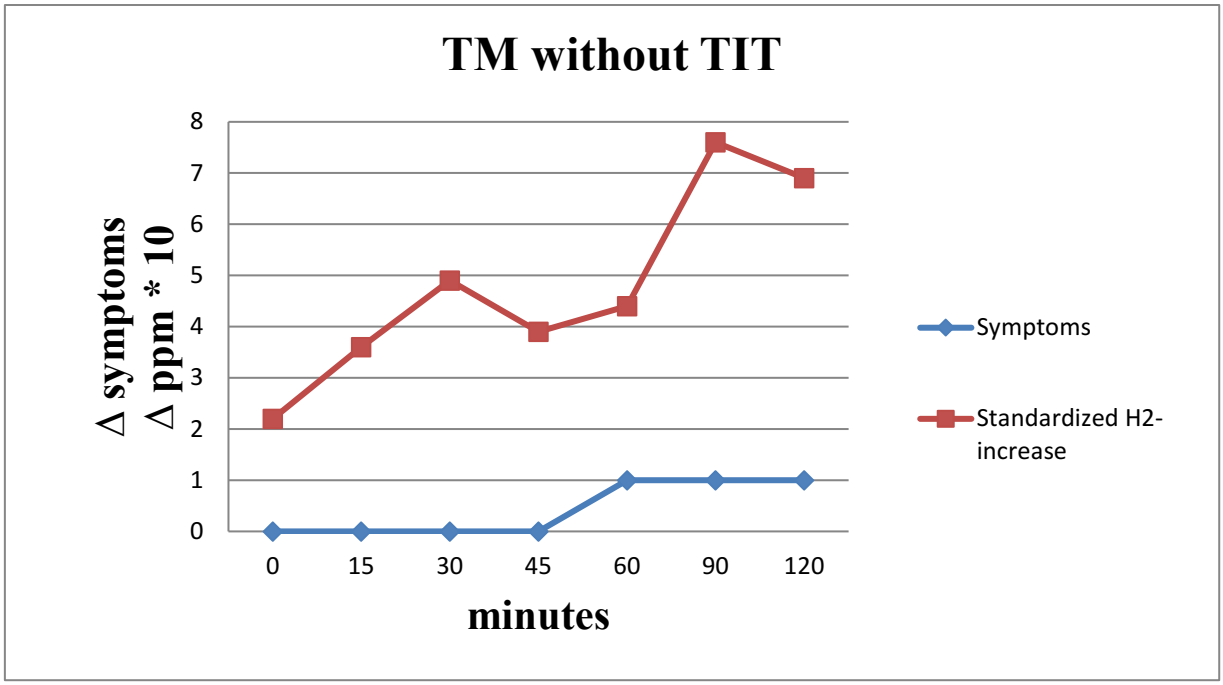

Figure 26: Hydrogen increase and symptom development in a patient suffering from TM without TIT

