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MOLECULAR PROFILE OF ORAL PROBIOTIC BACTERIA TO BE USED WITH FUNCTIONAL FOODS

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Abstract:

In this work, we present thermal and IR spectral profile and thereby molecular information about the probiotic bacteria- *Streptococcus salivarius* K12 (SsK12) to be further used with functional foods. In this context, for the first time, we characterized the global cellular constituents of this bacteria i.e. its proteins, lipids, polysaccharides, ribosomes, nucleic acids and cell wall by powerful calorimetric (differential scanning calorimetry) and infrared spectroscopic techniques, which are widely applied bioanalytical methods especially in the food microbiology and industry. Our results can be used by food specialists for the development of probiotic functional foods such as cheese, kefir, yogurt, buttermilk, pickle and chewing gum enriched with viable lyophilized bacteria to preserve oral health. Furthermore, the information we present here, would contribute for the development of prophylactic strategies and for the generation of recombinant probiotic SsK12 strains, which would help to launch out a new public health-care action plans.

Keywords: Probiotics, FTIR, Differential Scanning Calorimetry, Functional Foods, Freeze Drying, *Streptococcus salivarius* K12 (SsK12)

Introduction

Recent literature on oral and nasopharyngeal infectious diseases advice the use of preventive approaches to eradicate them before appearance of the symptoms rather than their symptomatic and post-symptomatic treatment (Tagg and Dierksen, 2003; Guglielmetti *et al.*, 2010; Santagati *et al.*, 2012; Yildiz, 2016). In this context many strategies have been developed and generally they are based on the application of different probiotic bacteria for the prevention and therapy of various diseases (Wescombe *et al.*, 2009; Wescombe *et al.*, 2010; Wescombe *et al.*, 2012; Di Pierro *et al.*, 2014). In this article we concentrated on one of these probiotic bacteria namely *Streptococcus salivarius* K12 (SsK12), by considering its strong preventive potential for infectious disease control (Tagg, 2004; Tagg, 2009). It should be noted that, this non-pathogenic strain has been successfully applied for the prevention and therapy of different bacterial and viral infectious diseases infecting oral and nasopharyngeal cavities (Power *et al.*, 2008; van Zon *et al.*, 2012; Di Pierro *et al.*, 2013; Di Pierro *et al.*, 2014; Di Pierro *et al.*, 2015). Children taking SsK12 appear to have had significantly fewer group A beta-hemolytic streptococci (GABHS) infections both during the 90-day period of prophylaxis and during the following 9 months (Gregori *et al.*, 2016). Another study also demonstrated its preventive role in reducing the incidence of both streptococcal (90 %) and viral pharyngitis (80 %) and/or tonsillitis in children (Di Pierro *et al.*, 2014). Patras *et al.* showed the effectiveness of SsK12, even in the preventive therapy against the vaginal colonization of group B streptococcus (GBS) and its spread to the newborn (Patras *et al.*, 2015). Interestingly, it has shown to increase the salivary interferon-γ and decrease the interleukin-8 levels in adults, affecting neither interleukin-1β nor the tumor necrosis factor-α (Mostefaoui *et al.*, 2004; Wescombe *et al.*, 2012). Although, a plenty of research have been conducted in the scientific and engineering community to study this strain, it is surprising that the global cellular constituents of this bacteria i.e. its proteins, lipids, polysaccharides, ribosomes, nucleic acids and cell wall have not been comprehensively characterized yet. Considering its great therapeutic potential and a lack of sufficient knowledge about its molecular constituents, we devoted effort to characterize SsK12 in a holistic way, by powerful calorimetric (differential scanning calorimetry- DSC) and infrared (IR) spectroscopic techniques for the first time, which are widely applied bioanalytical methods especially in the food microbiology and industry for their ultimate and explicit capacities for providing qualitative and quantitative information about a specimen simultaneously (Naumann *et al.*, 1991).

DSC is a thermal fingerprint technique measuring thermally induced conformational changes and phase transitions in the molecules (Kaletunç *et al.*, 2004; Abuladze *et al.*, 2009). Several endothermic transitions are being observed in the microorganisms under the thermal effect which correspond to the conformational alterations in the molecular ingredients of the studied microorganism such as lipids, proteins, nucleic acids and cell wall components (Lee and Kaletunc, 2002; Chiu and Prenner, 2011). DSC measures these thermal events and delivers the unique structural and functional information about the microbial molecules and various intracellular processes (Mackey *et al.*, 1991; Brannan *et al.*, 2015). DSC profiles reminds the gel electrophoresis profiles, however in DSC the specific peaks are identified according to the thermal stability rather than the mass and charge (Lepock *et al.*, 1990). Although, DSC has been mostly used for the analysis of single molecules, sophisticated biological systems like mammalian cells (Lepock *et al.*, 1990), vegetative bacterial cells (Miles *et al.*, 1986; Anderson *et al.*, 1991; Mackey *et al.*, 1991) and bacterial spores (Maeda *et al.*, 1978; Miles *et al.*, 1986; Anderson *et al.*, 1991) have also been studied.

