Growth and Gas Production of a Novel Obligatory Heterofermentative Cheddar Cheese Nonstarter Lactobacilli Species on Ribose and Galactose

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Interpretive Summary

Growth and Gas Production of a Novel Obligatory Heterofermentative Cheddar Cheese Nonstarter Lactobacilli Species on Ribose and Galactose. *Ortakci*. A novel lactic acid bacterium *Lactobacillus wasatchii*[1] sp. nov. isolated from a "gassy" Cheddar cheese, was studied. *Lb. wasatchii* is obligatory heterofermentative and grows rapidly on ribose compared to galactose, however, it co-utilizes both sugars well. Gas production occurs when *Lb. wasatchii* is grown in the presence of galactose. *Lb. wasatchii* survived HTST pasteurization and grows under salt and pH conditions typical to Cheddar cheese. The presence of nonstarter bacteria such as *Lb. wasatchii* can lead to unwanted gas-production and formation of slits and cracks that results in down-grading of cheese and subsequent economic losses to cheese manufacturers.

ABSTRACT

An obligatory heterofermentative lactic acid bacterium *Lactobacillus wasatchii* sp. nov. isolated from gassy Cheddar cheese was studied for growth, gas formation, salt tolerance and survival against pasteurization treatments at 63°C and 72°C. Initially, *Lb. wasatchii* was thought to only use ribose as a sugar source and we were interested in whether it could utilize galactose. Experiments to determine rate and extent of growth and gas production in carbohydrate restricted (CR) de Man, Rogosa, and Sharpe (MRS) medium under anaerobic conditions with various combinations of ribose and galactose at 12, 23, and 37°C were conducted with 23°C being the more optimum growth temperature of *Lb. wasatchii*. When grown on ribose (0.1%, 0.5%, and 1%), maximum specific growth rates (μ_{max}) within each temperature were similar. When galactose was the only sugar, μ_{max} was 2 to 4 times lower than with ribose. At all temperatures, highest final cell densities (OD_{640}) of *Lb. wasatchii* were achieved in CR-MRS plus 1% ribose, 0.5% ribose and 0.5% galactose, or 1% ribose combined with 1% galactose. Similar μ_{max} values

[1] The name of this bacterium was subsequently revised to *Lactobacillus wasatchensis.*

and final cell densities were achieved when 50% of ribose in CR-MRS was substituted with galactose. Such enhanced utilization of galactose in the presence of ribose to support bacterial growth has not previously been reported. It appears that *Lb. wasatchii* co-metabolizes ribose and galactose, utilizing ribose for energy and galactose for other functions such as cell wall biosynthesis. Co-utilization of both sugars could be an adaptation mechanism of *Lb. wasatchii* to the cheese environment to efficiently ferment available sugars for maximizing metabolism and growth. As expected, gas formation by the heterofermenter was observed only when galactose was present in the media. Growth experiments with MRS plus 1.5% ribose at pH 5.2 or 6.5, with 0, 1, 2, 3, 4, or 5% NaCl revealed that *Lb. wasatchii* is able to grow under salt and pH conditions typical of Cheddar cheese (4 to 5% salt-in-moisture, ~pH 5.2). Finally, we found *Lb. wasatchii* cannot survive LTLT pasteurization but survives HTST lab pasteurization with 4.5 log reduction occurred. The ability of *Lb. wasatchii* to survive HTST pasteurization and grow under cheese ripening conditions implies that the presence of this nonstarter lactic acid bacteria can be a serious contributor to gas formation and textural defects in Cheddar cheese.
KEYWORDS: nonstarter lactic acid bacteria, late blowing, ribose, cofermentation,

INTRODUCTION

Lactic acid bacteria (**LAB**) present in ripening cheese include deliberately added starter LAB and a variety of adventitious LAB referred to as nonstarter LAB (**NSLAB**). The NSLAB gain access to cheese through the milk or processing environment (Naylor and Sharpe, 1958; Peterson and Marshall, 1990; Martley and Crow, 1993; Somers et al., 2000).

The predominant NSLAB in Cheddar cheese are facultative heterofermentative (**FHF**) lactobacilli and, less frequently pediococci or obligatory heterofermentative (**OHF)** lactobacilli

(Jordan and Cogan, 1993; Crow et al., 2001; Banks and Williams, 2004). Presence of OHF lactobacilli are a particular concern because these microbes may promote the development of undesirable flavor and body defects including gas formation in Cheddar cheese (Dacre, 1953; Laleye et al., 1987; Khalid and Marth, 1990). Unwanted gas formation in Cheddar cheese is a recurrent and wide-spread problem in dairy industry that has probably affected most cheese plants (Michael and Mullan, 2000). Our group recently isolated a new *Lactobacillus* species from a ‘’gassy’’ Cheddar cheese after incubation on de Man, Rogosa, and Sharpe (**MRS**) agar for 35 d at 6 °C. This bacterium was designated *Lactobacillus wasatchii* sp. nov. (Oberg et al., 2015).

Lactobacillus wasatchii is an OHF species and therefore uses the pentose phosphate pathway (**PP**) to generate energy from pentose and hexose sugars. Its preferred sugar is ribose, though hexoses such as galactose are also potential energy source in cheese. More importantly, hexose sugars can be fermented by OHF to lactate, acetate/ethanol plus CO_2, making *Lb. wasatchii* a potential contributor to gassy defect in Cheddar cheese.

This study examined growth characteristics of *Lb. wasatchii* with respect to ribose and galactose utilization, gas formation, tolerance to the salt and pH values found in Cheddar cheese, and its ability to survive pasteurization treatments. To our knowledge, this is the first report on growth and gas formation of a slow growing OHF lactobacilli species isolated as a NSLAB from a ‘’gassy’’ Cheddar cheese.