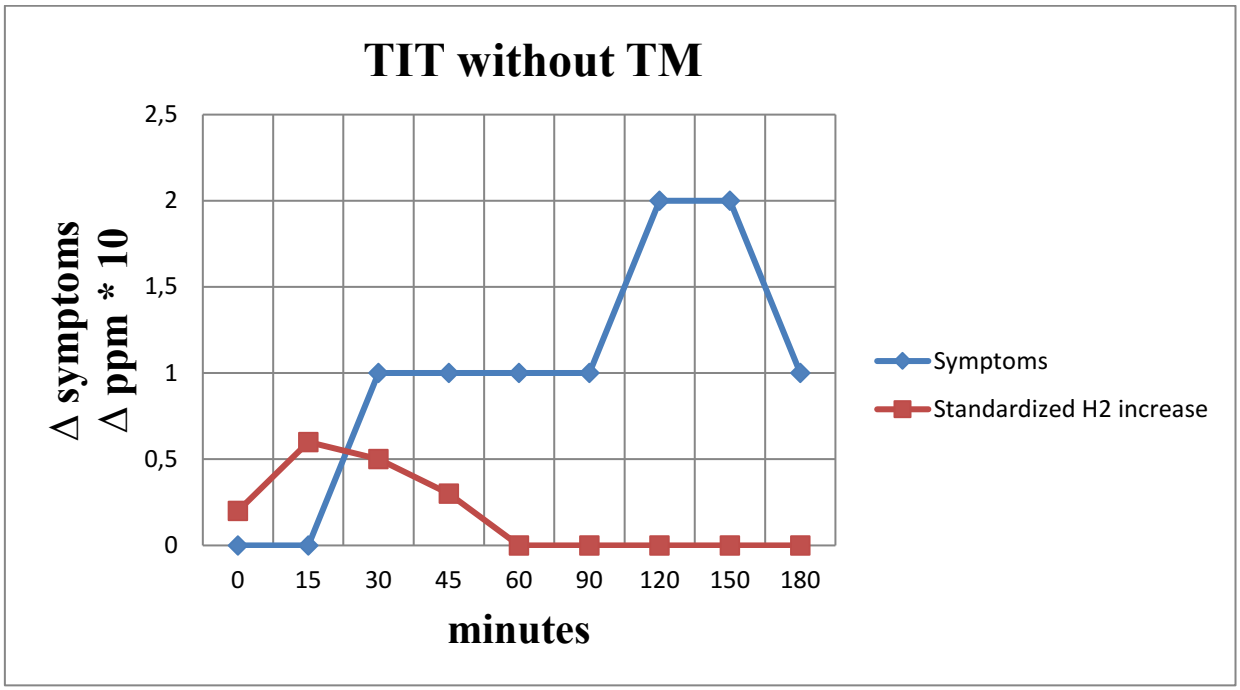

Figure 27: Hydrogen increase and symptom development in a patient suffering from TIT without TM

Relation between H_2- increase (trehalose malabsorption) and symptom development (trehalose intolerance)

Five of nine patients with trehalose intolerance (55%) did not have trehalose malabsorption (H_2 < 20 ppm). On the other hand five of nine trehalose malabsorbers (55%) did not report symptoms during H_2 breath test (See table 3).

Relation between trehalose malabsorption and malabsorption of lactose or fructose

In eight of thirty study participants (27 %) lactose malabsorption and in one fructose malabsorption (3 %) had been diagnosed previously. One of those patients had combined malabsorption of lactose and fructose (3 %). Three of eight patients with lactose malabsorption (37,5 %) showed an increase in hydrogen greater 20 ppm after the oral ingestion of 50 g trehalose, suggesting trehalose malabsorption. Six of 22 patients without lactose malabsorption (27%) had an increase in hydrogen > 20 ppm after trehalose. The persons with fructose malabsorption, respectively combined malabsorption of fructose and lactose also showed malabsorption of trehalose. Eight of 29 patients without fructose malabsorption (28%) were trehalose malabsorbers.

Table 4 shows the relation between the occurrence of malabsorption of lactose, fructose and trehalose.

	LM yes	**FM yes**	**LM no**	**FM no**
TM yes	3	1	6	8
TM no	5	0	16	21
Total	8	1	22	29

Table 4: Relation between trehalose malabsorption (TM) and malabsorption of lactose (LM) or fructose (FM)

Relation between trehalose intolerance and intolerance of lactose or fructose

Eleven patients reported lactose intolerance (37 %) and three subjects reported an intolerance of fructose (10%). Four of eleven patients with lactose intolerance (36 %) reported symptoms > 2 after the oral admission of the trehalose. Five of nineteen patients without lactose intolerance reported symptoms > Δ 2. One of three patients with fructose intolerance reported symptoms > Δ 2 after the administration of trehalose. Eight of 27 patients without fructose intolerance showed trehalose intolerance.

Table 5 shows the relation between the occurrence of intolerance of lactose, fructose and trehalose.

	LIT yes	**FIT yes**	**LIT no**	**FIT no**
TIT yes	4	1	5	8
TIT no	7	2	14	19
Total	11	3	19	27

Table 5: Relation between trehalose intolerance (TIT) and intolerance of lactose (LIT) or fructose (FIT)

Relation between trehalose malabsorption (TM), trehalose intolerance (TIT) and intolerance of mushrooms

In our study population in five patients a history of mushroom intolerance was documented. Three of these patients had trehalose malabsorption (TM), two had trehalose intolerance, respectively one patient showed a combined malabsorption and intolerance of trehalose.