IR spectroscopy has been extensively used as a tool for the classification, identification and discrimination of microorganisms by producing unique finger-prints of whole-organism (Wenning and Scherer, 2013). For example, Dziuba *et al.* used IR spectroscopy to identify several lactic acid bacteria at the genus level (Dziuba *et al.*, 2007), while Erukhimovitch *et al.* showed that it can be used for rapid discimination of bacterial infections from fungal infections (Erukhimovitch *et al.*, 2005). This technique is a quick, relatively inexpensive, easy to use, non-destructive and sensitive method, requiring a relatively small amount of sample with no excessive sample preparation steps like amplification, labelling or staining (Naumann *et*

al., 1991; Haris and Severcan, 1999; Maquelin *et al.*, 2003).

In this study, for the first time, we obtained thermal and IR spectral profile and thereby molecular information about the SsK12 cell which can be exploited in the development of preventive treatment and post-disease prophylactic strategies and/or for the generation of recombinant probiotic SsK12 strains. In its turn, this would help to launch out a new public health-care action plan and reduce the treatment expenses and antibiotic consumption significantly.

Materials and Methods

Bacteria growth conditions, determination of colony forming units (CFU) and lyophilization experiments

SsK12 was obtained from BLIS Technologies Ltd. manufactured as a fast-melt tablet (BLIS K12) and was grown 37ºC temperature in an orbital shaker (*Zhicheng*, CN) at 150 rpm for 40 h. Skim milk (1L for a tablet) was used as a growth medium. The incubated medium was poured into 300 mL Fast-Freeze Flasks (*Labconco*, US) which were autoclaved before and lyophilized in a benchtop Freeze Dry System (*FreeZone 6 Liter*, *Labconco*, US). Before and after lyophilization the grown culture was inoculated to nutrient agar (NA) for the determination of CFU in order to calculate the viable cell counts and thereby evaluate the effect of lyophilization on bacteria.

DSC experiments

Lyophilized bacteria cells were grown at 37 ^{0}C temperature in an orbital shaker (*Zhicheng*, CN) at 150 rpm for 24 h. Nutrient broth (NB) medium was used as a growth medium. All bacteriological procedures were performed in sterile conditions under a laminar flow hood (*Esco*, US). Bacteria cells were centrifuged (*Spectrafug 6C*, *LABNET*, US) at 4.000 rpm for 15 minutes in 15 mL volumes at a final optical density (OD_{600}) of 1 using UV-visible spectrophotometer (*UV-5100*, *SOIF*, CN). To provide isotonic environment for the cells and to discard the dead and clamped cells, the obtained pellet was washed twice with 1 mL of Phosphate buffered saline (PBS) buffer solution. The pellet was centrifuged (*Sigma 1-14 Microfuge*, *SciQuip*, UK) again at 4.000 rpm for 1 minute in 1 mL volume and all the remaining water was removed by a micropipette. The pellets were prepared in the aluminum hermetic DSC sample pans and the pans were sealed for the experiment. To avoid the calorimetric pan effect the empty and sealed pan was used as a reference. DSC thermograms were obtained at a thermal region from 30ºC to 110ºC at a rate of 2ºC/min. Enthalpies (ΔH° J/g) were calculated by dividing the peak area to the sample weight. All the experiments and analyses of the obtained thermograms were performed using DSC Q2000 instrument (*TA Instruments*, US) and thermal analysis software (*Universal Analysis 2000, TA Instruments*, US), respectively.

IR spectroscopy experiments and data analyses

The IR spectra of bacteria were obtained using Frontier FTIR Spectrometer (*PerkinElmer*, US) equipped with a universal ATR Miracle accessory. The spectrum of air was used as a reference. 10 µL of sample was placed on a diamond/ZnSe crystal plate (*PerkinElmer*, US) and dried with a mild nitrogen flux for 2 minutes. The samples were scanned over the spectral range 4000 to 650 cm^{-1}, at room temperature, with a resolution of 4 cm^{-1} and 32 scans.

In data analyses, the second derivative and vector-normalized IR spectra were used in order to increase accuracy during the determination of band positions. For visual demonstration of the changes in the spectra in the figures, the absolute band intensities were expressed as percent intensity and normalized to 100 %. *Opus 5.5* software (*Bruker*, US) was utilized for all data pre-processing.

Results and Discussion

Before lyophilization CFU was calculated as 1.7 X 10^8 CFU/mL for fast-melt tablet form of SsK12 grown in skim milk medium. After lyophilization CFU was calculated as 3.3 X 10^9 CFU/mL. On the upshot, it seems that lyophilization process did not affect the viability and activity of the bacteria and can be utilized for the production of dry SsK12 pellets safely. Dry pellet form of probiotics is important in food industry since they increase the shelf-life of the product and keep the bacteria in an active state. In this framework, the administration of lyophilization process to probiotic bacteria has also many advantages such as convenient and economical handling, transport and storage at ambient conditions (Dianawati *et al.*, 2013).

The positions and enthalpies of the DSC thermal peaks for SsK12 were assigned according to a literature and summarized at Table 1. As shown in the table, the thermal profile of bacteria was divided into three regions. In the literature these regions are respectively referred as ribosomal proteins, nucleic acids and cell wall components. Totally 9 thermal peaks were found for SsK12. The peak located at the 64.0 °C was found to be emerging from the degradation and/or denaturation of 50S ribosomes. Enthalpy value ΔH° for this peak was determined as 0.5000 J/g. Although the peaks specific for other ribosomes were expected, they were not located at SsK12 thermogram. This finding points out a significant denaturation of cellular proteins as a part of sophisticated ribosome disintegration (Mackey *et al.*, 1991; Mohácsi-Farkas *et al.*, 1999).

