Survival of Bifidobacterium animalis ssp. lactis DSMZ 10140 and Bifidobacterium animalis ssp. animalis ATCC 25527 during manufacture and storage of ice cream.

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Abstract

Probiotic bacteria are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2002); Based on this definition, to exert health benefits the organisms must be viable when consumed. In this study, survival of *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 and *B. animalis* ssp. *lactis* DSMZ 10140 was studied in ice cream over time by measuring viable counts on MRS agar plates incubated anaerobically at 37°C. These organisms were chosen because of their close genetic similarity and reported differences in technological properties. Subspecies specific PCR was used to verify the identity of colonies counted. Results show growth patterns of *B. animalis* ssp. *lactis* DSMZ 10140 and *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 were very similar suggesting survival in ice cream is not subspecies dependent.

Introduction

Probotics are live microorganisms which when administered in adequate amount confer a health benefit on the host (FAO/WHO 2002).
Probiotics have been proven to reduce duration and occurrence of diarrhea through maintenance of the gut microbiota, maintenance of guy homeostasis or stimulation of the immune system. Other reported health benefits associated with probiotics are the ability to stimulate the immune system, regulate the inflammatory response and alleviate allergic reactions. Probiotics may decrease inflammatory bowel disease and modify serum cholesterol levels in humans and reduce the risk of cardiovascular disease.

Bifidobacterium species represent a significant proportion of the probiotic cultures used in the food industry (P. Langhendries, J. Detry, J. van Hees and J.M. Lamboray, 1995). Members of the genus *Bifidobacterium* are among the most common microorganisms in the human colon, and they are considered to be important in maintaining a well balanced intestinal microflora (P. Langhendries, J. Detry, J. van Hees and J.M. Lamboray, 1995). Bifidobacteria have been shown to have beneficial properties, such as preventing diarrhea and intestinal infections, alleviation of constipation, and production of antimicrobials against harmful intestinal bacteria, and immunostimulation

(M. Saavedra, A. Abi-Hanna, N. Moore and R. Yolken, 1998). Some strains have been linked to probiotic function when added as living cells into milk products or supplied in animal food or as capsules. *Bifidobacterium* are considered to be obligate anaerobic bacteria (B. Biavati, b. Sgorbati, V. Scardovi, 1991). However, strains of *B. animalis* ssp. *lactis* are described as being able to grow at low oxygen concentrations in liquid medium (L. Meile, K. Fischer, T. Leisinger, 1995). These aerotolerant bifidobacteria species possess some of the oxygen or oxygen radicals scavenging mechanisms which are not detected in other strictly anaerobic bacteria, such as NADH oxidases, peroxidases, or superoxide dismutase (L. Meile, K. Fischer, T. Leisinger, 1995).

One of the most common subspecies of *Bifidobacterium* used in probiotics is *Bifidobacterium animalis* ssp. *lactis* (*B. animalis* ssp. *lactis)* because of its oxygen tolerance which allows for better stability in probiotic containing production. *Bifidobacterium animalis* ssp. *lactis* is gram-positive, non motile, non-sporeforming, irregularly rod-shaped cell, sometimes arranged in pairs, but not in chains. *B. animalis* ssp. *lactis* is anaerobic; no growth occurring on agar plates exposed to air. However, the *B. animalis* ssp. *lactis* can tolerate 10% of oxygen in the headspace atmosphere above liquid media. The optimum growth temperature is between 39°C and 42°C no growth has been observed above 46°C (Y. Cai, M. Matasumoto, Y. Benno. 1997). The other subspecies of *Bifidobacterium* being evaluated in this study is *Bifidobacterium animalis* ssp. *animalis (B. animalis* ssp. *animalis)* which is highly similar to *B. animalis* ssp. *lactis.* The sequence similarity is defined by analysis of the Idh gene which shows that *B. animalis* ssp. *animalis* is the most closely related strain to *B. animalis* ssp. *lactis*. The genetic relatedness of strains can be assessed using 16S rDNA gene sequencing which can differentiate between species.