4 DISCUSSION

Trehalose, a naturally occurring disaccharide in mushrooms, insects, seafood, algae, plants, bacteria, baker's and brewer's yeast has become of interest to the food industry (1). Due to the sugar's stability against heat, drought, dehydration, freezing and oxidation, it contributes to favored properties in food products such as an improvement of food consistency, the elongation of shelf life of products and the preservation of flavors and taste (3) (19) (31). In 2001 trehalose was registered as food ingredient and novel food in the European Union (1) Since then it has been increasingly incorporated into many products e.g. confectionery, pastries and freeze-dried products (10) (31). In most parts trehalose is not declared when added to foods.
For hydrolysis in the small intestine trehalose requires the disaccharidase trehalase (1). If trehalase levels are low, trehalose cannot be digested and undergoes a fermentative process in the colon. Subsequent gas production may lead to symptoms of carbohydrate intolerance e.g. abdominal distension, bloating, abdominal cramps or diarrhea (33).

Trehalase deficiency versus trehalose malabsorption

A mutation in the TREH gene may result in the metabolic condition trehalase deficiency (48). Trehalase deficiency, a reduced expression or activity of the disaccharidase trehalase in the small intestine, may lead to malabsorption of trehalose (33) (49). At the department of Gastroenterology/Hepatology at the University hospital Graz trehalose malabsorption can be diagnosed by a H_2 increase > 20 ppm over the basal hydrogen value in hydrogen breath test.

Trehalose malabsorption versus trehalose intolerance

Trehalose malabsorption may lead to symptoms of trehalose intolerance (33). However symptom development (intolerance) is not necessarily associated with a positive hydrogen breath test (malabsorption) (43). Development of symptoms is depending on the amount of maldigested trehalose passing into the colon and subsequent gas production by colonic bacterial flora. Subsequent water flow into the colon due to osmotic effects may cause diarrhea (33) (49).Occurrence of symptoms depends on the patient's individual tolerance and sensitivity of the gastrointestinal tract and may be aggravated in the presence of irritable bowel syndrome (43).

On the contrary trehalose intolerance (symptoms following trehalose ingestion) can also be present without a positive breath test for trehalose malabsorption. This can be due to irritable bowel syndrome or due to a difficulty to digest FODMAPS (fermentable oligosaccharides, disaccharides, monosaccharides and polyols) (43) (50). Another reason for trehalose intolerance without trehalose malabsorption is H_2 nonexcretion (41).
The prevalence of trehalose deficiency varies around the world, with the highest prevalence in Greenland. In 1988 Gudmand Hoyer et al. examined 97 biopsy samples for trehalase activity and demonstrated an incidence of trehalase deficiency of 8 % (2). Less is known about the prevalence of trehalose malabsorption and intolerance. At this point of time no studies exist concerning the prevalence of trehalose malabsorption and trehalose intolerance in patients presenting with symptoms suggestive of carbohydrate malabsorption. Our present study was the first clinical trial examining trehalose malabsorption and intolerance in symptomatic patients.
Referring to the study of Gudmand Hoyer et al. we considered to evaluate thirty patients in order to find n= 3 affected patients.

4.1 KEY RESULTS

The aim of this clinical trial was the evaluation of the prevalence of trehalose malabsorption (TM) and trehalose intolerance (TIT) in patients with symptoms suggestive of carbohydrate intolerance who were referred for a hydrogen breath test, to evaluate the clinical suspicion of lactose or fructose intolerance.
This current study demonstrated the importance of the diagnostic evaluation of this specific carbohydrate malabsorption. Thirty percent of study participants (9 out of 30 patients) showed malabsorption of trehalose, defined by an increase in breath hydrogen > Δ 20 ppm. Equally thirty percent of study participants (9 out of 30 patients) showed trehalose intolerance, defined by an increase in symptoms > Δ 2.
However in our study population trehalose malabsorption did not necessarily lead to symptoms of trehalose intolerance. In addition patients with trehalose intolerance did not necessarily show trehalose malabsorption (increase in hydrogen > Δ 20 ppm). Only four out of nine patients with TM showed symptoms following trehalose ingestion. Four out of nine patients with TIT showed a simultaneous increase in hydrogen concentration > Δ 2.