At the nucleic acid region of the thermogram the peaks located at 81.5°C (ΔH° 0.2000**)** and 86.3°C (ΔH° 1.0300) were assigned for the denaturation of RNA, while the peaks at 96.2°C (ΔH° 0.0004**)**, 97.5°C (ΔH° 0.0003**)**, 98.4°C (ΔH° 0.0005**)** and 99.2°C (ΔH° 0.0009**)** were considered specific for DNA denaturation. Cell wall components were melted and degraded at 104.0°C and 109.0°C with ΔH° 0.0100 and 0.0600, respectively.

The whole thermogram was also shown in the Figure 1A, in which all the peaks were labelled accordingly. In addition, 90.0-110.0°C region was shown in Figure 1B for the better illustration and resolution of thermal peaks at that region.

For efficient food production and processing, special considerations should be taken in account related to thermal inactivation of microorganisms. In these technologies, the heat-associated destruction of bacteria is primarily measured through denaturation of important major cellular constituents (cell wall, proteins, lipids and nucleic acids) by using DSC (Mohácsi-Farkas *et al.*, 1999). Knowledge on thermal profile of vegetative SsK12 as a part of functional food can be useful for food industry to concurrently check the adequacy of probiotic product before their marketing. This is very important problem related to probiotic functional foods since according to newest clinical reports and numerous complaints of consumers to European Food Safety Authority (EFSA), these products are not effective at all, most probably due to the lack of viable probiotic bacteria in content of these foods.

Table 1. Thermal profile of *Streptococcus salivarius* K12 (Anderson *et al.*, 1991; Mackey *et al.*, 1991; Alpas *et al.*, 2003; Kaletunç *et al.*, 2004; Tunick *et al.*, 2006; Brannan *et al.*, 2015).

Thermal Region (°C)	**#**	**Peak position** (°C)	**Enthalpy- ΔH°** (J/g)	**Assignment**
Ribosomal proteins 45-75	1	64.0	0.5000	Degradation and/or denaturation of 50S ribosomes
Nucleic acids 75-100	2	81.5	0.2000	RNA denaturation
	3	86.3	1.0300	
	4	96.2	0.0004	DNA denaturation
	5	97.5	0.0003	
	6	98.4	0.0005	
	7	99.2	0.0009	
Cell wall 100-110	8	104.0	0.0100	Melting and degradation of cell wall components
	9	109.0	0.0600	