Some differences between *B. animalis* ssp. *lactis* and *B. animalis* ssp. *animalis* is that they possess different abilities to grow on milk-based medium and in milk-based products. *B. animalis* ssp. *lactis* strains, such as DSMZ 10140, and very few *B. animalis* ssp. *animalis* strains are able to grow in milk. *B. animalis* ssp. *animalis* 25527 grows very poorly in milk (M. Ventura, R. Zink. 2002). This distinction is one of the reasons why *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis* are considered to be different subspecies and not synonyms.

In this experiment, survival of *B. animalis* ssp. *lactis* DSMZ 10140 and *B. animalis* ssp. *animalis* ATCC 25527 during freezing and storage in ice cream will be tested. Since *B. animalis* ssp. *lactis* and *animalis* are both anaerobic bacteria, the aerobic environment in the ice cream may be a potential problem in survival. The very cold temperatures may cause cell injury and/or death and also present problems with survival. However, rapid freezing resulting in small ice crystals may preserve the cells.

The objective of this study is to compare survival of *B. animalis* ssp. *animalis* 25527 and *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 during freezing and storage of ice cream. The primary methods of analysis are enumeration of Bifidobacterium from ice cream by viable cell counting followed by subspecies confirmation using PCR.

Materials and Methods

1. Media Preparation

MRS plates and broth was prepared using manufacturers directions, sterilized. MRS agar ((reference number: 288130, lot number: 9051993, 4.5g Difco agar (reference number: 214530, lot number: 8035322)) was poured and hardened. After preparation, MRS media was autoclaved for 20 minutes, cooled, and refrigerated until use.

2. **Acquisition of *bifidobacterial* strains stock culture preparation.**

   Isolates of *Bifidobacterium animalis* ssp. *lactis DSMZ 10140* and *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 were be obtained and made into stock culture using glycerol.

3. **Genus identification of *Bifidobacterium animalis* ssp. *lactis DSMZ 10140* and *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 by PCR.**

   A single or partial colony from each plate was deposited in the bottom of a 0.2ml thin-walled PCR tube (Corning Inc., Corning, NY) using a sterile inoculating needle. (Briczinski, 2004) Cells were lysed by microwaving the PCR tubes at power level six for seven minutes. *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 strains were identified by amplification of a sequence in the 16S rDNA spacer regions using primers Bflac2 and Bflac5 (Ventura, Reniero et al. 2001) *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 were identified by amplification of a sequence in the 23S rDNA and 16S-23S r DNA spacer regions with primers Ban 2 and 23Si (Ventura and Zink 2002).A negative PCR control was included using no template DNA. Following amplification, the reaction mixtures were separated on a 1.0% agarose gel (Promega Corporation, Madison, WI) using .5x TBE buffer (45mM tris, 45mM boric acid, 1mM EDTA, pH 8.0). Electrophoresis was performed using a submerged horizontal gel electrophoresis system at approximately 70 volts. The gel was stained using a solution of ethidium bromide (0.5µg/ml) and water and de-staining using distilled water. Bands were visualized on a UV transilluminator at 260 nm and images were captured with an AlphaImager 3300 Gel Documentation System (Alpha Innotech Corp., San Leandro, CA)

4. **Stock culture preparation**

   A portion of thawed samples of *Bifidobacterium animalis* ssp. *lactis DSMZ 10140* and *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 were streaked for isolation on MRS agar. From each bacteria sample, an isolated colony from MRS agar was transferred to 10ml of MRS broth and incubated at 37°C until turbid (18-24 hours). The turbid broth was mixed with an equal volume of sterile 20% glycerol (w/v) and distilled water (Briczinski, 2007). Ten portions of 5.0ml *Bifidobacterium animalis* ssp. *lactis DSMZ 10140* and *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 suspensions were dispensed into sterile cryovials (Nalgene, Rochester, NY) and frozen as stock culture at -70°C. Working cultures of the bacteria were prepared by transferring 200 µL of stock culture to a 10 ml volume of fresh broth or by transferring an isolated colony from an agar plate streaked with the stock culture to 10ml of MRS broth.

