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Development and Production of Extruded Food and Feed Products Containing Probiotic Microorganisms

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology, has not been submitted for any degree.

Aram Kazarjan

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Probiootilisi mikroorganisme sisaldavate ekstrudeeritud toiduainete ja loomasöötade tootmistehnoloogia väljatöötamine ja rakendamine

ARAM KAZARJAN

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KIRJASTUS

Dedicated to my Anna

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and patents, which are referred to by Roman numbers within the text:

I. International application published under the patent cooperation treaty (PCT), Title: Probiotic oil suspension and use thereof, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122107 A1 International publication date: 28.10.2010.

II. International application published under the patent cooperation treaty (PCT), Title: A vacuum infusion production plant, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122155 A1 International publication date: 28.10.2010.

III. International application published under the patent cooperation treaty (PCT), Title: Extruded food products compromising probiotic microorganisms, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122106 A1 International publication date: 28.10.2010.

IV. International application published under the patent cooperation treaty (PCT), Title: A method for the management of fecal consistency in dogs, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122104 A1 International publication date: 28.10.2010.

V. N. Kabanova, **A. Kazarjan**, I. Stulova, R. Vilu, Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentrations in broth, Thermochimica Acta, Volume 496, Issues 1-2, 10 December 2009, Pages 87-92.

VI. **A. Kazarjan**, N. Kabanova, R. Vilu, Microcalorimetric study of extruded dog food containing probiotic microorganisms, Advances in Microbiology, Volume 2, Number 4, December 2012, (in press).

ABBREVIATIONS

AO	Antioxidant
ASTM	American Society for Testing and Materials
cP	Centipoise
CFU	Cell Forming Units
cgs	Centimeter gram second
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
HPLC	High Pressure Liquid Chromatography
IBC	Intermediate Bulk Container
Pa·s	Pascal-Second
N_{tot}	Total number of bacteria
N_{exp}	Number of bacteria evolved during the exponential phase
OD	Optical Density
PCT	Patent Cooperation Treaty
Q_{tot}	Total heat
Q_{exp}	Heat produced in exponential phase
RPM	Revolutions Per Minute
RT	Rushton Turbine, impeller
RH	Relative Humidity
S_{exp}	Heat produced during exponential phase
S_{tot}	Heat produced during total growth
TVC	Total Viable Count
UK	United Kingdom
UPP	United Petfood Producers plc, full scale production factory
WIPO	World Intellectual Property Organization
Y_q	Yield coefficient
μ	Specific growth rate of bacteria
μ_{max}	Maximum specific growth rate of bacteria

INTRODUCTION

Various commercial attempts have been made to incorporate probiotic microorganisms into food or feed compositions with prolonged viability for long-term storage. Many of these do not provide sufficient levels of viable probiotic microorganism because standard manufacturing procedures such as extrusion are harmful to the microorganisms. As one example, efforts to coat or fill standard pet food kibbles and ready-to-eat breakfast cereals with probiotic microorganisms have been suggested but often prove impractical in practice.

The microorganisms used as probiotics in food and feed products are very sensitive to various physical/chemical influences such as temperature, moisture, extreme pH levels, organic acids etc. Various food and feed manufacturing processes include heat-treatment, which leads to loss of viability of the probiotic bacteria at the manufacturing stage, and thus also in the final product. Food and feed product manufacturing may include treatment with chemical compounds, serving as ingredients and/or preservatives, which also may have negative effects on the viability and stability of the probiotic microorganisms. Thus, treatments that are harmful to microorganisms ought to occur prior to their inclusion and it is thus not recommended to process food or feed products that contain probiotic ingredients after the inclusion stage. The ingredients used in product formulations should also not have a negative influence on probiotic viability.

Figure 1. Formula ProBiotic – a super premium line of probiotic pet food produced by Bacterfield Ltd.

Modern production facilities, logistics networks, and markets do not yet have a clear understanding of the handling requirements of extruded food and feed products that contain probiotic microorganisms. This dissertation contains a detailed description of the development and implementation of an original, protected by patent applications family, vacuum-infusion-based technology to produce extruded food and feed products that contain probiotic microorganisms and probiotic ingredients. This dissertation places specific emphasis on the preservation of viability to a sufficient level over the entire shelf-life of these products.

The Estonian company *Bacterfield* Ltd., developed two super premium quality pet food lines under the brand names of *ProBiotic LIVE (http://www.livefordogs.com)* and *Formula ProBiotic (http://www.bacterfield.com/formula-probiotic)* (see Figure 1) containing probiotic microorganisms at concentrations of 10^9 CFU/kg in their products over a 15 month shelf-life. The products have been produced since 2008, and they are available in various *European Union* states such as Belgium, Denmark, Slovakia, Germany, the United Kingdom and others.

The industrial technology of producing dry extruded pet food containing probiotic microorganisms used by *Bacterfield* Ltd., was developed and implemented as a result of the work reported in this dissertation.

AIMS OF THE STUDY

The main goals of this dissertation are to develop extruded food and feed products that contain probiotic microorganisms, to evaluate all of the technological aspects/problems that arise during production, maximize the viability of the probiotic ingredients over the shelf-life of the products, and minimize the technological stress factors during production. In order to achieve these goals, the project was divided into several steps:

- Development of an effective vehicle (probiotic ingredient carrier) for the introduction of the probiotics into food and feed products using vacuum infusion that allows for the preservation of probiotic ingredient viability and end-product health benefits, together with optimizing the technological aspects of using the vehicle.

- Development of a novel method of vacuum infusion to coat extruded products to incorporate probiotic bacteria into the product matrix without adversely affecting the viability and stability of the bacteria. This work has resulted in a family of patent applications to protect this technology.

- Development of a production scheme to manufacture extruded probiotic products on an industrial scale with the purpose of maximizing viability of the probiotic components.

- Determination of the physical, biological, and chemical properties of the products characterizing the processes that influence the viability of the probiotic ingredient during storage (shelf-life of the product) while developing formulations that provide maximal probiotic health benefits without sacrificing overall product quality.

- Development of a microcalorimetric method for the analysis of activity and viability of microbial ingredients in the extruded food and feed products during the shelf-life of the products.

- Evaluation of possible advantages and benefits to the health of animals fed with extruded feed products containing probiotic microorganism in animal feed trials.

1. LITERATURE SURVEY

Much has been written on the physiology and properties of possibly health promoting microorganisms (probiotics), and on the mechanisms of their interactions with host organisms, including humans. The topics outlined above are being actively studied, especially since the development of high throughput sequencing methods that have led to the development of metagenomics, nutrigenomics etc. There are also a huge number of preparations whose health promoting effects have been studied in cell cultures, mice, rats and humans [1-10].

However, there are not as many industrial level technologies for the large-scale production of food and feed products that have well established market positions. The majority of the success stories concern dairy products – yogurts, fermented milk products etc. Strains of *Acidophilus*, *Lactobacillus* etc. are perhaps the most well known ingredients of the health promoting dairy products. Use of probiotics in non-dairy food and feed products is currently lagging behind the dairy industry.

The sections below contain a thorough overview of the critical factors that ought to be taken into account when producing extruded foods and feed products containing probiotic microorganisms. Using these together intelligently maximizes the preservation of the probiotic count/viability in the final product over its entire shelf-life.

Because the work carried out during the course of this doctoral thesis involves the development of industrial processes, specifically for the production of extruded animal feed products, the technologies and processes applied in industrial practice are introduced and analysed.

1.1 Production of an extruded feed products

The main production phases of the extruded feed and food products using vacuum infusion are presented in Figure 2.

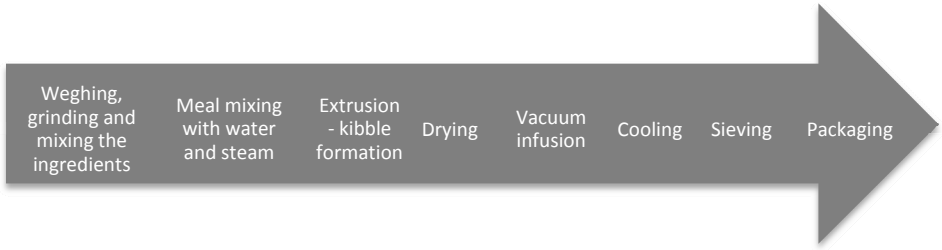

Figure 2. Production scheme of the extruded feed and food products.

Initially, all major ingredients are weighed, ground, and thoroughly mixed together to form a meal. The meal is then mixed with water and steam and extruded. The quantity of water and steam added influence both the shape and density (porosity) of the extruded product (kibbles). The full-scale factory extruder typically has four sections, each regulated up to 115°C. Eventually, the extruded meal is pushed through a die with a desired cross-section (holes). Rotating blades at the exit of the die slice the kibbles into an appropriate size.

Because the kibbles have a very high moisture content after extrusion, a drying process follows where the moisture content is adjusted to 6-8% by controlling the air temperature and speed in drying chambers. This moisture level ensures good penetration of the liquid ingredients introduced in downstream processes (such as oils, chicken digest as a smell enhancer, vitamins etc.). The liquid ingredients, such as oils, can be considered as vehicles for introduction of the dry compounds (powders) into the kibbles, if mixed together in the form of suspension.

The next phase of the production involves vacuum coating or vacuum infusion, where kibbles enter a hermetically sealed chamber. In this chamber liquid ingredients are sprayed on top of the kibbles that are continuously mixed. These penetrate into the matrix (pores) of the kibbles with the aid of a pressure swing from 200 mbar up to 950 mbar. The stepwise decrease and then increase in pressure pushes the liquids and liquid suspensions into matrix (pores) of the extruded product.

The vacuum coated kibbles are then cooled, where the temperature of the kibbles is gradually decreased to ambient temperature. Before the vacuum infusion process, the kibbles are sieved using a vibrating net with fixed cell sizes to remove oversized kibble(s). At this state the product is ready for packing.

A typical method that is widely used to introduce various functional ingredients into food and feed products is simply spraying them onto the surface of the product. The disadvantages of this technique are discussed in section 1.2. The use of vacuum infusion technology to introduce functional ingredients, including probiotic microorganisms, in the form of suspensions, opens up the possibility of developing a variety of extruded food and feed products enriched with various functional ingredients. This technology distributes the functional ingredients throughout the entire product matrix (pores), and the use of oil suspensions as a vehicle is an original method developed during the course of this doctoral work.

The general technological scheme described above will now be analysed in detail to establish a precisely defined terminology to discuss the results presented herein.

1.1.1 Food and feed products

The term “food and feed product” refers to an extruded porous product where probiotics are to be added. These may include breakfast cereals, pet food or treats intended for human and/or animal consumption. The food product may be a particulate based food or food ingredient, such as extruded snack products, tortilla chips, breakfast cereals, cookies, crisp bread, food foams, rice crisps, blends of peanuts, soybeans and corn, puffed wheat, low density foamed corn, rice breakfast cereal, co-extruded products, muesli bars, or any other extruded product that has a porous matrix structure.

1.1.2 Extrusion, extruded

The terms “extrusion” or “extruded” (see Figure 2) in this dissertation refers to “cooking extrusion” which is a combination of heating and extruding to create a cooked and shaped food product. This is a process in which moistened, starchy, proteinaceous foods are cooked and worked into viscous, plastic-like dough. The results of cooking the food ingredients during extrusion are: 1) gelatinization of starch, 2) denaturation of protein, 3) inactivation of raw food enzymes, 4) destruction of naturally occurring toxic substances, and 5) reducing microbial counts in the pre-extruded product. Upon discharge through the die, the hot, plastic extrudate expands rapidly with loss of moisture and heat because of the sudden decrease in pressure. After expansion, cooling, and drying, the extruded product develops a rigid structure and maintains a porous texture. A further objective of the extrusion is to eliminate any bacteria present in the ingredients (comparable with autoclaving). Thus addition of the probiotic ingredient should take place after the extrusion process.

1.1.3 Vacuum infusion

The term “vacuum infusion” (see Figure 2) refers to inclusion of a substance and dispersion of the substance throughout the body of an object (matrix) with the aid of vacuum (pressure lower than 1 bar). For example, vacuum infusion is an infusion of a suspension (comprising a liquid carrier serving as a vehicle and at least one probiotic ingredient) into the porous food matrix such as a pet food kibble or ready-to-eat breakfast cereal by means of a vacuum (vacuum infusion process).

1.1.3.1 Oil

This dissertation refers to “oil” as any edible vegetable and/or animal oil. In this context oil is in a viscous liquid state (“oily”) at room temperature. Oil includes fatty acids, which are carboxylic acids often with a long unbranched aliphatic tail (chain), which is either saturated or unsaturated (such as monounsaturated or polyunsaturated). The ratio of saturated to unsaturated fatty acids varies among

oils. For example, flaxseed oil is comprised of 9% saturated fatty acids, 18% monounsaturated fatty acids, and 73% polyunsaturated fatty acids. In contrast, coconut oil is comprised of 91% saturated fatty acids, 7% mono-unsaturated fatty acids, and 2% poly-unsaturated fatty acids.

With respect to health and diet, oils rich in unsaturated fatty acids are highly preferred over saturated fatty acids. Thus, in order to retain the health benefits of good oil, the products developed in this dissertation contain a high level of unsaturated fatty acids. Fish oils fall within this definition. Fish oils include, but are not limited to, salmon oil, mackerel oil, lake trout oil, herring oil, sardine oil, albacore tuna oil, cod liver, oil, sand eel oil (*Ammodytes tobianus*), and menhaden oil. Vegetable oils also fall within this definition. Vegetable oils include, but are not limited to, flaxseed oil, linseed oil, olive oil, and rice bran oil.

1.1.3.2 Suspension

Suspension refers to a fluid (such as an oil) containing particles that will not dissolve in the fluid and are sufficiently large to sediment. Freeze-dried microorganisms (probiotic ingredient) in form of a dry powder fall into this category. A homogeneous suspension refers to a suspension, wherein the particles are dispersed throughout the external phase (the fluid) through mechanical agitation (such as mixing). The suspended particles (e.g. microorganisms) are visible under a microscope and will settle over time if left undisturbed.

1.1.3.3 Vehicle

“Vehicle” or “carrier“ refers to a fluid component (such as an oil) that carries at least one substance. In this dissertation oil is used as a vehicle for vacuum infusion of at least one probiotic microorganism into an extruded food or feed product. The vehicle may have the additional function of preserving probiotic microorganism(s) embedded in the extruded product. Accordingly, at least one oil is used in the technology as a vehicle to infuse probiotic microorganisms during the manufacture of extruded food or feed products. Manufacturing is performed at room temperature in order to optimize the probiotic count, characterized by colony forming units (CFU), in the final food product. In this respect the viscous properties of the oil (e.g. dynamic viscosity) influence whether or not the oil is suitable for vacuum infusion into the food or feed product matrix. Oils that have an optimal viscosity at elevated temperatures may not be suitable for use at room temperature due to the change in viscosity.

1.1.3.4 Oil suspension as a vehicle for probiotic microorganisms

It is important that the probiotic ingredient is dispersed evenly in the oil suspension throughout the vacuum infusion process so that it may be evenly dispersed throughout the food or feed product matrix. In order to accomplish this the dynamic viscosity of the oil and suspension containing a probiotic microorganisms should be carefully selected. The suspension, in its final form, should enable an efficient vacuum infusion process without interfering with other manufacturing processes. For example, some oils and suspensions depending on the viscosity can have a tendency to clot inside the fluidic system and clog the nozzle used for spraying the suspension onto the product in the vacuum coater/vacuum infusion tank. Downtime of the equipment due to this type of problems must be avoided during production in the factory.

One key parameter is the viscosity of the probiotic oil suspension used in the vacuum infusion process. The only way to avoid unfortunate terminations of the production is to adjust the viscosity of the oil suspension to the optimal level.

1.1.3.5 Viscosity

The term "viscosity" refers to the resistance of a fluid to deformation by either a shear stress or extensional stress. In colloquial language, viscosity is "thickness" of a fluid. The coefficient of viscosity is often used as a value for viscosity. Both, shear viscosity and dynamic viscosity (or absolute viscosity) are frequently used. Viscosity generally decreases (or, alternatively, its fluidity generally increases) as temperature increases. Thus, close temperature control of the fluid is essential for accurate measurement of this fluid property, particularly in materials such as lubricants, whose viscosity can double with a change of only 5°C. Dynamic viscosity is measured with various types of rheometers.

1.2 Analysis of existing technologies

Various attempts have been made to develop marketable food and feed compositions containing probiotic microorganisms with prolonged shelf-life [[11]]. Many of these do not provide sufficient levels of viable probiotic microorganisms, and the microorganisms included are adversely affected by manufacturing procedures such as extrusion etc. For example, efforts to coat or fill standard pet food kibbles and ready-to-eat breakfast cereals with probiotic microorganisms have been suggested, but are often impractical in practice. This is confirmed also in case of various processes protected by patents that have been granted for production of probiotic infused foods and feed products.

For example, patent WO 01/95745 [12] of *Mars* Inc. provides a method to produce a food product (kibbles) characterized by a porous structure, comprising an instable substrate such as a probiotic microorganism in an oil solution, which are included in a flowable form into the product by means of "partial vacuum" step (applying a flow through pressure) followed by normalizing the pressure and releasing an inert gas into the vessel. The use of inert gases may influence the taste and smell of food and feed products. Considering that animals such as dogs can smell microscopic taste differences and refuse to take off smelling products, this is a serious problem. This problem is compounded by the fact that dogs sense 90% of the taste of the feed by smell alone.

Another patent, WO 05/070232 [13] of *Mars* Inc. provides a method to produce a food product similar to WO 01/95745 [12], and further stipulates that the oil should have a solid fat index of at least 20. WO 05/070232 [13] discloses that it is essential that the fat used in the vehicle have a solid fat index of at least 20 at 20˚C, and that the preferred vehicles are palm oil followed by coconut oil. With such a high fat index, the usage of coconut and palm oil are considered to be the most unhealthy oils because they have the highest rate of poly-saturated fatty acids (see Section 1.1.3.1) which lead to a reduction in the overall health benefits of the entire product, even if it contains probiotic bacteria.

A patent WO 03/009710 [14] of *Procter & Gamble* Inc. discloses an apparatus and method for on-line mixing and application of surface coating/introduction of liquids (potentially comprising the probiotics) onto food products. The surface coating or surface introduction technique is an inefficient way of introducing probiotic ingredients into the product because the probiotics lie at the product surface and the stability of such ingredient can be influenced by an environmental factors (open air, humidity etc.). These formulations generally tend to have shorter shelf lives.

Another patent, EP 0862863 B2 [15] of *Nestle* S.A., discloses a method to incorporate bacteria with an extruded formulation by a surface introduction technique (mixing with the product) that uses various carriers (fat, water, digest) without analysis of the carriers as such. According to the patent, EP 0862863 B2 [15] of *Nestle* S.A. the preservation of viability of bacteria was achieved only up to 8 weeks, which is not a sufficient shelf-life to be considered a successful technique for the preservation of probiotic viability.

Additionally, the choice of the bacteria intended to be included into the product should be based on the *EU Commission Regulations* for feed and food products correspondingly and bacteria used for the feed product should be registered in the *EU Community Register of Feed Additives* pursuant to *Regulation (EC) No 1831/2003*.

The disadvantages of the above methods open up an opportunity to develop an improved production technique to incorporate probiotics into food and feed products by means of a vacuum infusion. In particular, a more efficient and/or reliable production plant setup is desired that incorporates probiotics into food and feed products and results in prolonged viability.

1.2.1 The main features of the developed technology

An efficient way of introducing a dry specific compound/ingredient such as a concentrated probiotic powder into an extruded feed or food products is through the use of vacuum infusion (vacuum coating of the extruded product). Following this, the coating should be carried into the food matrix (pores) using a suitable liquid vehicle (the probiotic ingredient carrier, such as oil) together with vacuum coating techniques. To achieve maximum performance, this solid/liquid mixture/suspension needs to have specific viscosity and surface tension properties to achieve homogeneous dispersion and maximum penetration of the probiotic powder into an extruded feed or food product matrix. All these key technological factors are analyzed in this dissertation.

1.3 Evaluation of the shelf-life

The viability of probiotic ingredients, in form of microorganisms added to the product, should be preserved to a minimum effective concentration (in case of feed products regulated by an *EU Commission Regulation*) over the entire period of the shelf-life of the product to guarantee the preservation of the effective and functional concentration of such ingredient at the point of consumption.

The following should be accounted for to test if an extruded food or feed product has a stable probiotic population over its shelf-life:

- Extrusion technology parameters and set-up used in production
- Introduction of the probiotic ingredient
    - Determination of a probiotic ingredient vehicle
    - Inclusion of the probiotic ingredient into the product matrix
- Determination of the probiotic ingredient viability and sufficient stability preservation (shelf-life)
- Determination of health benefits of the extruded feed or food product containing a probiotic ingredient.

Many techniques, such as plate surface inoculation, are not sufficient to precisely determine all the aspects describing the stability of probiotic microorganisms and cannot effectively evaluate the shelf-life of probiotic products. Surface inoculation techniques on agar plates or gels provide only an initial indication of the concentration and viability of probiotic microorganisms. To improve upon these

techniques, microcalorimetry was employed to understand the various factors that influence the concentration and viability of the probiotic microorganisms.

1.3.1 Microcalorimetry for determination of viability and growth characteristics of microorganisms

Living organisms invariably and continuously dissipate energy. As this is virtually always coupled with the continuous production and exchange of heat, calorimeters are used to monitor living systems. It has been shown that production of heat by microbial cultures can be used for on-line monitoring of growth and metabolism of cells [16–20]. Calorimetry is especially useful to study the growth of cells in opaque media, for example solid media where agar, gelatine, etc. are used as jellifying agents [21], and it has been successfully applied to study spoilage processes and to determine shelf-life [20,22–24], usually when optical and other physical methods are not applicable [25,26]. Because heat effects are non-specific, they should be combined with other methods such as measuring the concentration of substrates and products, and outplating to correctly interpret the calorimetry results [27,28]. Additional techniques and measurements allow us to attribute special meaning to the results of calorimetric measurements.

Knowledge of the conversion and yield coefficients allows us to derive a number of characteristics of the growth of bacteria from the power–time curves. Microcalorimetry has been used for the experimental study of bacterial growth for over 50 years. The first experiments were carried out with *Streptococcus faecalis* and it was shown that the rate of heat production was coupled with the rate of biomass growth [29]. Later, growth of other bacteria was studied, including *Escherichia coli* in anaerobic conditions with low substrates and high inoculation concentrations [27], and growth of *Lactobacillus helveticus* in different environmental conditions [30]. Further studies carried out have elucidated the coupling of kinetic and thermodynamic processes [31–37]. It has been shown that calorimetry could provide information about different metabolic changes such as the shift from one substrate/type of catabolism to another, and the occurrence of limitations and inhibitions [16,25,37,38]. The enthalpy balance elaborated using calorimetric measurements can reveal the formation of unknown, unexpected by-products [35,38], or it can be used to study the synthesis of intermediate products of metabolism, and their effect on bacterial growth [39]. Production of ethanol by *Saccharomyces cerevisiae* has also been studied [17], along with the effect of added environmental toxicants on bacterial growth [40]. Calorimetric power–time curves could be used also as imprints of (complicated) microbial processes, because the shape and the number of phases of the curves depend on the composition of the bacterial consortia [18] and reflect complicated patterns of multi-stage growth processes. Despite the noted advantages, use of microcalorimetry was until recently limited in practice because of the absence of multichannel instruments. Carrying out experiments which take days, weeks or even months in 2–4 channel

instruments was not a very attractive possibility, especially in our age of high throughput methods. However, this situation has changed with the appearance of the multichannel TAM III [41] (*TA Instruments*). This instrument has the capacity to carry out up to 48 parallel microcalorimetric measurements simultaneously.

The TAM III, which is a type of heat conduction multi-channel microcalorimeter was used to determine the growth of cells [38]. TAM III is designed to monitor continuously a wide variety of processes and complex systems over the temperature range 15–150°C. TAM III is a multichannel instrument with maximum 48 channels. A 24-channel instrument was used in our experiments. A heat flow calorimeter works by channeling the heat produced or consumed by a reaction in the sample through heatflow sensors comprised of thermoelectric modules. When a temperature gradient is imposed (or formed) across the thermoelectric module, a voltage is created in accordance with the *Seebeck* effect. This voltage is proportional to the heat flow through the thermoelectric module and hence proportional to the rate of heat production or consumption by the sample. One side of the thermoelectric module is in contact with the sample and the other is kept isothermal by a heat sink, which is in contact with the TAM III thermostat (a liquid-based system). TAM III maintains outstanding sensitivity because of the excellent stability of the thermostat (<±100 μK/24 h) even over long periods of time (TA Instruments [41]). The detection limit of 0,5 μW and the baseline stability (over a period of 24 h) of ±0,2 μW characterize the instrument.

1.4 Advantages of probiotic products on an animal health level

The well-being of domestic animals is closely related to their feeding. Correct feeding should result in a fit and healthy pet. In addition to providing nutritional value, feed composition influences the intestinal microflora and may lead to or prevent gastrointestinal disorders. Therefore, knowledge of the gastrointestinal tract and digestion processes of healthy animals is integral to the understanding of a practical feeding practice. As carnivores, cats and dogs are characterized by a short digestive tract and a rapid flow rate of the bolus of food. The number and composition of this endogenous flora tend to be rather stable, although age and, to a lesser degree, feed may modify it. Gastric acidity, bile, intestinal peristaltics and local immunity are factors thought to be important in the regulation of bacterial flora in the small intestine of human beings and various other mammals.

Often canine and feline gastrointestinal disorders are linked to bacterial overgrowth and the production of enterotoxins produced by pathogenic bacteria. During the last few years, research has focused on some valuable strains of Lactic Acid Bacteria (LAB) and their potential use as probiotic agents. Probiotics are considered to be viable microbial preparations, which promote mammalian health by preserving the natural microflora in the intestine. Probiotics are believed to attach to the intestinal mucosa, colonize the intestinal tract and thereby prevent attachment of harmful

microorganisms thereon. A prerequisite for their action resides in that they have to reach the gut's mucosa in a proper and viable form and especially do not get destroyed by the influence of the low pH prevailing in the stomach. In particular, the physiology of the digestive tract of cats and dogs differs from humans. For example, the average pH in the stomach is about 2,4 for 5 dogs (dependently on breed type) and 4,2 for cats. Needless to say that there is a need to provide pet food compositions that are still able to quickly restore the loose feces conditions (defecation and diarrhea) of the pets after shelf-life of months. Rapid recovery of defecation and diarrhea to more neutral stool conditions is of both great health and general nutritional concern.

2. EXPERIMENTAL METHODS USED

2.1 Method for the viscosity measurements of oils

The SI physical unit of dynamic viscosity is the Pascal-second (Pa·s), which, in base SI units, is kg·m−1·s−1. If a fluid with a viscosity of one Pa·s is placed between two plates, and one plate is pushed sideways with a shear stress of one Pascal, it moves a distance equal to the thickness of the layer between the plates in one second. The cgs physical unit for dynamic viscosity is the poise. It is more commonly expressed, particularly in ASTM standards, as centipoise (cP). The relation between poise and Pascal-seconds is: 1 cP = 0,001 Pa·s = 1 mPa·s. Water at 20°C has a viscosity of 1,0020 cP.