One explanation for these discrepant results is H_2 nonexcretion. A former study by Hammer et al. demonstrated a rate of hydrogen nonexcretion of 18 % in the area around Graz (41). With reference to this data five of thirty study participants could be expected to be hydrogen nonexcretors. Consequently two of nine patients with trehalose intolerance could be expected to be H_2 nonexcretors. This suggestion leaves three patients with TIT without TM unexplained. These discrepant results in three patients with TIT without TM, who showed symptoms within thirty minutes, might be due to symptom origin in the small intestine. In fact these subjects might not be trehalose malabsorbers, but trehalose ingestion might have resulted in water accumulation in the small intestine resulting in intestinal distension causing abdominal bloating and pain.

Five of nine trehalose malabsorbers did not report symptoms during hydrogen breath test. We assumed these patients to be insensitive against the ingested amount of trehalose and to the products of bacterial metabolism. We concluded that trehalose malabsorption and trehalose intolerance are not necessarily associated with each other as is the case in lactose malabsorption and lactose intolerance (43).

Referring to lactose- and fructose malabsorption no relation between trehalose malabsorption and either of these conditions could be observed.

Equally no relation between trehalose intolerance and intolerance of lactose or fructose could be demonstrated.

Because trehalose is a major component of mushrooms, study participants were asked for a history of mushroom intolerance (1). No significant correlation between a history of mushroom intolerance and malabsorption or intolerance of trehalose could be obtained.

4.2 LIMITATIONS OF THE STUDY

This present study was a controlled, non-blinded single center study consisting of thirty study participants and following a standardized approach.
All study participants performed hydrogen breath test under the same conditions, respective to the dose of the administered sugar (50 g trehalose dissolved in 200 ml water), the frequency of measurements (every 15 minutes for the first hour, every 30 minutes for the last two hours), the period over which H_2 was measured (3 hours) and the record of symptoms on a scale from 0-5 (diarrhea and abdominal cramps/bloating were recorded). Hydrogen breath test and the assessment of symptoms were interpreted equally and under the same conditions. A positive breath test was defined by an increase in hydrogen > Δ 20 ppm (trehalose malabsorption), a positive symptom assessment was defined by an increase in symptoms > Δ 2 (trehalose intolerance). Exclusion criteria were defined strictly, such as a known gastrointestinal disease, the intake of antibiotics for the previous four weeks, the intake of lactulose for the precedent 3 days, a colonoscopy within the precedent four weeks, smoking prior hydrogen breath test, basal hydrogen concentration > 10 ppm and not fasting for at least 12 hours.

Possibility of non-adherence to preparation criteria

Hydrogen breath test was performed after a twelve hour fast. Patients were instructed to avoid eating slowly absorbable carbohydrates e.g. bread, pastries, beans, fibers one day prior to the test, because this might result in high basal hydrogen levels (35). Smoking had to be avoided prior to the test. Medications like antibiotics, pro- and anti-motility drugs had to be documented and discontinued prior to the test. In addition patients were instructed to brush teeth with water prior to the test. Despite a preparation protocol it cannot be guaranteed that study participants were adherent to these instructions making it a potential source of error.

Hydrogen breath test

Breath samples were only analysed for hydrogen. An increase in accuracy of test results would have been achieved by additional measurement of methane. Analysis for methane in breath samples would have detected hydrogen nonexcretors as well. However a device for the measurement of breath CH_4 was not available at the time of the study.

Equally additional biopsies of the small intestine determining trehalase levels would have eliminated other sources of error e.g. small intestinal bacterial overgrowth. However this would have involved an invasive procedure, that is endoscopy with biopsy.

No transferability to a healthy population in Austria

The results of this clinical trial are not transferable to a healthy population, because our study population consisted of symptomatic patients following carbohydrate ingestion.

Low number of study participants

This present study was aimed to be a pilot study for a potential larger study. Even with such a small number of patients a considerable proportion of patients with trehalose malabsorption (TM) and trehalose intolerance (TIT) could be identified.