5. **Inoculum Preparation:**

From stock culture, 100 µl of *Bifidobacterium animalis* ssp. *lactis* and *Bifidobacterium animalis* ssp. *animalis*, respectively were inoculated into 10 ml of MRS broth and incubated anaerobically for 24 hours. Sterile MRS broth was inoculated with 100 µl MRS broth containing *B. animalis ssp. animals* and *B. animalis ssp. lactis* respectively and incubated anaerobically for 14-16 hours. After incubation, 1.0 ml of inoculated MRS broth was aliquot into 100 ml of sterile broth and anerobically incubated for 24 hours. Then 5.0 ml of the previous inoculated MRS broth was aliquot into each 500 ml plastic dilution bottle and incubated for 24 hours. After incubation, the inoculated broth from each 500 ml bottle was diluted to 10^{-6} and plated in duplicates on MRS agar to 10^{-8} to determine a viable count.

6. **Centrifugation**

Six bottles, each containing 151 ml of inoculated MRS broth from the 500 ml bottles was transferred into 200 ml centrifuge bottles, balanced and centrifuged at 4°C and 6000 rpm for 20 minutes. Supernatant from each bottle was aseptically poured off into a beaker. Cells were re-suspended in 100 ml of sterile 0.1% Bacto Peptone (reference # 211677, Lot # 2177375) water. Bottles were then re-centrifuged, using the same conditions. After aseptically discarding supernatant, 20 ml of milk was added to each bottle and vortexed to re-suspend the cells resulting in 60 ml of concentrated, washed cells. Cells from this suspension were plated at dilutions of 10^{-5} $10^{-6,}$ and 10^{-7} to establish a viable count. Cell input and cell output were calculated to determine a total cell loss.

7. **Preparation and analysis of inoculated ice cream.**

Creamery ice cream mix was obtained (Table 2). 15 kg of mix was split into 5.0kg lots using a balance and sanitized holding containers. A Taylor batch freezer was rinsed and sanitized using XY-12 prior to processing and between each treatment. Prior to freezing, 20ml of the concentrated suspension of Bifidobacterium *animalis ssp. lactis DSMZ 10140* or *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 to the treated samples (nothing into the control). Following addition samples were mixed well to disperse the culture and then frozen under agitation. Ice cream temperature was measured immediately after ejection and weights were measured for calculation of overrun. Overrun was calculated by subtracting the weight of ice cream mix from the weight of ice cream divided by the weight of ice cream multiplied by 100. After freezing, the ice cream was ejected into three-gallon tubs, and then packaged into sanitized pint containers for hardening and stored in a freezer at -25°C. Ice cream mix composition was obtained (Bonnie Ford).

8. **Sampling Procedures**

11g of each ice cream mix treatment was aliquot into a sterile 99ml dilution blank for plating on MRS agar to $10^{-5,}$ 10^{-6}, and 10^{-7} in duplicate and anaerobically incubated for 72 hours. After hardening for 24 hours, 1g of melted ice cream was plated for viable cell counting on MRS agar and coliform testing on Violet-red bile agar. Each product was diluted and plated on MRS agar at dilutions of $10^{-5,}$ $10^{-6,}$ and 10^{-7} in duplicate and anaerobically incubated for 72 hours. After growth, viable cells were counted. Random colonies of bifidobacteria were picked from an isolated colony and using colony PCR, subspecies identification was confirmed.

Results and Discussion

Stock Culture preparation and PCR subspecies verification

A total of ten stock culture vials were prepared with glycerol and stored in a freezer at -70°C. PCR was done with inoculated MRS media used during the growth curve, after ice cream mix inoculation and after ice cream hardening. Agarose gel was analyzed using an Alpha Imager under UV light. The gel's results showed that there was no contamination. Each colony analyzed, negative and positive control gave the expected reaction (Figure 2). *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 results were about 680 base pairs and *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 results were about 467 base pairs. This matched up with the expected reaction on the 100 base pair DNA ladder showing the results were correct.