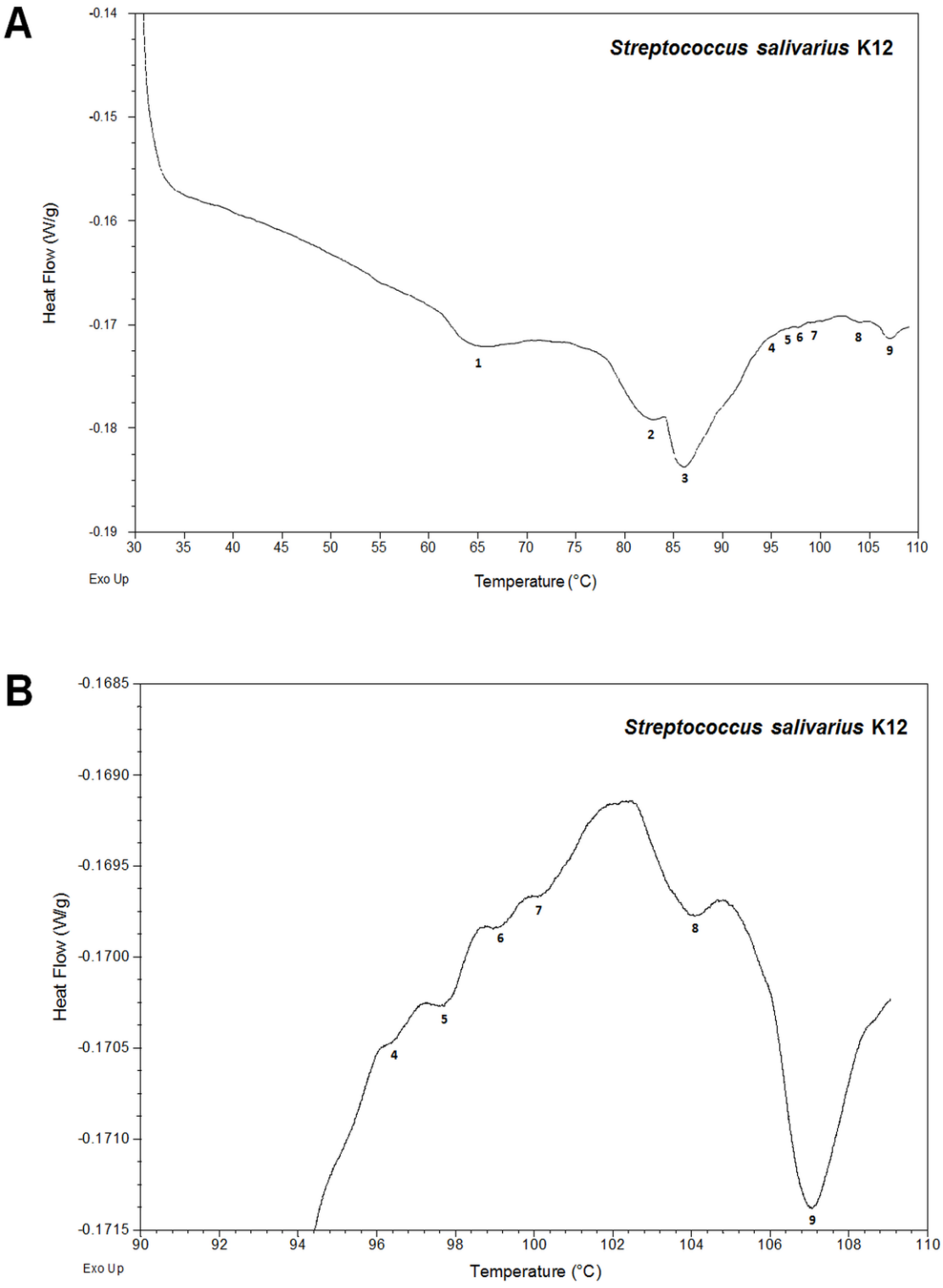


Figure 1. DSC thermograms of *Streptococcus salivarius* K12 A) at 30-110 °C and B) at 90-110 °C thermal regions.

IR spectra of SsK12 are demonstrated in Figure 2A and B, at C-H (3000-2800 cm^{-1}) and fingerprint (1800-650 cm^{-1}) IR regions, respectively. The spectra contains many bands which give molecular information on different functional groups emerging from lipids, polysaccharides, cholesterol, proteins and nucleic acids. The main bands which were assigned according to literature are labeled and also specified at Table 2. These molecular informations are important for the characterization of spesific strains. Since every bacteria has its own molecular IR fingerprint, these molecules can be spotted in any kind of heteregenous environment i.e. food products and used as biomarkers for the identification of SsK12 and simultaneous quality check i.e. probiotic capacity of particular functional food for oral health.

IR spectrum of SsK12 at 1750-1700 cm^{-1} region originating from ester CO stretchings of polyester storage compounds is shown in Figure 3A. The bands located at 1746 and 1713 cm^{-1} are specified for polyhydroxyalkanoates (PHAs), while the bands at 1739 and 1731 cm^{-1} are assigned for polyhydroxyoctanoates (PHOs). The band at 1721 cm^{-1} is qualified for polyhydroxybutyrate (PHB) (Naumann *et al.*, 1994; Helm and Naumann, 1995; Randriamahefa *et al.*, 2003; Kamnev, 2008). The percent (%) intensities, i.e. % concentration of these polyester storage compounds are demonstrated in Figure 3B. As shown in the figure, the total % concentrations of PHAs, PHOs and PHB are calculated as 52.2 %, 28.3% and 19.5 %, respectively for bacteria. Polyester storage compounds are the main carbon and energy reservioirs of most bacteria. During the starvation or stress circumstances the bacteria utilizes these polysaccarides to survive and maintain at these life-limiting conditions. In addition, these high moleular weight sugars are also known as extracellular polysaccarides (EPS) which is the most important sugar molecule maintaning removal of toxic compounds by directly binding and disabling their actions. The production of these compounds in SsK12 can give clues on how and when it interacts and combats with other pathogenic microorganisms in spesific environment which can be targeted to design smart applications considering probiotic functional foods.

IR spectrum of SsK12 at amide I (1700-1600cm^{-1}) region corresponding to proteins is presented in Figure 4A. As can be seen from the figure, the different secondary structures of proteins are measured as antiparallel β-sheet (1691 cm^{-1}), β-turns (1680 cm^{-1}), α-helices (1660 and 1650 cm^{-1}) and β-sheet (1622 cm^{-1}) for SsK12 (Kong and Yu, 2007; Yang *et al.*, 2015; Gurbanov *et al.*, 2016). The % intensities of these protein conformational structures are provided in Figure 4B. It was found that the proteins of SsK12 are predominantly in α-helix conformation (49.5 %). The proteins at β-sheet conformation was measured as 28.8 %, while antiparallel β-sheet and β-turn conformations were calculated as 12.5 % and 9.2 %, respectively. SsK12 mainly produces peptide/ protein origin, new generation and broad-spectrum lantobiotics known as salivaricins (A, A2, B and etc.) to fight againist infections by secreting them outside of the cell. Determination of protein composition of intact bacteria and also protein secondary structures of its proteins can be helpful for the understanding antibiotic production mechanisms and thereby developing better treatment systems for infectious diseases using probiotics as a component of functional fermented foods. In addition, knowledge about the secondary structures of salivaricins can be used for the design of recombinant or synthetic lantobiotics to get a prompt, effective and safe response in clinics.

IR spectrum of SsK12 at nucleic acid (1270-1210 cm^{-1}) region emerging from PO_2^- antisymmetric stretchings of different conformational forms of DNA is illustrated in Figure 5A. As shown, *A*-form (at 1241 and 1234 cm^{-1}) and *B*-form (at 1219 cm^{-1}) DNA conformations were found at spectrum (Banyay *et al.*, 2003; Whelan *et al.*, 2011; Whelan *et al.*, 2014). The % intensities of DNA conformational forms are given in Figure 5B. The total *A*-form DNA was measured as 55.0 %, while total *B*-form DNA was found as 45.0 %.

As known, the DNA and RNA encodes for all metabolic activities happening in the cell. Protein synthesis are controlled by nucleic acids, so the information we obtained can be interpreted to extract the relationship between transcription and translation processes for full active salivaricins. Here in special attention should be paid for conformational forms of DNA, since the predominance of particular conformational form (*A* or *B*-form) and/or supercoiling degrees directly affects the protein synthesis pathways for these small molecule and heat-stable compounds.

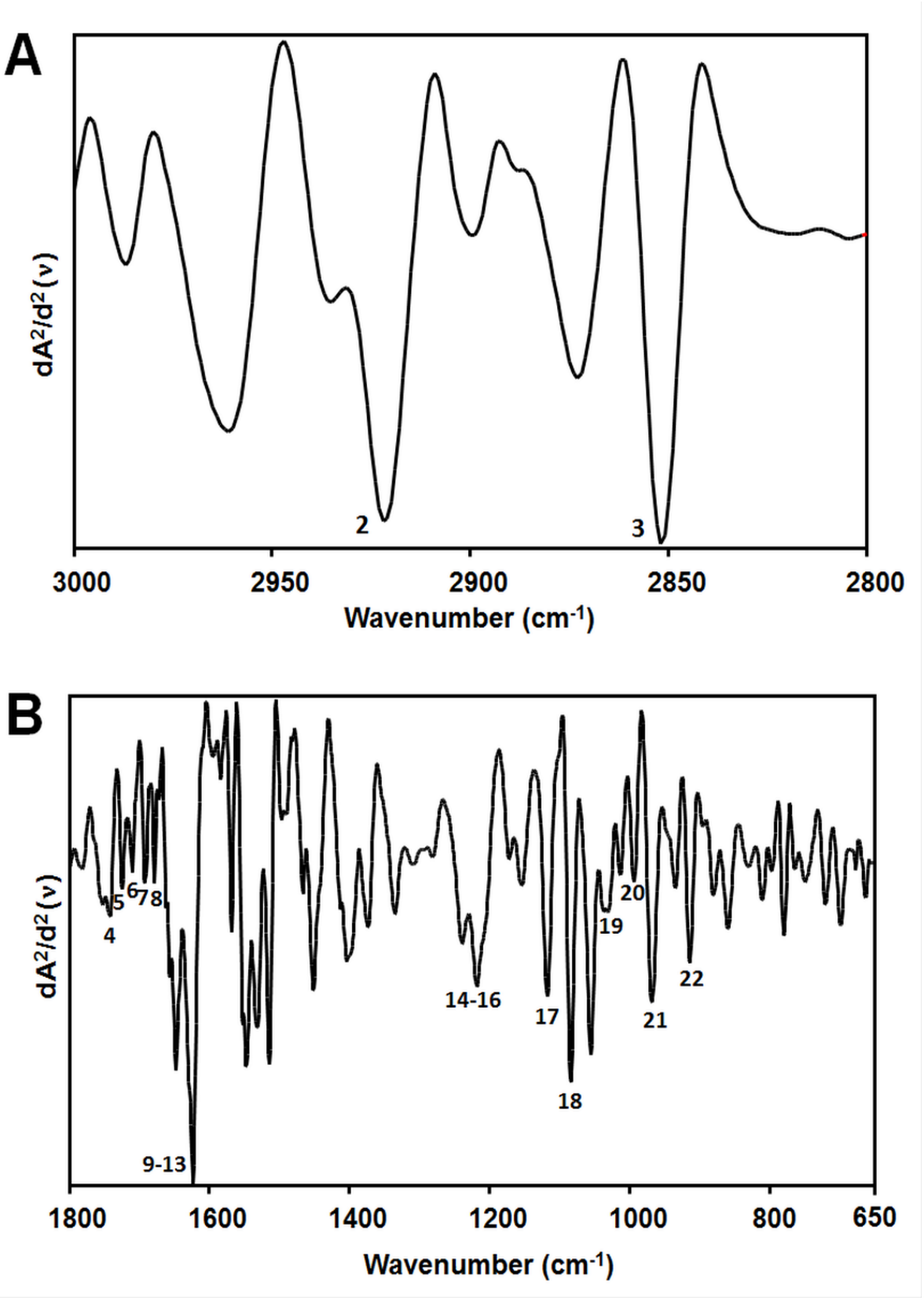


Figure 2. IR spectra of *Streptococcus salivarius* K12 at C-H (A) and fingerprint (B) IR regions.

Table 2. IR spectral profile of *Streptococcus salivarius* K12 (Naumann *et al.*, 1991; Naumann *et al.*, 1994; Helm and Naumann, 1995; Haris and Severcan, 1999; Banyay *et al.*, 2003; Randriamahefa *et al.*, 2003; Kamnev, 2008; Whelan *et al.*, 2011; Wenning and Scherer, 2013; Wenning *et al.*, 2014; Whelan *et al.*, 2014; Gurbanov *et al.*, 2015).