MATERIALS AND METHODS

Materials

Lactobacilli MRS broth, proteose peptone, polypeptone, beef extract, yeast extract, GasPak™ EZ, and agar were purchased from Becton Dickinson Inc. (Sparks, MD); ribose was donated by Bioenergy Life Science Inc. (Ham Lake, MN), UHT milk was from Gossner Foods Inc. (Logan, UT), Tween-80 and bromcresol purple were from Sigma-Aldrich Inc. (St. Louis, MO), dipotassium phosphate was from Fisher Scientific Inc. (Fair Lawn, NJ), sodium acetate trihydrate and diammonium citrate were from MallincKrodt Baker Inc. (Paris, KY), galactose, and triamonium citrate were from Alfa Aesar Inc. (Ward Hill, MA), magnesium sulfate was from Alfa Aesar Inc. (Heysham, England).

A carbohydrate-restricted version of MRS (**CR-MRS**) was prepared by the omission of glucose from the MRS broth formula. To 2 L of deionized water was added 20.0 g proteose peptone No. 3, 20.0 g beef extract, 10.0 g yeast extract, 2.0 g Tween-80, 4.0 g ammonium citrate, 10.0 g sodium acetate, 0.2 g magnesium sulfate, 0.1 g manganese sulfate, and 4.0 g dipotassium phosphate. The CR-MRS was supplemented with different levels of ribose and galactose to study growth properties of *Lb. wasatchii*.

Bacterium and Growth

Stock cultures of *Lb. wasatchii* were maintained at -80°C in MRS broth supplemented with 1.5% Ribose (**MRS+R**) and 10% glycerol. Working cultures was prepared by two successive transfers into 10 ml of MRS+R broth, with anaerobic incubation using GasPak™ EZ at 23°C for 32 h after each transfer. Growth of *Lb. wasatchii* was evaluated by inoculation of the working culture into 10 ml CR-MRS broth acidified to pH 5.20 with HCl and supplemented with 0.5 % galactose or ribose, 1.0% galactose, ribose, or a 0.50:0.50 combination) or 2.0% sugar (1% ribose plus 1% galactose). Optical density of the cell suspensions were followed at 640 nm after

inoculation and every 12 h thereafter at 12, 23, or 37°C and during anaerobic incubation in jars containing GasPak™ EZ. Maximum specific growth rate (μ_{max}) was calculated as the slope of the steepest linear portion of the growth rate curves. Broth samples containing Durham tubes were similarly prepared, inoculated, and incubated to test for gas production. Working cultures were prepared in duplicate for conducting growth curves and gas formation experiments.

To test NaCl tolerance of *Lb. wasatchii* at pH 5.2 or 6.5, *Lb. wasatchii* working cultures were prepared in triplicate and inoculated into MRS+R broth containing 0, 1, 2, 3, 4, or 5% (wt/wt) NaCl. Growth at 23°C under anaerobic conditions was followed by spectrophotometrical (OD_{600}) measurements every 8 h until the stationary phase was reached.

Thermotolerance

The ability of *Lb. wasatchii* to withstand pasteurization treatment was assayed by heating 9.9 ml of UHT milk to 63°C and 72°C in sterile polypropylene tubes. Once the desired temperature was reached, each tube was inoculated with 0.1 ml of *Lb. wasatchii* working culture (prepared in triplicate) containing ~6 x 10^8 cfu/ml of and the samples held at 63°C and 72°C for 30 min or 15 s, respectively, then placed in a 31°C water bath (the set temperature commonly used for making Cheddar cheese) for 2 h. These treatments were designed to mimic the high temperature short time (**HTST**) continuous pasteurization used in large-scale cheese operations and the low temperature long time (**LTLT**) batch pasteurization often used by small-scale artisan cheese makers. Samples were then plated on MRS+R agar in duplicate and incubated at 23°C anaerobically for 5 days.

Statistical Analysis

Statistical analysis of the effect of different temperature, sugar, pH, and NaCl treatments on μ_{max} and final cell density of *Lb. wasatchii* were performed using PROC GLM in SAS (version 9.1, SAS Institute, Cary, NC) and differences between means determined using REGWQ multiple range test and Tukey Least Squares Means.

RESULTS

Growth

Ribose. Growth curves for *Lb. wasatchii* at 23, 37, and 12 °C in CR-MRS with ribose at pH 5.20 are represented in Figures 1A, 2A, and 3A, respectively. Within each temperature, significantly higher μ_{max} values were observed when *Lb. wasatchii* was grown on CR-MRS plus ribose ($P<0.05$) compared to galactose as the sole sugar (Table 1). In the presence of 1% ribose, μ_{max} of *Lb. wasatchii* was 23°C > 37°C = 12°C. When grown in the presence of 1.0% ribose at 12 an 23°C, exponential growth continued until final OD_{640} levels of ~1.3 to 1.4 were reached (Table 2), with lower OD_{640} achieved at lower sugar levels, indicating available sugars was a limiting factor on extent of growth. Less cell growth occurred at 37°C with OD_{640} only reaching 0.75. Assuming that exponential growth ends when the sugars are depleted, the lower final cell density at 37°C may be indicative that more of the energy obtained via fermentation is being used to maintain cell viability because of energy-intensive stress responses at the higher temperature.

Galactose. When galactose was the only sugar, growth of *Lb. wasatchii* was slow (Figures 1B, 2B and 3B) with similar μ_{max} of less than 0.01 at all temperatures (Table 1). Final

cell densities were lower ($P < 0.05$) than when *Lb. wasatchii* was grown with ribose except when grown with the lowest sugar level (0.1%) at 12 and 37°C (Table 2). Slower utilization of galactose by *Lb. wasatchii* in the absence of ribose was expected, as we had previously seen that galactose did not provide a positive response on the API 50 CHL test even when held for longer than 48 h. With slower growth occurring with galactose as the only sugar, stationary phase in CR-MRS plus 0.5% galactose was only reached after 156 h at 23°C compared to 24 h in CR-MRS plus 0.5% ribose. At 37°C, the extent of bacterial growth remained low (final $OD_{640} \leq 0.22$) even when galactose level was increased to 1% (Table 2).

Combined Ribose and Galactose. When a 1:1 blend of galactose and ribose was used, μ_{max} rate was not significantly different than for ribose alone ($P<0.05$), with only a slight difference observed when grown at 23°C (Figures 1, 2, and 3 and Table 1). In general, final cell densities were similar when total sugar content was the same (Table 2). This indicates that galactose utilization by *Lb. wasatchii* is slower when there is no ribose present but provides almost the same rate of growth as ribose when both sugars are present.