Growth of *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 *and Bifidobacterium animalis* ssp. *animalis* ATCC 25527

Optical density in respect to time of *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 *and Bifidobacterium animalis* ssp. *animalis* ATCC 25527 are shown in table 2. Optical density measurements were taken every hour for fourteen hours and a last measurement was taken after twenty-four hours using a Spectrometer 21; between each measurement MRS broth was anerobically incubated. This data shows that there was a constant growth for both *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 *and Bifidobacterium animalis* ssp. *animalis* ATCC 25527 during the course of measurements taken. Growth rates of both organisms were similar as expected, both organisms went through lag and a slight log phase. This could have been due to possible injury of cells; because *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 *and Bifidobacterium animalis* ssp. *animalis* ATCC 25527 are both anaerobic organisms the hourly exposure to oxygen could have had an effect on the growth rate. *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 is however 10% oxygen tolerant, therefore this minimum amount of exposure to oxygen should not have significantly affected the growth of this organism.

Cell harvest and concentration efficiency

Viable cell counts were usedto evaluate the survival of each subspecies. Before inoculating the ice cream mix with the respective organism the initial count had to be calculated (Table 1). When cells were concentrated the percent recovery was calculated from initial viable cell counts and viable cell counts after centrifugation. *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 had a 110.63% recovery while *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 only had a 48.73% recovery. This difference in cell

recovery could have been due to the extensive exposure to oxygen, and *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 is oxygen tolerant allowing it to, in this case, grow while being in an aerobic environment, where as there was only a 48.73% recovery in *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 because it is not oxygen tolerant. *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 also favors growth in dairy products whereas *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 does not. Loss in cells in *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 could have also been due to the extreme agitation to the centrifuge bottles, when being centrifuged and when trying to re-suspend the cells in the 0.1% Bacto Peptone as well as skim milk. The temperature of the centrifuge could have also caused some cell injury and loss due to the optimum growth temperature of both organisms being 37 to 42°C and the temperature they were being centrifuged at was between 4 and 10°C. The results overall suggest there was little loss of *B. animalis ssp. animalis* and *B. animalis ssp. lactis* during harvesting.

Survival of *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 and *Bifidobacterium animalis ssp. lactis DSMZ 10140* in ice cream.

Bifidobacterium animalis ssp. *animalis* ATCC 25527 had a lower cell count than *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140, this was expected due to the aerobic environment, and the survival of *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 is more favorable. After freezing, ice cream treatments were diluted and plated to 10^8; counts were recorded after 24 hours, 5 days, 7 days, and 11 days in freezer storage at -25°C. Using the initial count of *B. animalis ssp. animalis* ATCC 25527 and *B. animalis ssp. lactis* DSMZ 10140 ice cream mix, viable cell counts and each viable cell count after plating, the percent log reduction was calculated. The log reduction value is a ratio of the initial log substance in cfu/g divided by the substance in cfu/g after storage. The percent log reduction shows that at 11 days *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 had a 0.18% reduction whereas *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 had a 0.79% reduction, which means *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 has had a greater cell loss since the ice cream mix inoculation initial count. This is unexpected because of the oxygen tolerant, lactose thriving characteristics of *B. animalis* ssp. *lactis* DSMZ 10140, however this could be due to the low temperatures in the storage freezer (-25°C) and *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 possibly being a more resilient subspecies. These results could also be due to the short observation time, if studied for a longer period of time, the expected results may show.

The percent overrun in each ice cream mix was calculated using the weights of the initial ice cream mix before freezing and the weight of the ice cream after freezing (Table 2). The control had the highest percent overrun at 78.1%, *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 had the lowest percent overrun at 73.3%, and *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 was in between with 75.3% overrun. Overrun is the measure of the amount of air mixed into an ice cream mix. The differences in the overrun percentages could have been due to the additional bacteria added to the treatment mixes and compared to nothing being added to the control which may have allowed more air to be mixed in. All ice cream treatments were mixed to 160 viscosities.

Conclusion

In conclusion, both organisms had a similar survival rate; there was not a significant difference in the populations of each organism in ice cream. The data presented in Table 4 and Figure 1 indicate little death of the organism during the time period of the study suggesting survival of both organism in ice cream is very similar. This could be due to the short length of storage or a result of the genetic similarity of the two stains studied. The hypothesis was proven incorrect and survival of *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 and *Bifidobacterium animalis* ssp. *animalis* ATCC 25527 does not appear to be subspecies dependent.