#	Wavenumber (cm^{-1})	Assignment
1	3008	**Olefinic = CH:** unsaturated lipids
2	2921	**CH_2 antisymmetric stretching:** mainly lipids
3	2852	**CH_2 symmetric stretching:** mainly lipids
4	1746	**Ester CO stretch:** polyester storage compounds, polyhydroxyalkanoates (PHAs)
5	1739	**Ester CO stretch:** polyester storage compounds, polyhydroxyoctanoates (PHOs)
6	1731	**Ester CO stretch:** polyester storage compounds, polyhydroxyoctanoates (PHOs)
7	1721	**Ester CO stretch:** polyester storage compounds, polyhydroxybutyrates (PHBs)
8	1713	**Ester CO stretch:** polyester storage compounds, polyhydroxyalkanoates (PHAs)
9	1691	**Amide protein region:** Antiparallel β-sheet
10	1680	**Amide protein region:** β-turns
11	1660	**Amide protein region:** α-helix
12	1650	**Amide protein region:** α-helix
13	1622	**Amide protein region:** β-sheet
14	1241	**PO_2^- antisymmetric stretching:** A-form DNA
15	1234	**PO_2^- antisymmetric stretching:** A-form DNA
16	1219	**PO_2^- antisymmetric stretching:** B-form DNA
17	1173	**CO—O—C antisymmetric stretching**: phospholipids, triglycerides and cholesterol esters
18	1118	**C-O stretching:** RNA ribose
19	1086	**PO_2^- symmetric stretching:** nucleic acids
20	1057	**C–O stretching**: polysaccharides
21	970	**C-C stretching:** DNA backbone
22	914	**Ribose ring vibrations:** RNA/DNA

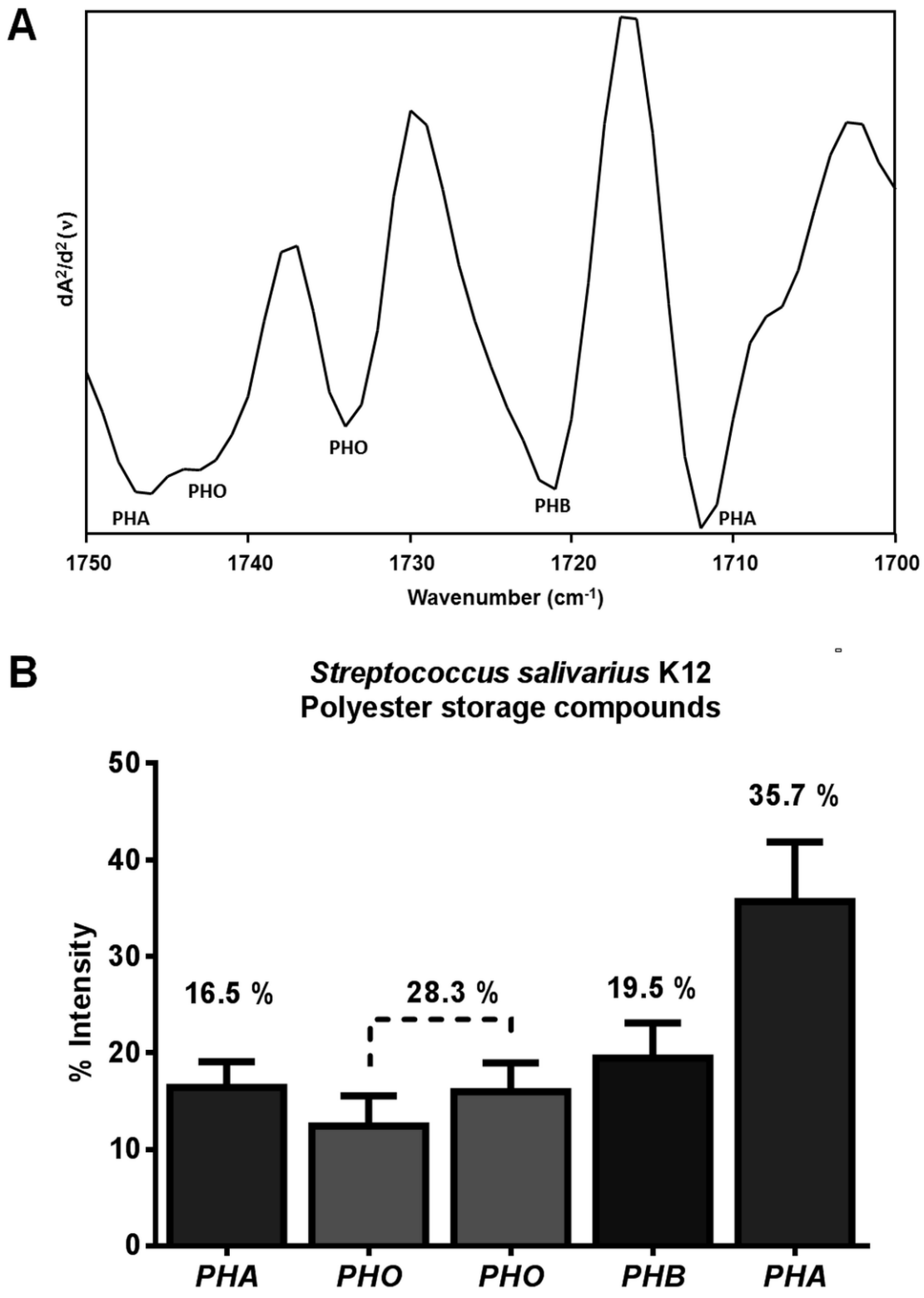
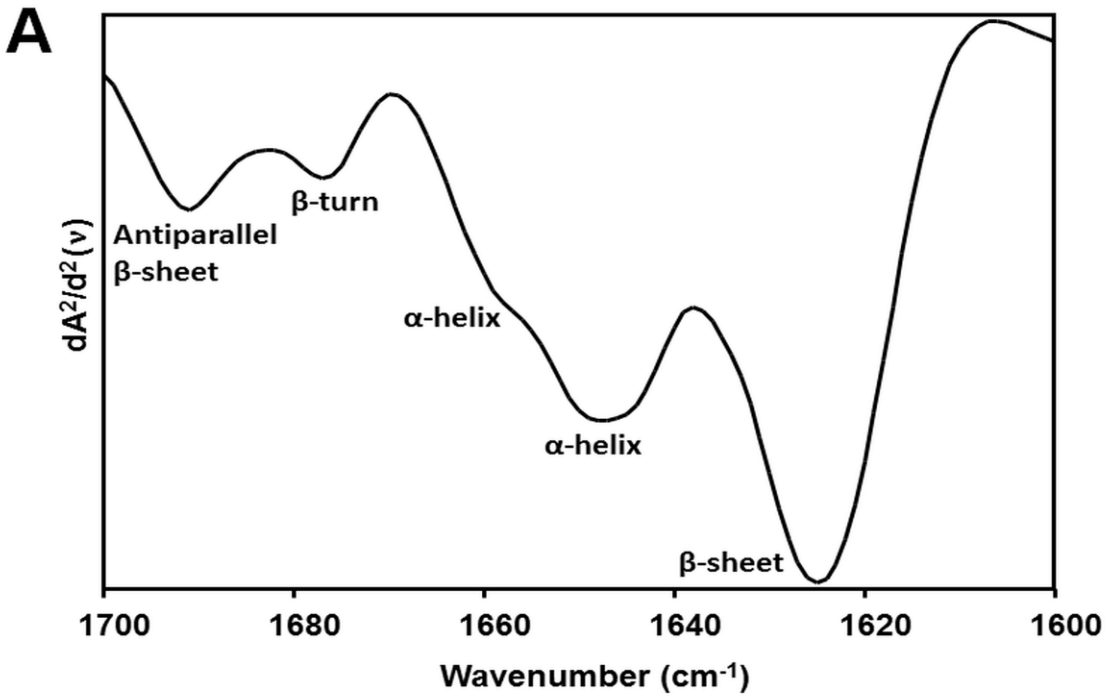


Figure 3. IR spectra of *Streptococcus salivarius* K12 at 1750-1700 cm^{-1} (A) and % intensities of polyester storage compounds (B).



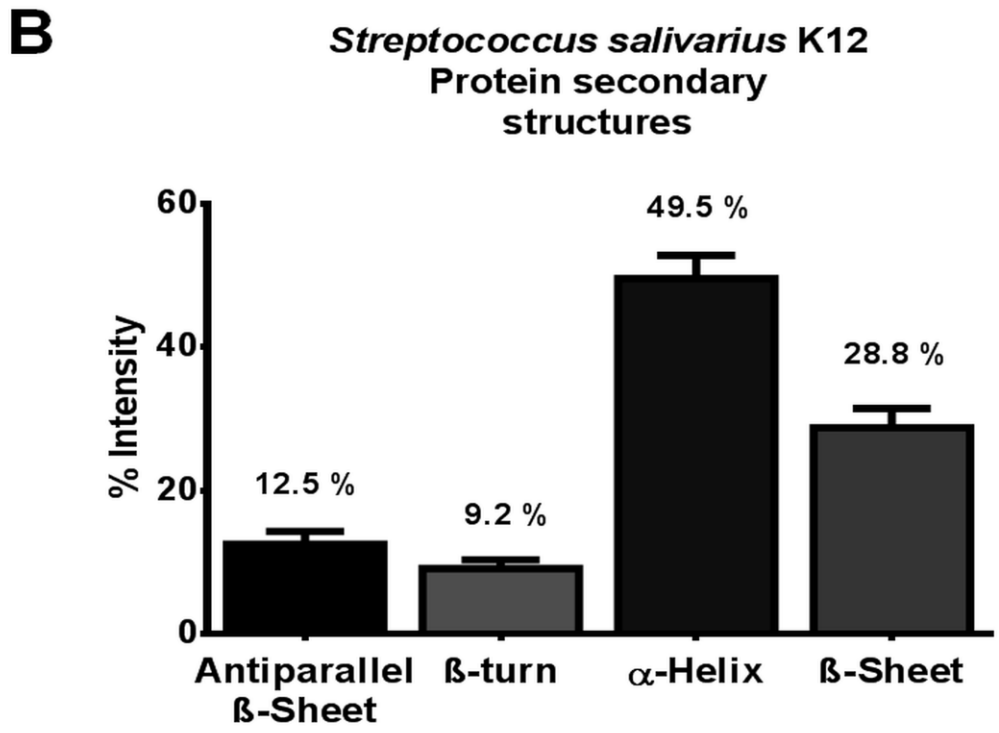


Figure 4. IR spectra of *Streptococcus salivarius* K12 at main protein (1700-1600 cm^{-1}) IR region (A) and % intensities of protein secondary structures (B).

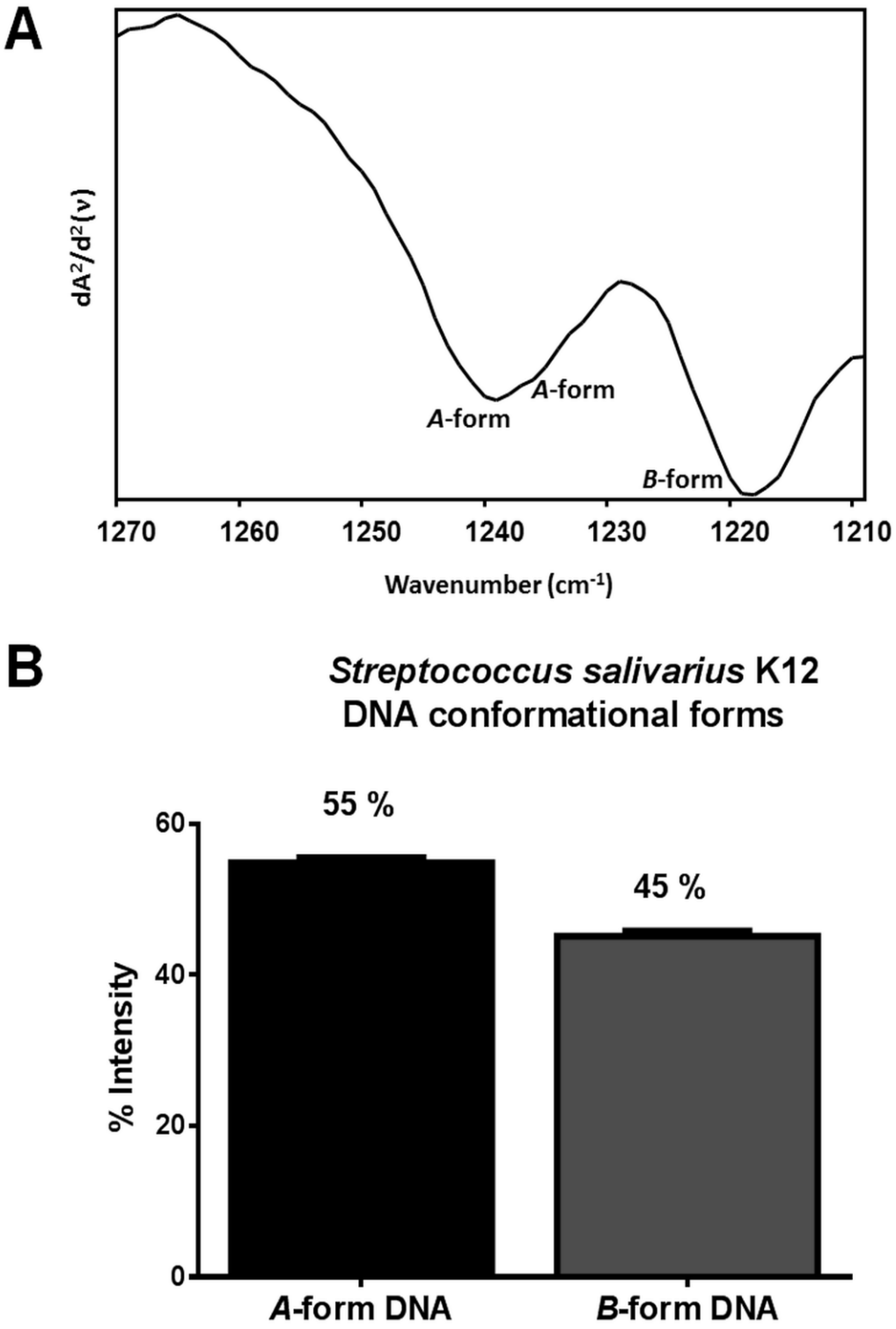


Figure 5. IR spectra of *Streptococcus salivarius* K12 at nucleic acid (1270-1210 cm^{-1}) IR region (A) and % intensities of DNA conformational forms (B).

Conclusion

SsK12 is an important non-pathogenic and probiotic bacteria especially for oral microbiota. It's beneficial and protective effects against infectious diseases have already been described in both clinical and laboratory studies. It colonizes in oral cavity, teeth and upper respiratory tract by forming chain and biofilm. In this study we determined the global molecular profile of SsK12 to better understand its cell biochemistry. Information we present here, can be used by food specialists for the development of probiotic functional foods such as cheese, kefir, yogurt, buttermilk, pickle and chewing gum. Functional foods especially fermented ones can be enriched with an adequate number of viable bacteria to be applied for the maintenance of oral health.

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