If not stated otherwise, this dissertation uses the term dynamic viscosity to mean dynamic viscosity at 20°C. Likewise, the change in dynamic viscosity of an oil is expressed as Δ Pa·s/°C. Also, the change in dynamic viscosity of an oil is described as the difference between the dynamic viscosity at 25°C and 20°C (Pa·s at 25°C - Pa·s at 20°C = ΔPa·s).

Equipment: Dynamic rheometer Physica MCR 301 (Anton Paar GmbH, Germany), C-PTD200 Peltier temperature control and CC27 coaxial cylinder measuring system (in/out diameter 26,66 and 28,92 mm).

Method: The viscosity of the oils was measured at a turning speed of 180 RPM; at temperature range of 5 to 50°C, heating rate was 0,5°C/min, viscosity was registered after each 1°C. Two parallels of samples were measured. The Table 1 lists the average viscosity (Pa·s) of the oils as follows: 1. Crude fish oil, 2. Salmon oil A, 3. Refined maize oil, 4. Cod liver oil, 5. Salmon oil B, 6. Soybean oil (with antioxidant), 7. Sunflower oil (with antioxidant), 8. Linseed oil, 9. Borage oil, 10. Salmon oil and bacteria suspension (heated), 11. Salmon oil and bacteria suspension (cooled), 12. Raw salmon oil (heated), 13. Raw salmon oil (cooled).

2.2 Determination of Total Viable Count

Total Viable Count (TVC) expressed in colony forming units (CFU) per gram of product was used to asses shelf-life and stability of the probiotic microorganism in the final product (see Figure 9). These were measured under the following conditions: refrigeration (6-8^{o}C), room temperature (21±3^{o}C) and accelerated temperature (37±1^{o}C). The relative humidity was also recorded: refrigeration 39% RH, room temperature 52% RH and accelerated temperature 54% RH.

As a general rule, under the accelerated temperature, one month of accelerated stability results is equivalent to 3 months of real time stability (x3 time faster).

2.3 Determination of the shelf-life of the products

2.3.1 Determination of the dog food shelf-life

Shelf-life test of the produced vacuum coated probiotic dog food was performed by usage of a surface inoculation (*outplating*) technique. Stability of the product was tested for Total Viable Count (TVC) to determine the number of Colony Forming Units (CFU) per gram of product with the usage of PCA (Plate Count Agar, Scharlau, Spain) under the following conditions:

- Refrigeration (6-8^{o}C) with 39% RH
- Room Temperature (21±3^{o}C) with 52% RH
- Accelerating temperature (37±1^{o}C) with 54% RH

40 grams of product sealed in final type of product packaging (i.e. foil sachet) was made available for each monthly test at and placed under the different temperature testing conditions given above.

The most important part of the sample preparation is that myristic acid is used as a diluent since this diluent ensures that the microorganisms contained within the oil component (probiotic vehicle) of the kibbles are 'dissolved' sufficiently in the final product sample.

Samples were taken at 0 day, 2 week, 1 month, 2 month, 3 month, 4 month and 5 month. Each measuring point was performed in three parallels and an average was calculated. Average TVC counts of probiotic vacuum coated pet food product during the 5 months shelf-life testing at different temperature conditions are presented in Figure 9.

2.3.2 Determination of the human product shelf-life

Coated with different suspensions (different oil carriers in different products) ready for consumption, probiotic infused products were sent to a laboratory (*Eclipse*, UK) to determine the Total Viable Count and a continuous probiotic viability measurements under the shelf-life trial of 2 months (see Table 6, 7). All samples were shipped in sterile *Falcon* tubes each containing approximately 5 g of sample. Each parallel was measured on 0 day (immediate) count, 2 weeks, 1 month, 2 months after the production. Each parallel was placed under 3 different storage conditions: refrigerated condition temperature of 6-8^{o}C, standard condition temperature of 18-24^{o}C, and high (accelerated) temperature conditions 36-38^{o}C. High (accelerated aging) temperature conditions are considered 3 times faster than processes at standard conditions, meaning that 2 months result at accelerated condition temperature equals to 6 months result at standard temperature conditions characterizing product stability at room temperature for 6 months.

2.3.3 Determination of the probiotic stability in the final product by microcalorimetry

Various extruded dog food formulations enriched with the probiotic ingredient *Enterococcus faecium* (NCIMB10415) produced by developed vacuum infusion were analysed *in-situ* by means of a multichannel microcalorimeter to evaluate the microbial stability and product shelf-life. A TAM III (24-channels, *TA Instruments*, US), a heat conduction multi-channel microcalorimeter was used to monitor the growth of cells in dog food and bacteria concentrate samples. The TAM III was used in static ampoule mode (batch process) (see *Wadsö* [20]). The calorimetric trials were performed at a fixed temperature of incubation at 37°C (equivalent of dogs body temperature [42]). All the samples/analytes were placed into autoclaved 3 mL microcalorimetric ammpules following all the aseptic rules. Ampoules were hermetically sealed with a cap and placed into the TAM III system. Before beginning the microcalorimetric experiments the TAM III microcalorimeter was calibrated and the baseline signal measured. All the calorimetric experiments were carried out in three parallels and the power-time curves obtained were normalized per gram of dog food. Averaged power-time curves of three parallel runs were used for the further analysis.

The maximum specific growth rate, heat produced during different growth phases, and lag–phase duration were all determined. The length of the lag-phase that can be used to determine the time necessary for the probiotic ingredient to restore its activity after consumption of probiotic containing extruded products was also measured. The calorimetric data of the extruded pet food product was analysed to increase the preservation of bacterial viability and product shelf-life.

Three commercially available dog food products differentiated by dog age (adult and senior) and by taste (salmon and chicken formulations) were used in the experiments: *ProBiotic LIVE Adult Chicken & Rice* (AC), *ProBiotic LIVE Adult Salmon & Rice* (AS) and *ProBiotic LIVE Senior Chicken & Rice* (SC). All dog food samples used in the experiments were initially received in hermetically closed separate 1,5 kg bags, with expiry date/lot. nr. 23.11.2012 and used freshly in the middle of the claimed product shelf-life period. All bags were aseptically opened immediately before the experiments. Additionally, a dry bacterial concentrate of the same strain of the bacteria as in the dog food (*Enterococcus faecium* NCIMB10415) provided by *Probiotics International* Ltd. (UK, Batch No BN 29094, expiry date 11.2013) was studied as a reference sample. The bacterial concentrate contained 10^{10} CFU per gram of the powder according to the producer.

Dry dog food kibbles of different types were ground and sifted to fine particles (less than 0,05 mm). Afterwards, samples were weighted in sterile plastic Falcon tubes. The mass of each sample was approximately ~0,5 g. Samples were aseptically transferred into 3 mL autoclaved microcalorimetric ampoules and

ampoules were weighted. Depending on the experimental conditions, sterile *MilliQ* water, adjusted to pH=2 (dog stomach equivalent pH [43]), or to pH=7 (neutral value), was added to the ground dry dog food in mass ratio 1:1. pH was measured with pH meter S20 Seven Easy Mettler Toledo with calibrated InLab 413 Mettler Toledo electrode. All the solutions were previously sterilized in an autoclave at 121°C for 15 min. As a reference, samples of dry non-diluted bacterial concentrate were also analysed. In order to determine the initial bacterial concentration in dog food kibbles outplating on PCA (Plate Count Agar, Scharlau, Spain) was carried out for all three dog food formulations and dry bacterial concentrate. Petfood calorimetric data are presented in the publication VI.

2.4 Means of pet feces management using probiotic pet food

Fecal management is an important issue in evaluation of a probiotic feed products. Special methodology for evaluating pet feces using developed probiotic pet food was worked out in the current thesis (see publication IV).

2.4.1 Purina and Bristol feces scoring system

The *Purina* feces scoring system was developed by *Nestle* S.A., *ProPlan Purina* for and similar scoring systems for pets and refers to the evaluation of stool samples based on visual characteristics, as the *Bristol Stool Chart* developed by *Dr. Ken Heaton* at the *University of Bristol* [44] as a medical aid designed to classify the form of human feces into seven categories. The scoring is from 1 to 7 going from hard and dry at 1 to no texture and watery at 7:

1-A) Very hard and dry, no residue left on the ground when picked up.
2-B) Firm, not hard. Little residue left on the ground when picked up.
3-C) Log-like, moist surface, leaves residues but holds form when picked up.
4-D) Very moist, long shape leaves residues and loses form if picked up.
5-E) Very moist, present in piles, distinct shape, leaves residues and loses form if picked up.
6-F) No defined shape, but has texture, occurs as spot or pile, leaves residues if picked up.
7-G) No texture, watery, flat, occurs as puddles.

The optimal score is considered to be 4. The probiotic product is considered to be successful in this dissertation if it improves the feces condition of a dog to score 3 and 4 of the *Purina* and *Bristol* feces scoring system (Figure 3 and 4).

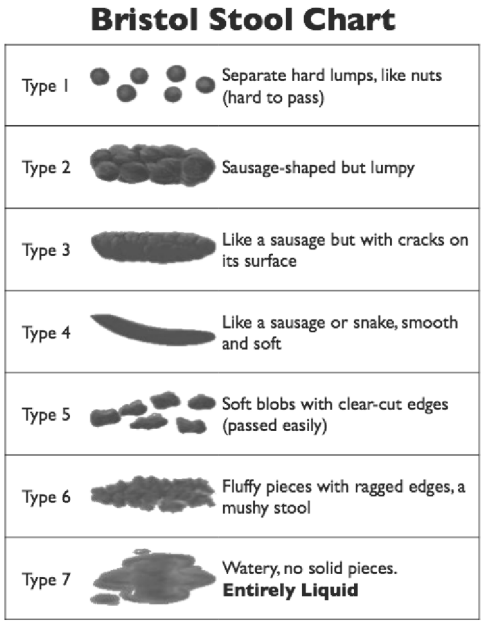

Figure 3. Bristol fecal 7 score stool chart [44].

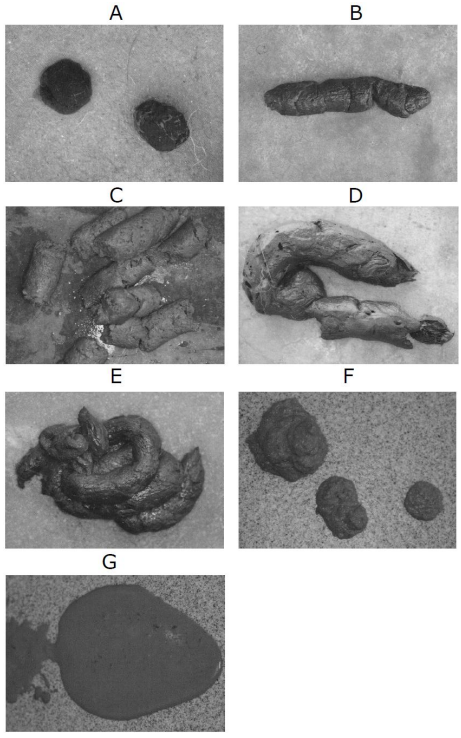

Figure 4. ProPlan Purina 7 score fecal stool scoring system (Brochure of Nestle S.A., Vevey, Switzerland, 2010).

2.4.2 Setup of the field - trial panel

Eight dogs were selected based on specific individual health problems. The nature of their disease, determined by a veterinarian, was the main criteria for selecting these dogs.

All dogs were adult dogs between 9 months and 4 years old, both males and females from the breeds *Boxers, Malinois Sheppard, Weimaraner*, and *English Bulldog*.

Time schedule of the trials: The selected dog panel received the extruded pet food kibbles for the entire duration of the 5 weeks testing period.

Controls during trials: For the entire duration of the trials the following people have checked that the protocol was exactly executed. Veterinarian: full check-up of all the dogs before the trials started, determination of the possible disease, at week 3 a complete health check-ups were performed on all dogs, evaluation of the feces during the entire period of the trials, control of the check-lists, evaluation of skin condition, evaluation of coat condition, evaluation of vitality level, taking pictures of dogs and all visual aspects of the trials. Feeding was checked daily by official investigators, weighing of feed was done by official controllers under supervision of the veterinarian. All data was filled into the appropriate forms by the investigators. The following aspects were checked during this test: feces quality based on the quotation system of *ProPlan Purina* and the *Bristol Stool Chart* similar to quotation system used (see Figure 3 and 4), general health improvement, skin condition, coat condition, behavior and vitality. Filed trial data is presented in the publication IV.

3. DEVELOPMENT OF THE TECHNOLOGY

3.1 Choice of the suitable probiotic microorganism

The microorganism *Enterococcus faecium* NCIMB10415 was used in the current work as a probiotic ingredient for feed product (dog food) manufacturing. This organism is on the list of *EU* authorized feed additives under the class of zootechnical additives (subclass of gut flora stabilizers). The minimum and maximum legal concentrations of this organism in feed products are $1x10^9$ CFU/kg and $3,5x10^{10}$ CFU/kg, respectively, according to *European Commission Regulation* (EC) No 1520/2007 of 19 December 2007 (Annex III) [45]. This means that the feed product should contain the above mentioned probiotic microorganism at concentrations of at least $1x10^9$ CFU/kg of the product over its shelf-life.

3.2 Choice of the suitable oil based on the viscosity measurements

Results: The measured viscosities of various oils typically used to produce feed and food productions are given in Table 1.

Table 1. Viscosity parameters (Pa·s) of various oils at different temperatures.

Temperature vs Oil type	5°C	10°C	15°C	20°C	25°C	30°C	35°C	40°C	45°C	50°C	Δ visc (20 - 25°C)
1. Crude fish oil	0,117	0,095	0,075	0,060	0,049	0,040	0,034	0,028	0,024	0,021	0,011
2. Salmon oil A	0,119	0,097	0,077	0,061	0,048	0,040	0,033	0,028	0,024	0,021	0,013
3. Refined maize oil	0,133	0,106	0,083	0,066	0,053	0,044	0,036	0,031	0,026	0,022	0,013
4. Cod liver oil	0,119	0,096	0,075	0,061	0,049	0,041	0,034	0,029	0,025	0,021	0,012
5. Salmon oil B	0,116	0,093	0,074	0,059	0,048	0,040	0,033	0,028	0,024	0,021	0,011
6. Soy bean oil (with AO)	0,115	0,092	0,073	0,059	0,048	0,040	0,033	0,028	0,024	0,021	0,011
7. Sunflower oil (with AO)	0,130	0,104	0,081	0,065	0,053	0,043	0,036	0,030	0,026	0,022	0,012
8. Linseed oil	0,101	0,082	0,065	0,053	0,043	0,036	0,030	0,026	0,022	0,019	0,010
9. Borage oil	0,114	0,092	0,072	0,058	0,047	0,039	0,033	0,028	0,024	0,020	0,011
10. Suspension (heating)	0,119	0,097	0,075	0,059	0,048	0,039	0,033	0,028	0,024	0,02	0,011
11. Suspension (cooling)	0,107	0,084	0,067	0,054	0,044	0,037	0,031	0,026	0,023	0,02	0,010
12. Raw salmon oil (heating)	0,155	0,095	0,073	0,058	0,047	0,039	0,032	0,027	0,023	0,02	0,011
13. Raw salmon oil (cooling)	0,106	0,083	0,066	0,053	0,044	0,036	0,031	0,026	0,022	0,02	0,009

One of the oils, *Salmon oil A* (supplied by *United Petfood Producers* (UPP) Belgium), displays unique viscosity properties in comparison with the other oils tested in the present experiment. Although the viscosity of *Salmon oil A* at refrigerating temperatures is higher than the remaining fish oils, in the temperature range of 20-25°C *Salmon oil A* loses viscosity faster (ΔPa·s/°C) with increasing temperature than the other oils tested (see Figure 6). The change in the viscosity (ΔPa·s/°C) of crude fish oil (supplied by *United Petfood Producers* (UPP) Belgium), cod liver oil (supplied by *United Petfood Producers* (UPP) Belgium) and *Salmon oil B* (*Vobra Special Petfoods* BV, Netherlands) is basically the same within the temperature range of 20-25°C.

Salmon oil A was chosen as the carrier oil (vehicle) for preparation of a probiotic/oil suspensions for manufacturing probiotic extrusion products by vacuum inclusion of the suspension. *Salmon oil A* was preferred due to the viscosity properties in the temperature range 20-25°C (see Figure 6) and its organoleptic/nutritional parameters (see Section 3.3.1.1). The manufacturing process is performed over a temperature range of 20-25°C and the use of *Salmon oil A* will avoid the clotting of spraying tips (nozzles) in the vacuum coater and thus aids in ensuring a homogenous distribution of probiotics in the carrier oil. Additionally, the oil/probiotic mixture is constantly mixed in the tank before introduction into a vacuum coater, and the use of *Salmon oil A* avoids the formation of a probiotic flakes (non suitable for a vacuum coating) during mixing.

The viscosity of the oils analysed are roughly equal at temperatures above 40°C, however, this temperature is too high to ensure the viability of probiotic bacteria, and consequently to preserve the probiotic load in the final product. The substances added to the oil affect the properties of the oils such as the viscosity. Accordingly, the properties of the oil must be taken into account when choosing a vehicle for the infusion of probiotic microorganisms. Care should also be taken to ensure that the substances added to the oil in the preparation of the oil/probiotic suspension do not severely affect other important parameters of the suspension, such as the viscosity.

3.3 Preparation of the probiotics and oil suspension - vehicle for the probiotic microorganisms

A suspension is obtained by mixing probiotic microorganisms in a dry powder form having a total concentration of between 10^9-10^{16} CFU/kg dry powder, into an oil. The inclusion rate for the final suspension should be 3,3-6,7 kg of the probiotic powder per 100 kg oil (choice of the *Salmon oil A* oil was described above in Section 3.2). The probiotics must be slowly mixed into oil to avoid precipitation. Thus, not all of the freeze- dried powder should be added at once. To maintain the viability of the probiotics, the temperature of the suspension should not exceed 30°C. The mixing may be performed in a mixing tank, such as an IBC container, under continuous stirring. Preferably, the final suspension is transferred to a

storage tank with a mixer. Transfer from the mixing tank to the storage tank is preferably done through a bottom outlet in the mixing tank into the storage tank via gravity to avoid excess shear in the pump. The suspension is then mixed in the storage tank at a temperature of 15-29°C, not exceeding 30°C (the mixing may be performed by rotation at 5-350 RPM) to obtain a suspension of homogenously dispersed probiotic microorganisms. The suspension should not be stored for longer than 3 hours in the storage tank before it is used in a vacuum infusion process. If the suspension is stored for a longer time the suspension may become contaminated.

The suspension is prepared as follows:

a) A powder with at least one probiotic microorganism in a dry powder freeze-dried form with a total concentration of 10^9-10^{16} CFU/kg is added to the oil,
b) An oil with a low peroxide value and a high ratio of poly-unsaturated fatty acids is used,
c) A suspension is prepared by adding 3,3 to 6,7 kg of the probiotic microorganism powder per 100 kg oil and stirring at room temperature,
d) The bacteria power is added gradually to the oil (probiotic vehicle) while continuously mixing/stirring the oil at room temperature in a storage tank.

The total concentration of at least one probiotic microorganism in the dry powder form should be between 10^{10} to 10^{13} CFU/kg. Between 3,3 to 6,7 kg of the probiotic microorganism powder per 100 kg is added to the oil in the container and mixed between one and three hours.

The suspension is applied to the feed product by vacuum infusion taking into account at least the concentration of the probiotic microorganisms in the suspension, loss of probiotics in the line of manufacturing, and calibrated accordingly to obtain a product within the range prescribed by the *EU Commission Regulation* (see Section 3.1).

The suspension obtains a final concentration of 10^9 - 10^{13} CFU/kg in the oil and has a dynamic viscosity of less than 0,061 Pascal-second (Pa·s) at 20°C.

The feed and food products developed in this dissertation are dog food products and ready-to-eat breakfast cereals for human consumption.

3.3.1 Suspension/vehicle parameters for extruded feed product manufacturing purposes

A good choice of oil as a probiotic compound carrier (oil vehicle) is based on the viscosity of the specific oil and the temperature, which is needed to achieve particular viscosity. It was shown experimentally at the full scale pet food factory

(UPP) during the development of the current technology that a suitable viscosity of the probiotic ingredient vehicle (suspension) for feed and food manufacturing purposes needs to be preserved at 0,061 Pa·s or less (see Figure 6). This viscosity of the probiotic ingredient vehicle helps to achieve a suitable pumping speed to spray the suspension onto the extruded product in the vacuum infusion tank, and thus achieve good dispersion of the probiotic carrier into the matrix of the product with minimal effects on the probiotic bacteria count and stability while minimizing production time. Together with the physical/chemical parameters of the oil, which can have an influence on the viability of the probiotics, the organoleptic properties of the specific oil is also an important factor determining the overall product taste and odour. In addition, nutritional parameters also need to be considered and will be analysed below.

3.3.1.1 Suspension/vehicle organoleptic, nutritional and viscosity parameters for extruded feed products

Organoleptic parameters: In case of a probiotic dog food, a salmon oil/bacteria suspension is used to produce an extruded dry dog food. The choice of the salmon oil was based on the eating preferences of dogs which are determined 90% by smell. Thus, it is very crucial to find an oil vehicle for a probiotic compound, which will not have an influence on a palatability of the final product (dog food). *Salmon oil A* (see Section 3.2) fulfils the organoleptic requirements of dogs.

Nutritional parameters: Together with the above mentioned parameters, the oil used as a vehicle for probiotics needs to be "healthy". High content of saturated fatty acids, trans fatty acids and etc. are generally considered as "unhealthy". The high concentration of saturated fats minimizes the probiotic effect of the ready product and increases the risk of coronary heart disease by raising levels of "bad" LDL cholesterol and lowering levels of "good" HDL cholesterol. Salmon oil is well known for its unique composition of polyunsaturated fatty acids (omega 3 and omega 6) and thus is generally considered "healthy".

Viscosity: To select a specific salmon oil variety that also fulfils the criteria for being suitable for vacuum infusion, the viscosity of different salmon oils were compared. As shown in Table 1, not all salmon oils have the same viscosity properties. The viscosity of *Salmon oil A* decreases faster between 20°C and 25°C than does *Salmon oil B* (difference in viscosity at 20-25^{o}C of both oils is 0,002 Pa·s). This provides an extra advantage of using *Salmon oil A* as a carrier (oil vehicle). Oils with lower viscosity in the desired temperature range improve the mixing ability of the suspension and spray more evenly onto the kibbles. In addition, lower viscosity reduces sedimentation and waste during manufacturing and improves probiotic stability within the finished product.

Considering all the data on *Salmon oil A,* this oil is considered suitable for the purposes discussed herein. It is to be understood that although the present example refers to dog food, *Salmon oil A* is also used in other products for animal consumption.

3.3.1.2 Viscosity of probiotic and oil suspension as vehicle for vacuum infusion of extruded feed product

Because the viscosity of the final suspension is a key parameter for vacuum infusion the influence of the bacteria on the viscosity of the oil must be considered. Table 1 (lines 10-13) and Figure 5 show that the influence of the bacteria on the final viscosity at different temperatures is minimal. The difference between the cooling and heating is likely due to residual heat in the analysed samples. Table 1 (lines 10-13) shows the viscosity of the raw salmon oil vs. suspension viscosity at heating from 5°C to 50°C and backwards cooling from 50°C to 5°C. At the current inclusion rate, which was used for the production of pet food, the viscosity difference between both samples (suspension and raw oil correspondingly, see Figure 5) is with average of 0,001 Pa·s at each temperature step. Δ visc. (20-25°C) of raw oil is 0,011 Pa·s at heating phase and 0,009 Pa·s at cooling phase. Δ visc. (20-25°C) of suspension is 0,011 Pa·s at heating phase and 0,010 Pa·s at cooling phase.

One can conclude that a change of Δ visc. (20-25°C) of both samples (suspension and raw oil) at cooling and heating phases induces a 0,01 Pa·s change in viscosity, on average. In general there will be a variation between different measurements of the viscosity of a specific type of oil between different batches. The results obtained in the current thesis show that the viscosity of the oil/suspension is an important parameter for the production of vacuum infused probiotic products.

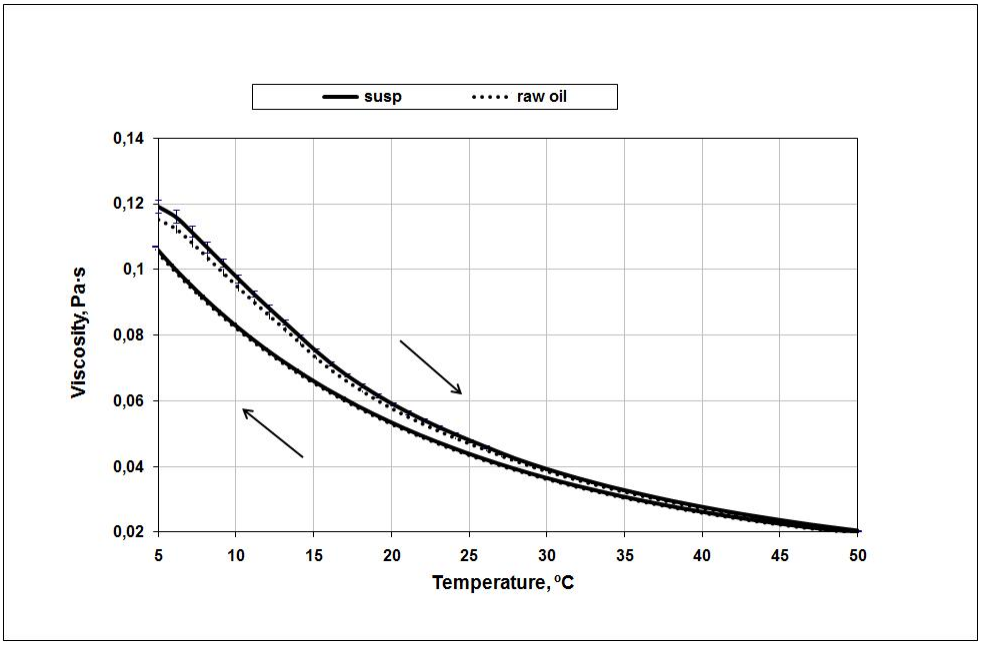

Figure 5. Viscosity of raw oil and suspension at various temperatures at cooling and heating. "Susp" (solid line) indicates the data on suspension of Salmon oil A with probiotics concentration/inclusion rate of 1,2 kg/ton of final product. Raw oil (dashed line) indicates the data of raw Salmon oil A without probiotics. Top lines show the viscosity when the temperature is increased from 5 to 50°C, whereas the bottom lines show the viscosity when the temperature is decreased from 50°C to 5°C. In the bottom lines the dashed and solid lines are practically positioned on top of each other.

To find a salmon oil, which also fulfils the criteria for being suitable for vacuum infusion, the viscosity of different salmon oils (including *Salmon oil A* and *Salmon oil B* from various producers) were compared. As shown in Figure 6, the viscosity of *Salmon oil A* decreases faster (see slope of the solid line) between 20°C and 25°C than does *Salmon oil B,* thus giving an extra advantage of usage of *Salmon oil A* as a carrier (oil vehicle) of probiotic compounds.

In Summary, *Salmon oil A* is a suitable oil vehicle for vacuum infusion of probiotics for an extruded food and feed products.