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Tables and Figures

Table 1: Recovery of cells during inoculum preparation.

Sample	1. Initial Population (CFU/g)	2. Total number of cells	3. Concentration of cells harvested (CFU/g)	4. Total number of cells harvested	5. Percent Recovery
DSMZ 10140	$1.08x10^{9}$	$4.89x10^{11}$	$1.72x10^{10}$	$1.03x10^{12}$	110.63%
ATCC 25527	$5.20x10^{8}$	$2.36x10^{11}$	$5.85x10^{9}$	$3.51x10^{11}$	48.73%

1. **Viable cell count of inoculum**
2. **Total number of cells= (viable cell count (cfu/ml) x total ml of cells)**
3. **Viable cell count of concentrated cells following centrifugation, washing, re-centrifugation and milk re-suspension.**
4. **Total Viable cells harvested= population of harvested cells x final volume**
5. **** % Recovery= (initial population- population) x 100**
   (initial population)

Table 2: Ice cream mix composition used for experimental ice creams

Ingredient	**Formula**
Total Fat	14.10%
MSNF	10.50%
Corn Syrup Solids	3.70%
Cane Sugar	*12.97%
Stabilizers	.500%

*** % Ingredient= lbs of ingredient x100**
Total # of lbs

Table 3: Overrun in experimental ice creams.

Product	Weight of mix (g)	Weight of Ice Cream (g)	* Percent Overrun
Control	518.5	291.2	78.1
DSMZ 10140	518.5	295.8	75.3
ATCC 25527	518.5	299.2	73.3

* $$\% \text{ Overrun} = \frac{(\text{weight of mix} - \text{weight of ice cream})}{(\text{weight of ice cream})} \times 100$$

Table 4: Survival of probiotic *Bifidobacteria* in ice cream stored over time at -25°C.

Time (days)	DSMZ 10140 (CFU/g)	% Log Reduction[1]	ATCC 25527 (CFU/g)	% Log Reduction
0 (mix)	$8.00x10^7$	n/a	$2.00x10^7$	n/a
1	$1.66x10^7$	0.68%	$1.46x10^7$	0.14%
5	$2.90x10^7$	0.44%	$2.01x10^7$	-0.002%
7	$4.50x10^7$	0.25%	$1.77x10^7$	0.05%
11	$1.31x10^7$	0.79%	$1.34x10^7$	0.17%
13	$1.59x10^7$	0.70%	$9.80x10^6$	0.31%

Percent log reduction is also shown.

[1] % Log Reduction: log (No/N) No =initial cfu/g; N=cfu/g after storage

Table 5: Primers used in PCR reaction.

Species	**Primers**
Bifidobacterium animalis ssp. *animalis* ATCC 25527	23si, Ban 2
Bifidobacterium animalis ssp. *lactis* DSMZ 10140	Bflac 2, Bflac 5
Bifidobacterium	Lm 3, Lm 26

Figure 1: Change in viable counts of DSMZ 10140 and ATCC 25527 in ice cream after 1, 5, 7, 11 and 13 days frozen storage.