4.3 CONCLUSION

In reference to this present study it is obvious that malabsorption and intolerance of trehalose plays a major role in contributing to gastrointestinal symptoms similar to those of lactose- or fructose intolerance.

It can be concluded that 30 % of patients with symptoms suggestive of carbohydrate malabsorption suffer of trehalose malabsorption and 30 % of patients suffer of trehalose intolerance. Contrary to our expectations we could not observe a significant correlation with malabsorption or intolerance of lactose or/and fructose or a history of mushroom intolerance.

However further research has to be done on this topic with a bigger study population, the use of a combined breath test for hydrogen and methane and the analysis of small intestinal trehalase activity.

Respective to the significant study results the addition of trehalose to foods has to be declared more accurate. By the means of declaring this sugar, people suffering of this metabolic condition would get the possibility to avoid these products and to improve symptoms.

5 BAKING EXPERIMENT

In the context of my diploma thesis I decided to do some baking experiments with trehalose and sucrose.
The aims of this baking experiments were to show the advantageous effects of trehalose, described in literature (e.g. improvement of the dough raise, reduced browning reaction, better consistency, preservation of flavors and taste, longer storage life compared to other sugars) (10) (3) (32) (31).

5.1 COMPARISON OF JELL ROLLS WITH SUCROSE AND MIXTURES OF SUCROSE AND TREHALOSE

In the first baking experiment I compared a normal jell roll with sucrose, a jell roll where 70 % of sucrose was replaced by trehalose and a jell roll where 50 % of sucrose was replaced by trehalose.
Both jell rolls with trehalose, especially the jell roll with 70 % trehalose, had a lighter and fluffier consistency making them more enjoyable for most of the testing subjects than the jell roll with sucrose. Contrary to descriptions in literature the trehalose rolls showed a decreased volume in comparison to the sucrose roll. The trehalose rolls were less sweet but showed a longer persistence in sweetness and showed a reduced browning reaction. With reference to the storage life the roll where 70 % of sucrose was replaced by trehalose had the longest shelf life and also remained fresh and juicy for the next days.

Figure 28: Comparison of jell rolls with sucrose and mixtures of sucrose and trehalose

Figure 29: Comparison of jell rolls with sucrose and mixtures of sucrose and trehalose – enlarged view

5.2 COMPARISON OF MUFFINS WITH SUCROSE AND A MIXTURE OF SUCROSE AND TREHALOSE

In the second baking experiment muffins with sucrose and muffins, where 50 % of sucrose were replaced by trehalose, were compared. The muffins, where 50 % of sucrose was replaced by trehalose, showed a reduced browning reaction, a more homogenous pattern, a lighter and fluffier consistency and a longer persistence in shelf life.

Figure 30: Comparison of muffins with sucrose and a mixture of sucrose and trehalose

5.3 COMPARISON OF YOGHURT CAKES WITH SUCROSE AND A MIXTURE OF SUCROSE AND TREHALOSE

In the third baking experiment a yoghurt cake with sucrose and a yoghurt cake, where 50 % of sucrose were replaced by trehalose, were compared. As demonstrated in the first two experiments the cake, where 50 % of sucrose was replaced by trehalose, also showed a lighter and fluffier consistency, a more homogenous pattern and a longer shelf life compared to the cake with sucrose.

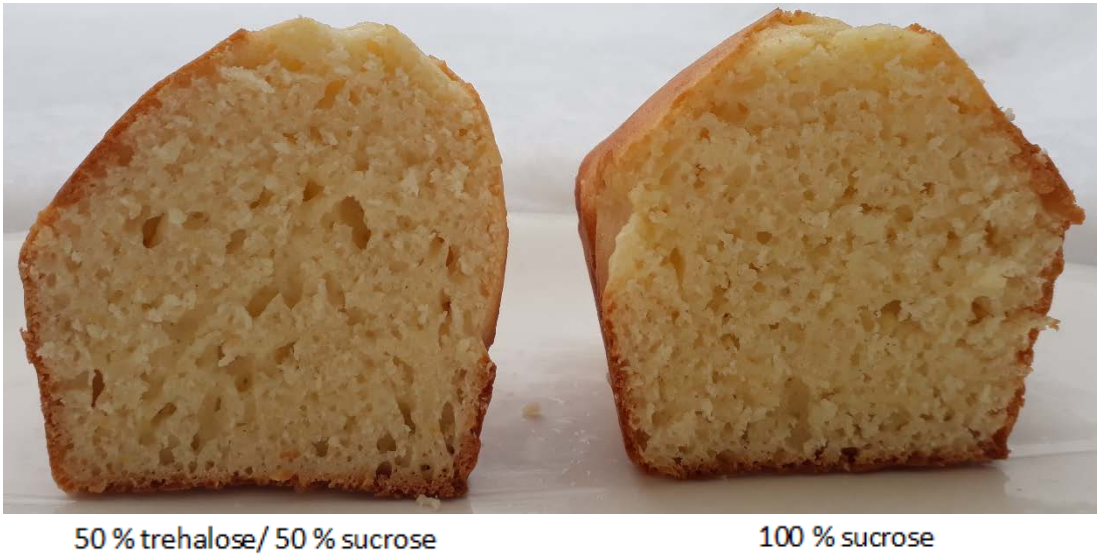

Figure 31: Comparison of yoghurt cakes with sucrose and a mixture of sucrose and trehalose

6 REFERENCES

1. Richards AB, Krakowka S, Dexter LB, Schmid H, Wolterbeek APM, Waalkens-Berendsen DH, et al. Trehalose: A review of properties, history of use and human tolerance, and results of multiple safety studies. Vol. 40, Food and Chemical Toxicology. 2002. p. 871–98.

2. Gudmand-Høsyer E, Fenger HJ, Skovbjerg H, Kern-Hansen P, Madsen PR. Trehalase deficiency in Greenland. Scand J Gastroenterol. 1988;23(7):775–8.

3. Elbein AD, Pan YT, Pastuszak I, Carroll D. New insights on trehalose: A multifunctional molecule. Vol. 13, Glycobiology. 2003.

4. Müller J, Boller T, Wiemken A. Trehalose and trehalase in plants: recent developments. Vol. 112, Plant Science. 1995. p. 1–9.

5. Horn F. Disaccharide. In: Berghold S, Grillhös C, Horn F, Lindenmeie G, editors. Biochemie des Menschen. 6th ed. Georg Thieme Verlag; 2015. p. 50.

6. Alvarez-Suarez JM, Tulipani S, Romandini S, Bertoli E, Battino M. Contribution of honey in nutrition and human health: A review. Vol. 3, Mediterranean Journal of Nutrition and Metabolism. 2010. p. 15–23.

7. Jain NK, Roy I. Effect of trehalose on protein structure. Vol. 18, Protein Science. 2009. p. 24–36.

8. Tamanna N, Mahmood N. Food processing and maillard reaction products: Effect on human health and nutrition. Vol. 2015, International Journal of Food Science. 2015.

9. Higashiyama T. Novel functions and applications of trehalose. Pure Appl Chem [Internet]. 2002;74(7). Available from: https://www.degruyter.com/view/j/pac.2002.74.issue-7/pac200274071263/pac200274071263.xml

10. Rosenplenter K, Nöhle U. Trehalose. In: Kurt Rosenplenter UN, editor. Handbuch

Süßungsmittel Eigenschaften und Anwendung. 2nd ed. Behr's Verlag GmbH & Co. KG; 2007. p. 242–58.

11. Lunn JE, Delorge I, Figueroa CM, Van Dijck P, Stitt M. Trehalose metabolism in plants. Plant J. 2014;79(4):544–67.

12. Mohammadi M, Dini M. Identification of manna sources, production mechanism and utilization in the iran. Iran J Med Aromat plants. 2003;(17):75–118.

13. Korsch H. Echinops spaerocephalus [Internet]. 2003. Available from: https://neobiota.bfn.de/handbuch/gefaesspflanzen/echinops-spaerocephalus.html

14. Stewart LC, Richtmyer NK, Hudson CS. The Preparation of Trehalose from Yeast. J Am Chem Soc. 1950;72(5):2059–61.

15. Myrothamnus [Internet]. Available from: https://de.wikipedia.org/wiki/Myrothamnus

16. Heimann W. Nicht reduzierende Disaccharide. In: Heimann W, editor. Grundzüge der Lebensmittelchemie. 3rd ed. Steinkopff- Verlag Heidelberg; 1976. p. 139–40.

17. Benaroudj N, Lee DH, Goldberg AL. Trehalose Accumulation during Cellular Stress Protects Cells and Cellular Proteins from Damage by Oxygen Radicals. J Biol Chem. 2001;276(26):24261–7.

18. Nishizaki Y, Yoshizane C, Toshimori Y, Arai N, Akamatsu S, Hanaya T, et al. Disaccharide-trehalose inhibits bone resorption in ovariectomized mice. Nutr Res [Internet]. 2000;20(5):653–64. Available from: http://www.sciencedirect.com/science/article/pii/S027153170000155X

19. Ohtake S, Wang YJ. Trehalose: Current use and future applications. Vol. 100, Journal of Pharmaceutical Sciences. 2011. p. 2020–53.

20. Luyckx J, Baudouin C. Trehalose: An intriguing disaccharide with potential for medical application in ophthalmology. Vol. 5, Clinical Ophthalmology. 2011. p. 577–81.

21. GmbH TP. Thealoz® duo [Internet]. Available from: http://www.thea-pharma.at/pta/thealozduo

22. Emanuele E. Can Trehalose Prevent Neurodegeneration? Insights from Experimental Studies. Curr Drug Targets [Internet]. 2014;15(5):551–7. Available from: http://www.eurekaselect.com/openurl/content.php?genre=article&issn=1389-4501&volume=15&issue=5&spage=551

23. Murray I a, Coupland K, Smith J a, Ansell ID, Long RG. Intestinal trehalase activity in a UK population: establishing a normal range and the effect of disease. Br J Nutr [Internet]. 2000;83(3):241–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10884712

24. GmbH GB. TREHALOSE – Ein multifunktioneller Zucker für die Backwarenindustrie. Food ingridients Pflanzl Nahrungsmittelrohstoffe. 2009;1–2.

25. Hoffmann W, Buzello A, Burghardt C. Untersuchungen zum möglichen Einsatz von Trehalose in Milchprodukten [Internet]. 2010. Available from: http://docplayer.org/7247608-Untersuchungen-zum-moeglichen-einsatz-von-trehalose-in-milchprodukten.html

26. Nutricia, Advanced Medical nutrition, Forticare [Internet]. Available from: http://www.nutricia.ie/products/view/forticare

27. KG HG& C. Haribo [Internet]. Available from: https://onlineshop.haribo.com/search?sSearch=Kracher.

28. Touger-Decker R, van Loveren C. Sugars and dental caries. Vol. 78, The American journal of clinical nutrition. 2003.

29. Canada H. Archived novel food information [Internet]. Available from: https://www.canada.ca/en/health-canada/corporate/contact-us.html

30. Roser B. Trehalose, a new approach to premium dried foods. Vol. 2, Trends in Food Science and Technology. 1991. p. 166–9.

31. Mitchell H. Sweeteners and Sugar Alternatives in Food Technology. 2nd ed. O'Donnell K, Kearsley MW, editors. Sweeteners and Sugar Alternatives in Food Technology. 2007. 1-413 p.

32. Eleutherio E, Panek A, De Mesquita JF, Trevisol E, Magalhães R. Revisiting yeast trehalose metabolism. Curr Genet. 2015;61(3):263–74.

33. Arola H, Koivula T, Karvonen AL, Jokela H, Ahola T, Isokoski M. Low trehalase activity is associated with abdominal symptoms caused by edible mushrooms. Scand J Gastroenterol. 1999;34(9):898–903.

34. Eisenmann A, Amann A, Said M, Datta B, Ledochowski M. Implementation and interpretation of hydrogen breath tests. J Breath Res. 2008;2(4):1–9.

35. Ghoshal UC. How to interpret hydrogen breath tests. J Neurogastroenterol Motil. 2011;17(3):312–7.

36. Braden B, Braden C, Klutz M, Lembcke B. Analysis of breath hydrogen (H2) in diagnosis of gastrointestinal function: validation of a pocket breath H2 test analyzer. Z Gastroenterol. 1993;31:242–5.

37. Hammer HF, Hammer J. Diarrhea Caused By Carbohydrate Malabsorption. Vol. 41, Gastroenterology Clinics of North America. 2012. p. 611–27.

38. Simrén M, Stotzer PO. Use and abuse of hydrogen breath tests. Vol. 55, Gut. 2006. p. 297–303.

39. Keller J, Franke a, Storr M, Wiedbrauck F, Schirra J. [Clinically relevant breath tests in gastroenterological diagnostics--recommendations of the German Society for Neurogastroenterology and Motility as well as the German Society for Digestive and Metabolic Diseases]. Z Gastroenterol [Internet]. 2005;43(9):1071–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16142616

40. Schneider ARJ, Jepp K, Murczynski L, Biniek U, Stein J. The inulin hydrogen breath test accurately reflects orocaecal transit time. Eur J Clin Invest. 2007;37(10):802–7.

41. Hammer HF, Petritsch W, Pristautz H, Krejs GJ. Assessment of the influence of hydrogen nonexcretion on the usefulness of the hydrogen breath test and lactose tolerance test. Wien Klin Wochenschr. 1996;108:137–41.

42. Romagnuolo J, Schiller D, Bailey RJ. Using breath tests wisely in a gastroenterology practice: An evidence-based review of indications and pitfalls in interpretation. Vol. 97, American Journal of Gastroenterology. 2002. p. 1113–26.

43. Misselwitz B, Pohl D, Frühauf H, Fried M, Vavricka SR, Fox M. Lactose malabsorption and intolerance: pathogenesis, diagnosis and treatment. United Eur Gastroenterol J. 2013;1(3):151–9.

44. Högenauer C, Hammer HF, Mellitzer K, Renner W, Krejs GJ, Toplak H. Evaluation of a new DNA test compared with the lactose hydrogen breath test for the diagnosis of lactase non-persistence. Eur J Gastroenterol Hepatol [Internet]. 2005;17(3):371–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15716664

45. Enattah NS, Sahi T, Savilahti E, Terwilliger JD, Peltonen L, Järvelä I. Identification of a variant associated with adult-type hypolactasia. Nat Genet. 2002;30(2):233–7.

46. Thiel S, Jesse N, Rosien U. Laktosemalabsorption und andere Enzymdefekte. In: Layer P, Rosien U, editors. Praktische Gastroenterologie. 4th ed. München: Elsevier, Urban & Fischer Verlag; 2011. p. 156.

47. The MathWorks I. Math Works [Internet]. Linear regression. Available from: http://de.mathworks.com/help/matlab/data_analysis/linear-regression.html?requestedDomain=www.mathworks.com.

48. Welsh JD, Poley JR, Bhatia M, Stevenson DE. Intestinal disaccharidase activities in relation to age, race, and mucosal damage. Gastroenterology. 1978;75:847–55.

49. Montalto M, Gallo a, Ojetti V, Gasbarrini a. Fructose, trehalose and sorbitol malabsorption. Eur Rev Med Pharmacol Sci [Internet]. 2013;17 Suppl 2(Suppl 2):26–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24443064

50. Gibson PR, Newnham E, Barrett JS, Shepherd SJ, Muir JG. Review article: Fructose malabsorption and the bigger picture. Vol. 25, Alimentary Pharmacology and Therapeutics. 2007. p. 349–63.

51. Kolago G. kolagen makrofotografia [Internet]. Larinus sp. Available from: https://kolagen.wordpress.com/category/chrzaszcze/ryjkowcowate/larinus-sp/

52. Pelser P. Asteraceae: Echinops sphaerocephalus [Internet]. 2009. Available from: http://phytoimages.siu.edu/imgs/pso/r/Asteraceae_Echinops_sphaerocephalus_15615.html.

53. Ergot of rye [Internet]. Available from: http://www.botany.hawaii.edu/faculty/wong/BOT135/LECT12.HTM