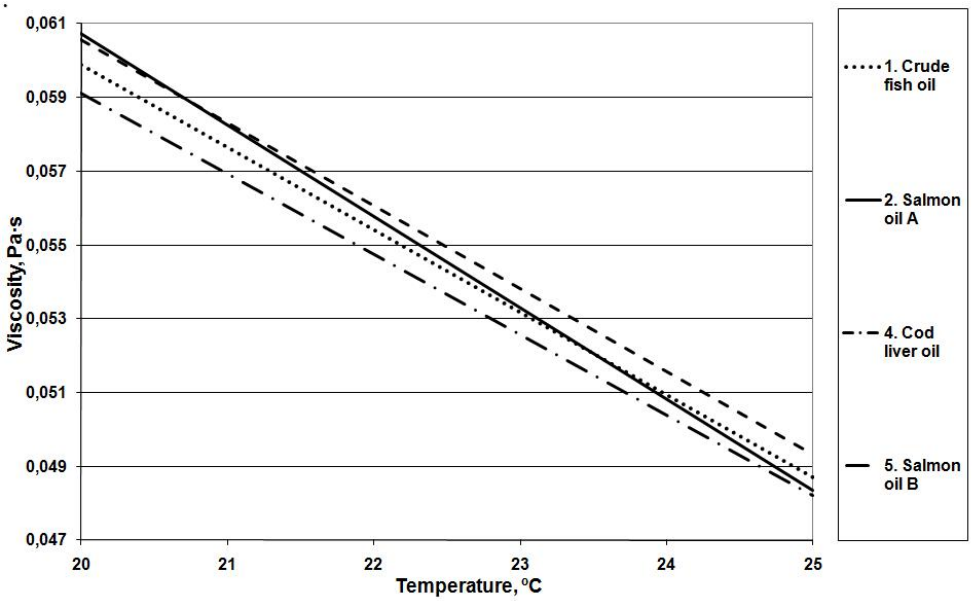

Figure 6. Viscosity of selected oil types versus temperature within the temperature interval of 20-25°C.

3.4 General scheme of production unit

A full-scale production plant of *United Petfood Producers plc* (UPP), *Ghent, Belgium* (see. Figure 7 and Figure 8, screen print-outs of the factory production control unit) was used to produce the probiotic pet food products described in this dissertation. The first part of the full-scale production plant consists of silos where different raw ingredients, such as various grains, pulp, meat meal and etc. are stored. The required amount of the ingredients, according to a confidential formula, are weighted, mixed and grinded to form a meal which is mixed with the water and steam and further then extruded (Figure 7, A). After extrusion, the kibbles are transferred to a drying chamber (Figure 7, B) where the moist of the kibbles is being regulated up to 6-8% for further vacuum coating procedure.

The production plant consists of a separate standing storage tank to hold the probiotic suspension, connected to a dosage tank (weighting box) for adding the probiotic suspension. The dosage tank is connected to a vacuum infusion tank (Figure 8, A) with spraying nozzles. To infuse more than two suspensions or solutions at a time without having to change the contents of the storage and dosage tanks we came up with the following scheme: three sets of storage and dosage tanks are used to dose a probiotic suspension, a fat solution, and a chicken digest (smell enhancer) solution. All of the dosage tanks are connected to a vacuum infusion tank by separate spraying nozzles leading into the vacuum infusion tank (Figure 8, A).

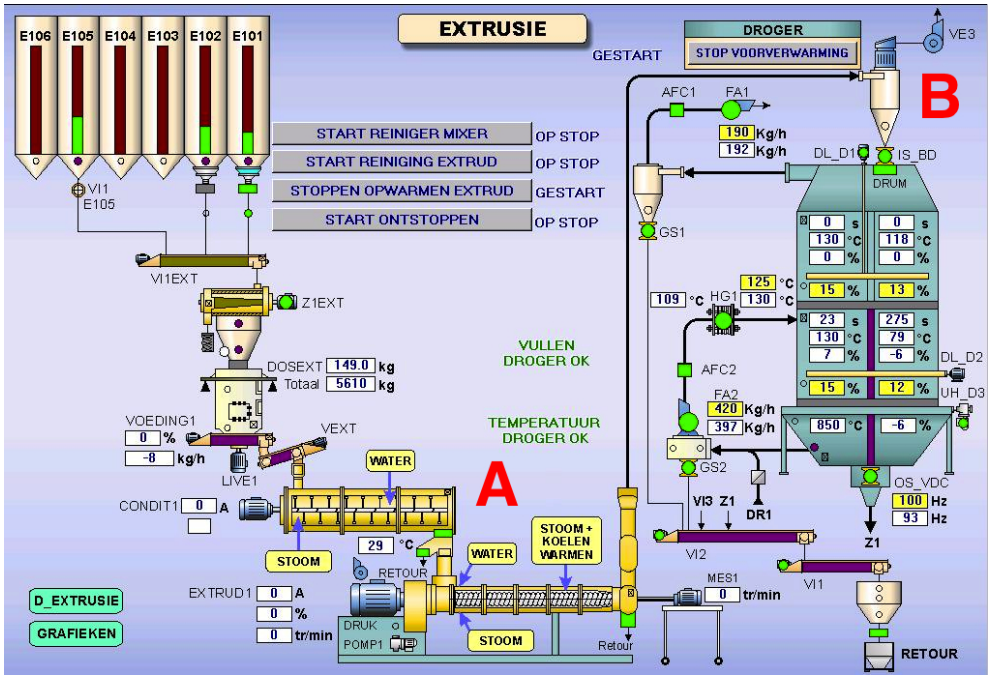

Figure 7. Full-scale production plant (UPP) – extrusion process.

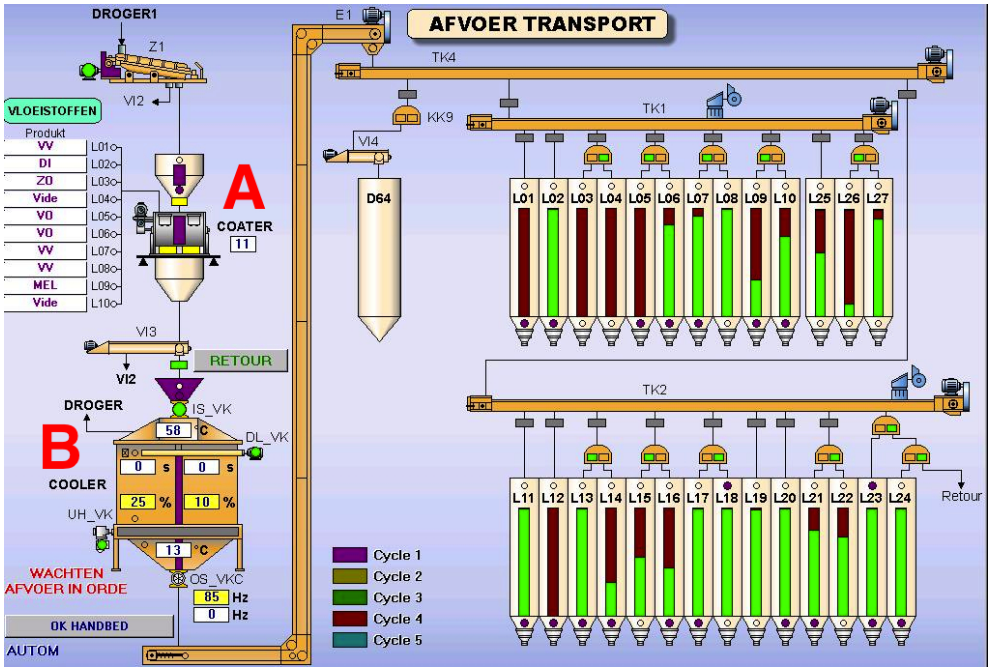

Figure 8. Full-scale production plant (UPP) – vacuum infusion process.

The probiotic suspension is kept separate from the other components to be vacuum infused into the product to ensure optimal viability of the probiotic suspension. Also, to avoid intermixing of the probiotic suspension with other liquid solutions, which may lower the viability of the probiotic microorganisms the probiotic suspension dosage tank (weighting box) is connected with its own set of spray nozzles to the vacuum infusion tank (Figure 8, A). This also avoids precipitation and clotting of the spraying nozzles. Additionally a rotational mixing tank of the probiotic suspension with an impeller is required to make and mix the probiotic suspension. By positioning the mixing/storage tank above the suspension dosage tank (weighting box), the suspension is transferred without the aid of a pump, which typically reduces bacterial viability.

The form and shape of the nozzles was optimized for the solution/suspension being vacuum infused. The vacuum infusion tank (Figure 8, A) typically has one or more openings to receive a food product. When the food product is in place in the tank the following occurs:

a) reduction of the pressure in the vacuum infusion tank to 0,2-0,95 bar,
b) introducing liquid solutions through the dosage controlling tanks (weighting box) and one or more sets of spraying nozzles at, e.g. a temperature of 15-29^{o}C,
c) restore pressure to 1 bar.

These steps are repeated with other components to be vacuum infused. Pressure cycling is important to ensure subsequent solutions are properly infused into the product. The release of the vacuum is usually performed slowly to avoid abrupt changes in pressure that may be harmful to the product and/or the probiotics.

To ensure an even distribution in the vacuum infusion tank, mixing of the product is required. Mixing is performed during the vacuum inclusion steps directly in the inclusion tank (Figure 8, A) and after the addition of each ingredient.

After the vacuum coating, the kibbles are transferred to a cooling chamber (Figure 8, B) where vacuum coated kibbles are cooled to the ambient temperature. At the end of this step product is ready for being packed into the consumable/commercial packaging.

3.4.1 Spraying nozzles orifice

Because different solutions are being sprayed onto the food or feed products, optimal spraying is required. The orifice of each of the spraying nozzles is located on top of the vacuum infusion tank (Figure 8, A). Each nozzle has a cross-sectional area of between 1-250 mm^2, which can be regulated to the optimal cross-section up to 1-3 mm^2 for achieving the best spraying with the optimal spraying angle. The importance of having optimal nozzles for each type of solution is that

the efficiency of spraying depends on the orifice of the spraying nozzles and the viscosity of the solution. Furthermore, spraying also depends on the speed that the solution is passed through the nozzle. Thus, not every infusion line needs to have the same type of spraying nozzle.

3.4.2 Mixing tank

To maintain the viability of the probiotics and optimal properties of the probiotic vehicle during the whole process of vacuum infusion, correct handling of the solution is required, including the use of gravity during probiotic suspension fluid transfer. When the suspension is transferred to the storage/mixing or dosage tank it is important not to supply too much force to the suspension because it may result in a loss of viability of the probiotics (flakes or precipitate formation).

3.4.3 Vacuum suction unit

The connection between the storage/mixing tank and the dosage tank (weighting box) should not be a vacuum or have positive pressure because pressure swings may be harmful to the viability of the probiotics. Furthermore, by minimizing the surfaces the probiotics come in contact with, loss of probiotics due to sticking to the surfaces of long tubes is avoided.

3.4.4 Mixing

It is important that the probiotics stay and become evenly distributed in the suspension when the suspension is held in the storage tank. To avoid sedimentation of the probiotics, this tank has mixing capabilities such as a rotating impeller, a rotating mixing tank, or a combination of an impeller and a rotating tank.

3.4.5 Opening for applying uncoated product

The vacuum infusion tank should also be able to receive the food product to be infused, typically through an opening directly connected to a drying device (Figure 7, B). This means that the food product to be infused may have a temperature above ambient when it enters the vacuum infusion tank (Figure 8, A). More solutions/suspensions infuse into the product when the product has a temperature of 20-50°C, typically 30-45°C, without resulting in significant loss of viability of the probiotics. The temperature of the product decreases during the coating of it with the liquid solutions, which have lower temperature than the products itself.

3.4.6 Control of pressure

The vacuum infusion tank (Figure 8, A) can be adjusted to pressures in the range of 0,01-1,5 bars, typically it is operated in the range of 0,2–0,95 bars. By having the possibility also to increase the pressure above 1 bar, a larger pressure difference may be achieved following pressure release, which may result in a better vacuum infusion.

3.4.7 Temperature control

It is important to control environmental conditions during production, and these should have a positive influence on viability of the probiotics. The probiotic suspension is kept in the temperature range of 15-29°C. Probiotics are, in general, sensitive to temperature variation so good control is required. To provide products, which have a constant viability count between different batches, temperature control of the tanks, which hold the probiotics is required.

It may be difficult to control the production plant manually, because many steps require multiple adjustments simultaneously. A control unit is used for controlling temperature throughout the process, the inlet and outlet valves, the amount of liquid sprayed through the nozzles, the pressure in the vacuum tank, and mixing time. Figure 7 and 8 are a screen print-outs of the factory production control unit in control of the production of the pet food at *UPP* full-scale factory.

3.5 Production process of extruded products

Taking all these factors into consideration, an effective production scheme using vacuum infusion technology was developed and extruded pet food kibbles with probiotic microorganisms present throughout the entire matrix of the product (pores) produced.

The factory set-up parameters are described below. All the technological aspects of the oil suspension preparation and production plant structure are covered within the proprietary technology owned by *Bacterfield* Ltd. and covered by patent applications I and II.

The required amounts of the ingredients (confidential commercial information) are weighed, ground, and mixed to form a meal. The meal is mixed with an appropriate amount of water and steam and followed an extrusion process were kibbles are formed. Dried kibbles are vacuum coated with liquid ingredients, including the salmon oil probiotic suspension. The vacuum coated kibbles are cooled (Figure 8, B) prior to packaging.

All raw ingredients used in the dry meal are ground with a sieve of 1 mm and the average particle size should not exceed 1,5 mm. The moisture level should be at 10,48% in the meal. The extrusion speed is set to 3800 kg/h to receive kibbles with a density from 360-380 g/L. The dryer temperature is set to 120°C and the moisture of kibbles after sieving stage is 6,20%. The ratio of probiotic bacteria in the end product is set at 1,2 kg per ton of product. Freeze-dried *Enterococcus faecium* NCIMB 10415 (E1707) probiotic bacteria powder with $1x10^{13}$ CFU/kg (from suppliers certificate of analysis) is pre-ordered for production (*Probiotics International* Ltd, UK). Laboratory analysis of freeze-dried *E. faecium* probiotic bacteria powder show an average concentration of $1,4x10^{13}$ CFU/kg in the raw probiotic powder used in the particular production.

The probiotic ingredient and its carrier, as well as the production batch and factory setup parameters, ensure that correct amounts of suspension are prepared for the vacuum infusion procedure. Suspension (in case of a pet food product) makes a 3% of the end product. *E. faecium* probiotic bacteria concentration in the suspension was measured prior to vacuum infusion procedure with an average of $1,08x10^{11}$ CFU/kg of the suspension.

Samples were taken immediately after vacuum infusion to determine the probiotic count and found to contain $2,05x10^{9}$ CFU/kg of product.

Vacuum infused dog food kibbles are cooled. After cooling, kibbles at 21°C contain more than $1,27x10^{9}$ CFU/kg. Moisture after cooling stage has been recorded at 8,50%.

During the last production stage the product is placed in a silo (upfront cleaned) before packaging and samples from the silo product are sent to an accredited Belgium laboratory for *Weende* analysis [46] (also known as proximate analysis, quantitative method to determine different macronutrients in feed). Results of *Weende* analysis are presented in Table 2.

Table 2. Weende analysis.

Moisture:	7,80%	Hygienic parameters:	
Dry matter:	92,20%	Salmonella (/25g):	absent
Crude ash:	7,52%	Enterobacteriaceae (/g):	< 10
Crude fiber:	2,31%	Clostridium perfringens (/g):	< 10
Crude protein:	24,68%		
Crude fat:	11,91%		
Sugar:	0,61%		
Starch:	47,37%		

The preparation of the suspension (oil and bacteria mixture) is carried out at the earliest one hour before the first vacuum infusion procedure to minimize the risk of oxidation. The mixing/storage tank of the suspension comprising an impeller should be completely empty and clean before filling it with the suspension.

Before production, the animal fat and digest are placed into separate storage tanks. For each batch, 500 kg of salmon oil and 18 kg of bacteria powder are added and the suspension is mixed for not less than 1 hour. The mixing speed is set to 180 RPM. At the stage of bacteria powder addition into the salmon oil, the temperature of the oil should be 26^{o}C and during mixing in storage tank, the suspension temperature should be not less than 22^{o}C. The oil used in this particular production batch was a Salmon oil (*International Quality Ingredients BV*, Netherlands). Pressure parameters for the vacuum infusion tank (Figure 8, A) were set up to 650 mbar for chicken fat and digest; and 850 mbar for the suspension. The spraying of the added liquids and suspension was performed in 3 stages:

Stage 1 – animal fat comprising chondroition & glucoseamine,
Stage 2 – salmon oil/bacteria suspension,
Stage 3 – chicken digest as a smell enhancer.

The animal fat and digest were pumped into separate weighting boxes (located at the top of the vacuum infusion tank) prior the vacuum infusion. The suspension was transferred into a separate weighting box (special dosage tank with an implemented impeller in it to keep the suspension homogeneous) until vacuum infusion in vacuum infusion tank. In this way the suspension never comes into contact with the digest and the fat before vacuum infusion in vacuum infusion tank.

3.7 Evaluation of the quality of the products

3.7.1 Post productional probiotic stability measurement

The final product was packed within 3 days after production to avoid all contact with air and any possible loss of bacteria quality/stability. The product was kept in a clean silo with controlled environmental parameters. The empty silo temperature was 19-20^{o}C, whereas the filled silo temperature was 22^{o}C with a product moisture level of 7,73%.

After production, the kibbles were submitted to different analyses in order to guarantee the quality of the products and the probiotic component. Analyses showed that the kibbles had an average concentration of probiotic bacteria within the range from 1,2x10^{9} CFU/kg to 1x10^{10} CFU/kg in the ready product.

Shelf-life test of the produced probiotic dog food confirmed the stability of the dog food for 15 months at room temperature. Probiotic dog food had a probiotic count on a level of $1{,}06x10^9$ CFU/kg in average over the product shelf-life.

Figure 9 provides the average TVC counts of probiotic vacuum coated pet food product over its 5 month shelf-life at different temperatures.

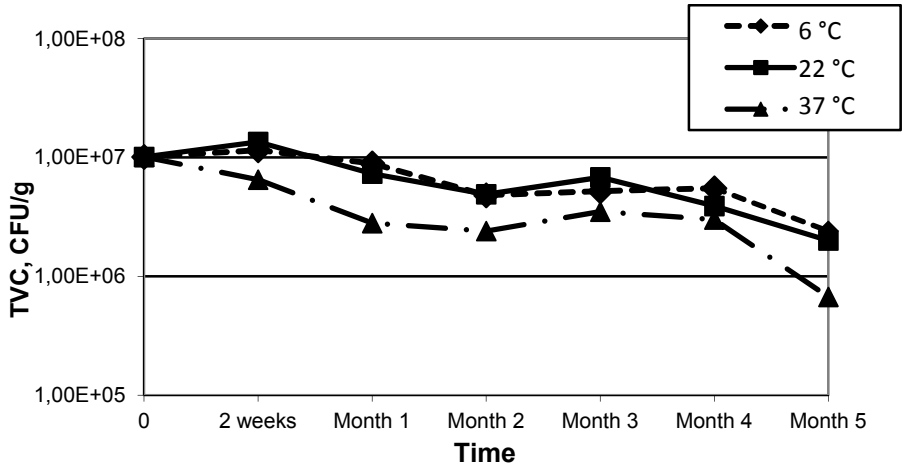

Figure 9. Probiotic bacteria average viability during the 5 month of shelf-life testing of a vacuum coated feed product.

3.7.2 Evaluation of the probiotic stability of the product

The aim was to produce a probiotic feed and food product that preserves the probiotic bacteria concentration and viability at the *EU Commission Regulation* dictated level for a period of one year or more, most preferably at room temperature to reduce storage and logistics costs.

The probiotic microorganism *Enterococcus faecium* NCIMB10415 was used in the current work for feed product manufacturing. Accordingly to the *EU Commission Regulation* the feed product (dog food) should contain the probiotic microorganism in its composition at the sufficient concentration at least $1x10^9$ CFU/kg of the product over the shelf-life of the product.

Total Viable Count (TVC) of vacuum infused dog food samples performed at various time points being kept at refrigeration, room and above ambient (accelerated) temperature conditions (see Section 2.3.1) gave a confirmation of the overall probiotic stability preservation in the final product (see Figure 9). Under the accelerated temperature (37 ± 1^oC), one month of accelerated stability results is equivalent to 3 months real time stability, so 5 months point at the accelerated temperature equals to the 15 month of shelf-life point at the room temperature

condition giving a 15 months probiotic load stability at room temperature in the vacuum infused dog foods produced.

The measured probiotic count on a level of $1{,}06 \times 10^9$ CFU/kg in average during the product shelf-life period corresponds with the product and probiotic stability during the shelf-life period of 15 months and fulfils the requirement of the *EU Commission Regulation* (EC) No 1520/2007 of 19 December 2007 [45].

For the further investigation and characterization of growth of bacteria in solid matrixes with a deeper interpretation of the probiotic stability and viability preservation not only in the final product but also during the passage throughout the gastrointestinal track of host organism a microcalorimetric technique for probiotic stability monitoring was developed and will be described below.

3.8 Development of the microcalorimetric technique for the probiotic stability monitoring

The purpose of this part of the work was the development of a microcalorimetry method to investigate and characterize the growth of bacteria in solid state matrices and opaque media (such as extruded pet food product). To evaluate major growth parameters, including the detectable bacteria concentration, the microcalorimetric trials were first conducted using Lactic Acid Bacteria as a model organism, using a liquid medium (see Publication V).

We studied the growth of *L. lactis* in CRM broth media (CRM - carbohydrate restricted medium) with glucose as the main carbon source using a multichannel thermal activity monitor TAM III. The optical density, concentrations of glucose and lactic acid in the culture media, pH of the culture media, and plate counts of the bacteria were measured in parallel to the calorimetric measurements to obtain the information necessary for the quantitative interpretation of the calorimetric power-time curves - including the peculiarities of growth processes that occur in the bacteria. The methods were applied for the study of growth of *L. lactis* in liquid cultures under different glucose concentrations (2–100 g/L). Growth of the bacteria during both the exponential phase and deceleration phase were analysed separately (deceleration is the phase that combines the very short stationary phase and death phase of cells together with the decrease (deceleration) of metabolic activities).

In all experiments heat flows (P, μW) were measured as power-time curves. The power-time curves obtained (see Figure 11 and 12) were divided into three phases, essentially as ordinary growth curves of bacteria (Figure 10(a)). A lag-phase which determines the adjustment period during which bacterial cells adapt to the new environment and start exponential growth [47] was defined in practice by the sensitivity of the microcalorimeter. In our case growth was observed if there were about 10^5 cells in the ampoule [48]. The second phase of the curve was the

exponential growth phase. In Figure 10(a) it was assumed that the transition to the stationary phase (deceleration phase of growth) was started from the maximum value of the power-time curves.

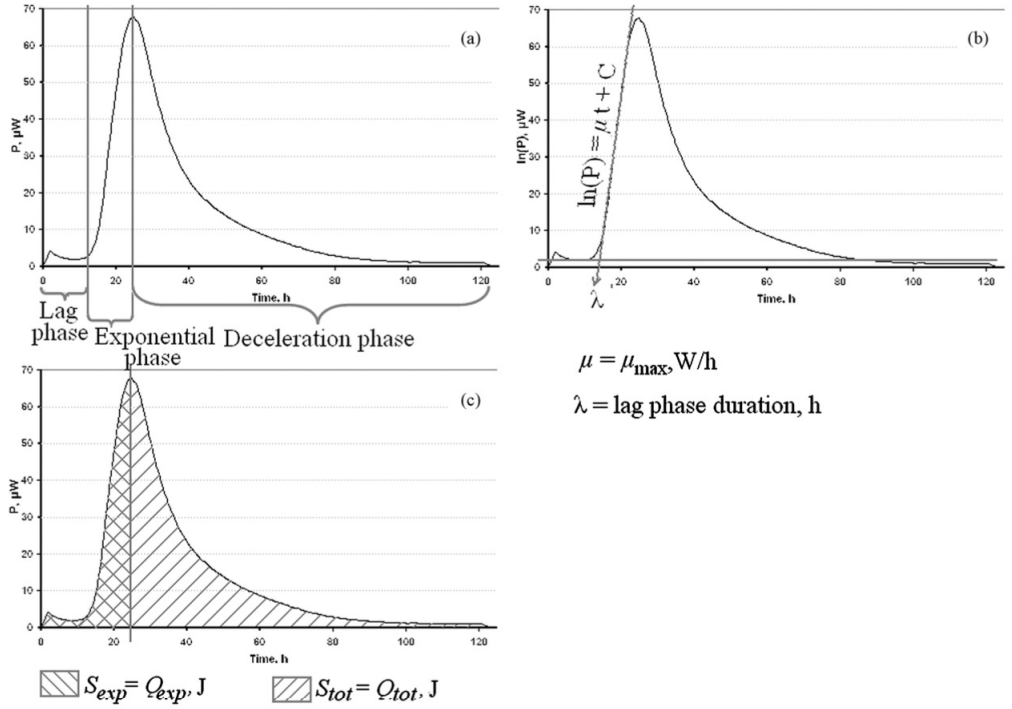

Figure 10. The explanation of the processing of bacteria calorimetric power-time curves: (a) the division of calorimetric power-time curves into three phases - lag phase, exponential growth phase, and deceleration phase; (b) determination of the maximum growth rate (μ_{max}, W/h) and the lag phase duration (λ, h); (c) heat amounts determined - Q_{exp} is the heat produced during the exponential phase and Q_{tot} - the total heat produced during the experiment.

The total heat produced during the growth process (area under the power-time curve, Q_{tot}, J) and the heat produced during the exponential growth phase (area under the power-time curve left of the vertical line, that goes through the peak, and baseline, Q_{exp}, J) (Figure 10(c)) as well as the average maximum growth rate (the slope of the tangent to the exponential phase, μ_{max}, W/h) and lag-phase duration (the crossing point of X-axis and the tangent, λ, h) (Figure 10(b)) were determined using *TAM Assistant* program (v 0.9.1012.40, *SciTech Software* AB, *Thermometric* AB).

Figure 11 provides power-time curves describe the growth of *L. lactis* IL1403 at different glucose concentrations (2, 20, 50, 100 g/L) at the same initial inoculation concentration of 10^2 CFU/mL. Each curve presents an average of three parallel

power-time curves which were measured for the different environmental conditions studied.