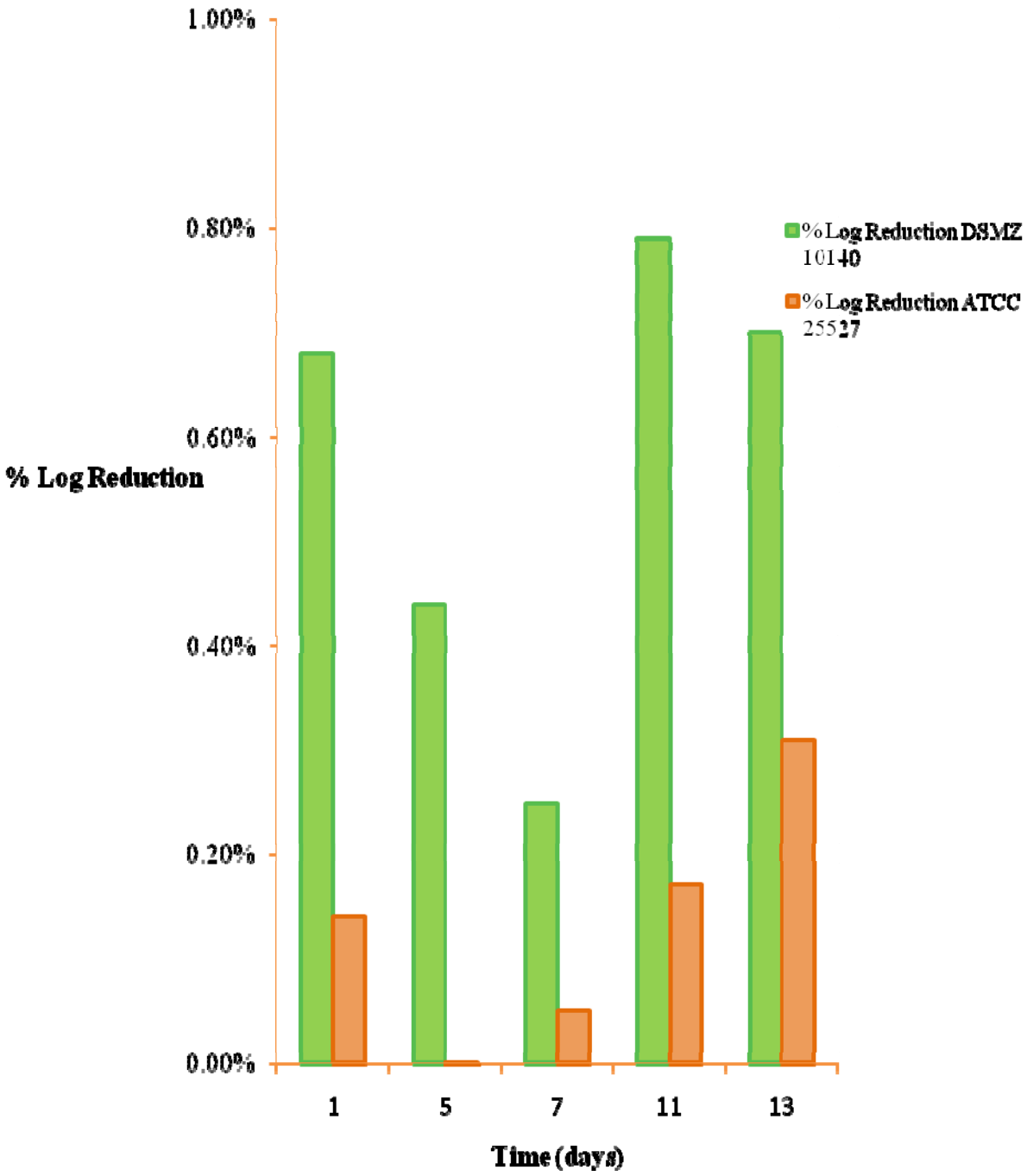
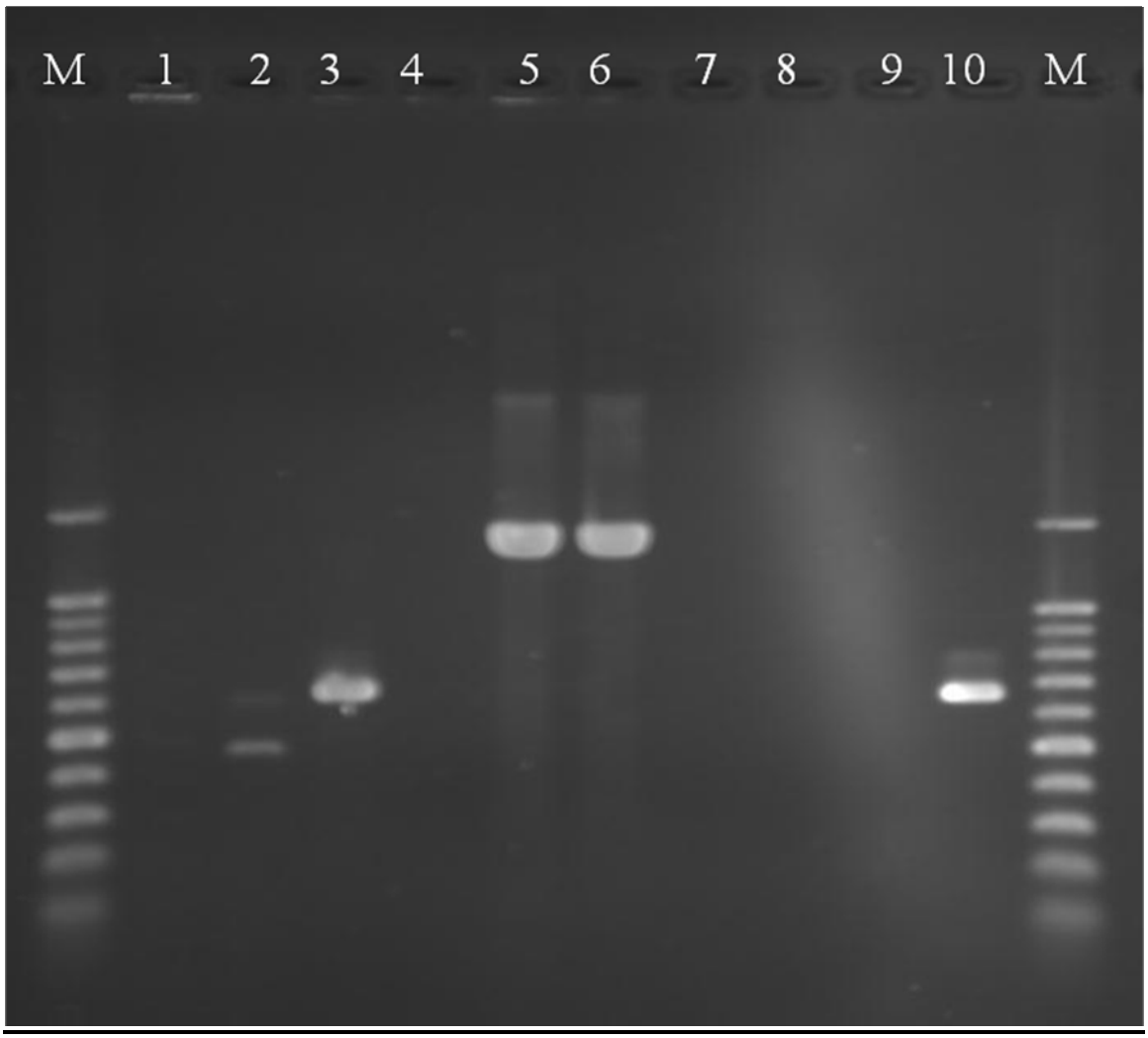