Salt Tolerance

The growth characteristics of *Lb. wasatchii* grown in MRS+R with 0 to 5% NaCl at pH 6.5 and 5.2 are shown in Figures 4A and 4B, respectively. After 48 h, an OD_{600} of 2.0 was reached in all media except for 5% salt at pH 5.2 which had an OD_{600} of 1.75 and only reached OD_{600} of 2.0 after 60 h. At pH 6.5, there was a slight decrease in μ_{max} when grown with 4% NaCl although this was not observed with 5% NaCl (Table 3). At pH 5.2, there was also significantly lower μ_{max} at both 4 and 5% NaCl ($P<0.05$). Final cell densities were the same ($OD_{600} = 2.0$) except at 5% NaCl with had final OD_{600} of 1.96 ($P<0.05$). A combination of salt and lower pH causes a decrease in μ_{max}, but *Lb. wasatchii* can grow in the same environment that occurs during

Cheddar cheese ripening (~pH 5.2, 4 to 5% salt-in-moisture). Such salt tolerance is expected for NSLAB isolated from Cheddar cheese, Jordan and Cogan (1993) observed growth of NSLAB such as *Lactobacillus casei*, *Lb. plantarum* and *Lb. curvatus* in 6% and some up to 8% (wt/wt) NaCl. Typically, at least 6% salt is needed to slow growth of NSLAB (Lane et al., 1997) and even then NSLAB populations in Cheddar cheese still reached about the same numbers at all salt levels (2.8 to 6.1%, salt-in-moisture) after 6 mo of storage. It is interesting to note that strains of *Lactobacillus danicus*, the NSLAB that is phylogenetically closest to *Lb. wasatchii*, was susceptible to salt and had negligible growth at 4% NaCl and none detected with 6.5% NaCl (Kask et al., 2003).

Gas Formation

Gas formation by *Lb. wasatchii* was only observed when galactose was present in the media. No gas formation was observed at 23°C when the sole sugar source was ribose or when the total sugar concentration, both ribose and galactose, was below 0.5%. At 12°C, no gas formation was observed at sugar contents of <1.0%. This may be because of the higher solubility of CO_2 at lower temperatures (CRC Handbook of Chemistry and Physics, 2009). At 37°C, gas formation was only detected in CR-MRS containing 1% ribose plus 1% galactose.

Thermotolerance

Subjecting *Lb. wasatchii* to HTST heat treatment (72°C for 15 s) resulted in~ 4.5-log reduction, from 6 x 10^6 cfu/ml to 9.2x10^1 cfu/ml surviving after cooling to 31°C. In contrast, no detectable colonies of *Lb. wasatchii* (i.e., <10^1 cfu/ml) were found after the LTLT treatment (63°C for 30 min). Survival of lactobacilli after milk pasteurization has been previously reported and underscores the potential for lactobacilli in milk to be a source of NSLAB in cheese made from pasteurized milk (Turner et al., 1986; Golnazarian, 2001; Beresford et al., 2001). The

finding that *Lb. wasatchii* can withstand HTST indicates the bacterium could gain access to cheese directly or produce biofilms in the cheese processing environment that provide a regular source of contamination.

DISCUSSION

Metabolic Capability

Lactobacillus wasatchii sp. nov. (Oberg et al., 2015) is an OHF lactobacilli closely related to *Lb. suebicus* (isolated from apple and pear mashes), *Lb. vaccinostercus* (isolated from cow dung), *Lb. hokkaidonensis* (isolated from timothy grass silage), *Lb. oligofermentans* (isolated from poultry) and *Lb. danicus* (isolated from cheese). None of these species is regularly isolated from cheese, which could be due to the fact that NSLAB isolation methods do not incorporate the long time, low temperature conditions used to isolate *Lb. wasatchii* and *Lb. danicus* (Kask et al., 2003; Oberg et al., 2011; Broadbent et al., 2013). Because its closest phylogenetic relatives are associated with plant materials and cow dung, we speculate that the origin of *Lb. wasatchii* was a dairy farm.

Lactobacillus wasatchii is an OHF lactobacilli possessing genes encoding phosphoketolase but lacking the genes encoding fructose-1,6-diphosphate aldolase. Thus, *Lb. wasatchii* ferments pentose and hexose sugars through the PP. Utilization of hexoses via OHF lactobacilli results in CO_2, lactate, and acetate/ethanol production, whereas pentose metabolism does not yield CO_2 (Axelsson, 2004). An OHF lifestyle corresponds with the finding that gas

formation was only observed when *Lb. wasatchii* was grown in CR-MRS plus galactose or CR-MRS plus ribose and galactose.

As opposed to common cheese NSLAB that are FHF lactobacilli, *Lb. wasatchii* preferentially utilizes ribose over glucose and other sugars (Oberg et al., 2015). Slow utilization of hexoses and active fermentation of pentoses was also reported for the OHF *Lb. vaccinostercus* KOZAKI and OKADA sp. nov. strains that were isolated from cow dung using a medium containing xylose as the sole carbon source (Okada et al., 1978). Another phylogenetic relative of *Lb. wasatchii*, *Lb. oligofermentans* sp. nov., also utilized glucose very weakly (Koort et al., 2005).

Ribose Fermentation

The heterolactic fermentation of ribose results in a slightly different end product pattern compared to galactose fermentation. No CO_2 is formed, and since no dehydrogenation steps are necessary to reach the intermediate xylulose-5-phosphate, the reduction of acetylphosphate to ethanol to regenerate NAD^+ becomes redundant. Instead, acetylphosphate can be converted by acetate kinase in a substrate-level phosphorylation step to acetate and ATP. Fermentation of ribose thus leads to production of equimolar amounts of lactic acid and acetic acid and net 2 mol ATP/mol ribose consumed (Axelsson, 2004).

Two amino sugars that are precursors to the peptidoglycan are N-acetylglucosamine and N-acetylmuramic acid. Both amino sugars are made from fructose-6-phosphate (**F6P**) that acts as the backbone molecule for cell wall synthesis (White, 2007). *Lactobacillus wasatchii* possesses a gene encoding transketolase that condenses two pentoses with F6P being one of the metabolic outputs with the remaining carbons eventually being converted into gylceraldehyde-6-phosphate. Based on this information, we speculate that when *Lb. wasatchii* is grown in CR-MRS plus

ribose, ribose is utilized for both cell wall synthesis and ATP generation to support cell division as shown in Figure 5 (Pathway directions {1}, {2} and {3}).

At higher concentrations of ribose, generally, the μ_{max} of *Lb. wasatchii* is the same as at lower concentrations. Thus, PP is operating as fast as possible in generating energy when *Lb. wasatchii* was grown in CR-MRS plus either ribose concentrations. It is interesting the similar μ_{max} values were achieved when a ribose-galactose mixture was used even at the low level of 0.05 % ribose plus 0.05% galactose (Table 1). The only notable change that was seen with increasing sugar concentration was that the time over which exponential growth occurs was lengthened and a higher final cell density was attained.

Galactose Fermentation

Lactobacillus wasatchii grew very slowly when galactose was the sole carbohydrate source of energy (μ_{max}= 0.005, 0.009, and 0.008 on 1% galactose at 12, 23, and 37°C, respectively). At 37°C, *Lb. wasatchii* showed only limited growth with a final OD_{640} of ~0.2 reached when galactose was the sole sugar (0.1% vs. 1%). It is interesting, that *Lb. wasatchii* reached significantly higher final cell densities when grown on ≥0.5% galactose at 12 and 23°C versus 37°C ($P<0.05$). Significantly lower final cell density at 37°C may be due to more of the ATP produced by fermentation being utilized to sustain cell viability because of energy-intensive stress responses at the higher temperature. Similar results were found by Adamberg et al. (2005) who reported slower growth of *Lb. danicus* with glucose or galactose at 30°C compared to 24°C. However, ribose utilization rates by *Lb. danicus* were the same at both temperatures. In comparison, utilization of hexose sugars by *Lb. casei*/*paracasei* was higher at 30°C compared to 24°C while ribose utilization did not change (Adamberg et al., 2005).

Analysis of the *Lb. wasatchii* genome suggests galactose enters the cell via a permease and then fermented into the Leloir pathway and converted to glucose-6-phosphate (**G6P**) as shown in Figure 5. The G6P then be utilized using PP via dehydrogenation to 6-phosphogluconate, followed by decarboxylation to ribulose-5-phosphate (**R5P**) and CO_2 (pathway direction {4}, {5}, {1}, {2}). Both of these steps require reduction of NAD^+ to NADH.

The R5P can then be further metabolized in the PP to lactate and acetate/ethanol with potential of generating up to net 2 ATP. However, the need to re-oxidize NADH may direct the pathway from acetylphosphate towards ethanol production rather than acetate. Thus, galactose utilization through Leloir and PP would supply 1 mol each of lactic acid, ethanol, and CO_2, and net 1 mol ATP/mol of galactose (Axelsson, 2004).

There are two possible explanations for the much slower growth of *Lb. wasatchii* on galactose compared to ribose: (1) there is a rate limiting step in the pathways leading to conversion of galactose into R5P, or (2) the need to re-oxidize NADH requires conversion of acetylphospate into ethanol rather than acetate so that only 1 mole of ATP per mole of galactose is produced as reported by Axelsson (2004).

Co-metabolism of Galactose with Ribose

There have been a few instances in which growth of lactobacilli is increased in the presence of two sugars compared to either of the sugars alone. Gobetti et al. (1995) reported that a fructose negative strain of *Lactobacillus sanfrancisco* (another OHF species) grows faster when it co-ferments fructose in the presence of maltose; maltose is consumed for energy and fructose serves as an external electron acceptor for re-oxidation of NADH. This does not seem to be the case for *Lb. wasatchii* as neither galactose nor ribose is known to function as an external electron acceptor.

In general, FHF lactobacilli such as *Lb. plantarum* can utilize both pentoses and hexoses although Westby (1989) and Westby et al. (1993) reported a strain of *Lb. plantarum* (NCIMB 8026) that was unable to utilize ribose in the absence of glucose. They offered two hypotheses to explain this observation: (1) *Lb. plantarum* NCIMB 8026 lacks the pathways to produce F6P from pentose sugars through transketolase or via fructose-1,6-bisphosphatase thus being unable to make C_6 units from C_5 sugars and needing an external source of C_6 units for biosynthesis of peptidoglycan and other cell building blocks; or (2) that phosphoenolpyruvate (**PEP**) production during pentose metabolism (compared to hexose fermentation via glycolysis) in *Lb. plantarum* NCIMB 8026 was insufficient to support the PEP-dependent uptake of ribose. According to Neidhardt et al. (1990) only one PEP molecule is produced per ribose molecule metabolized (versus two PEP molecules per glucose) leaving no PEP molecules for the other cellular functions such as peptidoglycan synthesis.

With *Lb. wasatchii,* transketolase is available to covert pentoses into F6P, thus producing the needed C_6 building blocks for peptidoglycan. Also, for *Lb. wasatchii* the uniqueness is improved utilization of a hexose in the presence of a pentose rather than the other way around. So, neither of these hypotheses explain the mechanism of galactose and ribose co-utilization by *Lb. wasatchii* (which appears highly adapted to ferment ribose). Ribose metabolism in *Lb. wasatchii* is more profitable than galactose (or other hexose) fermentation in terms of energy production. Fred et al. (1921) reported that certain groups of pentose-fermenting LAB commonly found in silage, sauerkraut, and related substances, showed high acid production from pentose sugars while, hexose sugars yielded low acid but high ethanol production. Once again, this observation is probably a reflection of the substrate energetics; with 2 ATP per pentose but only

1 ATP from hexoses due to the need to re-oxidize NADH to NAD^+ using the ethanol branch of PP.

To explain growth attributes of *Lb. wasatchii* during co-utilization of ribose and galactose, it is necessary to consider the potential fates of each sugar with regard to energy yield and cellular building blocks. Since the similar μ_{max}, and final cell densities were observed when *Lb. wasatchii* is grown in the presence of ribose plus galactose or ribose alone, the rate of energy production and cell wall synthesis is likely the same. Given that *Lb. wasatchii* has the gene for G6P isomerase; it can convert G6P to F6P and utilizes galactose as a ready source of hexose for peptidoglycan synthesis (Figure 5, pathway direction {4}, {6}).

In a parallel manner, final cell densities of *Lb. wasatchii* is identical for cells grown in ribose or with 50% of the ribose replaced with galactose (except for 0.5% ribose vs. 0.25% ribose plus 0.25% galactose at 23°C). This further suggests that only ribose is being used for energy production and that an insignificant amount of ribose is being diverted for peptidoglycan synthesis by transketolase conversion of pentoses to F6P (Figure 5, pathway direction {1}, {2}). This hypothesis is supported by findings in *Bifidobacterium breve* where Degnan and McFarlane (1991) found cells grown in the presence of ^{14}C arabinose (a pentose) and glucose (a hexose) did not incorporate carbon from arabinose into cellular macromolecules.

We propose that when an OHF LAB such as *Lb. wasatchii* has both ribose and hexoses available for growth, that the ribose is primarily utilized for ATP production via the lower portion of the PP (Figure 5, pathway direction {1}, {2}), while the hexose is utilized for synthesis of peptidoglycans and other cellular macromolecules (Figure 5, pathway direction {4}, {6}). This has the advantage of maximizing ATP production as the need to re-oxidize NADH is minimized when only ribose is fermented. The extent of ribose that is diverted from the PP for

peptidoglycan synthesis would depend on the relative amounts of hexoses present. A consequence of such simultaneous co-metabolism is that acetate would be expected as the end produce rather than ethanol from acetylphosphate. When ribose is depleted, then galactose would need to be fermented down the PP to provide energy to the cell. This corresponds with our observations that gas production occurred towards the end of exponential growth or early stationary phase (after 48h at 23°C).

Our results clearly demonstrate that *Lb. wasatchii* can co-utilize ribose and galactose which are two potential substrates for NSLAB (Tinson et al., 1982; Thomas, 1987; Rapposch et al., 1999; Michel and Martley, 2001) in Cheddar cheese. We also have shown that *Lb. wasatchii* is quite tolerant to salt and pH conditions that usually exist in ripening Cheddar cheese. The ability to readily consume mixed putative cheese sugars, grow at cheese ripening temperatures as well as survival against harsh environment of cheese, support our hypothesis that *Lb. wasatchii* contributes late gas blowing and textural defects in Cheddar cheese. To better understand the adaptation of *Lb. wasatchii* to cheese microenvironment, it would be desirable to study whether other sugars in milk and cheese (e.g., lactose, N-acetylgalactosamine, N-acetyl neuraminic acid, mannose, fucose, N-acetylglucosamine) can also be co-utilized by *Lb. wasatchii* in the presence of ribose. When describing carbohydrate utilization abilities of bacteria, such co-utilization should also be considered as our initial testing of *Lb. wasatchii* led us to believe that it was not capable of utilizing galactose.

CONCLUSIONS

A new obligatory heterofermentative nonstarter lactic acid bacterium, *Lactobacillus wasatchii* sp. nov. (isolated from a blown Cheddar cheese) was shown to require ribose for rapid

growth unlike other cheese NSLAB that grow well on glucose. Due to its OHF nature, *Lb. wasatchii* utilizes six and five carbon sugars through the pentose phosphate pathway. Fermentation of hexoses such as galactose will produce CO_2, so OHF have been implicated in late blowing of Cheddar cheese. We speculate that when ribose and galactose are both available, *Lb. wasatchii* uses ribose to produce energy and galactose for peptidoglycan synthesis and growth. This capability is well suited to cheese ripening and we have shown that *Lb. wasatchii* can grow under cheese-like stress conditions of low pH (5.2), and at least up to 5% salt content. It also has the potential to survive the HTST pasteurization used in large scale dairy processing, which may explain how it gains entry to the milk processing environment.

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Figure Legends

Figure 1. Growth of *Lb. wasatchii* (OD_{640}) at 23°C in carbohydrate restricted MRS adjusted to pH 5.2 and supplemented with ribose (panel A), galactose (panel B), or a mixture of ribose and galactose (panel C). Numbers for each symbol represent the percent concentration (wt/vol) of sugar added to the medium. Error bars = SE (n=2).

Figure 2. Growth of *Lb. wasatchii* (OD_{640}) at 37°C in carbohydrate restricted MRS adjusted to pH 5.2 and supplemented with ribose (panel A), galactose (panel B), or a mixture of ribose and galactose (panel C). Numbers for each symbol represent the percent concentration (wt/vol) of sugar added to the medium. Error bars = SE (n=2).

Figure 3. Growth of *Lb. wasatchii* (OD_{640}) at 12°C in carbohydrate restricted MRS adjusted to pH 5.2 and supplemented with ribose (panel A), galactose (panel B), or a mixture of ribose and galactose (panel C). Numbers for each symbol represent the percent concentration (wt/vol) of sugar added to the medium. Error bars = SE (n=2).

Figure 4. Growth of *Lb. wasatchii* (OD_{600}) in regular MRS broth supplemented with 1.5% ribose (wt/vol) plus 0 to 5% NaCl and adjusted to pH 6.5 (panel A) or pH 5.2 (panel B). Error bars = SE (n=3).

Figure 5. Proposed pathways for ribose and galactose utilization by *Lb. wasatchii.*

Table 1. Maximum specific growth rate (μ_{max}) of *Lb. wasatchii* at 12, 23, or 37°C when grown in carbohydrate restricted MRS broth with various levels of ribose, and galactose.

Sugar		μ_{max}		
Ribose	**Galactose**	**12°C**	**23°C**	**37°C**
--------(% wt/vol)-------		----------(OD$_{640}$/h) ----------		
0.1	0	0.0085^{hij}	0.0235^{cd}	0.019^{de}
0.5	0	0.0145^{efghi}	0.0365^{a}	0.0195^{de}
1.0	0	0.0155^{efgh}	0.0355^{ab}	0.0215^{de}
0	0.1	0.003^{j}	0.0095^{ghij}	0.008^{ij}
0	0.5	0.006^{j}	0.0095^{ghij}	0.0075^{ij}
0	1.0	0.005^{j}	0.009^{ghij}	0.008^{ij}
0.05	0.05	0.010^{fghij}	0.021^{de}	0.0185^{de}
0.25	0.25	0.016^{efg}	0.0285^{bc}	0.021^{e}
0.5	0.5	0.018^{de}	0.0385^{a}	0.0205^{de}
1.0	1.0	0.017^{def}	0.0375^{a}	0.0195^{de}

$^{a-j}$Means values with the same letter are not significantly different from each other (α = 0.05).

Table 2. Final cell density of *Lb. wasatchii* measured as optical density at 640 nm when grown at 12, 23, or 37°C in carbohydrate restricted MRS broth with various levels of ribose and galactose.

Sugar		Final Cell Density[1]		
Ribose	**Galactose**	**12°C**	**23°C**	**37°C**
------(% wt/vol)--------		----------(OD_{640})----------		
0.1	0	0.3^{j}	0.35^{j}	0.225^{kl}
0.5	0	0.795^{de}	0.8^{de}	0.705^{fg}
1.0	0	1.44^{a}	1.37^{b}	0.75^{ef}
0	0.1	0.298^{j}	0.214^{l}	0.205^{l}
0	0.5	0.68^{gh}	0.69^{fgh}	0.215^{l}
0	1.0	0.58^{i}	0.755^{ef}	0.195^{l}
0.05	0.05	0.335^{j}	0.335^{j}	0.300^{j}
0.25	0.25	0.83^{d}	0.83^{d}	0.62^{hi}
0.5	0.5	1.36^{b}	1.285^{c}	0.835^{d}
1.0	1.0	1.42^{ab}	1.4^{ab}	0.72^{fg}

[1]Measured as OD_{640} after incubation for 72 h for growth at 23 and 37°C for medium containing ribose, and after 204 h for all samples incubated at 12°C and those with only galactose at 23 and 37°C.

[a-l]Means values with the same letter are not significantly different from each other (α=0.05).

Table 3. Maximum specific growth rate (μ_{max}) of *Lb. wasatchii* cells grown at 23°C in MRS broth supplemented with 1.5% ribose as a function of salt and pH.

	μ_{max}	
NaCl	**pH 5.2**	**pH 6.5**
(% wt/wt)	----------(OD_{600}/h) ----------	
0	0.05^{cde}	0.061^{abcd}
1	0.064^{abc}	0.076^{a}
2	0.058^{bcde}	0.056^{bcde}
3	0.057^{bcde}	0.068^{ab}
4	0.044^{e}	0.048^{de}
5	0.044^{e}	0.053^{bcde}

[a-e]Means values with the same letter are not significantly different from each other (α=0.05).

Figure 1. Ortakci..

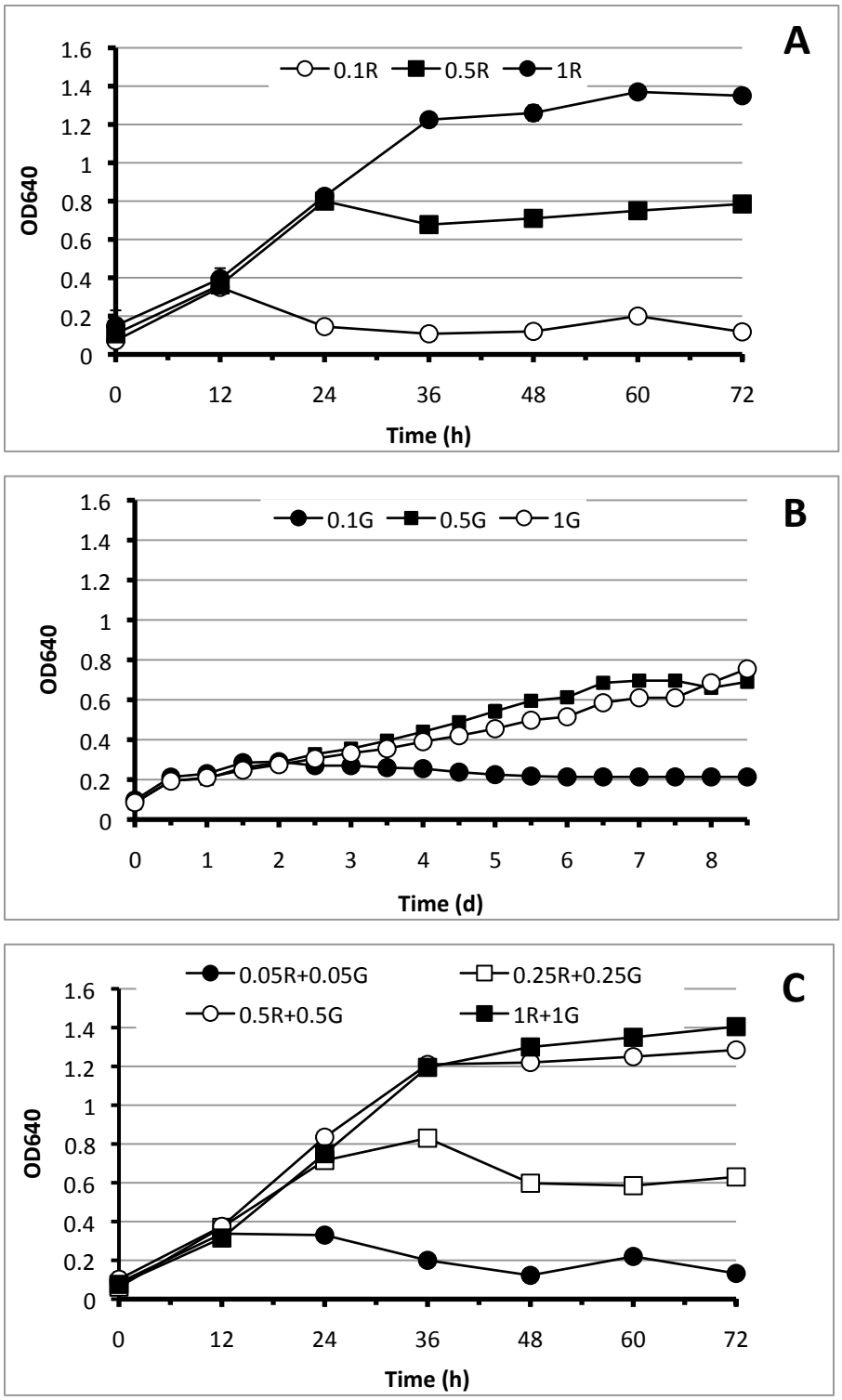


Figure 2. Ortakci..

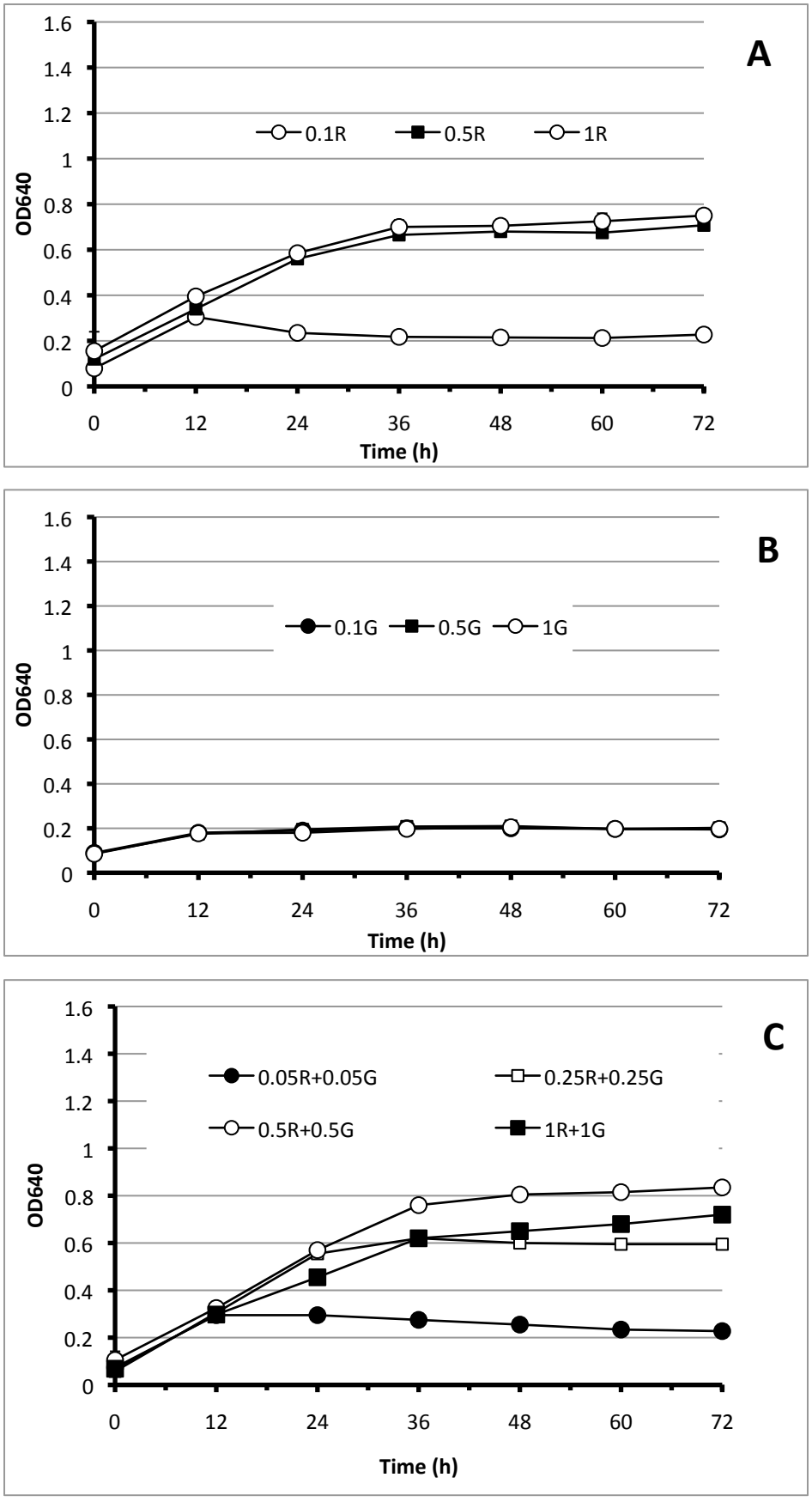


Figure 3. Ortakci..

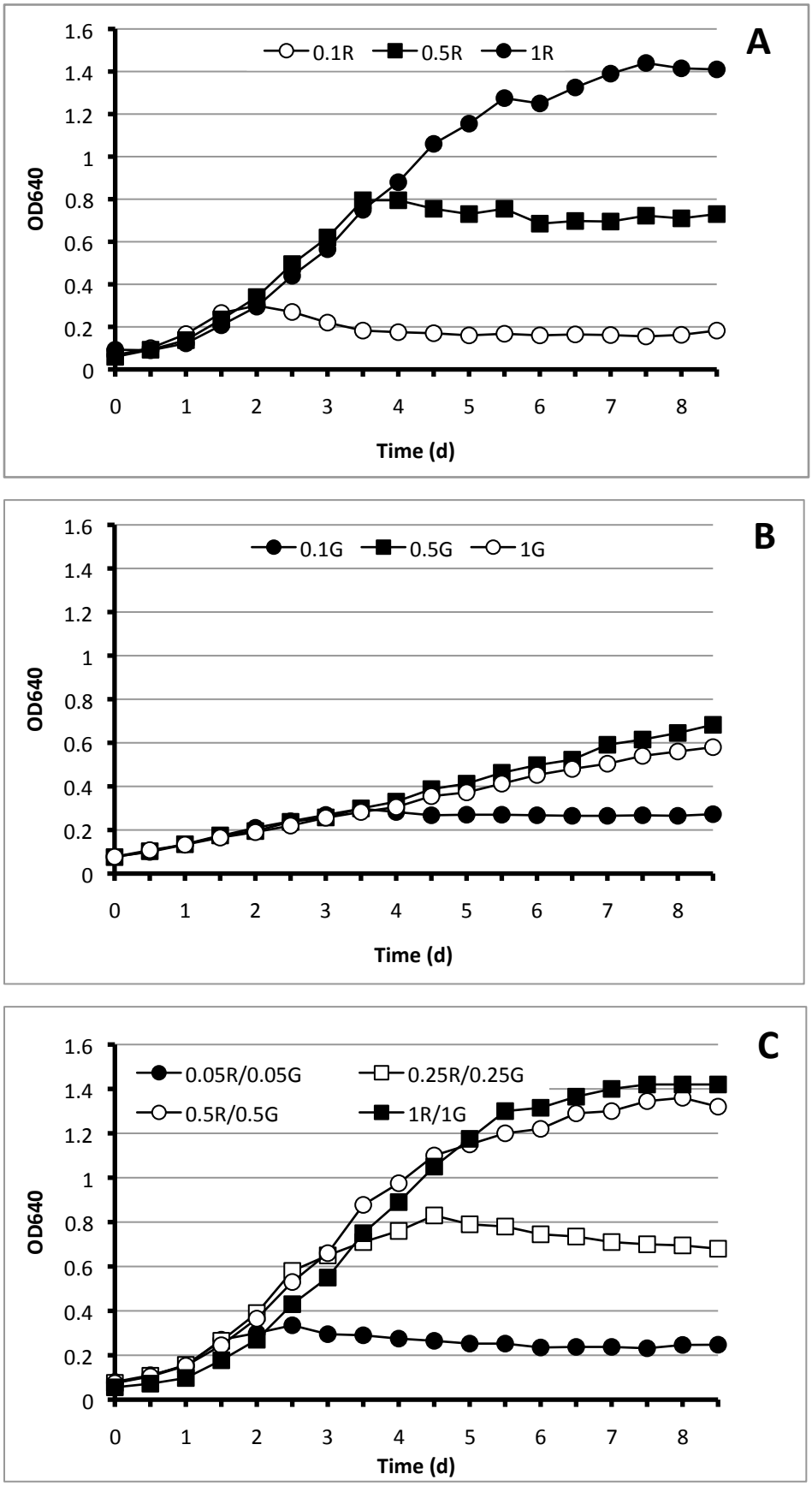
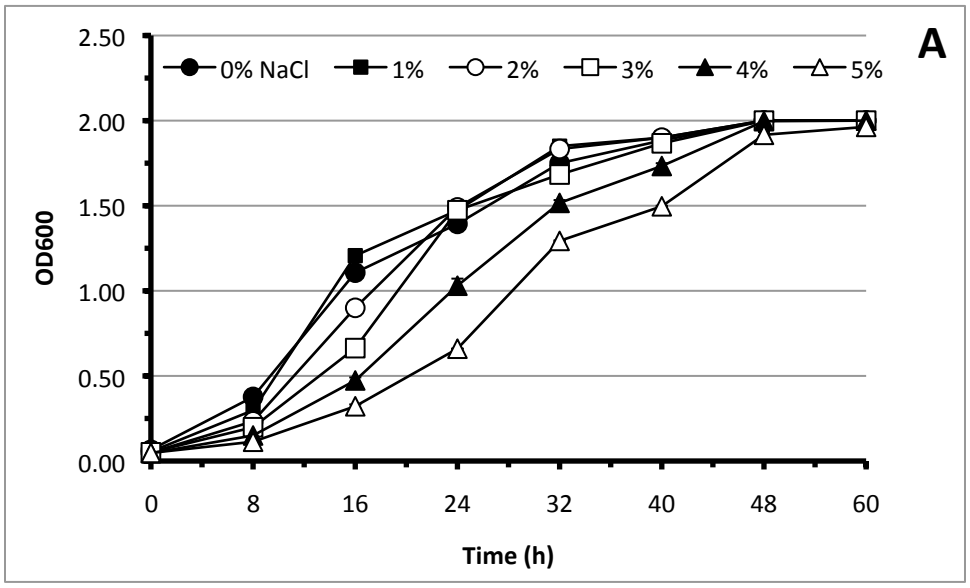


Figure 4. Ortakci..



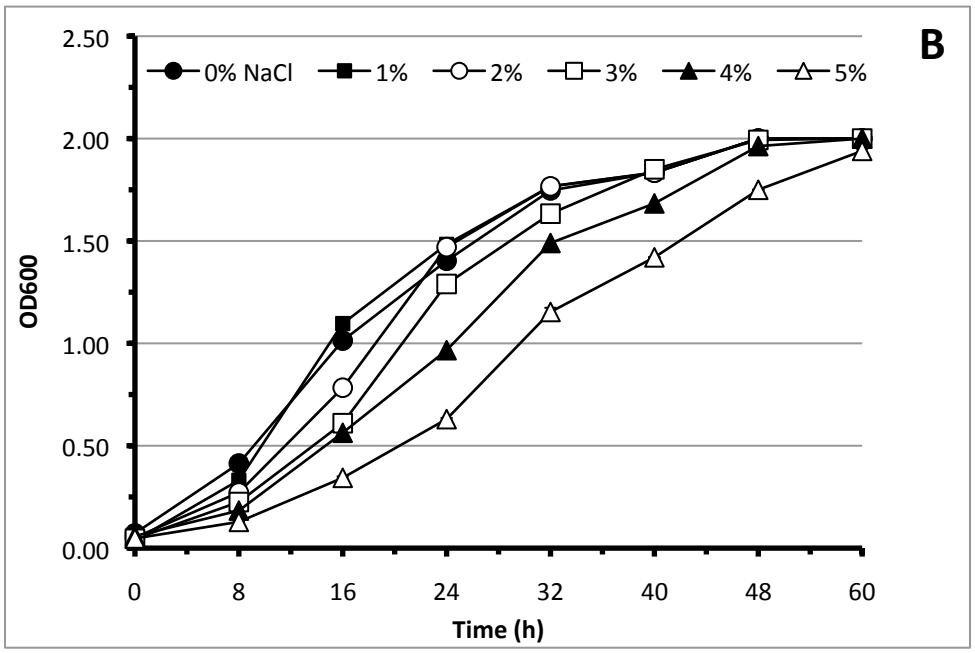


Figure 5. Ortakci.