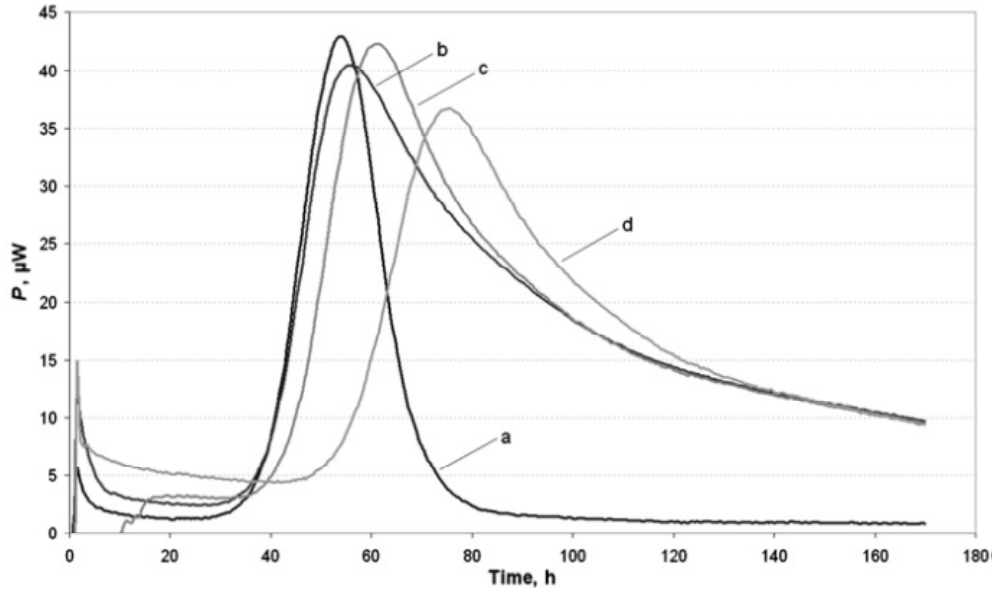

Figure 11. Bacterial growth monitored by calorimetric power-time curves with differing initial substrate (glucose) concentration: a - 2 g/L; b - 20 g/L; c - 50 g/L; d - 100 g/L.

It is evident from Figure 11 that the calorimetric power-time curves measured were different in the case of different initial glucose concentrations. It was shown that the exponential growth of the bacteria was stopped due to the exhaustion of glucose, which occurs at 2 g/L in this strain of *L. lactis*. At higher glucose concentrations, up to 50-100 g/L, exponential growth of the bacteria was terminated at pH 4,3. A number of conversion coefficients were determined which are used in the interpretation of power-time curves in this and possibly future studies of *L. lactis* under other experimental conditions, including solid-state growth.

The results show that calorimetry is a reproducible and sensitive method for the continuous monitoring of bacterial growth. This technique can be used to study bacterial viability and stability measurements as in the various production mixes and in the final product such as extruded kibble. This microcalorimetric method, using power-time curve analysis, was applied to investigate and characterize the growth of bacteria to assess probiotic stability of vacuum infused dog food.

3.8.1 Analysis of the probiotic stability in the final product by microcalorimetry means

Figure 12 presents average power-time curves of *E. faecium* (NCIMB10415) for different dog food formulations with both dry and moisturized samples at two different pH values.

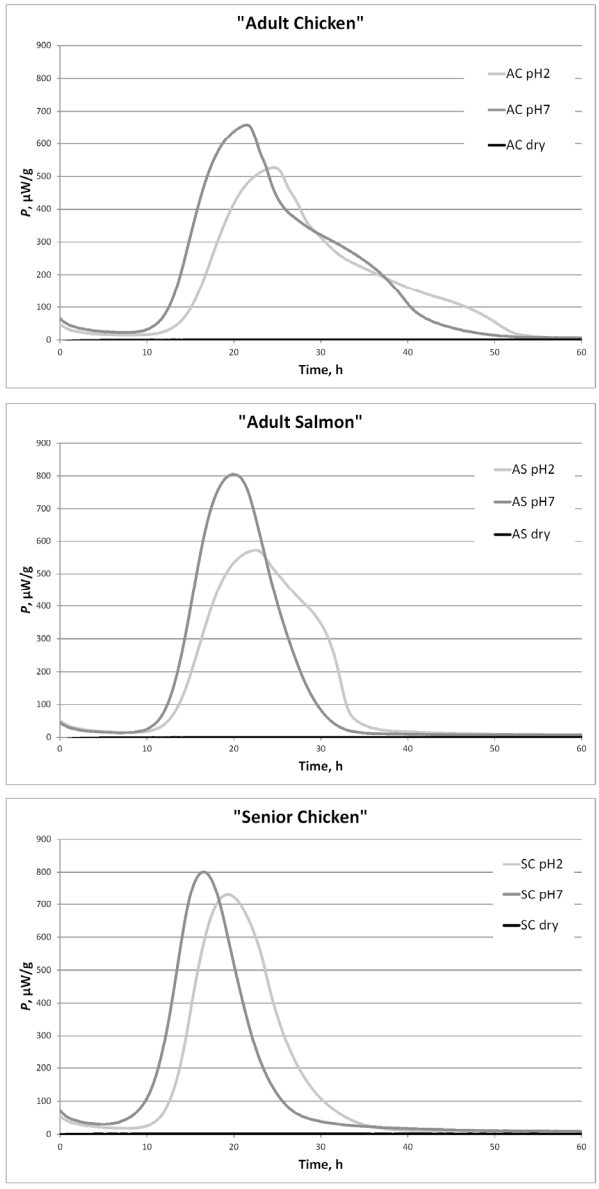

Figure 12. Power-time curves of growth of probiotic bacteria in three different dog food: dry and moisturized samples (AC, AS and SC) at two different pH (pH=2 and pH=7) values.

The standard deviation of the averaged power-time curves is 0,55 μW, thus confirming the high reproducibility of this calorimetric method. Table 3 presents the analysis of these power-time curves.

Table 3. Parameters describing bacterial growth of three dog food formulations (AC, AS, SC) at two different pH (pH=2 and pH=7) levels obtained from the processed power-time curves: lag phase duration (λ, h), maximal specific growth rate (μ_{max}, W/h), heat produced during exponential growth phase (Q_{exp}, J/g) and during the total growth (Q_{tot}, J/g) and the respective numbers of bacteria produced during the both growth phases (N_{exp}, CFU/g and N_{tot}, CFU/g respectively).

		λ, h	σ, h	μ_{max}, W/h	σ, W/h	Q_{exp}, J/g	σ, J/g	N_{exp}, CFU/g	Q_{tot}, J/g	σ, J/g	N_{tot}, CFU/g
pH 2	AC	4,94	0,77	0,45	0,05	14,28	0,86	$5,53x10^9$	37,09	8,58	$1,44x10^{10}$
	AS	5,08	0,49	0,53	0,01	13,89	4,96	$5,38x10^9$	32,18	7,77	$1,25x10^{10}$
	SC	4,68	0,39	0,58	0,00	12,85	0,92	$4,97x10^9$	32,37	1,74	$1,25x10^{10}$
pH 7	AC	3,54	0,33	0,50	0,03	17,24	1,13	$6,67x10^9$	41,62	3,78	$1,61x10^{10}$
	AS	4,73	0,09	0,60	0,04	15,52	1,11	$6,01x10^9$	34,45	2,10	$1,33x10^{10}$
	SC	3,42	0,50	0,51	0,17	13,21	0,47	$5,11x10^9$	31,68	3,40	$1,23x10^{10}$

The length of the lag phase was 3,9±0,7 hours (in average) at pH 7 (neutral pH). The lag phase was prolonged by 1 hour, up to 4,9±0,2 hours (in average) at pH 2 (low pH), which is the pH level in dog stomach [43]. Prolongation of the lag-phase at low pH can be explained by acidic stress effect on the probiotic bacteria.

The average specific maximum growth rate (μ_{max}, W/h) of this *Enterococcus faecium* bacteria was practically the same at low and neutral pH levels (0,52±0,06 W/h and 0,54±0,05 W/h correspondingly).

The average heat produced during the exponential phase (Q_{exp}, J/g) at neutral pH was 15,33±0,90 J/g and 13,67±0,74 J/g at under acidic conditions. Using the Y_Q value $2,58±0,44x10^{-9}$ J/CFU determined by us for *L. lactis* IL1403 [49], the average number of bacteria grown during the exponential growth (N_{exp}, CFU/g - $5,93x10^9$ CFU/g at neutral pH and $5,29x10^9$ CFU/g at low pH) was calculated - see Table 3.

The total heat produced during the entire period of thermodynamic activity (full length of power-time curves registered, all bacteria growth phases included - Q_{tot},

J/g) was also practically the same at both pH values studied. The average total heat produced at neutral pH was 35,92±0,78 J/g and 33,88±1,23 J/g at low pH, which corresponds to $1{,}39 \times 10^{10}$ CFU/g and $1{,}31 \times 10^{10}$ CFU/g, respectively. The numbers of bacteria grown during the exponential growth phase (N_{exp}, CFU/g) and bacteria grown during the complete growth (N_{tot}, CFU/g) achieved the same levels of 10^9 and 10^{10} CFU/g correspondingly at the two pH values.

All three formulations of *ProBiotic LIVE* dog food (AC: Adult Chicken & Rice, AS: Adult Salmon & Rice and AS: Senior Chicken & Rice) enriched with probiotic bacteria of *Enterococcus faecium* (NCIMB10415) had the same initial bacterial count with an average of 10^6 CFU/g confirmed by *outplating* of the samples on the PCA presented in Table 4. Average concentrations of bacteria in all three dog food formulations was $2{,}86 \times 10^6 \pm 5{,}31 \times 10^5$ CFU/g according to the results presented in Table 4. This confirms the claim by the producer that the average *E. faecium* load in the product is of 10^6 CFU per gram.

Table 4. Plate counts of the bacteria on PCA and pH values of different dog food formulations and bacteria concentrate.

Sample	N, CFU/g	σ, CFU/g	Bulk pH
AC	$2{,}35 \times 10^6$	$2{,}19 \times 10^4$	5,77
AS	$3{,}41 \times 10^6$	$6{,}17 \times 10^4$	5,87
SC	$2{,}82 \times 10^6$	$5{,}14 \times 10^4$	5,94
Bacteria concentrate	$2{,}24 \times 10^{10}$	$3{,}28 \times 10^8$	5,23

It is known that the low acidic pH in the stomach of dogs is considered to be a barrier for probiotic bacteria. The ability to preserve viability during the passage through the stomach acidic barrier is a prerequisite for the further successful colonization of the probiotic bacteria in the intestinal system of the host. The *Enterococcus faecium* bacteria present in the dog food of *ProBiotic LIVE* formulations were able to show metabolic activity at low pH value, which confirmed that *Enterococcus faecium* can tolerate the passage through the acidic barrier of the dog's stomach and populate the intestine afterwards. The ability of the probiotic bacteria *Enterococcus faecium* (NCIMB10415), to preserve its activity throughout the passage through the stomach acidic barrier of dogs was also confirmed in trials of the *European Food Safety Authority* (EFSA, [50]). Analysis of the power-time curves revealed that the duration of the time needed for the probiotic bacteria to regain their activity in the host organism after consumption (the length of the lag-phase) was about 5 hours (see Table 3). This time is sufficient for the digest (feed) flow to pass the acidic barrier of stomachs of dogs.

There was no bacterial metabolic activity observed in the case of non-diluted dry dog food samples analysed, see Figure 12. Also the freeze-dried *Enterococcus*

faecium (NCIMB10415) bacteria powder concentrate (both dry and diluted samples) showed no thermal activity (data not shown).

The results show *Enterococcus faecim* (NCIMB10145) bacteria, when present in extruded dog food kibbles, is able to preserve its viability and growth characteristics under acidic and neutral pH. This indicates that the extruded kibbles containing *E. faecium* bacteria are able to tolerate the dog's stomach acidic barrier with further successful colonization in the intestine of dog.

Publication VI shows that the calorimetric technique developed in this doctoral work is a sensitive method for the continuous *in-situ* monitoring of bacterial activity in complicated matrices such as extruded feed or food products, and could be applied to assess the shelf-life of probiotic ingredients in the products claiming probiotic activity.

3.9 Evaluation of the possible advantages of probiotic product on an animal health level

Table 5 presents the results of a 32 day probiotic vacuum infused dog food feeding trial based on the fecal score evaluation on a daily basis.

Table 5. 9 dog fecal scores evaluation during 32 day feeding field trial.

Dog day	Nr. 1 score	Nr. 2 score	Nr. 3 score	Nr. 4 score	Nr. 5 score	Nr. 6 score	Nr. 7 score	Nr. 8 score	Nr. 9 score	Average
1	7	5	4	5	4	6	6	7	4	5,3
2	6	5	4	5	5	6	6	4	4	5,0
3	5	5	4	5	5	6	5	6	4	5,0
4	7	5	4	5	5	6	5	6	4	5,2
5	5	5	4	4	4	5	5	5	4	4,6
6	5	5	4	4	4	5	4	5	4	4,4
7	5	5	4	4	4	5	5	5	4	4,6
8	5	4	4	4	4	5	5	5	4	4,4
9	4	4	4	4	4	5	5	5	4	4,3
10	5	4	4	4	4	5	5	5	4	4,4
11	5	5	4	4	4	5	5	5	4	4,6
12	5	4	4	4	4	5	5	4	5	4,4
13	5	4	4	4	4	5	5	4	5	4,4
14	4	4	4	4	4	5	5	5	4	4,3
15	6	4	4	4	4	5	5	5	4	4,6
16	4	5	3	5	4	5	5	5	4	4,4
17	4	5	4	4	4	5	5	5	4	4,4
18	4	3	3	4	4	6	6	5	4	4,3
19	4	4	3	4	4	5	5	5	4	4,2
20	4	4	3	4	3	5	5	5	4	4,1
21	4	3	3	4	3	5	5	5	4	4,0
22	4	3	3	4	3	5	5	5	4	4,0
23	4	3	3	4	3	5	4	4	3	3,7
24	4	3	3	5	3	5	4	5	4	4,0
25	3	4	4	4	3	5	4	4	3	3,8
26	3	4	3	4	3	5	4	3	4	3,7
27	3	3	3	4	3	5	4	3	4	3,6
28	3	4	2	4	3	5	4	3	4	3,6
29	3	3	2	4	4	5	4	4	4	3,7
30	4	3	2	3	3	5	4	5	4	3,7
31	3	3	3	4	3	5	4	4	4	3,7
32	3	3	3	3	3	5	4	3	4	3,4

Based on the results fecal score evaluation of 9 dogs obtained during the 32 day probiotic vacuum infused dog food feeding trial from Table 5 an average fecal score points were taken and a chart was drawn to visualize the fecal score tendency and change over the trial duration of the entire probiotic dog food feeding period (see Figure 13).

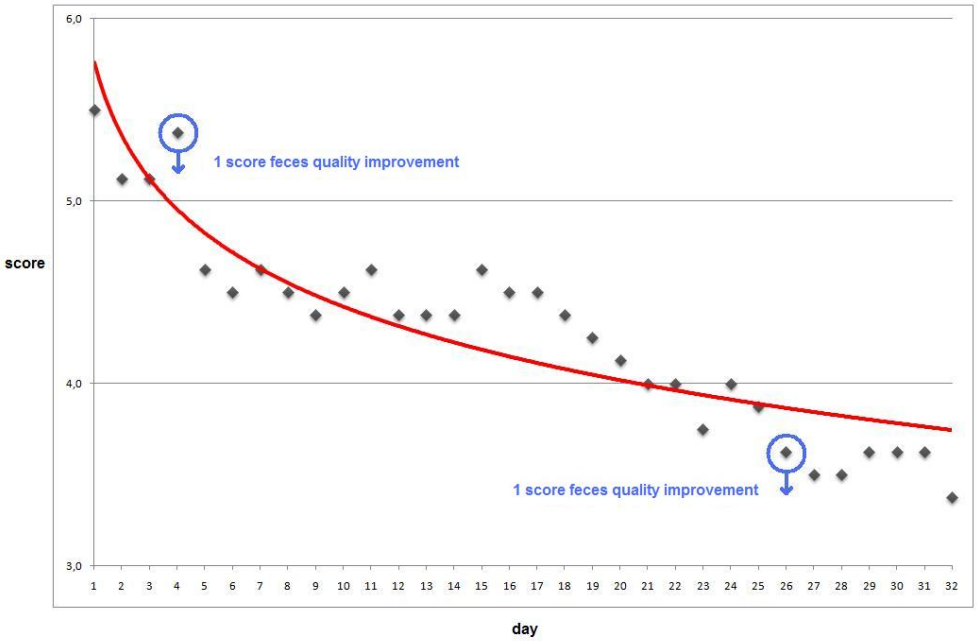

Figure 13. Average fecal score during 32 day of dog feeding field trial.

A logarithmic trend line (in red) can describe the change in fecal consistency over time. Figure 13 shows that the feces quality score improved by one point after the 4th day of consuming the extruded probiotic pet food and one point further improvement after 26 days of consumption. This clearly indicates that consumption of the extruded probiotic pet food reduces the quality scores of diarrhea in fecess by 2 points.

The analysis of the health effects of the extruded dog food with compromising probiotic microorganism during the feeding field trial was based on the following diseases and symptoms:

Diarrhea: several dogs, which were used in these trials were diagnosed with diarrhea. The purpose was to demonstrate the effect of the probiotic ingredient on the intestinal flora of these dogs and thus show that we could improve fecal quality.

Stress diarrhea: the effect of extruded pet food with compromising probiotic microorganism on stress diarrhea has been proven through different dogs, where

we were able to drastically improve fecal quality. Stress diarrhea is very common with dogs that live in breeding farms or dogs which participate in competitions.

Chronic diarrhea: the reasons for chronic diarrhea are often related to food intolerance, which leads to a bad state of the digestive system. These are very common problems with modern dogs, especially when they are pure breed. During these trials we solved almost all problem cases.

Food intolerance: it is only when we have a food intolerance towards one ingredient that the probiotic ingredient is not sufficient. To be able to prove the effect on food intolerance we were advised to select dogs, with the allergy determined without any doubt.

Bad digestive system: there are several breeds of dogs, which have a bad digestive system. The main breeds where this problem occurs are for example the *Sheppard* breeds, the *Bulldog* and *Boxers*. For this reason these dogs were all included in the trials. These dogs have a short digestive system, which causes them to have problems to completely digest their food. As we have seen through these test results, the probiotic ingredient helps these dogs to digest their food better. It also allows the dog owner to give lesser quantities of the food. So we can conclude that the ability of the dog to retain nutrients within the food is much better.

Skin and coat problems: the main cause of skin problems is immunity deficiency. The first result on a dog with a low immunity level is the poor skin quality. The results of these trials show that the probiotic ingredient cannot take away an actual disease such as *demodex* (results not shown). But we were able to reduce the symptoms and the inconvenience for the dog. When we look to the overall state of the skin and coat of the different dogs, which participated in these trails, we can conclude that all dogs showed a better skin and coat quality at the end of the trials.

Vitality problems: dogs with a low immunity level show a low vitality level, however, this is a subjectively determined quality that is close to impossible to objectively measure. For this reason we had to rely on the opinion of the breeder. The overall opinion of the several breeders, which participated in these trials was that the vitality of the dogs was good. On the dog, which received only half of his daily feeding, thanks to the extruded petfood with compromising probiotic microorganism, we were able to see a significant improvement in vitality.

Results of the feeding field trials confirmed (*in-vivo*) the health benefits of the probiotic vacuum infused dog food product on the host organism.

4. TESTING OF THE TECHOLOGY FOR THE HUMAN FOOD PRODUCT PRODUCTION

4.1.Viscosity of probiotic oil suspension as vehicle for vacuum infusion of extruded food products

Because of specific taste parameters demanded by human consumers, vegetable oils were preferred as suitable oil vehicles for vacuum infusion of probiotics into extruded food products. As shown in Figure 14, the viscosity of vegetable oils widely used in human extruded food measured between 20°C to 25°C shows clear differences. Typical oils include maize oil, soybean oil, sunflower oil, linseed oil, and borage oil. As shown in Figure 14 the viscosity of linseed oil decreases faster between 20°C and 25°C than all the other given vegetable oils - at 20°C it has viscosity of less than 0,060 Pascal-second (Pa·s). In addition to the unique physical/chemical and organoleptic parameters of linseed oil such as a slightly nutty taste and fish oil like poly-unsaturated fatty acid profile, there are additional advantages in using linseed oils as a probiotic carrier (oil vehicle) to produce extruded human food product. Linseed oil contains 9% saturated fatty acids, 18% monounsaturated fatty acids, and 73% polyunsaturated fatty acids. For dietary application, oils rich in unsaturated fatty acids are highly preferred due to their health benefits.

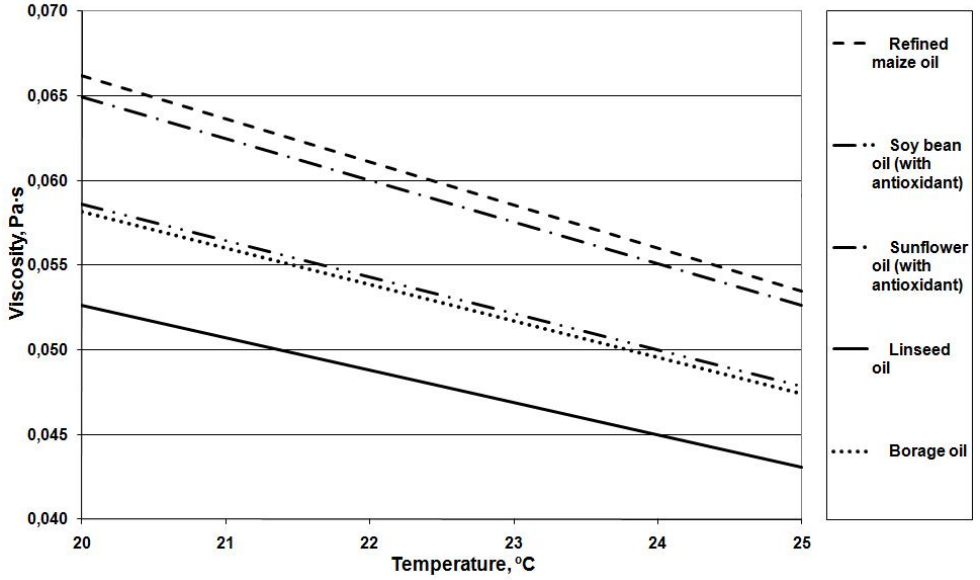

Figure 14. Viscosity of selected vegetable oil types versus temperature within the temperature interval of 20–25°C.

4.1.1 Organoleptic, nutritional and viscosity parameters of suspensions/vehicles for extruded food products

Organoleptic parameters: Use of animal fats/oils in a human product is limited because of the organoleptic parameters, which can have an overall effect on palatability of the final product. Thus, animal oils, such as those found in different types of fish, are not preferred by the end consumers, even if the oil meets other health criteria in terms of the content of poly-unsaturated fatty acids. Thus, the oil used as a probiotic oil vehicle in a human product needs to meet the viscosity criteria required for optimal vacuum infusion but with different organoleptic parameters than the oils used for animal products. Vegetable oils may be suitable candidates.

Nutritional parameters: Instead of using animal oil it may be advantageous also to be able to have a suitable oil vehicle of vegetable origin. Several vegetable oils have positive health parameters. Linseed oil (*Vobra Special Petfoods* BV, Netherlands) compared with soybean oil, maize oil, and sunflower oil is considered as a "healthy" oil with a high concentration of poly-unsaturated fatty acids (omega 3 and omega 6) and mild nutty taste. These parameters make linseed oil a suitable candidate as an oil vehicle for human product manufacturing.

Viscosity: When comparing the viscosity of different oils with oils of vegetable origin in the range of 20°C and 25°C it became apparent that linseed oil has unique properties for its use as an oil vehicle for vacuum infusion of probiotics (see Figure 14). Linseed oil has the lowest viscosity at both 20°C and 25°C out of the vegetable oils analysed. The change in viscosity with temperature of linseed oil is relatively small compared with the other oils tested, however it also has a low viscosity when compared to the other oils. Even when compared to the animal oils (Figure 6), linseed oil has the lowest viscosity at both 20°C and 25°C. Taken together, the viscosity of linseed oil together with its unique physical/chemical and organoleptic parameters makes this oil a good candidate for use as a probiotic oil vehicle for human product manufacturing.

4.2 A pilot trial for production of probiotic product for human consumption using the vacuum infusion technology

The following commercially available formulations were chosen to produce two ready-to-eat extruded cereal products for pilot testing during this doctoral work. These products were infused with products using vacuum infusion technology.

Breakfast cereal 1: Four grain snack (kibbles) breakfast cereal "*Neljavilja-krõbuskid*" (AS *BalSnack International Holding*, Estonia) - ingredients: four grain flakes 70% (rye, oat, wheat, barley flakes), oat brans 20%, fibre (inulin), multivitamins, salt 0,4%. 100 g of the first product contains in average: energy

289,4 kcal (1224,5 kJ), protein 11,3 g, carbohydrates 44,4 g, fat 3,5 g, fibre 10,6 g, sodium 0,004 g.

Breakfast cereal 2: Flakes (pillows) with cinnamon "*Oho*" (UAB *Naujasis Nevėžis*, Lithuania) - ingredients: whole grain wheat and rice flour, sugar, vegetable fats, fructose, cinnamon 1%, salt. 100 g of second product contains in average: energy 438 kcal (1845 kJ), protein 8,0 g, carbohydrates 70,2 g (of which sugars 20,1 g), fat 13,9 g (of which saturated 5,8 g), fibre 2,2 g, sodium 0,36 g.

Both products were vacuum infused by using a probiotic/linseed oil suspension. Oil used in particular pilot trial was linseed oil (OÜ *Tervix*, Estonia). The probiotic vacuum infused product was finally coated with low glycemic index syrup. The commercially available probiotic bacteria formulation *Protexin Balance* (*Protexin Health Care*, UK) and two different low glycemic index syrups of Agave (*Allos* GmbH, Germany) and Maple (*Cofradex* ApS, Denmark) were used. Vacuum infusion was carried out using *Zepter* VG-010 *Vacsy Vacuum Pump* with glass container VG- 011-19 (*Zepter International Group*).

Methods: 150 grams of breakfast cereals per product and per batch were used. Daily dose of probiotics (1 capsules containing $1x10^8$ CFU, accordingly to the producer of probiotic compound) was added per 4,5 g of the carrier (linseed oil or water) making a 3% (usual production ratio) out of the product amount to be infused. *Protexin Balance* multi strain probiotic bacteria - ingredients: FOS (Fructooligosaccharide), probiotic cultures: *Lactobacillus casei, Lactobacillus rhamnosus, Streptococcus thermophilus, Bifidobacterium breve, Lactobacillus acidophilus, Bifidobacterium longum, Lactobacillus bulgaricus*- daily dose of 200 million CFU/per day/2 capsules; $2x10^8$ CFU/day (*Protexin Health Care*, UK, Best before 11.2011, lot nr BN 22962) was gradually introduced into the oil or water carrier to create a homogeneous suspension. Prepared suspensions (oil or water with probiotics) were continuously mixed on a Vortex mixer prior to spraying in order to guarantee the homogeneity of the suspensions.

The spraying of the suspensions and syrups was accomplished using sprinklers. Before the vacuum infusion process, the amount of spray coating (by weight) was determined to receive a 3% coating by the bacteria suspension and 5% coating by the agave or maple syrup coating as a final layer (ratio taken from usual production data of dog food). Prepared suspensions were used for vacuum infusion into the matrix of ready-to-consume (extruded) human products. Multi-strain probiotics containing different suspensions (oil or water) were sprayed on different breakfast cereals appropriately in a ratio of 4,5 g to 150 g of the product (3%). Afterwards, the product was coated with different syrups (agave or maple) sprayed onto different breakfast cereals appropriately in a ratio of 7,5 g to 150 g of the product (5%). The product was mixed during spraying to guarantee an equal dispersion of sprayed suspensions and coating syrups onto the different products used in the pilot

trial. Spraying of the suspensions and mixing was done in one and the same vacuum infusion glass bowl sterilized prior the pilot trial to eliminate the probiotic count reduction and contamination between intermediate processes. The glass bowel was closed with a special vacuum control lid and vacuum atmosphere of 500 mbar by 630 liters/s vacuum pump was created for approximately 40 seconds in the glass bowl containing the product.

All syrups used for the final layer coating (2 stage coating, see below) in pilot trials were preheated to 50^{o}C prior to coating to ensure an appropriate viscosity for spraying. Spraying of appropriate suspensions and the final coating layers were performed in 2 separate stages corresponding to the suspension (linseed oil or water) and syrup type (agave or maple).

Stage 1 (3% of product weight). During the process of vacuum coating, the prepared probiotic suspensions were vaporized onto the appropriate product and vacuum pressure of 500 mbar was created for approximately 40 seconds. Normal atmospheric pressure (1 bar) conditions were restored inside the vacuum infusion device (glass bowl) by gradual opening of the pressure control system.

Stage 2 (5% of product weight). Preheated up to 50^{o}C final coating layer (agave or maple syrup) was vaporized onto the product and vacuum pressure of 500 mbar was created for 20 seconds. Normal atmospheric pressure (1 bar) conditions were restored inside the vacuum infusion device (glass bowl) by gradual opening of the pressure control system.

All different product coatings with different suspensions were performed in 3 parallel experiments. All experiments were performed at room temperature. The products coated with different suspensions were sent to a laboratory for a Total Viable Count (TVC) determination and analysis of a shelf-life during 2 months. All samples were shipped in sterile Falcon tubes each containing approximately 5 g of sample.

4.2.1 Measured parameters of probiotic human food product

Measurements: Bacterial counts in each parallel was measured immediately after production (day 0) and subsequently, 2 weeks, 1 month, and 2 months after production. Each parallel was placed under 3 different storage conditions: refrigerated conditions of 6-8^{o}C, standard conditions of 18-24^{o}C, and accelerated conditions 36-38^{o}C. Accelerated temperature conditions induced 3 times faster aging, meaning that after 1 month of storage the bacterial count was the same as after 3 months of storage under standard conditions. This test shows that the product is stabile at room temperature for 6 months. All the TVC measurements of the raw materials used are presented in Table 6 and all TVC measurements of

performed shelf-life analyses are presented in Table 7. Results in Table 6 and Table 7 are averages of 3 parallels per suspension and final coating layer of both products (Breakfast cereal 1 and 2) analysed in these pilot trials.

Table 6. TVC measurements of used raw materials, CFU/g.

Breakfast cereal 1 bulk	10	Linseed/bacteria suspension	$1{,}60 \times 10^8$
Breakfast cereal 2 bulk	40	Water/bacteria suspension	$1{,}20 \times 10^8$
Raw bacteria powder	$9{,}00 \times 10^{10}$		

Table 7. TVC measurements of shelf-life analysis, pillows and kibbles, CFU/g.

Breakfast cereal 1 - kibbles, TVC, CFU/g						
Probiotic suspension carrier	Final coating layer	Storage condition temperature, °C	Bacteria count at different time stages			
			Day 0	2 weeks	1 month	2 months
Water	Agave	6-8	$8{,}67 \times 10^5$	$9{,}83 \times 10^5$	$1{,}18 \times 10^6$	$1{,}17 \times 10^6$
		18-24	$8{,}67 \times 10^5$	$9{,}03 \times 10^5$	$8{,}73 \times 10^5$	$7{,}67 \times 10^5$
		36-38	$8{,}67 \times 10^5$	$7{,}07 \times 10^5$	$4{,}87 \times 10^5$	$2{,}13 \times 10^5$
Linseed oil	Agave	6-8	$7{,}73 \times 10^5$	$7{,}97 \times 10^5$	$7{,}47 \times 10^5$	$8{,}13 \times 10^5$
		18-24	$7{,}73 \times 10^5$	$8{,}80 \times 10^5$	$9{,}93 \times 10^5$	$9{,}13 \times 10^5$
		36-38	$7{,}73 \times 10^5$	$5{,}63 \times 10^5$	$4{,}63 \times 10^5$	$6{,}70 \times 10^5$
	Maple	6-8	$1{,}63 \times 10^6$	$1{,}16 \times 10^6$	$1{,}13 \times 10^6$	$1{,}14 \times 10^6$
		18-24	$1{,}63 \times 10^6$	$1{,}00 \times 10^6$	$1{,}02 \times 10^6$	$9{,}23 \times 10^5$
		36-38	$1{,}63 \times 10^6$	$6{,}53 \times 10^5$	$5{,}57 \times 10^5$	$3{,}37 \times 10^5$
Breakfast cereal 2 - pillows, TVC, CFU/g						
Probiotic suspension carrier	Final coating layer	Storage condition temperature, °C	Bacteria count at different time stages			
			Day 0	2 weeks	1 month	2 months
Water	Agave	6-8	$9{,}00 \times 10^6$	$5{,}00 \times 10^6$	$3{,}45 \times 10^6$	$3{,}66 \times 10^6$
		18-24	$9{,}00 \times 10^6$	$3{,}00 \times 10^6$	$2{,}63 \times 10^6$	$3{,}51 \times 10^6$
		36-38	$9{,}00 \times 10^6$	$1{,}03 \times 10^6$	$8{,}73 \times 10^5$	$5{,}17 \times 10^5$
Linseed oil	Agave	6-8	$9{,}60 \times 10^5$	$9{,}20 \times 10^5$	$9{,}13 \times 10^5$	$8{,}53 \times 10^5$
		18-24	$9{,}60 \times 10^5$	$1{,}00 \times 10^6$	$1{,}25 \times 10^6$	$1{,}10 \times 10^6$
		36-38	$9{,}60 \times 10^5$	$7{,}23 \times 10^5$	$6{,}47 \times 10^5$	$2{,}60 \times 10^5$
	Maple	6-8	$3{,}16 \times 10^6$	$2{,}23 \times 10^6$	$1{,}50 \times 10^6$	$1{,}51 \times 10^6$
		18-24	$3{,}16 \times 10^6$	$2{,}54 \times 10^6$	$1{,}95 \times 10^6$	$1{,}67 \times 10^6$
		36-38	$3{,}16 \times 10^6$	$8{,}93 \times 10^5$	$8{,}07 \times 10^5$	$5{,}07 \times 10^5$

4.3 Evaluation of probiotic human food products

The pilot trial indicates that the initial Total Viable Count of bulk commercially available breakfast products (see Table 6, Breakfast cereal 1 and 2 bulk) show dramatically lower counts than at the end of the pilot trial after introducing the probiotic bacteria suspension into the matrix of products (Table 7, 0 day count). This indicates that the particular technology used for the vacuum infusion of the breakfast products (kibbles and pillows) developed in this work is also suitable for the manufacture of probiotic breakfast products. Additionally, the shelf-life study results (see Table 7) indicate that both products used in particular pilot trial (Breakfast cereal 1 and 2) have good stability (up to 6 months at the room temperature) and all the Total Viable Count (TVC) fluctuations at different storage temperatures of different suspensions (probiotic vehicles) and final coating layers stay within 1 log value from the initial amount.

In summary, the pilot trial results (see Publication III), of all the products used in current pilot trial, together with different suspensions (probiotic vehicles), and final coating layers used, are able to maintain a probiotic count at a sufficient level up to 6 months. These results indicate that different types of extruded food products for human consumption (e.g. Breakfast cereal 1 and 2) may be vacuum infused with probiotics by using the technology developed in this dissertation while maintaining a high TVC over a reasonable shelf-life.

5. CONCLUSIONS

1. During the course of this work, technology to produce extruded food and feed products containing probiotic microorganisms was developed and implemented commercially. The products are marketed as super premium pet food products in various *European Union* countries under the brand names of *ProBiotic LIVE* and *Formula ProBiotic*.

2. A novel vacuum infusion method using oil suspensions as carriers was developed to incorporate probiotic bacteria into the product matrix without causing negative impacts on other production steps. An optimal oil viscosity in the range of 0,061 - 0,048 Pa·s was determined for the vacuum infusion process. Other properties of the carriers, which were both essential to ensure that the probiotic ingredient were sufficiently viable, and that the end product had marketable properties, were also identified and taken into account.

3. The optimal parameters for the maximal preservation of the probiotic viability of the end products were determined, and realized in the production processes. The viability of the probiotic ingredients was found to be stable for 15 months storage at room temperature with the average concentration of probiotic bacteria exceeding 10^9 CFU/kg, thus meeting the requirements of the *EU Commission Regulation*.

4. Evaluation of the quality of the extruded feed product containing probiotic microorganisms was carried out in animal trials.

5. A novel microcalorimetry method was developed to confirm the stability of the probiotic ingredient and product shelf-life. The calorimetric data confirmed preservation of microbial viability/activity and load *in-situ* during the storage time specified by the producer. The microcalorimetric method for the evaluation of viability and activity of the probiotic microorganisms in the functional food and feed products developed is widely applicable in the field.

6. Efficacy of the developed vacuum infusion technology was confirmed on a pilot scale trial carried out with breakfast cereals currently available on the market for human consumption.

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ABSTRACT

We report the development of technology to produce extruded food and feed products containing probiotic microorganisms incorporated by means of vacuum infusion of an oil suspension. The production scheme involves preparing a probiotic microorganism suspension taking into account also organoleptic and nutritional components. It was found that the viscosity parameters of the oil suspension needed to be 0,061 Pa·s or lower to function correctly in the processes equipment. The development and delivery of this suspension was optimized to increase the shelf-life of the products and the probiotic count and viability.

The production scheme and operating conditions were optimized. The factory scale production scheme of an extruded vacuum infused food products was developed and implemented, and the product is currently being marketed under the brand names of *ProBiotic LIVE* and *Formula ProBiotic*. Development of the production technology involved evaluating the health benefits of the product on a animal health level (diarrhea feces/stool quality improved to a score of 4).

A human food product pilot trial run on a laboratory scale based on the same vacuum infusion methodology was performed and optimized. It was shown that in this case the viscosity of the oil suspension should be 0,061 Pa·s or lower. The trials included measuring also the overall food products organoleptic and nutritional parameters, the probiotic bacteria count, and preservation of viability over the shelf-life of the products.

Designing the production scheme involved understanding the relationships between probiotic ingredients with all the other ingredients, as many interactions have an influence on the overall probiotic stability of the products. The count and viability change over the time, which influence the total health benefits should be controlled and maintained.

A microcalorimetric method for bacteria viability monitoring and determination of the shelf-life of these products was developed. The microcalorimetry based method may be applied widely also in other areas of the probiotic food and feed sector.

KOKKUVÕTE

Käesolevas töös on välja töötatud probiootilisi baktereid sisaldavate toiduainete ja loomasöötade tootmise tehnoloogia, mis põhineb bakterite õlisuspensiooni vaakuminfusioonil. Uurimised näitasid, et vaakuminfusiooniks sobiva probiootiliste bakterite õlisuspensiooni valmistamiseks on oluline, et suspensiooni viskoossus oleks väiksem 0,061 Pa·s. Töös optimeeriti samuti õlisuspensiooni kasutamise ja vaakuminfusiooni tingimused, et saavutada toodete vajalikud organoleptilised omadused ja eluiga.

Töötati välja probiootilisi baktereid sisaldavate ekstrudeeritud toodete tööstusliku tootmise tehnoloogia ning see ka realiseeriti. Kasutades väljatöötatud tehnoloogiat toodetakse erinevatele koertele sobivaid ekstrudeeritud "krõbuskeid" rahvusvahelisele turule kaubamärkide *ProBiotic LIVE* and *Formula ProBiotic* all. Toodete hindamine söötmiskatsetes näitas, et nad on kõrge kvaliteediga ning tervistavate omadustega.

Inimtoiduks sobivate probiootilisi baktereid sisaldavate ekstrudeeritud toitude tootmise pilootkatsed näitasid, et väljatöötatud vaakuminfusiooni tehnoloogia sobib ka nende valmistamiseks. Inimtoiduks sobivate toitude tootmiseks töötati välja selleks sobivad õlisuspensioonid ning leiti nii organoleptiliste omaduste seisukohalt kui ka toodete säilivusaja pikendamiseks optimaalsed tingimused.

Väljatöötatud tehnoloogiale on taotletud intellektuaalomandi kaitset optimaalselt disainitud PCT patendiperekonna näol, mis praeguseks ajaks on viidud rahvuslikku faasi USAs, Jaapanis, Euroopa Liidus ja mujal.

Probiootilisi baktereid sisaldavate toodete aktiivsuse ja eluea hindamiseks võeti kasutusele mikrokalorimeetria ning töötati välja vajalikud metoodilised alused. Kasutades mikrokalorimeetriat, näidati, et väljatöötatud tehnoloogiaga toodetud "krõbuskid" sisaldavad elujõulisi baktereid vastavalt tootja poolt antud spetsifikatsioonidele. Töös saadud tulemused näitavad, et mikrokalorimeetria kasutuselevõtmine probiootiliste bakterite elulemuse ja aktiivsuse hindamisel neid sisaldavate toitude ja muude preparaatide puhul on suure perspektiiviga ettevõtmine.

PUBLICATION I

International application published under the patent cooperation treaty (PCT), Title: Probiotic oil suspension and use thereof, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122107 A1 International publication date: 28.10.2010.

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(54) Title: PROBIOTIC OIL SUSPENSION AND USE THEREOF

(57) Abstract: The present invention provides probiotic/oil suspension for vacuum infusion of an extruded probiotic food product characterized by balancing the requirements of a high concentration of the probiotic micro-organism in the suspension and physical properties that makes the suspension applicable for vacuum infusion. Further provided are methods of preparing said suspension and use thereof in the manufacture of an extruded probiotic food product.

Probiotic oil suspension and use thereof

Technical field of the invention

The present invention relates to the provision of a probiotic/oil suspension for vacuum infusion of an extruded probiotic food product. Further, the invention relates to a method of preparing the probiotic/oil suspension. Finally, the invention relates to a method of using the probiotic/oil suspension in the manufacturing of extruded probiotic food product and the extruded probiotic food product obtainable by the method.

Background of the invention

Various commercial attempts have been made to achieve food compositions containing probiotic micro-organisms with prolonged viability for long term storage, many of these do not provide sufficient efficacious levels of viable probiotic micro-organism due to issues associated with susceptibility of the micro-organism to standard commercial pet food manufacturing procedures such as extrusion. For example, efforts of coating or filling standard pet food kibbles with probiotic micro-organisms have been suggested but, in practice, often prove impractical.

WO 01/95745 provides a method of producing a food product (kibbles) characterised by a porous structure, comprising an instable substrate such as a probiotic micro-organism in an oil solution, which are included in a flowable form into the product by means of a step of "partial vacuum" followed by normalizing the pressure by releasing an inert gas into the vessel.

WO 05/070232 provides a method of producing a food product similar to WO 01/95745, further characterized in that the oil should have a solid fat index of at least 20. WO 05/070232 discloses the essential use of fat with the solid fat index of the vehicle is at least 20 at 20°C and the preferred vehicle are coconut oil and even more preferred palm oil.

WO 03/009710 discloses system and method for on-line mixing and application of surface coating compositions for food products; an apparatus is also disclosed. The apparatus comprises a dry matter – liquid mixing module (wherein the dry matter may be probiotics) connected inline to a liquid – liquid mixing module,

wherein one or more liquid can be mixed into the first liquid (potentially comprising the probiotics).

Summary of the invention

Thus, an object of the present invention relates to the provision of probiotic/oil suspension for vacuum infusion of an extruded probiotic food product characterized by balancing the requirements of a high concentration of the probiotic micro-organism in the suspension and physical properties that makes the suspension applicable for vacuum infusion.

Thus, one aspect of the invention relates to a suspension for vacuum infusion of an extruded probiotic food product, wherein said suspension comprises an oil and at least one probiotic micro-organism in the concentration of 10^6-10^{16} CFU/kg of said oil, and said suspension having a dynamic viscosity of less than 0.08 pascal-second (Pa·s) at 20°C.

In an alternative aspect the invention relates to a suspension for vacuum infusion of an extruded probiotic food product, wherein said suspension comprises an oil and at least one probiotic micro-organism in the concentration of 10^6-10^{16} CFU/kg of said oil, and wherein said oil having a dynamic viscosity of less than 0.08 pascal-second (Pa·s) at 20°C.

The suspension of the invention is adapted to the vacuum infusion process by selecting suitable components for the suspension and balancing the components in the suspension to obtain a suspension suitable for application of the suspension on the food product by spraying the suspension on the product under vacuum.

Another aspect of the present invention relates to a method of preparing a suspension of the invention, said method comprising:

a) providing at least one probiotic micro-organism in a dry powder form having a total concentration of 10^9-10^{17} CFU/kg dry powder

b) providing an oil

c) adding 0.3 to 15 kg of said probiotic micro-organism powder per 100 kg oil to said oil in an container at continuously stirring at RT to make a suspension premix

d) transferring the suspension premix of c) to a storage tank comprising mixing means with the proviso that the transfer is not by vacuum suction

e) mixing said premix suspension RT to obtain a suspension of homogenously dispersed probiotic micro-organism

and obtaining said suspension.

Yet another aspect of the present invention is to provide a method of producing an extruded food product comprising at least one probiotic micro-organism, wherein said probiotic micro-organism is homogenously distributed throughout the structure of the food by vacuum inclusion of the suspension of the invention.

Still another aspect of the present invention is to provide an extruded probiotic food product obtained by said method of producing an extruded food product.

A final aspect of the present invention relates to use of the suspension of the invention for the preparation of a extruded probiotic food product, wherein said suspension comprises an oil and at least one probiotic micro-organism in the concentration of 10^7-10^{17} CFU/kg of said oil and said suspension having a dynamic viscosity of less than 0.08 pascal-second (Pa·s) at 20°C.

Claims

1. A suspension for vacuum infusion of an extruded probiotic food product, wherein said suspension comprises an oil and at least one probiotic micro-organism in the concentration of 10^6-10^{16} CFU/kg of said oil, and said suspension having a dynamic viscosity of less than 0.08 pascal-second (Pa·s) at 20°C.

2. The suspension according to claim 1, wherein the peroxide level of said oil is not more than 2 meq O_2/kg.

3. The suspension according to any of the preceding claims further comprising an antioxidant.

4. The suspension according to claim 3, wherein said antioxidant is selected from the group consisting of natural antioxidants and synthetic antioxidants.

5. The suspension according to claim 4, wherein said natural antioxidant is an antioxidant selected from the group consisting of flavanoids, cartonoids, tocotrienol, tocopherol and terpenes.

6. The suspension according to claim 5, wherein the antioxidant is astaxanthin.

7. The suspension according to claim 4, wherein the antioxidant is selected from the group consisting of BHA and BHT.

8. The suspension according to any of the preceding claims, wherein the oil is rich in unsaturated fatty acids.

9. The suspension according to any of the preceding claims, wherein the unsaturated fatty acids are n−3 fatty acids and/or n-6 fatty acids.

10. The suspension according to any of the preceding claims, wherein the oil is selected from the group consisting of fish oil, vegetable oil, and a combination thereof.

11. The suspension according to claim 10, wherein the fish oil is selected from the group consisting of salmon oil, mackerel oil, lake trout oil, herring oil, sardine oil, albacore tuna oil, cod liver oil, sand eel oil (*Ammodytes tobianus*), and menhaden oil.

12. The suspension according to any of claims 1-10, wherein the oil is a vegetable oil.

13. The suspension according to claim 12, wherein the oil is selected from the group consisting of linseed oil, olive oil, borage oil, lin oil, camelina oil, grape seed oil, chia oil, kiwifruit seeds oil, perilla oil, lingonberry, purslane oil, seabuckthorn oil, hemp oil and soybean oil.

14. The suspension according to any of the preceding claims, wherein the at least one probiotic micro-organism is selected from the group consisting of bacteria, yeast and mold.

15. The suspension according to claim 14, wherein said at least one probiotic micro-organism is bacteria selected from the group consisting of Bifidobacterium, Bacteroides, Clostridium, Fusobacterium, Melissococcus, Propionibacterium, Streptococcus, Enterococcus, Lactococcus, Kocuriaw, Staphylococcus, Peptostrepococcus, Bacillus, Pediococcus, Micrococcus, Leuconostoc, Weissella, Aerococcus, Oenococcus and Lactobacillus.

16. The suspension according to claim 15, wherein said at least one probiotic micro-organism is *Enterococcus faecium*.

17. The suspension according to claim 15, wherein said at least one probiotic micro-organism is the NCIMB 10415 strain of *Enterococcus faecium*.

18. The suspension according to claim 14, wherein said at least one probiotic is a yeast selected from the group consisting of Saccharomyces, Debaromyces, Candidaw Pichia and Torulopsis.

19. The suspension according to claim 14, wherein said at least one probiotic is a mold selected from the group consisting of Aspergillus, Rhizopus, Mucor, and Penicillium and Torulopsis.

20. The suspension according to claim 14, wherein the probiotic micro-organism is selected from the group consisting of *Aspergillus niger, A. oryzae, Bacillus coagulans, B. lentus, B. licheniformis, B. mesentericus, B. pumilus, B. subtilis, B. natto, Bacteroides amylophilus, Bac. capillosus, Bac. ruminocola, Bac. suis, Bifidobacterium adolescentis, B. animalis, B. breve, B. bifidum, B. infantis, B. lactis, B. longum, B. pseudolongum, B. thermophilum, Candida pintolepesii, Clostridium butyricum, Enterococcus cremoris, E. diacetylactis, E. faecium, E. intermedius, E. lactis, E. muntdi, E. thermophilus, Escherichic coli, Kluyveromyces fragilis, Lactobacillus acidophilus, L. alimentarius, L. amylovorus, L. crispatus, L. brevis, L. casei L. curvatus, L. cellobiosus, L. delbrueckii ss. bulgaricus, L farciminis, L. fermentum, L. gasseri, L. helveticus, L. lactis, L. plantarum, L. johnsonii, L. reuteri, L. rhamnosus, L. sakei, L. salivarius, Leuconostoc mesenteroides, P. cereviseae (damnosus), Pediococcus acidilactici, P pentosaceus, Propionibacterium freuclenreichii, Prop. shertnanii, Saccharontyces cereviseae, Staphylococcus carnosus, Staph. xylosus, Streptococcus infantarius, Strep. Salivarius ss. thermophilus, Strep. thermophilus, Strep. lactis.*

21. The suspension according to any of the preceding claims, wherein the dynamic viscosity of said suspension is less than 0.06 pascal-second (Pa·s) at 20°C.

22. The suspension according to any of the preceding claims, wherein the dynamic viscosity of said suspension is in the range of 0.04 to 0.06 pascal-second (Pa·s) at 20°C.

23. The suspension according to any of the preceding claims, wherein the suspension has a Δ Pa·s between 20°C and 25°C of at least 0.009 Pa·s.

24. A method of preparing a suspension according to any of the preceding claims, said method comprising:

a) providing at least one probiotic micro-organism in a dry powder form having a total concentration of 10^9-10^{17} CFU/kg dry powder

b) providing an oil.

c) adding 0.3 to 15 kg of said probiotic micro-organism powder per 100 kg oil to said oil in an container at continuously stirring at RT to make a suspension premix

d) transferring the suspension premix of c) to a "storage tank" comprising mixing means with the proviso that the transfer is not by vacuum suction

e) mixing said premix suspension RT to obtain a suspension of homogenously dispersed probiotic micro-organism

and obtaining said suspension.

25. The method according to claim 24, wherein said the total concentration of the at least one probiotic micro-organism in said dry powder form is in the range of 10^{10} to 10^{13} CFU/kg dry powder.

26. The method according to claims 24 or 25, wherein 3.3 to 6.7 kg of said probiotic micro-organism powder per 100 kg is added to said oil in the container.

27. The method according to any of claims 24-26, wherein said premix is mixed for no more than 3 hours of mixing.

28. The method according to any of claims 24-27, wherein said premix is mixed for not less than 1 hour of mixing.

29. A method of producing an extruded food product comprising at least one probiotic micro-organism, wherein said probiotic micro-organism is homogenously distributed throughout the structure of the food by vacuum inclusion of the suspension according to any of the preceding claims.

30. The method according to claim 29, wherein said food product is a pet food product.

31. The method according to claim 29, wherein said food product is a human food product.

32. An extruded probiotic food product obtained by a method according to any of the claims 29 to 31.

33. The food product of claim 31, wherein the minimal amount of the probiotic in the product is in the range of 10^5 CFU/Kg to 10^{15} CFU/Kg.

34. The food product of claim 32 or 33, wherein product % moisture is above 7%.

35. The use of the suspension according to any of the preceding claims for the preparation of a extruded probiotic food product, wherein said suspension comprises an oil and at least one probiotic micro-organism in the concentration of 10^6-10^{16} CFU/kg of said oil and said suspension having a dynamic viscosity of less than 0.08 pascal-second (Pa·s) at 20°C.

PUBLICATION II

International application published under the patent cooperation treaty (PCT), Title: A vacuum infusion production plant, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122155 A1 International publication date: 28.10.2010.

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(54) **Title**: A VACUUM INFUSION PRODUCTION PLANT

(57) **Abstract**: The invention relates toa production plant for vacuum infusing a food product comprising at least a first storage tank for storing a probiotic suspension, said first storage tank being connected to a first dosage tank for dosing a probiotic suspension, a second storage tank for storing a second solution, said second storage tank being connected to a second dosage tank for dosing the second solution, and wherein the first dosage tank and the second dosage tank are connected to a vacuum infusion tank by one or more spraying nozzles leading into the vacuum infusion tank, and wherein at least the first dosage tank is individually connected to the vacuum infusion tank by one or more first spraying nozzles leading into the vacuum infusion tank.

A vacuum infusion production plant

Technical field of the invention

The present invention relates to a vacuum infusion production plant. In particular the present invention relates to a vacuum infusion production plant for infusing probiotic micro-organisms into extruded food products.

Background of the invention

Various commercial attempts have been made to achieve food compositions containing probiotic micro-organisms with prolonged viability for long term storage, many of these do not provide sufficient efficacious levels of viable probiotic micro-organism due to issues associated with susceptibility of the micro-organism to standard commercial pet food manufacturing procedures such as extrusion. For example, efforts of coating or filling standard pet food kibbles with probiotic micro-organisms have been suggested but, in practice, often prove impractical.

WO 01/95745 provides a method of producing a food product (kibbles) characterised by a porous structure, comprising an instable substrate such as a probiotic micro-organism in an oil solution, which are included in a flowable form into the product by means of a step of "partial vacuum" followed by normalizing the pressure by releasing an inert gas into the vessel.

WO 05/070232 provides a method of producing a food product similar to WO 01/95745, further characterized in that the oil should have a solid fat index of at least 20. WO 05/070232 discloses the essential use of fat with the solid fat index of the vehicle is at least 20 at 20°C and the preferred vehicle are coconut oil and even more preferred palm oil.

WO 03/009710 discloses system and method for on-line mixing and application of surface coating compositions for food products; an apparatus is also disclosed. The apparatus comprises a dry matter – liquid mixing module (wherein the dry matter may be probiotics) connected inline to a liquid – liquid mixing module, wherein one or more liquid can be mixed into the first liquid (potentially comprising the probiotics)

Hence, an improved production plant for incorporating probiotics into food products would be advantageous, and in particular a more efficient and/or reliable production plant for incorporating probiotics into food products prolonging the viability of the probiotics would be advantageous.

Summary of the invention

A first aspect the invention relates to a production plant for vacuum infusing a food product comprising

- a first storage tank for storing a probiotic suspension, connected to a first dosage tank for dosing a probiotic suspension,

wherein the first dosage tank is connected to a vacuum infusion tank by one or more spraying nozzles leading into the vacuum infusion tank.

A second aspect relates to a production plant for vacuum infusing a food product comprising at least

- a first storage tank for storing a probiotic suspension, said first storage tank being connected to a first dosage tank for dosing a probiotic suspension,
- a second storage tank for storing a second solution, said second storage tank being connected to a second dosage tank for dosing the second solution,

wherein the first dosage tank and the second dosage tank are connected to a vacuum infusion tank by one or more spraying nozzles leading into the vacuum infusion tank, and wherein at least the first dosage tank is individually connected to the vacuum infusion tank by one or more first spraying nozzles leading into the vacuum infusion tank.

It may be advantageously to be able to vacuum infuse more than two suspension/solution without having to change the content of the storage tank and the dosage tank. Thus, in a third aspect the invention relates to a production plant for vacuum infusing a food product comprising at least

- a first storage tank for storing a probiotic suspension, said first storage tank being connected to a first dosage tank (6) for dosing a probiotic suspension,

- a second storage tank for storing a fat solution, said second storage tank being connected to a second dosage tank for dosing a fat solution,

- a third storage tank for storing a digest solution, said third storage tank being connected to a third dosage tank for dosing a digest solution, and

wherein the first dosage tank, the second dosage tank and the third dosage tank are connected to a vacuum infusion tank by one or more spraying nozzles leading into the vacuum infusion tank, and wherein at least the first dosage tank is individually connected to the vacuum infusion tank by one or more first spraying nozzles leading into the vacuum infusion tank.

In the production plant of the disclosed invention the probiotic suspension is kept separate from the other components which are going to be vacuum infused into the product. This is done having the first dosage tank individually connected to the vacuum infusion tank. An advantage is that optimal viability of the probiotics is maintained when the probiotic oil/fat suspension is applied without getting in contact with the other ingredients before it reach the vacuum tank.

The solutions in the second dosage tank and the third dosage tanks may be connected to the vacuum infusion tank through a joined connection, which may make the plant simpler to construct.

Claims

1. A production plant for vacuum infusing a food product comprising at least

- a first storage tank (2) for storing a probiotic suspension, said first storage tank (2) being connected to a first dosage tank (6) for dosing a probiotic suspension,

- a second storage tank (3) for storing a second solution, said second storage tank being connected to a second dosage tank (7) for dosing the second solution,

wherein the first dosage tank and the second dosage tank are connected to a vacuum infusion tank (14) by one or more spraying nozzles leading into the vacuum infusion tank, and wherein at least the first dosage tank (6) is individually connected to the vacuum infusion tank (14) by one or more first spraying nozzles leading into the vacuum infusion tank.

2. A production plant according to claim 1, further comprising at least a third storage tank (4) for storing a solution, said third storage tank (4) being connected to a third dosage tank (8) for dosing the third solution through one or more spraying nozzles.

3. A production plant according to claim 2, further comprising at least a fourth storage tank (5) for storing a solution, said fourth storage tank (5) being connected to a fourth dosage tank (9) for dosing a solution through one or more spraying nozzles.

4. A production plant according to any of claims 1-3, wherein at least one of the following dosage tanks also is individually connected to the vacuum infusion tank (14) by one or more spraying nozzles: the second dosage tank (7), the third dosage tank (8) and the fourth dosage tank (9).

5. A production plant according to any of claims 1-4, wherein each of the following dosage tanks also is individually connected to the vacuum infusion tank (14) by one or more spraying nozzles: the second dosage tank (7), the third dosage tank (8) and the fourth dosage tank (9).

6. A production plant according to any of claims 1-5, wherein the orifice of each of the spraying nozzles has a cross-sectional area of 1-250 mm^2, possibly 1-200

mm^2, such as 1-150 mm^2, or 1-100 mm^2, or 1-50 mm^2, or 1-25 mm^2, or 1-15 mm^2 or 1-10 mm^2 or 1-5 mm^2 or 1-3 mm^2.

7. A production plant according to any of claims 1-6, wherein a first mixing tank (1) is connected to the first storage tank (2) through a bottom outlet in the first mixing tank, and where the probiotic suspension is intended for being passed from the first mixing tank (1) to the first storage tank (2) at least by means of gravity, possibly by means of gravity only.

8. A production plant according to claim 7, wherein the connection between the first mixing tank (1) and the first storage tank (2) does not comprise a vacuum suction unit.

9. A production plant according to claims 7 or 8, wherein the connection between the first mixing tank (1) and the first storage tank (2) does not comprise a positive displacement unit.

10. A production plant according to any of claims 1-9, wherein the first storage tank (2) comprises at least one of the following means for mixing: a rotating impeller, a rotating mixing tank.

11. A production plant according to any of claims 1-10, wherein the vacuum infusion tank (14) comprises at least one of the following means for mixing: a rotating impeller, a rotating mixing tank.

12. A production plant according to any of claims 1-11, wherein the vacuum infusion tank (14) comprises at least one opening (17) for applying the uncoated food product to said vacuum infusion tank (14).

13. A production plant according to any of claims 1-12, wherein the vacuum infusion tank (14) is further connected to an collection tank (15) for passing the coated food product from the infusion tank (14) to the collection tank (15), and wherein the collection tank (15) is further connected to at least one vessel (16) containing one or more substances to be applied to the collection vessel.

14. A production plant according to claim 13, wherein the collection tank (15) comprises at least one of the following means for mixing: a rotating impeller, a rotating mixing tank.

15. A production plant according to any of claims 1 to 14, wherein at least the first storage tank (2) and the first dosage tank (3) comprise means for maintaining the temperature of the probiotic suspension in the range of 15°C to 29°C.

16. A production plant according to any of claims 1-15, wherein the plant further comprises a control unit for controlling at least one of the activities selected from the group consisting of: controlling the temperature in at least one of the storage tanks, controlling the temperature in at least one of the dosage tanks, controlling opening and closing of inlets and outlets between two or more of the tanks, controlling the amount of liquid sprayed through the nozzles, controlling the pressure in the vacuum tank and controlling the mixing time.

PUBLICATION III

International application published under the patent cooperation treaty (PCT), Title: Extruded food products compromising probiotic microorganisms, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122106 A1 International publication date: 28.10.2010.

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(54) **Title**: EXTRUDED FOOD PRODUCTS COMPRISING PROBIOTIC MICRO-ORGANISMS

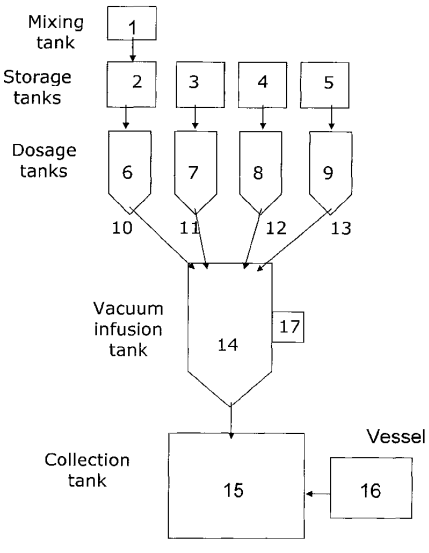

Fig. 1

(57) **Abstract**: The present invention relates to a vacuum infused synbiotic human extruded food product having 1) a density of 1 g/L to 1000 g/L at RT, 2) a sugar content of less than 10 wt%, 3) a total content of at least one of inulin and/or FOS ranging from 2.5-10 wt%, 4) a ratio between saturated to unsaturated fatty acids in total fat content of less than 20/1, and wherein at least one strain of probiotics is evenly distributed in said food product in an oil vehicle and wherein the food product has a probiotic count of at least 10^6 CFU/kg of dry matter. The invention furthermore relates to methods for obtaining the product and production plants for producing the product.

WO 2010/122106 A1

Extruded food products comprising probiotic micro-organisms

Technical field of the invention

The present invention relates to human food compositions, methods for obtaining the food compositions and production plants for producing the food compositions. In particular the invention relates to extruded cereal product comprising probiotics for human consumption.

Background of the invention

Various commercial attempts have been made to achieve food compositions containing probiotic micro-organisms with prolonged viability for long term storage, many of these do not provide sufficient efficacious levels of viable probiotic micro-organism due to issues associated with susceptibility of the micro-organism to standard commercial food manufacturing procedures such as extrusion. For example, efforts of coating or filling standard pet food kibbles with probiotic microorganisms have been suggested but, in practice, often prove impractical.

WO 01/95745 provides a method of producing a food product (kibbles) characterised by a porous structure, comprising an instable substrate such as a probiotic micro-organism in an oil solution, which are included in a flowable form into the product by means of a step of "partial vacuum" followed by normalizing the pressure by releasing an inert gas into the vessel.

WO 05/070232 provides a method of producing a food product similar to WO 01/95745, further characterized in that the oil should have a solid fat index of at least 20. WO 05/070232 discloses the essential use of fat with the solid fat index of the vehicle is at least 20 at 20°C and the preferred vehicle are coconut oil and even more preferred palm oil.

WO 03/009710 discloses system and method for on-line mixing and application of surface coating compositions for food products; an apparatus is also disclosed. The apparatus comprises a dry matter – liquid mixing module (wherein the dry matter may be probiotics) connected inline to a liquid – liquid mixing module, wherein one or more liquid can be mixed into the first liquid (potentially

comprising the probiotics). Hence, an improved production plant for incorporating probiotics into food products would be advantageous, and in particular a more efficient and/or reliable production plant for incorporating probiotics into food products prolonging the viability of the probiotics would be advantageous.

Summary of the invention

Micro-organisms used as probiotics in a food products are very sensitive to various physical/chemical factors such as temperatures, moist, levels of pH, organic acids etc. Various food manufacturing processes include a heat-treatment, which leads to loss of viability of the probiotic bacteria at the manufacturing stage. Other stages of food product manufacturing include treatment with chemical compounds, serving as an ingredients and/or preservatives, which might have negative effect on the probiotic micro-organism viability. Such treatments of the product shall be allowed only prior to the inclusion of the probiotic micro-organisms, nevertheless any leftovers or defects of the product matrix will have a negative effect on a stability of the product in the future. Therefore it is not recommended to process the food product with probiotic ingredient after the inclusion stage. Ingredients used as a part of the formulation of the ready product should not have a negative influence on probiotic viability. WO 03/009710 teaches away from keeping the suspension comprising the probiotics separate from all other liquids until the reach the solid food product.

To minimize loss of viability of the probiotics during the production stage it may be advantageously to use freeze-dried or any similar way treated probiotic micro-organism.

Another general health problem with food products comprising probiotics is that often sugars are used as a preservative to maintain the viability of the probiotics. Though the viability of the probiotics may be increased the overall health benefits of such products is low at the point regarding the sugar content.

The present invention solves the above problem by disclosing a food product comprising the beneficial effects of a high content of viable probiotics and at the same time having a low content of sugars.

This invention describes extruded ready to eat products for human consumption that includes a fat/oil suspension comprising probiotic micro-organisms, wherein the probiotic compound is vacuum infused throughout the matrix of the product and additionally may be protected by an extra layer of honey or similar compound deriving from natural sources.

Despite the fact that oils/fats that are rich in unsaturated fatty acids are generally considered healthy, these compounds are commonly avoided in food products comprising probiotic micro-organisms. The reason being that these fats are considered to be liquid and less stable therefore not suitable for preserving probiotic micro-organisms for a longer period of time. Surprisingly, it has been found that using the production method of the invention good viability of the probiotics can be maintained in a food product, even when unsaturated fatty acids are present in the disclosed levels.

Thus, in a first aspect the invention relates to a vacuum infused synbiotic extruded food product for humans having;

1) a density of 1 g/L to 1000 g/L at RT,

2) a total sugar content of less than 10 wt%,

3) a total content of at least one of inulin and FOS ranging from 2.5-10 wt%,

4) a ratio of saturated to unsaturated fatty acids in total fat content of less than 20/1, and

wherein at least one strain of probiotics is evenly distributed in said food product, in an oil vehicle and wherein the food product has a probiotic count of at least 10^6 CFU/kg of dry matter.

The food product described in this invention should initially be extruded as part of conventional production process since extruded products develop a rigid structure and maintain a porous texture. The density of the vacuum infused products may vary depending on the type of product which has been vacuum infused. Thus, in an embodiment the density is 200 g/L to 1000 g/L, such as 400 g/L to 1000 g/L, such as 600 g/L to 1000 g/L, such as 1 g/L to 500 g/L or such as 100 g/L to 500 g/L.

To sustain the health benefits of the food product described in this invention the final product preferably not include sugar. If the product should not comprise sugar the level in the final food becomes 0%. If the product should comprise sugar the total amount may range from 0.1-10 wt%. Thus, in another embodiment the content of sugar is 0-10%, such as 0.1-8 wt%, such as 0.1-6 wt%, such as 0.1-4 wt%, such as 0.5-4 wt%, or such as 1-4 wt%, or such as 2-3 wt%.

The product may also comprise fructo-oligosaccharides (FOS) and/or inulin at a concentration of not less than 2.5 wt%. Thus, in a further embodiment the content of fructo-oligosaccharides is 2.5-10 wt%, such as 2.5-8 wt%, such as 2.5-6 wt%, such as 2.5-4 wt%, or such as 2.5-3 wt%.

The ratio between saturated to unsaturated fatty acids in total fat content also influences the health benefits of the product. In order to sustain the key health benefits and features of the food product, the product described in this invention may comprise a high level of unsaturated fatty acids. Furthermore, the total amount of fats in the food product may range from 0.5 wt% till 45 wt% of net weight of the product, where preferably the ratio of saturated to unsaturated fats within the total fat content may range from 20/1 – 1/12. Thus, in yet an embodiment of the invention, the ratio of saturated to unsaturated fatty acids is 20/1 to 1/12, such as 15/1 to 1/10, such as 10/1 to 1/1, such as 5/1 to 1/1, such as 3/1 to 1/1, or such as 1/1.

Known health beneficial unsaturated fatty acids are omega-3 fatty acids such as α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) and omega-6 fatty acids such as linoleic acid and arachidonic acid. Thus in yet an embodiment the unsaturated fatty acids in the product comprises at least one of α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid and arachidonic acid.

Claims

1. A vacuum infused synbiotic human extruded food product having;

1) a density of 1 g/L to 1000 g/L at RT,

2) a sugar content of less than 10 wt%,

3) a total content of at least one of inulin and/or FOS ranging from 2.5-10 wt%,

4) a ratio between saturated to unsaturated fatty acids in total fat content of less than 20/1, and

wherein at least one strain of probiotics is evenly distributed in said food product in an oil vehicle and wherein the food product has a probiotic count of at least 10^6 CFU/kg of dry matter.

2. The food product according to claim 1, wherein the product is selected from the group consisting of extruded snack products, tortilla chips, breakfast cereals, cookies, crisp bread, food foams, rice brokens, blend of peanut, soybean and corn, puffed wheat, low density foamed corn and rice breakfast, co-extruded products, muesli bars and any other extruded food products that are formed by extrusion process.

3. The food product according to claims 1 or 2, with the proviso that the food product does not comprise a synthetic sweetener.

4. The food product according to any of the preceding claims, wherein the count of at least one probiotic is 10^6-10^{19} CFU/kg.

5. The food product according to any of the preceding claims, wherein the glycemic index is 1-55.

6. The food product according to any of the preceding claims, wherein the oil is fish oil.

7. The food product according to any of the preceding claims, wherein the oil is selected from the group consisting of salmon oil, mackerel oil, lake trout oil,

herring oil, sardine oil, albacore tuna oil, sand eel oil, *Ammodytes tobianus* oil, menhaden oil, flax oil, flax and seed oil.

8. The food product according to claim 7, wherein the oil is salmon oil.

9. The food product according to any of claims 1-5, wherein the oil is a vegetable oil.

10. The food product according to claim 9, wherein the oil is selected from the group consisting of linseed oil, olive oil, borage oil, lin oil, camelina oil, grape seed oil, chia oil, kiwifruit seeds oil, perilla oil, lingonberry, purslane oil, seabuckthorn oil, hemp oil.

11. The food product according to claims 9 or 10, wherein the oil is linseed oil.

12. The food product according to any of the preceding claims, wherein the oil vehicle has a dynamic viscosity of less than 0.08 pascal-second (Pa·s) at 20°C.

13. The food product according to any of the preceding claims, wherein said at least one probiotic micro-organism is bacteria selected from the group consisting of Bifidobacterium, Bacteroides, Clostridium, Fusobacterium, Melissococcus, Propionibacterium, Streptococcus, Enterococcus, Lactococcus, Kocuriaw, Staphylococcus, Peptostrepococcus, Bacillus, Pediococcus, Micrococcus, Leuconostoc, Weissella, Aerococcus, Oenococcus and Lactobacillus.

14. The food product according to claim 13, wherein said at least one probiotic micro-organism is *Enterococcus faecium*.

15. The food product according to claim 13 or 14, wherein said at least one probiotic micro-organism is the NCIMB 10415 strain of *Enterococcus faecium*.

16. The food product according to any of claim 1-12, wherein said at least one probiotic is a yeast selected from the group consisting of Saccharomyces, Debaromyces, Candidaw Pichia and Torulopsis.

17. The food product according to claim 16, wherein said at least one probiotic is a mold selected from the group consisting of Aspergillus, Rhizopus, Mucor, and Penicillium and Torulopsis.

18. The food product according to claim 16 or 17, wherein the probiotic micro-organism is selected from the group consisting of *Aspergillus niger, A. oryzae, Bacillus coagulans, B. lentus, B. licheniformis, B. mesentericus, B. pumilus, B. subtilis, B. natto, Bacteroides amylophilus, Bac. capillosus, Bac. ruminocola, Bac. suis, Bifidobacterium adolescentis, B. animalis, B. breve, B. bifidum, B. infantis, B. lactis, B. longum, B. pseudolongum, B. thermophilum, Candida pintolepesii, Clostridium butyricum, Enterococcus cremoris, E. diacetylactis, E. faecium, E. intermedius, E. lactis, E. muntdi, E. thermophilus, Escherichic coli, Kluyveromyces fragilis, Lactobacillus acidophilus, L. alimentarius, L. amylovorus, L. crispatus, L. brevis, L. Casei, L. curvatus, L. cellobiosus, L. delbrueckii ss. bulgaricus, L farciminis, L. fermentum, L. gasseri, L. helveticus, L. lactis, L. plantarum, L. johnsonii, L. reuteri, L. rhamnosus, L. sakei, L. salivarius, Leuconostoc mesenteroides, P. cereviseae (damnosus), Pediococcus acidilactici, P pentosaceus, Propionibacterium freuclenreichii, Prop. shertnanii, Saccharontyces cereviseae, Staphylococcus carnosus, Staph. xylosus, Streptococcus infantarius, Strep. Salivarius ss. thermophilus, Strep. thermophilus, Strep. lactis.*

19. The food product according to any of the preceding claims, wherein at least part of the saccharides are at least one of natural sourced fructo-oligosacharide and natural sourced inulin.

20. The food product according to any of the preceding claims, having the taste, texture and appearance of a conventional product of the same type without probiotics.

21. The food product according to any of the preceding claims, wherein the food product further comprises honey.

22. A method for producing synbiotic extruded human food product with a ratio between saturated to unsaturated fatty acids of the total fat content of less than 20/1, said method comprising the steps of

- providing a first extruded component having a mono-saccharide content of less than 10% and a density of 1 g/L to 1000 g/L at RT,
- providing a suspension having a dynamic viscosity of less than 0.08 pascal-second (Pa·s) at 20°C, wherein said suspension comprises an oil/fat and at

least one probiotic micro-organism having a concentration of 10^7-10^{17} CFU/kg of the oil/fat,

- providing a source of inulin and/or FOS,

adding the first components to a vacuum infusion tank, and

- a) reduce the pressure in the vacuum infusion tank to [0.2-0.95 bar]
- b) vaporize the suspension at a temperature of 15-30°C,
- c) restore pressure to [1 bar], and

coating the product obtained in c) with the source of inulin and/or FOS.

23. The method for producing synbiotic extruded human food product according to claim 22, wherein the first component is an extruded cereal product.

24. The method for producing synbiotic extruded human food product according to claims 22 or 23, wherein the cereal product is selected from the group consisting of extruded snack products, tortilla chips, breakfast cereals, cookies, crisp bread, food foams, Rice brokens, blend of peanut, soybean and corn, puffed wheat, low density foamed corn and rice breakfast, co-extruded products, muesli bars and any other extruded products that are formed by an extrusion process.

25. The method for producing synbiotic extruded human food product according to any of claims 22-24, wherein the extruded component has a temperature above 15°C when the suspension is vaporized on the extruded component.

26. The method for producing synbiotic extruded human food product according to any of claims 22-25, wherein the oil is tasteless and odourless.

27. The method for producing synbiotic extruded human food product according to any of claims 22-26, wherein the oil is selected from the group consisting of oil from mackerel, lake trout, herring, sardines, salmon or albacore tuna, sand eel, *Ammodytes tobianus*, and menhaden.

28. The method for producing synbiotic extruded human food product according to any of claims 22-27, wherein the suspension is introduced on a product within 5 hours after the mixing of the oil/fat with the probiotics.

29. The method for producing synbiotic extruded human food product according to any of claims 22-28, wherein the saccharides comprise at least one of fructo-oligosaccharides and/or inulin.

30. The method for producing synbiotic extruded human food product according to any of claims 22-29, wherein at least part of the fructo-oligosaccharides and/or inulin are comprised in honey.

31. The method for producing synbiotic extruded human food products according to any of claims 22 to 30, wherein the fructo-oligosaccharides and/or inulin are coated on the food product.

32. A production plant for vacuum infusing a human cereal food product comprising

- a first storage tank (2) for storing a probiotic suspension, connected to a first dosage tank (7) for dosing a probiotic suspension,

wherein the first dosage tank (7) is connected to a vacuum infusion tank (13) by one or more spraying nozzles leading into the vacuum infusion tank (13).

33. The production plant according to claim 32, wherein the orifice of each of the spraying nozzles has a cross-sectional area of 1-250 mm^2, possibly 1-200 mm^2, such as 1-150 mm^2, or 1-100 mm^2, or 1-50 mm^2, or 1-25 mm^2, or 1-15 mm^2 or 1-10 mm^2 or 1-5 mm^2 or 1-3 mm^2.

34. The production plant according to claims 32 or 33, wherein a first mixing tank (1) is connected to the first storage tank (2) through a bottom outlet in the first mixing tank, and where the probiotic suspension is intended for being passed from the first mixing tank (1) to the first storage tank (2) at least by means of gravity, possibly by means of gravity only.

35. The production plant according to any of claims 32-34, wherein the connection between the first mixing tank (1) and the first storage tank (2) does not comprise a vacuum suction unit.

36. The production plant according to any of claims 32 to 35, wherein the connection between the first mixing tank (1) and the first storage tank (2) does not comprise a positive displacement unit.

37. The production plant according to any of claims 32 to 36, wherein the first storage tank (2) comprises at least one means for mixing selected from the list consisting of a rotational impeller and a rotational mixing tank.

38. A production plant according to any of claims 32 to 37, wherein the vacuum infusion tank (13) comprises at least one opening (16) for applying the uncoated food product to said vacuum infusion tank (13).

39. The production plant according to any of claims 32-38, wherein the vacuum infusion tank (13) is further connected to an collection tank (14) for passing the coated food product from (13) to (14), and wherein the collection tank (14) is further connected to at least one vessel (15) containing one or more substances to be applied to the collection vessel.

40. The production plant according to claim 39, wherein the collection tank (14) comprises at least one means for mixing selected from the list consisting of a rotational impeller and a rotational mixing tank.

41. The production plant according to any of claims 32 to 40, wherein at least the first storage tank (2) and the first dosage tank (7) comprise means for maintaining the temperature of the probiotic suspension in the range of 15°C to 29°C.

42. A production plant according to any of claims 32 to 41, further comprising

- a second storage tank (3) for storing a second solution, connected to a second dosage tank (8) for dosing the second solution,

wherein the second dosage tank is connected to a vacuum infusion tank (13) by one or more spraying nozzles (11) leading into the vacuum infusion tank (13), and wherein the first dosage tank (7) is individually connected to a vacuum infusion tank (13) by one or more spraying nozzles (10) leading into the vacuum infusion tank (13).

43. The production plant according to claim 42, further comprising

- a third storage tank (4) for storing a third solution, connected to a third dosage tank (8) for dosing a third solution through one or more spraying nozzles, and

wherein the third dosage tank is connected to a vacuum infusion tank (13) by one or more spraying nozzles leading into the vacuum infusion tank (13).

44. The production plant according to claim 43, further comprising

- a fourth storage tank (5) for storing a fourth solution, connected to a third or second dosage tank (9 or 8) for dosing the fourth solution,

wherein the fourth dosage tank is connected to a vacuum infusion tank (13) by one or more spraying nozzles leading into the vacuum infusion tank (13).

45. The production plant according to any of claims 32-44, wherein the plant further comprises a control unit for controlling at least one of the activities selected from the group consisting of: controlling the temperature in at least one of the storage tanks, controlling the temperature in at least one of the dosage tanks, controlling opening and closing of inlets and outlets between two or more of the tanks, controlling the amount of liquid sprayed through the nozzles, controlling the pressure in the vacuum tank and controlling the mixing time.

46. The vacuum infused synbiotic human extruded food product obtainable by the method of any of claims 22-31.

47. A probiotic cereal food product comprising at least one probiotic micro-organism, wherein said probiotic micro-organism is infused in said food product, obtainable by a method according to any of the proceeding claims.

PUBLICATION IV

International application published under the patent cooperation treaty (PCT), Title: A method for the management of fecal consistency in dogs, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122104 A1 International publication date: 28.10.2010.

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(54) **Title**: A METHOD FOR THE MANAGEMENT OF FAECAL CONSISTENCY IN DOGS

(57) **Abstract**: The present invention relates to means for defecation and/or faeces management in a pet comprising feeding the pet with a vacuum infused pet food kibble comprising within the kibble structure a mixture of probiotic micro-organisms and an oil or similar vehicle.

A METHOD FOR THE MANAGEMENT OF FAECAL CONSISTENCY IN DOGS

Technical field of the invention

The present invention relates to defecation management and improvements of the faeces consistency and faeces condition in pets. The vacuum infused pet food kibbles of the present invention changes and optimises the various scores of the PURINA system surprisingly well under high volume manufacturing and modern logistics.

Background of the invention

The well-being of domestic animals is closely related to their feeding. Correct feeding should result in a fit and healthy pet. In addition to providing nutritional value, food composition influences the intestinal microflora equilibrium and may lead to or prevent gastrointestinal disorders. Therefore, knowledge on the gastro-intestinal tract and digestion processes of healthy animals is integral to the understanding of a practical feeding practice. As carnivores, cats and dogs are e.g. characterized by a short digestive tract and a rapid flow rate of the bolus of food.

The number and composition of this endogenous flora tend to be rather stable, although age and, to a lesser degree, food may modify it. Gastric acidity, bile, intestinal peristalsis and local immunity are factors thought to be important in the regulation of bacterial flora in the small intestine of human beings and various other mammals.

Often canine and feline gastrointestinal disorders are linked to bacterial overgrowth and the production of enterotoxins produced by pathogenic bacteria.

During the last few years, research has focused on some valuable strains of Lactic Acid Bacteria (LAB) and their potential use as probiotic agents. Probiotics are considered to be viable microbial preparations which promote mammalian health by preserving the natural microflora in the intestine. Probiotics are believed to attach to the intestinal mucosa, colonize the intestinal tract and thereby prevent attachment of harmful micro-organisms thereon. A prerequisite for their action

resides in that they have to reach the gut's mucosa in a proper and viable form and especially do not get destroyed by the influence of the low pH prevailing in the stomach. In particular, the physiology of the digestive tract of cats and dogs differs from humans. For example, the average pH in the stomach is about 3.4 for dogs and 4.2 for cats.

Although many references discloses the inclusion of a probiotic micro-organism in a pet food, the available art is does not disclose the effects of products with sufficient CFUs (Colony Forming Units) in the feed which comply with high volume manufacturing and modern logistics.

Consequently, there is a need to provide pet food compositions that are still after shell life of months able to quickly levelling the faeces conditions of the pets. Rapid levelling of defecation and diarrhoea to more neutral stool conditions is of both great health and general nutritional concern.

Summary of the invention

Thus, an object of the present invention relates to means for defecation and/or faeces management in a pet comprising feeding the pet with a vacuum infused pet food kibble comprising within the kibble structure a mixture of probiotic micro-organisms and an oil or similar vehicle.

In particular, it is an object of the present invention to provide a method for defecation management in a dog comprising feeding the dog with a vacuum infused pet food kibble comprising within the kibble structure a mixture of probiotic micro-organisms and a fish oil that solves the above mentioned problems of the prior art with high volume manufacturing and modern logistics and still retaining the beneficial effects of the product.

Other aspects and preferred embodiments will be apparent from the following detailed description of the invention.

Claims

1. A method for defecation management in a dog comprising feeding the dog with a vacuum infused pet food kibble comprising within the kibble structure a mixture of probiotic micro-organisms and an oil.

2. The method according to claim 1, wherein the faeces condition of the dog is levelled to score 3 and 4 of the PURINA feces scoring system.

3. The method according to claim 1 or 2, wherein the oil has dynamic viscosity of less than 0.08 pascal-second (Pa·s) at 20°C.

4. The method according to any of the preceding claims, wherein the kibble contains 10^6-10^{19} CFU/kg.

5. The method according to any of the preceding claims, wherein the change in feces consistency is reached in less than 14 days.

6. The method according to any of the preceding claims, wherein the change in faeces consistency is achievable 12 months after the production date of the kibble.

7. The method according to any of the preceding claims, wherein the kibble is not stored under refrigerated conditions.

8. Use of a pet food kibble comprising within the kibble structure a mixture of probiotic micro-organisms and an oil for the manufacture of a composition for improving the faeces condition of a dog to score 3 and 4 of the PURINA feces scoring system.

9. Use of a pet food kibble comprising within the kibble structure a mixture of probiotic micro-organisms and an oil for the manufacture of a composition for maintaining a faeces condition of a dog at score 3 and 4 of the PURINA faeces scoring system.

10. A vacuum infused pet food kibble comprising within the kibble structure a mixture of probiotic micro-organisms and an oil for use in improving the faeces condition of a dog to score 3 and 4 of the PURINA faeces scoring system.

11. A vacuum infused pet food kibble comprising within the kibble structure a mixture of probiotic micro-organisms and an oil for use in maintaining the faeces condition of a dog at score 3 and 4 of the PURINA faeces scoring system.

12. The method according to any of the preceding claims, wherein the oil is selected from the group consisting of fish oils, vegetable oils, and a combination thereof.

13. The method according to any of the preceding claims, wherein the fish oil is selected from the group consisting of salmon oil, mackerel oil, lake trout oil, herring oil, sardine oil, albacore tuna oil, cod liver oil, sand eel oil (*Ammodytes tobianus*), and menhaden oil.

14. The method according to claim 12, wherein the oil is a vegetable oil.

15. The method according to claim 14, wherein the oil is selected from the group consisting of linseed oil, olive oil, borage oil, lin oil, camelina oil, grape seed oil, chia oil, kiwifruit seeds oil, perilla oil, lingonberry, purslane oil, seabuckthorn oil, hemp oil and soybean oil.

PUBLICATION V

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Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentrations in broth

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ABSTRACT

Growth of bacteria *Lactococcus lactis* IL1403 in broth was studied using microcalorimeter in combination with HPLC, *plate count*, pH and OD (optical density) measurements. On the basis of the calorimetric data maximum specific growth rate (μ_{max}, W h^{-1}), yield coefficients (Y_{XS}, g g^{-1}; Y_Q, J cfu^{-1}), produced heat (Q, J), lag–phase duration (λ, h), etc. were calculated for the bacteria. Power–time curves measured allowed to analyze growth curves as consisting of two phases—exponential phase and "deceleration phase". Active growth of biomass/active metabolic processes are taking place during the exponential phase of growth, and the second, "deceleration phase" combines the stationary phase of bacterial growth curve together with the deceleration of the metabolism of bacteria. The numerical data obtained for the analysis of the bacterial growth in broth form a necessary basis for further studies of *L. lactis* using calorimetric method.

1. Introduction

Due to their irreversible nature, life processes invariably and continuously dissipate Gibbs energy. As this is virtually always coupled with the continuous production and exchange of heat, calorimeters could be readily used for the monitoring of life processes. It has been shown that generation of heat by microbial cultures can be used for *on-line* monitoring of growth and metabolism of cells [1–5]. Calorimetry is especially helpful in the studies of growth in opaque media, for example solid media where agar, gelatine, etc. are used as jellifying agents [6], but it could be successfully applied also for the studies of spoilage processes and shelf-life determination [5,7–9]—everywhere where the possibilities of optical and other physical methods are limited due to the properties of non-transparent media [10,11]. But as heat effects are unspecific, they should be combined for more rich interpretation with the results of parallel experiments using other methods like measurements of concentration of substrates and products, outplating, for the determination of the number of bacteria, etc. [12,13]. In fact these parallel experiments allow to give a special meaning to the results of calorimetric measurements. Taking into account these circumstances a "rich use" of calorimetric method is possible after power–time curves have been complemented with the data of parallel experiments mentioned above. Knowledge of necessary conversion, yield, etc. coefficients allows to derive a number of characteristics of the growth of bacteria from the power–time curves. Carrying out of this type of preparatory study was one of the aims of the present work.

Microcalorimetry has been used for the experimental study of bacterial growth during the last 50 years. The first experiments were carried out with *Streptococcus faecalis* and it was shown that the rate of heat production was coupled with the rate of biomass growth [14]. Later growth of other bacteria was studied—growth of *Escherichia coli* in anaerobic conditions in case of low substrate and high inoculation concentrations [12], growth of *Lactobacillus helveticus* in different environmental conditions [15], etc. Further studies carried out have elaborated the coupling of kinetic and thermodynamic processes [16–22]. It has been shown that calorimetry could provide information about different metabolic changes such as shift from one substrate/type of catabolism to another, occurrence of limitations and inhibitions [1,9,23,24]. The enthalpy balance elaborated using calorimetric measurements can reveal the formation of unknown, unexpected by-products [19,24], or can be used in studying the synthesis of intermediate products of metabolism, and their effect on bacterial growth [25]. Production of ethanol by *Saccharomyces cerevisiae* has been studied [2], effect of added environmental toxicants on bacterial growth has been investigated [26], etc. Calorimetric power–time curves could be used also as imprints of (complicated) microbial processes, as the shape and the number of phases of the curves depend on the composition of the bacterial consortia [3] and reflect complicated patterns of multi-stage growth processes.

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Despite the noted advantages use of microcalorimetry was until recently limited in practice because of the absence of multichannel instruments. Carrying out experiments which take days, weeks or even months in 2–4 channel instruments was not a very attractive possibility, especially in the age of wide use of high throughput methods. However, the situation was changed with the appearance of the multichannel TAM III on the market—since the introduction of multichannel instrument it had been feasible to carry out microcalorimetric studies as regular investigations.

Growth of *L. lactis* in CRM broth media (CRM—*carbohydrate restricted medium*) with glucose as a main carbon source was studied in the present work using a multichannel thermal activity monitor TAM III. Optical density, concentrations of glucose and lactic acid in the culture media, *pH* of the culture media and *plate counts* of the bacteria were measured in parallel to the calorimetric measurements to obtain information, necessary for the quantitative interpretation of the calorimetric power–time curves in terms of peculiarities of growth processes of the bacteria. The methods were applied for the study of growth of *L. lactis* in liquid cultures on different concentrations of glucose (2–100 g L^{-1}). Growth of the bacteria during the exponential phase of growth and during the deceleration phase (the phase that combines the very short stationary phase and death phase of cells together with the decrease (deceleration) of metabolic activities) were analyzed separately. It was shown that the exponential growth of the bacteria was stopped due to the exhaustion of glucose at low concentration of glucose (regarding the growth of *L. lactis* at glucose concentration 2 g L^{-1}), whereas at higher glucose concentrations—up to 50–100 g L^{-1}, exponential growth of the bacteria was terminated by pH 4.3. A number of conversion coefficients were determined which have been used in the interpretation of the obtained power–time curves. These conversion coefficients could be used also in further studies of *L. lactis* in other experimental conditions including solid-state growth.

2. Materials and methods

2.1. Inoculum preparation

The cultivated strain *Lactococcus lactis* subsp. *lactis* IL1403 was kindly provided by Dr. Ogier from INRA (French National Institute for Agricultural Research, Jouy-en-Josas, France). Frozen storage cultures of *L. lactis* IL1403 were thawn and pregrown on Petri dishes with M17 Agar (LAB M, UK) for 24 h at 35 °C. One colony from a pregrown Petri dish was used as an inoculum for a 10 mL culture in liquid M17 Broth at 35 °C. 1 mL of bacterial suspension grown overnight (exactly 24 h) was used as inoculum for the next liquid 10 mL M17 Broth (FLUKA) medium and grown 14–16 h which allowed the *L. lactis* IL1403 bacteria to reach the middle of the exponential growth phase. The number of bacteria was determined by plating on M17 Agar and incubating for 3 days at 35 °C, and average number of bacteria in the mid-exponential culture was $(4.16 \pm 0.1601)\,10^8$ cfu mL^{-1}. The inoculum needed for the experiments was prepared by diluting of samples of the mid-exponential culture in peptone water. The calculated inoculation was in calorimetric and in parallel growth experiments 10^2 cfu mL^{-1}.

2.2. Growth experiments

CRM broth for growth media was prepared in a 800 mL Erlenmeyer flasks, sterilized by autoclaving at 121 °C for 15 min and after that shared to the 200 mL screw capped glass bottles where the glucose was added according to the desired media compositions—2, 20, 50, 100 g L^{-1}. A definite amount (1%, v/v) of the inoculum was added to the bottles with media solutions to get an inoculation rate of 10^2 cfu mL^{-1}, and after the inoculation the suspensions were intensively stirred. 2 mL of the culture from each bottle was put into 3 mL calorimetric ampoules and run at +35 °C. All the calorimetric growth experiments were carried out in three parallels.

The 200 mL bottles with bacterial suspensions were placed into thermostat at +35 °C. Glucose and lactic acid concentrations, pH, OD were determined in 4 mL samples, *outplating* was carried out using 100 μL samples at regular time intervals determined by bacterial calorimetric growth curves.

2.3. Analytical methods

HPLC (High Performance Liquid Chromatography) Separations Module Waters 2695 Alliance was used with a Refractive Index Detector 2414 and column BioRad HPX-87H Organic Acid Analysis Aminex Ion Exclusion Column 300 × 7.8 mm for measuring glucose and lactate concentrations (solvent—sulfuric acid solution (2.2 mL per 1 L MilliQ water), flow rate 0.6 mL min^{-1}, measuring temperature 35 °C). Biomass was calculated from optical density data measured at 540 nm using a spectrophotometer (PHOTOMETR KFK-3, USSR). The pH was measured with pH meter (S20 Seven Easy Mettler Toledo with InLab 413 Mettler Toledo electrode).

2.4. Compositions of the media used

The composition of the balanced M17 Agar (LAB M, UK) was as follows: peptone 5.0 g L^{-1}, soya peptone 5.0 g L^{-1}, yeast extract 2.5 g L^{-1}, beef extract 5.0 g L^{-1}, lactose 5.0 g L^{-1}, sodium glycerophosphate 19.0 g L^{-1}, magnesium sulfate 0.25 g L^{-1}, ascorbic acid 0.5 g L^{-1}, agar No. 2 15 g L^{-1}.

The composition of the balanced M17 Broth (FLUKA) was as follows: tryptone 2.5 g L^{-1}, meat peptone (peptic) 2.5 g L^{-1}, soya peptone (papainic) 5.0 g L^{-1}, yeast extract 2.5 g L^{-1}, meat extract 5.0 g L^{-1}, glucose 20.0 g L^{-1}, sodium glycerophosphate 19.0 g L^{-1}, magnesium sulfate 0.25 g L^{-1}, Tween 80 1.08 g L^{-1}, ascorbic acid 0.5 g L^{-1}, lactose 5.0 g L^{-1}).

The composition of the peptone water used for the dilution of the inoculum was as follows: NaCl 8.5 g L^{-1}, bacteriological peptone (LABM, UK) 1 g L^{-1}.

CRM broth growth medium of the following composition was used: casitone (Difco) 20 g L^{-1}, Tween 80 1.08 g L^{-1}, sodium chloride (MERCK) 25 g L^{-1}, magnesium sulfate 0.58 g L^{-1}, manganese sulfate 0.3 g L^{-1}, ferric sulfate 0.06 g L^{-1}, B-vitamin solution (SIGMA) 10 ml L^{-1}, MES (2-[N-morpholino]ethanesulfonic acid, SIGMA) 8.5 g L^{-1}.

2.5. TAM III [27]

The TAM III, which is a type of heat conduction multi-channel microcalorimeter was used to determine the growth of cells [28]. TAM III is designed to monitor continuously a wide variety of processes and complex systems over the temperature range 15–150 °C. TAM III is a multichannel instrument with maximum 48 channels. We used 24-channel instrument in our experiments. A heat flow calorimeter works by channeling the heat produced or consumed by a reaction in the sample through heat flow sensors comprised of thermoelectric modules. When a temperature gradient is imposed (or formed) across the thermoelectric module, a voltage is created in accordance with the Seebeck effect. This voltage is proportional to the heat flow through the thermoelectric module and hence proportional to the rate of heat production or consumption by the sample. One side of the thermoelectric module is in contact with the sample and the other is kept isothermal by a heat sink which is in contact with the TAM III thermostat (a liquid-based system). TAM III maintains outstanding sensitivity because of the excellent stability of the thermostat (<±100 μK/24 h) even over long periods of time (*TA Instruments* [27]). The detection limit of 0.5 μW and the

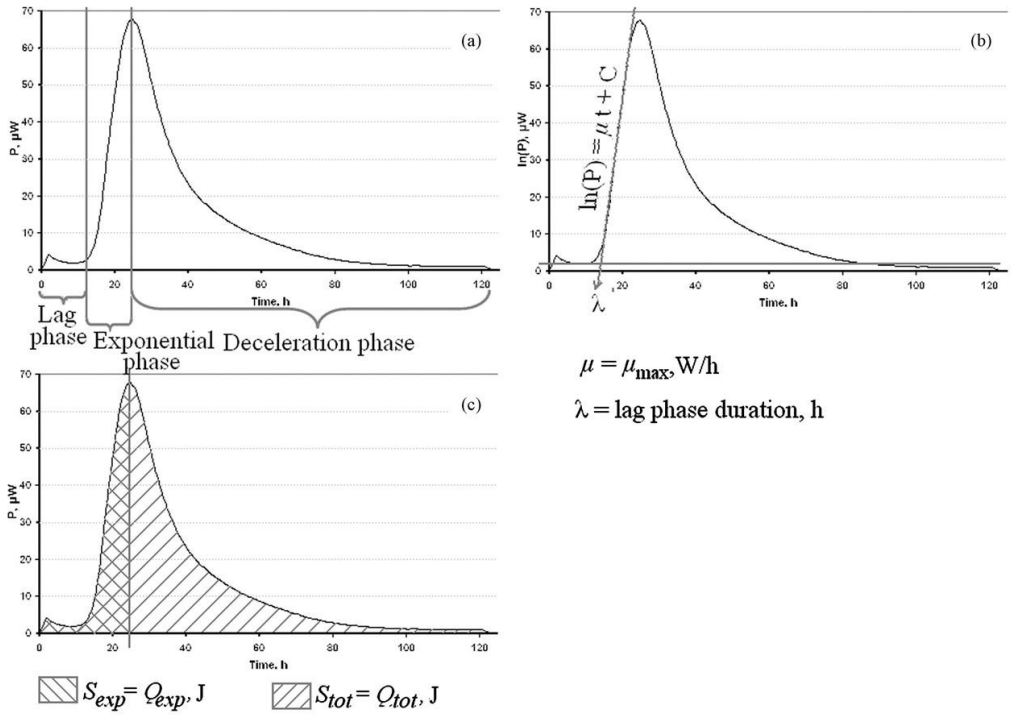

Fig. 1. The explanation of the processing of calorimetric power–time curves; (a) the division of calorimetric power–time curves into three phases–lag phase, exponential growth phase, and deceleration phase; (b) determination of the maximum growth rate (μ_{max}, h^{-1}) [3,16,17] and the lag phase duration (λ, h); (c) heat amounts determined—Q_{exp} is the heat evolved during the exponential phase and Q_{tot}—the total heat produced during the experiment.

baseline stability (over a period of 24 h) of ±0.2 μW characterize the instrument. The TAM III was operated in static ampoule mode (batch process) (see Wadsö [28]).

2.6. Analysis of power–time curves

3 mL ampoules with prepared cultures (see above) were placed into the TAM III. In all experiments heat flows (dQ/dt, μW) were measured. The curves that are the output of microcalorimeter are called power–time curves. Power–time curves obtained (see Fig. 1) were divided into three phases, essentially as ordinary growth curves of bacteria (Fig. 1(a)). Lag-phase which determines the adjustment period during which bacterial cells adapt to the new environment and start exponential growth [29] was defined in practice by the sensitivity of the microcalorimeter. In our case the growth was observed if there were about 10^5 cells in the ampoule (unpublished data). The second phase of the curve was exponential growth phase. In Fig. 1(a) it was assumed that the transition to the stationary phase (deceleration phase of growth) was started from the maximum value of the power–time curves.

The total heat produced during the whole process of growth (area between the power–time curve and baseline, Q_{tot}, J) and the heat evolved during the exponential growth phase (area between the power–time curve, vertical line, that goes through the peak, and baseline, Q_{exp}, J) (Fig. 1(c)) as well as the average maximum growth rate (the slope of the tangent to the exponential phase, μ_{max}, W h^{-1}) and lag-phase duration (the crossing point of X-axis and the tangent, λ, h) (Fig. 1(b)) were determined using *TAM Assistant* program (v 0.9.1012.40, *SciTech Software AB*, *Thermometric AB*). All the multiple Y-axis graphs were drawn using *Microcal* (*TM*) *Origin* (v 6, *Microcal Software*, *Inc*) program (Fig. 3).

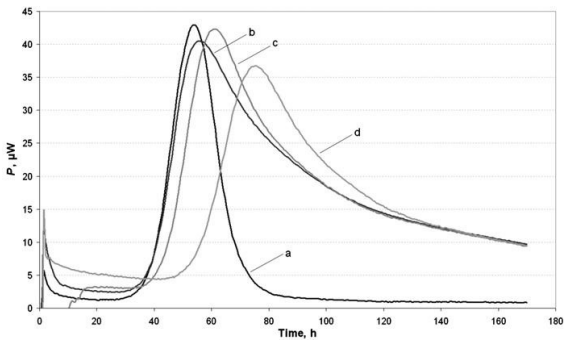

Fig. 2. Bacterial growth monitored by calorimetric power–time curves with differing initial substrate (glucose) concentration: a—2 g L^{-1}; b—20 g L^{-1}; c—50 g L^{-1}; d—100 g L^{-1}.

3. Results and discussion

3.1. Calorimetric growth curves in broth

The power–time curves describing growth of *L. lactis* IL1403 at different glucose concentrations (2, 20, 50, 100 g L^{-1}) and the same initial inoculation rate of 10^2 cfu mL^{-1} are presented in Fig. 2. Each curve presents average of three parallel power–time curves which were measured for the different environmental conditions studied.

Table 1
Parameters describing bacterial growth at different glucose concentration in CRM broth obtained from the processed power–time curves.

Glucose concentration, g L^{-1}	Maximum specific growth rate, μ_{max}, W h^{-1}	Evolved heat during the exponential growth phase Q_{exp}, J	Evolved total heat Q_{tot}, J	Lag-phase duration λ, h
2	0.2055[a]	1.4819	3.4544	29.71
2	0.2147[a]	1.5695	3.6604	29.42
20	0.1730[a]	1.7971	11.226	27.54
20	0.1732[a]	1.9582	11.302	27.80
50	0.1530[a]	2.1029	10.921	29.82
50	0.1533[a]	2.0755	10.825	29.29
100	0.1075[a]	2.3109	9.8581	34.86
100	0.1032[a]	1.9670	9.2376	33.93

[a] $R = 0.999$.

The standard deviation of the averaged curves was 0.355 μW which was quite low, confirming high reproducibility of the calorimetric method.

It is evident from Fig. 2 that calorimetric power–time curves measured were different in the case of different initial glucose concentrations. The power–time curve observed at low glucose concentration 2 g L^{-1} was almost like a Gaussian curve – symmetric – after rapid exponential growth phase similarly fast deceleration phase was taking place. With the increase of glucose concentration the power–time curves lost their symmetry, and growth deceleration phase became extended and prolonged. The power–time curves were processed as described in Section 2 and the numerical results obtained were presented in Table 1. The data obtained showed that the maximum specific growth rate decreased with the glucose concentration increasing which is in a good agreement with the observations made also earlier [30,31]. As seen from Table 1 the maximum specific growth rate (μ_{max}, W h^{-1}) decreased from >0.2 W h^{-1} to 0.1 W h^{-1} on the increase of glucose concentration from 2 g L^{-1} to 100 g L^{-1}.

The heat evolved during the exponential phase increased only slightly (from 1.5 J to 2 J) on the same change of glucose concentration. Assuming that growing bacterial cells produce equal amount of heat at different glucose concentrations it could be deduced that the number of bacteria produced during the exponential phase was almost the same in case of all substrate concentrations studied (see also Fig. 4).

The total evolved heat (Q_{tot}, J) was the smallest at 2 g L^{-1} glucose concentration (about 3.5 J) and it was approximately three times higher (about 10–11 J) in case of higher substrate concentrations studied (20, 50 and 100 g L^{-1}). Taking into account also the results of the additional experiments (see below) it could be assumed that at small glucose concentration the growth of the bacteria was limited by substrate (glucose) exhaustion, whereas at higher glucose concentrations physiological activities of the cells associated with the evolution of heat were continued even after the intensive growth of the biomass was stopped. At low glucose concentration the heat evolved during the exponential phase was 43% of the total, whereas at higher glucose concentrations the contribution of the exponential phase decreased to 17–21%. As noticed, the heat evolved during the exponential phase was practically the same in all growth conditions studied.

The duration of the lag-phase was the longest—34 h at the highest glucose concentration used. The shortest lag-phase was observed at glucose concentration 20 g L^{-1}, where it was equal to 27 h (Table 1).

In parallel experiments with the measurements of power–time curves growth of the bacteria was monitored also through the measurements of pH, lactic acid (*LA*, g L^{-1}) and glucose (*GLC*, g L^{-1}) concentrations changes in time in case of different initial glucose concentrations (Fig. 3) (see also Section 2). All the data measured in these parallel experiments were splined [32].

As seen from Fig. 3 the microbial growth curves could be divided into two groups also on the bases of the change of environmental parameters (pH, glucose and lactate concentrations): (a) in the case of low concentration of glucose (2 g L^{-1}) the substrate was exhausted in the end of the growth, and (b) the glucose was not exhausted in the end of growth in the case of all other concentrations studied.

In the case of low concentration of glucose (2 g L^{-1}) the active growth (see calorimetric power–time curve, black line) was observed starting from the 29th hour of cultivation, the maximum value of the power–time curve 22 μW mL^{-1} was observed at the 50th hour, and then the sharp decrease of heat production followed. The concentration of glucose decreased rapidly in parallel with the growth, and glucose (red line) was exhausted at the 60th hour. As expected, the production of lactic acid (blue line), and the change of pH (green line) were observed in parallel to the growth. The amount of lactic acid produced during the growth (∼1.8 g L^{-1} or 0.02 mol L^{-1}) was almost the same as the amount of glucose consumed (∼2 g L^{-1} or 0.01 mol L^{-1}) which was in a good agreement with the stoichiometry of lactic acid homofermentative metabolism according to which from 1 mole of glucose 2 moles of lactic acid are produced, i.e. the ratio of the masses of glucose consumed and lactate produced should be 1:1. When growth was fully stopped the pH was 4.3 (see Fig. 3).

As mentioned earlier in association with the analysis of power–time curves (see Fig. 2) the amount of heat produced during the exponential growth phase was practically the same in different growth conditions (about 2 J). As seen from the curves presented in Fig. 3 the maximum of the power–time curves was observed in all the cases, except the low concentration of glucose (2 g L^{-1}), at pH 4.3. The maximum of the power–time curve at low concentration of glucose was observed at pH 4.7 (according to Andersen et al. [30] the rate of glucolysis is considerably affected by acidity of the growth media starting with already pH 5). These facts obtained indicated that the exponential growth phase of the cultures of *L. lactis* most probably was terminated by pH (pH 4.3) at high initial concentrations of glucose in the culture media, and by exhaustion of glucose at the initial glucose concentration 2 g L^{-1}. The results obtained by us are in agreement with the results obtained earlier [33].

As seen from Fig. 3 the extended deceleration phases of heat production took place in parallel with the glucose consumption and lactic acid production at high initial concentrations of glucose. pH reached the minimum value pH 3.2 at around 100 h considerably earlier than glucose consumption or lactate production were stopped. These facts indicated that the final pH was determined by the buffering capacity of the growth media, rather than by the concentration of lactic acid in the media.

It must be noted that the consumption of glucose and production of lactic acid and dissipation of noticeable amount of heat was taking place during the post-exponential growth phase of cultivation which indicated that bacteria were slightly active even at pH 3.2 in the end of the experiments [21,23]. However, it should be noticed also that at higher concentrations of glucose, and especially during the post-exponential growth phase the ratio of lactic acid produced

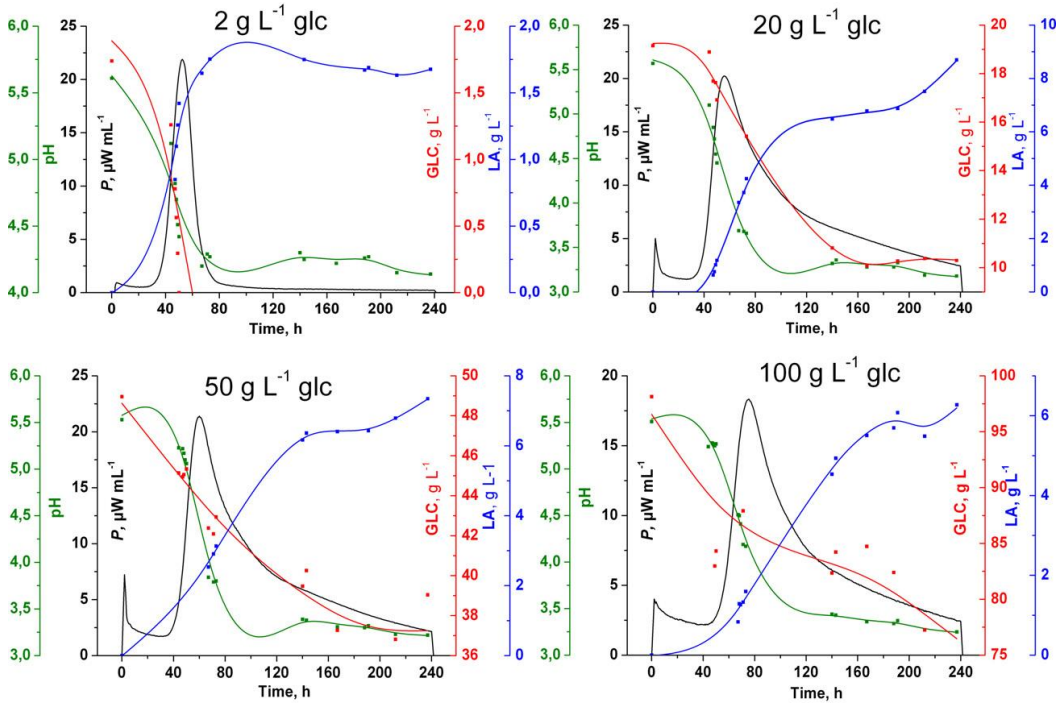

Fig. 3. Calorimetric power–time curves, pH change, lactic acid production and glucose consumption curves describing the growth of the bacteria in case of different initial glucose concentrations 2, 20, 50 and 100 g L^{-1}.

to glucose consumed was remarkably less than expected in the case of homolactic fermentation—lactic acid produced made up only 62% of the glucose consumed in case of 50 g L^{-1} GLC, and 22% in case of 100 g L^{-1} glucose.

All the data referred showed that the post-exponential growth phase (deceleration phase) is a complicated pattern of metabolic changes taking place after the exponential growth phase was stopped. Details of these patterns deserve certainly careful further studies using in addition to calorimetry also other methods.

Growth of the bacteria *L. lactis* was studied also using in addition to the measurements of power–time curves optical density and *plate-count* measurements. The data obtained were splined and shown in Fig. 4. During the growth in exponential phase the number of bacteria was proportionally increasing with the growth of OD. Exponential growth was ended at $1.5–2.5 \times 10^8$ cfu mL^{-1}; and OD = 0.40–0.68. During the post-exponential growth phase the OD values were staying practically constant, whereas the numbers of bacteria decreased about 10 times. The different behavior of growth characteristics in the post-exponential growth phase could be explained by assuming that OD showed not only living bacterial biomass which were able to multiply, but also partially autolysed or "dead" biomass. *Plate-count* method showed only viable cells.

Exponential phase curves measured by *outplating* or OD measurements were in a good agreement with the exponential phases of the power–time curves indicating that the latter could be used for the study of the bacterial growth during the exponential growth. A more complicated was the situation with the declining parts of the power–time curves. It is reasonable to assume that at least part of the heat evolved during the post-exponential growth phase was not reflecting the growth of biomass but some other physiological processes.

3.2. Conversion coefficients

If the stoichiometry of the biomass growth does not change during the growth, the rate and amount of biomass formation, (dX/dt) and $(X - X_0)$, are proportional to the rate and amount of heat production, dQ/dt and Q respectively. In this case Y_Q (J cfu^{-1}), the conversion, yield coefficient–amount of heat produced per cell synthesized, could be determined:

$$\frac{dX}{dt} = Y_Q \frac{dQ}{dt} \quad (1)$$

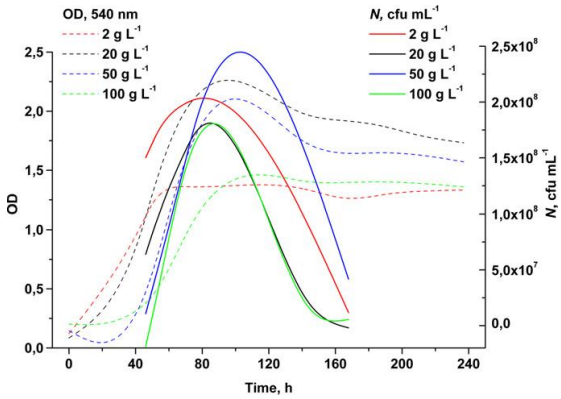

Fig. 4. The change of bacterial number (cfu mL^{-1}) and optical density (OD) in broth media with different substrate concentration (glucose 2, 20, 50, 100 g L^{-1}).

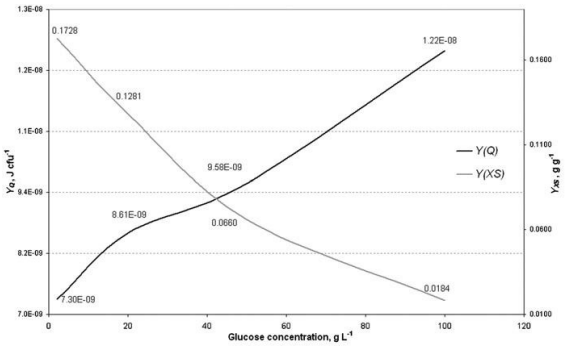

Fig. 5. Dependence of heat yield coefficient Y_Q (J cfu^{-1}) and Y_{XS} (g g^{-1}) determined for the exponential growth phase on different glucose concentrations 2, 20, 50, 100 g L^{-1}.

Knowing the value of Y_Q makes possible calculation of cell numbers and biomass from the power–time curves. For the calculation of Y_Q power–time curves and growth curves of biomass should be measured. Usually biomass is evaluated by optical density of the culture (OD), by number of cells in the culture broth determined by *outplating* (*N—bacterial number*, cfu mL^{-1}), and/or determined measuring dry biomass (*X*, g).

For the determination of constant k_1 which characterizes the relationship between optical density and bacterial cell number (cfu mL^{-1}) (Eq. (2)) the growth of *L. lactis* IL1403 was monitored in chemostat.

$$\Delta N = k_1 \times \Delta OD \quad (2)$$

monitored in chemostat. On the basis of the data collected the value of the constant was determined $k_1 = 1.63 \times 10^9 \pm 4.01 \times 10^8$ cfu. As known, bacteria are very actively growing in chemostat. The value of the same constant determined from the batch experiments presented in this paper was in exponential phase four times lower than in chemostat ($4.53 \times 10^8 \pm 7.7 \times 10^7$ cfu OD^{-1}).

The amount of biomass was estimated in chemostat experiments also through measurements of dry biomass. The value of the coefficient k_2 (Eq. (3)) was determined $k_2 = 0.32 \pm 3.05 \times 10^{-3}$ g OD^{-1}.

$$\Delta X = k_2 \times \Delta OD \quad (3)$$

On the basis of calorimetric *on-line* measurements of the power–time curves, *plate count* and OD (optical density) measurements the yield coefficients Y_Q, Y_{XS} were calculated. Both, Y_Q and Y_{XS} were calculated using the data of the exponential phase of the growth curves and corresponding intervals of the power–time curves in case of different glucose concentrations studied (Fig. 5).

As seen from Fig. 5, the heat yield was the lowest (7.30×10^{-9} J cfu^{-1}) at low glucose concentration (2 g L^{-1}). The heat yield value for the bacteria was growing practically linearly up to 1.22×10^{-8} J cfu^{-1} on increase of the glucose concentration. In full agreement with the observed behavior of the Y_Q the yield coefficient Y_{XS} which characterizes efficiency of biomass formation was decreasing on the increase of the glucose concentration—see Fig. 5. The value of Y_{XS} determined at the initial substrate concentration of 2 g L^{-1} was comparable to the values determined in the literature [31,34]. However, the values of Y_{XS} decreased remarkably on the increase of the initial glucose concentrations. It may indicate that homolactic growth was changed for some other scheme of fermentation of glucose (see de Vos and Hugenholtz [35]).

It should be emphasized that the calculation of Y_Q and Y_{XS} and their physical status is different here from the growth of bacteria on a single carbon source mineral media. It has been shown that bacteria *L. lactis* do not grow on mineral media. They need complex media for supporting their growth. However, there do not exist a medium where the consumption of the other than glucose components of the complex media could be measured. We have estimated that the consumption of other components (amino acids, vitamins, etc.) did not exceed 10% of the consumption of glucose in the chemostat experiments. Calculation of the values of parameters defined as yield coefficients Y_Q, Y_{XS}, etc. in these circumstances gives indicative values of the conversion coefficients, which, however, could be used in the interpretation of the experimental results obtained in the similar conditions.

4. Conclusion

The results obtained showed clearly that calorimetry is a reproducible and sensitive method for the continuous monitoring of anaerobic growth of *L. lactis*. Supplemented and combined with the results of parallel measurements of HPLC, *OD*, number of bacteria, etc. multichannel calorimetry is becoming a powerful method for the quantitative studies of bacterial growth.

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PUBLICATION VI

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Microcalorimetric study of extruded dog food containing probiotic micro-organisms

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Abstract

Extruded dry dog food products claimed to have a probiotic ingredient of *Enterococcus faecium* (NCIMB10415) in the commercial available formulations under the brand name of ProBiotic LIVE (Bacterfield S.A., Luxembourg) were studied in the present work using a multichannel thermal activity monitor TAM III. Maximum specific growth rate, heat produced during different growth phases, and lag–phase duration were determined. The length of the lag-phase that can be used to determine the time necessary for the probiotic ingredient to restore its activity after consumption of probiotic containing extruded products was also measured. The calorimetric data confirmed the ability of the *Enterococcus faecium* to grow at the acidic pH conditions, modeling conditions of gastro-intestinal tract of dogs, and preserve its metabolic activity (viability) at the same level as at the neutral pH. The results obtained indicated that microcalorimetry was a precise and convenient tool for monitoring probiotic activity in complicated solid-state matrices.

Keywords: *Enterococcus faecium*, probiotic dog food, microcalorimetry

1. Introduction

Various attempts have been made to produce extruded feed or food product(s) with stable and viable probiotic bacteria count over the entire shelf-life period of the carrier product [1-3]. However, determination of the number of bacteria, and especially evaluation of their growth and metabolic activity in extruded food and feed products by means of *outplating* etc. cannot be considered sufficiently precise and convenient.

It has been shown that generation of heat by microbial cultures can be used for on-line monitoring of growth and metabolism of cells [4–10]. Very sensitive measurements of heat flows using microcalorimetry is one of the most attractive techniques for studying and monitoring of the metabolic activity of bacteria in opaque liquid media, and solid matrices [11-13]. It could be successfully applied in the studies of spoilage processes and shelf-life determination of products containing microbes [8,14–16], in fact in all cases where the possibilities of optical and other physical methods are limited due to the non-transparency of media [17-18].

Growth of bacteria in extruded dry dog food products under the brand name of ProBiotic LIVE (Bacterfield S.A., Luxembourg) claimed to contain a probiotic ingredient of *Enterococcus faecium* (NCIMB10415) were studied in the present work using a multichannel thermal activity monitor TAM III.

2. Materials and methods

2.1. Samples

The experiments were performed with dry dog food in the form of extruded kibbles under a brand name of ProBiotic LIVE (Bacterfield S.A., Luxembourg) containing according to the producer viable probiotic bacteria *Enterococcus faecium* (NCIMB10415) during the entire period of product shelf-life (15 months) at room temperature. The concentration of bacteria as declared on the packaging is 10^6 cfu per gram of dog food in average. Three commercially available dog food products differentiated by dog age (adult and senior) and by taste (salmon and chicken formulations) were used in the experiments: ProBiotic LIVE Adult Chicken & Rice (AC), ProBiotic LIVE Adult Salmon & Rice (AS) and ProBiotic LIVE Senior Chicken & Rice (SC). All dog food samples used in the experiments were initially received in hermetically closed separate 1.5 kg bags, with expiry date/lot. nr. 23.11.2012 and used freshly in the middle of

the claimed product shelf-life. All the bags were aseptically opened immediately before the experiments. Additionally, dry bacterial concentrate of the same strain of the bacteria as in the dog food (*Enterococcus faecium* NCIMB10415) provided by Probiotics International Ltd. (UK, Batch No BN 29094, expiry date 11.2013) was studied as a reference sample to dog food formulations. The bacterial concentrate contained 10^{10} cfu per gram of the powder according to the producer.

2.2. Sample preparation and experimental conditions

Dry dog food kibbles of different types were ground and sifted to fine particles (less than 0,05 mm). Afterwards samples were weighed in sterile plastic Falcon tubes. The mass of each sample was approximately ~0.5 g. Samples were aseptically transferred into 3 mL autoclaved microcalorimetric ampoules. Depending on experimental conditions sterile MilliQ water, adjusted to pH=2 (dog stomach equivalent pH [19]), or to pH=7 (neutral value), was added to the ground dry dog food in mass ratio 1:1. pH was measured with pH meter S20 Seven Easy Mettler Toledo with calibrated InLab 413 Mettler Toledo electrode. All the solutions were previously sterilized by autoclaving at 121°C for 15 min. As a reference, samples of dry non-diluted bacterial concentrate were also analyzed.

In order to determine the initial bacterial concentration in dog food kibbles *outplating* on PCA (Plate Count Agar, Scharlau, Spain) was carried out for all three dog food formulations and dry bacterial concentrate.

2.3. Microcalorimetry

A TAM III (24-channels, TA Instruments, US), a heat conduction multi-channel microcalorimeter was used to monitor the growth of cells in dog food and bacteria concentrate samples. The construction and functioning of the TAM III calorimeter were described earlier by Wadsö [8]. Microcalorimetric power-time curves were analyzed as described by Kabanova [10]. The calorimetric trials were performed at fixed temperature of incubation at 37 °C (equivalent of dogs body temperature [20]) using 3 mL calorimetric ampoules. All the calorimetric experiments were carried out in three parallels and power-time curves obtained were normalized per gram of dog food. Averaged power-time curves of three parallel runs were used for the further analysis.

3. Results and discussion

The averaged power–time curves of growth of *Enterococcus faecium* (NCIMB10415) in different formulations of dog food in case of dry samples and in moisturized samples at two different pH values are presented in **Figure 1**.

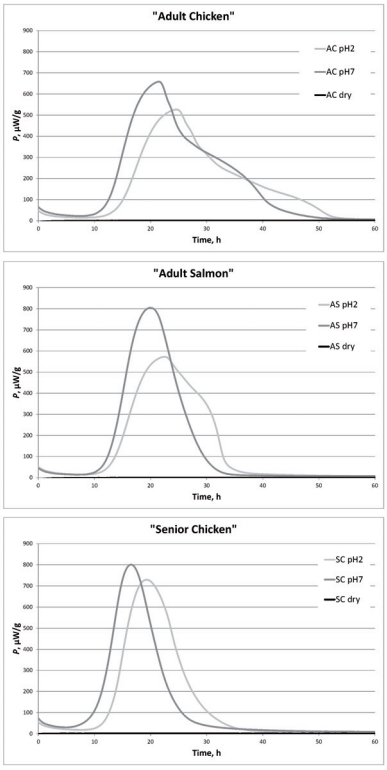

Figure 1. Power-time curves of growth of probiotic bacteria in three different dog food: dry and moisturized samples (AC, AS and SC) at two different pH (pH=2 and pH=7) values.

The standard deviation of the averaged power-time curves was 0.55 μW, which was quite low, confirming high reproducibility of the calorimetric method.

Power-time curves were analyzed according to the Kabanova [9] and all numerical values of processed power-time curves are presented in **Table 1**.

Table 1. Parameters describing bacterial growth of three dog food formulations (AC, AS, SC) at two different pH (pH=2 and pH=7) levels obtained from the processed power–time curves: lag phase duration (λ, h), maximal specific growth rate (μ_{max}, W/h), heat produced during exponential growth phase (S_{exp}, J/g) and during the total growth (S_{tot}, J/g) and the respective numbers of bacteria produced during the both growth phases (N_{exp}, cfu/g and N_{tot}, cfu/g respectively).

		λ, h	σ, h	μ_{max}, W/h	σ, W/h	S_{exp}, J/g	σ, J/g	N_{exp}, cfu/g	S_{tot}, J/g	σ, J/g	N_{tot}, cfu/g
pH 2	AC	4,94	0,77	0,4547	0,05	14,28	0,86	5,53E+09	37,09	8,58	1,44E+10
	AS	5,08	0,49	0,5271	0,01	13,89	4,96	5,38E+09	32,18	7,77	1,25E+10
	SC	4,68	0,39	0,5809	0,00	12,85	0,92	4,97E+09	32,37	1,74	1,25E+10
pH 7	AC	3,54	0,33	0,5039	0,03	17,24	1,13	6,67E+09	41,62	3,78	1,61E+10
	AS	4,73	0,09	0,6012	0,04	15,52	1,11	6,01E+09	34,45	2,10	1,33E+10
	SC	3,42	0,50	0,5123	0,17	13,21	0,47	5,11E+09	31,68	3,40	1,23E+10

The length of the lag phase was 3.9 ± 0.72 hours (in average) at pH 7 (neutral pH). The lag phase was prolonged by 1 hour, up to 4.9 ± 0.2 hours (in average) at pH 2 (low pH), which is the pH level in the stomach of dogs [13]. Prolongation of the lag-phase at low pH can be explained by acidic stress effect on the probiotic bacteria.

The average specific maximum growth rate (μ_{max}. W/h) of the *Enterococcus faecium* bacteria was practically the same at low and neutral pH levels (0.5209 ± 0.0634 W/h and 0,5391 ± 0.0539 W/h correspondingly).

The average heat produced during the exponential phase (S_{exp}. J/g) at neutral pH was 15,33 ± 0.90 J/g and 13,67 ± 0.74 J/g at acidic pH condition. Using the Y_Q value 2.58 ± 0.44 * 10^{-9} J/cfu determined by us for *L. lactis* IL1403 [10] the average number of bacteria grown during the exponential growth (N_{exp}, cfu/g – $5.93*10^9$ cfu/g at neutral pH and $5.29*10^9$ cfu/g at low pH) was calculated – see **Table 1**.

The estimates of biomass obtained should be considered reliable, as the values of Y_Q have been practically the same in the different papers: $5,53*10^{-8}$ J/cfu for soil samples [21], $4,22*10^{-9}$ J/cfu and $3,4*10^{-8}$ J/cfu for *Staphylococcus aureus* and *E. coli* respectively [22].

The total heat produced during the entire period of thermodynamic activity (full length of power-time curves registered, all bacteria growth phases included - S_{tot}. J/g) was also practically the same at both pH-s studied. The average total heat produced at neutral pH was 35,92 ± 0.78 J/g and 33,88 ± 1.23 J/g at low pH, which correspond to $1.39*10^9$ cfu/g and $1.31*10^9$ cfu/g respectively. The numbers of bacteria grown during the exponential growth phase (N_{exp}, cfu/g) and bacteria grown during the complete growth (N_{tot}, cfu/g) achieved the same levels of 10^9 and 10^{10} cfu/g correspondingly at the two pH values.

All three formulations of ProBiotic LIVE dog food (AC: Adult Chicken & Rice, AS: Adult Salmon & Rice and AS: Senior Chicken & Rice) enriched with probiotic bacteria of *Enterococcus faecium* (NCIMB10415) had the same initial bacterial count in average 10^6 cfu/g confirmed by *outplating* of the samples on the PCA presented in **Table 2**. Average concentration of bacteria in all the three dog food formulations was $2,86*10^6$ ± $5,31*10^5$ cfu/g according to the results presented in **Table 2**. This confirms the claimed by a producer average *E. faecium* load of 10^6 cfu per gram in the product.

Table 2. Plate counts of the bacteria on PCA and pH values of different dog food formulations and bacteria concentrate.

Sample	N, cfu/g	σ, cfu/g	Bulk pH
AC	2,35E+06	2,19E+04	5,77
AS	3,41E+06	6,17E+04	5,87
SC	2,82E+06	5,14E+04	5,94
Bact. concentrate	2,24E+10	3,28E+08	5,23

It is known that the low acidic pH in the stomach of dogs is considered as a barrier for the probiotic bacteria. Ability to preserve the viability during the passage through the stomach acidic barrier is a prerequisite for the further successful colonization of the host intestine by the probiotic bacteria. The *Enterococcus faecium* bacteria present in the dog food of ProBiotic LIVE formulations were able to show strong metabolic activity at low acidic pH value, which confirmed the fact that *Enterococcus faecium* can tolerate the passage through the acidic barrier of the dog's stomach and populate the intestine afterwards. The ability of the probiotic bacteria *Enterococcus faecium* (NCIMB10415), to preserve the activity throughout the passage through the stomach acidic barrier of dogs was also confirmed in the trials of the European Food Safety Authority (EFSA, [23]).

Analysis of the power-time curves revealed that the duration of the time needed for the probiotic bacteria to regain their activity in the host organism after consumption, the length of the lag-phase, was about 5 hours (see **Table 1**). This time is sufficient for the digest flow to pass the acidic barrier of stomachs of dogs.

There was no bacterial metabolic activity observed in the case of non-diluted dry dog food samples analyzed, see **Figure 1**. Also the freeze-dried *Enterococcus faecium* (NCIMB10415) bacteria powder concentrate (both dry and diluted samples) showed no thermal activity (data not shown).

4. Conclusion

The results obtained showed clearly the ability of the probiotic *Enterococcus faecim* (NCIMB10145) bacteria strain present in the extruded dog food kibbles to preserve its viability and growth characteristics at acidic and neutral pH. This indicated that the extruded kibbles containing *E. faecium* bacteria are able to tolerate the dog's stomach acidic barrier with further successful colonization of the dog's intestine.

The results obtained showed that calorimetric technique is a prospective and sensitive method for the continuous *in-situ* monitoring of bacterial activity in such complicated matrices as extruded feed or food products, and for shelf-life determination of probiotic ingredients in the products.

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5. **Courses & Conferences**

1st Baltic Conference on Food Science and Technology, FOODBALT, Kaunas, Lithuania, **2006**

Quantum Eesti AS seminar: Vapor sterilization basics and laboratory autoclave structured individuality dependently on sterilization processes, Tallinn, Estonia, **2006**

XXVIII National Conference on Calorimetry, Thermal Analysis and Chemical Thermodynamics, Milan, Italy, **2006**

The 3rd International Scientific Conference on Probiotics and Prebiotics - IPC2008, High Tatras, Slovakia, **2008**

The 4st International Scientific Conference on Probiotics and Prebiotics - IPC2010, Kosice, Slovakia, **2010**

Annual Conference on a regulatory update on animal feed & feed additives in the EU, London, United Kingdom, **2010**

Seminar „Pet food usage and placing on the market", Estonian Ministry of Agriculture Tallinn, Estonia **2011**

The 5th International Scientific Conference on Probiotics and Prebiotics - IPC2011, Kosice, Slovakia, **2011**

6. Professional employment

2005 - 2007 Tallinn University of Technology, Department of Chemistry, Chair of Biotechnology, research fellow

2007 - …. Bacterfield Ltd., technical manager

7. List of patents and articles

- N. Kabanova, **A. Kazarjan**, I. Stulova, R. Vilu, Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentrations in broth, Thermochimica Acta, Volume 496, Issues 1-2, 10 December **2009**, Pages 87-92

- International application published under the patent cooperation treaty (PCT), Title: A method for the management of fecal consistency in dogs, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122104 A1 **(2010)**

- International application published under the patent cooperation treaty (PCT), Title: A vacuum infusion production plant, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122155 A1 **(2010)**

- International application published under the patent cooperation treaty (PCT), Title: Probiotic oil suspension and use thereof, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122107 A1 **(2010)**

- International application published under the patent cooperation treaty (PCT), Title: Extruded food products compromising probiotic microorganisms, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122106 A1 **(2010)**

- **A. Kazarjan**, N. Kabanova, R. Vilu, Microcalorimetric study of extruded dog food containing probiotic microorganisms, Advances in Microbiology, Volume 2, Number 4, December **2012**, (in press)

ELULOOKIRJELDUS

1. **Isikuandmed**

Eesnimi	Aram
Perekonnanimi	Kazarjan
Sünniaeg ja -koht	10.03.1983, Tallinn
Kodakondsus	Eesti

2. **Kontaktandmed**

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3. **Hariduskäik**

1990 - 2002	Tallinna Linnamäe Kool
2002 - 2005	TTÜ, rakenduskeemia ja biotehnoloogia, B.Sc.
2005 - 2007	TTÜ, rakenduskeemia ja biotehnoloogia, M.Sc.

4. **Keelteoskus**

Armeenia keel	emakeel
Eesti keel	kõrgtase
Inglise keel	kõrgtase
Vene keel	kõrgtase

5. **Täinedusõpe ja konverentsid**

1st Baltic Conference on Food Science and Technology, FOODBALT – 2006, Kaunas, Lithuania, **2006**

Quantum Eesti AS seminar: Vapor sterilization basics and laboratory autoclave structured individuality dependently on sterilization processes, Tallinn, Estonia, **2006**

XXVIII National Conference on Calorimetry, Thermal Analysis and Chemical Thermodynamics, Milaano, Itaalia, **2006**

The 3rd International Scientific Conference on Probiotics and Prebiotics - IPC2008, High Tatras, Slovakia, **2008**

The 4st International Scientific Conference on Probiotics and Prebiotics - IPC2010, Kosice, Slovakia, **2010**

Annual Conference on a regulatory update on animal feed & feed additives in the EU, London, Inglismaa, **2010**

Seminar „Lemmikloomatoidu turuleviimine ja kasutamine", Põllumajandusministeerium, Tallinn, Estonia **2011**

The 5th International Scientific Conference on Probiotics and Prebiotics - IPC2011, Kosice, Slovakia, **2011**

6. Teenistuskäik

2005 - 2007 Tallinna Tehnikaülikool, Rakenduskeemia ja biotehnoloogia õppetool, laborant

2007 - …. Bacterfield OÜ, tehniline juht

7. Publikatsioonide loetelu

- N. Kabanova, **A. Kazarjan**, I. Stulova, R. Vilu, Microcalorimetric study of growth of *Lactococcus lactis* IL1403 at different glucose concentrations in broth, Thermochimica Acta, Volume 496, Issues 1-2, 10 December **2009**, Pages 87-92

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- International application published under the patent cooperation treaty (PCT), Title: Extruded food products compromising probiotic microorganisms, Inventors: Kirejevas, Vygantas and **Kazarjan, Aram**. International publication Nr.: WO 2010/122106 A1 **(2010)**

- **A. Kazarjan**, N. Kabanova, R. Vilu, Microcalorimetric study of extruded dog food containing probiotic microorganisms, Advances in Microbiology, Volume 2, Number 4, December **2012**, (in press)

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