Figure 2: Colony PCR done with genus subspecies specific primers and one isolated colonly of bacteriafor identity confirmation of *Bifidobacterium animalis* ssp. *lactis* DSMZ 10140 *and Bifidobacterium animalis* ssp. *animalis* ATCC 25527 done 24 hours after freezing

Lane	Sample	Expected Reaction
M	DNA Ladder	100 bp Ladder
1	DSMZ 10140 + *animalis* subspecies primers[1]	Negative
2	ATCC 25527 +*animalis* subspecies primers	467 bp
3	DSMZ 10140 + *lactis* subspecies primers[2]	680 bp
4	ATCC 25527 + *lactis* subspecies primers	Negative
5	DSMZ 10140 + *Bifidobacterium* genus primers[3]	1350 bp
6	ATCC 25527 + *Bifidobacterium* genus primers	1350 bp
7	ATCC 25527+ no primers (negative control)	Negative
8	DSMZ 10140 + no primers (negative control)	Negative
9	*Bifidobacterium* + no primers (negative control)	Negative
10	Isolated DSMZ 10140 DNA + *lactis* subspecies primers (positive control)	680 bp
M	DNA Ladder	100 bp Ladder


M 1 2 3 4 5 6 7 8 9 10 M

<u>Figure 3:</u> Pictorial Flow Diagram of ice cream experiment: